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Quinton Burnham
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Systematics and biogeography of the Australian burrowing freshwater crayfish genus *Engaewa* Riek (Decapoda: Parastacidae)

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the degree of Doctor of Philosophy, April 2014

The knowledge of anything,
since all things have causes,
is not acquired or complete
unless it is known by its causes.

Abū ʿAlī al-Ḥusayn ibn ʿAbd Allāh ibn Sīnā
(Avicenna) (c.980-1037)
Philosopher.

Happy is he who gets to know
the reasons for things.

Virgil (70-19 BCE)
Poet.

ABSTRACT

The overall aim of this study was to explore the systematics and biogeographic patterns of the freshwater crayfish genus *Engaewa* Riek, a strongly burrowing freshwater crayfish restricted to the coastal corner of south-western Australia (SWA). The genus *Engaewa* is a Gondwanan relict with great potential as a marker of historical processes, due to its high habitat specificity and low dispersal ability. This study comprises an extensive taxonomic and phylogenetic revision of the genus *Engaewa* (using both molecular and morphological data), a detailed study of its distribution and uses the knowledge gained to explore biogeographic patterns in the biodiversity hotspot of SWA.

The molecular analyses undertaken in this project support the monophyly of the genus *Engaewa*. They also, combined with a re-evaluation of morphological characters, support the recognition of (at least) two new species in addition to the five currently described species. Diagnostic morphological characters for the current species and two additional previously undescribed species, along with an updated taxonomic key, are presented. *Engaewa* species possess a genetic structure that is highly unusual and is characterised by particularly low intra-population diversity, and very high inter-population diversity on a scale seemingly not observed in freshwater crayfish before. Based on the updated species designations, the ecology, distribution and conservation status of each species level lineage are also reviewed in this study.

A biogeographic interpretation of the phylogenetic trees and population analyses/summary statistics from the genetic data is consistent with a scenario wherein lineages within this genus have undergone cyclical periods of expansion followed by contraction into refugia, in response to repeated changes in both climate and sea-level. This cyclical process concurs with the Taxon Pulse Hypothesis and has driven lineage diversification, via vicariant speciation, causing rapid bursts of speciation within the genus. This study has identified a number of refugia (from periods of inhospitable climate) centered on locations within the Cape-to-Cape region of SWA (i.e. between Cape Naturaliste and Cape Leeuwin) and on the south coast (specifically the region around the town of Walpole), which are supported by information from other taxa

likewise adapted to mesic habitat, such as other freshwater crayfish, slaters, frogs, orchids and sedges. Not only does this study recognise biogeographic concordance between these taxa in SWA, it highlights a possible central role for *Engaewa* in creating habitat for other taxa.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

    i)   incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

    ii)   contain any material previously published or written by another person except where due reference is made in the text of this thesis; or,

    iii)   contain any defamatory material.

Signed: ………………………………………………
             Quinton Burnham

ACKNOWLEDGEMENTS

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1) THE GENUS *ENGAEWA*

1.1 Introduction

Freshwater crayfish provide a useful organism for a wide range of scientific endeavours and have done so at least since Huxley (1880) used them as the subject for his work *The Crayfish: an introduction to the study of biology*. They have been the focus of studies ranging from ecology to anatomy and physiology, molecular evolution, population genetics, speciation and conservation. Freshwater crayfish possess many characteristics that make them suitable for a variety of studies, not least of which is their availability, as they are widely distributed, naturally occurring on every continent except continental Africa, the Indian sub-continent and Antarctica (Crandall & Buhay, 2008; Nystrom, 2002; Scholtz, 2002). They are also often highly abundant and dominate the invertebrate biomass in many freshwater systems (Nystrom, 2002; Whitledge & Rabeni, 1997).

This study reviews the systematics of the freshwater crayfish genus *Engaewa* Riek, 1967 and then uses it to investigate fine-scale biogeographic patterns in the coastal regions of south-western Australia. This investigation aims to explain the distribution of lineages within the genus *Engaewa*, in an attempt to understand the impact of historical climatic changes on taxa adapted to mesic habitats within the region. This information will be used to identify areas of particular biogeographical significance, specifically those that have acted as refugia[*] for moisture dependent taxa. If *Engaewa* is to be the focus of a biogeographic study it is necessary to understand this genus in relation to other freshwater crayfish, firstly in terms of both phylogeny and distribution, and then in terms of its unique anatomical, physiological and behavioural characteristics. These topics are addressed in this introduction prior to the research questions, hypotheses and thesis structure being expounded.

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[*] The Latin terms 'refugium' (singular) and 'refugia' (plural) are used in relation to habitats that allow for long-term persistence of populations during unfavourable periods, and are distinguished here from the term 'refuge(s)', considered to be any situation that alleviates temporary unfavourable conditions (following Keppel *et al*., 2012).

1.1.1 Freshwater crayfish

All freshwater crayfish belong to the decapod infraorder Astacidea Latreille, 1803, which is considered an ancient lineage, having originated in the early Carboniferous ~325 million years ago (MYA) (Hasiotis, 2002; Porter, Perez-Losada & Crandall, 2005), with the astacid and Nephropoidea Dana, 1852 lineages separating ~280 MYA (Porter *et al.*, 2005). The Astacidea contains two superfamilies of freshwater crayfish, Astacoidea De Haan 1841 and Parastacoidea Huxley 1879, which have a non-overlapping northern and southern hemisphere distribution, respectively. It was assumed that this distribution pattern represented separate freshwater origins, if not polyphyly, of the freshwater crayfish (Huxley, 1880), however, it now seems unlikely that this is the case. A monophyletic grouping is supported by the presence of several apomorphic characters, as well as features of postembryonic development (reviewed in Scholtz, 2002) and genetic data (Crandall, Harris & Fetzner Jr., 2000b). It appears that the ancestor of all freshwater crayfish entered freshwater environments during the Triassic at the latest and, with the separation of Pangaea ~185 MYA, evolved into the Astacoidea in Laurasia (containing the families Astacidae Latreille, 1802 and Cambaridae Hobbs, 1942) and Parastacoidea in Gondwana (composed of a single family, the Parastacidae Huxley, 1879) (Crandall & Buhay, 2008; Holdich, 2002; Scholtz, 2002).

Recent fossil (Bedatou, Melchor, Bellosi & Genise, 2008; Martin *et al.*, 2008) and molecular (Crandall *et al.*, 2000b; Scholtz, 2002; Toon *et al.*, 2010) evidence suggests the lineages within the parastacids are considerably older than the radiations in the astacid and cambarid crayfish. The superfamily Astacoidea appears to have begun to radiate ~90 MYA, whereas the superfamily Parastacoidea began to radiate earlier at ~135 MYA (Porter *et al.*, 2005). Currently there are over 640 described species in the Astacidea with the Cambaridae accounting for over 420 species in 12 genera and the Astacidae containing 39 species in 6 genera, whilst the Parastacidae are composed of over 170 species within 15 genera (Crandall & Buhay, 2008). The Parastacoidea can be distinguished from the Astacoidea on the basis of gill structure, the absence of both the first pleopods and a seminal receptacle (Hobbs Jr., 1988), and by molecular sequence data (Crandall & Buhay, 2008; Crandall *et al.*, 2000b).

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The Southern Hemisphere distribution of the parastacids was noted by Huxley (1880) and Ortmann (1902), which led to them being referred to as having a Gondwanan origin (e.g. Williams, 1981) despite not being present in India and southern Africa (Hobbs Jr., 1988). Riek (1972) suggested that the Parastacidae are a monophyletic group (contrary to Riek, 1959) and that the family originated in south-eastern Australia, where the family currently has its greatest diversity, with eight of the ten recognised extant Australian genera present (Schultz *et al.*, 2009). In total, fifteen genera are presently recognised in the family Parastacidae (their distribution is shown in Figure 1.1). Nine genera are found only in Australia (*Astacopsis* Clark, *Engaeus* Erichson, *Engaewa*, *Euastacus* Clark, *Geocharax* Clark, *Gramastacus* Riek, *Ombrastacoides* Hansen and Richardson, *Spinastacoides* Hansen and Richardson and *Tenuibranchiurus* Riek (Hansen & Richardson, 2006; Hobbs Jr., 1988; Riek, 1969, 1972)), whilst the genus *Cherax* Erichson occurs in Australia, New Guinea, and on nearby islands (Clark, 1936; Holthius, 1986). Outside of Australia *Astacoides* Guerin is located in Madagascar (Hobbs Jr., 1987), *Parastacus* Huxley, *Samastacus* Riek and *Virilastacus* Hobbs all occur in South America (Crandall, Fetzner Jr., Jara & Buckup, 2000a; Riek, 1971) and *Paranephrops* White is found in New Zealand (Archey, 1915; Hopkins, 1970).

Whilst Australia has a large number of crayfish species and they are a common component of the freshwater macroinvertebrate assemblages in many waterways, they are far from homogeneously distributed, with over 90% of species found in the eastern and (to a much lesser extent) northern coastal regions of Australia (Figure 1.1). Central and western Australia are comparatively impoverished except for south-western Australia (SWA), where eleven species of freshwater crayfish are endemic (with an additional non-native established species). The eleven endemic crayfish species in SWA are divided between two genera, with six species belonging to the relatively well known and broadly distributed *Cherax* and five species belonging to the locally endemic genus *Engaewa*. *Engaewa* is, therefore, a significant component of Australia's freshwater crayfish, as it is the only locally endemic genus outside of eastern Australia and represents almost half of the species found in the western portion of the country.
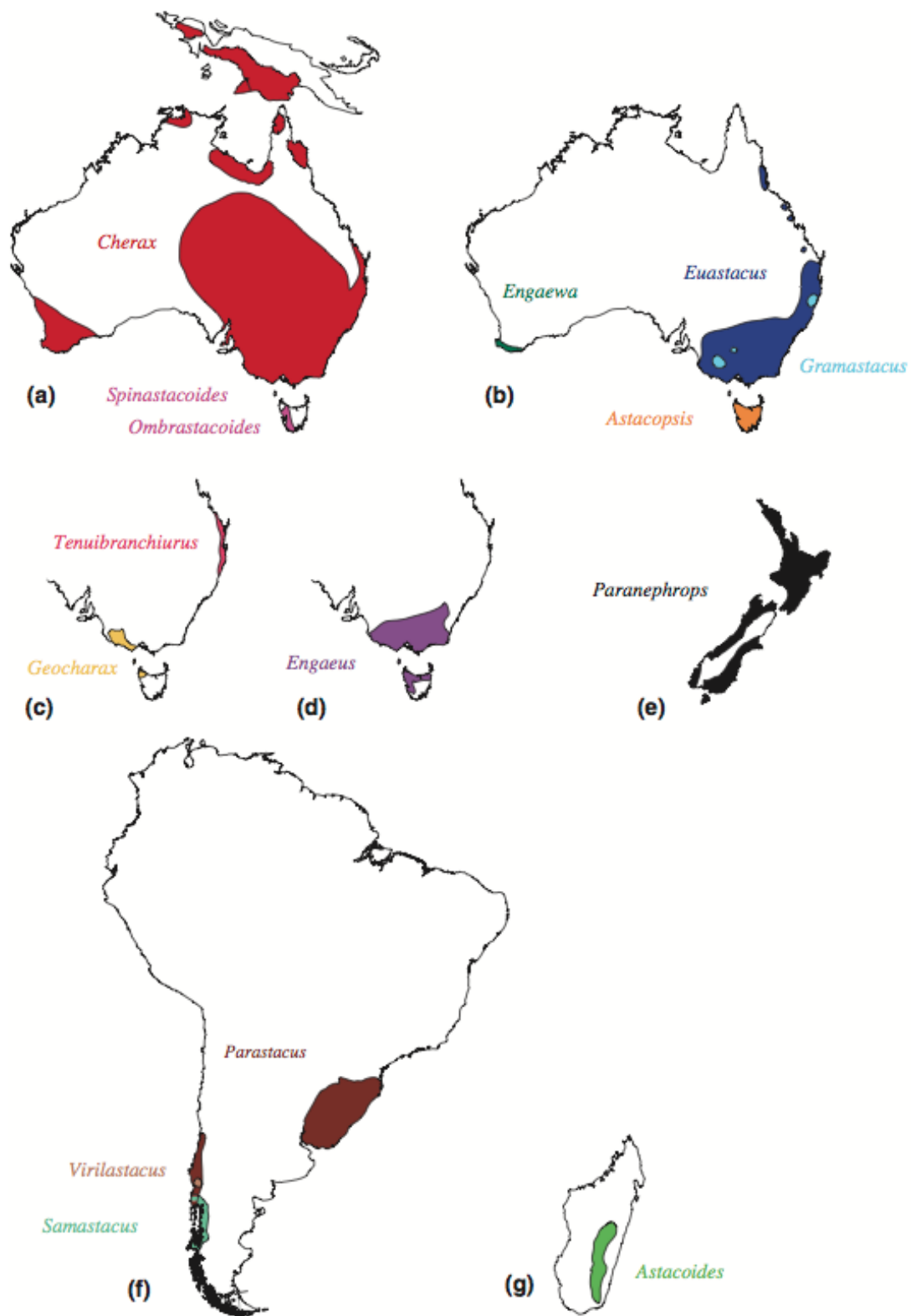
Figure 1.1 Distribution maps of the parastacid crayfish from Australia (a-d), New Zealand (e), South America (f), and Madagascar (g). Distributions are based on Crandall *et al*. (1999, 2000a), Dawkins *et al*. (2010), Hansen & Richardson (2006), Horwitz (1994), Horwitz & Adams (2000), and Shull *et al*. (2005). Reproduced from Toon *et al.* (2010).

1.1.2 The genus *Engaewa*

Engaewa species occupy a coastal distribution throughout the High Rainfall Zone (HRZ) of SWA (the region that receives in excess of 800 mm annual rainfall; after Hopper, 1979), with the extent of their combined distributions approximately matching the Warren Bioregion, as defined by the Interim Biogeographic Regionalisation for Australia (Thackway & Cresswell, 1995) (Figure 1.2). The first known collections of this crayfish were made in the late 1950s and, in 1967, Riek published a formal description of three species in a new genus, *Engaewa* (*Engaewa similis* Riek, *Engaewa reducta* Riek and *Engaewa subcoerulea* Riek) (Riek, 1967a). The three species erected by Riek were based on specimens from only one locality each and Riek noted that there were likely to be more species throughout the region (Riek, 1967a). In 2000, Horwitz and Adams published a more thorough review where they provided support for Riek's three species, using a combination of morphological characters and allozymes, whilst describing an additional two species (*Engaewa pseudoreducta* Horwitz and Adams and *Engaewa walpolea* Horwitz and Adams) from newly sampled localities (Horwitz & Adams, 2000). Despite expanding the number of species recognised within the genus, Horwitz and Adams (2000) noted that there still remained populations requiring further taxonomic review and that considerable morphological variation existed within the currently defined species.

Engaewa species are differentiated from 'typical' freshwater crayfish due primarily to adaptations, both physical and behavioural, resulting from their burrowing lifestyle. This has seen them grouped along with crayfish from genera such as *Engaeus* and *Tenuibranchiurus* from eastern Australia in a category of strong or primary burrowers and described as terrestrial or near terrestrial (e.g. Horwitz & Richardson, 1986; Riek, 1972). The characteristic features of burrowing crayfish species include a reduction in size of the abdomen, with a narrowing of the first abdominal somite (Hobbs Jr., 1975), and chelae that are depressed, shortened and broadened, with fingers moving in a vertical plane (Hobbs Jr., 1975; Holdich, 2002). All of these are considered adaptations to a burrowing lifestyle as they allow for easier passage through burrows and are more effective in blocking the burrow against invaders (Holdich, 2002; Richardson, 2007). Burrowing crayfish also display vaulting and lengthening of the

cephalothorax, which results in an increased size of the gill chamber (Hobbs Jr., 1969; 1975). This is most likely an adaptation that allows for increased oxygen transfer from the air, due to the often low oxygen content of water in the burrow systems (Reynolds, Souty-Grosset & Richardson, 2012a; Richardson, 2007).
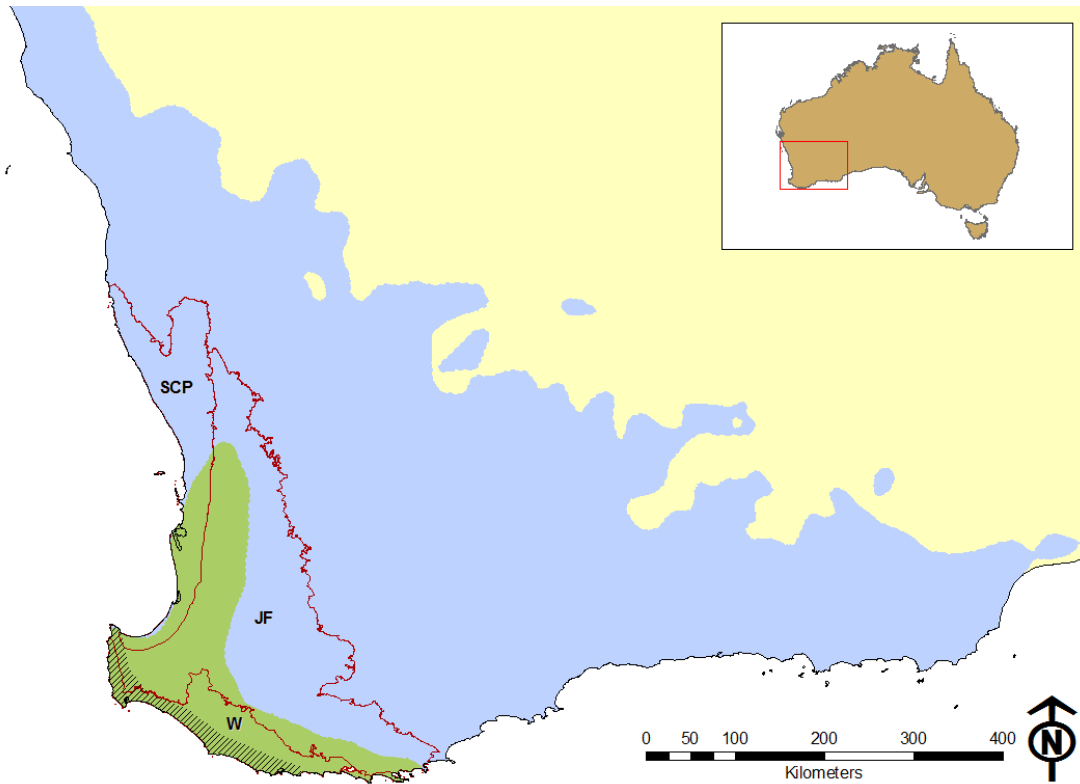
Figure 1.2 South-western Australia is shown with the High Rainfall Zone (Green), Transitional Rainfall Zone (Blue) and Arid Zone (Yellow) of Hopper (1979) and the IBRA Warren (W), Jarrah Forest (JF) and Swan Coastal Plain (SCP) regions' boundaries overlain in red. Shading shows the approximate distribution of *Engaewa*. For sources of GIS data see Section 2.5.

An additional identifying characteristic of burrowing crayfish, first described by Horwitz (1988b; 1990), is an abdominal flap on females, which may serve to shield the eggs and maintain a moist microclimate around them. This characteristic was recorded in the genera *Engaewa, Engaeus, Geocharax, Gramastacus* and *Tenuibranchiurus*, and was assumed by Horwitz (1988b; 1990) to be the result of common ancestry between these species (i.e. that they form a phylogenetic clade to the exclusion of the other Australian parastacids). More recently Schultz *et al.*, (2009) supported this conclusion of Horwitz (1988b) on the basis of molecular data. As a result of these studies the aforementioned genera shall be collectively referred to as the '*burrowing clade*' in the

Australian crayfish fauna throughout this thesis. Though they are grouped, it is important to note that species in each of these genera, and the genera themselves, show considerable variation in the degree to which they display burrowing adaptations, the type of burrow constructed and the placement of burrows in the landscape (for examples of the variety of burrows constructed see Horwitz & Richardson, 1986). It should also be noted that all crayfish seek shelter of some type and that most (if not all) crayfish are capable of constructing at least rudimentary burrows (Berrill & Chenoweth, 1982; Hobbs Jr., 1981; Riek, 1969).

The swamp systems in coastal SWA that provide suitable habitat for *Engaewa* are relatively continuous in the far south-western corner but become increasingly fragmented further north in the distribution of the genus; this is one factor postulated to have resulted in *Engaewa* species having limited ranges and the distribution of populations within species being largely disjunct (Horwitz & Adams, 2000). The most accurate representation of the distribution of *Engaewa* species currently available was presented by Horwitz and Adams (2000), which records the occurrence of 41 populations (based on their distribution map – reproduced here in Figure 1.3). The distribution boundaries for each species are generally poorly defined and many of the historical records lack detailed locality information, however, the records available prior to this study suggest that:

- *E. reducta* is limited to the northern part of the region from Cape Naturaliste to the Margaret River;
- *E. pseudoreducta* is found only within a single reserve just north of the Margaret River;
- *E. similis* has a range with a northern boundary at the Margaret River and extending south-east along the coast to somewhere in the vicinity of Windy Harbour;
- *E. subcoerulea*'s range commences approximately where that of *E. similis* finishes and continues east to just past Walpole; and
- *E. walpolea* is limited to the immediate vicinity of the Walpole townsite on the south coast.
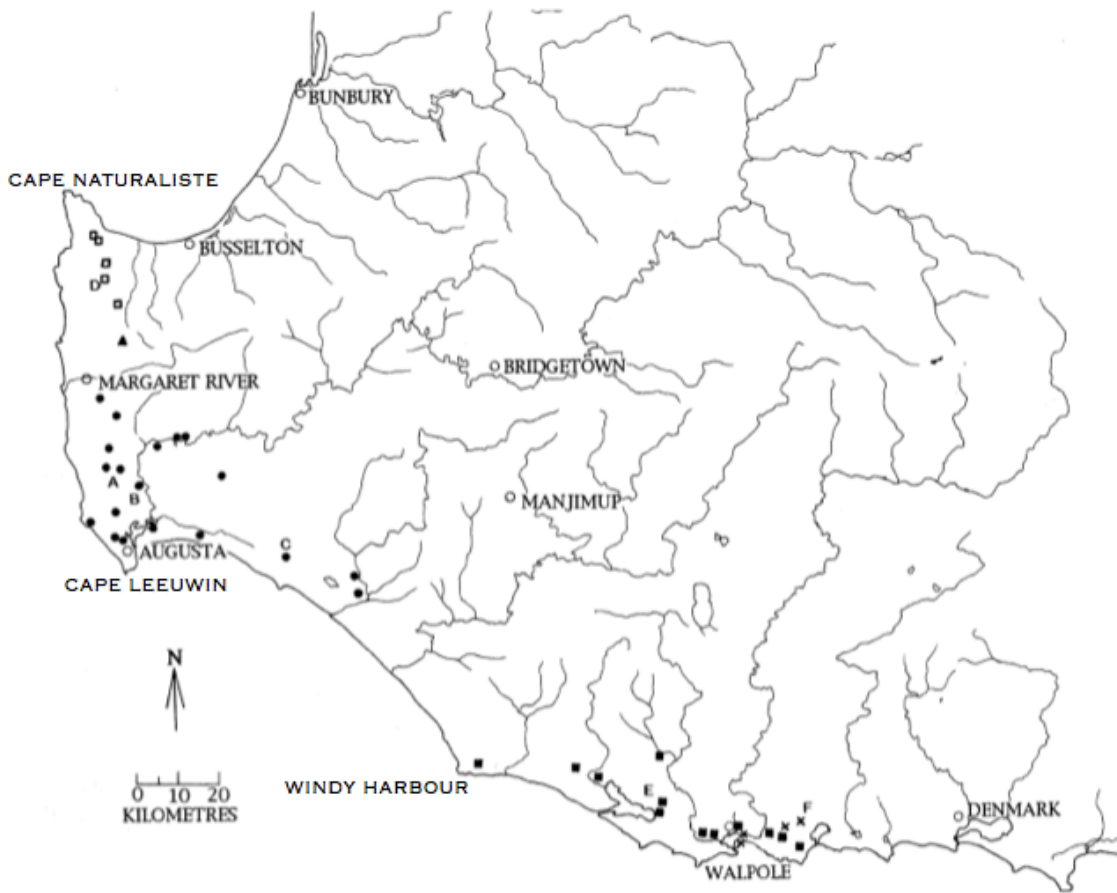
Figure 1.3 Distribution of the five currently recognised species within the genus *Engaewa* prior to undertaking sampling for this project (adapted from Horwitz & Adams, 2000). Populations of each species are represented by the various shapes (Open square- *Engaewa reducta*; Triangle- *Engaewa pseudoreducta*; Circle- *Engaewa similis*; Closed square- *Engaewa subcoerulea*; Cross- *Engaewa walpolea*) and the letters A-F refer to specimens used for allozyme electrophoresis by Horwitz and Adams (2000).

Prior to the commencement of this study no sites of sympatry had been recorded within the genus *Engaewa* and the only species with overlapping distributions were *E. subcoerulea* and *E. walpolea* (Horwitz & Adams, 2000). This situation opens up a range of questions as to how, and why, the species became, and have subsequently remained, geographically separated; these questions will be addressed in this thesis. Whilst there appears to be a lack of sympatry between *Engaewa* species the same cannot be said for the (relatively) ecologically similar and phylogenetically closely-related genus *Engaeus*, which is much more diverse in terms of species numbers and covers a larger area of the continent in the south-eastern corner. Together these comparisons suggest that SWA and south-eastern Australia may have undergone significantly different historical events.

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All species in the genus show narrow geographical distributions with *E. pseudoreducta* being the quintessential example – its known range prior to this study constituted significantly less than 3 km^2. It had also been eradicated from its type locality, and habitat degradation at its only other known location had created serious concerns over its survival (Horwitz & Adams, 2000). *Engaewa walpolea* is known from only a handful of locations and *E. reducta* is also of conservation concern due to a limited and fragmented range (Horwitz & Adams, 2000). *Engaewa similis* and *E. subcoerulea* have larger ranges and are both well represented in National Parks and, as such, although some populations are under threat the species as a whole were not considered to be in any immediate danger of extinction (Horwitz & Adams, 2000). Concerns regarding the persistence of *Engaewa* species led to the recognition that there may be a need to formally list one or more species as threatened with extinction.

Despite some *Engaewa* species being considered rare (based on their limited distributions), members of this genus appear to exert a disproportionate impact on their habitat. This can occur as the type of rarity (within or between ecosystems) determines whether a rare species has the capability to influence community processes (Lamont, 1992). Based on Rabinowitz' 'seven forms of rarity' *Engaewa* certainly fall into the category characterised by 'small geographic range' and 'narrow habitat specificity' but also probably under the local population size of 'large dominant somewhere', for many populations (i.e. endemics) (Rabinowitz, 1981). Thus, despite being rare on a larger scale, some species (such as *Engaewa* spp.) can be locally prominent or abundant and therefore can still be central to the functioning of the ecological community of which they are a part.

Of particular interest in this sense is the role of the burrows that *Engaewa* individuals dig. It is known that crayfish burrows facilitate the movement of oxygen, water and nutrients through the soil profile (Horwitz & Knott, 1983), transfer microorganisms (Richardson, 1983), provide drainage during wet periods and a water reservoir during dry periods (Richardson & Wong, 1995) and generally extend surface effects deeper than their normal range (Richardson, 1983). Burrows may be lined with rootlets and fungal hyphae, due to the increased oxygen levels in burrows (Richardson,

1983) and, as most crayfish are considered omnivorous and are known to utilise several food items including both delicate and robust plant material (Nystrom, 2002), it is likely that root grazing occurs (Richardson & Wong, 1995; Riek, 1972).

Engaewa species generally dig their burrows in sandy or loamy soils in heathlands that are dominated by myrtaceous shrubs, although there is considerable variation in the habitat that they will utilise (Horwitz & Adams, 2000). They can be found in seasonally inundated basins (sumplands), channels (creeks) and flats (floodplains) and seasonally waterlogged basins (damplands), channels (troughs), flats (palusplains) and slopes (paluslopes) (*sensu* Semeniuk & Semeniuk, 1995). The depths to which the burrows can reach vary from just below the surface to well in excess of two metres; they can also branch repeatedly, be ramified laterally and extensively, and can be acutely slanted (Horwitz & Adams, 2000; pers. obs.).

In addition to the likely physical and chemical transformations in the soil, and the associated effects on plant growth related to *Engaewa* burrows, the burrows themselves also directly provide habitat for pholeteros (a collective term for a suite of species living in the burrows (*sensu* Lake, 1977)) (Hansen, Adams, Krasnicki & Richardson, 2001; Horwitz, 1995; Horwitz & Rogan, 2003). This provision of habitat may be important to the biological diversity of SWA as the aquatic invertebrates in crayfish burrows contain a greater than expected level of local or restricted endemism (Horwitz, 1997). The ability of a species to influence environmental conditions and resource availability significantly compared to the surrounding unmodified environment and in doing so create, maintain or destroy habitat for other species qualifies them as an ecosystem engineer (Jones, Lawton & Shachak, 1994; 1997). Crayfish have been acknowledged as being ecosystem engineers (Creed & Reed, 2004; Richardson, 1983; Usio, 2002) and, as the habitat they create results in a positive feedback for the crayfish itself, the engineering process represents Dawkins' (1982) extended phenotype engineering (Jones *et al.*, 1994; 1997). *Engaewa* species likely represent an example of an allogenic engineer, as their actions may alter the availability of resources for other species and in doing so create, modify and maintain habitat (Lawton, 1997). Bertness (1985) demonstrated how the removal of a 'keystone species' (in this case burrowing

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fiddler crabs, *Uca pugnax* (Smith)) can alter factors such as drainage, decomposition, sedimentation, erosion and primary production; all of which are possible outcomes that may result from the removal (or extirpation) of *Engaewa* from swamp communities if they too prove to be a keystone species.

1.1.3 Biogeographic significance

South-western Australia is considered a discrete biogeographic region, separated from other regions in Australia by differences in climate and associated drainage system patterns (Johnstone, Lowry & Quilty, 1973). It is characterised by low relief and subdued topography, with matching low erosion rates and mature/sluggish drainage (Mulcahy, 1967). The vegetation of the HRZ of SWA encompasses multiple types, ranging from tall open-forests to coastal heath (Gardner, 1959) and whilst the topography may be muted, the expression of ecological variation is not, with sharp ecotones being a feature of the southern part of the HRZ (Wardell-Johnson, Inions & Annels, 1989). Lentic environments on the coastal plains and inland regions are often surface expressions of extensive groundwater systems, whereas many swamps and seasonal damplands in the extreme southwest occur as a result of poor drainage (V. & C. Semeniuk Research Group, 1997). Thus, many of the surface water environments within SWA are commonly temporary and/or seasonal in nature, including those inhabited by *Engaewa*.

It has been noted that in order to explore the biogeography of a region it is necessary to understand the evolutionary history of various groups, so that taxonomically significant species (such as those from ancient or distinct lineages) can be identified and focused upon (Samways, 1994). In Australia, the significance of unique and restricted taxa of Gondwanan origin has been increasingly recognised (Hopper *et al.*, 1996). Parastacids have been strongly linked to studies of Gondwanan lineages (e.g. Toon *et al.*, 2010) and *Engaewa* specifically has been described as a Gondwanan relict (Horwitz & Adams, 2000). Furthermore, Horwitz and Adams (2000) suggested that *Engaewa*'s distribution might be significant in terms of exploring biogeographic hypotheses relating to the flora and fauna of SWA. Thus, it will be argued throughout this thesis that there is much to be gained by investigating the

biogeography of *Engaewa*. However, in order to understand the significance of these species, there is a need to understand the geologic and climatic history of SWA.

For the past ~60 MYR the global climate has generally been cooling, to the point where the Quaternary Period has been dominated by Ice Ages (Hewitt, 2004). The end of the Eocene, transitioning into the Oligocene (~35 MYA), saw the environment cool significantly (Macphail, Alley, Truswell & Sluiter, 1994) with the initial formation of the Antarctic ice sheet ~35 MYA (Hewitt, 2004) following the rifting of Australia from Antarctica and the associated opening and expansion of the Southern Ocean as Australia moved northwards (Frakes, 1999). This cooling process resulted in both the southeast and southwest of Australia experiencing a climatic shift from an aseasonal wet tropical/subtropical biome to a drier one with winter seasonal rainfall (Hocking, Moors & van de Graff, 1987).

The general drying process that occurred in Australia probably did not develop contemporaneously across the continent (Kemp, 1981). In the case of SWA, it has been assumed that significant drying occurred before the middle to late Miocene (Archer, 1996), with the north-western coast of Australia experiencing arid conditions even earlier than the southwest (Beard, 1977; Kemp, 1981). This would have effectively cut off the north-west as a potential migration route to tropical regions in northern Australia for tropical/subtropical adapted species (Hopper, 1979). Therefore, the only sanctuary open to these species would have been in the high-rainfall areas of the southwest (Hopper, 1979), or to retreat underground (e.g. more recent stygofaunal groups such as the subterranean diving beetles invaded the groundwater ~5-8 MYA (Humphreys, 2000; Leys, Watts, Cooper & Humphreys, 2003)). For such taxa, even during periods of aridity, a high-rainfall zone existed on the south-western coast of Australia; primarily as a result of moisture-laden winds (Hopper, 1979). Cold, dry air masses heading north from Antarctica spend days over the relatively warm ocean, thus gaining moisture, which could result in orographic precipitation upon reaching the SWA coastline (Markgraf, McGlone & Hope, 1995).

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Although a HRZ would have persisted even during relatively arid periods, at times the wet habitat remnants would have been reduced to such a degree that only small numbers of species survived as relicts of the original Miocene rainforests (Archer, 1996; Galloway & Kemp, 1981). It has been suggested (e.g. Archer, 1996) that this explains the lack of relictual mammalian fauna, however, numerous plant and invertebrate species have persisted through generally inhospitable periods, as these two groups have characteristics that permit them to exist in small populations in suitable microhabitats. Therefore, the aridification that occurred in SWA, combined with the current extreme seasonal variability in water availability within SWA, limits many of the once widespread, mesic-adapted, Gondwanan species to highly restricted and patchy distributions (Hopper *et al.*, 1996; Harvey, 2002b). Whilst water availability is strongly seasonal, Mucina and Wardell-Johnson (2011) suggested that there remained a predictable nature to climatic seasonality (over evolutionary timescales), which would have allowed the persistence of old pre-Pleistocene lineages.

These restricted and relictual lineages can be thought of as climate relicts, and the habitats that maintain them as climatic refugia. Originally identified in relation to Quaternary glaciations in the Northern Hemisphere (particularly Europe) (Keppel *et al.*, 2012), the concept of climatic refugia has been extended to any area that is largely buffered from the impacts of climate change (Ashcroft, 2010). Specifically, Dobrowski (2011, p. 1023) defined refugia on climatic grounds as "physiographic settings that can support once prevalent regional climates that have been lost (or are being lost) due to climate shifts" and Hampe and Jump (2011, p. 317) defined refugia on biological grounds as "any place that harbours a climate relict population".

Although not being impacted directly by the physical processes of glaciation, areas within Australia would still have been impacted by the events of glacial periods, which most notably include increased aridification (Fuijoka, Chappell, Fitfield & Rhodes, 2009). Often refugia are related to complex landscape topography (i.e. mountains and valleys) (Médail & Diadema, 2009), however, they are increasingly being recognised in regions of subdued topography (Byrne, 2008; Hopper, 1979),

although these remain somewhat cryptic (Keppel *et al.*, 2012). This study aims to identify, and further characterise, these types of refugia within SWA.

Invertebrates generally are well suited to exploring a range of biogeographic questions. Due to their small size and often specialised behaviour, invertebrates are often confined to topographically or geographically restricted areas and specialised microhabitats (Main, 1996b) and certain invertebrate groups also have limited dispersal capabilities (Harvey, 2002b). These characteristics are particularly prevalent for groups that represent relictual lineages from an earlier environment (i.e. Gondwanan) (Main, 1996b). Species that are highly sedentary (or face significant barriers to dispersal) are of particular interest to biogeography as they generally have deep population structure that is not overwritten by high gene flow (Koizumi *et al.*, 2012). In addition to this, the microhabitats they occupy are particularly vulnerable to artificial disturbances resulting from human activities and constructions (Main, 1996b), and it has been noted that ongoing local extinctions, combined with species transplantations (whether intentional or not), are essentially erasing biological information that can provide insights into past environments (Koizumi *et al.*, 2012).

There are a number of factors that make *Engaewa* potentially a suitable taxon for a biogeographic study of SWA, and particularly so for understanding the influence of climate on taxa that are wedded to freshwater and/or moist microhabitats. *Engaewa* are only able to disperse significantly during periods of very specific environmental conditions, thus recent and/or regular movements of individuals between populations are unlikely to have masked deeper patterns. Their potential for overland dispersal is limited and whereas many other freshwater invertebrates have a winged stage to facilitate dispersal, *Engaewa* do not. Furthermore, as they live underground they are unlikely to be transported by other vectors (humans, water birds, mammals etc.). All of these factors are significant, as they likely have acted to preserve the historical signal of periods of expansion and retraction associated with aridity within the genus.

1.2 Research aims and thesis format

The overall aim of this thesis is:

"To undertake a systematic and biogeographic investigation of the genus *Engaewa*, an extreme burrowing freshwater crayfish endemic to the coastal corner of south-western Australia, and to provide new insights into the biogeography of the region."

Prior to this work, only five peer-reviewed journal publications were available that either specifically or significantly relate to the genus *Engaewa*. Of the publications that discuss topics relating to *Engaewa* in detail, one is related to taxonomy (Riek, 1967a) and three to phylogeny (Schultz *et al.*, 2009 and to a lesser extent Crandall *et al.*, 1999 and Toon *et al.*, 2010), whilst only Horwitz and Adams (2000) address other aspects such as taxonomy, ecology, biogeography and conservation. The information provided in these documents makes it evident that there are significant gaps in our knowledge relating to the systematics, distribution patterns and ecology of *Engaewa*.

The systematics of *Engaewa* are reviewed in detail in the third chapter of this thesis (the second chapter being devoted to the methods of data acquisition) using both molecular and morphological data. The molecular data were used both to delimit species and to elucidate the genetic structure and diversity contained within and between species. Therefore, the third chapter of this thesis covers the phylogenetic placement of the genus *Engaewa*, reviews the taxonomy of the genus, and also explores the degree and distribution of genetic and morphological variability within the genus.

Following the systematic revision, insights into the natural history of *Engaewa* accumulated during the collecting of specimens for this project are presented in Chapter 4. Understanding the type and extent of habitat that *Engaewa* species utilise, contributes towards an explanation of the geographic distributions of lineages. A concomitant motivation to study the genus *Engaewa* is that, like many restricted aquatic invertebrates, its populations are facing increasing survival pressures (Horwitz & Adams, 2000). As well as covering natural history, the fourth chapter of this thesis also

addresses conservation issues faced by the genus *Engaewa*. The taxonomic and evolutionary understanding of *Engaewa* derived from this study, enables the identification of conservation units, which can be prioritised so as to maintain their evolutionary processes and preserve genetic resources (Frankham, Ballou & Briscoe, 2002). This work involved extensive field collections to improve our knowledge of the distributions of each species, which is a significant component of the assessment of each species' conservation status (Lamont & Connell, 1996; Samways, 1994) and reliant upon sufficient sampling of specimens to account for geographic variation (Sites & Crandall, 1997).

The fifth chapter of this thesis addresses the biogeography of the genus. This chapter consists of the explanation and testing of a number of *a priori* hypotheses related to the distribution of *Engaewa* lineages (in relation to the information presented in Chapters 3 and 4), followed by a discussion on how the lineages within the genus have arisen. Each hypothesis was tested against the pattern derived from the systematics, in an attempt to uncover a proposed process (a historical framework for vicariance and dispersal mechanisms among and within lineages within this genus) that would produce an outcome that matches most closely the observed pattern. One significant challenge of this approach is to unravel the effect of more recent changes, to ensure that firstly the true underlying historical pattern can be recognised, before the more recent ecological driven changes can then be incorporated.

The sixth chapter of this thesis uses the biogeographical insights gained in relation to *Engaewa* to highlight biogeographic boundaries and refugial areas for other taxa in the region. In doing so, an assessment will be made as to whether conclusions can be reached regarding a generalised response of taxa (specifically those adapted to mesic habitats) in SWA to climatic shifts in the past. The location and nature of refugia within SWA will be discussed in this chapter, in order to better define them.

In summary, in order to address the aim of this thesis I will initially review the systematics of the genus *Engaewa* and then seek to: (a) understand the diversification events that have occurred within the genus *Engaewa* (i.e. the pattern); (b) propose and test models that can provide an explanation of a process that results in the observed pattern; (c) identify areas within SWA that are of a refugial nature (specifically climatic refugia) (i.e. the pattern); and, (d) explain how and when such refugia operate to derive the process that creates the observed pattern.

2) GENERAL METHODS

2.1 Introduction

Few *Engaewa* specimens have been previously collected, and only a handful have been used for molecular analyses; therefore, substantial effort was dedicated to collecting a sufficient number of specimens, and then to obtain DNA sequences. Neither the methods for collecting specimens, nor obtaining DNA sequences, for this genus have been described in detail anywhere prior to this study. In addition to these methods, morphological characters significant in relation to the taxonomy and/or phylogeny and/or ecology of these crayfish are presented here. Finally, the methods and data sources used to characterise the distribution of these crayfish and the type and extent of suitable habitat are also provided. Thus, this chapter constitutes a summary of the basic methods relevant to this study.

2.2 Specimen collection

As *Engaewa* spend virtually their entire life below ground, one immediate challenge is to confirm their presence at a site. In order to direct collection efforts, in the first instance potential habitat throughout the search area was identified using a combination of maps and satellite imagery, looking for small creeks or potentially larger swamp systems that possessed a significant canopy of native vegetation. As areas that have experienced habitat degradation (most commonly resulting from the clearing of native vegetation and/or the presence of cattle and/or altered hydrological conditions) generally will not support populations of *Engaewa* (Horwitz & Adams, 2000) the presence of native vegetation was seen as a proxy for habitat that was likely to be relatively undisturbed.

The search area was initially defined as being the region from which *Engaewa* had previously been recorded (i.e. between Cape Naturaliste and Bow Bridge – see Figure 1.3). An expanded search along the coastline in both directions was subsequently conducted, until no evidence of *Engaewa* was repeatedly and predictably found in apparently suitable habitat. As well as a linear extension along the coastline, the search area also was extended to a distance of approximately 20 km inland from the coast, again until there was reason to assume that *Engaewa* were likely not present at or past this area. This approach created a list of potential habitat sites that required groundtruthing. Areas that appeared to be suitable from maps and imagery were often not so once visited, hence the only reliable way to assess an area was to visit it. Virtually every accessible creek, drainage line, swamp or seepage as well as numerous roadside ditches and artificial water bodies within the search area were examined. The presence of *Engaewa* was confirmed for 62 sites throughout this study (Figure 2.1).

An important corollary here is that non-detection of *Engaewa* at a particular site cannot be taken as definitive evidence of its absence (i.e. it might be a false negative), which is true of all presence/absence records (MacKenzie, 2005). Although every attempt was made to be accurate in recording presence/absence, the cryptic nature of these animals precludes confidence in statements regarding absence, whereas a single crayfish confirms presence (for that particular species at least), at that specific time. While I am confident that the search was extensive, it is possible that one or more isolated, small populations remain in this fragmented landscape. As such, additional sampling should continue in the region at every opportunity and always as a part of any assessment for future infrastructure development. Notable impediments to detection of *Engaewa* are the species' cryptic, burrowing nature, the difficulty seeing and accessing burrows in often dense vegetation and gaining access (by road) to potential sites, particularly during the wet season when the animals are assumed to be closest to the surface and the soil is most suitable for excavating crayfish burrows.
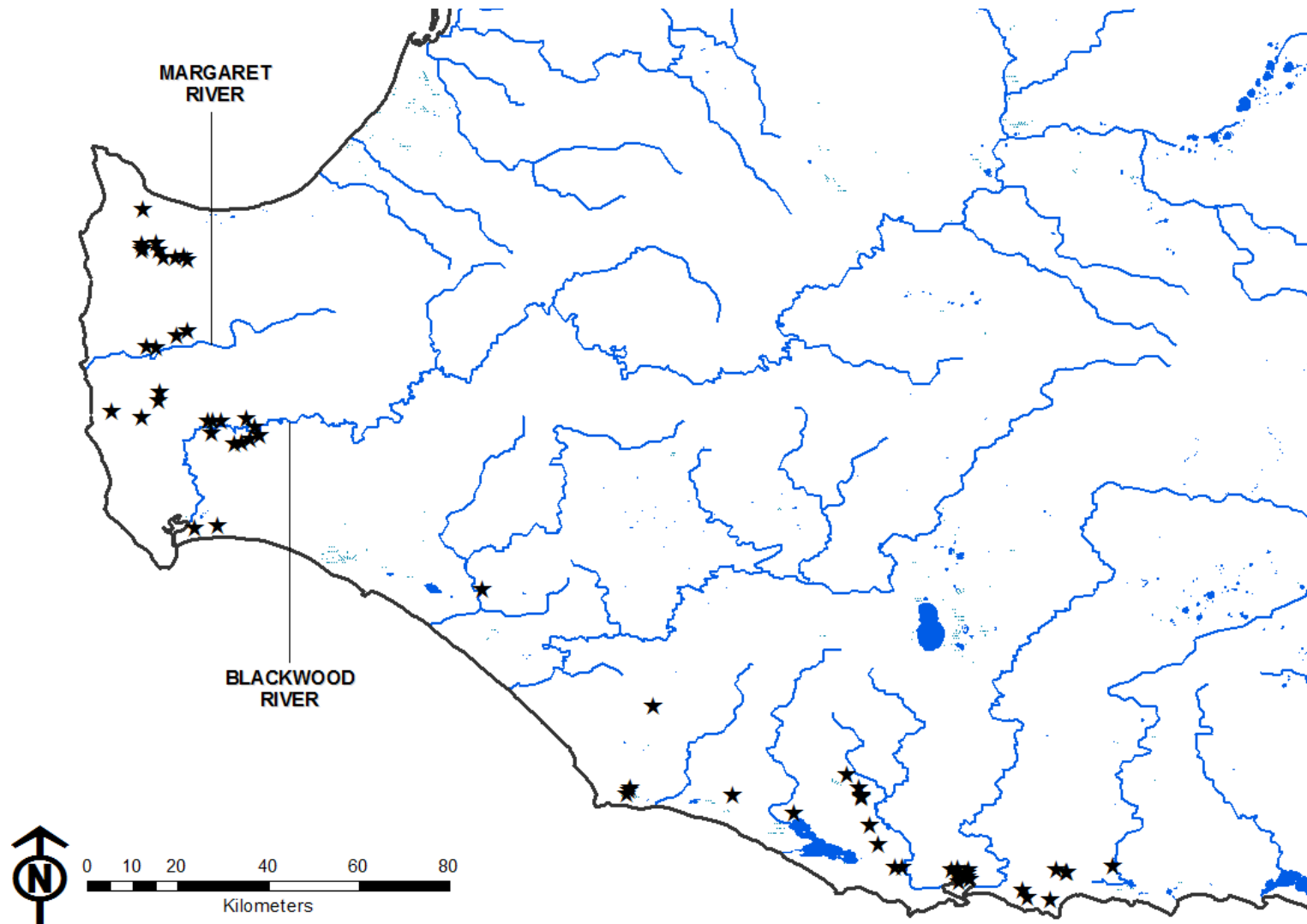
Figure 2.1 Location of all *Engaewa* specimens collected during this study (black stars). Major drainages are shown in blue.

Potential habitat was assessed for the presence/absence of these crayfish by looking firstly for characteristic chimneys of soil, which can signal the entrance to a burrow system (Figures 2.2 & 2.3). These chimneys are formed by the crayfish from the accumulation of small spherical pellets of soil that have been expelled as the tunnel systems have been excavated. Pellets can be up to 1-2 cm in diameter, though generally they are much smaller. Chimneys can range from less than half a dozen small pellets surrounding a small hole to a conical shaped chimney up to ~35 cm high and formed from tens or even hundreds of individual pellets. The soil forming the chimney may be distinctly pelleted or it may appear as a simple mound of soil, due to the effect of weathering. Where obvious chimneys were lacking, closer attention was paid to any patches of different coloured soil, or even simple holes in the ground that may also signal the entrance to a burrow. Newly formed chimneys appear rapidly during the winter months (i.e. the rainy season) throughout the entire range of the genus (as seen in the example provided in Figure 2.4).

Crayfish of the genus *Cherax* also create burrows in the same region as *Engaewa* and it is not always easy to distinguish between the chimneys produced by these different crayfish. As a general rule *Cherax* species typically dig shorter, straight tunnels and have small chimneys with much larger pellets. *Cherax* chimneys also often form a miniature 'caldera', whereas *Engaewa* chimneys almost always appear conical. The diameter of the tunnel extending vertically from the chimney is also characteristic, as *Engaewa* burrows are much smaller in diameter (approximately a little finger in width) when compared to a *Cherax* burrow (often in the range of middle finger to thumb in width and, at times, larger). Chimneys from both genera may either appear to be open (with a hole leading into the burrow system clearly visible) or closed (where the chimney appears to be 'plugged' by soil). Furthermore, if excavation of a burrow commences and tunnels are found that repeatedly bifurcate it can generally be assumed that the burrow belongs to an *Engaewa* species, due to the relative simplicity of *Cherax* burrows.

Figure 2.2 Two 'chimneys', formed by spherical pellets of soil, which indicate the entrance to an *Engaewa* burrow system (or systems).

Figure 2.3 A number of 'chimneys' indicating the entrance to one or more *Engaewa* burrow systems can clearly be seen along the roadside as the grass has recently been slashed.

Figure 2.4 An example of chimney construction occurring throughout winter at a site in Yelverton with burrows absent in March (a) and present in October (b). The burrows are evident within the red circle.

Collecting crayfish with the standard digging method involved digging a hole centred on a chimney or group of chimneys and then following any tunnels. A combination of shovelling and exploring tunnels by hand was used as the excavation of the burrow system proceeded, often bailing water out of the hole as the excavation continued. Using this method, crayfish were located either in a tunnel and pulled out by hand, in the water in the hole and bailed out, or shovelled out with the soil. A variety of different sized holes were excavated ranging from 0.2 m x 0.2 m to 4 m x 2 m, although most holes were less than 0.5 m x 1.0 m and whilst the deepest holes were 1.5-2 m deep (for an example see Figure 2.5), they were rarely dug deeper than 1 m. Regarding burrow structure, the previously reported depth range of 0.3 to >2 m (Horwitz & Adams, 2000) was supported, as 2 metres was the greatest depth dug to in this study (on the Scott Coastal Plains (*E. similis*)) and the burrow system was seen to continue deeper. Lateral burrows were also found to extend for many metres on occasion (e.g. Figure 2.6). The actual depth to which each species digs may vary and generally may reflect the maximum depth to which the water table is lowered throughout the year.

The success rate of this standard method varied greatly, with between zero and four crayfish collected from any single excavation. Due to the size and complexity of tunnel systems, often no crayfish were found, as they may have been able to escape, quite probably by reaching the water table where they could rapidly descend into a deeper chamber. The majority of crayfish were taken from the layer of soil corresponding to the level of the ground water, with few successfully collected deeper in the water and very few found closer to the surface.

Figure 2.5 Excavated *Engaewa* burrow systems showing the depth at which groundwater was located, and also where specimens were collected.

Figure 2.6 Partially excavated *Engaewa* burrow system showing the length of a lateral burrow.

Collecting *Engaewa* in this manner is a difficult task and often the vegetation is very dense, meaning attempts to locate and excavate burrows are hindered both by the vegetation above ground and by the root system below. Many potential *Engaewa* sites are difficult to access as they are either remote, or where accessible, are on private land and require landowner permission. As most species have some level of protection for conservation reasons the number of specimens that could be taken was limited and the amount of habitat damage caused by both access and digging needed to be minimised. In order to make sure damage to the habitat was minimised, all soil was returned to the hole once crayfish collection had finished, with particular attention paid to ensuring the upper layer of soil was both removed and replaced intact (as much as possible). Vegetation was replaced and additional vegetative material placed over the disturbed area to prevent increased evaporation from the soil. An example of a restored and replanted burrow excavation is shown in Figure 2.7. A further consideration was the possibility of spreading *Phytophthora cinnamomi* Rands (dieback), thus all digging equipment, boots and car tyres had to be sterilised after exiting a possible dieback infected area.

Figure 2.7 An example of a refilled and replanted burrow excavation (in Spearwood Creek). Small *Engaewa* chimneys (a few centimetres in height) were found on the edge of the previously dug hole after returning to a site sampled one month prior. There was no obvious lasting damage caused by sampling to the immediate microenvironment and the density of burrows in the vicinity had not appeared to diminish.

As previously mentioned, collection of crayfish was generally achieved by digging out the burrow system; however, on (very) rare occasions it was possible to collect them by spotlighting in shallow puddles or channels at night. Spotlighting can be used for a wide range of crayfish species and involves examining shallow water bodies at night via torchlight, when crayfish are often more active. The occurrence of *Engaewa* outside of their burrow has only been reported in the literature once previously. Horwitz and Adams (2000, p. 676) stated that; "The species was first found in a gently sloping block of land just logged; individuals were collected from water-filled tractor tyre ruts in May 1981 following recent heavy rain". The spotlighting method was attempted repeatedly for all species, however, it was only found to be regularly successful for collecting *E. walpolea* (the same species collected by Horwitz and Adams) and on one occasion for collecting specimens from Spearwood Creek (nominally *E. similis* though their species status will be reviewed in the next chapter).

Engaewa walpolea individuals were regularly found by spotlighting in very shallow puddles and channels, including roadside ditches (e.g. Figure 2.8), and a very recently moulted *E. walpolea* individual was found by spotlighting (it was still soft and its exuvia was present) in early October. Multiple crayfish could be found in close proximity within puddles, which often also had small *Cherax* present, although during the relatively brief periods of observing these crayfish whilst collecting specimens no interactions were witnessed between individuals of the two different crayfish genera. The collection made at Spearwood Creek was during a particularly large thunderstorm late at night with torrential rain and sheet water flowing across the ground down the broad valley floor. Multiple specimens were found walking through the shallow water amongst the vegetation. It is unclear whether the other species leave their burrows. At other sites *Engaewa* body parts (most often claws) were found rarely, though it was unclear if these have been shed when moulting or if the crayfish had been predated upon. This may suggest that even where *Engaewa* have not been found on the surface they may, under certain conditions (i.e. if the water table is sufficiently high or there is a significant rain event), leave their burrows.

Figure 2.8 An example of puddles in which *Engaewa walpolea* specimens were collected at night via the spotlighting method; the native vegetation is usually very dense but had been cleared for access to powerlines.

2.3 DNA sequence data

The selection of markers for this study was based on a thorough review of the relevant literature, with the scale of the study (i.e. primarily inter- and intra-specific) in mind and with the aim to maximise the comparability of results with studies of closely aligned genera (i.e. Schultz *et al.*, 2008; Schultz *et al.*, 2009; Schultz *et al.*, 2007). Molecular markers with different rates of substitution capture signatures of population processes at different times in evolutionary history (Avise *et al.*, 1987; Thomson, Wang & Johnson, 2010) as increasing genetic variability provides greater power to detect recent branching events but reduced power to assess deeper phylogenetic history, due to high rates of substitution overwriting signatures of very old events. The distribution of genetic variation results from a combination of molecular and population-level processes, including mutation, genetic drift, gene flow, dispersal and demography (Wang, 2010).

With the above considerations taken into account, the nuclear large-subunit ribosomal RNA gene (LSU, also known as 28S), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 12S rRNA (12S), part of the highly variable internal-transcribed spacer section of the ribosomal RNA cistron (ITS), the mitochondrial cytochrome c oxidase 1 (COI) gene and the mitochondrial r16S (16S) were chosen as potentially suitable markers to be screened.

Particular mention should be made of the mitochondrial protein-coding COI gene as it has been touted as a potential universal, stand-alone marker for barcoding endeavours (Hebert, Cywinska, Ball & deWaard, 2003; International Barcode of Life http://ibol.org; Barcode of Life Data System, http://www.barcodesystems.org). An issue that has arisen with the use of COI is the presence of multiple, paralogous copies of COI in the genome. These nuclear mitochondrial pseudogenes (commonly referred to as numts) are mitochondrial DNA sequences that have been transferred to the nucleus (Lopez *et al.*, 1994) and have been reported in numerous crayfish studies (Buhay, 2009; Nguyen, Murphy & Austin, 2002; Song, Buhay, Whiting & Crandall, 2008). As a result, for any chromatogram that displayed possible numts the associated sequence was excluded from the data set used in this study.

Each of the markers trialled was successfully sequenced for *Engaewa* specimens, however, not all markers could be included in the analyses presented as the cost of sequencing all of them across a large data set was prohibitive. Therefore, it was decided that ITS and 12S would be excluded. ITS was excluded as it appeared to be evolving in *Engaewa* by insertion and deletion rather than substitution, making it difficult to align and complicating phylogenetic reconstruction methods; a pattern that has also been documented in other taxa (Blaxter, 2004). The 12S gene was excluded as the primers used produced relatively short sequences of approximately 310 base pairs before editing. The details of primers and sequencing conditions for these two markers are still presented below should they prove useful for future studies.

In order to extract DNA from the ethanol preserved crayfish used in this study, either a small incision was made on the ventral surface of the tail and muscle tissue was removed with tweezers, or tweezers were slid along the inside of the carapace and a gill grasped and gently extracted, taking care not to damage the internal or external structures of the crayfish so as not to interfere with later morphological analyses. Total DNA was isolated from the tissue samples using a QIAGEN Blood and Tissue Kit and PCR was used to amplify the sequence of interest using the total genomic DNA as a template, with the primers listed in Table 2.1.

Table 2.1 Primer names and sequences successfully used in this study to produce sequences from *Engaewa* samples.

Marker	Primer	(5'→3')	Reference
16S	1471 1472	CCTGTTTANCAAAAACAT AGATAGAAACCAACCTGG	Crandall, Lawler & Austin (1995) Crandall & Fitzpatrick Jr. (1996)
COI	CR-COI-F CR-COI-R	CWACMAAYCATAAGAYATTGG GCRGANGTRAARTARGCTCG	Cook, Pringle & Hughes (2008)
LSU	LSUF2 LSUR	ACAAGTACCDTRAGGGAAAGTTG TACTAGAAGGTTCGATTAGTC	Sonnenberg, Nolte & Tautz (2007)
GAPDH	G3PCQ157F G3PCQ981R	TGACCCCTTCATTGCTCTTGACTA ATTACACGGGTAGAATAGCCAAACTC	Buhay, Moni, Mann & Crandall (2007)
12S	12S-1F 12S-1R	CTTKAAATTYAAARAATTTGGCGG AGCGACGGGCGATATGTAC	Shull *et al.* (2005)
ITS	ITS4 ITS1L	TCCTCCGCTTATTGATATGC TCCGTAGGTGAACCTGCGGAAGGAT	White, Bruns, Lee & Taylor (1990)

The PCR reaction mixture contained 2μL of template DNA (the concentration of DNA in samples varied widely, however, 2μL worked well for the vast majority of samples regardless), 10μL of HotStarTaq Plus Master Mix (QIAGEN), 1.5μL of both the forward and reverse primers and 10μL of water, to make a total volume of 25μL. The general cycling conditions were an initial denaturing step (94°C for 5 min), 35 cycles of denaturing (94°C for 30 sec), annealing (46°C for 30 sec) and extension (72°C for 45 sec), and a final extension step (72°C for 7 min), with slight variations being used to troubleshoot difficult samples. PCR products were run on a 1.5 % agarose gel containing SybrSafe (Invitrogen), in order to visualise and photograph PCR products. Samples from which a fragment of the correct size was clearly visible were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing, where they were sequenced using ABI BigDye chemistry. A subset of sequences was entered into a BLASTn search in GenBank, to ensure that the correct region of DNA had been amplified and to verify homology with other parastacid sequences.

Chromatograms were checked and edited by comparing the sequence derived from both the forward and reverse primers for each sample in FinchTV v.1.4.0 (www.geospiza.com). The consensus sequences resulting from this process were then aligned using Muscle (Edgar, 2004) as implemented in MEGA5 (Tamura *et al.*, 2011) with the default parameters, and the alignments were revised by eye in an effort to maximise the positional homology and rectify any obvious errors. Sequences for protein coding regions were also translated into amino acid sequences in MEGA5 using the invertebrate mitochondrial code within the program to ensure there were no stop codons present in the reading frame. Coding genes were also tested for saturation in order to check that the phylogenetic signal was not overwhelmed by substitutions using the program Dambe v. 5.3.21 (Xia, 2013). No coding genes were found to have experienced substitution saturation, and no stop codons were found in any genes.

The number of *Engaewa* samples successfully sequenced and the number of sites they are from (shown in parenthesis) for each marker were as follows: 16S - 82 (53); COI - 79 (45); GAPDH - 31 (26); LSU - 65 (50) (specimen details can be found in Appendix 1). The success rate for sequencing samples varied across markers, however,

some specimens repeatedly failed despite many attempts at optimising the procedure; it is unclear why this was the case but it may be related to sample handling prior to DNA extraction (i.e. time between sample collection and adequate preservation, and subsequent storage temperature; Quicke, Lopez-Vaamonde & Belshaw, 1999; Vink *et al.*, 2005). For those analyses where markers were combined, the datasets were reduced according to the number of sequences for the same specimen available across data sets, so that the total number of samples (and number of locations in parenthesis) were 60 (41) for the mtDNA markers combined and 29 (26) for all markers combined. After editing, sequence lengths (number of base pairs) were as follows: 16S - 394; COI - 719; GAPDH - 733; LSU - 949.

2.4 Morphological characters

The morphological characters considered for use in this study included those previously noted by Horwitz and Adams (2000) and Burnham (2005) as being potentially informative for these crayfish (either taxonomically/phylogenetically, or in relation to ecology). One issue with assessing *Engaewa* species morphologically is that many characters tend to develop with size and assessing juvenile specimens is difficult as the characters are often either not yet developed or are too small to be clearly distinguished. This is not surprising as crayfish development is known to be either isometric or allometric (i.e. parts of the body may increase either proportionately or disproportionately to the rest, respectively) and sexual dimorphism of secondary characters may become apparent only upon reaching sexual maturity (Reynolds, 2002).

The issue of scaling and dimorphism is especially pertinent to analyses of the chelae, which have been recognised as possessing highly significant diagnostic characters within *Engaewa* previously (Burnham, 2005; Horwitz & Adams, 2000) and are the major morphological structures discussed in this study. Horwitz (1990) outlined two important characteristics of the chelae of *Engaeus* species, which are also important to *Engaewa* species: structural dimorphism (homochelosity or heterochelosity), and sexual dimorphism. In this thesis (and following the terminology of Horwitz, 1990) homochelosity is where chelae do not differ markedly in their proportions, even though

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their sizes may be different, whereas heterochelosity is where chelae differ in any or all of their proportions, setation, tuberculation and the form of the cutting edges. Heterochelous individuals are referred to as having a 'small dimorphic chela' and 'large dimorphic chela', whereas homochelous individuals are described as having 'isomorphic chelae'. Various forms of sexual dimorphism are found in most species of *Engaeus* (Horwitz, 1990), however, prior to this study no examples of sexual dimorphism were recognised in *Engaewa*.

The morphological characters on the chelae that are utilised as diagnostic characters for delineating species in this study are the pattern of setation and granulations/tubercles (or lack therefore). In addition to these characters on the chelae, the presence or absence of sternal pores, and their shape, are also highly significant in delineating species. These characters will be discussed in detail in Chapter 3, both in terms of how they relate to species delineation (and the phylogenetic significance of this) and also their possible function.

2.5 Distribution and habitat description

When the presence of *Engaewa* was confirmed at a site, positional data were recorded using hand-held GPS or locality descriptions were made GIS-compatible through retrospective georeferencing using Google Maps (www.maps.google.com) and Google Earth (version 4.2.0198.2451 beta). These data were used to produce distribution maps and to calculate the extent of occurrence in ESRI ArcGIS 10.0 in order to assess the conservation status of each species. These distributions greatly overestimate the actual distribution of each species (i.e. the area of occupancy) as they are simply recorded as the minimum polygon size encompassing all recorded sample sites. Whilst this approach may potentially exclude a small number of sites it will undoubtedly include vast tracts of unsuitable habitat.

The characteristics of the habitat occupied by each species were described based primarily on field observations, but also on later analyses using ESRI ArcGIS 10.0 and Google Earth (version 4.2.0198.2451 beta). Data were obtained from Australian

Government Geoscience Australia (www.ga.gov.au), Australian Government Department of Environment (www.environment.gov.au), Bureau of Meteorology (www.bom.gov.au), Australian Soil Resource Information System (www.asris.csiro.au), and Western Australia Department of Parks and Wildlife (WA) (www.dpaw.wa.gov.au), with the specific GIS shapefiles used listed below. These data sources were also used to produce a series of maps throughout this thesis; thus any figures that do not have a source provided in the figure titles were created by the author using these data in ESRI ArcGIS 10.0.

- Geoscience Australia: 2005 National Marine Bioregionalisation of Australia (63466); Vegetation – Pre-European Settlement 1788 (42356); Vegetation – Post-European Settlement 1988 (42357); GEODATA 9 Second Digital Elevation Model (DEM-9S)
- Department of Environment: Interim Biogeographic Regionalisation for Australia (IBRA) Version 7 (Regions)
- Bureau of Meteorology: Geofabric Surface Cartography; Average Annual Rainfall (IDCJCM004)
- Australian Soil Resource Information System: Digital Atlas of Australian Soils (soilAtlas2M)
- Department of Parks and Wildlife: Landscape Conservation Units

There is still much uncertainty regarding the palaeoclimate in SWA, however, the basic outline of temperature and sea-level used in this study follows that provided in Figure 5.1. Reconstructed palaeocoastlines were developed to provide the best estimates of possible high and low sea-level stands, and are incorporated into the biogeographic hypotheses examined. An important caveat of this approach, however, is that using present day contours does not account for the impacts of events such as tectonic uplift and subsidence, tidal scouring or the accumulation of sediments (Voris, 2000). Palaeodrainage networks were modelled using the 2005 National Marine Bioregionalisation of Australia data (63466) (Geoscience Australia). In a similar approach to that adopted by Schultz *et al.* (2008), palaeodrainages were calculated using the Stream Order extension in ESRI ArcGIS 10.0, which uses the dataset to determine flow direction and accumulation and identifies the most likely path for palaeodrainages.

3) SYSTEMATICS OF THE GENUS *ENGAEWA*

3.1 Introduction

Before revising the systematics of *Engaewa* and quantifying the degree and distribution of genetic variation within the genus, it is worthwhile briefly outlining its taxonomic history (both in terms of the type and extent of data reviewed), as this will provide the starting point for this study and highlight where revision is most likely to be needed. The general approach for such studies on other freshwater crayfish taxa shall also be briefly discussed, as this information will justify the approach taken in this study. Firstly however, it is important to clearly define what will be considered to represent a species and how it will be defined for the purposes of this thesis.

The groups of organisms commonly defined as 'species' have become the currency of numerous scientific endeavours and are widely acknowledged as being the basic unit of analysis in biogeography, ecology and conservation biology (Hausdorf, 2011; Sites & Marshall, 2004). Despite the importance of species, a definition of what actually constitutes a species has been contentious (see Hey, 2006; Mayden, 1997). Without a universally accepted definition (Abbott, Ritchie & Hollingsworth, 2008; George & Mayden, 2005) a large body of literature has focused not only on producing a conceptual definition but also a practical methodology (Sites & Marshall, 2004); yet it has been noted that very few taxonomists explicitly state the criteria or evidence that forms the basis of their species concept (Schlick-Steiner *et al.*, 2010). This can have significant impacts, as it has been shown that the application of different species concepts can lead to the recognition of different species boundaries (e.g. Agapow *et al.*, 2004; Wiens & Penkrot, 2002). Therefore, I will explicitly state both the theoretical framework and the operational methodology employed to delimit species (Wiens, 2004a).

De Queiroz (1998) formulated a General Lineage Concept of Species (GLC), which acts to unite all other concepts under a single umbrella and will be the species concept adopted in this thesis. As species are conceptualised as (segments of) separately evolving lineages, any evidence of lineage separation can be evidence for the existence of different species (de Queiroz, 2007). By unifying previously disparate species concepts, the properties emphasised under the alternative concepts each become valid lines of evidence for species delimitation under the GLC and identifying more than one property adds additional lines of evidence and a higher degree of corroboration (de Queiroz, 2007).

In order to increase the rigour of species delineated in this study, and to avoid criticisms levelled at species identification based on a single line of evidence, 'iterative taxonomy' will be employed (Yeates et $al.$, 2011). This involves forming an initial hypothesis of species boundaries based on one data source and using a repeatable protocol (H_0), which is then tested with results from a different dataset with taxon sampling based on H_0 to produce H_1. If H_0 and H_1 concur then the species boundaries have survived testing and iteration ends. If H_0 and H_1 propose different species boundaries then a biological or evolutionary explanation will be sought for the source of discordance and the species boundaries refined accordingly to produce a new hypothesis of species boundaries (H_2). Details of this procedure are outlined in the species delineation methodology (3.2.2).

3.1.1 Taxonomic history of *Engaewa*

When Riek (1967a) initially erected the genus *Engaewa* and described the first three species he did so based on very limited sampling (a total of four sites were recorded, with material examined from three) and a limited number of morphological characters (essentially three characters were used to distinguish between *E. subcoerulea* and *E. reducta*/*E. similis* and a further six characters used to distinguish between *E. reducta* and *E. similis*). This is not an unusual situation for such 'alpha taxonomy' as it has been acknowledged that "in poorly known groups, initial inferences about species boundaries are almost always based on a subset of characters and methods of analysis that by contemporary standards would be judged inadequate, and this problem is often

compounded by inadequate sampling of specimens and localities" (Morando, Avila & Sites Jr, 2003, p. 159). Horwitz and Adams (2000) made significant improvements in the understanding of species boundaries and diversity within the genus, however, they also acknowledged that there remained unaccounted-for diversity – this chapter seeks to address this issue.

It has been widely accepted that *Engaewa* is a monophyletic genus (e.g. Horwitz & Adams, 2000; Riek, 1972; Schultz *et al.*, 2009) despite the morphological characters Riek (1967a) cited when erecting the genus being shared with some members of *Engaeus* (Horwitz, 1990). The morphological similarities between the five genera of the burrowing clade (as defined in 1.1.2) mean that whilst there is actually considerable diversity, there are also numerous overlapping characters. Furthermore, the genera *Engaewa* and *Engaeus* share numerous morphological characters and there is no single morphological character that distinguishes between them (Horwitz, 1990; Horwitz & Adams, 2000). Whilst they may superficially be considered to be morphologically and ecologically similar, *Engaeus* actually has a much wider range of morphologies and occupies a wider range of ecological niches. Horwitz (1990, p. 438) described *Engaeus* as "spanning the range from the so-called land-crabs, with for instance greatly reduced abdomens, antennae and antennules, which occupy type 3 burrows, to species which spend most of their lives in surface waters in type 1a or 1b burrows with morphological features superficially not unlike a yabby in the genus *Cherax*.".

Engaeus laevis (Clark) exhibits characters that blur the distinction between *Engaeus* and *Gramastacus*, with a similar situation occurring between *Engaeus lyelli* Clark and *Tenuibranchiurus* (Horwitz, 1990). As a result of these examples Horwitz (1990, p. 438) suggested "It is quite possible that the existing five generic divisions within this related group may not be the most accurate representation of either their phylogenetic relationships or their morphological characteristics". It appears that this prediction was accurate as, based on molecular data, Schultz *et al*. (2009; 2007) have subsequently suggested *E. lyelli* should be recognised as a new genus, and, based on both molecular and morphological data, the currently recognised genus *Tenuibranchiurus* may actually be composed of two genera (Dawkins, pers. com.).

Horwitz and Adams (2000, p. 677) listed seven morphological characters, which are shared by all members of *Engaewa* (Table 3.1), and cited the commonality of these as evidence for the monophyly of the genus. However, the authors also acknowledged the presence of these seven characters in species from the genera *Engaeus* and *Tenuibranchiurus*, though no single species possessed more than four (Horwitz & Adams, 2000). Horwitz and Adams (2000) also conducted an analysis of 17 allozymes, from which they further deduced that *Engaewa* was monophyletic as the genus exhibited a maximum level of divergence of 44% of fixed differences (FD) in the enzymes reviewed compared to a minimum of 71% FD for the four outgroups (maximum of 88% and average of 73.5% FD).

Riek (1967a) originally recognised only two of the three northern species (*E. reducta* and *E. similis*) on the basis of six morphological characters (Table 3.2). Of these Horwitz and Adams (2000) considered the rostral tip apex, the rostral carinae and the lateral spination of tail fan elements to be useful, though not always consistently expressed. They found the shape of the inner ramus of the uropod to be useful, but variability due to sex and maturity needs to be accounted for; the degree of eye pigmentation reliable only for some populations, not the species as a whole; and the mesial margin of the carpus to be unreliable. *Engaewa pseudoreducta* (which was later described by Horwitz and Adams (2000)), possesses both states for the rostral tip apex, the rostral carina is absent and it has full lateral spination of tail fan elements, with the exception of the telson where it is obsolete. Horwitz and Adams (2000) proposed three additional characters that are useful for delineating the three northern species, namely the setation pattern on the chelae, the terminal spine on the antennal scale and the lateral processes and keel of the sternum (Table 3.3) (a number of the major diagnostic characters can also be seen in the reproductions of Horwitz and Adams (2000) presented in Figure 3.1).

Of the northern species only *E. reducta* and *E. similis* were included in the allozyme analysis of Horwitz and Adams (2000), where it was recorded that these two species exhibited fixed differences at two loci (12% fixed gene differences (FD)). Whilst it has been suggested that 15% FD is needed between allopatric populations to

recognise distinct species (Horwitz, Adams & Baverstock, 1990; Richardson, Baverstock & Adams, 1986), the presence of additional supporting data (in the form of morphological characters) in this case was seen as sufficient support for species status (Horwitz & Adams, 2000). For the two southern species, *E. subcoerulea* is distinguished by a U-shaped cervical groove, the nature of the dorsal edge of the propodus of the chela and pores on the lateral processes of the sternum at the 1st, 2nd, 3rd & 4th pereopod (Horwitz & Adams, 2000), and *E. walpolea* is recognised by a combination of absent rostral carinae, a granulate carina on the ventral edge of the propodus and an anteriorly pointed sternal keel at the lateral processes of the 3rd pereopod (Horwitz & Adams, 2000). Both of these species were also clearly supported by the allozyme data of Horwitz and Adams (2000).

Table 3.1 Morphological characters proposed by Horwitz and Adams (2000) to distinguish members of the genus *Engaewa* from other closely related crayfish species.

Reduced rostral carinae, and absent postorbital carinae.
Fully developed exopodite of the third maxilliped.
Forward extension of second abdominal pleura only partially overlapping first.
Propodal palm smooth laterally, and ventrally carinate.
Supplementary tubercle(s) on cutting edge of propodal finger.
Elongate pore underneath lip of lateral processes of 4th pereopod.
Swollen keel of the sternum between the third and fourth pereopods.

Table 3.2 Morphological characters proposed by Riek (1967a) to distinguish between *Engaewa reducta* and *E. similis*.

Character	*reducta*	*similis*
Rostral tip apex	rounded	pointed
Rostral carinae	distinct anteriorly only	distinct over entire rostrum
Mesial margin of the carpus	convex	straight
Shape of the inner ramus of uropod	rounded	truncate
Lateral spination of tail fan elements	very reduced	full
Degree of eye pigmentation	very reduced	full

Table 3.3 Summary of the review by Horwitz and Adams (2000) of the morphological characters proposed by Riek (1967) to distinguish between the *Engaewa reducta* and *E. similis*, extended to include *pseudoreducta* and additional diagnostic characters.

Character	*reducta*	*similis*	*pseudoreducta*
Rostral tip apex	usually rounded	usually a blunt point	either rounded or with a point
Rostral carinae	usually distinct anteriorly only	usually distinct over entire rostrum	usually absent
Mesial margin of the carpus	not useful for distinguishing between species		
Shape of the inner ramus of uropod	generally rounded but varies according to age and sex	generally truncate but varies according to age and sex	generally rounded but varies according to age and sex
Lateral spination of tail fan elements	generally full spination	generally reduced spination	full spination, except absent or obsolete on the telson
Degree of eye pigmentation	not useful for distinguishing between species		
Setation pattern on the chelae	diagnostic but can be inconsistently expressed, also depends upon age of crayfish and type of chelae		
Terminal spine on the antennal scale	usually small	usually large, although may be lacking entirely	usually small
Lateral processes and keel of the sternum	pore on the lateral process of the 3rd pereopod	no pores	no pores
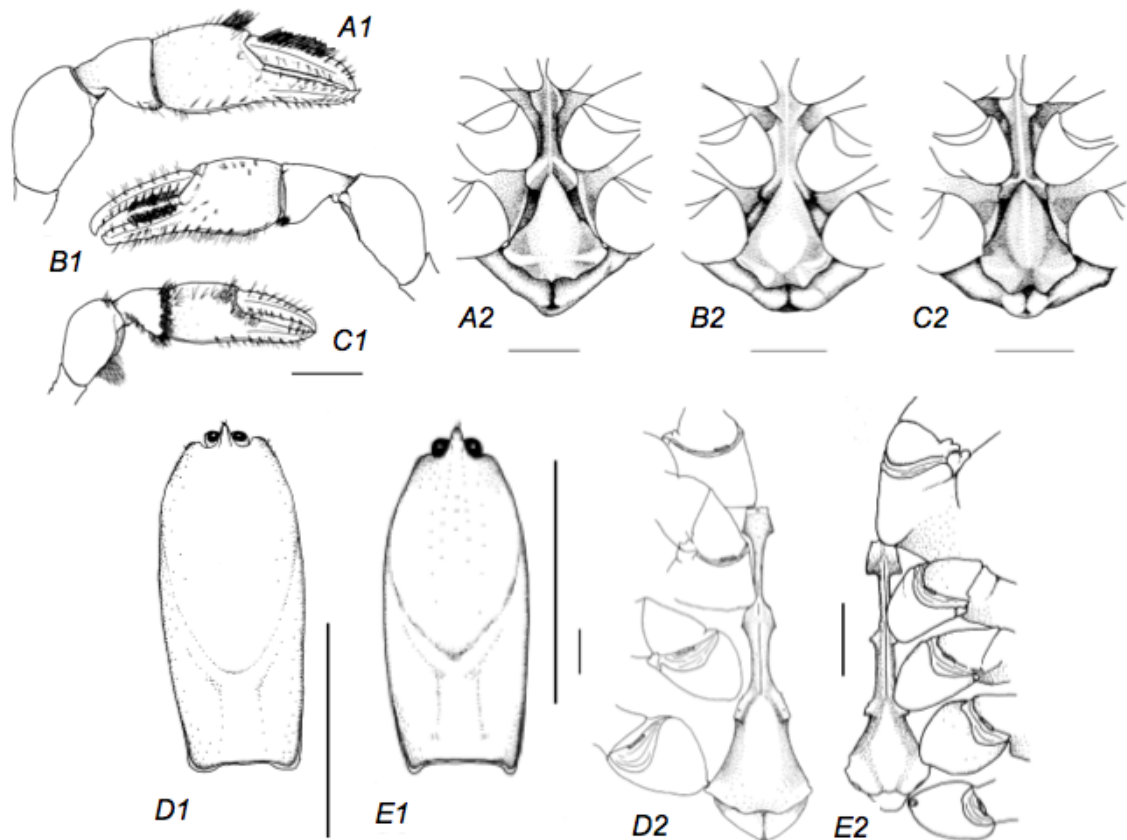
Figure 3.1 Chelae and sternal keel of *Engaewa reducta* (*A1&2*), *Engaewa similis* (*B1&2*) and *Engaewa pseudoreducta* (*C1&2*) (scale bars represent 2 mm for *A-C1* and 5 mm for *A-C2*). Carapace and sternal keel of *Engaewa subcoerulea* (*D1&2*) and *Engaewa walpolea* (*E1&2*) (scale bars represent 10 mm(*D1&E1*), 1 mm(*D2*), 2 mm(*E2*)). Reproduced from Horwitz and Adams (2000).

3.1.2 Crayfish systematics

Mirroring the difficulties of defining and delineating species as previously discussed, crayfish taxonomy has also often been in a state of flux, with different understandings of morphological and habitat variation within freshwater crayfish common. It has been recognised that there can be a high degree of morphological variation in some cases and morphological conservatism in others (Hansen *et al.*, 2001) and, as noted by Austin and Knott (1996), taxonomic characters may be more variable than realised, morphological and habitat differences may not equate with specific distinctions and genetically distinct species may not necessarily be morphologically distinct.

Studies utilising molecular data to examine systematics for freshwater crayfish with morphology that is ambiguous or difficult to interpret (e.g. Austin & Knott, 1996; Campbell, Geddes & Adams, 1994; Horwitz *et al.*, 1990; Zeidler & Adams, 1990) suggest that solely morphologically based taxonomic studies of freshwater crayfish need to be interpreted with caution. For example, Sokol (1988) discussed the possibility of morphological plasticity due to environmental factors in freshwater crayfish (specifically *Cherax destructor* Clark) and illustrated the difficulties in making taxonomic judgements in the absence of additional non-morphological data. Further examples of environmentally induced morphological plasticity have been provided by Austin (1996) and Austin and Knott (1996) who also suggested that species from the genus *Cherax* may display differing morphological phenotypes in relation to varying freshwater habitats. *Cherax crassimanus* Riek, *Cherax quinquecarinatus* Gray and *Cherax preissii* Erichson each utilise an extremely wide range of freshwater habitats, ranging from deeper, permanent rivers to semi-permanent swamps (Austin & Knott, 1996). Austin and Knott (1996) found a direct correlation between this habitat variation and a large component of the morphological variation observed both within and between the currently recognised species. The morphological characters appearing to respond to habitat were made up of a diverse range of traits, including characters such as the length of claw and rostrum, the development of ridges, and the size of the abdomen and head (Austin & Knott, 1996); characters that had previously been considered to be of taxonomic importance (e.g. Riek, 1967a, 1969).

The implication of these insights is that the conventional approach to the taxonomy of freshwater crayfish, where small anatomical differences are assumed to be reliable guides to specific distinctions, both in the Southern Hemisphere (e.g. Clark, 1936, 1941; Hobbs Jr., 1987; Morgan, 1986, 1988; Riek, 1967a, 1967b, 1972; Sumner, 1978; Swain, Richardson & Hortle, 1982) and Northern Hemisphere (Hobbs Jr., 1989 and references therein) crayfish fauna may be flawed and, therefore, so too the existing systematics of freshwater crayfish. Furthermore, the presence of potential morphological plasticity within freshwater crayfish suggests that, where habitat characteristics have been used as supporting information for the delineation of freshwater crayfish (based on an assumption that crayfish species tend to occupy narrow

and distinct habitats) these errors may have been compounded (Austin & Knott, 1996). Clearly the use of such convergent characteristics, interpreted as the result of descent from a common ancestor, will result in the construction of erroneous taxonomies and phylogenies (Fetzner & Crandall, 2002).

Addressing taxonomic and phylogenetic questions via the utilisation of non-morphological characters (e.g. serology and genetics) has a long history in astacological research (e.g. Austin, 1996; Austin & Knott, 1996; Clark & Burnet, 1942; Patak & Baldwin, 1984; Patak, Baldwin & Lake, 1989) and more recently has been acknowledged in playing an important role in conservation biology through ensuring accurate definitions of species boundaries, facilitating detection of cryptic species, and providing boundaries for management units within species (Cataudella *et al.*, 2010). A variety of different DNA regions have been used in crayfish studies and the selection of which one(s) to utilise in a particular study depends largely on the temporal scale of relationships being looked at and the specific aims of the project undertaken. For instance, the circular mitochondrial genome (mtDNA) generally has a higher substitution rate than the nuclear coding genome (nuDNA) and is therefore most suited to exploring shallow (e.g. intra- and inter-specific level) systematic relationships (Avise, 2000).

The vast majority of DNA sequence based studies of crustacean phylogenetic and phylogeographic relations have utilised the mitochondrial r16S and/or subunit I of the cytochrome c oxidase (COI) gene regions (Schubart, 2009). These two markers have been used widely for inferring crayfish relationships in North America (e.g. Barriga-Sosa *et al.*, 2010; Dillman, Wagner & Wood, 2010; Taylor & Hardman, 2002), South America (e.g. Crandall *et al.*, 2000a), Europe (e.g. Cataudella *et al.*, 2010; Grandjean, Frelon-Raimond & Souty-Grosset, 2002; Grandjean, Harris, Souty-Grosset & Crandall, 2000; Pedraza-Lara, Alda, Carranza & Doadrio, 2010), Australia (e.g. Crandall *et al.*, 1999; Dawkins *et al.*, 2010; Gouws, Stewart & Daniels, 2006; Hansen & Richardson, 2006; Ponniah & Hughes, 2006; Schultz *et al.*, 2007; Shull *et al.*, 2005) and New Zealand (e.g. Apte, Smith & Wallis, 2007).

Whilst 16S and COI are by far the most commonly employed markers they are not the only ones used, with published studies also including the mitochondrial markers 12S rRNA (Breinholt, Porter & Crandall, 2012; Buhay & Crandall, 2008; Buhay *et al.*, 2007; Munasinghe, Murphy & Austin, 2003; Shull *et al.*, 2005), adenosine triphosphatase 6 (ATPase 6) (Nguyen & Austin, 2005), and Cytochrome b (Cyt b) (Munasinghe *et al.*, 2003), as well as the nuclear markers glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Buhay *et al.*, 2007; Schultz *et al.*, 2009), Histone H3 (Buhay *et al.*, 2007), the Internal Transcribed Spacer region 2 (ITS2) (Bentley, Schmidt & Hughes, 2010), and 28S rRNA (LSU) (Breinholt *et al.*, 2012; Shull *et al.*, 2005).

In light of the reported issues when using morphological characters as the basis of taxonomy for some crayfish groups, not only will a range of morphological characters be considered in this study, but also multiple nuclear and mitochondrial DNA markers selected from those above. Just as there is no single best data on which to base a systematic revision, there is also no single best method to delineate species. Thus, a range of different methods for delineating species will be considered (as outlined in 3.2.2) to assess whether the various data and methodologies are congruent. The data obtained to undertake the systematic revision have additional value to this study, as the biogeographic analyses are based on the distribution and variety of genetic lineages (not just species) as well as dating of lineage splits.

3.2 Methods

3.2.1 Phylogenetic reconstructions

The samples included in this study span the known distribution range of *Engaewa* and include representatives of all currently described species. Throughout the analyses presented, individual specimens will be referred to by their specimen codes; these codes are composed of three letters and/or numbers that act as a site identifier (e.g. TT2), followed by a two-digit collection number (e.g. TT201). The codes, details of the collection site and species status for each specimen are provided in Appendix 1. This approach was deliberately adopted so as to avoid attempting to place specimens into a geographic or species context until after the completion of the systematic revision. In

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order to facilitate the evaluation and discussion of phylogenetic relationships and the taxonomic implications, identifiable lineages have been colour-coded on the trees produced.

Crayfish from the genera *Engaeus*, *Tenuibranchiurus*, *Geocharax* and *Gramastacus* were selected as outgroups for most analyses, as they appear to be the most closely related to *Engaewa* based on the phylogenies of Australian freshwater crayfish as presented in Schultz *et al.* (2009) and Toon *et al.* (2010). However, the relationships between *Engaewa* and these genera, and their monophyly, were also tested. Furthermore, the assumption of a sister relationship between *Engaewa* and *Engaeus* (based on the aforementioned phylogenies) was tested, as any grouping found whereby they do not form a clade to the exclusion of all other taxa will refute this hypothesis.

Six datasets were constructed for phylogenetic analyses (the specific samples used for each can be found in Appendix 1):

- 16S mitochondrial DNA sequences (16S);
- COI mitochondrial DNA sequences (COI);
- GAPDH nuclear DNA sequences (GAPDH);
- LSU nuclear DNA sequences (LSU);
- Combined mitochondrial DNA sequences (mtC);
- All sequences combined (allC).

Additional datasets were also constructed by adding sequences retrieved from Genbank (see Appendix 2 for details), in order to re-evaluate the findings of other studies once the *Engaewa* dataset from this study was included. These datasets were:

- Combined 16S and GAPDH, with data from Schultz *et al.* (2009) (16S_GAP);
- 16S, with additional sequences retrieved from GenBank (16S_GB).

jModelTest ver. 3.7 (Posada & Crandall, 1998) was run for each of the above data sets, to identify the models of nucleotide substitution (i.e. evolutionary model) that best fit the data, as assessed by the Akaike Information Criterion (Akaike, 1974) and including all of the models offered by the program (Table 3.4). Prior to performing tree-

based analyses, all but one representative of each haplotype was filtered from the alignment using Collapse ver. 1.2 (Posada, 2004) with the filtered taxa added to the trees after performing the analyses. All phylogenetic trees produced were visualised with Figtree ver. 1.4.0 (Rambaut, 2012).

Table 3.4 Model selected by AIC in jModelTest and the associated settings for each model for the eight non-partitioned datasets analysed in the phylogenetic analyses.

Dataset	16S	COI	GAPDH	LSU	mtC	allC	16S_GAP	16S_GB
Model	GTR+G	TrN+I+G	TPM2+G	TIM2+G	TIM1+I+G	TIM+I+G	TIM3+I+G	TrN+I+G

The outgroup criterion was used to root all phylogenies, with outgroups chosen based on previous hypotheses of parastacid phylogeny (as described above). All phylogenetic trees were rooted by incorporating outgroup sequences from species within the genera *Engaeus*, *Tenuibranchiurus*, *Geocharax* and *Gramastacus* except for 16S_GAP, which was rooted with outgroup sequences from the more distantly related genera *Euastacus* and *Paranephrops*, as this dataset was used to explore relationships between *Engaewa* and the genera used as outgroups for the other phylogenies.

Whether multiple lines of evidence should be combined before phylogenetic reconstruction or analysed separately and combined *a posteriori* is at the heart of the total evidence debate (*sensu* Kluge (1989) in a phylogenetic context – and referring to the earlier coining of the term by Carnap (1950)). The two competing approaches, total evidence versus consensus, can be seen as representing character congruence and taxonomic congruence, respectively (*sensu* Mickevich (1978)). Supporters of character congruence claim that all data should be combined for phylogenetic analysis (e.g. Kluge, 1989, 1998; Kluge & Wolf, 1993), whereas proponents of taxonomic congruence insist that independent datasets should be analysed separately and combined by means of consensus techniques *a posteriori* (e.g. Bull *et al.*, 1993; Miyamoto & Fitch, 1995; Swofford, 1991). A third alternative in this debate, and the one accepted in this thesis, is an intermediate solution where the decision of whether to combine data is based on the results of statistical heterogeneity tests (e.g. Farris, Källersjö, Kluge & Bult, 1995; Huelsenbeck & Bull, 1996; Mickevich & Farris, 1981; Rodrigo, Kelly-Borges, Bergquist & Bergquist, 1993).

An Incongruence Length Difference (also known as a partition-homogeneity) test (Farris, Källersjö, Kluge & Bult, 1994) was performed for the combined data sets (mtC, allC and 16S_GAP) using 100 replicates of 1000 addition sequence replicates, as implemented in PAUP* ver. 4.10b (Swofford, 2002), in order to examine whether they could be combined into larger data matrices. The results of the partition-homogeneity tests were not significant indicating no conflict between the different markers ($P = 0.36$, $P = 0.97$, $P = 0.21$ for mtC, allC and 16S_GAP respectively), so they were each combined and analysed to produce a single phylogeny per dataset. Depending on the specific analysis, the combined datasets were analysed either with the model of evolution partitioned by gene, or with a single model of evolution for the concatenated genes. When partitioned, the dataset had a lower case 'p' attached to its identifying label to indicate partitioning (e.g. mtCp, for the combined mitochondrial dataset partitioned by gene). Phylogenetic incongruence between markers was also further examined by analysing datasets separately and comparing nodal support values on a node-by-node basis, as comparing the estimated measures of support (bootstrap (BS) and posterior probability (PP) values) can detect conflicts between their topologies based on highly supported clades (de Queiroz, 1993; Mason-Gamer & Kellogg, 1996; Wiens, 1998).

Maximum-Likelihood (ML) analyses were performed both using PhyML ver. 2.4.4 (Guindon & Gascuel, 2003) and RAxML (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008) at the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010) in order to test whether the two programs would produce the same topology and comparable support values[*]. The PhyML analyses used the best substitution model selected by jModeltest ver. 3.7 (Posada, 2008) under the Akaike Information Criterion (Akaike, 1974) with support for nodes assessed by non-parametric bootstrap (Felsenstein, 1985) with 1000 bootstrap replicates. RAxML uses only variations of the General Time Reversible (GTR) model of nucleotide substitution, as the author (Stamatakis, 2006) believes that GTR is the most common and general model for DNA

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[*] As noted in the RAxML manual likelihood values cannot be directly compared to likelihood values of other ML programs, however, the likelihood values obtained by other programs are expected to be similar. The purpose of comparison here is simply to ensure that there are no major conflicts, rather than attempting to rigorously compare the outcomes produced by different programs, data partitions, models of substitution etc.

analysis and using a simpler model would only make sense with respect to the computational cost. RAxML, therefore, is designed to efficiently implement and optimize GTR instead of offering a plethora of distinct models, which are only special cases of GTR but are programmed in a generic and thus inefficient way (Stamatakis, 2006). Programs such as jModeltest will propose the usage of a simpler model if the likelihood of a fixed topology under that simpler model is not significantly worse than that obtained by GTR based on a likelihood ratio test.

Parameters were estimated for each gene independently and support for nodes of the resulting ML trees was assessed by non-parametric bootstrap (Felsenstein, 1985) allowing RAxML to determine the optimum number of bootstrap replicates. For combined datasets the ML analyses were performed both as a single analysis and as partitioned datasets in RAxML, but only as a single analysis in PhyML as it does not currently allow for partitioned data sets. For all ML analyses, topologies with BS values >70% were considered to be highly supported, and those with values between 50% and 70% were considered to be weakly supported (Huelsenbeck & Hillis, 1993).

Bayesian analyses (BA) were performed using MrBayes ver. 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). All Bayesian analyses were started from a random tree, with flat default priors. Analyses were run with two searches for a minimum of 1 million generations using four Markov chains (one cold, three hot) for each search, sampling every 1000 generations. Initially the temperature was left at the default value of 0.2, however, if convergence was not reached and acceptance rates of the Metropolis proposals were lower than recommended (10–70% with low acceptance rates for the swaps between adjacent Markov chains) the temperature setting was decreased, following the recommendations of the MrBayes ver. 3.1 User Manual (Ronquist, Huelsenbeck & van der Mark, 2005). Model parameters were optimised, based on the results of jModelTest, using the Lset and Pset options, while for partitioned datasets all other parameters were unlinked across partitions using the 'unlink' command (transition/transversion rate ratio (tratio), substitution rates (revmat), character state frequencies (statefreq), gamma shape parameter (shape) and proportion of invariable sites (pinvar)). Runs were stopped only after the standard deviation of split

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frequencies fell below 0.01, and ensuring there was an effective sample size (ESS) >200 for all parameters and a potential scale reduction factor (PSRF) near to 1. Plots of log-likelihoods were examined graphically, using Tracer ver. 1.3 (Rambaut & Drummond, 2003) to ensure that they reached stationarity, and the first 25% of samples were discarded as burnin. The remaining trees were used to create a consensus tree and to estimate Bayesian posterior probabilities. Bayesian posterior probabilities greater than 0.95 were considered significant support for a clade (Huelsenbeck & Rannala, 2004). The same analysis was performed at least twice to verify topological convergence and homogeneity of posterior clade probabilities between runs (Huelsenbeck, Larget, Miller & Ronquist, 2002).

The rate and timing of lineage splitting events may be of interest to the field of phylogenetics. Fluctuations in the rate of diversification within a phylogeny can be visualised by plotting the number of lineages through time (Nee, Mooers & Harvey, 1992). To test for evidence of an explosive radiation at any stage within the *Engaewa* clade, a lineage through time (LTT) plot was produced and the constant rates test (CR or sometimes also called the MCCR) of Pybus and Harvey (2000) was used to test for a significant deviation in the rate of clade formation. The first step was to make an ultrametric tree from the combined marker phylogenetic tree (allC) with the program PATHd8 (Britton *et al.*, 2007). This ultrametric tree, with branch lengths proportional to time, was read in Ape (Paradis, Claude & Strimmer, 2004) and the ladderise function used to order the branches to produce a ladderised tree. Where there were polytomies they were randomly resolved in Ape. To calculate the gamma test statistic the gammaStat function in Ape was used and the LTT plot produced. The gamma statistic (Pybus & Harvey, 2000) describes the average distance of weighting times from the midpoint of the tree. Negative gamma values indicate that almost all branching events have occurred earlier in the tree and positive gamma values indicate that splits have tended to occur closer to the tips of the tree. The obtained gamma statistics were tested in Ape with one-tailed tests, to see if they were significantly different from constant rates of clade diversification.

Phylogenetic analyses were undertaken on the 16S_GB dataset via TREE-PUZZLE (Schmidt, Strimmer, Vingron & von Haeseler, 2002), which was used to conduct quartet puzzling, via a three-step procedure. Firstly, maximum-likelihood trees were reconstructed for all possible quartets (maximum-likelihood step). Next, the quartet trees were combined to create an overall tree, by sequentially adding sequences in random order to an already-existing subtree with the position of a new sequence determined by a voting procedure (puzzling step). Once an intermediate tree relating all sequences was obtained, the puzzling step was repeated several times, thereby elucidating the landscape of possible optimal trees (Strimmer & von Haeseler, 1996). These data were then visualised via likelihood mapping (MLM). MLM places the outcome of each quartet into one of the 'seven basins of attraction', which provides a simple representation of the percentage of fully resolved trees, net-like regions (where two alternative tress cannot be selected between) and star phylogenies, uncovered in the quartet puzzling step (Figure 3.2) (Strimmer & von Haeseler, 1997). MLM thereby provides a simple means of visualising a summary of phylogenetic content of a dataset, along with the relative frequencies of the likelihoods for each topology represented as one point inside an equilateral triangle.
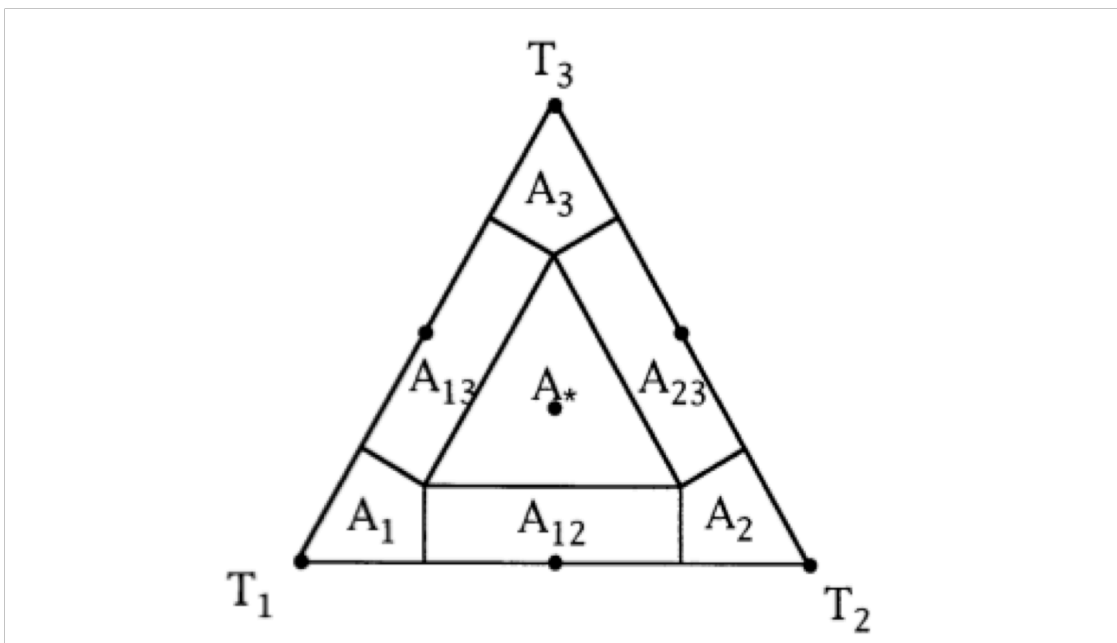
Figure 3.2 A representation of the seven basins of attraction in likelihood mapping. The dots indicate the corresponding seven attractors. A_1, A_2, A_3 show the tree-like regions. A_{12}, A_{13}, A_{23} represent the net-like regions and A_* displays the star-like area. Reproduced from Strimmer and von Haesler (1997).

The results of the quartet puzzling were displayed via likelihood maps, in order to visualise the phylogenetic content of the various markers and to test assumptions of monophyly and sister group relationships. In order to conduct the analysis, likelihood mapping was selected in TREE-PUZZLE with the sequences grouped into four clusters (corresponding to one for each of *Engaewa*, *Engaeus sensu stricto*, *Engaeus lyelli* and the rest of the burrowing clade (i.e. *Tenuibranchiurus*, *Geocharax* and *Gramastacus*)). Ten thousand quartets were constructed and all codon positions were selected. Based on the outcome of jModelTest, the TN model (Tamura & Nei, 1993) was employed and a gamma distributed model of rate heterogeneity with four gamma rate categories and the gamma distribution parameter alpha estimated from the data set. TREE-PUZZLE estimated the transition/transversion parameter and nucleotide frequencies directly from the data set.

3.2.2 Species delineation

Multilocus data and a range of methods have been used on the assumption that if consistent delineation of species across these data and methods occurs it provides stronger support for the results (Niemiller, Near & Fitzpatrick, 2012). The form of iterative taxonomy adopted in this study was conducted via five steps:

1) A *prima facie* (H_0) estimate of species boundaries was based on one data source using a repeatable protocol.

2) These boundaries were tested with results from a different dataset with taxon sampling based on H_0, to produce H_1.

3) If H_0 and H_1 are the same, the species boundaries proposed will have survived testing and iteration will end, with these becoming the currently accepted species designations.

4) If H_0 and H_1 propose different species boundaries, biological or evolutionary explanations will be sought for the source of discordance.

5) Based on the results of step 4, species boundaries will be suggested to produce a new hypothesis of species boundaries, H_2. The H_2 species boundaries will be accepted and iteration will end within this thesis. However, it will be suggested that efforts be made in the future to add additional data in the hope of deriving both a H_0 and H_1 that are in agreement.

Following the protocol described above to produce H_0, the first step was to identify supported monophyletic clades from the phylogenetic trees. H_0 was then tested by seeking diagnostic morphological characters (with taxon sampling based on H_0) to produce H_1. The morphological characters considered as being potentially informative included those outlined in the morphological data acquisition section of the previous chapter (section 2.4). If H_0 and H_1 propose the same boundaries these will be accepted, if not H_2 will be formulated. The final hypothesis of species boundaries is presented as a phylogenetic tree, which is considered to be the 'accepted tree' in this study (i.e. the most highly supported topology based on the maximum amount of information available, and believed to represent the best possible estimate of the true phylogenetic relationships within the genus; presented in 3.3.2). Any lineages that could be attributed to previously described species (on the basis of the identifying characteristics listed by Horwitz and Adams (2000)) have the species name attached and previously undescribed monophyletic groups of crayfish identified in this study are termed *clades* with a letter attached as a unique identifier in the text and figures (e.g. *E. clade A*).

Whilst the approach being employed in this study to delineate species only requires a single method to produce H_0 (i.e. monophyletic groupings from the phylogenetic trees) and a second method to produce H_1 (morphology), additional methods of delineation using the genetic data were compared to see whether these different methods recognise the same species boundaries based on the same data. Therefore, further clarification of species boundaries was sought via seeking a difference in inter- and intra-species genetic distances (akin to a barcoding gap), AMOVA and haplotype networks.

Mean net sequence divergences within and between species groups were calculated for both 16S and COI using Maximum Composite Likelihood with 1000 bootstrap replicates in MEGA5 (Tamura *et al.*, 2011). However, the suggestion by Hebert, Stoeckle, Zemlak and Francis (2004b) that a standard threshold to recognise species level divergence be used was not followed, rather any distinction, regardless of size, was considered as potential evidence of species level groupings. It has been argued that the divergence-threshold method lacks strong biological support and is unsuitable

as a universal criterion for animal species delineation (Hickerson, Meyer & Moritz, 2006; Meyer & Paulay, 2005; Wiemers & Fiedler, 2007). The justification for this is that there has been shown to be vastly different rates of divergence for different markers and across different taxa (Arbogast *et al.*, 2002).

COI and 16S variation within and among clusters of sequences were analysed using analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) as implemented in ARLEQUIN v.3.11. This can be used as an approach to delineate taxa based on population genetics analyses, as it is possible to interpret the AMOVA results used to calculate intra- versus inter-cluster variation in a way analogous to F-statistics (Wright, 1978). An AMOVA approach employing the Tamura-Nei model (which estimates the differential rates of transitional substitution between purines and between pyrimidines and the proportion of transversional differences (Tamura & Nei, 1993)) was used to compute F_{CT} (the amount of variation among groups relative to the total variance based on haplotype frequency) and Φ_{CT} (the amount of variation among groups relative to the total variance based on haplotype frequency and genetic divergence) for various groupings. Monaghan, Balke, Gregory and Vogler (2005) suggested a F_{CT} value >0.95 can represent evidence for accurate species grouping (meaning that >95% of the total genetic variation in the dataset arises from differences among groups). This suggestion was tested via repeating the AMOVA process at different hierarchical levels and with different groupings (e.g. haplotypes, populations, and various groupings of the clades identified in the phylogenetic tree (characterised by their general geographic distribution)). Levels of significance of statistics characterising variation at different hierarchical levels was assessed through 10,000 permutations.

In a somewhat similar approach to the F_{CT} method of delineation some authors (particularly those studying taxa in marine systems) have argued that distinct haplotype networks can be interpreted as species groups (e.g. Addison & Hart, 2005; Baratti, Goti & Messana, 2005; Jolly *et al.*, 2005; Tarjuelo *et al.*, 2004). Haplotype networks represent the reticulate structure of gene flow within species, as opposed to the hierarchical structure between higher taxon levels. Hart, Keever, Dartnall and Byrne (2006) suggest that the connection of haplotype networks represents the extent of

lineage sorting, whereby multiple networks suggest multiple species that have been separated long enough to become distinct lineages. Thus this may provide a simple and objective method of using molecular data to draw species boundaries based on 95% connection limits in parsimony haplotype networks (Hart *et al.*, 2006). Furthermore, assigning sequences to either tips or interior nodes within the network is also informative as interior nodes are assumed to be older, and therefore ancestral, to the tip haplotypes (Posada & Crandall, 2001). Haplotype network construction was achieved by constructing a parsimony network with TCS 1.21 (Clement, Posada & Crandall, 2000) initially using a 0.95 limit and gaps as missing data, however, due to the fact that very deep divergences were found between most unique haplotypes, limits to parsimony were relaxed to 0.90. TCS collapses sequences into haplotypes and calculates frequencies of shared haplotypes and uses these to construct networks based on the probability of parsimony for pairwise comparisons of haplotypes until the probability exceeds the specified cut-off value (Clement *et al*., 2000).

3.2.3 Divergence dating

If sequences evolve in a clocklike manner, genetic distances between taxa can be used to approximate dates for nodes of the inferred phylogenetic trees, however, the concept of a universal, strict molecular clock has fallen out of favour (Ayala, 1997; Bromham & Penny, 2003; Kumar, 2005; Li, 1993) as it is now widely recognised that nucleotide and amino acid substitutions do not generally accumulate at a constant and universal rate (Duffy, Shackelton & Holmes, 2008; Smith & Donoghue, 2008; Thomas, Welch, Lanfear & Bromham, 2010). Errors arise due to rate heterogeneity at three levels: taxa (Yang, Goldman & Friday, 1994); loci (Swofford, Olsen, Waddell & Hillis, 1996); and nucleotide sites within a locus (Nei & Li, 1979). Therefore, a likelihood ratio test (LRT; Huelsenbeck & Crandall, 1997) was used to assess whether clocklike evolution could be assumed for sequences used in this study.

The LRT assesses the statistical significance between the log-likelihood of trees calculated with different assumptions; in this case, the test was used to compare between enforcing a molecular clock or not. If twice the difference between the likelihoods is not significant, it may indicate that the dataset tested is evolving in a

clocklike manner; i.e. (LR = $2(\ln L_1 - \ln L_0)$), where $\ln L_0$ is the maximised log likelihood of the null hypothesis (i.e., the clocklike tree) and $\ln L_1$ of the alternative hypothesis (i.e., the non-clocklike tree)). The LRT test statistic was calculated based on unconstrained and clock-enforced phylogenies via two separate maximum likelihood analyses conducted in PAUP* using the model settings as selected in jModeltest (Posada & Crandall, 1998). TREE-PUZZLE also was used to reconfirm the clocklike or non-clocklike nature of evolution in the datasets, first tested via the LRT as employed in PAUP. As both the LRT and TREE-PUZZLE rejected the null hypothesis of clocklike sequence evolution for the dataset, alternative methods were needed in order to estimate nodal dates.

To account for the lack of a strict molecular clock, relaxed molecular clock models (introduced by Sanderson (1997, 2002) and Thorne, Kishino and Painter (1998)), have been developed, which lie on a continuum between strict-clock inference models (assumes a constant evolutionary rate), and time-free inference models (do not incorporate evolutionary rates). Relaxed clock models employed via Bayesian inference (as implemented in the software MULTIDIVTIME (Thorne & Kishino, 2003) or BEAST (Drummond & Rambaut, 2007) for instance) can provide a powerful alternative to calculations made under local-clock or no-clock models (Drummond, Ho, Phillips & Rambaut, 2006; Pulquerio & Nichols, 2007; Pybus, 2006).

Using MULTIDIVTIME (the approach of Toon *et al.* (2010)) requires calibration points, based on either fossil or geological evidence, and it has been shown that the number and distribution of the calibration points throughout the tree is vital for accurate estimation (e.g. Porter *et al.*, 2005; Thorne & Kishino, 2002; Yang & Yoder, 2003). There are no obvious calibration points available within *Engaewa*, as there are no fossils and geological data are seemingly too coarse, both spatially and temporally. Although it has been argued that the formation of the Nullabor Plain may provide an absolute lower boundary for splits within moisture dependent taxa dispersed across eastern and western Australia (e.g. Roberts & Maxson, 1985b; Unmack, 2001), it alone is unlikely to provide any reasonable confidence estimates for the split between *Engaewa* and *Engaeus*, let alone within *Engaewa*.

Thus, the program *BEAST was used to date nodes within the phylogeny presented in this study. *BEAST, an extension of the program BEAST (as used by Schultz *et al.* (2009)), combines previous methods in order to jointly infer both gene and species tree topologies, divergence times, and population sizes (Heled & Drummond, 2010). Using a relaxed or strict molecular clock, the roots of the individual gene trees are estimated and combined using the multispecies coalescent (rather than concatenation as in BEAST) to estimate the species tree root (Heled & Drummond, 2010). Using the lineages identified in this study (i.e. in 3.3.2), a species tree was created from the combined 16S and GAPDH dataset using *BEAST ver. 1.7.5 (Heled & Drummond, 2010). An uncorrelated lognormal relaxed clock model was enforced for both genes. Uninformative uniform priors (0-100) were used on the ucld.mean for the nuclear gene (GAPDH), and an informed uniform prior for the ucld.mean for 16S (0.00265-0.0045, mean=0.00325). The informed prior was determined using reported substitution rates for similar organisms, and represent the range of different values stated in the literature for 16S (0.53-0.90%) (e.g. Schubart, Diesel & Hedges, 1998; Stillman & Reeb, 2001; Sturmbauer, Leninton & Christy, 1996). Both ucld.stdev were adjusted to represent a plausible distribution (exponential, initial value=2, mean=0.5) and the ploidy type (nuclear or mitochondrial) specified.

Two runs were performed from random starting trees and were combined (using Logcombiner (Drummond & Rambaut, 2007)) to give a total of 200 million generations, sampling every 1000 generations. An 'empty alignment' was also run (i.e. without nucleotide data, using only the set priors), to examine the influence of the assigned priors on the parameters. All runs were checked for convergence, the ESS values examined (>200 was considered appropriate), and the burnin determined using the program Tracer ver. 1.5 (Rambaut & Drummond, 2007). The post-burnin trees were annotated using TreeAnnotator ver. 1.7.5 (Drummond & Rambaut, 2007) and visualised using Figtree. The final species tree produced by *BEAST also provides an estimate of divergence dates between nodes, based on the rates of evolution entered for 16S (and estimated by the program for GAPDH). These dates, and the associated 95% HPD values (akin to confidence intervals) were displayed, again using Figtree.

3.2.4 Analyses of genetic diversity

It is important to note before describing the genetic diversity found at the population level in *Engaewa*, that in order to correctly represent the demographic history of populations it is necessary to accurately identify groups of samples that do, in fact, represent populations (Fazalova *et al.*, 2010); yet this seemingly simple task has proved difficult to achieve for these crayfish. The characteristics of suitable habitat patches are only loosely defined and the conditions that may promote dispersal between habitat patches are yet to be defined. Furthermore, the dispersal capabilities of individuals in the genus have not been documented, although they can be reasonably assumed to be limited (based on their burrowing habit and the associated morphological specialisation). These issues make understanding what represents a population for *Engaewa* species difficult. Thus, 'populations' have been determined for this study as being represented by specimens collected from a single 'site' – with a site being defined as the areal extent of connected habitat as determined whilst undertaking sampling. Therefore, it is entirely possible that some collections of animals from different sites should actually be grouped together into a more accurate biological population. Furthermore, on a number of occasions multiple specimens were collected from a very small area within a single site, raising the question of whether they may have been closely related individuals (i.e. siblings in a shared burrow), which potentially could result in an underestimate of diversity.

Due to practical constraints collecting specimens, and the conservation concern regarding the species in the genus, it was evident that opportunities to undertake population level analyses were limited. Despite this, a number of analyses of genetic diversity were performed on the 16S and/or COI data sets, as they represented the most complete data available and provide direct comparisons to other studies that present data for a range of parastacids. All tests were performed using DnaSP ver. 5.0 (Librado & Rozas, 2009) unless otherwise stated. Firstly basic summary statistics including the number of polymorphic sites (s), haplotype diversity (h), nucleotide diversity (π), average number of pairwise differences (k), and number of unique haplotypes (# hap) were calculated for both 16S and COI, as these diversity measures do not depend on

sample size and are, therefore, particularly appropriate for this type of study (Nei, 1987; Nei & Kumar, 2000; Nei & Li, 1979).

The non-synonymous/synonymous rate ratio ($\omega=d_N:d_S$) was calculated for COI as it is an important indicator of selective pressure at the protein level, with $\omega=1$ representing neutral mutations, $\omega<1$ purifying selection, and $\omega>1$ diversifying positive selection (Yang, Nielsen, Goldman & Pedersen, 2000). This ratio is a powerful test of the neutral model of evolution as it requires few assumptions, however, it does require a rather strong signal in order to detect selection (Yang & Bielawski, 2000). Next, a combination of methods was used to allow for the independent evaluation of inferences tied to population growth by statistical tests based on different assumptions. All of the following tests were performed on all samples pooled together, and on *E. reducta, E. similis, E. subcoerulea* and *E. walpolea* separately, but not the other species/clades as they had few samples.

A mismatch analysis was performed on both 16S and COI. This analysis plots the distribution of the observed and expected number of differences between pairs of sequences, which will appear smooth and often unimodal where there has been population expansion, while stable population sizes will produce ragged and often multi-modal distributions (Rogers *et al.*, 1996; Rogers & Harpending, 1992). The initial and final θ were set to 0 and 9999999 respectively, which allows DnaSP to estimate the appropriate values for an expansion-decline model. The date of growth or decline measured in units of mutational time was defined as $\tau = 2\mu t$ with μ being the divergence rate per sequence per generation and t the time in generations. As an approximation of the divergence rate, the averages of two reported crustacean 16S rates (0.53% – 0.9% MYR (Stillman & Reeb, 2001; Sturmbauer *et al.*, 1996)) and COI rates (1.4% – 2.6% MYR (Knowlton & Weigt, 1998; Schneider-Broussard, Felder, Chlan & Neigel, 1998)) were used and the generation time was defined as two years, based on a generalisation for freshwater crayfish species (Hobbs Jr., 1991).

The COI dataset was used to calculate current genetic diversity ($\theta\pi$) (Tajima, 1983) (computed by pairwise differences between sequences) and historical-based genetic diversity (θ_W) (Watterson, 1975) (based on the number of segregating sites). The comparison of these represents Tajima's D and gives an indication of changes in genetic diversity, with recent losses of diversity (e.g. through selective sweeps or population bottlenecks) typically represented by $\theta\pi<\theta_W$, whereas recent increases in genetic diversity (e.g. through population growth) show $\theta\pi>\theta_W$. Tajima's D should be near zero if population sizes have been stable. An excess of singletons will produce negative values and can result from either population growth (as this tends to produce a coalescent with long pendant edges (star-shaped genealogy)), or as a result of deleterious mutations producing very low frequency alleles (Durrett, 2008). Positive values usually result from either population isolation delaying deepest coalescence, or balancing selection producing an excess of heterozygotes (Durrett, 2008). Therefore, significantly negative values of Tajima's D are often thought to be evidence of expanding populations and significant positive values of recently contracted populations (Tajima, 1989).

Further to Tajima's D, the neutrality tests Fu's F_S (Fu, 1997), R_2 (Ramos-Onsins & Rozas, 2002), and Fu and Li's F* and D* (Fu & Li, 1993) were also calculated. These neutrality tests were used to detect departures from the mutation-drift equilibrium. Fu's F_S is especially sensitive to an excess of low-frequency polymorphisms (rare haplotypes) (Fu, 1997; Ramos-Onsins & Rozas, 2002), for which it takes on low or negative values. A significant negative departure of F_S from zero is often taken as evidence of recent demographic expansions or population bottlenecks in situations where no selective advantage among haplotypes exists (Fu, 1997; Rogers & Harpending, 1992). R_2 uses information from segregating sites, and is more powerful for small numbers of segregating sites (Ramos-Onsins & Rozas, 2002). Non-significant values of F* and D* in combination with significant F_S is indicative of recent population growth or range expansion (Fu, 1997). These tests were calculated using both the total number of mutations (η) and the number of segregating sites (S). Coalescent simulations were performed, using 1000 replicates, for the above analyses to further test if any of the values obtained were statistically significant. These tests have

been shown empirically to be the most powerful methods for detecting recent rapid population growth (Ramos-Onsins & Rozas, 2002).

3.2.5 Geographic mapping of clade boundaries

Haplotype networks were produced via the parsimony method for species delineation (described in 3.2.2) but also via a median-joining (MJ) approach (Bandelt, Forster & Rohl, 1999) to further explore the distribution of genetic diversity within *E. similis*. DNA haplotype sequences of closely related taxa can be joined into a network, based on mutational steps, producing a form of gene tree. This gene tree can then be placed in a geographical context to explore the pattern and process of diversification (Avise *et al.*, 1987). As with phylogenetic tree reconstructions there is also no single best method for accurately recreating haplotype networks and different approaches may produce different results (Cassens *et al.*, 2003). Whilst for many datasets statistical parsimony and MJ approaches produce similar results, under the conditions of many missing node haplotypes (i.e. the haplotypes in the analyses are relatively distantly related) MJ generates significantly less errors (Cassens, Mardulyn & Milinkovitch, 2005). Thus, the MJ method is also used here as it has been noted as being particularly applicable in situations where genetic distances are too large for statistical parsimony (Trontelj, Machino & Sket, 2005); as is the case with the data from this study.

The program Network ver. 4.6.1.1 (www.fluxus-engineering.com) was used to infer the most parsimonious solution ('Steiner trees') of the median joining network. Networks take the form of reticulate graphs, where ambiguities in the data resulting from factors such as homoplasious character change create loops, which are indicative of alternative genealogical pathways (Cassens *et al.*, 2005). Numerous population level phenomena act to create reticulate genealogical relationships, such as recombination, gene conversion, lineage sorting, deep coalescence, etc. (Posada & Crandall, 2001). Whereas strict consensus trees provide only one possible topology, it is actually a less resolved representation of the data, and networks can therefore convey more information; however, highly complex networks containing all possible solutions become difficult to interpret due to multidimensionality and high numbers of inferred missing haplotypes. Four different options were trialled in Network to see if they

affected the outcome. Firstly MJ was completed using two alternate distance calculation methods (Connection Cost and Greedy FHP) with default settings. Next (for the Connection Cost method only) the parameter ε (a weighted genetic distance measure) was adjusted from the default value of 0 to 10 to test its effect on the network produced. If epsilon is set low in theory it may not produce a full median network, however, increasing epsilon greatly increases the computing time and the complexity of the network produced. Despite this, the Network manual suggests that a value of 0 or 10 usually produces a good network (Fluxus Technology, 2012). Finally the Reduced Median (RM) algorithm was used to again see whether it would change the network produced. A reduced median network uses only binary data (multistate nucleotide positions are excluded) and can be used to improve the clarity of large data sets or as a means of validating a MJ network (Fluxus Technology, 2012).

3.3 Results

3.3.1 Phylogenetic reconstructions

The ML tree utilising the combined 16S and GAPDH dataset and including the sequences used in Schultz *et al.* (2009) strongly supports a bifurcation at the node separating the lineage that gives rise to *Engaewa* and *Engaeus* (both *Engaeus sensu stricto* and *Engaeus lyelli* – as defined by Schultz *et al.*, 2009) from the other crayfish of the burrowing clade (BS value = 100)(Figure 3.3). However, it only provides weak support for a divergence between *Engaeus lyelli* and the *Engaeus sensu stricto*/*Engaewa* lineage, and the node indicating a sister relationship between *Engaeus sensu stricto* and *Engaewa* is not supported (Figure 3.3). It also weakly supports the hypothesis of a burrowing clade (containing *Engaeus, Engaewa, Geocharax, Gramastacus* and *Tenuibranchiurus*)(Figure 3.3), as suggested by Horwitz (1988b). Based on this result the procedure was repeated with the highly divergent *Engaeus lyelli* sequences excluded, as long branch lengths may obscure the true phylogeny (for example, see Wägele & Mayer, 2007). Exclusion of these sequences resulted in supported monophyly, and a sister relationship, for *Engaewa* and *Engaeus*; although it was only weakly supported (BS value = 67) (Figure 3.3), as a bootstrap value of 70 is required for strong support. The node where the *Engaewa*/*Engaeus* lineage and the

lineage leading to the rest of the burrowing clade diverge still had a bootstrap value of 100, and the node separating these taxa from *Cherax* was still weakly supported (BS value = 59) (Figure 3.3). Thus, it is evident that *Engaewa* are more closely related to these east coast genera than to the genus *Cherax*, including those species that *Engaewa* can be found in sympatry with.
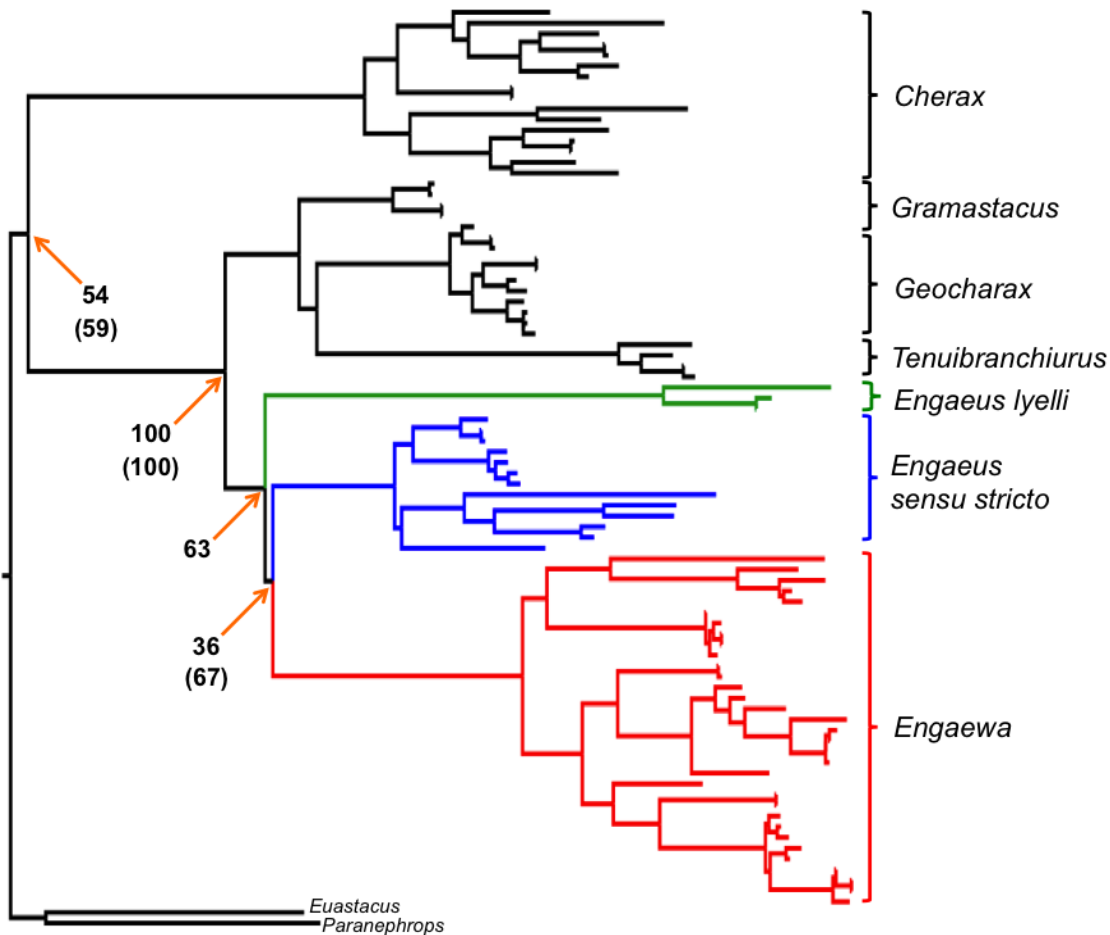
Figure 3.3 RAxML Maximum-likelihood combined 16S and GAPDH tree showing the relationship between members of the burrowing clade of Australian parastacids using data from this study and that of Schultz *et al*. (2009). Bootstrap support values for important nodes (in the context of this study) are given. *Engaewa* spp, *Engaeus sensu stricto* and *Engaeus lyelli* (red, blue and green, respectively) are highlighted. This analysis was repeated excluding the *Engaeus lyelli* sequences and the resulting bootstraps are shown in parentheses.

Maximum-likelihood maps (MLM) were created by quartet puzzling using the 16S dataset of this study and additional sequences retrieved from GenBank. Firstly, the sequences from the burrowing clade matching those used by Schultz *et al.* (2009) (as per Figure 3.3) were tested and showed 5% of resolved quartets support a grouping of (*Engaeus sensu stricto*(*Engaewa*:*Engaeus lyelli*)), whereas the grouping of (*Engaeus lyelli*(*Engaewa*:*Engaeus sensu stricto*)) was supported by 35% of quartets and there was 31% support for the grouping of (*Engaewa*(*Engaeus sensu stricto*:*Engaeus lyelli*)), with 25% of quartets remaining unresolved (Figure 3.4a). When this process was repeated using all available sequences for the study species from GenBank, the percentage of resolved quartets favouring the grouping of (*Engaewa*(*Engaeus sensu stricto*:*Engaeus lyelli*)) increased to 53% and the percentage unresolved increased to 36% (Figure 3.4b). Support for the groupings of (*Engaeus sensu stricto*(*Engaewa*:*Engaeus lyelli*)) and (*Engaeus lyelli*(*Engaewa*:*Engaeus sensu stricto*)) were only 1% and 2.5%, respectively (Figure 3.4b).
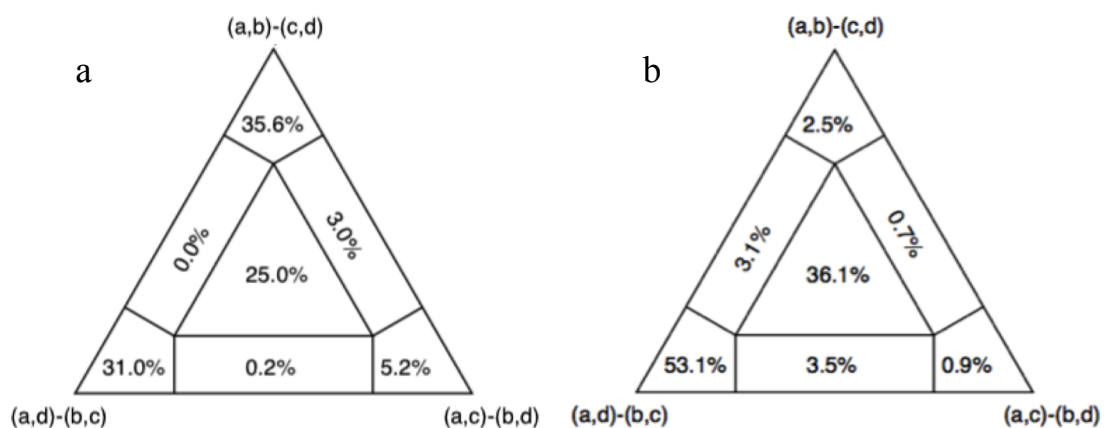
Figure 3.4 Maximum-likelihood mapping of quartet puzzling of the burrowing clade of Australian parastacids using (a) 16S data from this study and that of Schultz *et al*. (2009); and (b) 16S data from this study and GenBank. a=*Engaewa* spp, b=*Engaeus sensu stricto*, c=*Engaeus lyelli*, d=all others (*Geocharax, Gramastacus, Tenuibranchiurus*). The values displayed represent the support value for the particular relationships found in the quartet puzzling trees.

When looking within the genus *Engaewa*, the phylogenetic trees produced from the various datasets and with different tree construction methods were largely congruent and generally suggest eight distinct lineages (Figures 3.5-3.11). The two likelihood-based programs (PhyML and RAxML) produced no conflict and only minor differences in bootstrap support values (as would be expected due to the stochastic nature of the bootstrap resampling method). This suggests that differences in the models employed (as previously mentioned RAxML only employs the GTR model) did not produce a different outcome. Whilst it is known that phylogenetic methods generally perform worse when the incorrect model is assumed (Bruno & Halpern, 1999; Felsenstein, 1978; Huelsenbeck, 1995; Huelsenbeck & Hillis, 1993) it is also known that ML is rather robust to the model used (Fukami-Kobayashi & Tateno, 1991; Gaut & Lewis, 1995). Therefore, only one tree method will be presented for each dataset. Likelihood and Bayesian analyses produced near identical topologies with differences involving only minor rearrangements of some terminals (individual specimens). Between two to five outgroups from closely related genera were used for each tree, with monophyly of *Engaewa* supported in all trees.

The mitochondrial datasets both individually and combined (16S, COI & mtC) provided high support for some branches yet failed to support many others regardless of the phylogenetic reconstruction method used (Figures 3.5-3.10). As the haplotypes in each lineage are highly consistent, the branches are colour coded to show the relationship between the various lineages across different trees. Significant changes to the haplotype level arrangement between the trees relate to individuals from the population BW3 and the relationship between individuals in the populations PRD and TT2. For the COI gene samples BW303/BW304 sit just outside the lineage to which they are assumed to belong, although this was not seen for sample BW305 in the 16S data. The significance of this cannot be deduced without additional data. Samples from PRD and TT2 have a sister relationship for 16S but not COI, though they are sister lineages in the combined data phylogeny. The initial branching point within the genus was the only node that was consistently placed and had high statistical support for all of the mitochondrial trees; thus the relationship between lineages is unclear.
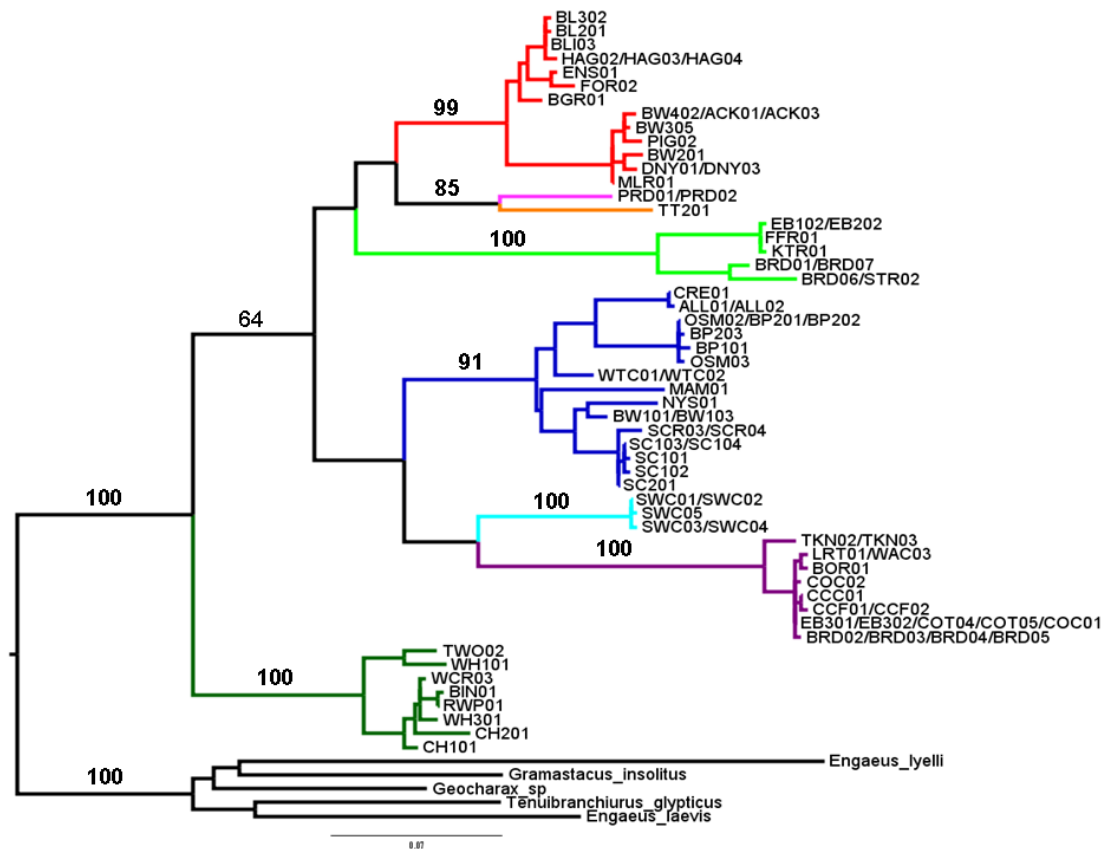
Figure 3.5 PhyML Maximum likelihood 16S tree with supported bootstrap values (>50%) shown above major branches (highly supported (>70%) shown in bold).
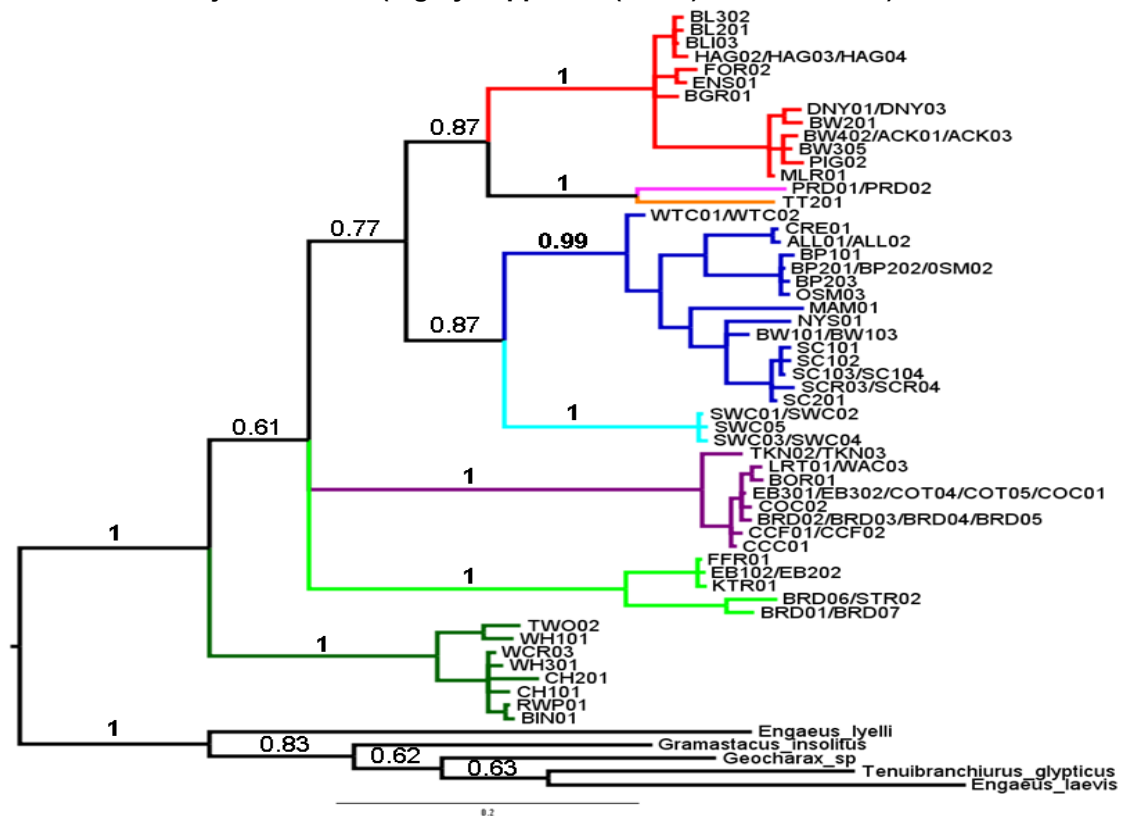
Figure 3.6 Bayesian 16S tree with posterior probabilities >0.50 shown above major branches (supported values (i.e. >0.95) are shown in bold).

65
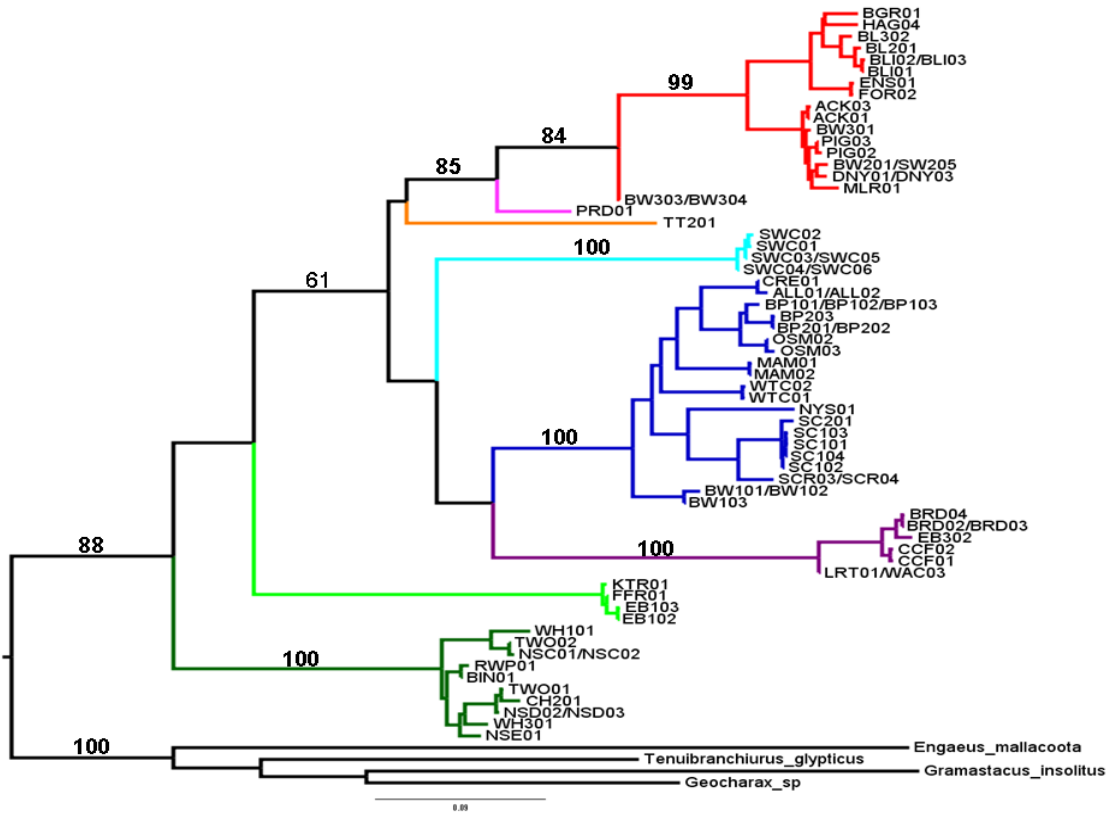
Figure 3.7 PhyML Maximum likelihood COI tree with supported bootstrap values (>50%) shown above major branches (highly supported (>70%) shown in bold).
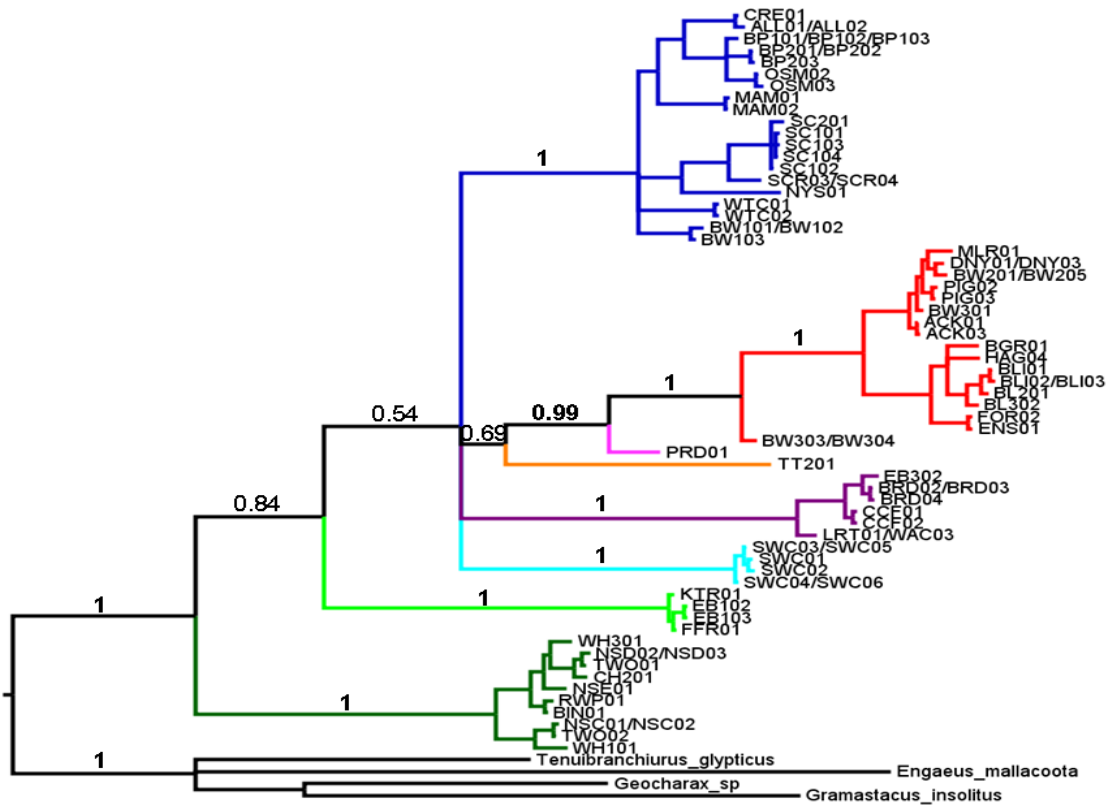
Figure 3.8 Bayesian COI tree with posterior probabilities >0.50 shown above major branches (supported values (i.e. >0.95) are shown in bold).

66
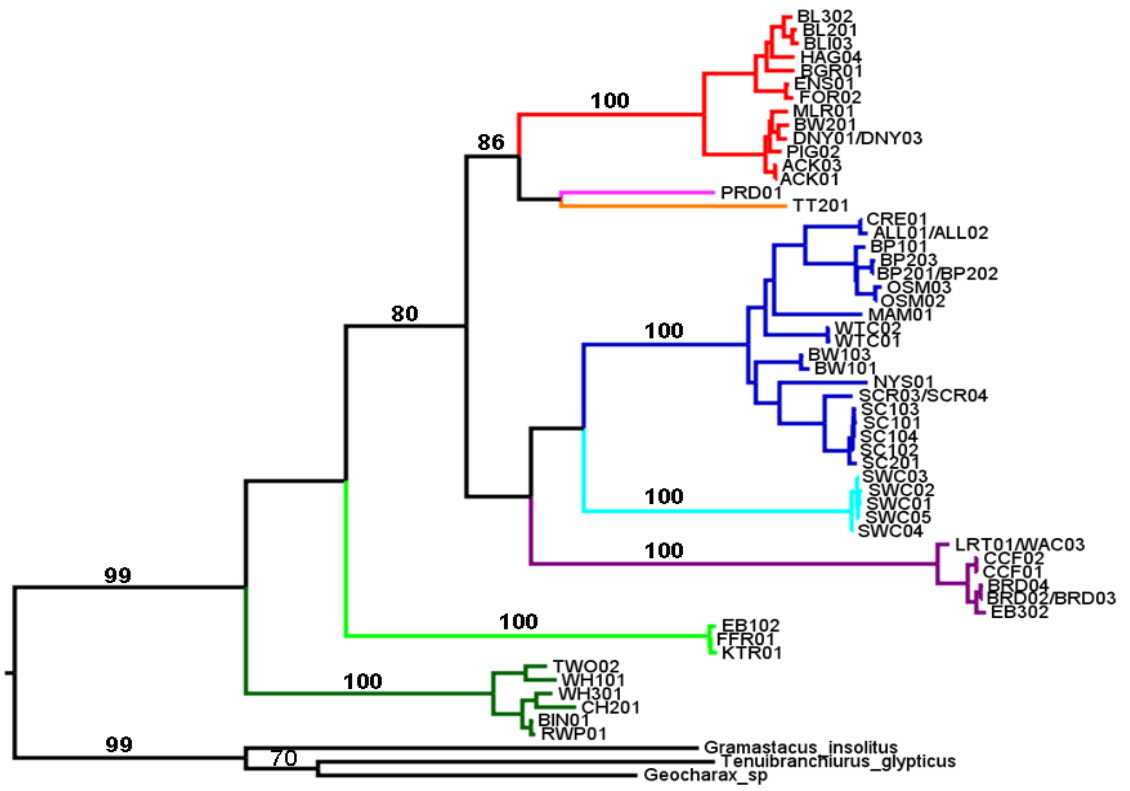
Figure 3.9 PhyML Maximum likelihood combined mtDNA (16S&COI) tree with supported bootstrap values (>50%) shown above major branches (highly supported (>70%) shown in bold).

When the nuclear datasets (GAPDH and LSU) were considered individually they provided very poor phylogenetic resolution, therefore only one tree is shown for the two datasets as an example (GAPDH using ML – Figure 3.10). Although GAPDH and LSU were not phylogenetically informative individually, when added to mitochondrial datasets they improve the resolution over mitochondrial markers alone (particularly for deeper branches).
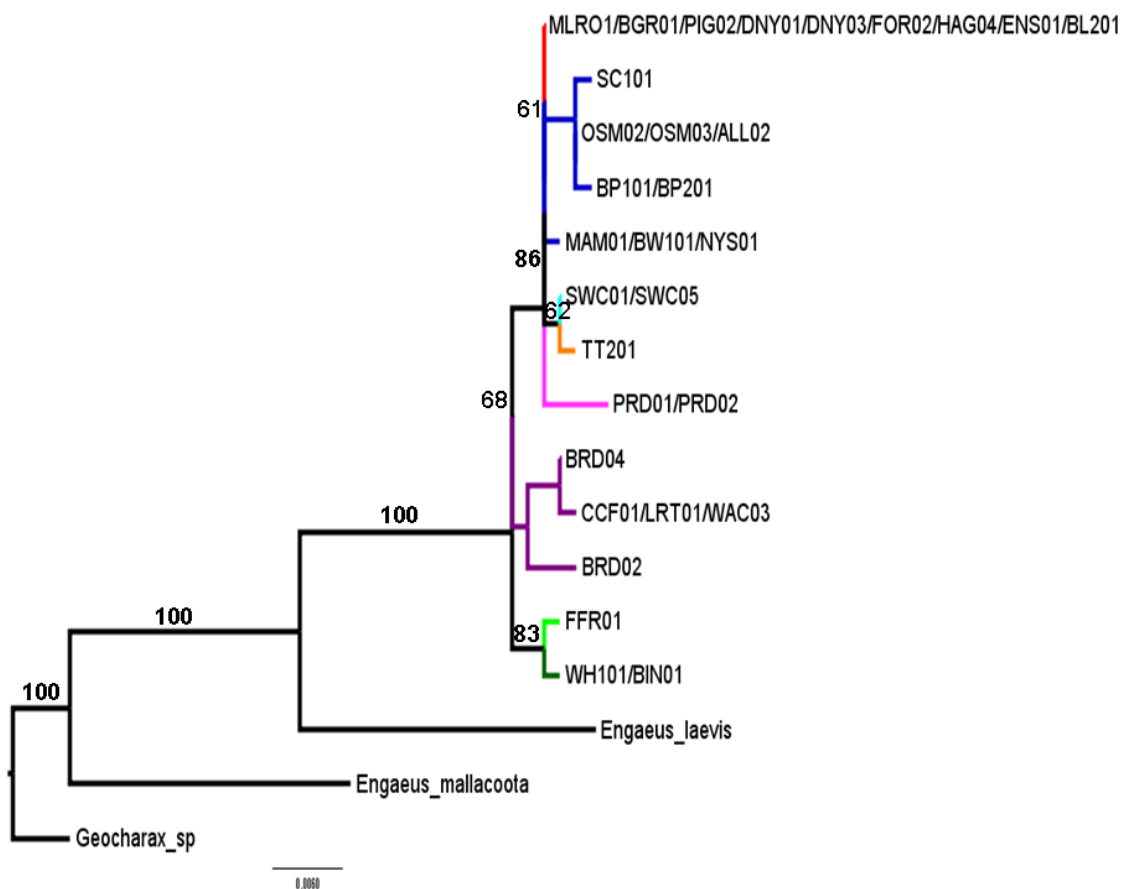
Figure 3.10 PhyML Maximum likelihood GAPDH tree with supported bootstrap values (>50%) shown above major branches (highly supported (>70%) shown in bold).

The combined partitioned Bayesian tree (allCp) provided highly significant support values (PP >0.95) for all branch points except for 3 splits near the tips and within a species group (i.e. population level) (Figure 3.11). Using the two ML tree reconstruction methods for the same combined dataset (though not partitioned in the case of PhyML, as partitioned analyses are not available) produced a combination of highly, weakly and non-supported bootstrap values, though there were no conflicts in the topology between Bayesian and ML (Figure 3.11). This is not surprising as both

empirical and simulation studies have found Bayesian posterior probabilities to be high relative to other measures of support, such as nonparametric bootstrap (Alfaro & Holder, 2006; Douady *et al.*, 2003; Suzuki, Glazko & Nei, 2002), though they tend to be correlated (Buckley, Arensburger, Simon & Chambers, 2002). The ML bootstrap support values shown on this tree are those taken from the tree produced by RAxML; though the actual values for both ML methods were very similar and the support levels (highly/weakly/non-supported) were identical. This tree is considered to represent H_0 for the species delineation (dealt with in the next section, 3.3.2).
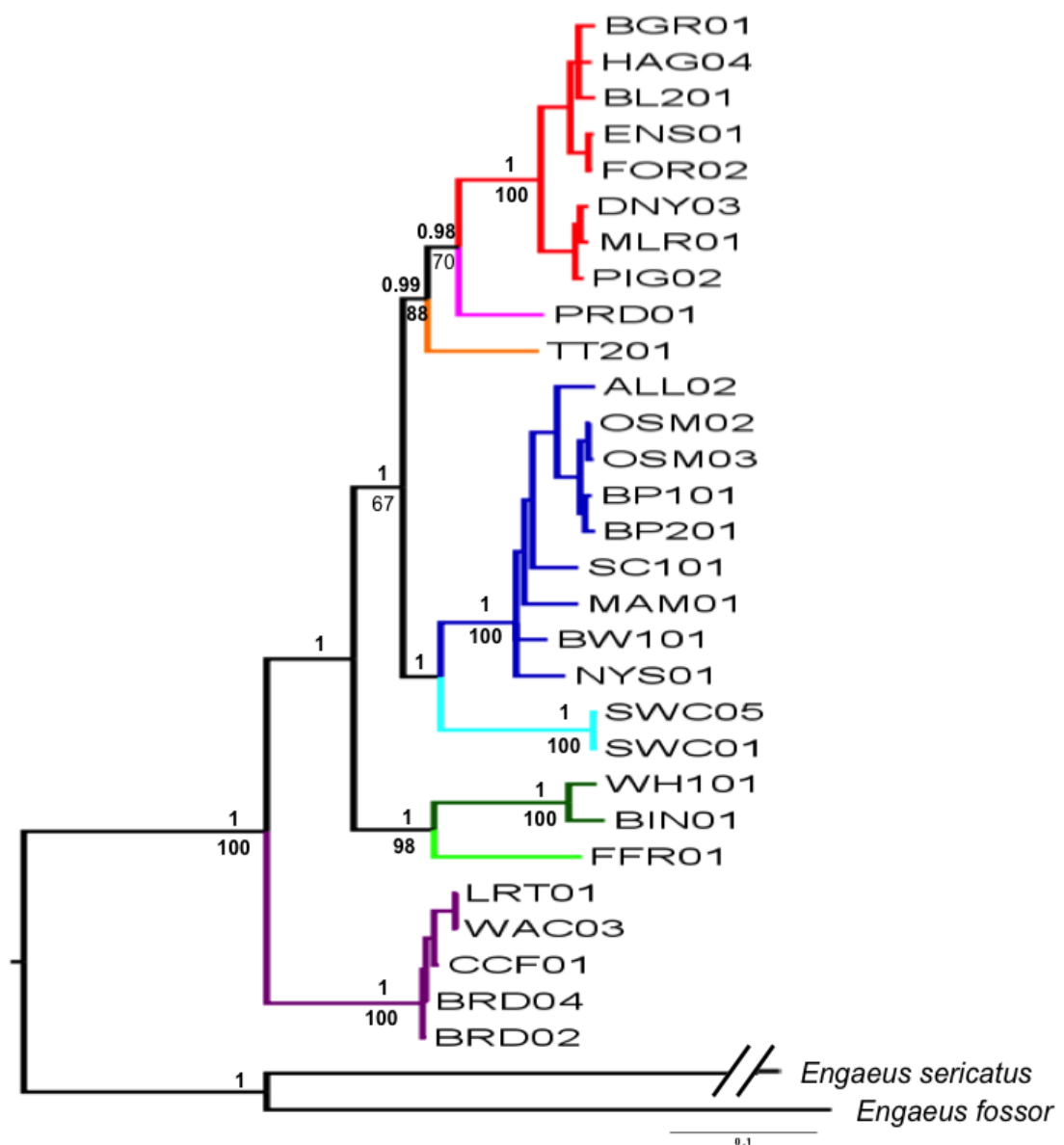
Figure 3.11 Bayesian combined tree with posterior probabilities shown above, and bootstrap values from the RAxML analysis shown below, major branches. Values are only shown for supported nodes.

Time since speciation events can have a significant effect on the reliability of phylogenetic trees (Nichols, 2001), thus a LTT plot was created for *Engaewa* spp. The LTT plot produced a highly significant (p=0.0001) gamma value of 3.8766, indicating that there is statistically significant evidence for an explosive radiation in relatively recent times (i.e. late in the taxon's history) (Figure 3.12). It is important to note that LTT plots can be misleading if all extant taxa are not sampled (Barraclough & Nee, 2001; Pybus & Harvey, 2000), as it will underestimate the number of lineages towards the present, potentially producing an erroneous appearance of slowing down in lineage diversification (Kozak, Weisrock & Larson, 2006). However, due to the extensive sampling undertaken in this study (and the fact there appears to be an increase rather than decrease in lineage formation towards the present) it is assumed that virtually all extant taxa (or at least a percentage high enough so as to not mislead the analysis) have been sampled in this study.
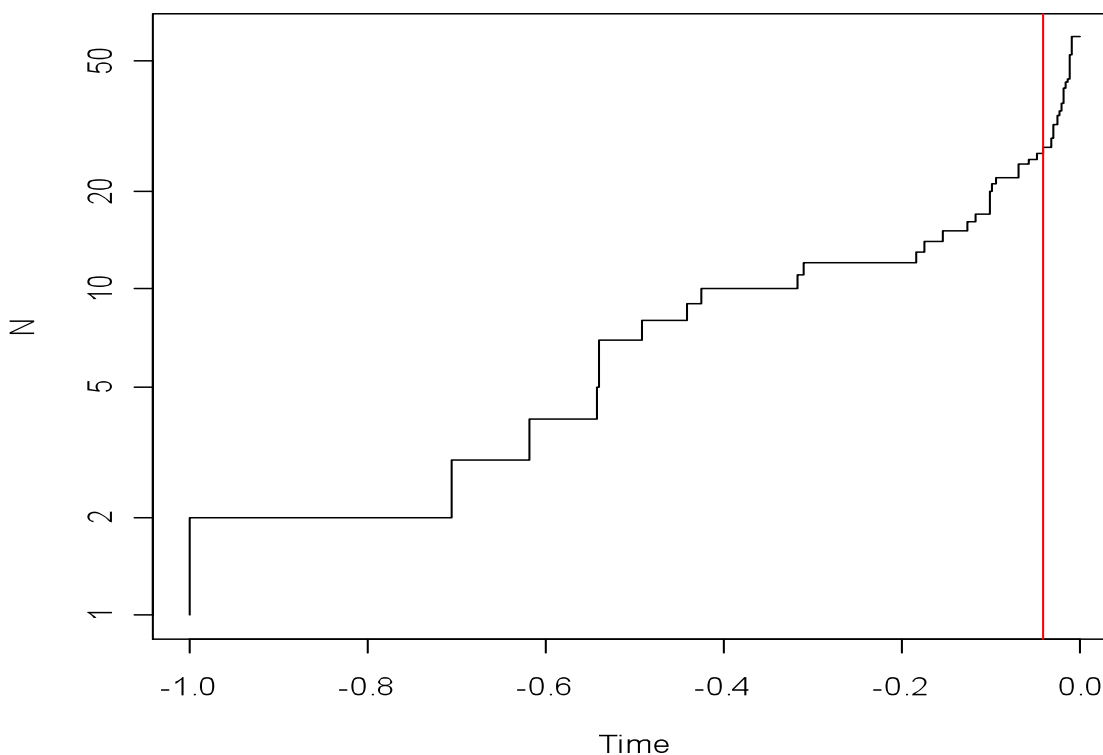
Figure 3.12 Lineage through time plot showing evidence of explosive radiation within the genus *Engaewa*. The red line signifies the shift from inter- to intra- species branching pattern.

3.3.2 Species delineation

The phylogenetic reconstructions in the preceding section suggest there are eight lineages that potentially represent species. Therefore, the eight colour coded lineages highlighted on the phylogenetic trees form the initial H_0. In order to produce the final species delineation via the methodology employed in this study it is also necessary to derive the morphologically based species delineation (H_1). Whilst a number of morphological characters were found to be useful for delineating between a subset of species, such as the shape of the interantennal scale, the shape of the rostrum and the degree to which the rostral carinae are raised, one group of characters in particular stood out as being most useful for delineating species – the nature and pattern of setation and tubercles/granulations on the chelae. Thus it is the nature of the various characteristics of the chelae that form the basis of the morphological evaluation of species boundaries presented as H_1.

Based on the descriptions of the chelae provided by Horwitz and Adams (2000) two of the lineages identified in H_0 could be attributed to the species *E. reducta* and *E. walpolea*. The character states of the chelae for *E. reducta* were found to be consistent for specimens placed in this lineage by the molecular data. For *E. walpolea* the current description generally held, except setae were recorded over all surfaces of the chelae of females only, a characteristic not described by Horwitz and Adams (2000). The recognition of this character represents the first case of sexual dimorphism recorded in *Engaewa.*

Based on characteristics of the chelae, two lineages identified in H_0 could be recognised as coinciding with *E. similis and E. subcoerulea* (based on the descriptions of Horwitz and Adams (2000)). However, each of these species was sister to a lineage in H_0 that could also be identified via unique characteristics of the chelae, which did not correspond to any of the descriptions of Horwitz and Adams (2000); these lineages represent *E. clade A* and *E. clade B* (being sister to *E. similis and E. subcoerulea*, respectively). A range of morphological characters that can be used to distinguish between the currently described species and their sister lineage are discussed below. In both cases the morphology of the type specimen fits the already described species, thus

there would be no nomenclatural instability with regards to the species names already in use. However, the recognition of the new lineages as species would remove some of the reported morphological variability associated with these species descriptions.

A chelae-related diagnostic character that can distinguish between *E. clade A* and *E. similis* is the presence of long setae on the ventral surfaces of both isomorphic and small dimorphic chelae for *E. similis*, which are absent in *E. clade A* (Figure 3.13). Other setae on the chelae are somewhat variable for these species, however, there is still a recognisable pattern. *Engaewa clade A* has long but relatively sparse setae on the lateral surfaces of the dactyl and propodal finger of the isomorphic and large dimorphic chelae, compared to *E. similis* which has a dense mat of short bristle setae on the lateral surfaces of the dactyl and propodal finger of isomorphic chelae and short bristle setae on the small dimorphic chelae (though when present on the small dimorphic chelae there may be some longer bristle setae as well) (Figure 3.13). Very rarely a greatly reduced dense mat may be present on the cutting edges of the large dimorphic chelae of *E. similis*. The nature of the tubercles on the dorsal edge of the dactyl of both isomorphic and large dimorphic chelae are also diagnostic as *E. similis* has two distinct rows of relatively large tubercles, whereas *E. clade A* has smaller tubercles over the entirety (i.e. not in rows). The tubercles along the dorsal edge of the propodus also varies between these two groups as they are large, although sparse and continuing along the entirety for *E. similis*, whereas they are greatly reduced but generally complete on *E. clade A*. An additional character that can be used to delineate between these two species is the degree to which the rostral carinae are raised. The rostral carinae are moderately raised on *E. similis* and continuing most or all the way along the rostrum, whereas in *E. clade A* they are generally absent or at most very weakly raised.

i

ii
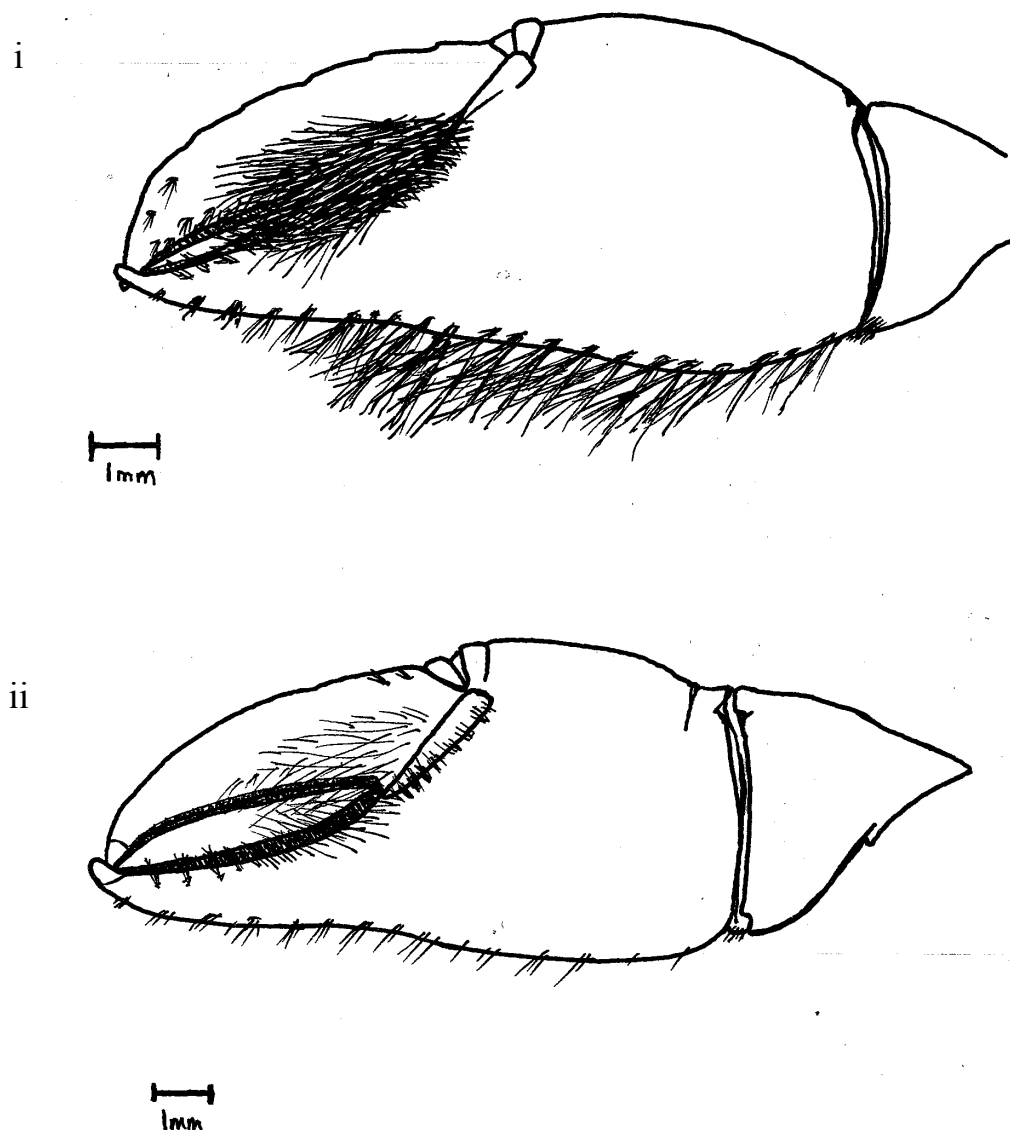
Figure 3.13 Lateral view of an isomorphic chela showing the diagnostic setation pattern of *Engaewa similis* (i) and *Engaewa clade A* (ii).

The most obvious diagnostic character that can be used to distinguish *E. clade B* from *E. subcoerulea* is the presence of three rows of long tufts of plumose setae along the entire length of the dactyl and continuing as a single row approximately half way along the propodus, with a small patch of short plumose setae ventrally at the propodal palm-carpal articulation, as well as three rows of tufts of long plumose setae ventrally along propodal palm and finger on the small dimorphic chelae (Figure 3.14). *Engaewa subcoerulea* has no such long setae. *Engaewa clade B* can also be distinguished from *E. subcoerulea* by the nature of the tubercles on the dorsal edge of the dactyl of the large dimorphic chelae as they occur as large tubercles in two sparse rows in *E. subcoerulea*,

whereas *E. clade B* has much denser and smaller tubercles over the entirety (i.e. not in rows). *Engaewa clade B* also has a very faint row of tubercles along the entire dorsal edge of the propodus, which are absent in *E. subcoerulea*. The degree of granulation of the propodal palm also varies, as the granulations are more dense and pronounced on *E. clade B* covering approximately the distal two-thirds, whereas in *E. subcoerulea* they are prominent over only one third. A further diagnostic character that can be used to delineate these two lineages is the shape of the sternal pores. This is most evident on the lateral process of the 2nd pereopod where *E. clade B* has an open elongate pore, and *E. subcoerulea* has a closed or open slit.

i   ii

2.4 mm
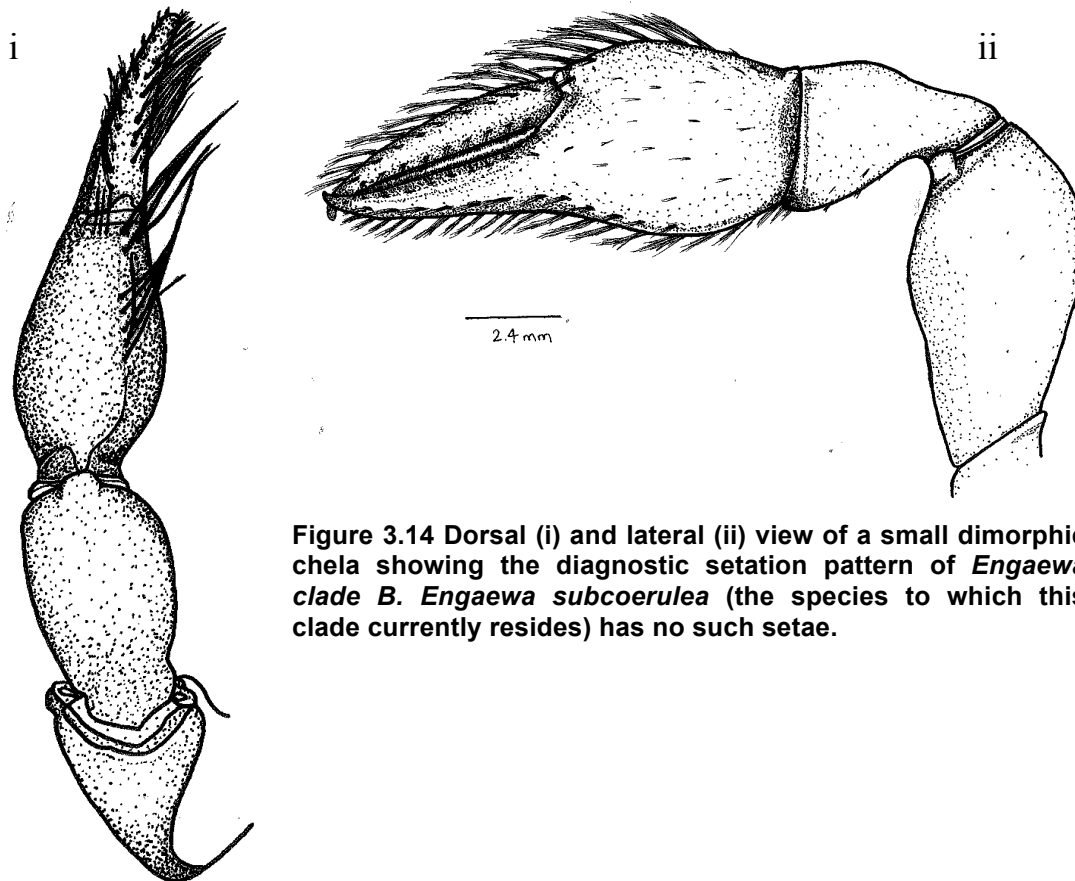
Figure 3.14 Dorsal (i) and lateral (ii) view of a small dimorphic chela showing the diagnostic setation pattern of *Engaewa clade B*. *Engaewa subcoerulea* (the species to which this clade currently resides) has no such setae.

As described above, six of the lineages identified in H_0 can be clearly identified following the method of H_1. However, the relationship of the two remaining genetic lineages is unclear. One of the genetic lineages was identified based on a single specimen (TT201). The chelae setation of this specimen is largely consistent with the

description of *E. pseudoreducta* and a number of museum specimens of this species that were examined, and it was collected from close to the type locality. The final genetic lineage was formed by two specimens (PRD01 & PRD02) collected from a site on Payne Road, 16.5 km north of the *E. pseudoreducta* type locality. The setation found in the Payne Road specimens was neither typical of *E. pseudoreducta* nor consistent when compared to each other, though it reflects the same pattern as the molecular data whereby they were most closely aligned to *E. pseudoreducta*. Based on the assessment of museum specimens there appears to be considerable variation in the degree of setation for *E. pseudoreducta* generally and, considering there are only three genetic specimens currently available across the two lineages, these two lineages from H_0 are conservatively grouped together in H_1.

Based on morphological analyses there are seven defined morphotypes forming H_1 (Table 3.5), thus H_0 and H_1 are not congruent. Following the species delineation methodology outlined in 3.2.2, H_2 will be formulated and will become the accepted hypothesis of species lineages. The close phylogenetic relationship between *E. pseudoreducta* specimens and those from Payne Road in addition to their morphological affinity suggests that both an evolutionary and biological logic exists for grouping these specimens together into a single lineage until such as time as further data may be available to reconcile H_0 and H_1. As no other incongruence exists between the lineages suggested by H_0 and H_1, H_2 for this study will be that seven lineages should be recognised. Five of the lineages identified by the phylogenetic analyses undertaken can be attributed to the currently defined species (*pseudoreducta* (including the Payne Road specimens)*, reducta, similis, subcoerulea, walpolea*), based on morphologic species designations (using the characters of Horwitz and Adams (2000)), with the additional recognition of *clade A* and *clade B*. Therefore, the seven lineages recognised at the species level in this study are; *pseudoreducta, reducta, similis, subcoerulea, walpolea, clade A* and *clade B*. The tree presented for H_0 is reproduced here with the various colour-coded lineages adjusted and having species names attached (with the labels *E. clade A* and *E. clade B* used for the previously unrecognised lineages) in agreement with H_2 (Figure 3.15).

Table 3.5 Diagnostic morphological characters (SD=small dimorphic chelae, LD=large dimorphic chelae, ISO=isomorphic chelae, LPxP=lateral process of the 'x' pereopod).

Species	Chelae setation	Chelae granulation	Sternal pores
pseudoreducta	ISO and SD: patches of short setae on ventral surface of merus, distal lateral and mesial edges of carpus, around cutting edges, and occasionally over parts of propodal palm(though highly variable).	-	-
reducta	ISO and SD: dense patch of short setae on proximal half of dorsal surface of dactyl, and often distal third of dorsal edge of propodus.	-	LP2P: no pore LP3P: with pit or pore
similis	ISO and SD: two rows of long setae on ventral surface. ISO: dense patch of setae on lateral surface of dactylus and propodal finger. SD: very short patch of bristle setae on lateral surface of dactylus and propodal finger.	ISO and LD: two rows of large tubercles on the dorsal dactylus surface; large sparse tubercles on entire dorsal propodal surface.	-
subcoerulea	None.	LD: two sparse rows of large tubercles on dorsal dactylus; granulations prominent on distal one-third of propodal palm.	LP2P: closed/open slit LP3P: with pore
walpolea	Setae only on female, where it is found sparsely over all surfaces.	-	-
clade A	ISO and LD: long sparse bristle setae on lateral surface of dactylus and propodal finger.	ISO and LD: small tubercles over entire dorsal dactylus surface (i.e. not in rows); generally complete but reduced tubercles on dorsal propodal surface.	-
clade B	SD: three rows of long plumose setae on ventral surface of propodal palm and finger, and on dactylus where it continues to a single row halfway along propodus; small patch of short plumose setae on ventral surface at propodal palm-carpal articulation.	LD: dense small tubercles over entire dorsal dactylus surface (i.e. not in rows); very faint row on dorsal propodus; dense and pronounced granulations on distal two-thirds of propodal palm.	LP2P: open and elongate pore LP3P: with pore
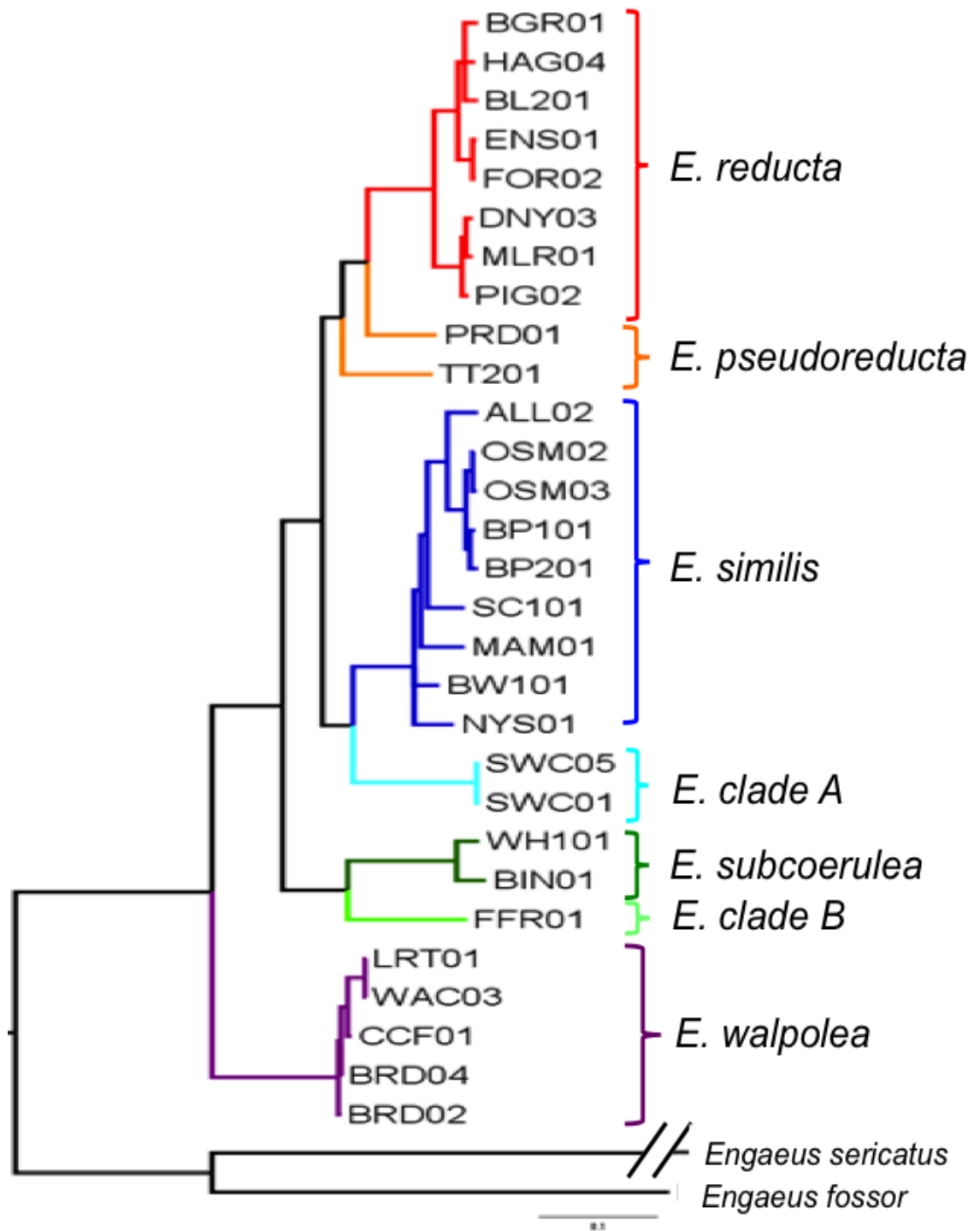
Figure 3.15 Bayesian combined tree (from 3.3.1) with species names shown for the colour-coded lineages that represent the species designation of this study (i.e. H₂).

The seven lineages representing H_2 are largely supported by a difference in inter- and intra-species genetic distances. Intra-lineage genetic distances for 16S ranged from 0.003 for *E. clade A* to 0.078 for *E. pseudoreducta*, and between lineage divergences ranged from 0.100 between *E. reducta* and *E. pseudoreducta* to 0.262 between *E. clade B* and *E. walpolea* (Table 3.6a). For COI, *E. clade A* again had the lowest diversity and *E. pseudoreducta* the highest (more than twice the next closest) (Table 3.6b). *Engaewa reducta* and *E. pseudoreducta* again had the lowest distance between lineages (0.057), though the highest estimate for COI was 0.276 between *E. clades A* and *B* (Table 3.6b). For some lineages and lineage comparisons COI displayed a larger divergence and for others 16S did, though generally both 16S and COI presented a similar picture (i.e. lineages that were highly divergent were so for both genes and vice versa). The most noticeable exceptions to this were the divergences within the *E. clade B* lineage, which was 0.048 for 16S but only 0.006 for COI, and within the *E. pseudoreducta* lineage, which was 0.078 for 16S and 0.167 for COI (Table 3.6a&b). Range size appears to roughly equate to diversity (very low in *E. clade A*, low in *E. walpolea*, but relatively similar for the other, geographically wide-spread lineages), with the exceptions as previously mentioned.

Table 3.6 Within and between group 16S (a) and COI (b) corrected genetic distances for the species groups (including the two new lineages highlighted in this study) in the genus *Engaewa*. Numbers in parentheses represent number of sequences. The within-clade distances are shown on the shaded diagonal (standard error ranged from 0.002-0.016 for 16S and 0.002-0.007 for all lineages except *pseudoreducta* which was 0.022 for COI (not shown)) and the net between-clade distances are shown below the diagonal with the associated standard error above.

16S (a)	clade A	clade B	pseudoreducta	reducta	similis	subcoerulea	walpolea
clade A (5)	0.003	0.038	0.027	0.031	0.024	0.038	0.032
clade B (8)	0.228	0.048	0.030	0.029	0.030	0.037	0.045
pseudoreducta (3)	0.151	0.168	0.078	0.020	0.024	0.036	0.039
reducta (18)	0.177	0.175	0.100	0.044	0.026	0.031	0.032
similis (22)	0.126	0.185	0.129	0.142	0.057	0.028	0.031
subcoerulea (8)	0.216	0.221	0.208	0.193	0.154	0.038	0.035
walpolea (18)	0.168	0.262	0.227	0.189	0.178	0.197	0.010

COI (b)	clade A	clade B	pseudoreducta	reducta	similis	subcoerulea	walpolea
clade A (6)	0.004	0.032	0.017	0.023	0.020	0.028	0.022
clade B (4)	0.276	0.006	0.022	0.030	0.024	0.026	0.027
pseudoreducta (2)	0.107	0.142	0.167	0.012	0.013	0.023	0.019
reducta (21)	0.183	0.239	0.057	0.061	0.018	0.024	0.025
similis (26)	0.162	0.197	0.062	0.145	0.073	0.026	0.018
subcoerulea (12)	0.237	0.204	0.147	0.202	0.210	0.040	0.029
walpolea (8)	0.192	0.233	0.118	0.196	0.150	0.243	0.024

As a method of further testing the species groupings based on genetic structure the ratios of F_{CT} (the amount of variation among groups relative to the total variance based on haplotype frequency) and Φ_{CT} (the amount of variation among groups relative to the total variance based on haplotype frequency and genetic divergence) were compared with a variety of different groupings. The F_{CT} values are based solely on haplotype frequency and as haplotype diversity essentially equalled 0 or 1 it was largely uninformative, only Φ_{CT} values are reported. Therefore, the original intention to use F_{CT} values in a way to demarcate species boundaries (following the suggestion of Monaghan, *et al*. (2005)) could not be followed, however, Φ_{CT} was instead analysed. It can be seen from Table 3.7 that the suggested 95% boundary for the partitioning of genetic variation between groups (Φ_{CT}) was never reached. It is also evident from this table that as the partitions were more narrowly defined the value of Φ_{CT} continued to rise. This is as a result of virtually every population representing a unique genetic group (discussed in more detail in the next section).

Table 3.7 Values of Φ_{CT} resulting from different partitioning of genetic diversity in the total diversity of *Engaewa*. Colours indicate divisions within each partitioning scheme. The divisions tested correspond to various species hypotheses, with "hap's" being the genetic diversity divided into connected MP haplotype networks (16S=21, COI=25)). The seven lineages suggested in this thesis are identified (Lineage) as well as further geographic subdivions of these lineages (Geo.Div. – nth MR (north of Margaret River); sth MR (south of Margaret River); treeton (population in the Treeton Forest Block); payne rd (population at Payne Road); MR - BR (between Margaret and Blackwood Rivers); sth BR (south of Blackwood River)) (refer to Figure 2.1 for the location of these geographic boundaries).

Lineage	Geo.Div.	Number of Divisions								
		2	3	4	5	7	8	9	11	hap's
reducta	nth MR									
	sth MR									
pseudoreducta	treeton									
	payne rd									
similis	nth MR									
	MR - BR									
	sth BR									
clade A										
subcoerulea										
clade B										
walpolea										
16S	ΦCT	0.267	0.463	0.590	0.618	0.774	0.782	0.815	0.853	0.928
COI	ΦCT	0.283	0.429	0.527	0.540	0.688	0.696	0.741	0.776	0.918

Whilst the 95% value cannot be used for these crayfish, it may be possible to employ the heuristic of Occam's Razor to identify a grouping that provides the most explanatory power with the fewest assumptions. This was tested by plotting the Φ_{CT} values for both 16S and COI and seeing if at a particular grouping (corresponding to a specific number of 'species') a transition in the slope could be identified, whereby further divisions would add little to the genetic partitioning (the Φ_{CT} value). With 16S and COI both showing a clear change in the slope at seven divisions it could be argued that this represents the 'best' division of the lineages, based on genetic distinctiveness, without over-splitting into non-meaningful groups (Figure 3.16). These seven divisions coincide with the seven lineages recognised by H_2.
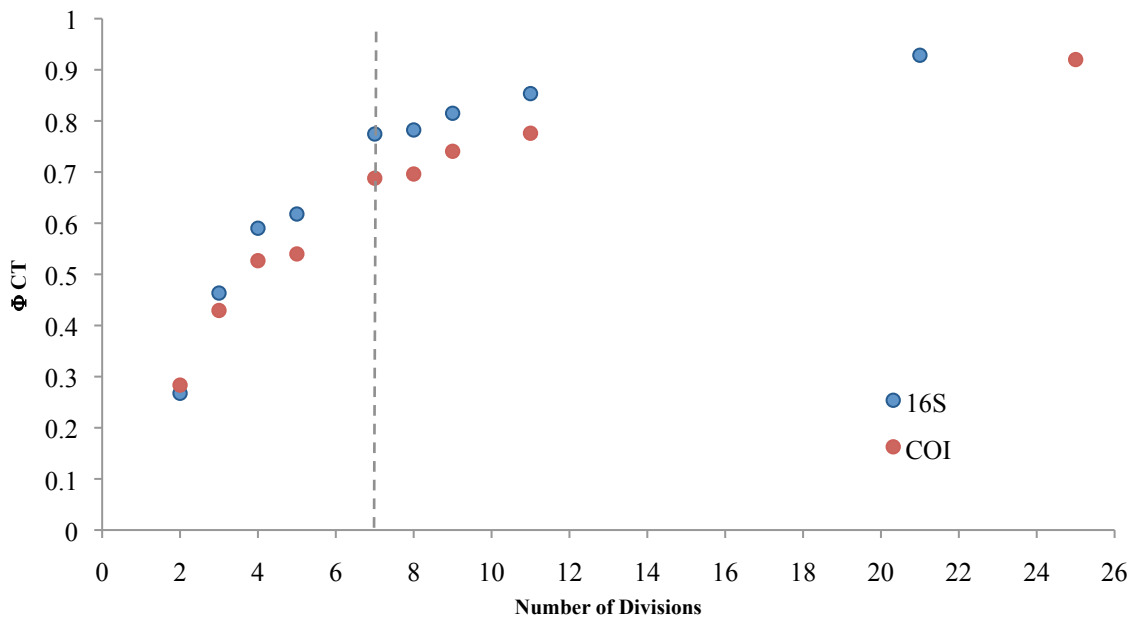
Figure 3.16 Values of ΦCT resulting from AMOVA assessments of a variety of divisions of the total pool of genetic diversity on *Engaewa* for the mitochondrial 16S and COI markers. The highlighted value at seven divisions maximises the between-group value whilst minimising the number of assumptions made (i.e. the most parsimonious explanation).

The use of haplotype networks to delineate species would result in a huge increase in the number of species recognised (networks shown in 3.3.5). Even at a 90% confidence level (rather than the 95% level normally used) it would result in nineteen species being recognised (including seven from the populations currently considered to represent *E. similis* alone) and, based on the genetic structure of these species (as

discussed in detail later), it appears with further sampling even more 'species' would be identified.

None of the additional genetic analyses discussed provide strong support for particular species groupings, or any significant evidence to dispute the delineation based on H_2. Therefore, the seven species level lineages that form H_2 are recognised in this study: *pseudoreducta, reducta, similis, subcoerulea, walpolea, clade A* and *clade B*.

3.3.3 Divergence dating

The divergence dates derived from the 16S and GAPDH *BEAST analysis places the basal node of the genus at ~122 MYA with 95% confidence intervals spanning from ~197-65 MYA (Figure 3.17). The topology of the species tree estimated by *BEAST differs slightly from the accepted topology in this thesis, as *E. walpolea* was placed in a clade with *E. subcoerulea/E. clade B* rather than being basal to the rest of the genus, whilst all other relationships in the tree are congruent with the accepted topology. Despite the discrepancy in topology, the dates for the nodes splitting the northern and southern species, and splitting *E. walpolea* from the other southern species, overlap significantly (~73 and 60 MYA with ranges of 112-45 and 95-32 MYA, respectively) (Figure 3.17). The split between *E. subcoerulea* and *E. clade B* is dated to approximately the same time as the split between the *E. reducta/E. pseudoreducta* and *E. similis/E. clade A* lineages (though with larger margins of confidence) (~45 and 42 MYA, respectively), while the splits between *E. reducta* and *E. pseudoreducta* and *E. similis* and *E. clade A* are slightly younger but largely synchronous (~33 and 32 MYA, respectively) (Figure 3.17).
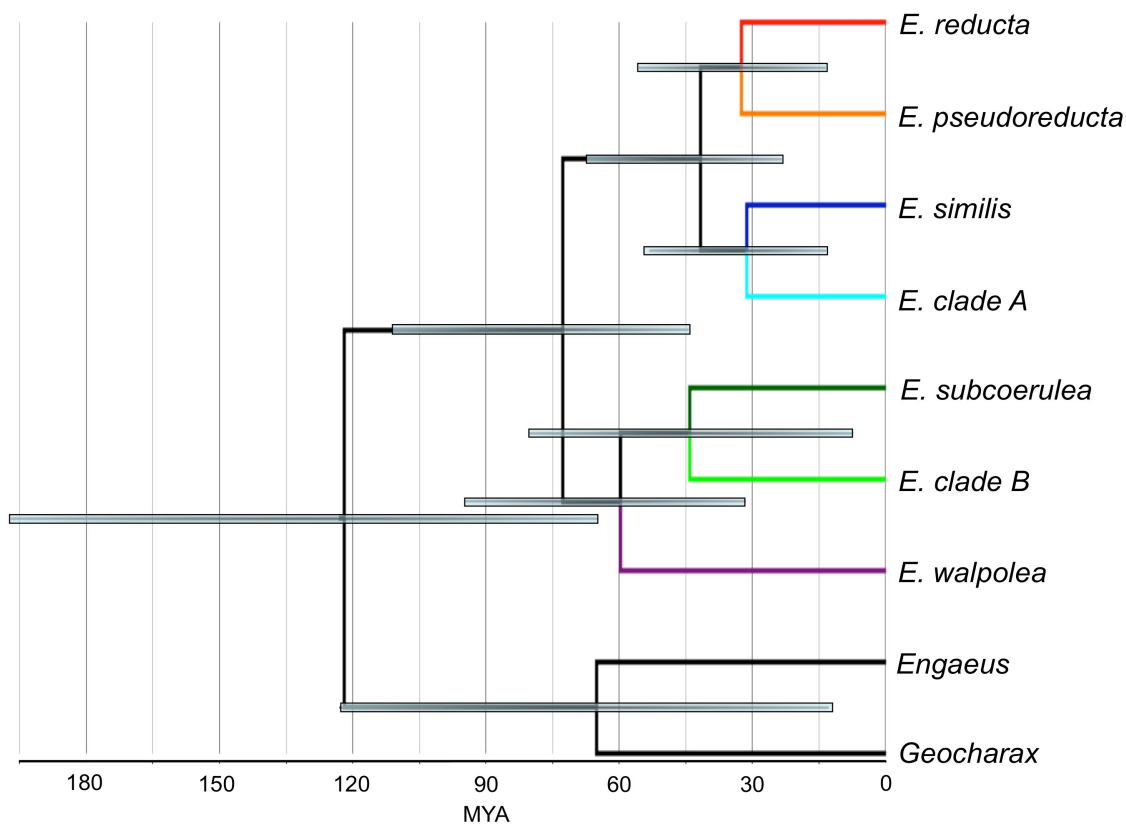
Figure 3.17 *BEAST analysis showing divergence dates of nodes between *Engaewa* lineages. Bars represent 95% highest posterior density intervals.

3.3.4 Analyses of genetic diversity

Before analysing genetic diversity, it is worth noting that few samples were sequenced per population in this study (see Appendix 1 and Figure 3.18), and it seems that adding more specimens would increase the number of haplotypes identified within each population. When the number of haplotypes is viewed in comparison to the number of sequences obtained per population it does not appear to have reached a plateau (Figure 3.18). In total 82 specimens were sequenced across 53 sites with 54 unique haplotypes identified for 16S and for COI 79 specimens across 46 sites yielded 63 haplotypes (Tables 3.8 & 3.9; populations with multiple and/or shared haplotypes can be viewed in the maps presented in 3.3.5).
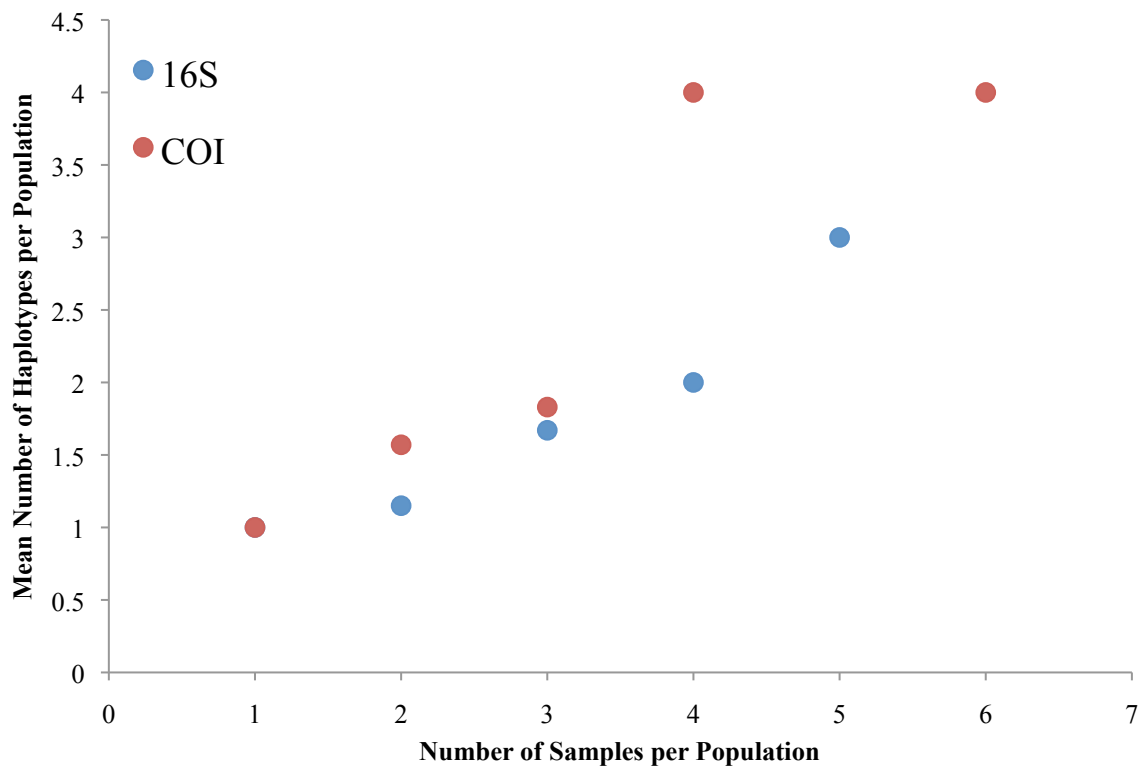
Figure 3.18 Mean number of haplotypes per population in relation to the number of *Engaewa* specimens sampled from each population, for 16S and COI sequences used in this study.

The genus overall had high haplotype diversity (0.988 and 0.994 for 16S and COI, respectively) and nucleotide diversity (0.128 and 0.134 for 16S and COI, respectively) (Tables 3.8 & 3.9). Haplotype diversity for COI was high for most species (0.800-1.000) with the only exception being *E. pseudoreducta* (0.667). The COI haplotype diversity corresponds to the two specimens at Payne Road sharing a haplotype, which differed from the haplotype of the one other *E. pseudoreducta* specimen. For 16S only one specimen sampled from Payne Road and it shared its haplotype with the other *E. pseudoreducta* sample, hence a haplotype diversity of 1.000. Despite having haplotype diversity values ≥0.800 for both 16S and COI, *E. clade A* had very low nucleotide diversity (0.003 and 0.004 for 16S and COI, respectively), meaning that most samples differed from each other but only by a very small number of mutational changes. A similar pattern to that seen in *E. clade A* can also be observed for *E. walpolea* for both 16S and COI, as well as *E. clade B* for COI only. All of the other lineages have both high haplotype diversity and relatively high nucleotide diversity.

Table 3.8 Sample and 16S fragment information and measures of molecular diversity.

Species	N	n	bp	*s*	*h*	π	*k*	# hap
All	82	53	394	147	0.988	0.128	47.555	54
clade A	5	1	394	2	0.800	0.003	1.000	3
clade B	8	6	394	33	0.893	0.043	16.393	5
pseudoreducta	3	2	394	36	0.667	0.063	24.000	2
reducta	18	14	394	43	0.949	0.042	16.184	13
similis	22	12	394	63	0.964	0.059	22.322	15
subcoerulea	8	8	394	38	1.000	0.038	14.250	8
walpolea	18	10	394	15	0.876	0.009	3.569	8

N = total number of individuals; n = number of sample sites; bp = number of nucleotide base pairs; *s* = number of segregating sites; *h* = haplotype diversity; π = nucleotide diversity; *k* = average number of pairwise differences; # hap = number of unique haplotypes.

Table 3.9 Sample and COI fragment information and measures of molecular diversity.

Species	N	n	bp	*s*	*h*	π	*k*	# hap
All	79	46	719	267	0.994	0.134	96.290	63
clade A	6	1	719	7	0.867	0.004	3.133	4
clade B	4	3	719	7	1.000	0.006	4.333	4
pseudoreducta	2	2	719	91	1.000	0.127	91.000	2
reducta	21	13	719	119	0.981	0.054	38.495	17
similis	26	12	719	145	0.977	0.065	46.379	20
subcoerulea	12	10	719	71	0.970	0.037	26.621	10
walpolea	8	5	719	38	0.929	0.023	16.179	6

N = total number of individuals; n = number of sample sites; bp = number of nucleotide base pairs; *s* = number of segregating sites; *h* = haplotype diversity; π = nucleotide diversity; *k* = average number of nucleotide differences; # hap = number of unique haplotypes.

The non-synonymous/synonymous rate ratio ($\omega=d_N{:}d_S$) was calculated for COI producing an overall value for all samples combined of 0.015 (with a maximum of 0.043 for any individual species), indicating no strong selection across the gene. The results of the mismatch analysis of all samples pooled together for both COI and 16S clearly produce ragged, multi-modal distributions rather than a smooth, unimodal pattern (Figure 3.19). This same pattern was also seen when the species that had a reasonable number of samples were tested individually (*E. reducta, E. similis, E. subcoerulea* and *E. walpolea*) (graphs not presented). In addition to the mismatch analysis, a number of other tests related to inferences of changes in population size were performed on the COI dataset, both based on the total number of mutations and number of segregating sites (Table 3.10). These tests included much more sensitive tests than the mismatch analysis (such as F_S and R_2), however, none of these tests were statistically significant.

Table 3.10 COI genetic diversity measures for four species of *Engaewa*. D = Tajima's D; *Fs* = Fu's Fs; *R2* = Ramos-Onsins & Rozas' R2; D* = Fu & Li's D*; F* = Fu & Li's F*. Measures calculated from total number of mutations and number of segregating sites are indicated by (η) and (*S*) respectively.

Species	D (η)	D (*S*)	*Fs*	*R₂*	D* (η)	D* (*S*)	F* (η)	F* (*S*)
reducta	0.301	0.669	0.475	0.127	0.703	0.609	0.678	0.733
similis	0.406	0.912	1.624	0.121	1.032	0.927	0.976	1.088
subcoerulea	0.540	0.613	0.837	0.149	0.255	0.236	0.377	0.382
walpolea	0.555	0.555	1.931	0.174	0.950	0.950	0.956	0.956
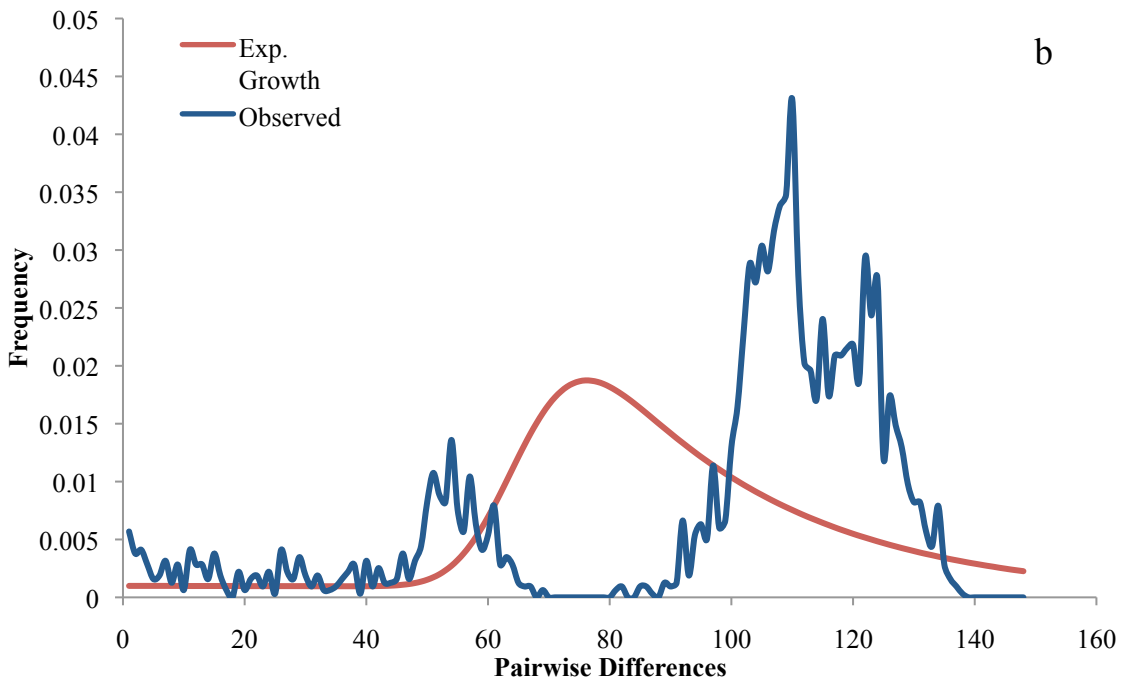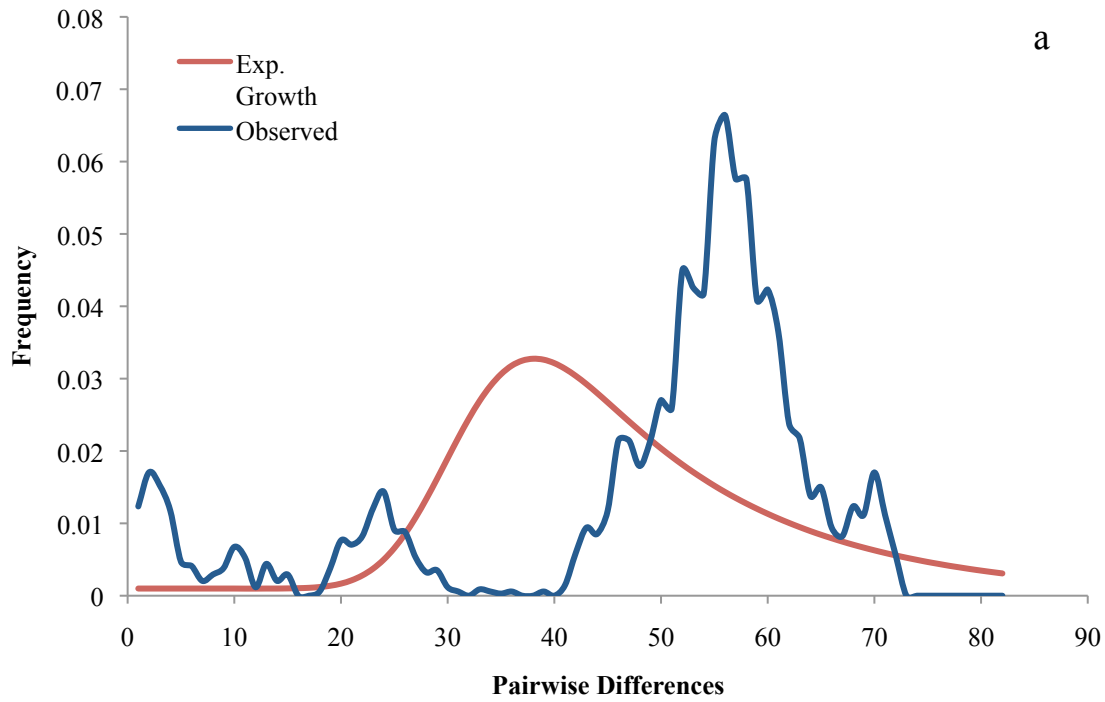
Figure 3.19 Mismatch distribution for (a) 16S sequences, and (b) COI sequences from the _Engaewa_ samples used in this study, showing the observed versus the expected distribution under an exponential growth model.

3.3.5 Geographic mapping of clade boundaries

As a result of the collections made for this study and the species groupings (including the two new clades) presented in 3.3.2, the species' geographic boundaries need to be redrawn to reflect these changes (presented in Figure 3.20). *Engaewa reducta* now has a disjunct distribution, with a group of populations found in tributaries of the Blackwood River added to the northern cluster; these Blackwood River populations overlap with the ranges of both *E. pseudoreducta* and *E. similis*. *Engaewa pseudoreducta* has an extra population in close vicinity to the previously known locality, plus an additional population further north (Payne Road – as highlighted in 3.3.2). The distributional range of *E. similis* has been extended north, past that of *E. pseudoreducta* and into the region previously assumed to contain only *E. reducta*. A population that had previously been considered to be a part of the range of *E. similis* is, as a result of this study, recognised as *E. clade A* and is found amongst the *E. reducta* populations near the Blackwood River. *Engaewa walpolea* has had no significant changes to its distribution though additional populations have been uncovered within its known range. The previously widespread *E. subcoerulea* has been divided into two species with the recognition of the new *E. clade B*, thus dividing its previously assumed range at the town of Walpole, with *E. subcoerulea* found to the west and *E. clade B* to the east.
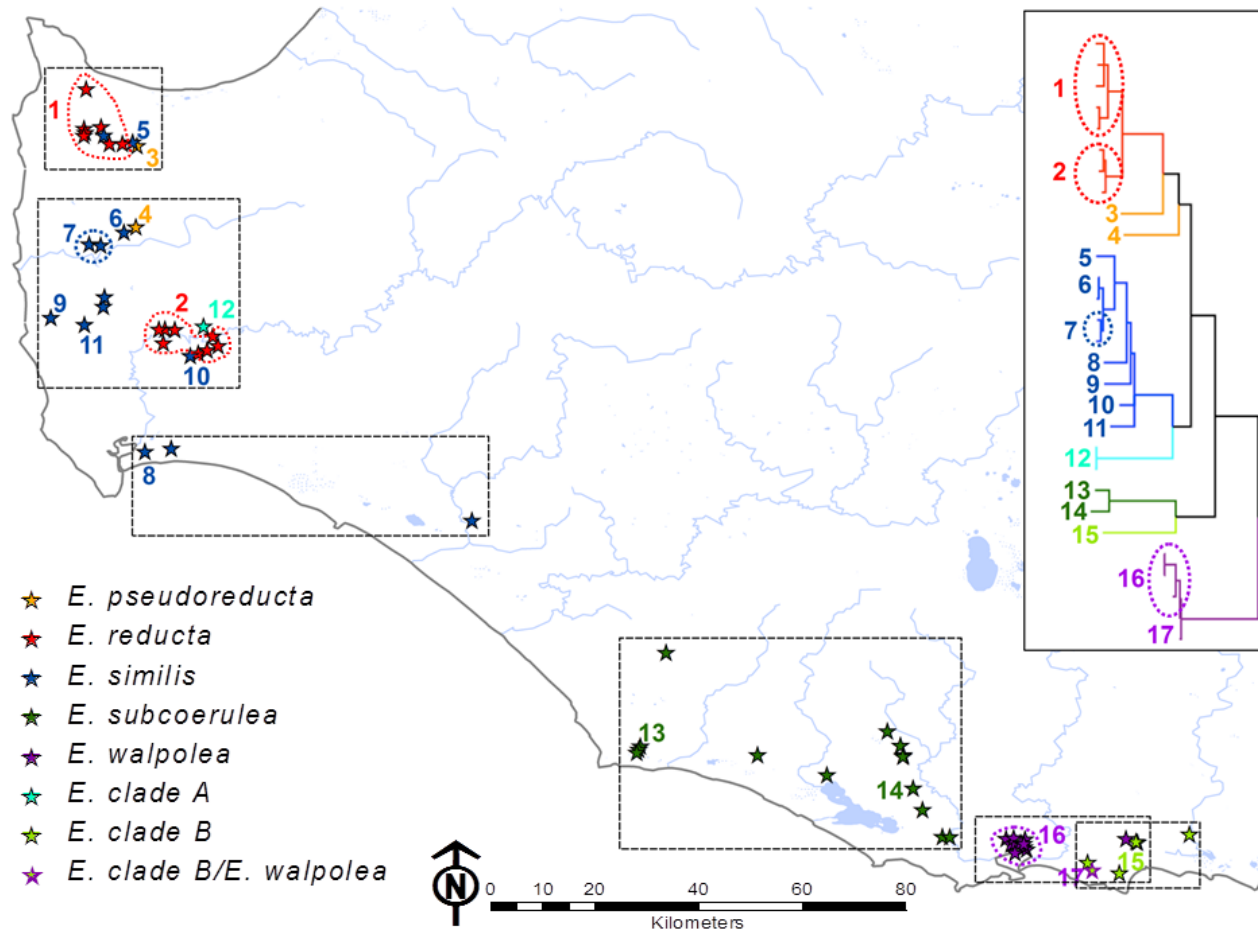
Figure 3.20 Location of all *Engaewa* specimens collected during this study and colour-coded based on the clades identified. The overall phylogenetic tree (from Figure 3.15) is shown for reference, with the numbers referring to the corresponding population on the map. Where a group of closely related haplotypes are present they are circled on both the tree and map. Number 17 refers to the *Engaewa walpolea* found in sympatry with *Engaewa clade B* and is shown by a two-tone star. Squares overlain on the map indicating where networks are shown in Figures 3.21-3.26.

Parsimony haplotype networks were also mapped directly, in order to visualise shared and unique haplotypes at the population level and as a method for understanding genetic connectivity of specific populations. The patterns of haplotype networks can be seen as representing both haplotype diversity (the number of samples per haplotype) and nucleotide diversity (the connectivity of haplotypes). The 16S haplotypes for *E. reducta* form two networks, one composed of all populations found in the formerly recognised northern range of this species (Figure 3.21), and the second containing all of the newly recognised populations to the south (Figure 3.22). Both the northern and southern populations of *E. reducta* show relatively high diversity of haplotypes, though in the northern populations the degree of diversification and the relationship between populations roughly equates to geography (Figure 3.21), whereas in the southern populations even those that are geographically proximate (i.e. in parallel drainages) are highly diverse, with the exception of the populations ACK and BW4 which are adjacent to each other, although on opposite sides of the Blackwood River, and share a haplotype (Figure 3.22).

Engaewa similis haplotypes also form two major networks, representing a northern and southern group of populations, however, this differs from *E. reducta* as there is also a number of additional unique haplotypes and a haplotype pair (Figures 3.21-3.23). Just north of Margaret River a group of three populations share four haplotypes, with BP2 and OSM each sharing a haplotype as well as each possessing a unique haplotype (Figure 3.22). All of the *E. similis* populations south of the Margaret River possess unique haplotypes, not connected to any other (Figure 3.22), until the populations on the Scott Coastal Plain where SC1 contains three haplotypes, which are all only one mutational step distant from the haplotype in SC2, which is an additional single step from the haplotype at SCR (Figure 3.23). *Engaewa clade A*, being a single population, forms a single network of three haplotypes, whilst the two *E. pseudoreducta* haplotypes do not connect (Figures 3.21-3.22).
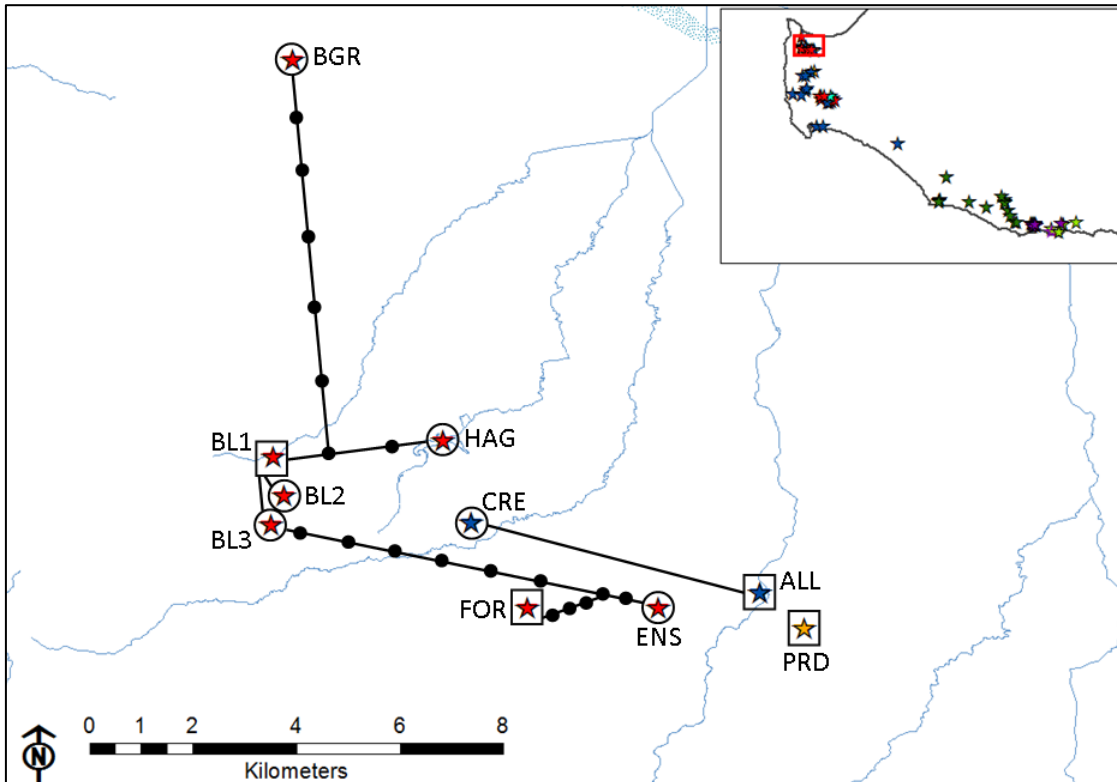
Figure 3.21 16S haplotype networks for northern populations of *Engaewa pseudoreducta* (yellow)*, Engaewa reducta* (red) and *Engaewa similis* (blue). Haplotypes are shown by squares (ancestral) and circles, and related haplotypes connected by lines with the number of mutational steps represented by black dots.
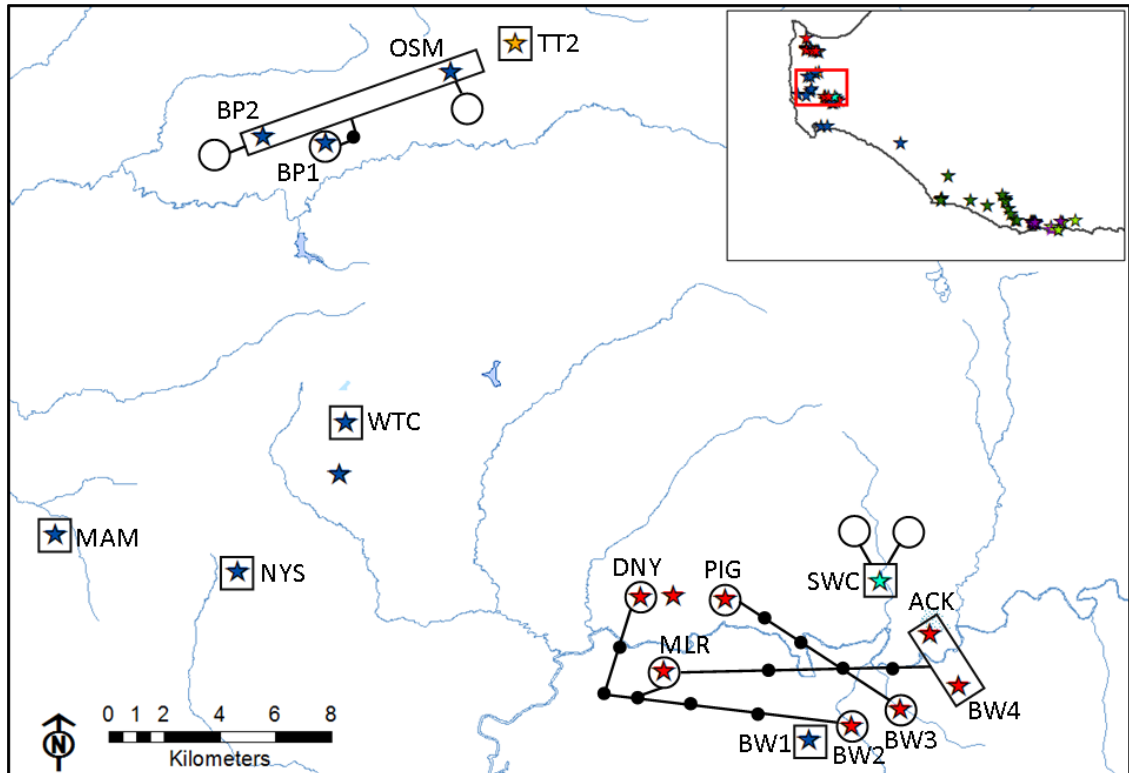
Figure 3.22 16S haplotype networks in the vicinity of the Margaret and Blackwood Rivers for _Engaewa pseudoreducta_ (yellow), _Engaewa reducta_ (red), _Engaewa similis_ (blue) and _Engaewa clade A_ (aqua). Haplotypes are shown by squares (ancestral) and circles, and related haplotypes connected by lines with the number of mutational steps represented by black dots. Haplotypes shared across populations are shown by extended squares/circles encompassing both populations. Where multiple haplotypes were found within populations they are indicated by empty circles branching off the population in question.
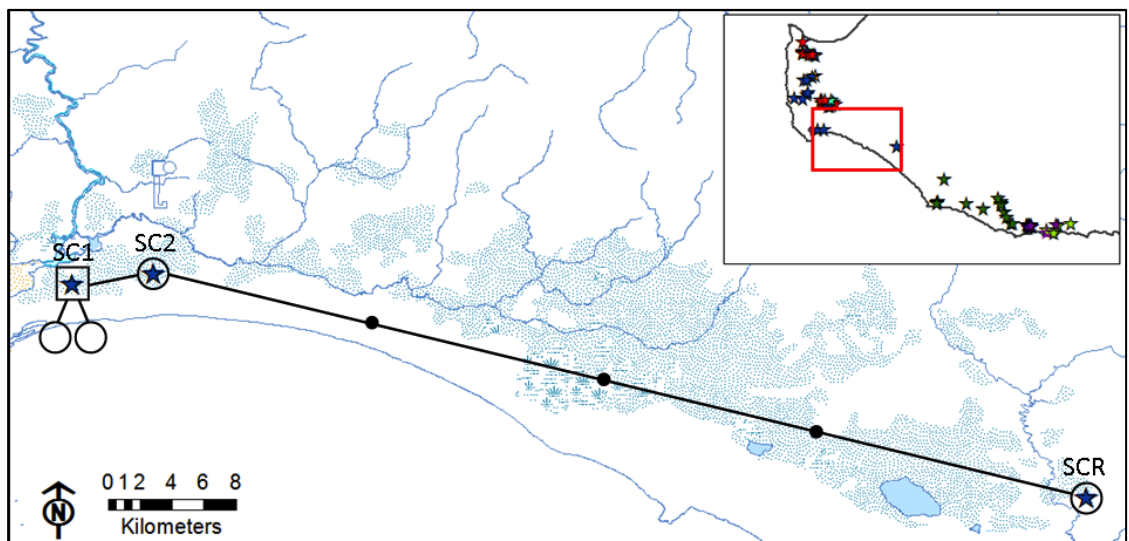
Figure 3.23 16S haplotype networks for southern populations of _Engaewa similis_. Haplotypes are shown by squares (ancestral) and circles, and related haplotypes connected by lines with the number of mutational steps represented by black dots. Where multiple haplotypes were found within a population they are indicated by empty circles branching off the population in question.

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Whilst most populations of *E. subcoerulea* sampled connect to make a single haplotype network (Figure 3.24), the relationship between haplotypes when compared with the relative distribution of populations is highly variable. There are two instances of unrelated haplotypes found in neighbouring populations (WH1 & WH3, TWO & RWP), as well as highly diverse haplotypes at CH1 and CH2. In contrast, there are also two closely related haplotypes (separated by three mutational steps) at the far ends of the species range (Figure 3.24).
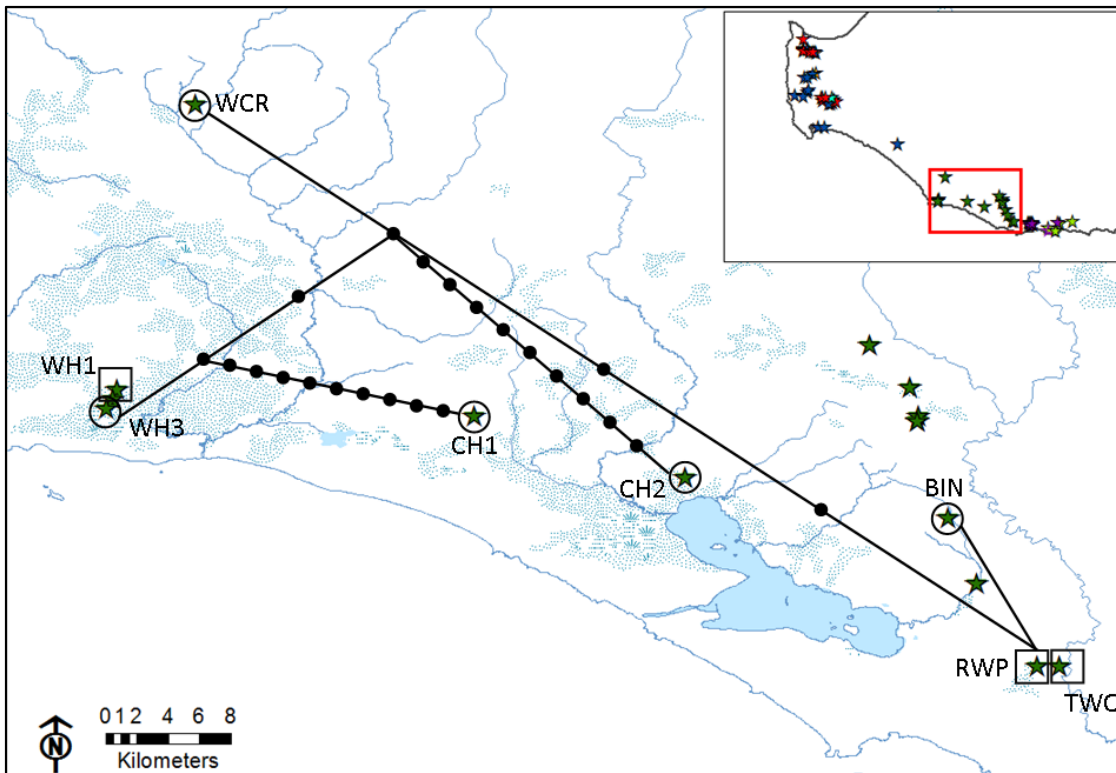
Figure 3.24 16S haplotype networks for *Engaewa subcoerulea*. Haplotypes are shown by squares (ancestral) and circles, and related haplotypes connected by lines with the number of mutational steps represented by black dots.

Engaewa walpolea has a number of closely related haplotypes, including a shared haplotype between three populations (Figure 3.25). One of these shared haplotypes is, by *Engaewa* standards, highly geographically disparate and one population has a haplotype that is significantly genetically distant from the rest of the network (TKN, with nine mutational steps to the nearest haplotype) (Figure 3.25). *Engaewa clade B* has one network of three closely related haplotypes across widely distributed populations, and two isolated haplotypes, one of which is unique to the BRD and the other which is shared between BRD and SRD (Figure 3.26).
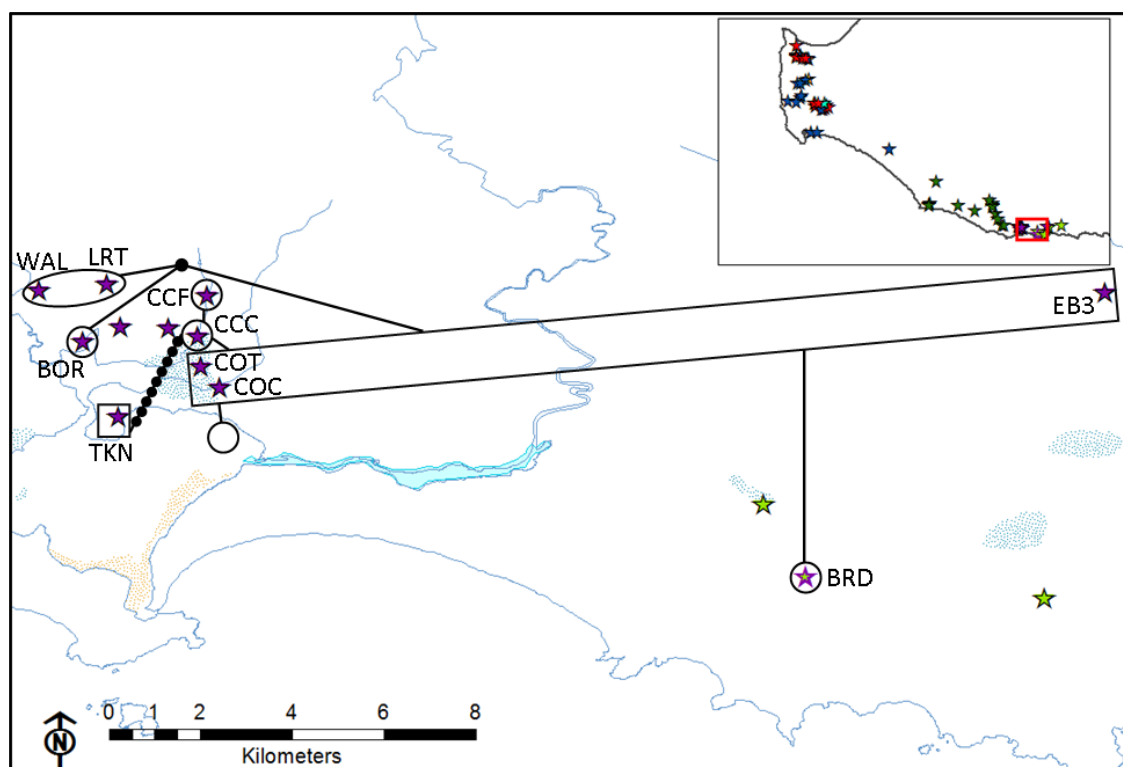
Figure 3.25 16S haplotype networks for *Engaewa walpolea*. Haplotypes are shown by squares (ancestral) and circles, and related haplotypes connected by lines with the number of mutational steps represented by black dots. Haplotypes shared across populations are shown by extended squares/circles encompassing both populations. Where multiple haplotypes were found within a population it is indicated by an empty circle branching off the population in question.
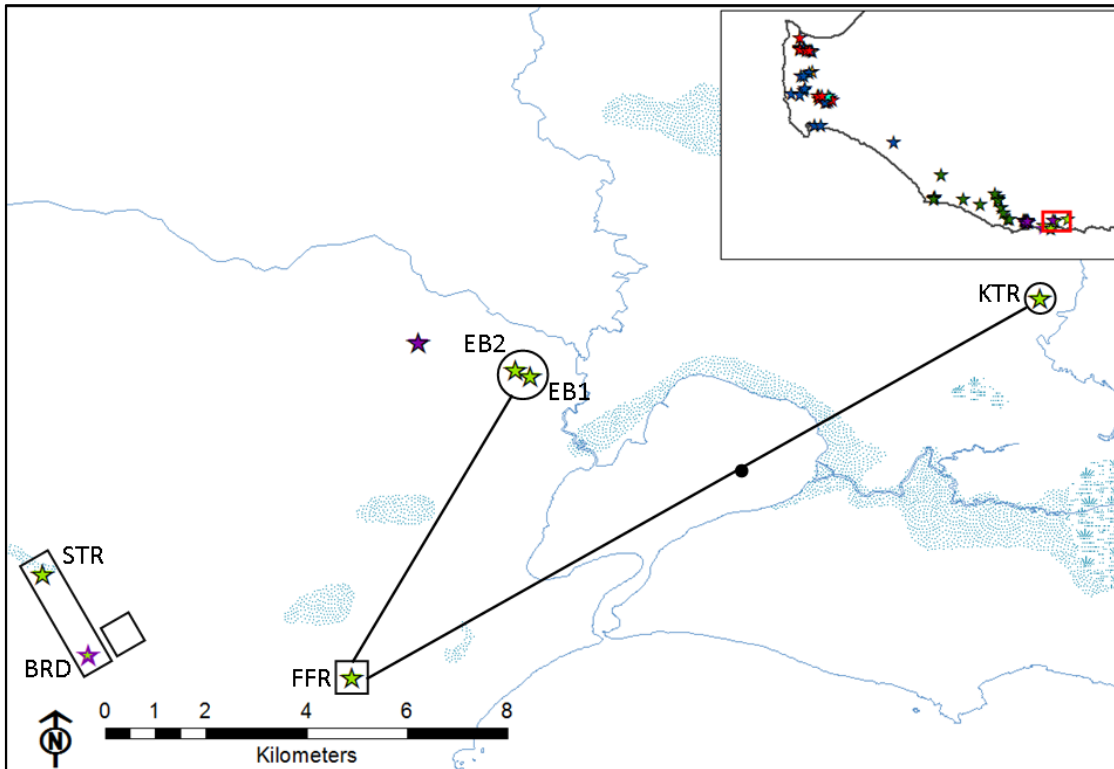
Figure 3.26 16S haplotype networks for *Engaewa clade B*. Haplotypes are shown by squares (ancestral) and circles, and related haplotypes connected by lines with the number of mutational steps represented by black dots. Haplotypes shared across populations are shown by extended squares/circles encompassing both populations. Where multiple haplotypes were found within a population they are indicated by an empty square beside the population in question.

The networks derived from COI are largely congruent with those from 16S, though slight differences were evident due to differences in individuals sequenced and, more biologically significantly, from the higher diversity found in COI. This higher diversity meant that within the northern *E. reducta* group specimens HAG04 & BGR01 and ALL01 & FOR02 formed pairs independent of the rest of the network, and in the southern group ACK01 & ACK02 formed a pair separate from the rest of the network. For *E. similis* SCR03 & SCR04 formed a pair that was not connected to the rest of the network as per 16S. Another significant difference related to *E. similis* is that whereas in 16S specimen OSM02 shares a haplotype with samples BP201 & BP202 it does not for COI. Further differences were also found in the COI network for *E. subcoerulea* with sample WH301 separated from the rest and RWP01 & BIN01 forming a pair separate to the rest, whilst sample TWO02 (which is by itself in the 16S networks) connects to NSC01 & NSC02, which are not in the 16S dataset. *Engaewa walpolea* has

one change between the COI and 16S networks with samples LRT01 & WAC03 being separate from the rest of the network for COI.

As the haplotype networks produced by statistical parsimony provided little insight into the complex distribution pattern of, and relationship between, *E. similis* populations and the *E. clade A* population, further analyses were conducted by using the median-joining method. The network produced by the Greedy-FHP criterion has both the fewest median vectors and smallest number of mutational steps and thus provides the most parsimonious result (Figure 3.27b). The increase in the value of epsilon produced significantly more median vectors and multidimensional reticulations (Figure 3.27d). The networks produced by MJ (and RM) provide connections within *E. similis* that largely coincide with their geographic distribution (i.e. the nearest genetic haplotype is closest to the nearest geographic haplotype) (Figure 3.27). These networks also suggest that the node representing the most recent common ancestor of the *E. similis/E. clade A* group may be located within the northern to central portion of the species extant distribution, while none of the methods used suggest a close connection between *E. clade A* and the nearby *E. similis* population south of the Blackwood River (Figure 3.27a-c).
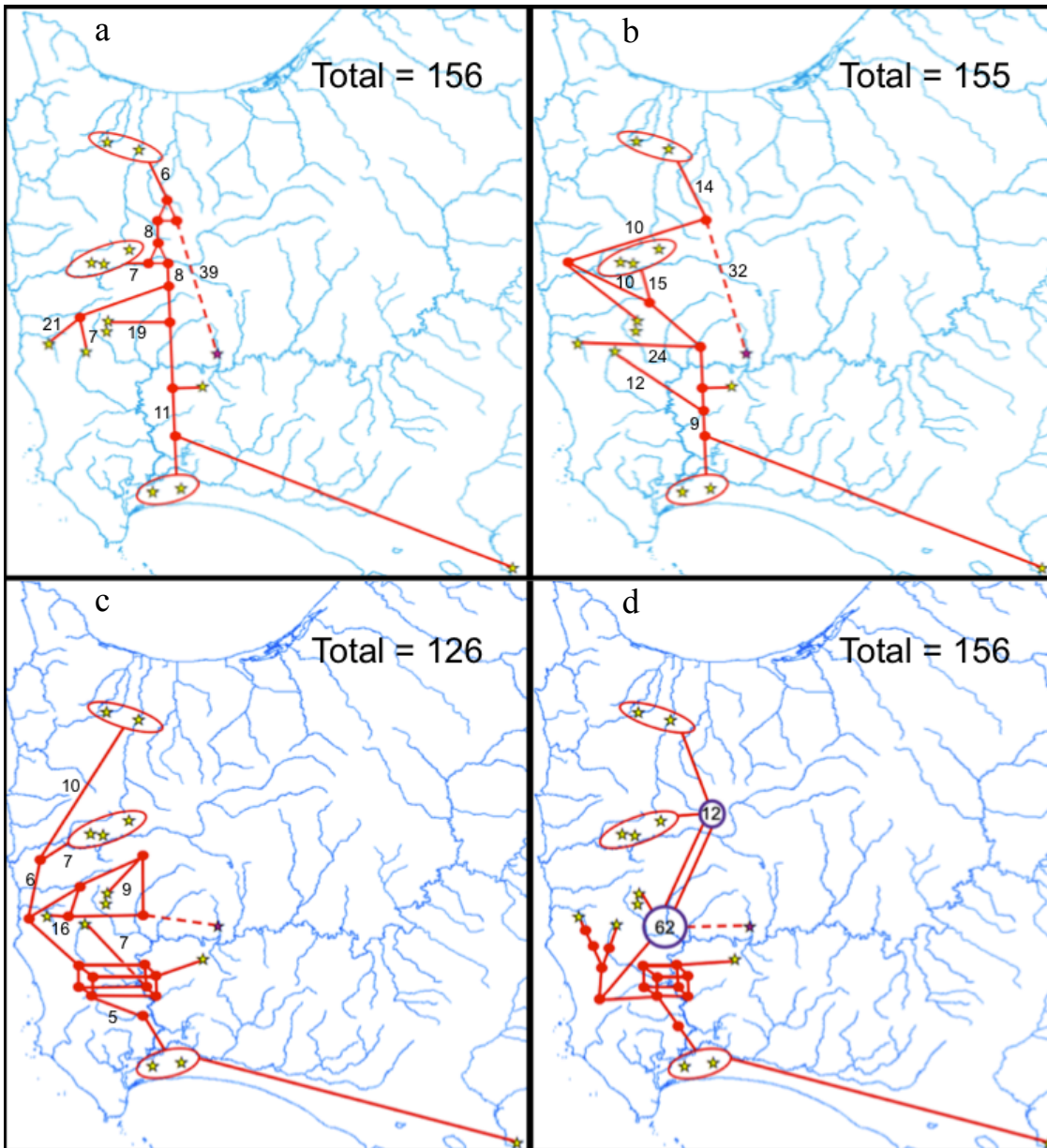
Figure 3.27 Geographic distribution of 16S haplotype networks for populations of *Engaewa similis* (yellow stars) and *Engaewa clade A* (purple star) using the criterion (a) Connection Cost, (b) Greedy-FHP, (c) Connection Cost ε=10, and (d) Reduced Median. Missing haplotypes (median vectors) along connecting lines are represented by red dots. Open red circles represent groups of closely related haplotypes (no more than one mutational step) across populations and purple circles represent large numbers of median vectors, with the number of vectors shown in the circle. For a-c the value of all connections greater than 5 mutational steps are shown.

It is difficult to explain the discrepancies between the various networks produced via the MJ and RM approach as, with relatively few taxa, it might be expected that the true network could be determined, or very nearly so. Interestingly, the RM network has more median vectors (including a cycle and cube) than the MJ networks.

This appears counterintuitive as the RM method is designed to simplify large, complex data sets. Whereas increasingly complex networks are generally considered to be a more accurate representation of the true pattern within the data, as they capture more of the uncertainty inherent in these relationships, the fact that the loss of data via the exclusion of 10 multistate positions created more complexity may actually be a sign that the true signal within the data was reduced (Figure 3.27c). It has been found that an increased number of sites in the analysis generally increases error, presumably because it increases the number of unique haplotypes and, therefore, the number of inferred trees with additional connections and internal nodes needed (thus creating uncertainty about how they are related) (Woolley, Posada & Crandall, 2008). Trontelj *et al.* (2005) suggest that (based on the Network instruction manual) long branches may make the analysis unreliable and that using RM can circumvent this issue, though no justification for this interpretation was found in the current manual. It has also been noted (e.g. Morrison, 2005) that the different median-joining options can produce vastly different networks, though there has been no quantitative assessment of these and it is unclear how to choose between them.

3.4 Discussion

3.4.1 Position of *Engaewa* within the parastacids

When formally describing and erecting the genus *Engaewa* in 1967, Riek noted morphological similarities between crayfish of this genus from SWA and members of another genus, *Pseudengaeus* Clark (subsequently synonymised with *Engaeus* by Riek in 1969) from south-eastern Australia, but justified the recognition of the new genus *Engaewa* on the basis of the form of the sternal keel and the pleural lobe of the first segment (Riek, 1967a). That the genus *Engaewa* is monophyletic was clearly assumed by Riek (1967a) (as he erected a new genus in which to place the three species he described) yet he also stated that it was allied to *Engaeus*. The monophyly of the genus was subsequently supported by Horwitz and Adams (2000) using morphological and allozyme data. The argument in favour of monophyly was further strengthened by Crandall *et al.* (1999), Schultz *et al.* (2009) and Toon *et al.* (2010) as these three studies

each supported reciprocal monophyly of the genus amongst the other Australian crayfish based on 16S rDNA sequence data.

A review of Australasian crayfish by Crandall *et al.* (1999) using the 16S region of the mitochondrial genome concluded that *Engaewa* comprises one of three major clades of freshwater crayfish in Australasia and is a sister group to all other Australasian genera. Unfortunately, Crandall *et al.* (1999) did not implement an outgroup with which to root their tree and although the tree they presented appeared to root *Engaewa* as the basal clade, the authors stressed that this conclusion should not be drawn from their data. An unrooted tree with three clades provides three possible arrangements, only one of which would place *Engaewa* as the ancestral clade. Crandall *et al.* (1999) presented their analysis based on two species of *Engaewa* (*E. similis* and *E. subcoerulea*), however, the *E. subcoerulea* sequence (Genbank Accession AF135983.1) they used in the analysis is erroneous, and is most likely a sequence for a North American crayfish, probably a species of *Orconectes* Cope or *Procambarus* Ortmann. Obviously the conclusions presented by Crandall *et al.* (1999) must be interpreted with considerable caution considering this error.

Crandall *et al.* (2000a), in a review of South American crayfish, also presented phylogenetic trees with additional South American samples added to the data from Crandall *et al.* (1999), which again appeared to suggest *Engaewa* is the basal clade. Toon *et al.* (2010), however, presented similar trees, though this time rooted with non-parastacid species, which no longer presented *Engaewa* as a basal clade. Both of these studies also suggest that the Australian parastacids are a non-monophyletic assemblage, with *Ombrastacoides* and *Spinastacoides* being sister to the New Zealand species, and a member of a clade also containing the Madagascan species.

The proposal by Crandall *et al.* (1999) that *Engaewa* forms a distinct lineage and is sister to the rest of the Australian parastacids, is also contradicted by other theories proposed, which have concentrated on ecologically related morphological adaptations. These include the reviews by Riek (1972), which placed *Engaewa* alongside *Engaeus* and *Tenuibranchiurus* as well as *Parastacus* from South America, and Horwitz (1988b)

in which *Engaewa* was grouped with *Engaeus, Tenuibranchiurus, Gramastacus,* and *Geocharax*. The relationships proposed by Riek (1972) and Horwitz (1988b) both rely primarily on the interpretation of morphological and behavioural adaptations to burrowing. Riek (1972) proposed that the clade to which *Engaewa* belongs constitutes 'strong burrowers', which are defined by holding the chelae and moving the finger in a vertical or sub-vertical plane. Horwitz's (1988b) grouping of *Engaewa* with *Engaeus, Tenuibranchiurus, Gramastacus,* and *Geocharax* was based on a number of characters with perhaps the most prominent, and from a cladistic point of view the most powerful, being the presence of a uniquely derived character in the form of a flap on the second abdominal pleonite of reproductively active females, which may be an adaptation that keeps the microenvironment around the eggs moist.

Interestingly, the hypothesis of Horwitz (1988b) includes a subset of genera ascribed to the category of a Type 2 burrower (those digging burrows connected to the water table, corresponding roughly to Hobbs' primary burrowers (Hobbs Jr., 1942)) by Horwitz and Richardson (1986). Horwitz and Richardson (1986) assigned species from the genera *Cherax, Engaeus, Engaewa, Parastacoides* Clark*, Tenuibranchiurus, Geocharax* and, to a lesser extent, those from the genera *Euastacus* and *Astacopsis* to this category. However, it is important to be aware that the authors noted different species from a single genus, or even different populations of a single species, may construct burrows that belong to different types and the burrowing habit itself is believed to be highly plastic (Austin & Knott, 1996). The notable discrepancy between the hypothesis of Horwitz (1988b) and the classification of Horwitz and Richardson (1986) is the position of *Gramastacus*. *Gramastacus insolitus* Riek does not burrow (McCormack, 2014; Zeidler, 1982), although the recently described *Gramastacus lacus* McCormack does (McCormack, 2014). *Gramastacus insolitus* has been shown to utilise the burrows of *Geocharax falcata* Clark and *Cherax destructor* in the Grampians National Park, Victoria (Johnston & Robson, 2009), and possesses the aforementioned flap on the second abdominal segment – a 'burrowing' adaptation. That the burrowing habit varies yet the reproductive flap described by Horwitz (1988b) is consistently expressed in certain genera suggests that, while it most likely developed as a burrowing

adaptation, it is probably shared as a result of common descent, rather than being separately derived in response to a particular habitat type.

In an attempt to provide further clarity to this issue, Schultz *et al.* (2009) revisited these relationships using analyses based on 16S DNA sequence data and found statistical support for the theory of Horwitz (1988b) and rejected the relationships proposed by Crandall *et al.* (1999) and Riek (1972). The same partitioning is also supported in the phylogeny presented by Toon *et al*. (2010) based on a combined analysis of 16S, COI, 18S and 28S DNA sequences. Interestingly, the phylogenetic relationships presented in these two studies suggest that the vertical orientation of the chelae (upon which Riek (1972) based his grouping) is homoplasious, with it either being lost twice (*Gramastacus* and *Geocharax*) or gained independently at least twice (once in the *Tenuibranchiurus* lineage and again in the lineage containing *Engaeus* and *Engaewa* spp). An alternative interpretation of the orientation of the chelae is that rather than being viewed as two states they should be considered as representing a continuum, which is directly linked to the degree to which the crayfish burrows (more vertical equating to stronger burrowing habitat) (Richardson, 2007). Riek (1972) did actually acknowledge that *Geocharax* and *Gramastacus* hold their claws on more of an oblique angle, but still considered them to open horizontally.

Based on the aforementioned studies and the data from this thesis, it appears likely that *Engaewa* is monophyletic and forms part of a clade with, or is at least nearest neighbour to, the other genera considered in this study to represent the burrowing clade of Australian crayfish from eastern Australia. The phylogenetic tree created from the combined 16S and GAPDH dataset, including the data from Schultz *et al*. (2009), presented in this thesis supports the conclusion that, with the exception of *Engaeus*, all of the other genera included (*Cherax, Engaewa, Geocharax, Gramastacus, Tenuibranchiurus*) are monophyletic (Figure 3.3). It also supports the suggestion that *Engaeus* appears to be composed of two distinct lineages, which arguably deserve generic level recognition – *Engaeus lyelli* and *Engaeus sensu stricto* (following Schultz *et al*., 2009).

A further point to be drawn from the phylogenetic reconstructions presented in this study is that no definitive statement can be made regarding the relationship between *Engaewa* and the two *Engaeus* clades, as there are no trees showing strong support for the branch nodes. Schultz *et al*. (2009) stated that the true grouping between *Engaewa* and the two *Engaeus* clades should be (*Engaeus sensu stricto*(*Engaeus lyelli*:*Engaewa*)) as the authors (2009, p. 586) stated that "*Engaeus lyelli* [is] seemingly closer to *Engaewa* than to other *Engaeus* species". However, the combined 16S and GAPDH tree of Schultz *et al*. (2009) provided non-supported values for the split between *Engaeus lyelli* and *Engaewa* (PP 0.56, BS <50%) and the sister relationship between *Engaeus sensu stricto* and an *Engaeus lyelli/Engaewa* clade was only weakly supported (PP 0.80, BS 55%). The tree from this study favours a relationship of (*Engaeus lyelli*(*Engaeus sensu stricto*:*Engaewa*)) (Figure 3.3), however, the split between *Engaeus sensu stricto* and *Engaewa* was not supported (BS <50%) and the sister relationship between *Engaeus lyelli* and an *Engaeus sensu stricto/Engaewa* clade only weakly supported (BS 63%).

The lack of resolution for this relationship between *Engaewa/Engaeus* is further highlighted by the presentation of maximum-likelihood maps (MLM). When all available sequences from GenBank and the sequences derived in this study were included, over 50% of quartets suggested *Engaewa* is sister to an *Engaeus sensu stricto/Engaeus lyelli* clade (Figure 3.5). The grouping suggested by Schultz *et al.* (2009) as being the most likely (i.e. (*Engaeus sensu stricto*(*Engaewa*:*Engaeus lyelli*))) is, based on this data, considered the least likely with support of only 1%. Over one-third of quartets remained unresolved, suggesting there is no clear phylogenetic signal in the data.

In order to truly understand the relationships between genera (as well as obviously being significant for the biogeography of the genus *Engaewa* – as shall be discussed later) it is valuable to derive estimates of divergence dates. However, two recent attempts to date various nodes within the freshwater crayfish have placed vastly different dates on the time to the most recent common ancestor (TMRCA) for *Engaewa*. Schultz *et al*. (2009) used a relaxed molecular clock analysis in the program BEAST,

which was based on published mutation rates for the 16S marker in crabs (Stillman & Reeb, 2001; Sturmbauer *et al.*, 1996). Toon *et al.* (2010) also used a Bayesian approach though they used the program MULTIDIVTIME and used fossils to calibrate a number of nodes within their phylogeny. It is unclear which of these two approaches is likely to yield the most accurate estimate, as both have widely acknowledged problems and potential errors involved (for example see Rutschmann, 2006). Furthermore, these two studies each used different data, both in terms of the taxa and gene regions included.

Schultz *et al.* (2009) and Toon *et al.* (2010) both provided estimates for the divergence dates between Australian crayfish genera using phylogenetic trees that place *Engaewa* basal to the rest of the burrowing clade. The tree presented by Toon *et al.* (2010) did provide statistical support for the basal placement, although the tree of Schultz *et al.* (2009) did not. The phylogeny of the Australian burrowing crayfish presented in this study (i.e. Figure 3.3) (like that of the accepted overall phylogeny of Schultz *et al.* (2009) (i.e. not the BEAST species tree)) placed *Engaewa* in a lineage with *Engaeus*, when trying to clarify the relationship of the burrowing taxa, but on the *BEAST (or BEAST in the case of Schultz *et al.* (2009)) tree used for dating, *Engaewa* represented its own lineage.

Schultz *et al.* (2009) suggested a date for TMRCA of *Engaewa* and the rest of the burrowing crayfish clade in the region of 50-20 MYA but Toon *et al.* (2010) presented an estimate that is some 100 million years earlier around 150-100 MYA. Breinholt, Perez-Losada and Crandall (2009) dated the split between *Engaeus* and *Geocharax* to ~145 MYA, however, the tree on which this study was based did not show the Australian crayfish as being monophyletic, nor did it include all genera, and thus the date is of doubtful accuracy. The dating undertaken in this study was intended primarily for looking at speciation events that have occurred within the genus, however, the node separating the ingroup from the outgroup (i.e. *Engaewa* and specimens from *Engaeus* and *Geocharax*) is largely consistent with that of Toon *et al.* (2010), placing the date at ~122 MYA (Figure 3.17). Whilst the date derived from this study admittedly has a very large 95% confidence range (spanning from ~197-65 MYA), even the lower boundary is earlier than the estimate of Schultz *et al.* (2009).

Fossilised burrows attributed to parastacids have been uncovered in the Otway and Strzelecki ranges in Victoria and dated to ~116-106 MYA (Martin *et al.*, 2008). These burrows are considered to most closely represent those made by *Engaeus* out of all the extant taxa (Martin *et al.*, 2008), suggesting strongly burrowing crayfish were present by then, thus further confirming the later date of the TMRCA of *Engaewa* provided by Toon *et al.* (2010) is at least plausible. Whilst it is difficult to draw any conclusion regarding the TMRCA of *Engaewa* from these studies considering their sizeable discrepancy, they do suggest that the *Engaewa* lineage is one of the oldest in the Australian crayfish and possibly the oldest within the burrowing group. The significance of the alternative hypotheses of the TMRCA for *Engaewa* is discussed in further detail in Chapter 5.

In summary, the phylogenetic trees and genetic distances reported in this study support the current assumption of monophyly of all genera recognised in the Australian members of the Family Parastacidae (with the caveat that the recognition of two lineages in *Engaeus* as proposed by Schultz *et al.* (2009) is accepted). The recognition of a burrowing clade within this fauna is also supported. The data also highlight the high genetic diversity contained within the genus *Engaewa*. Further to this, a close phylogenetic affinity between *Engaewa* and *Engaeus* (both *sensu stricto* and *lyelli*) is reaffirmed. Both the phylogenetic trees and the MLM fail to provide convincing evidence for a specific relationship between the *Engaewa*/*Engaeus* groups, although there is a suggestion in this study that the relationship proposed by Schultz *et al.* (2009) is the least likely. A sister relationship between *Engaewa* and *Engaeus sensu stricto* was hinted at by the smaller dataset, but once all available sequences were included a sister relationship between the two *Engaeus* lineages appeared more likely. In order to attempt to resolve this relationship additional genetic markers and/or morphological characters will need to be utilised. This relationship, including the order of branching between the three groups (or the presence of a hard polytomy) and the date at which they may have split, is highly significant from a biogeographical standpoint and will be discussed further in Chapter 5.

3.4.2 Species delineation and morphology

The first step in the species delineation procedure followed in this study was to produce a phylogenetic representation of relationships based on a number of molecular markers. Whilst there is still debate regarding the optimal strategy for producing a 'true' phylogeny (due to issues such as gene trees versus species trees, combining markers, partitioning strategy, tree reconstruction methods, coalescent approaches, etc. (for examples see Avise, 2004)), studies have shown that conducting a total simultaneous analysis can result in a highly resolved tree with high support values for the maximum number of nodes (e.g. Baker, Wilkinson & DeSalle, 2001; Creer, Malhotra & Thorpe, 2003; Flynn & Nedbal, 1998). It has been shown that by concatenating enough data from individual incongruent markers a tree with 100% bootstrap support for all nodes can be achieved (Rokas, Williams, King & Carroll, 2003), whereas seeking congruence through conducting separate analyses, which are then combined can result in an almost total loss of phylogenetic signal (Creer *et al.*, 2003). Such a loss of resolution has been one of the key criticisms of consensus approaches (Allard, Farris & Carpenter, 1999; Eernisse & Kluge, 1993; Nixon & Carpenter, 1996) as, although phylogenetic signal in data is additive, noise is actually averaged over all data partitions (Wenzel & Siddall, 1999).

There is general acceptance that the use of multiple data sources (i.e. mitochondrial and nuclear genes) adds to the number of independent markers in the dataset making it more likely that inaccuracies in individual genetic markers will be overcome, thus improving the likelihood of reconstructing the true species phylogeny (Niemiller *et al.*, 2012; O'Meara, 2010; Toon, Finley, Staples & Crandall, 2009; Yang & Rannala, 2010). As the phylogenetic tree produced by Bayesian analysis from the combined dataset has highly significant support values (all PP ≥0.98 – see Figure 3.11) and there is no conflict with the tree produced via ML, nor the species groupings evident on the mitochondrial based trees, it is suggested that this represents the most robust estimate of the phylogeny of *Engaewa* currently available. Therefore, each of the colour-coded clades within the phylogenetic trees presented in Section 3.3.1 appear to represent a distinct evolutionary lineage.

The accepted tree (Figure 3.15) places *E. walpolea* as the basal clade in the genus, followed by *E. subcoerulea*/*E. clade B* and then the *E. reducta* complex (containing the species *E. reducta*, *E. similis* and *E. pseudoreducta* (following Horwitz & Adams, 2000) and the previously unrecognised *E. clade A*); a finding that is in agreement with the phenogram produced by Horwitz and Adams (2000) based upon analysis of 17 allozymes. The topologies from the BA and ML trees were essentially the same, although ML did not produce highly significant statistical support for a small number of branching points within the tree (Figure 3.12). Analyses of individual markers did not contradict the accepted tree with regards to the groupings of species level clades, however, the mitochondrial markers did place *E. subcoerulea* as the basal clade within the genus (and was highly supported) but failed to produce a clearly supported pattern for the other species. Discordance between mtDNA and nuDNA trees is a common phenomenon and well documented in the literature (for examples see Buckley, Cordeiro, Marshall & Simon, 2006). Discordance between different gene trees and species trees can be explained by genetic polymorphism (gene duplication events), introgression between related species or differentiated lineages (introgressive hybridisation) or incomplete lineage sorting (incomplete and stochastic sorting of ancestral polymorphisms) (reviewed in, for example, Maddison (1997)). Incomplete lineage sorting may represent a problem for organismal phylogeny if the time needed for haplotypes within a lineage to coalesce is greater than the time between successive speciation events (Page & Holmes, 2000).

Concerns have been raised that partitioned Bayesian analyses can provide strong posterior probability values for short interior nodes, which may be only weakly supported by ML bootstrap analysis (e.g. Leache & McGuire, 2006). It has previously been demonstrated that when confronted with increasingly short internodes, posterior probability values can become unpredictable and Bayesian analyses may place strong support on an arbitrarily resolved hard polytomy (Lewis, Holder & Holsinger, 2005). However, partitioned Bayesian analyses may allow for more precise specification of models than is possible for ML analysis, and better modelling of the substitution process improves phylogenetic signal, thus resulting in higher support (Brandley, Schmitz & Reeder, 2005; Leache & McGuire, 2006). Thus, mixed-model phylogenetic

methods may reduce systematic error and more realistic modelling of the heterogeneous nature of DNA evolution could resolve difficult phylogenetic problems (e.g. a rapid radiation) that other approaches cannot (Brandley *et al.*, 2005). The lack of resolution in the mitochondrial trees, combined with the lineage through time plot and statistics and the *BEAST estimates of nodal ages, suggest a relatively recent and rapid pattern of radiation. This may mean that there has been insufficient time for lineage sorting and coalescent processes to have occurred for these markers, thus obscuring the true phylogeny. Whilst some markers do not provide support for the arrangement of various nodes, the species groupings on all trees are consistent.

There is also support for the clades identified on the trees to be considered as distinct lineages from the inter- and intra-clade genetic divergences. The barcoding gap was originally formulated based on the difference between intraspecific and mean interspecific, congeneric distances, however, numerous authors have pointed out that using the smallest interspecific distances would be more appropriate (e.g. Meier, Shiyang, Vaidya & Ng, 2006; Meier, Zhang & Ali, 2008; Meyer & Paulay, 2005; Moritz & Cicero, 2004; Vences, Thomas, Bonett & Vieites, 2005a; Vences *et al.*, 2005b). Rather than working with a predefined, standard level of genetic divergence that can recognise species (as per the original formulation of the barcoding concept) many authors have compared the divergences between candidate species to that between already described species, on the assumption that this may distinguish cryptic species. This approach has been applied to a wide range of taxa, including crayfish (e.g. Coughran, Dawkins, Hobson & Furse, 2012; Furse, Dawkins & Coughran, 2013).

The highest intra-specific genetic distance for 16S (see Table 3.6a) for the lineages identified was 0.078 for *E. pseudoreducta*. This may be misleading as the relationship between the two populations (a 'known' *E. pseudoreducta* site and the site at Payne Road) included in this analysis is somewhat unclear (as described in 3.3.2), however, they are being conservatively lumped together until more data are available. The next most divergent lineage is *E. similis* at 0.057, which is just over half of the lowest between lineage comparison (0.100 between *E. reducta* and *E. pseudoreducta*) although as previously mentioned the comparisons involving *E. pseudoreducta* may not

be a true reflection of the diversity. The next lowest value is 0.126 between *E. similis* and *E. clade A*, which prior to this study were not recognised as separate lineages. These results suggest that for 16S there is a clear gap between the within and between species diversity which ranges from approximately 1.5 times the distance to over 50 times, and suggests that it may be possible to define a barcoding gap.

The situation for COI is less clear (see Table 3.6b), although this again seems to be due to the two divergent populations within the *E. pseudoreducta* clade. The value within *E. pseudoreducta* is 0.167, higher than many of the other between species comparisons, whilst species pair comparisons including *E. pseudoreducta* are as low as 0.057 between it and *E. reducta*. If the *E. pseudoreducta* clade is excluded there again appears to be a clear gap between inter- and intra-species divergences with the highest within species value being 0.073 and the lowest between species pairwise comparison being approximately double at 0.145 (between *E. similis* and *E. reducta*) and the largest differences are again approximately 50 times higher.

There is a clear gap between the intraspecific and interspecific divergence values for both 16S and COI in *Engaewa* based on the mean values, for 16S based on the raw values and for the raw values of COI when *E. pseudoreducta* is excluded. By recognising seven distinct species level lineages intraspecific divergence values were approximately <1-8% for 16S with a mean of 4% and <1-17% for COI with a mean of 5%. If the potentially misleading '*pseudoreducta*' data were excluded it made only a slight difference to the mean intraspecific divergence values as they became 3% for 16S and 4% for COI. Interspecific divergence values were 10-26% with a mean of 18% for 16S and 6-28% also with a mean of 18% for COI. Again, excluding the *pseudoreducta* data made only a slight change with the means increasing to 19% for 16S and 21% for COI. A summary table of these values is presented in Table 3.11.

Table 3.11 Summary of corrected intra- and inter- specific genetic divergences (presented as percentages) for both 16S and COI for the *Engaewa* samples used in this study. A potential barcoding gap is identified between the highest intraspecific distances and lowest interspecific distances.

	16S				COI			
	Intra-specific %		Inter-specific %		Intra-specific %		Inter-specific %	
	Highest	Mean	Lowest	Mean	Highest	Mean	Lowest	Mean
Including *pseudoreducta*	7.8	4.0	10.0	18.1	16.7	5.4	5.7	17.6
Excluding *pseudoreducta*	5.7	3.3	12.6	18.9	7.3	3.5	14.5	20.5

The intraspecific values reported in this study are comparable to those reported for European (Grandjean *et al.*, 2000), North American (Crandall, 1996), and Japanese crayfish (Koizumi *et al.*, 2012). The values are also similar to the uncorrected p-distances in amphibians reported by Vences *et al.* (2005a) for COI and Vences *et al.* (2005b) for 16S. Vences *et al.* (2005a) concluded that applying the 10X proposal of Hebert *et al.* (2004b) would require threshold values in the region of 40-50% (similar to what would be required for *Engaewa*) and suggested that this is unreasonable, as it is above the saturation plateau of COI and exceeds the highest divergence values observed among any pair of amphibian species. Instead they proposed tentative guidelines for amphibian species of 5% for 16S and 10% for COI divergence (Vences *et al.*, 2005a).

To test whether there is a standard divergence value similar to that reported here for *Engaewa* that can be used to delineate species in other crayfish, this approach was tested for the closely related and speciose crayfish genus *Engaeus*. All *Engaeus* 16S sequences from GenBank (excluding *Engaeus lyelli*) were used to calculate genetic distances between species as per the procedure for *Engaewa*. Pairwise interspecific distances were found to range from 0.1% (between *Engaeus merosetosus* Horwitz and *Engaeus sericatus* Clark) to 36% (between *Engaeus disjuncticus* Horwitz and *Engaeus rostrogaleatus* Horwitz), with a mean of 17%. Whilst the average divergence between species of *Engaeus* was similar to that of *Engaewa* there were many cases of very small distances in *Engaeus*. Thirty-six species pairs of *Engaeus* had a lower divergence than the highest intraspecific divergence for *Engaewa* (*E. pseudoreducta*). Three possible conclusions can be drawn from this result; there may not be a divergence value (for

16S) that can be applied across multiple crayfish groups as a standard for recognising species (perhaps due to different rates of molecular evolution in different lineages – as suggested by the branch lengths in Figure 3.3 for instance), and/or, the current species delineations for a number of *Engaeus* species may need to be revised, and/or, *Engaewa* contains a number of (possibly cryptic) species that cannot be distinguished based on the methodology this study (which, as previously outlined, is conservative).

Another alternative method of species delineation also tested was the use of haplotype networks. Use of this method for *Engaewa* would result in a great many species, as at a 90% confidence interval (rather than the suggested 95%) nineteen species would be recognised. This is a surprising result as examples abound within the crayfish literature of closely related species that can be connected via haplotype networks at a 95% confidence interval (e.g. Buhay & Crandall, 2005; Buhay *et al.*, 2007). Therefore, this method appears neither suitable for delineating species within the genus *Engaewa* specifically, or crayfish generally.

The lineages identified on the phylogenetic trees and supported by the genetic divergences, can be seen as corresponding to the 'Candidate Species' of Vences *et al.* (2005a; 2005b) or to the 'Unconfirmed Candidate Species' of Vieites *et al.* (2009). Situations where divergent genetic lineages within a single described species are detected have often, after subsequent revision of the morphology, been found to represent previously overlooked species level entities (Hebert *et al.*, 2004a; Hebert *et al.*, 2004b). This appears to be the case in this study.

The discovery of *E. clade A* as a unique lineage was somewhat pre-empted by Horwitz and Adams (2000 p.673) who noted that "three specimens from Spearwood Creek near the Blackwood River are regarded as errant individuals for the purposes of this review: they display character states which are different to each other, new to the species, and allied to one or other species. Their exact status, and those of all populations north of the Blackwood River and east of Margaret River township, must await a detailed genetic and morphological treatment of more individuals".

The presence of *E. clade B* was not suspected prior to this study, although Horwitz and Adams (2000 p. 664) stated that for *E. subcoerulea* "considerable variation exists on the general diagnostic theme presented". Much of the variation noted within the description of *E. subcoerulea* by Horwitz and Adams (2000) can now be viewed as differences between these two lineages, rather than variation within a single species. Specimens now placed in both *E. subcoerulea* and *E. clade B* were examined by Horwitz and Adams (2000), however, the diagnostic setae of *E. clade B* were not described. In the species '*Diagnosis*' for *E. subcoerulea*, diagnostic characters were only attributed to the large dimorph and thus only this chela was described. The only description of the small dimorphic chelae for *E. subcoerulea* comes under '*Morphological Variation*' where Horwitz and Adams (2000 p. 665) state, "For small dimorphs, the propodus and dactyl bear more bristle setae …". This perhaps suggests that the now diagnostic setae were observed in some specimens, but were not considered important.

The significance of the chelae setae, from either a phylogenetic or functional view, is hard to determine. Setae on the pereopods of decapods have been interpreted as functional structures that contribute to the relatively complex grooming behaviour of these animals. However, the specialisation of grooming appendages in macrurans is intermediate within the decapods, and is associated with a transition from free swimming (high grooming) to walking (low grooming) (Bauer, 1986). This is believed to be due to the high significance of epizoic body fouling influencing swimming in natant decapods (Bauer, 1981). Further to this, Bauer (1981) hypothesised that epizoic fouling pressures would be lower in decapods that burrow directly into sediments. In decapod crustaceans, general body grooming is performed by minor chelipeds and by brushes on the posterior walking legs (Bauer, 1981). Astacidea do not have antennal grooming brushes on the 1st pereopod, as some other decapods do, and instead generally use the third maxilliped (Bauer, 1986), while the second and third pairs of chelipeds have been reported to pick at the exoskeleton and the carapace is often scraped by pereopods four and five (Bauer, 1981; Thomas, 1970).

Reynolds, Souty-Grosset and Richardson (2012a; 2012b) refer to the presence of numerous sensory setae in strongly burrowing species; though no reference for the setae performing this function is given, nor any further detail provided. Regardless of whether the diagnostic setae on the chelae were found to be either sensory or to perform a cleaning function, there is no obvious reason why each species would have developed its own unique pattern. If some other functional use, such as playing a role in digging burrows, could be determined then it may be possible to find a correlation to soil type for instance. Whilst there are likely some species-specific habitat characteristics, they are unlikely to be considerable enough to explain how a totally unique setae pattern could be selected for in different species. The fact that the setation patterns do not vary within a species, even when there are overlapping and disjunct distributions, suggest they are not plastic and are not being somehow guided by the immediate environmental conditions encountered by each population. Where no functionality can be attributed to a morphological structure that is largely invariable within a species, it may simply be the result of drift followed by fixation. However, with the high degree of genetic and morphological variability between populations it might be reasonable to expect the setation pattern to be more variable than has been noted. If this character is neither functional, nor experiencing drift in highly isolated populations, it may be that some other form of stabilising selection is taking place, such as sexual selection. Sexual selection, however, generally results in increasing sexual dimorphism as the character will be prevalent in one sex only. However, the setation pattern only appears to vary between the sexes in *Engaewa* in one species (*E. walpolea*). This ratio of sexual dimorphism of the chelae setae appears to be similar to that seen in the genus *Engaeus* (Horwitz, 1990).

Another morphological structure particularly worthy of note is the sternum, which includes a ridge that runs along the ventral surface of the crayfish, and lateral processes between the pereopods; in *Engaewa*, between the 3rd, 4th and 5th pereopods these processes appear as swollen lobes. Horwitz and Adams (2000, p.677) suggested that the character complex "holds possibly the most phylogenetic information since it is assumed to be both a reproductive and a derived feature, with more potential to be non-convergent than other derived character states owing to the conservative nature of

reproduction". Whilst this may be true it is also a complicated structure, which makes defining independent characters and character states difficult and it also shows a considerable degree of variation (Burnham, 2005). Despite these difficulties the sternal keel does provide one highly significant and clearly expressed character; the presence or absence of sternal pores. *Engaewa* spp. possess varying numbers of sternal pores ranging from *E. subcoerulea* and *E. clade B*, which possess sternal pores in all lateral processes, to *E. similis*, *E. pseudoreducta*, *E. walpolea* and *E. clade A*, which possess pores only in the lateral process of the 4th pereopod. *Engaewa reducta* possesses pores in the lateral process of the 3rd as well as in the 4th pereopod.

As with the chelae setation, it is difficult to define the functional and phylogenetic significance of the sternal pores. In crayfish, various functions relating to reproduction have been have been suggested for the role of the sternal pores, including sperm receptacles, cement glands and pheromone secretors (Suter, 1975). The possession of pores in both sexes would appear to contradict the notion of them acting as either a sperm receptacle or as cement glands for attaching the eggs to the pleopods (Suter, 1975). Suter (1975) also argued against the pores performing a role in the production and subsequent release of pheromones, as they are present in some, but not all, species of *Engaeus*. All *Engaewa* species do possess at least one set of pores so this possibility cannot be dismissed outright for this genus. Pheromones are always involved in crayfish mating (Reynolds *et al.*, 2012b), however, the effectiveness of this in burrow systems is unclear. It has been noted that chemical stimulation of crayfish caused by the presence of food creates general restlessness, rather than directing the crayfish along a diffusion gradient to the food (Bell, 1906; Bovbjerg, 1956). This extra movement may be enough to bring the crayfish into contact with the food, however, their inability to directly find food from a distance possibly brings into question how well pheromones released into the water within a branching burrow system would function in bringing potential mates together. Still, as with the example related to food, secretion of pheromones may be enough to simply excite the potential mating partner into an exploration that could bring them together. Bechler (1995) suggested that burrowing crayfish might mark the entrance to burrow systems with some form of pheromone, which could either attract mates or ward off rival males. However, this would only be

relevant to *Engaewa* if they find sexual partners via overland dispersal, which is, as yet, unknown.

Suter (1975) promoted the hypothesis that, for *Engaeus*, the pores may secrete a substance that is used to line the burrow and make it impervious to water. This theory would explain the possession of the character by only some species but by both sexes. This is also a plausible explanation for their presence in *Engaewa*, as the number of pores possessed by each species does appear to coincide with differences in the soil types predominantly utilised by each species, and the size of their burrow systems. Soils and burrowing are discussed in detail in the next chapter; however, species that generally inhabit sandier soils and dig larger burrow systems possess more sternal pores.

Sternal pores are present (to varying degrees) in both *Engaeus* and *Engaewa* species but rarely in other parastacids; thus it may be plesiomorphic for the parastacids and has regularly been lost (so that it appears as a symplesiomorphy), or it may be a synapomorphy of *Engaewa*/*Engaeus* that has arisen independently within other lineages and is, therefore, homoplastic (assuming these 'sternal pores' in different taxa are homologous). Regardless, the presence of pores on all lateral processes likely represents the plesiomorphic state for the ancestral *Engaewa* and they have been lost from various lateral processes multiple times throughout their history. The pore on the 1st and 2nd lateral process must have been lost at least twice (on the lineage leading to *E. walpolea* and again on the lineage leading to the *E. reducta* complex) and lost from the 3rd lateral process at least three times (*E. walpolea*, *E. similis*/*E. clade A* and *E. pseudoreducta*) (Figure 3.28b). This would total seven individual losses. An alternative option is that the loss or gain of the pores may be relatively plastic within these burrowing crayfish and that there were four additions from an ancestor possessing the pore only on the 4th lateral process (Figure 3.28c) (though it would also have necessitated three initial losses if the *Engaewa/Engaeus* ancestor had a complete complement of pores). This may be less likely as it is often accepted for many characters that repeated independent losses are more likely than repeated independent gains (i.e. it would be considered a Dollo character).
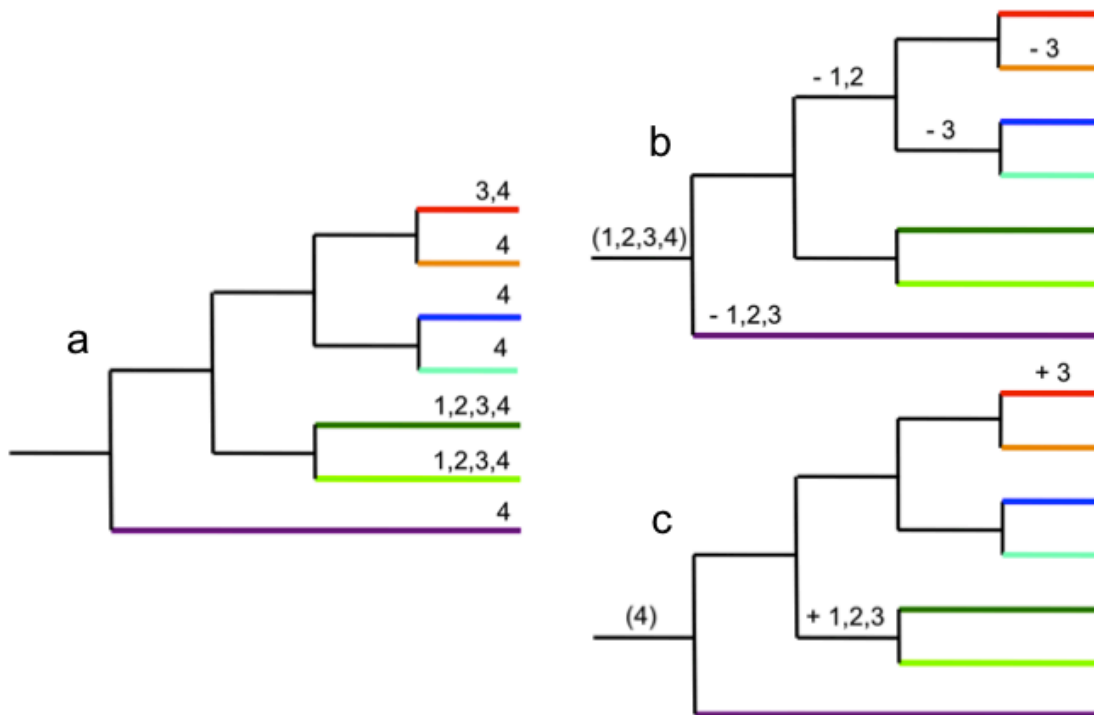
Figure 3.28 (a) The lateral processes on which sternal pores are present for each extant lineage of *Engaewa* recognised in this thesis: (b) & (c) show alternative hypotheses of loss/gain of sternal pores along lineages, with the hypothesised ancestral state shown in brackets. (b) Changes along the lineages if only loss of pores occurred. (c) Least number of changes to the number of sternal pores along the lineages needed, allowing for both loss and gain of pores.

With the recognition of two new lineages and a revised and improved understanding of morphological diversity within the genus in mind, a new taxonomic key has been formulated building on the most recently published key (Horwitz and Adams 2000, which itself was built on the key of Riek 1967a) and is presented in Appendix 3. One further point directly related to taxonomy that warrants clarification is the recognition of the *E. reducta* complex, which was highlighted by Horwitz and Adams (2000) on the basis of allozyme and morphological similarity.

Horwitz and Adams (2000) suggested that the *E. reducta* complex represents incipient speciation. Cases where divergences can be seen within a particular trait (whether morphological, molecular or ecological) between closely related groups, and these divergences are anticipated to increase in magnitude and/or frequency, are often assumed to represent incipient speciation. By definition, incipient speciation means that

populations are on their way to becoming species (Butlin, Galindo & Grahame, 2008), however, the term is also often used in situations where there are species (or subspecies) that are weakly divergent and assumedly have only recently separated (as in the case of the *E. reducta* complex). Some authors (e.g. Butlin *et al.*, 2008) have suggested that the use of the terms incipient species/speciation be avoided, as claiming that populations are undergoing incipient speciation assumes a knowledge of the future (i.e. that the divergence process will continue through to full speciation) when, in fact, it is entirely possible that conditions could change and the divergent populations may homogenise.

If a definition of an incipient species being 'diverging populations *beginning* to speciate' is accepted, then the suggestion by Horwitz and Adams (2000) that the *E. reducta* complex is made up of distinct species, yet it represents incipient speciation, is conflicting. The relationship between *E. pseudoreducta* and the population at Payne Road may represent incipient speciation (with the caveat noted by Butlin *et al.* (2008) that the divergence is expected to continue) or they may, in fact, already represent distinct species. The geographic subdivisions within both *E. reducta* and *E. similis* and the presence of multiple haplotype networks within these species may also represent the early stages of speciation. The identification of hybrid zones between *Cherax tenuimanus* (Smith) and *Cherax cainii* Austin and Ryan (Austin & Ryan, 2002) and between possible species level lineages within *Geocrinia rosea* (Harrison) (Driscoll & Roberts, 2008) in SWA suggests that there may be numerous taxa in the region that have 'speciated' but have not, as yet, achieved reproductive isolation from their congenerics.

These examples may be indicative of conditions in SWA that have prompted relatively recent divergence in multiple taxa (this will be discussed when addressing biogeography in Chapter 5), and that the use of the incipient speciation concept may be particularly warranted in this situation. The lineage-through-time plot presented in Section 3.3.1 shows that there may have been a recent increase in the number of lineages within *Engaewa*. It can be seen therefore, that the concept of incipient speciation is also largely dependent on the species concept employed, as some definitions of species will require only a single line of evidence, whereas others require

multiple supporting characters. Incipient speciation can perhaps best be thought of as those 'species' that have achieved differentiation in a small number of characters. This is true also of the use of the subspecies category. Opinions on how to define subspecies vary, however, Wilke and Pfenninger (2002, p. 1445) suggested that the concept is best suited to populations that are "isolated reproductively (usually by geographical barriers) and that exhibit recognisable phylogenetic partitioning due to the time-dependent accumulation of genetic differences". However, when using a lineage based species concept, subspecies as well as incipient species become unwarranted categories.

This work also recognises significantly different distributional patterns to those proposed in Horwitz and Adams (2000). Previously (starting with Riek 1967a and then Horwitz and Adams 2000) all species, with the exception of *E. subcoerulea* and *E. walpolea*, were considered to have discrete, non-overlapping distributional ranges. Thus, geographic information previously had been considered likely to provide enough information to make a reasonable assumption as to the species designation of a collected specimen. The splitting of *E. subcoerulea* and *E. clade B* actually removes the previously supposed example of species overlap (as the divide between these species is essentially the distribution of *E. walpolea*). The presence of southern populations of *E. reducta* and northern populations of *E. similis* creates a new overlap between these species as well as with *E. pseudoreducta*. The significance of this in terms of the biogeography of the genus is discussed in detail in Chapter 5.

It is important to note that at least one, and possibly two, species level entities (*E. clade A* and potentially Payne Road) are known from only a single locality and *E. pseudoreducta* (excluding Payne Road) is confirmed from only two very small adjacent creek lines (having been extirpated from its type locality). *Engaewa clade A* is found in a single locality and all surrounding habitat was surveyed and found to contain other species, bringing into question whether it exists outside of this single creek line. This finding suggests that, although the likelihood of finding new species is believed to be very low (based on how few species were found in comparison to the number of sites surveyed and the lack of any significant areas of, as yet, unsurveyed potential habitat), any populations found should be considered as potential new species and thoroughly

examined. This, along with the recognition of species with overlapping distributions in this study, is significant as previously for this genus it was assumed that distribution could (with a reasonable degree of certainty) be used to predict which species would be found at a particular site.

3.4.3 Degree and distribution of diversity within *Engaewa*

One point from this study that requires consideration before the level and distribution of diversity within the genus can be discussed, is whether increasing sample-sizes within localities would dramatically alter the estimate of within-locality genetic diversity and/or connectivity between populations. Lindblom (2009) suggested that the identification of haplotypes within a study can be thought of in terms of haplotype richness, and likened this to accumulating species richness in a biological survey. It has long been recognised that recording every single species, and their relative abundance, in a survey is often impossible and a decision must be made as to the trade-off between the time and effort involved in the sampling process and the likelihood of finding more species (Lindblom, 2009). The rate at which additional sampling adds to the richness and abundance of species provides important information about overall diversity (Magurran, 2004), so too does the accumulation of haplotype richness (Lindblom, 2009). It has been demonstrated through both simulated and real data that there is no standard sample size that can be used to detect all haplotypes within a population or species (Zhang *et al.*, 2010). Further to this, routinely used sample sizes in DNA barcoding projects (in the range of 5-10 individuals) may, on occasion, capture a reasonable percentage of haplotypes, however, they generally do not even come close to capturing all diversity and often many hundreds of samples are needed (Zhang *et al.*, 2010).

It could be assumed that limited sampling would result in an underestimation of genetic diversity, however, in this study the populations that had more individuals sampled did not produce more nucleotide diversity (e.g. for COI six individuals were sequenced from the Spearwood Creek population but there was still only a divergence value of 0.004). In contrast, Figure 3.18 shows that the number of haplotypes found in relation to the number of individuals sampled may not have reached a plateau, thus

hinting that diversity, based on haplotype number, may be underestimated. Despite the under-representation of unique haplotypes in this study generally, in populations where multiple haplotypes were detected they vary little from each other (i.e. due to the aforementioned low nucleotide diversity). Horwitz and Adams (2000) also demonstrated a lack of within population variation using allozyme data, as only two cases of a possible 54 showed polymorphisms. These were malate dehydrogenase for one *E. reducta* population and lactate dehydrogenase for *E. subcoerulea*, both of which had low levels of variation, with the more common allozyme being present at percentages of 87% and 80%, respectively.

By combining the information obtained in this study relating to haplotype and nucleotide diversity it becomes apparent that additional sampling within currently known populations is unlikely to fill in the substantial gaps in the haplotype networks (presented in Section 3.3.5). This is due to the large number of mutational steps between haplotypes found in different populations and the small number of mutational steps between haplotypes within populations. It is also unlikely that sampling of additional populations (if they even exist) would connect haplotypes to any significant degree, as even geographically proximate populations can be highly divergent. For example, there are populations within *E. subcoerulea* (which is one of the more highly connected species) that are only hundreds of meters apart but do not connect within a 90% parsimony network.

The low numbers of shared haplotypes across populations identified in this study suggests that significantly more within population sampling effort would be unlikely to increase the number of haplotypes shared between disjunct populations. If there was a widespread ancestral haplotype it would be expected that random sampling would identify this most common haplotype most often, and that unique haplotypes occurring at a low frequency would be more likely to be missed. It can therefore be deduced that the sampling conducted in this study is under-representative of the haplotype and (to a lesser extent) nucleotide diversity present in this genus, though arguably it would not drastically alter the inferred patterns of diversity and connectivity. Unfortunately, there is no way to avoid under-sampling of these species due to the conservation concern

surrounding them. There is limited material held in museums and attempts were made to extract and amplify DNA from these, however, much of the material has been stored in conditions that make successful DNA extraction incredibly difficult.

A similar conclusion regarding within population variability was drawn from a phylogeographic study of *Engaeus sericatus* by Schultz *et al.* (2008), where it was found that genetic variation between individuals within localities was minimal, as haplotypes were identical within 21 of 23 multi-sample localities and differed by only one base pair within two of 23 multi-sample localities (two individuals were sampled from each of 19 localities, three from three localities, and five from one locality). Due to the high numbers of zero within-locality variability the authors concluded that increasing sample-sizes within localities would be unlikely to significantly add to estimates of within-locality diversity.

When looking at the haplotype networks, the large genetic distances and associated homoplasious character changes between the sampled populations make it difficult to distinguish a clear pattern in the data, and suggest that there are many missing haplotypes and possible genealogical pathways. Due to the intense sampling effort undertaken in this project it must be assumed that the large number of missing haplotypes inferred in all network methods results from many population extirpations. If persistence through time was high we would expect to find many shared haplotypes between populations and closely related haplotypes connecting populations, as haplotypes would arise and spread throughout the species and be maintained. If, however, there had been severe bottlenecks in the past, much diversity would be lost.

Very small population numbers and sizes may result in each population possessing relatively few haplotypes and, in a spatially highly structured species, there may be few shared haplotypes (as the locally prominent varieties have the statistically greater chance of persisting), thus low haplotype diversity within populations can be due to bottlenecks (vicariance) or the founder effect (dispersal). Patterns of high haplotype diversity but low nucleotide diversity are often seen as evidence for expansion following a bottleneck (Avise, 2000), however, this was not supported by

any of the measures used in this study (i.e. the mismatch distributions do not follow the expected growth curve shown in Figures 3.19a&b and none of the test statistics were significant). Coalescent theory suggests that the most common haplotype and/or the one with the most branches connected to it will represent the most likely root (i.e. the ancestral haplotype) of a population level phylogeny (Morrison, 2005). However, it is clear that all haplotypes in these analyses occur as pendant haplotypes (i.e. they branch off the main trunk of the network) and link only to haplotypes from the same or an adjoining population so that no ancestral haplotype(s) can be identified.

It is clear from the haplotype networks that along the southern coast of Western Australia populations are genetically far more connected than in the northern portion of the distribution of this genus. This can be attributed to the differences in habitat connectivity between the two regions. North of the Blackwood River, suitable habitat appears to be both historically less connected and further fragmented in recent times due to anthropogenic influence. In comparison, starting from the mouth of the Blackwood River and continuing east along the southern coast, the habitat is much more connected through an extensive connected wetland system associated with the coastal plains. The differences seen in the haplotype networks may, therefore, be seen as a result of both historical isolation between populations in the north (resulting in larger genetic distances between populations) combined with the loss of populations (i.e. haplotypes) (creating gaps within the networks), as opposed to the more connected southern populations. However, while populations on the south coast are more connected, there are still numerous geographically proximate populations that are not closely related genetically. This finding suggests that a simple explanation based on habitat connectivity and/or proximity cannot provide a complete explanation.

3.4.4 Geographic partitioning of diversity in freshwater crayfish

A review of the trends in early crayfish genetic studies was provided by Fetzner & Crandall (2002). In this review the authors suggested that variation within species for the 16S marker might be generally low in parastacids, but that the variation among species within genera and particularly between genera is high in this family, when compared to the other freshwater crayfish. A number of studies have supported the

supposition of Fetzner & Crandall (2002) and have shown low levels of genetic variation within many Australian freshwater crayfish species (e.g. Munasinghe, Burridge & Austin, 2004b; Munasinghe *et al.*, 2003; Schultz *et al.*, 2008; Schultz *et al.*, 2007; Sinclair *et al.*, 2011; Versteegen & Lawler, 1996), even across independent river drainages and recognised faunal breaks. The genetic distances within species of *Engaewa* reported in this study span from the lowest end of the parastacid spectrum (in *E. clade A* and *E. walpolea*) to some of the highest reported (in *E. pseudoreducta* and *E. similis*).

The results from this study can also be compared to Astacidae in Europe and Cambaridae in North America. Trontelj *et al*. (2005) investigated the genetic structure of two *Austropotamobius* Skorikow species distributed widely across Europe and reported levels of nucleotide diversity for COI of 0.043 for *Austropotamobius pallipes* (Lereboullet) and 0.037 for *Austropotamobius torrentium* (Schrank). It could be expected that a species spread across a large geographical region such as the *Austropotamobius* species would have high diversity, as there is ample opportunity for localised diversification, and assumedly geographical structuring. However, the values reported by Trontelj *et al*. (2005) fall well within the range of COI values for *Engaewa* species (even excluding the highly divergent *E. pseudoreducta* value; 0.004-0.065). The phylogeographic networks for these two *Austropotamobius* species produced by Trontelj *et al*. (2005) and Chiesa *et al*. (2011) also recovered a similar number of mutational steps between haplotypes as the *E. similis/E. clade A* network presented in 3.3.5, despite covering much of western Europe. Buhay and Crandall (2005) investigated the genetic structure of four subterranean species and two surface dwelling species of *Orconectes* from the eastern U.S.A. and reported levels of nucleotide diversity for 16S of 0.00238 to 0.00894 for the subterranean species and 0.00394 and 0.02501 for the surface dwelling species. Again these values essentially fall within the range of values for *Engaewa* species (0.003-0.063).

The pattern of low diversity within populations and high diversity between populations that is characteristic of *Engaewa* species is also seen for *Cambaroides japonicus* (De Haan) in northern Japan. Koizumi *et al.* (2012) suggested that *C. japonicus* has one of the highest levels of genetic differentiation reported for any organism ($F_{ST} = 0.96$), with 69% of the populations sampled having single haplotypes. A significant difference between the situation for *Engaewa* and *C. japonicus* is that while both have populations that are small and generally have unique haplotypes, there is a low frequency of missing haplotypes in *C. japonicus*. For *Engaewa* it is hypothesised that there have been many population level extinctions, thus creating the large gaps seen in the haplotype networks, combined with bottlenecks and drift in small populations, however, there must be a different process occurring in *C. japonicus* that has prevented this pattern from forming. While populations of *C. japonicus* have small effective population sizes and most likely have experienced bottlenecks, there must be a mechanism that allows long-term persistence of small populations, or that extinction-colonisation dynamics can maintain genetic variation (Koizumi *et al.*, 2012).

Based on the above discussions on diversity, it seems that *Engaewa* species encapsulate the entire range of diversity values reported in other crayfish species. This can be explained by the unusual situation for *Engaewa*. The species of this genus range from those existing as a single population and possessing very little diversity, to those that are relatively widespread but with highly disjunct populations that are essentially evolving as isolated units. That all populations are evolving in this manner and that there is no connectivity between catchments, and often not even within drainages, demonstrates that *Engaewa* species correspond to the Death Valley Geographic Model (DVM) (*sensu* Meffe & Vrijenhoek, 1988).

The DVM occurs when individuals are unable to migrate between catchments and, therefore, populations become highly differentiated without the homogenising influence of inter-population gene exchange (Huey, Baker & Hughes, 2010; Meffe & Vrijenhoek, 1988). It was originally proposed for the situation of species living in isolated pools that have no hydrological connection, and the species themselves have no ability to connect via overland dispersal (Meffe & Vrijenhoek, 1988). The obvious

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prediction for these species is that they would be isolated for long periods of time, which would, in turn, lead to them becoming highly differentiated. Isolation, combined with probable small local population size, would lead to genetic drift being the dominant force shaping the genetic structure of these species (Hughes, Schmidt & Finn, 2009). This would result in a pattern where spatial genetic structure does not correspond to boundaries such as drainages and catchments and, therefore, there would not necessarily be a correlation between genetic and geographic distance (Hughes *et al.*, 2009).

The DVM essentially lies at one extreme of a continuum of models proposed to explain the geographic partitioning of genetic diversity in freshwater systems. At one extreme in this continuum is the DVM, where there is no gene flow between populations, and at the other is panmixia, which has complete mixing. In between are a number of alternative models that rely on the characteristics of drainage systems (i.e. a hierarchical dendritic stream network) and the varying dispersal abilities of taxa. In theory these models can allow for predictions to be made regarding the degree and distribution of genetic diversity across the landscape if the species biology is understood, or if the pattern of within and between catchment diversity is understood then assumptions can be made regarding the species biology. Following the recently proposed key of Hughes, Huey and Schmidt (2013), that is designed to predict the model of connectivity for Australian freshwater species, *Engaewa*'s potential connectivity would fit the DVM.

The DVM in many ways mirrors the situation faced by populations that occur on currently isolated 'islands'. There are numerous situations where populations/species can be considered to exist in a situation that replicates those found on actual islands. In this case it is expected that geographically isolated populations will, over time, experience considerable divergence. These 'islands' can take a number of forms, but one of the most commonly considered examples is taxa that are restricted by altitude. An example of this comes from many of the species within the Australian crayfish genus *Euastacus*, which are found on isolated elevated peaks along the eastern coastline (Ponniah & Hughes, 2004).

Another (perhaps less obvious) example of a habitat island comes from *Mandarina* snail species in Japan that are restricted to arboreal habitats. Davison and Chiba (2008) have shown that the arboreal snail species have less genetic exchange between populations than their ground-dwelling relatives. Currently these snails are highly geographically subdivided, due to limited gene flow and low effective population size, and the fine-scale differentiation has resulted in most populations possessing unique haplotypes (Watanabe & Chiba, 2001). It was hypothesised that the genetic structure of arboreal *Mandarina* Pilsbry could be best explained by the circumstances whereby extremely structured subpopulations actually maintained overall diversity within species through bottlenecks (Davison & Chiba, 2008). This would result from the independence of each 'deme' meaning that selection cannot act across the entire population, only the local subpopulations. If they had greater mobility (and therefore reduced population structure) selective sweeps would occur across the entire population, reducing overall diversity. This example may reflect the situation for *Engaewa*, where there are highly divergent (essentially unique) populations that contain little diversity.

3.4.5 Summary

Engaewa is a highly diverse genus, containing many divergent lineages, particularly when considered in relation to the limited distribution of this taxon. It is clear from the systematic revision undertaken that additional species level lineages should be identified in this genus (from five species to seven, with the possibility of an eighth – or more). The phylogeny utilising the maximum amount of available data (i.e. all genetic markers combined) was highly supported, with well-defined relationships between the major lineages. Whilst many aspects of the morphology of these crayfish are variable, the setation pattern of the chelae appears to be highly conserved, species-specific, and readily identifiable, thus is a useful character for species delineation. Yet despite the taxonomic significance of this character, its function and the selection forces acting upon it are poorly understood.

The pattern of genetic diversity within species of this crayfish is highly unusual and likely results from the extreme burrowing habit and low dispersal ability of these species. The general pattern identified from the genetic data is that *Engaewa* species are composed of genetically depauperate, yet highly differentiated, populations. Based on this observation, the number of populations within each species becomes the defining characteristic of the overall diversity within each species, as each additional population greatly increases the diversity within the species. This has resulted in species that have similar, if not greater, diversity than much more widely distributed species of freshwater crayfish (and many fauna generally). Thus, the unique genetic structuring of *Engaewa* can be defined by low intra-population diversity, with very few shared haplotypes between populations and high genetic differentiation between populations and species.

This outcome, whilst unusual, is not surprising. It has been suggested that isolated relict populations lacking connectivity with the main range of a species will possess low genetic diversity, due to the influence of bottlenecks, drift and selection (Sepulveda-Villet & Stepien, 2012), and (as shall be shown in the next chapter) the ecology of these crayfish, combined with the availability (or lack thereof) of habitat makes such an outcome likely. The molecular dating undertaken in this study suggest the genus is ancient and that the species themselves are not newly derived, however, the LTT suggests there has been a significant increase in lineage formation in more recent times. It will be argued in the biogeographic treatment of this genus in Chapter 5 that this can be explained by the contraction of formerly more widespread species into highly isolated populations. Divergence in isolation, combined with many population extirpations, can explain the increase in lineages and would produce the unusual, disconnected haplotype networks seen.

That lineages within these crayfish are genetically diverse, highly restricted, and ancient, has been established in this chapter. These data also support the supposition that they are likely to be habitat specific and sedentary (as will be considered in more detail in the next chapter). These characteristics suggest this taxon will prove to be a useful model organism for a biogeographic study, which should allow for the overall aim of this thesis to be addressed.

4) HABITAT & CONSERVATION OF THE GENUS *ENGAEWA*

4.1 Introduction

The distribution of a species is dictated by the combination of three classes of factors: (1) intrinsic factors such as dispersal ability and habitat tolerance; (2) extrinsic factors such as availability of habitat and opportunity for dispersal, or interactions with other taxa; and (3) historical factors that may have shaped their distribution in the past (Ponder & Colgan, 2002). This chapter relates specifically to factors incorporated in (1) and (2) above, whilst (3) is largely the domain of biogeography and will be dealt with primarily in the next chapter. To provide the type of data that are needed to understand the biogeographic history of lineages within the genus *Engaewa*, the habitat occupied by each species will be described in this chapter in terms of landform, vegetation, hydrology, soil, elevation, and aspect. For each species observations of the type of burrow constructed will also be provided, as well as any additional relevant notes in the form of observations made whilst collecting specimens. As habitat tolerance and availability can have a direct effect on the distribution of species, it is necessary to consider the similarities and differences in these for different *Engaewa* species before the major aims of this thesis can be addressed (i.e. in order to test biogeographic hypotheses).

Along with an analysis of habitat, an assessment of the conservation status of all *Engaewa* species is required, as conservation and management decisions should be based on sound knowledge of the taxonomy, biological diversity and distributions of species (Linkem, Hesed, Diesmos & Brown, 2010). Thus, the need for an accurate taxonomy when undertaking conservation efforts dictates that whenever the systematics of a group of organisms is reviewed so too should its conservation status, or, conversely, if there is a desire to review the conservation status of a group then its systematics should be also reviewed. Species diversity is often the focus for practical conservation efforts and makes the assumption that genetic diversity (the basic unit of

biodiversity[*]) will also be conserved (Byrne, 2007). Whilst this assumption may often hold, especially where species diversity is low and boundaries are well defined, there will be many cases where it does not. Where species taxonomy is not adequately aligned with phylogenetic diversity inappropriate conservation actions may be taken (Linkem *et al.*, 2010), as shown for some endangered animal species (Ryder, Chemnick, Schafer & Shima, 1989; Templeton, 1986; Zink & Kale, 1995). However, before any conservation status for each species level lineage is addressed, the threats faced by *Engaewa* generally will be outlined.

4.1.1 Threats impacting on *Engaewa* species

South-western Australia has been acknowledged as being a prominent centre of biodiversity. In addition to the recognition of exceptional biological richness in the region it has also been recognised as being under exceptional threat, thus qualifying it as one of the 25 global biodiversity hotspots of Myers *et al.* (2000). Broad-scale disturbance regimes in SWA are made possible by the subdued topography, which has led to much of the region having been transformed (in little over a century) from predominantly natural vegetation to predominantly agricultural land for wheat and sheep (Hobbs, 1992). In fact, over 80% of the transitional-rainfall zone (TRZ) has been cleared for agriculture (Hobbs, 1992) (Figure 4.1). The HRZ still retains a much greater proportion of native vegetation than the TRZ (Wardell-Johnson & Horwitz, 1996), though the degree of land clearing in the Warren Bioregion is still significant; with estimates of the proportion of cleared land ranging from 13.2% (Shepherd, Beeston & Hopkins, 2002) to 31% (Beard & Sprenger, 1984).

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[*] Biological diversity, or biodiversity, is a 'pseudocognate' term (Salt, 1979), however, a definition along the line of that given by McNeely *et al.* (1990, p. 17) – "It is an umbrella term for the degree of nature's variety, including both the number and frequency of ecosystems, species, or genes in a given assemblage" – is commonly accepted and used here.
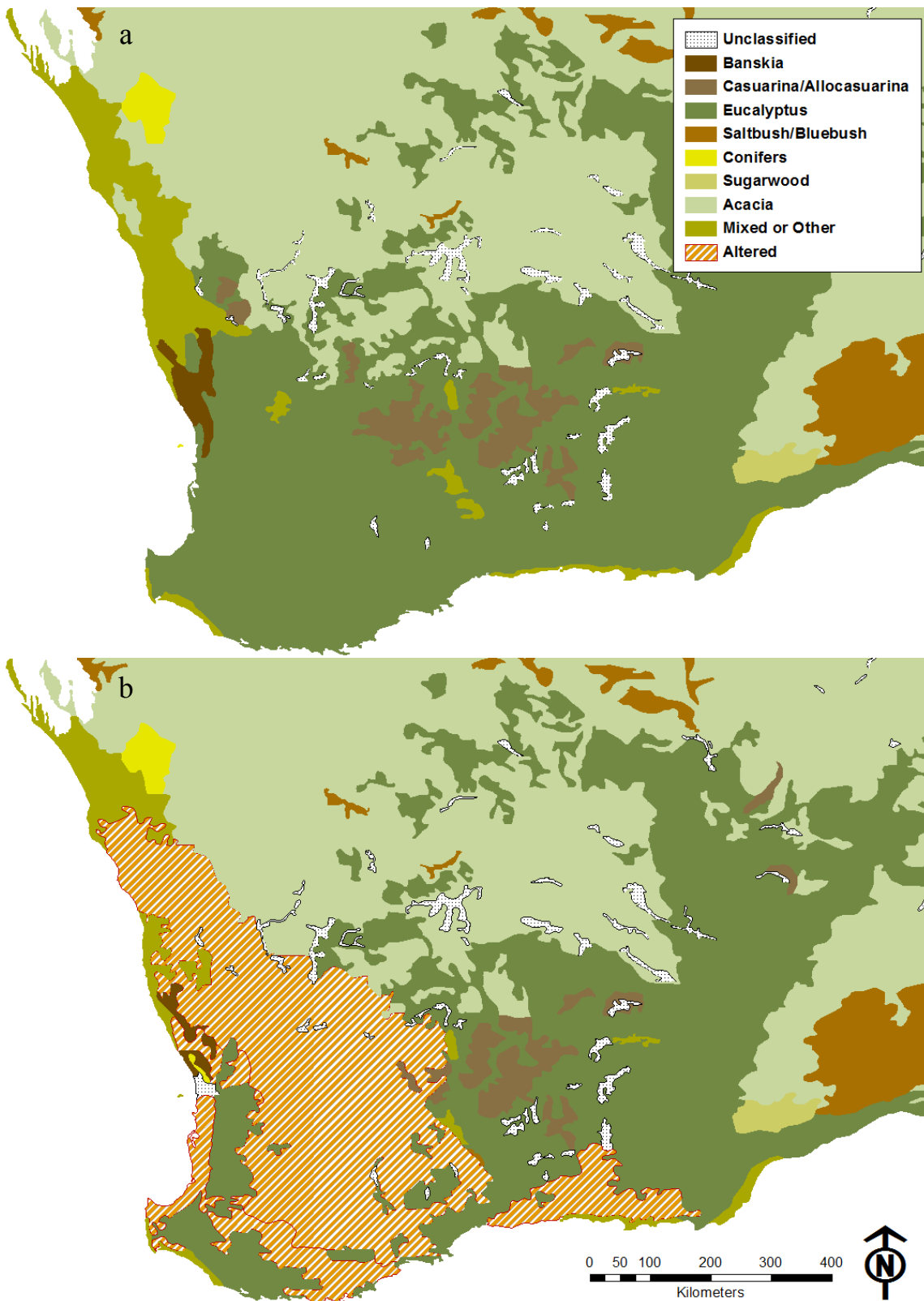
Figure 4.1 Major vegetation types of south-west Australia (a) pre-European settlement (1788), and (b) 200 years post-European settlement (1988), showing the approximate degree of land alteration. For sources of GIS data see Section 2.5.

The large forested areas of the extreme south-west have undergone dramatic modification since European settlement (Hobbs, 1992) and alterations to the natural environment, resulting from changing land use, have particularly intensified since the 1960s, dramatically increasing fragmentation within the region (Wardell-Johnson & Horwitz, 1996). Whilst the non-cleared areas may be considered to be covered by native vegetation, much of this area has experienced degradation and there now exists many small remnant areas in SWA supporting biota that will probably not be able to persist in the long term without direct intervention to ameliorate the effects of habitat reduction and isolation (Hobbs, 1992).

As well as resulting in a general degradation of the landscape, land use activities in SWA during the past 200 years have also negatively impacted aquatic environments specifically (Yen & Butcher, 1997). Threats that have been specifically noted to be affecting important wetlands of the Warren bioregion include vegetation clearing, changed fire regimes, changed hydrology, exotic weeds, feral animals, pathogens, pollution, eutrophication, mining, and plantation harvesting (Dept. Sustainability Environment Water Population and Communities, 2009). Thus, the freshwater aquatic biodiversity of SWA is facing significant and increasing survival pressure, which is particularly evident in the coastal, wetter margins (Horwitz *et al.*, 2008).

The threats that are impacting on aquatic systems in SWA create a variety of direct and second order effects. Clearing or logging of vegetation, building of roads, bridges, dams etc., and the construction of firebreaks, can all alter surface and/or subsurface water flow. These outcomes will potentially increase sediment deposition, water logging and flooding (Trayler, Davis, Horwitz & Morgan, 1996; Wardell-Johnson, Roberts, Driscoll & Williams, 1995), and can also increase the spread of fungal pathogens (Trayler *et al.*, 1996). The extraction of groundwater or alteration of natural flows, particularly when combined with periodic drought, may cause acidification and toxification of wetlands (Horwitz & Rogan, 2003; Sommer & Horwitz, 2001), whilst clearing of land for agriculture or logging can increase salinity in wetlands (Bunn & Davies, 1992; Trayler *et al.*, 1996; Wardell-Johnson *et al.*, 1995) and water quality can also suffer due to the deliberate, or accidental, addition of pollutants,

such as fertilisers, pesticides, herbicides and heavy metals (McComb & Davis, 1993; Nystrom, 2002; Wardell-Johnson *et al.*, 1995). Furthermore, the introduction of exotic animals to these regions has produced considerable impacts on the native fauna and flora, with hoofed exotics (i.e. grazing cattle), in particular, exerting a negative influence on wetlands, as they compact soils, impairing both permeability and water holding capacity (Main, 1992). Although feral pigs (*Sus scrofa* Linnaeus) occur in low densities throughout SWA, they are highly mobile and can cause intense localised disturbance, particularly during the summer months when their digging activities are concentrated within riparian zones (Wardell-Johnson *et al.*, 1995).

There have been few discussions specifically on the impact of fire on swamps and peatlands, and how this affects the associated fauna (e.g. Horwitz, Judd & Sommer, 2003), despite the suggestion that Gondwanan relicts may be particularly susceptible to disturbances caused by fire (Main, 1996a). Fire can threaten swamp fauna in a number of ways, both directly and indirectly. Burning of organic sediments can either remove sediments completely, or alter their water holding capacity, and result in microhabitats that are no longer moisture rich (Horwitz & Rogan, 2003). The underlying mineral soil may also be lost, which will alter local hydrology by either creating surface pools or, conversely, by increasing drainage (Horwitz & Rogan, 2003; Trayler *et al.*, 1996). This can result in acidification and metal toxification, which is caused by drying and re-wetting of soils (Horwitz *et al.*, 2003). Additional issues that arise from fire in swamps are related to activities that are actually undertaken to minimise the impact of the fire and include the application of fire retardants, which can contaminate water and soil, and digging trenches to contain the fire, which damages the soil and alters drainage patterns (Horwitz *et al.*, 2003).

Many of the above threats have been specifically noted in relation to *Engaewa* as the peatlands and swamps they inhabit require the moisture to be maintained, and are particularly vulnerable to degradation (Wardell-Johnson & Horwitz, 1996). Whilst disturbance ("the relatively sudden and discrete loss of biomass, structure or function" (Walker, 2011, p. 916)) occurs as a part of natural systems, humans have added a number of anthropogenic factors that can be either entirely novel or act to ameliorate or

exacerbate natural processes (Walker, 2011). Identified threats to *Engaewa* include drainage for agriculture and peat or sand mining, water extraction from bores, dam construction, road and bridge construction, grazing of cattle, exposure and subsequent hydration of acid sulphate soils, activities of feral pigs, the use of pesticides and herbicides, and fire in or around wetlands (Horwitz, 1995; Horwitz & Adams, 2000; Horwitz & Rogan, 2003).

As well as the above threats the direct impact of declining availability of habitat appears to be a major factor fuelling the current conservation concern surrounding some *Engaewa* species (Horwitz & Adams, 2000). This is not surprising as habitat destruction has been identified as the major cause of species extinctions (Pimm & Raven, 2000). Furthermore, habitat fragmentation (which encompasses habitat loss, habitat reduction, and the increased isolation of habitats (Bennett, 2003)), has been recognised as one of the most common outcomes of human activities (Frankham *et al.*, 2002) and a fragmented distribution is considered to be characteristic of most plants and animal populations at risk of extinction (Caballero, Rodríguez-Ramilo, Ávila & Fernández, 2010). Major effects of habitat fragmentation include altered microclimates and increased external influences (e.g. invasion or predation), as well as the isolation of habitat patches (Saunders, Hobbs & Margules, 1991).

Even for species that are not facing extinction in the immediate future, habitat loss will often cause severe reductions of both populations and individuals (Browning *et al.*, 2001) and destruction and fragmentation of habitat can create isolated populations (Browning *et al.*, 2001; Lande, 1988). This is of serious concern as, wherever human activities have eliminated populations and reduced the ranges of species, a combination of fragmentation and limited dispersal ability means many species (particularly freshwater species) are unable to migrate across the landscape to re-establish local populations (which also increases concerns relating to predicted climate change) (Strayer & Dudgeon, 2010). This may also mean that extirpations and extinctions could yet occur for these isolated populations due to the effects of past fragmentation preventing species dispersal in the future (e.g. Fagan, 2002; Fagan, Unmack, Burgess & Minckley, 2002; Matthews & Marsh-Matthews, 2007).

Not only have there been recent and severe human induced alterations to the natural character of SWA, but there are also anticipated to be (and arguably already have been) impacts directly related to climate change. The taxa that populate the Earth today are those that have evolved and persisted throughout an atypical period in the Earth's history, as the past several million years have been unusually cold, and have also experienced numerous dramatic, and fairly regular, climatic oscillations (i.e. Milankovitch cycles) (Peterson & Lieberman, 2012). This is significant, as it has been suggested that the climate is changing rapidly and may warm by as much as 4°C by 2100, with species' distribution shifts having already been recorded in response to warming (both pole-wards and to higher elevations) (Thuiller *et al.*, 2005). Climate change will almost inevitably result in changes to rainfall regimes, which is anticipated to reduce ecosystem net primary productivity and potentially result in shifts in community composition (Knapp *et al.*, 2002). Soil moisture dynamics are directly responsible for plant productivity, soil biogeochemistry and water stress, therefore changes to the frequency and amount of rainfall events will modify soil moisture dynamics, and the temporal structure (i.e., intensity, duration, and frequency) of periods of water stress (Porporato, Daly & Rodriguez-Iturbe, 2004). Even if total rainfall remains the same, changed intensity and frequency of rainfall events (i.e. heavier individual rain events but occurring less often) will affect soil moisture dynamics and plant conditions (Porporato *et al.*, 2004). It is clear that the many, and varied, interrelated impacts of climate change now need to be added to the list of threats to *Engaewa*. Considering the range of threats faced by *Engaewa* it is important to understand the current environmental conditions in SWA, and the anticipated changes in the future arising from predicted climate change, in order to both explain the distribution of *Engaewa* and for effective conservation planning.

Currently SWA has a Mediterranean climate, with over 80% of the total rainfall occurring between April and October (Bates *et al.*, 2008), and rainfall being highest and most reliable in the extreme southwest (Bates *et al.*, 2008; Gentilli, 1972; Hodgkin & Hesp, 1998). Whilst summers are generally dry, deteriorating cyclonic storms and thunderstorms can infrequently produce often heavy and localised precipitation in January to May (Hodgkin & Hesp, 1998). SWA has received relatively consistent

annual rainfall since records began in the 19th century (Gentilli, 1972), however, there is evidence that the climate in SWA has changed in recent times, and it is projected to continue to change, with a prediction of overall drying (Hughes, 2003; Solomon, Plattner, Knutti & Friedlingstein, 2009). Over the past century rainfall in SWA has declined significantly (Figure 4.2), with a 10% decline in the number of rainy days, a 25% decrease in total rainfall in winter, and a decrease in summer heavy rainfall events (Hughes, 2003). Decreasing rainfall in SWA is considered likely to continue, as more than 90% of the coupled climate models submitted for the IPCC Fourth Assessment Report simulate a rainfall decline (Hope & Ganter, 2010) and predictions suggest that annual average rainfall may decrease by up to as much as 60% by 2070 (Hughes, 2003).
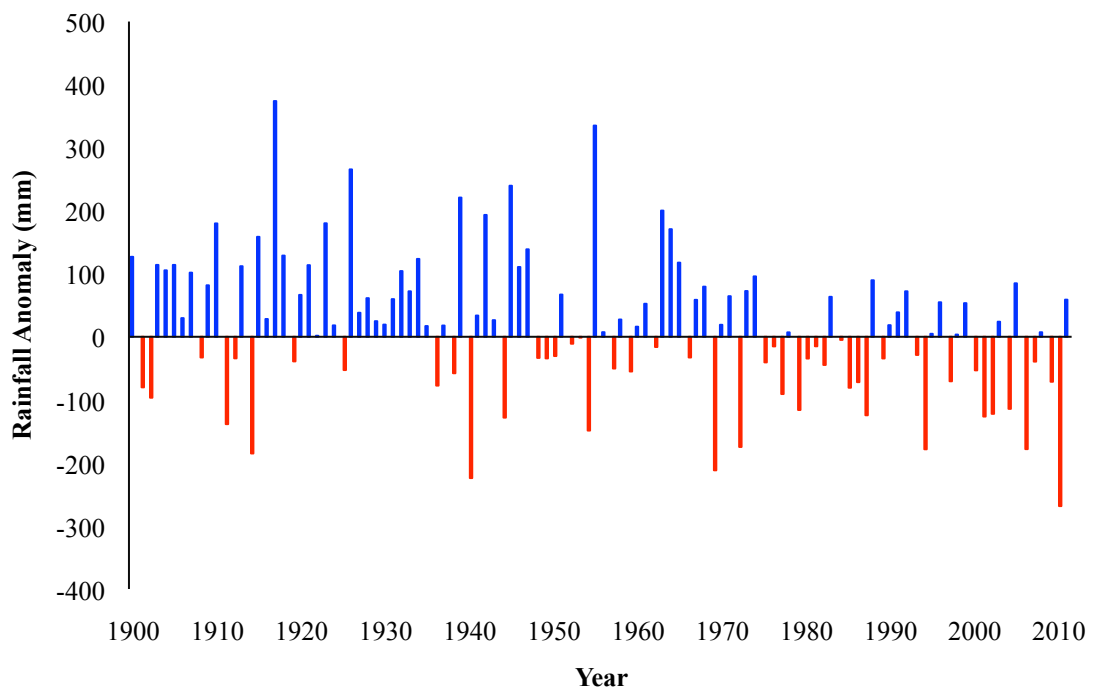
Figure 4.2 Trend in rainfall anomaly (mm) for Southwestern Australia from 1900-2011 (adapted from Bureau of Meteorology, 2013).

Mean temperatures across Australia have increased 0.1-0.2°C per decade since 1951, with the southern half of Western Australia (along with inland Queensland) seeing the greatest rises (Hughes, 2003) (Figure 4.3). Since the 1970s annual mean temperatures in SWA have increased at a rate of about 0.15°C per decade (Bates *et al*., 2008). Increased temperatures, and decreased rainfall, are likely to cause an increase in the number of days with high to extreme fire danger over much of Australia, due to an

increase in fuel dryness, reduced relative humidity, and potentially increased fuel load if CO_2 levels increase under a greenhouse scenario (although limited water availability may offset this 'CO$_2$ fertilisation effect') (Hughes, 2003). Evaporation is a significant factor in soil moisture dynamics, and is greater than rainfall in all areas of SWA except the extreme south-west (Hodgkin & Hesp, 1998). Evaporation is predicted to rise by up to 8% per degree of global warming across most of Australia, resulting in a decrease in annual moisture balance of ~40-120 mm per degree of global warming (Hughes, 2003). Of less obvious effect (at least over short time scales), but still potentially significant, are changes to sea-levels. Global sea-level rises have been projected to reach ~60 cm by 2100, due to glacier melting and ocean warming, although it may reach as much as 1 m due to the effects of accelerated decline of polar ice sheet mass (Nicholls & Cazenave, 2010); though admittedly this is likely to be less pronounced along the Australian coastline (Hughes, 2003).
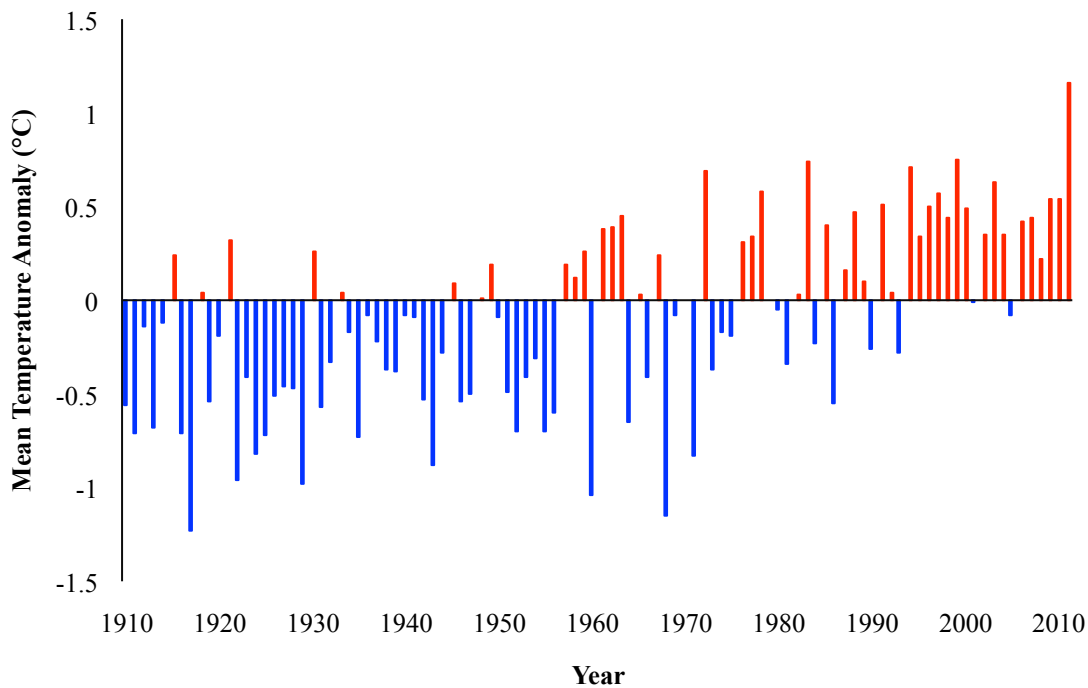
Figure 4.3 Trend in mean temperature anomaly (°C) for south-western Australia from 1910-2010 (adapted from Bureau of Meteorology, 2013).

All of the above mentioned threats make it clear that *Engaewa* species are likely to face significant survival pressure, both in the short term directly from human influences, and in the long term from climatic shifts. Furthermore, it is obvious that these two factors will interact and have an even greater cumulative negative effect. Thus, this chapter fulfils a number of important roles. The systematic revision undertaken in the preceding chapter demands a re-assessment of the conservation status of each species level lineage, as does an improved understanding of both habitat utilisation and threats being faced by each species. Furthermore, a biogeographic treatment of this taxon (especially considering the emphasis of this study on climatic refugia) requires an understanding of the important habitat characteristics for each species, and if there are any significant ecological differences between species.

4.2 Habitat overview for the genus *Engaewa*

It seems intuitively obvious that water availability would be the most important factor determining the distribution of a freshwater crayfish; however, *Engaewa*'s current distribution does not strongly correlate with a specific rainfall boundary. Currently the maximum annual rainfall in SWA is ~1600 mm, which decreases both to the north and east, as well as with distance from the coast (Figure 4.4). A comparison of rainfall gradient to the distribution of the genus suggests that, whilst rainfall may be significant, there must be other factors involved in explaining *Engaewa*'s current distribution. This suggests that the influence of (any or all of) topography, hydrology, and soils must be significant. The distributional boundaries of *Engaewa* species largely coincide with an elevation limit of ~90 m (Figure 4.4), at which point there is often an obvious change in the local geomorphology. This transition point can be thought of as demarcating the boundary between low-lying coastal landforms (that are predominantly composed of peaty or sandy soils) and those that are more highly incised with exposed or underlying rock (and are unsuitable for *Engaewa*). This suggests that *Engaewa* has limited ability to penetrate higher reaches of the drainages that extend inland from the flat, low lying coastal plains that are favoured by these crayfish. Furthermore, soil units that have a predominantly hilly aspect or are dominated by coastal dunes appear to limit the distribution of *Engaewa* (Figure 4.4).
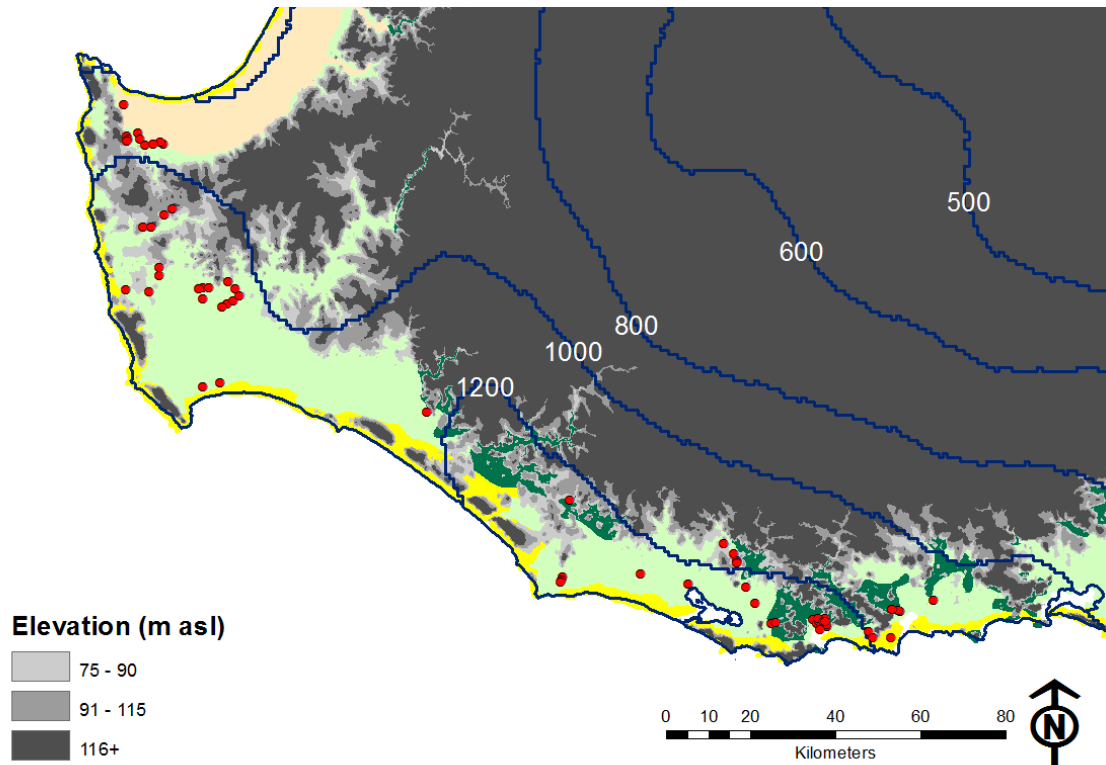
Figure 4.4 *Engaewa* collection localities (red dots) in relation to elevation and soil units: predominantly hilly (dark green); primarily composed of coastal dunes (yellow); and suitable soil units for *Engaewa* species (light green). The Swan Coastal Plain is also highlighted (beige). Blue lines and associated numbers represent annual mean rainfall gradients. For sources of GIS data see Section 2.5.

Engaewa are generally found digging in deep, leached sands in low-lying areas, which are somewhat acidic, with low nutrients and of uniformly coarse texture (based on the Atlas of Australian Soils (McKenzie, Jacquier, Ashton & Cresswell, 2000)). Whilst the mapping scheme used in the Atlas of Australian Soils is admittedly coarse, it does provide a fair description of 'typical' soils in which *Engaewa* are found. However, on a local scale they actually appear capable of burrowing in virtually any soil type, except the lateritic gravels common to much of SWA (for example see Figure 4.5, where *E. similis* had burrowed into hard white clay, gravely sand and peaty loam all within a distance of 25 m). Not surprisingly, the altitudinal limit for *Engaewa* identified in this study and the soil units that define their distribution often coincide (Figure 4.4). Therefore, it is likely that topography is the main factor determining distribution, as elevated areas are generally composed of dry ridges and where there are drainage lines they tend to be heavily incised and steeply falling; thus, they are unlikely to provide the deep and moist soils needed by these crayfish.

Figure 4.5 Three different soil types at a single site in which *Engaewa* had dug burrow systems. (a) peaty layer with high organic content overlaying fine sand, (b) pale heavy clay, and (c) coarse sand with gravel (possibly originating from road construction).

Regardless of the soil type occupied, newly formed chimneys generally appear rapidly during the winter months (i.e. the rainy season), a pattern that is also seen in both *Engaeus* (Suter & Richardson, 1977) and *Parastacus* (Noro & Buckup, 2010) species, as well as North American crayfish (Hobbs Jr., 1981). Although this may indicate a peak in digging activity, it is possible that it may simply represent a change in the depth at which soil is being removed and/or deposited, as much of the digging that is occurring may actually be simply redistributing soil underground. They may dig only at or below the water level, or they may be clearing out silt and soil deposits resulting from water running into the burrow or the water table rising.

Whether burrows are open at the top or are 'plugged' by soil varies, both throughout the year, and within and between sites. It has been suggested that the presence of plugged burrow entrances is a deliberate act by the crayfish to preserve humidity inside the burrow (Horwitz & Knott, 1983; Noro & Buckup, 2010), especially in regions (like SWA) where surface waters are rarely permanent and ambient temperatures are high (Horwitz & Knott, 1983). The summer period has been recognised as the period of highest stress for burrowing crayfish, due not only to limited water availability, but also because dissolved oxygen and pH within the burrow water are likely to be lowest at these times (Noro & Buckup, 2010). Strongly burrowing crayfish have adaptations that permit them to utilise aerial respiration, and it has been suggested that in summer oxygen may not be derived from water present in the burrow; rather the main function of the water is to maintain humidity (Suter & Richardson, 1977). Hobbs Jr. (1981) recorded secondary and tertiary burrowing crayfish apparently in torpor above the level of the water table and assumed that the humidity was at, or close to, saturation. He also proposed (at least for the North American burrowing crayfish) that the chimney might actually act to create airflow through the burrow, thereby increasing oxygen levels.

When considering the distribution of *Engaewa*, roads and roadside ditches are worthy of particular consideration as they are a recent feature within the landscape that can directly influence local hydrology. Roads cut through various habitat types and where a swamp or seepage containing *Engaewa* is bisected by the construction of a road, two events can occur. Firstly, there may be a partial (or even total) cessation of gene flow from one side of the road to the other if the habitat is not linked via a pipeline under the road (or occasional flooding). Secondly, roadside ditches are designed specifically to channel water and, as such, they can create an artificial seasonally waterlogged environment. This can allow *Engaewa* species to disperse from a previously isolated watercourse, where they can either (a) be restricted to the habitat contained along the roadside, or (b) if the ditch contacts another area suitable for the persistence of these crayfish then they may disperse into this new habitat. In scenario (b), the habitat with which they come into contact may be devoid of *Engaewa*, might contain another population of the same species, or might contain a different species.

Evidence for the significance of roadside ditches comes from Conspicuous Beach Road (Figure 4.6) where both *E. clade B* and *E. walpolea* were collected. It appears that the site from which (BRD in the distribution maps presented in Chapter 3) the two species were collected was (prior to the influence of the road) a site where *E. clade B* resided and that the *E. walpolea* specimens have subsequently entered via the roadside ditch. This assumption is based on the habitat type, which is characteristic of *E. clade B* but not *E. walpolea,* and the fact that the *E. clade B* specimens were dug out of burrows leading away from the ditch and into the native habitat, whereas the *E. walpolea* specimens were collected from the ditch itself (via spotlighting).

Figure 4.6 The situation where two *Engaewa* species were found in sympatry. (a) shows the road along which the crayfish were found (in the ditch to the left of the photo). In (b) the proximity of the roadside ditch (containing *Engaewa walpolea,* red arrow) to the burrows being excavated (containing *Engaewa clade B*).

Whether the current state of sympatry between these two species will remain a sustainable situation in the future depends on a number of factors. Firstly, if one species is in the act of replacing the other, via direct competition or competitive exclusion, then the location will revert to containing a single species. However, microhabitat separation may allow both species to persist at this location. This is a relatively common occurrence in freshwater crayfish where the two species in question will utilise slightly different habitat (e.g. Growns & Marsden, 1998; Horwitz, 1994; Jones & Bergey, 2007; Morgan, 1986; Suter & Richardson, 1977), suggesting this is a feasible scenario for these *Engaewa* species. Whilst microhabitat separation may allow them to remain in this state for a while, it may not allow for long-term persistence of both species. This is due to the potential vagaries of water availability in a roadside ditch, when compared to natural habitat. It can be assumed that individuals of *E. clade B* have the ability to withstand significant dry periods due to their strongly burrowing habit. However, as *E. walpolea* appears less capable of burrowing, a prolonged dry period combined with the exposed nature of, and gravelly soil in, the ditch may be enough to drive the population of this species to extirpation, whereas in their 'natural' habitat the combination of hydrology, topology and vegetation would allow them to persist.

The example above is the only known occurrence of sympatry between *Engaewa* spp., yet (as discussed above) this occurrence is relatively common in freshwater crayfish. Even if it is assumed that extant *Engaewa* lineages speciated in an allopatric manner, the question remains: why have they not subsequently invaded the habitat of other species? When closely related species remain allopatric it may suggest that they are too ecologically similar to coexist (Zink, 2012), possibly due to niche conservatism (where lineages conserve their ecological niche through time to maximise their success) (Wiens, 2004b; Wiens & Donoghue, 2004), or there may be competitive exclusion (Cole, 1960; Hardin, 1960), due to factors such as an antagonistic behaviour inhibiting access to a limiting resource. Another possibility that must be considered is whether they simply cannot come into contact with each other due to environmental barriers. All of these scenarios will be considered throughout this chapter, and the next, as they relate to *Engaewa*.

With only one case of sympatry known, it is worth considering in more detail the mechanisms that may prohibit it. Wilke and Pfenninger (2002) suggested six possible mechanisms that may be responsible for maintaining allopatry in the *Hydrobia* Hartmann snails they were studying, which may provide a useful framework for exploring the issue in relation to *Engaewa*. These six mechanisms are; (1) geographical barriers, (2) biogeographical boundaries, (3) competition, (4) minimum viable population size, (5) host–parasite relationship, and (6) temporal population stability.

- Mechanisms 1 and 3 are both obvious factors that could ensure allopatry in *Engaewa*. Geographical barriers are undoubtedly important to *Engaewa* with there being many obvious habitat discontinuities across the range of the genus. Although the occurrence of competition between *Engaewa* species is unknown, it is at least plausible.

- It is unclear why Wilke and Pfenninger (2002) suggested that biogeographical boundaries (mechanism 2) could be responsible for maintaining allopatry, as biogeographic boundaries are merely a recognition of a boundary where multiple species turnover. Clearly species encounter some factor at a boundary that causes a divergence (or has historically), although the boundary is not a cause of such a divergence. The specific example given by Wilke and Pfenninger (2002) is that different ecological conditions across the biogeographical boundary may prevent the dispersal of species across the boundary. Whilst a biogeographical boundary does not ensure allopatry, an ecological boundary may be responsible for turnover in *Engaewa* species.

- Wilke and Pfenninger (2002) suggested minimum viable population size (mechanism 4) is significant as (particularly for the mud snails they were studying) a large number of individuals are needed to create a self-sustaining population. Therefore, the occasional dispersal of a few individuals into the habitat of another species would not result in sympatry. This may also be significant for *Engaewa* as they would need to enter the new habitat, create burrow systems and still find a mate of the same species in order to reproduce.

- Mechanism 5 is not likely to be important for *Engaewa* as there is no reason to suggest that these species would be differentially affected by parasites, as they are found over a relatively small area, making the occurrence of different parasites unlikely (in comparison to other species that may occur across separate continents for instance).

- Mechanism 6 will be most important in situations where habitat is largely ephemeral and local extinction events occur. In this scenario, if a sympatric situation had developed then both species may be extirpated and re-establishment will most likely occur via recruitment from neighbouring populations; thus potentially reverting to a single species situation. The significance of this situation to *Engaewa* is not clear as there is likely to be little opportunity for re-establishments in the short term (i.e. no metapopulation process) due to their low vagility and dispersal ability, however, over longer time scales this scenario may occur.

Based on the above considerations it is proposed that the 'natural' state for *Engaewa* species is that of allopatry resulting from abiotic factors that produced both historical and contemporary geographic boundaries. However, it is likely that this is (and historically has been) reinforced by the effect of ecological influences. This may indicate that *Engaewa* is largely in a 'Goldilocks' situation, whereby the species generally show niche conservatism, though slight differences develop that are just sufficient to prevent sympatry occurring. Whilst the complexity of these differences is not well understood, the general habitat characteristics for each species highlight the gross variation within and between species (Table 4.1 – with species specific detail provided in the next section (4.3)).

Table 4.1 Habitat and landform summary of typical conditions for significant regional groups within the *Engaewa* species.

Species	Soil	Vegetation	Hydrology (during wet season)	Elevation (m asl)	Aspect[*]
E. pseudoreducta	Clay	Dense shrub strata, minimal tree strata	Surface water present that can cover burrows	110-120	Southerly
E. pseudoreducta (Payne Road)	Sand	Open and low shrub strata, no tree strata	Surface water present in highly defined shallow channels that can cover some burrows	40	No discernable aspect
E. reducta (north)	Sand with or without organics	Dense shrub strata, minimal tree strata	With or without surface water nearby to burrows	20-75	Variable
E. reducta (south)	Sand/Clay	Dense or open shrub strata, dense or open tree strata	With or without surface water nearby to burrows	25-50	Variable
E. similis	Peat	Dense or open shrub strata, dense tree strata	Often with surface water nearby to burrows	25-115	Variable
E. similis (Scott Coastal Plain)	Sand	Low and open shrub strata, no tree strata	No surface water present	10-30	No discernable aspect
E. subcoerulea	Sand with or without organics	Low and open shrub strata, lacking tree strata	No surface water present	10-90	No discernable aspect
E. walpolea	Peat	Dense shrub strata, minimal tree strata	Often surface water covering burrows	0-30	Variable (mostly southerly)
E. clade A	Peat	Dense shrub strata, no tree strata	Often without surface water nearby to burrows	35	Southerly/ South-eastern
E. clade B	Sand with organics	Dense shrub strata, no tree strata	With or without surface water nearby to burrows	10-60	Variable

[*] As a general rule, all species exhibit higher densities on south/south-eastern aspects except where the vegetation is particularly dense or on large, flat coastal plains.

4.3 Habitat and conservation status of *Engaewa* species

In the assessment of each species/clade recognised in this study provided below, the current conservation status is listed (IUCN, Federal and State) and then a recommendation is made, considering the additional information acquired during this study and based on the IUCN Red List categories and criteria (IUCN, 2012). There are five basic criteria (A-E, with numerous further criteria contained within each of these) by which taxa must be assessed for inclusion on the IUCN Red List; A. Declining population (past, present and/or projected) B. Geographic range size, and fragmentation, decline or fluctuations C. Small population size and fragmentation, decline, or fluctuations D. Very small population or very restricted distribution E. Quantitative analysis of extinction risk (e.g., Population Viability Analysis). It is important to note that currently *Engaewa* species can only be assessed under criteria B or D (Table 4.2), which relate to distribution, as there is insufficient knowledge to assess them based on population sizes or a quantitative analysis of extinction risk. Criterion B is further divided into B1 and B2, which are Extent of Occurrence (EOO) and Area of Occupancy (AOO), respectively, however due to the difficulty of determining AOO for *Engaewa* species only EOO will be considered. For all species the EOO polygons presented are conservative (i.e. they are far more likely to be an overestimation than an underestimation), as they encapsulate all likely habitat geographically linking known sites and including a degree of additional buffering, and there is little doubt that using an AOO to assess these species would result in the same, or higher, threat category.

Climate change is obviously of considerable relevance to species that appear to be climate relicts, however, there are noted difficulties in assessing the relationship of climate change with the various criteria in the IUCN Red List document (IUCN, 2013). Under the criteria relating to geographic range, which are the most relevant to *Engaewa*, the impacts of climate change can be used to satisfy criterion B1b or B2b (i.e. a continuing decline, observed, inferred or projected, in any of the following: extent of occurrence - area of occupancy - area, extent and/or quality of habitat - number of locations or subpopulations - number of mature individuals), though not criterion (B1a or B2a) (i.e. severely fragmented).

Table 4.2 IUCN Red List criteria B and D that are used to assess the conservation status of *Engaewa* species (IUCN, 2012).

B. Geographic range in the form of either B1 (extent of occurrence) AND/OR B2 (area of occupancy)			
	Critically Endangered	**Endangered**	**Vulnerable**
B1. Extent of occurrence (EOO)	< 100 km²	< 5,000 km²	< 20,000 km²
B2. Area of occupancy (AOO)	< 10 km²	< 500 km²	< 2,000 km²
AND at least 2 of the following 3 conditions:			
(a) Severely fragmented OR Number of locations	= 1	≤ 5	≤ 10
(b) Continuing decline observed, estimated, inferred or projected in any of: (i) extent of occurrence; (ii) area of occupancy; (iii) area, extent and/or quality of habitat; (iv) number of locations or subpopulations; (v) number of mature individuals			
(c) Extreme fluctuations in any of: (i) extent of occurrence; (ii) area of occupancy; (iii) number of locations or subpopulations; (iv) number of mature individuals			
D. Very small or restricted population			
	Critically Endangered	**Endangered**	**Vulnerable**
D. Number of mature individuals	< 50	< 250	D1. < 1,000
D2. *Only applies to the VU category* Restricted area of occupancy or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.	-	-	D2. typically: AOO < 20 km² or number of locations ≤ 5

4.3.1 *Engaewa pseudoreducta*

HABITAT

The type locality and most known populations of *E. pseudoreducta* occur in very narrow headwater drainages, with a dense vegetation structure and heavy clay soils, in and adjoining State Forest No. 62 (Figure 4.7). The burrows found during this study were identified by small piles of soil that differed slightly in colour from the surrounding soil. This soil is likely to have represented small, washed down chimneys, as there had been significant rainfall and the burrows were within a narrow, but wet, creek line. Small chimneys may be indicative of a combination of small burrows, little maintenance occurring (due to the clay holding its shape well), and a high degree of weathering in the area. As the water table was essentially at ground level at the time of collecting this species, the burrow systems were not fully explored though they appeared to branch laterally at a shallow depth, as well as possessing tunnels proceeding deeper. It may be that *E. pseudoreducta* burrows are relatively small by *Engaewa* standards, suggesting a general pattern of burrow size relating to body size, as *E. pseudoreducta* specimens are relatively small.

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Figure 4.7 Habitat at the Treeton site where *Engaewa pseudoreducta* specimens were collected.

The population that was discovered further north of the species' previously recognised distribution (at Payne Road) was found in habitat that is significantly different. Whereas the typical *E. pseudoreducta* habitat is in narrow, sloping depressions in clay soils, the Payne Road site is a larger flat plain type habitat (although much smaller than the coastal plains), with very small dendritic channels amongst sparse and stunted vegetation (Figure 4.8a). The soil at this site is predominantly coarse white sand, rather than clay (Figure 4.8b).
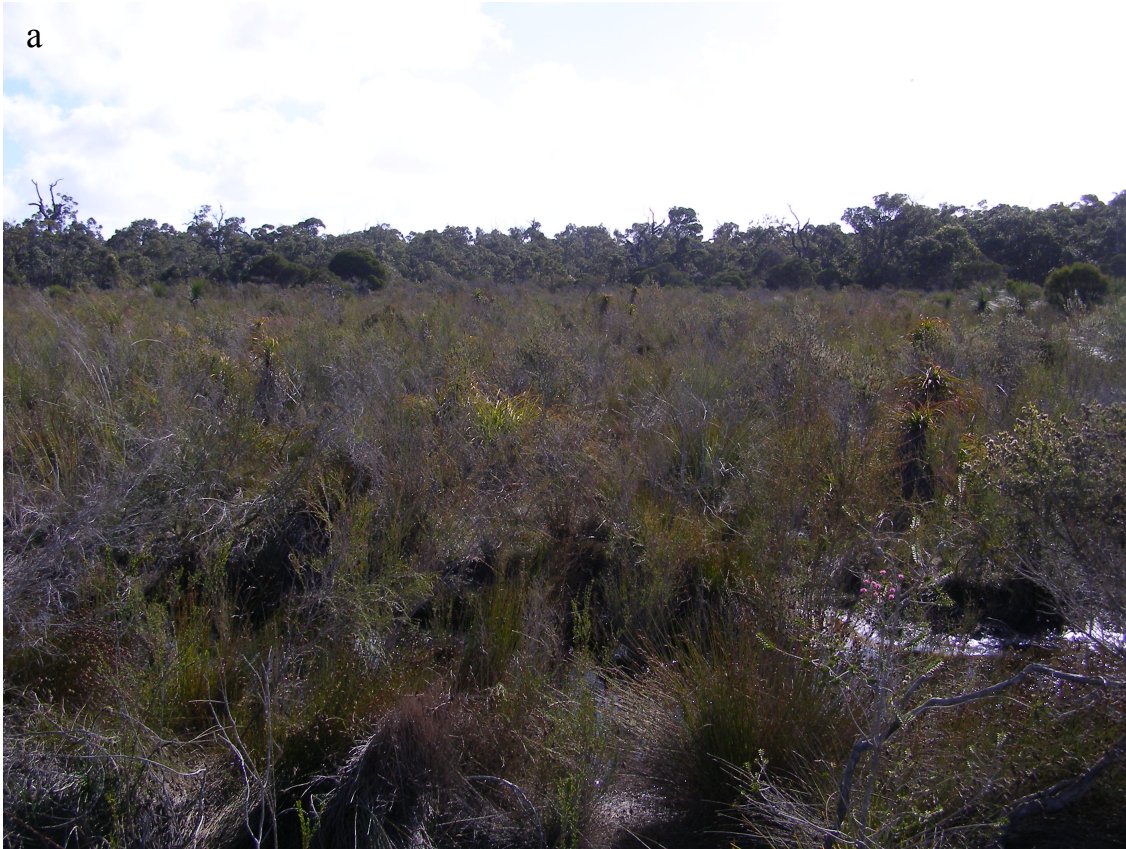
Figure 4.8 Habitat at Payne Road (vegetation (a), and soil (b)), where *Engaewa pseudoreducta* specimens were collected.

CONSERVATION STATUS

Current status: (IUCN)   Critically Endangered

                      (Federal)   Critically Endangered

                      (State)   Critically Endangered

Suggested status: Critically Endangered (B1a,b(iii))

Concern for *E. pseudoreducta* was raised by Horwitz and Adams (2000) since it could no longer be found at the type locality, which was converted to a farm dam and the local catchment converted to a blue gum plantation, had only been found at one other site, and the known range of the species prior to this study constituted less than 3 km^2. *Engaewa pseudoreducta* was subsequently gazetted on Schedule 1 as fauna that is rare or is likely to become extinct (Wildlife Conservation (Specially Protected Fauna) Notice 2006), under the *Western Australian Wildlife Conservation Act 1950*, on the criteria that it had very restricted areas of occurrence and occupancy, with extreme fluctuations in area, extent and/or quality of habitat, and number of locations or subpopulations. Recovery planning by the State's Department of Environment and Conservation commenced in 2007 for this species, during which time nominations were prepared for federal recognition and in 2009 the species was gazetted as Critically Endangered under the Commonwealth of Australia's EPBC Act (1999). This species has also been assessed as Critically Endangered on the IUCN Red List (Burnham, 2010a).

As noted in Chapter 3 two individuals whose taxonomic status is unclear were collected from a site on Payne Road. The Payne Road population is represented by only two individuals and *E. pseudoreducta* is represented by a single specimen (in the genetic data), however, they appear to be most closely related to each other, both in terms of the genetic study presented here and based upon a morphological designation. Therefore, following the methodology of this study the Payne Road specimens are conservatively considered to represent a divergent lineage contained within the species *E. pseudoreducta*. As well as a taxonomic motivation for this decision (i.e. making a designation based on only two specimens from a single populations is difficult, and arguably irresponsible), there is also a sound conservation basis for this decision. As *E.*

pseudoreducta currently has the highest possible recognition from a conservation viewpoint it seems prudent to group these two lineages so that the Payne Road population receives the same conservation protection until further revisions can be undertaken.

That additional populations were uncovered during sampling potentially bodes well for the survival of the species as a whole, although an increase from one to three populations is obviously not a reason to reduce concern, particularly as downstream habitat alteration has isolated all populations into small pockets of suitable habitat. The genetic divergence between these two samples hints at the possibility that interpopulation mtDNA diversity is extremely high for *E. pseudoreducta*, thus making these existing populations even more significant from a conservation viewpoint. Therefore, the currently acknowledged geographic range of *E. pseudoreducta* should include the drainage system from which the original description was made and be extended to include the population at Payne Road, some 16 km north (Figure 4.9). Including the population at Payne Road increases the known range of *E. pseudoreducta* to ~76 km^2 (Figure 4.9).
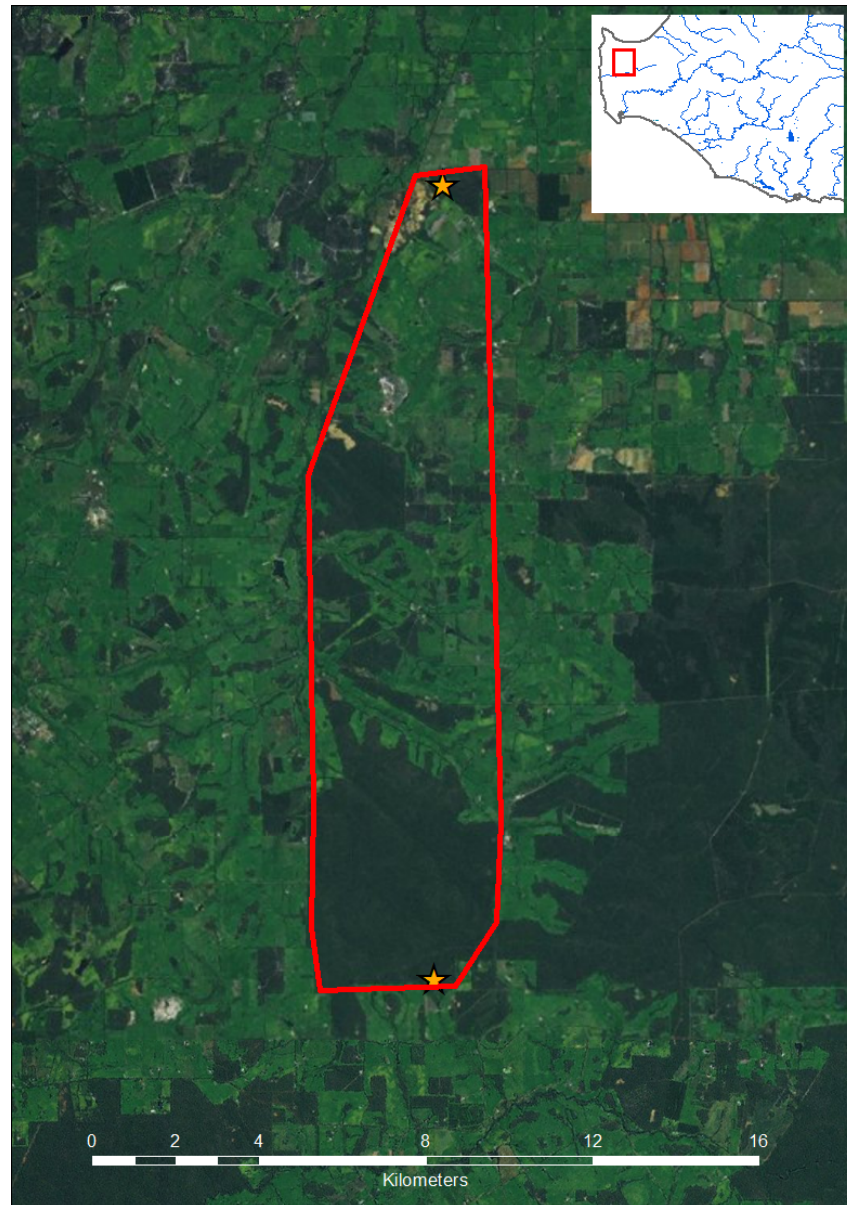
Figure 4.9 Distribution of *Engaewa pseudoreducta*, with the polygon used to assess the Extent of Occurrence for this species shown. For sources of GIS data see Section 2.5.

Based on the data presented in this study it appears that the conservation status of *E. pseudoreducta* should remain unchanged (Critically Endangered). The geographic range of this species, in terms of the EOO (criterion B1), falls well within the boundaries set within the criteria document for Critically Endangered (<100 km^2). Furthermore to fully satisfy the criterion of B1, and thus be validly considered as Critically Endangered, the species in question must conform to at least two of three further requirements. Whilst *E. pseudoreducta* is no longer believed to exist at only a single location there is no doubt that the distribution of populations is severely

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fragmented, thus satisfying criterion B1a. Despite the increase of EOO resulting from this study, the loss of the population at the type locality and threats faced by remaining populations (particularly in the form of changed hydrological regimes, resulting from human activities such as damning of surface waters and extraction of groundwater), satisfies criterion B1b(iii) (decline of area, extent and/or quality of habitat) as it can be reasonably argued that all of these can be 'inferred or projected' based on the past and on-going anthropogenic impacts in the area, particularly when combined with suggested impacts of future climate change for the region (Horwitz *et al.*, 2008).

4.3.2 *Engaewa reducta*
<u>**HABITAT**</u>

The distribution of *E. reducta* is divided into two areas. In the northern extent of the distribution of the genus, the extreme habitat modification that has occurred makes it difficult to assess the variety of habitat that may have previously been utilised. Currently *E. reducta* is found primarily in sandy, deep draining soils in relatively narrow drainages, where they dig expansive burrow systems and produce large sandy chimneys (Figure 4.10a). The vegetation in these habitats is generally open with little to no tree canopy (Figure 4.10b); however, they may also be present in tea-tree swamps in the far north of their range. In the southern portion of their range (around the Blackwood River) they also dig large burrow systems often in sand (Figure 4.11a), however, the clay content of the soil may be much higher (Figure 4.11b). The vegetation in this region is again generally of an open nature. In both the northern and southern populations burrows are found both in areas of habitat where the water table would, or almost would, reach the surface and would periodically be shallowly flooded (whether by the water table or surface run-off) (as can be seen in Figure 4.10a), and in areas where the water table would rarely, if ever, reach the surface.

Figure 4.10 Habitat (soil (a), and vegetation (b)) typical of *Engaewa reducta*.

Figure 4.11 Differences in the soil types from the habitats occupied by *Engaewa reducta* in the southern portion of its range (i.e. near the Blackwood River).

CONSERVATION STATUS

Current status: (IUCN)   Endangered

                        (Federal)   Critically Endangered

                        (State)   Endangered

Suggested status: Least Concern

The current IUCN Red List assessment for *E. reducta* determined that the species should be listed as Endangered based on its geographic range (criteria B1, EOO <5000 km^2) (Burnham, 2010b). Based on the result of this study the range of *E. reducta* is greatly expanded, though the EOO still totals only ~939 km^2 (Figure 4.12). Clearly this species still meets this basic requirement to be considered Endangered by the Red List, however, there are further requirements to be met.

In the IUCN assessment it was determined that the distribution of populations is severely fragmented, with the fragmentation of habitat being caused by cattle grazing and large-scale water abstraction – thus satisfying criterion B1a. Criterion B1b was also met as there was an observed decline of area of occupancy, area, extent and/or quality of habitat, as well as number of locations or subpopulations. This was largely based on Horwitz and Adams (2000) report of *E. reducta* having been extirpated from its type locality, due to the impact of cattle, water hole construction and hydrological changes associated with encroaching urbanisation.
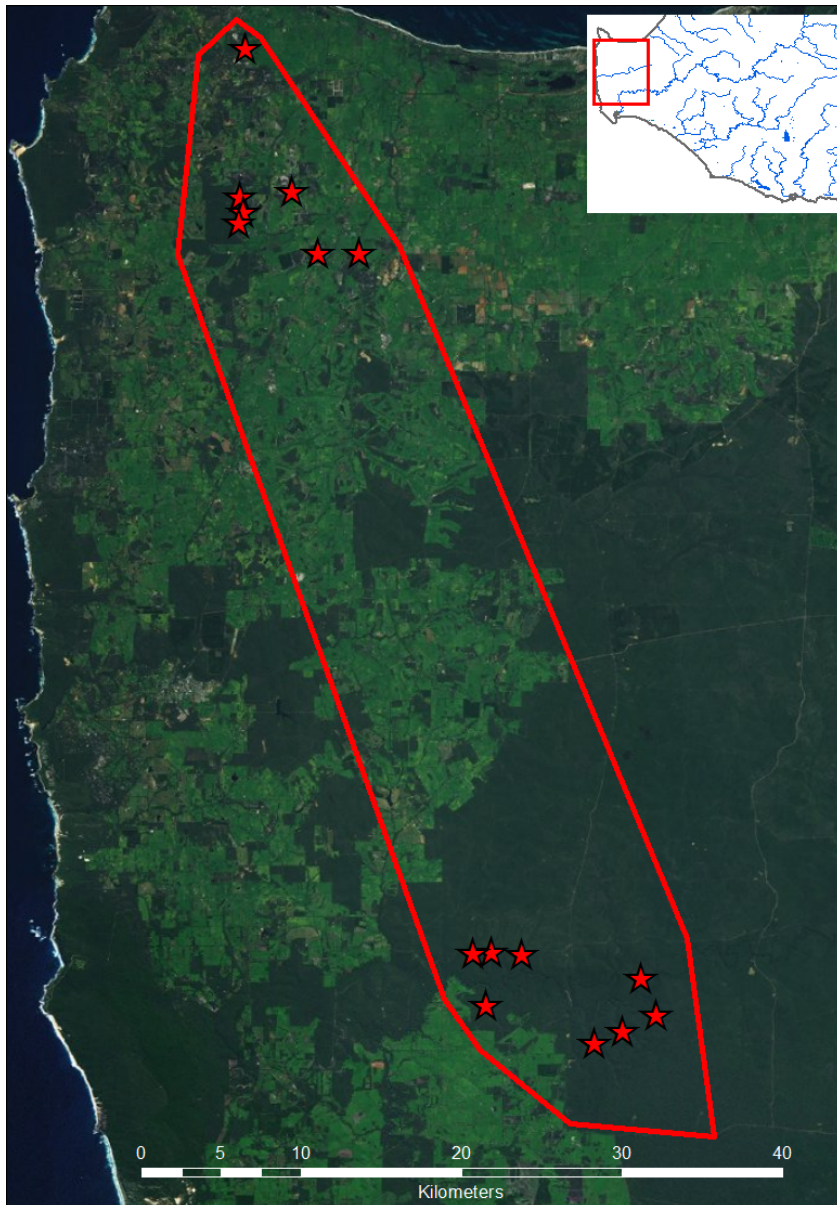
Figure 4.12 Distribution of *Engaewa reducta*, with the polygon used to assess the Extent of Occurrence for this species shown. For sources of GIS data see Section 2.5.

The discovery of two divergent lineages within *E. reducta* occurring in two distinct distribution centres poses interesting questions for the conservation of this species. The newly uncovered southern populations are entirely contained within State Forest No. 32 and are in relatively pristine habitat (with the exception of a road crossing each drainage), whilst there are northern populations known to occur within the Haag Nature Reserve and Timber Reserve No. 139 25 south-east of Dunsborough. However, much of the habitat in the northern area is highly fragmented and a largely agricultural matrix surrounds many of the populations. There is little doubt that on-going impacts

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(as outlined in the current assessment) still meet the requirements of B1b, however, B1a (severely fragmented) can no longer be met. In order to be severely fragmented "more than half of the individuals (or, more than half of the occupied habitat area) must be in small and isolated patches" (IUCN, 2013), which is not the case for *E. reducta* once the newly recognised southern populations are considered. Without being severely fragmented, B1a can only be met based on the number of locations. Due to the likelihood of future extirpations (especially if, as suggested here, the current conservation protection afforded this species be rescinded) this species may in the relatively near future meet the criteria to be listed as Vulnerable (≤10 locations). It is also possible that species can be assessed as Vulnerable under criteria D2 if they have an AOO <20 km^2. Therefore, before any changes are made to the conservation status of this species it is suggested further detailed surveying to accurately determine the AOO of this species should be undertaken.

4.3.3 *Engaewa similis*
<u>**HABITAT**</u>

The habitat occupied by *E. similis* can be divided into two distinct types, based on geographic region. In the southern part of their distribution (i.e. the Scott Coastal Plain) they are found predominantly in deeply draining sandy coastal soils, with vegetation that is either stunted and sparse, or with thick low shrub (e.g. Figure 4.13). Throughout much of this region the water table rarely reaches the surface and the crayfish dig substantial burrow systems, reaching in excess of 2 m in depth. In the northern portion of the distribution of this species (from Augusta northward) *E. similis* can be found most commonly in moist peaty loam soils around the edges of drainage lines, where they dig burrow systems that often branch laterally close to the surface. The vegetation structure in these habitats generally includes a significant canopy of small trees (Figure 4.14).

Figure 4.13 Habitat typical of that occupied by *Engaewa similis* in the southern portion of its range (i.e. the Scott Coastal Plains).

Figure 4.14 Habitat typical of *Engaewa similis* in the northern portion of its range (i.e. north of Augusta).

CONSERVATION STATUS

Current status:	(IUCN)	Least Concern
	(Federal)	Not Listed
	(State)	Not Listed
Suggested status:		Least Concern

Horwitz and Adams (2000) suggested *E. similis* was not considered to be of conservation concern, due to its relatively wide distribution, large number of populations and their occurrence within protected areas (Scott and D'Entrecasteaux National Parks and the Gingilup Swamps Nature Reserve). It was also suggested in the most recent Red List assessment that, although there have been impacts due to human activities related to urbanisation and land drainage, there has been significantly less impact in recent years and this crayfish is abundant where still persisting (Burnham, 2010c). This was mirrored in the status of Least Concern in the Red List. Despite this, it was suggested that this assessment was largely due to a lack of understanding related to the degree of fragmentation between populations, and that its known extent would qualify the species as Endangered (EOO <5000 km^2).

This species is relatively widely distributed in the southern part of its range, whilst simultaneously facing increasing pressure in the more northern areas (specifically from Augusta northward) due to numerous human endeavours, such as drainage, urbanisation and grazing of cattle. Currently the EOO of ~4275 km^2 (Figure 4.15) would qualify this species to be listed as Endangered (criteria B1, <5000 km^2), however (as previously stated), there is a need to meet two of the three additional requirements; for *Engaewa* species this (for reasons discussed earlier) would be B1a and/or B1b. *Engaewa similis* cannot be listed under B1a, for either being severely fragmented or for the number of locations. The large area of habitat in the southern region means that the majority of the total AOO (and most likely the majority of individuals) is not in isolated habitat patches, thus this species cannot be considered severely fragmented, and there are more than 10 locations, which is the largest number that would allow for listing under a threat category (i.e. Vulnerable). Thus, despite the likelihood that many

populations may be lost in the near future, the status of *E. similis* as Least Concern should remain.

Figure 4.15 Distribution of *Engaewa similis*, with the polygon used to assess the Extent of Occurrence for this species shown. For sources of GIS data see Section 2.5.

4.3.4 *Engaewa subcoerulea*
<u>**HABITAT**</u>

Engaewa subcoerulea predominantly inhabit large coastal plains where they dig expansive burrow systems, which will often reach 2 m or more along both the horizontal and vertical axes. They are generally found in sandy soils with or without organic matter and they are also common in gravely road edges. Corresponding to the large burrow systems, the chimneys of this species tend to be extremely large, although once the sand dries it often crumbles, losing the typical pelleted appearance. In these areas the vegetation is either stunted and sparse (Figure 4.16a), or dense and composed of shrubs up to 2.5 m high (Figure 4.16b). Based on the earlier described pattern of topography/soil unit as a determinant of distribution there is one population of *E. subcoerulea* that occurs in an unexpected location. This population was identified on the outskirts of the townsite of Northcliffe in a site that did not conform to the general characteristics of the habitat typical of this species. It was in a site that had a dark peaty loam soil overlaying deeper sand, with a closed canopy of vegetation and was much wetter than typical *E. subcoerulea* habitat.

Figure 4.16 (a) Coastal plain type habitat typical of *Engaewa subcoerulea*. The site shown in (b) is along a road edge that had recently been slashed and the dark soil of many *Engaewa* 'chimneys' is visible.

CONSERVATION STATUS

Current status:	(IUCN)	Least Concern
	(Federal)	Not Listed
	(State)	Not Listed
Suggested status:		Least Concern

Of all *Engaewa* species *E. subcoerulea* appears to be the most secure from a conservation viewpoint, at least for the near future, and was assessed for the IUCN Red List as belonging to the category of Least Concern (Burnham, 2010d). It is relatively widespread in comparison to other *Engaewa* species (although less so once the distinction is made between *E. subcoerulea* and *E. clade B*), but would still qualify as Endangered based on its EOO (~2113 km^2) (Figure 4.17). However, as this species neither appears to be severely fragmented, nor existing in a small number of locations, it does not meet the criteria for B1a. This species also has many large populations existing in protected areas (D'Entrecasteaux, Shannon, & Walpole-Nornalup National Parks and Gladstone, Keystone & Pingerup State Forest), which are currently experiencing little habitat disturbance, beyond the relatively minor and localised impacts caused by introduced feral pigs, thus it does not meet any of the criteria for B1b (although the impacts resulting from climate change should be considered in future assessments). Therefore, the outcomes of this study suggest that there should be no change to current Red List status for this species.
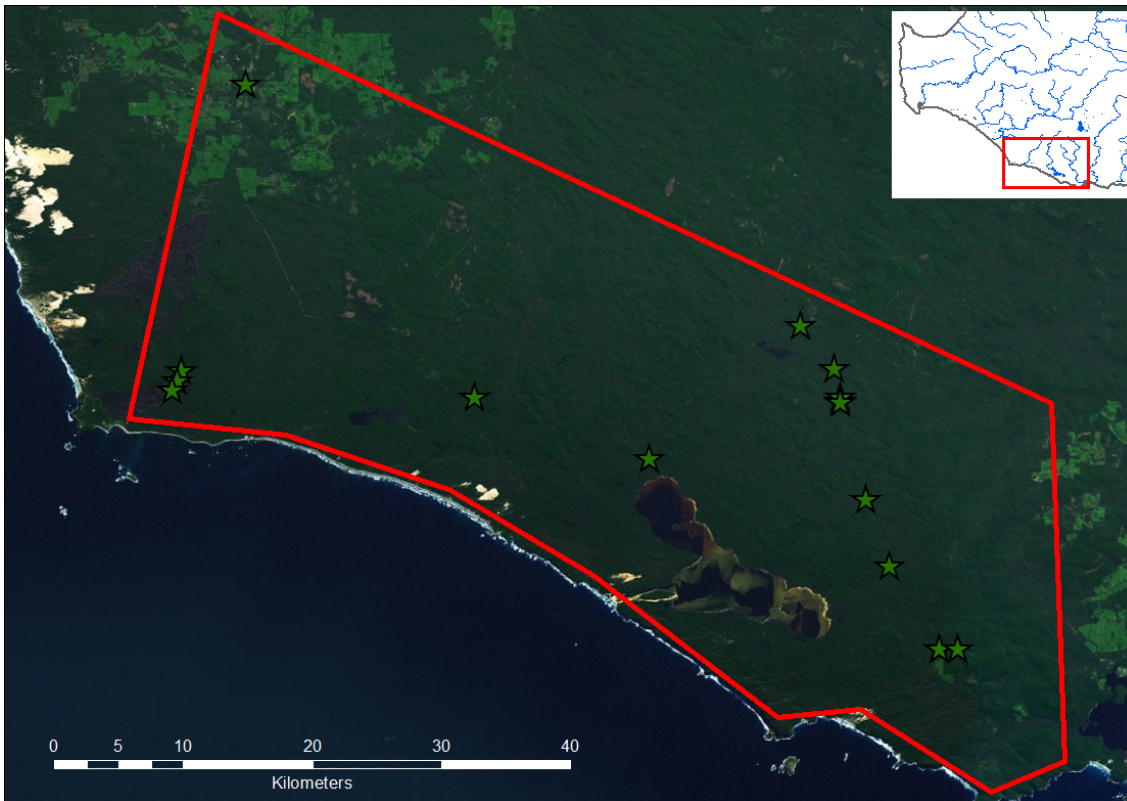
Figure 4.17 Distribution of *Engaewa subcoerulea*, with the polygon used to assess the Extent of Occurrence for this species shown. For sources of GIS data see Section 2.5.

4.3.5 *Engaewa walpolea*
<u>**HABITAT**</u>

Engaewa walpolea tend to be found in areas that are particularly wet by *Engaewa* standards, including in the bottom of depressions or in channels that become completely submerged for significant periods of time. These areas generally have very dense vegetation, of varying heights (e.g. Figure 4.18a). The burrow systems of *E. walpolea* are generally quite shallow (many appeared to end around 40 cm below the surface) and relatively simple compared to the other *Engaewa* species; hence this species generally creates only small chimneys (Figure 4.18b), or often there will be no chimney at all and the burrow can only be identified by small holes. Specimens of this species collected from burrows were found within a maximum of 50 cm from the surface, though most were found significantly closer to the surface (in the range of 10-20 cm). As this species is small this again supports a correlation between body size (and possibly chelae size) and degree of burrowing. *Engaewa walpolea* were also found occupying burrows with small *Cherax*, perhaps digging short tunnels off the main

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Cherax burrow; further suggesting this species may not be as strongly burrowing as the other species. Associations between *Engaewa* and *Cherax* species have previously been recorded by both Riek (1967, 1969) (*E. subcoerulea* with *C. crassimanus*) and Horwitz and Adams (2000) (*E. subcoerulea* with both *C. crassimanus* and *C. preissi*, *E, pseudoreducta* with *Cherax glaber* Riek and *C. quinquecarinatus* also noted at the site, and *E. similis* with *C. crassimanus*), though it seems most pronounced in this species.

It seems that *E. walpolea* burrows are predominantly found in shallow dark peaty loam soils, some with a higher proportion of sand, overlaying a layer of gravel and/or clay. Soil profiles were examined at two sites where *E. walpolea* were present. An impenetrable layer of gravel and/or clay was encountered at 1.30 m and 1.65 m below the ground level, assumedly representing a shallow occluding layer, which would restrict further digging. By comparison, soils examined where other *Engaewa* species were present continued to depths in excess of 2 m at a number of sites and in some cases deeper than 3 m. This occluding layer, if largely impervious to water, may create a perched water table, allowing the crayfish to remain in contact with the groundwater without needing to burrow deeply.

When collected via spotlighting *E. walpolea* specimens appear to be much more energetic than those of other species and regularly attempt to "tail flip" (the caridoid escape reaction; where the abdominal muscles are rapidly contracted, causing the crayfish to be propelled rapidly backward (Wiersma, 1947)). However, it has been suggested that strongly burrowing crayfish do not perform tailflips (Reynolds *et al.*, 2012b; Richardson, 2007) and specimens from the other *Engaewa* species are docile when collected, even when placed into water. Whether this is related to species-specific differences or due to the different collecting method is not clear, however, on the single occasion that specimens were collected by spotlighting at Spearwood Creek (*E. clade A*) they were not noted to perform tail flips. Two berried females of *E. walpolea* were collected via spotlighting in September and were seen to be holding their tail in a manner that curled around the eggs and sealed them from view (a habit also recorded by Horwitz (1988b) for species of *Engaeus*). An additional berried female was found in a very shallow burrow.

Figure 4.18 Habitat typical of *Engaewa walpolea* (a). Very small chimneys (compared to those formed by most other *Engaewa* species), such as seen in (b), are characteristic of *Engaewa walpolea*.

CONSERVATION STATUS

Current status: (IUCN)   Endangered

                        (Federal)   Endangered

                        (State)   Vulnerable

Suggested status: Endangered (B1a,b(iii))

The current IUCN Red List assessment lists *E. walpolea* as Endangered, based on the geographic range of this species (EOO – criteria B1), it persisting in ≤5 locations (B1a), and a continuing decline of extent of occurrence, and of area, extent and/or quality of habitat (B1b(i,iii)). However, it was stated that the "distribution is fragmented, but the degree of fragmentation is unclear. If it were in fact found that the sites were severely fragmented, then this species would qualify for a listing under Critically Endangered" (Burnham, 2010e) as the EOO was actually under this threshold (i.e. to qualify as Critically Endangered under B1a the species needs to be either severely fragmented or have number of locations = 1).

The EOO of this species has increased based on the sampling undertaken in this study (from 28 km^2 to ~127 km^2 – Figure 4.19), which still qualifies it as Endangered, however its EOO is now slightly larger than that required to qualify as Critically Endangered (EOO <100 km^2) under criteria B1 (although as outlined earlier in this chapter the EOO's defined in this study are more likely than not slight overestimations). Once again, as for all *Engaewa* species, its actual AOO is far less than its EOO and if it could accurately be calculated would likely fall under the guidelines for being considered Critically Endangered (<10 km^2).
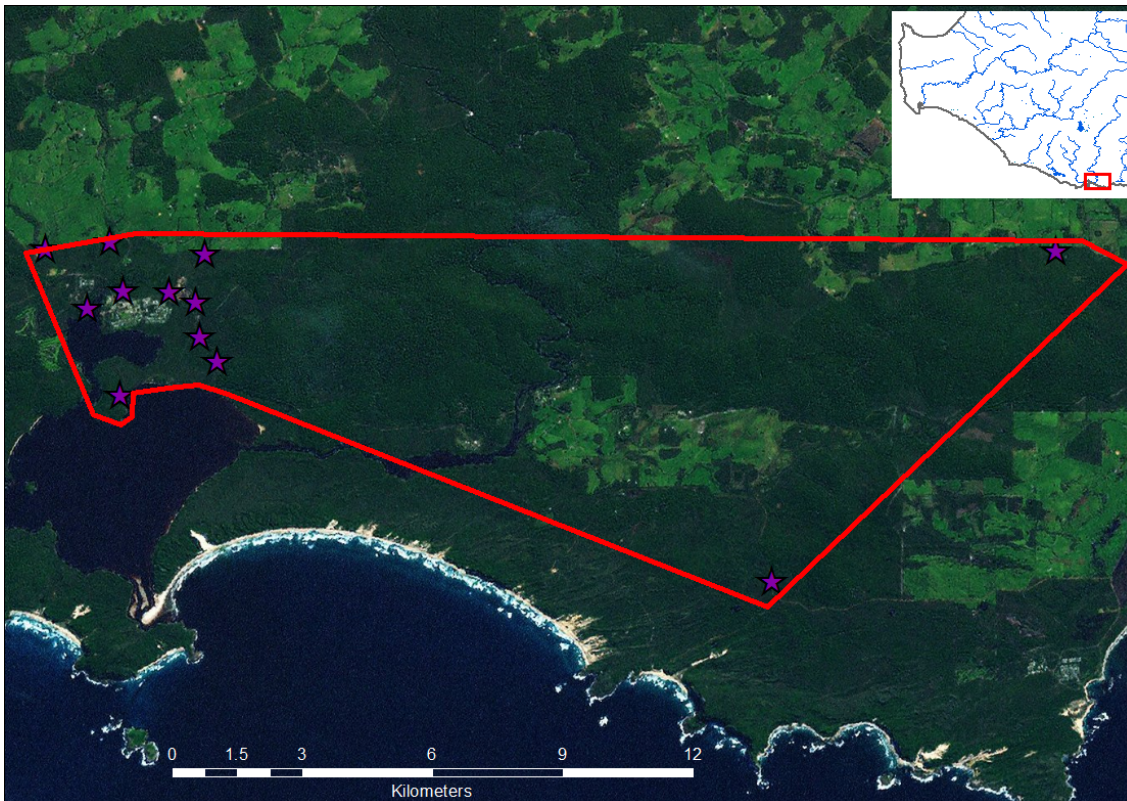
Figure 4.19 Distribution of *Engaewa walpolea*, with the polygon used to assess the Extent of Occurrence for this species shown. For sources of GIS data see Section 2.5.

To be listed under the Red List criteria B the range of this species also needs to be considered severely fragmented, or the number of locations must fall under any of the values required for the relevant threat category. This is difficult to assess, as it is hard to define fragmentation for *E. walpolea*, as the connectivity between the geographically proximate sampling sites is poorly understood. The haplotype networks that were produced in Chapter 3 suggested that most sampling sites for this species have historically been reasonably connected with a shared haplotype covering much of the species range. As this species appears to have greater dispersal ability than other *Engaewa* species, and much of the habitat is (at least potentially) connected it does not appear that this species should be considered severely fragmented. However, it can be argued that the immediate proximity of most sampling sites to the town of Walpole means that they should be considered as one location (based on the IUCN definition – "a geographically or ecologically distinct area in which a single threatening event can rapidly affect all individuals of the taxon present" (IUCN, 2013 p.41) and including the

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few disparate locations from which this species has been recorded means that it meets the criteria for Endangered under (B1a), based on locations ≤5.

Under the current IUCN assessment it was determined that there was a continuing decline of extent of occurrence (B1b(i)), based largely on their extirpation from the type locality and on-going development in the area (Horwitz & Adams, 2000). However, this study has increased the EOO for this species, and there does not appear to have been more recent losses of populations. Whilst there have not been noted decreases to the EOO there have undoubtedly been on-going declines to the area, extent and/or quality of habitat for these crayfish; thus satisfying B1b(iii). Although some populations occur in protected areas (Keystone and Tingle State Forests), most habitat has been degraded to some degree through impacts resulting from farming practices (including leaching of pesticides, herbicides and fertilisers, alterations to surface and subsurface water flows, and increased siltation of creeks), logging activities, land clearing, road construction and maintenance, increasing urbanisation, altered hydrological flows, feral animals and weeds, and possibly through shifting fire regimes (although the exact impact of this is poorly known).

4.3.6 *Engaewa clade A*
<u>HABITAT</u>

Engaewa clade A is currently known only from Spearwood Creek, which is a large U shaped paluslope valley into which the Leederville aquifer discharges (thus maintaining a relatively high water table all year round). As previously mentioned, this site is the only one from which crayfish other than *E. walpolea* were collected via spotlighting. Collection via this method occurred during a thunderstorm when the entire valley slope was saturated with a few centimetres of surface water present. When compared to the other *Engaewa* habitats that are found parallel to Spearwood Creek, it is much broader and has a more open vegetation structure (Figure 4.20a), with a peaty soil (Figure 4.20b) compared to either sandy or clay-based soils (Figure 4.11a&b). On the opposite side of the Blackwood River the habitat is largely open floodplains, as opposed to the peat paluslopes that form Spearwood Creek.

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Figure 4.20 Vegetation (a) and soil (b) at Spearwood Creek where *Engaewa clade A* are found.

CONSERVATION STATUS

Current status: Not Assessed

Suggested status: Vulnerable (D2)

Engaewa clade A is known only from a single location and the neighbouring parallel creek lines have all been found to possess *E. reducta*. The surrounding area was thoroughly searched during this study and all habitats that were deemed suitable for *Engaewa* were found to contain another *Engaewa* species. This situation suggests there

may not actually be any additional populations of this 'candidate species'. The single known population is within the State Forest No. 32 (proposed National Park) and occurs along with Threatened Ecological Communities, but any species known from a single population obviously faces an extremely high extinction risk, due purely to stochastic processes.

Assuming there is no wide-scale disturbance (e.g. a large-scale groundwater extraction scheme in the region as was recently proposed – though later rejected) then the immediate conservation of this population (and by extension the entire species), beyond possible stochastic occurrences appears assured in the short term, as the only threatening process likely to occur in the short-term is the possible disturbance to the habitat caused by feral pigs (although the impact of fire should be considered). On a longer time scale alterations to the region's character resulting from climate change may be a significant issue, as this population may not have the capability to migrate in response, and should remain a consideration.

Despite the apparently stable current situation this 'candidate species' should be reviewed for conservation listing, assuming it will be formally described as a species, as any species existing as a single population faces an obvious danger of extinction. Applying the listing scheme of the IUCN the highest threat category this candidate species would be eligible for is Vulnerable. The IUCN Red List Categories and Criteria: Version 3.1. (2012 p.20) states that "A taxon is Vulnerable when the best available evidence indicates that it … [is] considered to be facing a high risk of extinction in the wild". *Engaewa clade A* meets criteria D for listing as Vulnerable as it exists as a "population with a very restricted area of occupancy (typically less than 20 km^2) or number of locations (typically five or fewer) such that it is prone to the effects of human activities or stochastic events within a very short time period in an uncertain future, and is thus capable of becoming Critically Endangered or even Extinct in a very short time period".

4.3.8 *Engaewa clade B*
HABITAT

Few *E. clade B* sites are known but this species appears to be at least similar to *E. subcoerulea* ecologically. *Engaewa clade B* are found in both large, flat coastal type habitats (i.e. sandy soils with low shrubby vegetation) although they are also found in both narrow and broad valleys (e.g. Bow Bridge and Kent River, respectively) where the soil is more peaty, the habitat generally wetter and the vegetation growing up to 2.5 m tall (Figure 4.21a&b). Where *E. clade B* have been found, the burrow density is usually high with large chimneys present, and at the time of collection the water table was close to the surface.
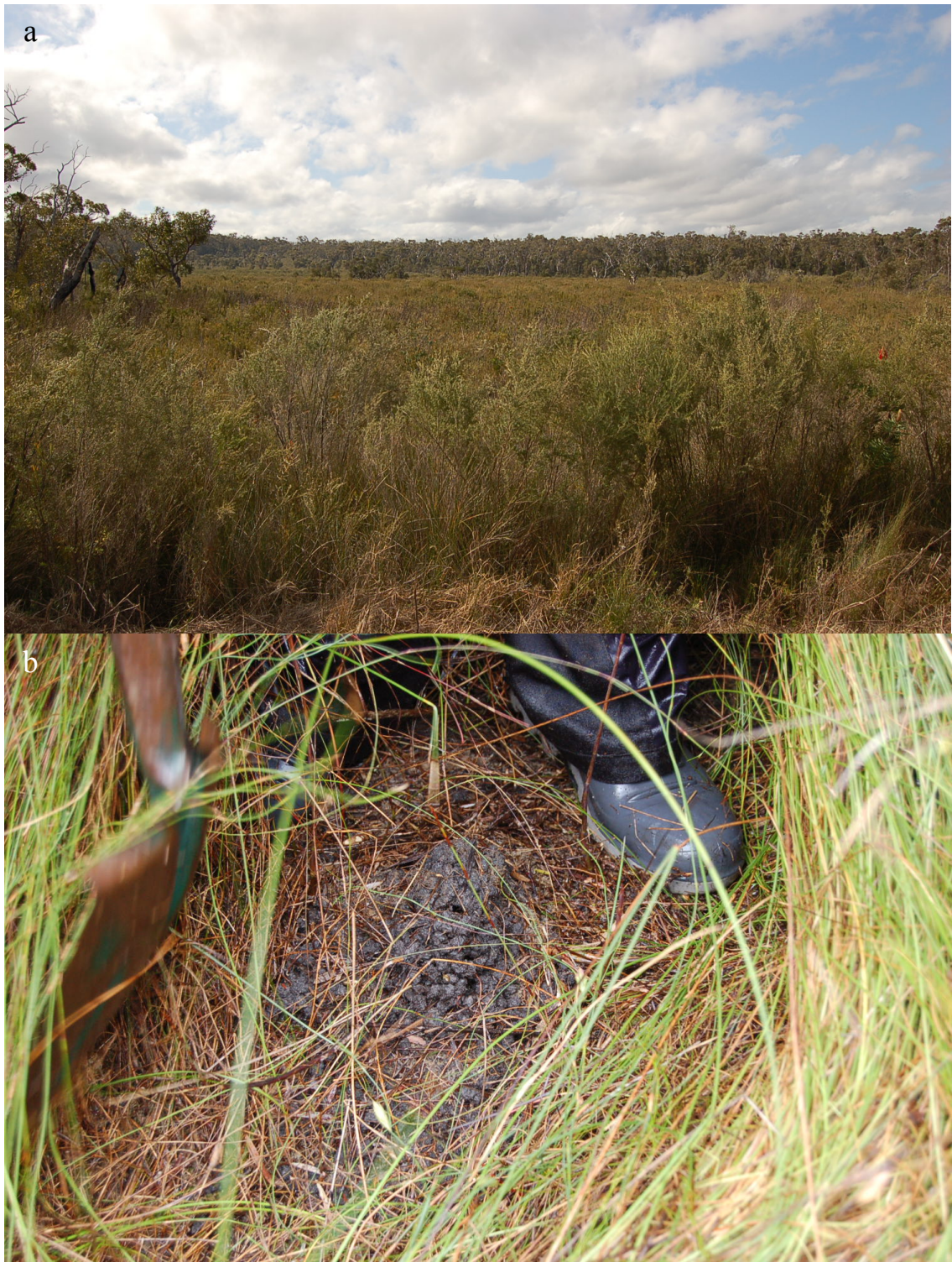
Figure 4.21 Typical vegetation (a) and soil (b) in which *Engaewa clade B* can be found.

CONSERVATION STATUS

Current status: Not Assessed

Suggested status: Least Concern

Engaewa clade B, much like *E. subcoerulea*, appears to be of minimal conservation concern at the present time. It is difficult to accurately define the EOO of this 'new' species without further fine-scale sampling in the eastern portion of its range, although in this study a conservative approach to defining the EOO has resulted in an estimate of ~244 km^2 (Figure 4.22). Based on distribution this candidate species would meet the requirements of Endangered for EOO and at least the same for AOO (<5000 km^2 and <500 km^2, respectively). Whilst a strict minimum convex polygon based on known sites would greatly reduce the estimate of EOO, it would not reduce it to the degree that would warrant a higher threat category (i.e. Critically Endangered requires an EOO <100 km^2). Although the EOO fits within the threat categories, *E. clade B* exists in a less disturbed region of the distribution of the genus and is known to occur in protected areas (Tingle State Forest), and is likely to occur in other protected areas (Quarram, & Owingup Swamp Nature Reserves and possibly William Bay National Park), that are currently experiencing little habitat disturbance beyond the relatively minor and localised impacts caused by introduced feral pigs. Although there clearly has been fragmentation and degradation of habitat of this species in the past, there is no evidence to suggest that its range should be considered severely fragmented or to be experiencing any on-going significant declines under the IUCN definitions. Thus if it were to be assessed for the IUCN Red List it would belong to the category of Least Concern, with the important caveat that it be reassessed based on increased development and/or impacts of climate change in the future.
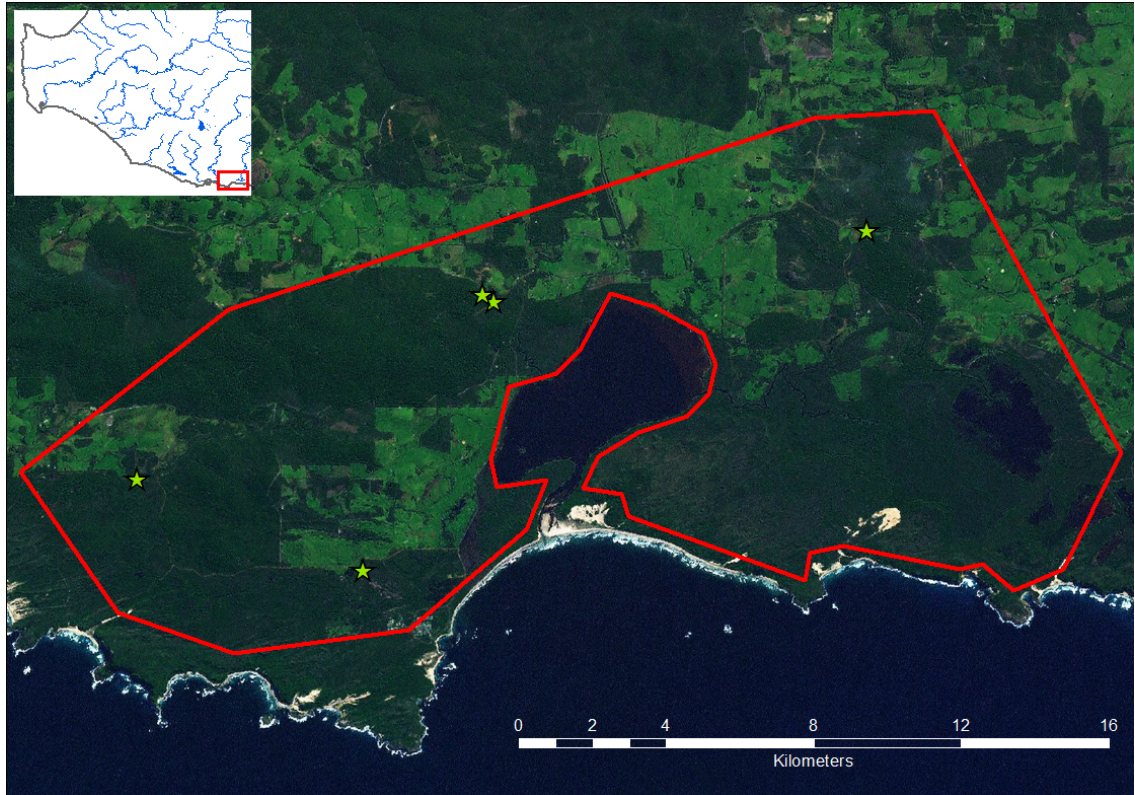
Figure 4.22 Distribution of *Engaewa clade B*, with the polygon used to assess the Extent of Occurrence for this species shown. For sources of GIS data see Section 2.5.

4.3.9 Summary

A comparison of the distributional range of (both the described and undescribed) *Engaewa* species identified in this study and the characteristics of the habitat they occupy highlights a trend within this genus, whereby they can be divided into two groups: (1) generalist and (relatively) widespread, and (2) specialist and restricted (Table 4.3). As discussed in Chapter 3, the ancestral state for *Engaewa* is believed to be strongly burrowing thus, in the context of *Engaewa*, generalist and specialist refer to species that would be highly specialised for burrowing compared to a more aquatic form, respectively, which would usually be reversed in relation to the basic ecology and morphology of freshwater crayfish generally. Such a 'regression' (from a burrowing form to a more conventional free-swimming form) is not unique, as it has previously been noted within the genus *Engaeus* (Horwitz, 1990). Generalist species occupy a range of habitats, but most commonly deeply draining sandy soils, often including coastal plains. Specialist species occupy habitat that is uncommon within the context of the range of this genus (i.e. shallow soils (*E. walpolea*), clay-based soils (*E.*

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pseudoreducta), or aquifer-fed creeks (*E. clade A*). These unusual habitats can all be seen to create highly localised conditions that retain water (clay-based or shallow soils) or remain wetter (aquifer fed). These specialist species generally exhibit less extreme burrowing morphology (and hence are specialised in the context of *Engaewa*), and tend to be smaller with smaller chelae (which are more often isomorphic) in relation to the generalist species. The significance of the habitats in which the specialist species are found to the persistence of these crayfish through climatic cycles (i.e. their role as refugia) will be discussed in the following chapters.

Table 4.3 Ecological type (generalist or specialist), approximate distribution (Extent of Occurrence), and comments for each of the *Engaewa* species and clades identified in this study.

Ecological type	Species	~EOO (km)	Comments
Generalist	*E. similis*	4275	Occupies a wide range of habitats
	E. subcoerulea	2113	Found almost exclusively in large, sandy coastal plains
	E. reducta	939	Occupies a wide range of habitats, though generally sandy soils
	E. clade B	244	Generally found in sandy coastal soils
Specialist	*E. walpolea*	127	Restricted to shallow soils and appearing to be more 'aquatic' than other species
	E. pseudoreducta	76	Restricted to clay-based soils near the type locality (though in sandy soils at the disjunct Payne Road site)
	E. clade A	1	Restricted to a single aquifer-fed drainage

Based on the information obtained in this study the conservation status (using the IUCN Red List Criteria) for each species has been reassessed. Apart from an assessment of the two clades identified in this study once they are formally described, *E. reducta* is the only species that needs to be reassessed for the Red List based on this data (Table 4.4). Due to the recognition of additional populations of this species, the EOO for *E. reducta* has been greatly expanded and, most importantly, these additional populations occur in a protected area largely removed from the threats faced by this species previously. As such, the proposed category in this study for this species is Least Concern, downgraded from the previous level of Endangered. Before such a change should officially occur however, further investigation is required to more accurately calculate the AOO of this species, as it may be that it can qualify as Vulnerable (based on Criterion D).

Table 4.4 Assessment of conservation status for all *Engaewa* species (and species level clades) recognised in this study (both current – based on the most recent IUCN Red List assessment – and proposed). Conservation status is based on the IUCN categories (CR= Critically Endangered; EN= Endangered; VU= Vulnerable; LC= Least Concern) with the criteria used for the proposed criteria shown.

Species	Current Category	Proposed Category	Criteria
E. pseudoreducta	CR	CR	B1a,b(iii)
E. reducta	EN	LC	-
E. similis	LC	LC	-
E. subcoerulea	LC	LC	-
E. walpolea	EN	EN	B1a,b(iii)
E. clade A	-	VU	D2
E. clade B	-	LC	-

5) BIOGEOGRAPHIC HISTORY OF THE GENUS *ENGAEWA*

5.1 Introduction

Biogeography is a broad and inclusive science providing a method of linking traditional single disciplines and a focus for interdisciplinary studies (Spellerberg & Sawyer, 1999), which can be seen both as its great strength and greatest difficulty (Knapp, 2005). The essential goals of biogeography can be summarised as being the discovery of the patterns of spatial distribution of biological groups on the Earth's surface, and the means (both mechanisms and processes) by which this distribution was achieved (Santos & Amorim, 2007). Discovering the geographic patterns of variation within and between lineages, in concert with well-substantiated phylogenetic hypotheses, can reveal the processes that generate and maintain biodiversity, as well as answer numerous important biological questions relating to the nature and tempo of speciation, the temporal and spatial occurrence of barriers to gene flow, the nature of demographic parameters through time, and the appropriate partitioning of diversity into taxonomic units (Esselstyn & Brown, 2009).

Biogeography reveals patterns of distribution that are the result of physiological and behavioural adaptations. These adaptations result from abiotic and biotic factors; that is, interactions with the environment as well as interactions with other organisms both intra- and inter-specific (in the form of competition); and also affect reproductive recruitment and dispersal mechanisms, which have a direct influence on species' distributions (Spellerberg & Sawyer, 1999). Superimposed on these more direct influences are the gradual effects resulting from large-scale processes occurring over geological timescales, such as climate, sea-level changes and plate tectonics (Spellerberg & Sawyer, 1999). To complicate this situation further, the impacts, both direct and indirect, of human activities may mimic these processes, except they occur on vastly different time scales (Spellerberg & Sawyer, 1999). Biogeography, therefore, can be thought of as an endeavour aimed at explaining the cumulative evolution of the Earth's biota.

Much biogeographical work has used continental or landmass scale areas as analytical units; for example, Gondwanan biogeography has been studied for over a century. The climatic, geological, and hydrological history of a region is often considered when investigating the processes that have generated present-day patterns of biodiversity (Gaston, 2000; Lomolino, Riddle & Brown, 2006) and given that the effects of these factors are most apparent when looking at large geographic scales, their influence on speciation is likewise typically observed over broad areas (Kozak, Blaine & Larson, 2006; Pyron & Burbrink, 2010). The lack of biogeographic studies looking at small scales is largely due to the difficulty of defining boundaries within landmasses as they may change over time, whereas continental boundaries are a more permanent feature (Crisp, Linder & Weston, 1995). Where smaller geographic regions have been analysed, this has generally been in order to examine island fauna and flora (i.e. situations with discrete geographic boundaries).

Due to a general focus of biogeographic studies on situations with well-defined boundaries, far less attention has been given to situations where species' ecology is intimately linked to environmental factors that are locally heterogeneous and can have profound effects on diversification at smaller spatial scales (Doebeli & Dieckmann, 2003; Huston, 1999; Kuchta, Parks & Wake, 2009). Finely resolved patterns for species with limited dispersal abilities are often entirely overlooked (Giribet & Edgecombe, 2006), despite sedentary invertebrates surviving in small, isolated populations, which acts to preserve the continuity of their phylogeographical signal (unlike many vertebrates that exist as metapopulations) (Price, Barker & Villet, 2010).

Whilst there have been a number of studies looking at taxonomic patterns of invertebrates in SWA (e.g. Harvey, 1996, 2002a, 2002b; Hopper *et al.*, 1996; Main, Harvey & Waldock, 2002; Main, 1996b, 1999; Moir, Brennan & Harvey, 2009; Reid, 2002; Rix, 2006, 2008; Rix, Harvey & Roberts, 2010), due in part to the recognition of the prevalence of high diversity and short-range endemism in the region (Harvey, 2002b), there have been few explicit, molecular phylogeographic studies. Thus, little is known about the biogeographic and speciation patterns for many invertebrate groups (Rix *et al.*, 2010). This chapter fills a gap in our knowledge, by documenting the

biogeography of a restricted taxon with limited dispersal abilities and fine-scale distributions.

5.1.1 Methodology

In this chapter a number of *a priori* models that may explain the speciation and biogeography of the genus *Engaewa* will be explored, firstly from a theoretical basis and then in comparison to the available molecular, morphological, biological, ecological and geological data. These hypotheses begin broadly, commencing with the initial occurrence of the genus in SWA, and become increasingly complicated. Before commencing the exploration of these hypotheses, the adopted approach will be discussed to clearly outline how this study fits within the current paradigm of hypothesis testing.

The approach relies upon interpreting the branching pattern of well-established phylogenies. Phylogenies represent the pattern of cladogenetic splits leading to present-day lineages and, therefore, an indirect record of the evolutionary history of speciation (Hennig, 1966). As such, it is a logical extension that the splitting of a lineage on a phylogenetic tree will (assuming speciation occurs due to allopatric processes) generally represent the splitting of its distribution in space (Pigot, Phillimore, Owens & Orme, 2010). Therefore, the sequence of branching can be read either as a sequence of dispersal, with taxa invading a new region, differentiating and then invading another region, or as a sequence of differentiation of lineages in an already widespread ancestor due to vicariance events (Heads, 2009).

Deciphering the biogeographic history of *Engaewa* will be achieved by modelling *a priori* hypotheses of various dispersal/vicariance scenarios and contrasting the predicted tree structure to that obtained from the genetic data in Chapter 3. As noted by Knowles (2004), hypotheses of these types need to be simple enough that they can be clearly represented by the data available, but not so simple as to lack any real biological significance. It has been suggested (e.g. Ponniah & Hughes, 2006) that this type of approach can be of particular benefit when there are little (or no) fossil or palaeoclimatic data available, as is the case for *Engaewa* in SWA.

Although this may seem a relatively simple and fool-proof method of determining the mode of speciation, current geographical distributions may not represent what they were at the time of speciation (Bishop, 1981), and if range movements are common or rapid the pattern can become confounded (Barraclough & Nee, 2001). It is also important to note that factors that currently maintain boundaries between species may be different to those that initially caused the disjunction (Wardell-Johnson & Roberts, 1993). Accordingly, as some boundaries may have very complex histories in relation to different taxa, many ancient biogeographic boundaries will likely require a combination of historical and ecological explanations (Glor & Warren, 2011). One way to overcome this possible limitation is to look at situations where range movements are unlikely to be problematic (Barraclough & Nee, 2001), such as is provided by *Engaewa*. An important caveat of all these analyses is that anthropogenic habitat alteration may have altered the defining characteristics of some of these areas, or created 'artificial' divisions within distributions, which may act to confound the signal in the biogeographic data.

The notion that phylogenetic relationships can be used to infer past geographic distributions is as old as evolutionary biology itself (Ronquist & Sanmartin, 2011), however, biogeography has been seen as lacking scientific rigor (Crisp, Trewick & Cook, 2011). As it deals with historical events that can neither be observed directly, nor manipulated experimentally, the traditional approach has been for researchers to observe and analyse the present-day pattern and, from this, provide an explanation in terms of historical processes ('pattern before process') (Crisp *et al.*, 2011). A commonly adopted approach is to look for correlations between distributional change through time and 'events', such as continental break-up or climate change, which are then generally inferred as being the causative agent for current distributions (Crisp *et al.*, 2011). A problem with such an approach is that a set of observations can be consistent with many alternative explanations and there is likely to be a degree of subjectivity or bias, particularly if the researcher makes implicit process assumptions (e.g. a proponent of dispersal over vicariance or vice-versa) (Crisp *et al.*, 2011). As a result of these issues, an inductive approach to biogeography has been criticised as akin to storytelling and

considered an unscientific endeavour that generates speculative theories related to the biogeography of a group, rather than testing hypotheses (Crisp *et al.*, 2011).

In order to shift from inductivism to Popperian science, Crisp *et al.* (2011) suggest that instead of creating hypotheses based on observed patterns, restrictive propositions are formulated and specific predictions that can rule out many of the alternative hypotheses are tested; this is the approach adopted in this study. An example of one of these hypotheses (which will be later formally introduced and discussed in detail) is that *the distribution of Engaewa species is a result of a 'stepping-stone' dispersal pattern that results in a ladderised tree, onto which species can be mapped in a geographically linear fashion*. This type of topology testing relies on both accurate modelling of possible trees resulting from the various vicariance/dispersal hypotheses, and a robust phylogeny of the taxa in question (Ponniah & Hughes, 2004).

There is no single best method of interpreting evolutionary history from molecular data. However, to do so there are two major assumptions that must be accepted: (1) that the current distribution of species reflects their mode of diversification, and (2) that the gene tree is congruent with the species tree, as substantiated by multiple independent molecular markers (Leray *et al.*, 2010). The first of these caveats will be addressed by considering environmental characteristics, life-history traits, and biological interactions gained from field observations, which can add additional information that can help elucidate the processes that drive diversification (Leray *et al.*, 2010) and suggest whether there are likely to have been significant range shifts since diversification. The second caveat is well known, as it is widely acknowledged that inferring species history from mtDNA only is fraught with danger (Knowles & Richards, 2005). Without assessing the impact of past demographic and biogeographic events on the pattern of genomic variation as well as mtDNA, there is a risk of misinterpreting the biogeographic and demographic past (Hey & Machado, 2003; Knowles, 2004) as these loci may not accurately reflect the species history, due to factors such as the stochastic nature of the lineage sorting process (Edwards & Beerli, 2000; Knowles & Maddison, 2002; Maddison, 1997; Takahata, 1989). Accordingly, in

this study the majority of analyses and the interpretation of the biogeographic hypotheses are based primarily upon the combined data.

The analyses of biogeographic hypotheses will also incorporate the dates derived in this study for the nodes between various lineages and whilst there is much uncertainty in relation to these dates, it will become evident that the scenario derived from the testing will be equally applicable to younger dates (in fact, due to the regular oscillations between glacial and interglacial periods, this would arguably be an easier proposition to explain). Many biogeographic studies have investigated the impact of cyclical climate changes throughout the relatively recent past, however, in this study a much longer time period must be accounted for, as the formation of the genus may have been (according to the dating presented in Chapter 3) during the Cretaceous. Therefore, the period considered in this study will include eustatic sea-level oscillations associated with the cooler interval at the Jurassic-Cretaceous boundary (Haq, Hardenbol & Vail, 1987; Price, 1999), continuing through the Early Eocene climatic optimum and the following cooling period, right up to the ongoing recent cyclical glacial and interglacial periods (Figure 5.1).

It has been suggested that during the Pleistocene, glacial periods account for approximately 80% of the time, and typically last for up to 90,000 years, whereas interglacials are relatively short periods of around 10,000 years (Rull, 2009). As glacial conditions have been the predominant condition throughout the Pleistocene, taxa adapted to these cold/dry conditions can be considered 'normal' and those that flourish during the warm/wet 'disturbances' are the exception. Although a general glacial/interglacial pattern of low sea-level/dry climate and high sea-level/wet climate, respectively, is considered the norm, there have been exceptions (for example see Kershaw & Nanson, 1993). Therefore, it is necessary to consider the alternative high sea-level/dry climate and low sea-level/wet climate when considering biogeographic hypotheses. In fact, the current period is similar to an interglacial wet period but is drier and less humid than is typical (Dodson & Ramrath, 2001), thus representing a high sea-level/dry climate scenario.
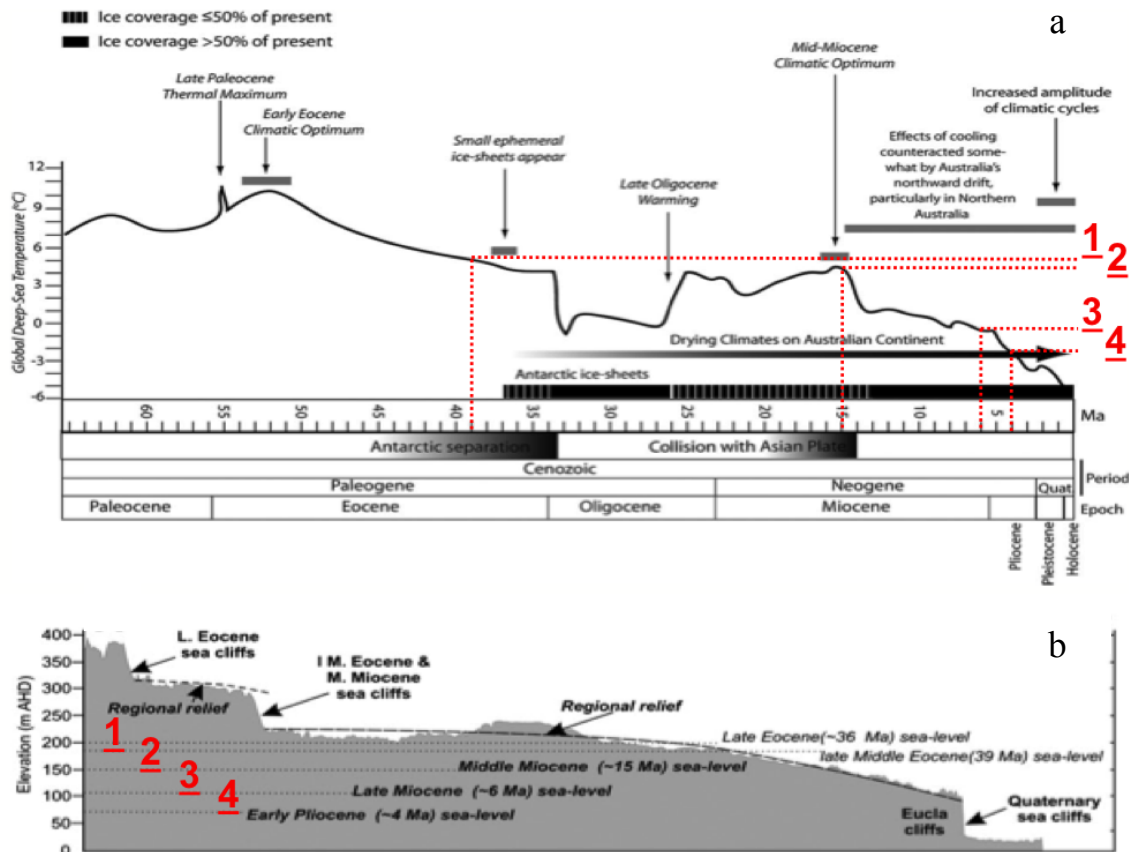
Figure 5.1 Global deep-sea temperature presented as a proxy for palaeoclimate of the Australian continent since 65 MYA ('Quat.' = Quaternary) (a), and a representation of the associated sea-level at the western end of the Eucla Basin (b). Red numbers highlight specific time periods showing the association between global temperature and sea-level. (Adapted from Byrne *et al*., 2011 and Hou *et al*., 2008).

In Australia, periodic episodes of wet/dry have affected species distributions by altering continuity of habitat and, by extension, gene flow (rather than in the northern hemisphere where cold/warm is considered more significant) (Keast, 1981). Climatic oscillations can be seen as having had a two-fold effect, as they would shift climate zones as well as raise and lower sea-level, which have caused the ranges of species to undergo cycles of movement, compression, expansion, subdivision, and even elimination (Zink, 2012). Periods of drying restrict mesic adapted taxa to refugial areas around the continental periphery that maintain adequate rainfall due to their geographic position and physiography (Keast, 1981). Whilst the HRZ as a whole can be considered a refugium from aridity, for particular taxa (i.e. those that are highly moisture dependent) there will have been specific microrefugia within this wider region. The concept of "refugia within refugia" has been discussed by Byrne (2008) in relation to Pleistocene climatic fluctuations across southern Australia (having previously been

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described in Europe by Gómez & Lunt, 2007 and others). Such refugia will be described for *Engaewa* in this chapter and discussed in detail in the following chapter.

As a final summary to the methodological considerations presented above, and to properly frame the setting in which this study is being conducted, a number of factors influencing the approach to hypothesis testing adopted in this study will be defined. In order to represent Popperian science each hypothesis mentioned will have an explicit statement attached, which can be falsified through comparing the phylogenies presented in this thesis to those that would be predicted on the basis of a logical extension of the explicit statement. I propose that the phylogenetic approach to biogeographic hypothesis testing (as proposed by Ponniah and Hughes (2004) and others) is most suitable for the data available, which are constrained by:

- a lack of significant geologic evidence, suitable to the scale of *Engaewa*'s distribution,
- the paucity of *Engaewa* samples available (both in terms of number of populations and number of individuals from each population) compared to many biogeographic studies,
- a lack of relevant fossils and/or ancient DNA,
- the low within population, and high between population, haplotype diversity preventing the use of network-based phylogeographic approaches, and,
- the relative paucity of other biogeographic studies of taxa within the region of *Engaewa*'s distribution, which could be contrasted to, and/or combined with, the data presented here.

Despite the difficulties noted above, *Engaewa* is a suitable model for formulating a biogeographic hypothesis for the coastal regions of SWA. The genus is likely to have persisted in the region for a long time but is unlikely to have recently dispersed considerably throughout the region, which should result in a strong historical signal within the genus that will not have been more recently overridden.

5.2 Hypothesis testing

5.2.1 Multiple invasions versus endemic speciation
Outline

The close relationship between crayfish of SWA and south-eastern Australia (SEA) is well established (i.e. Riek, 1972; Schultz *et al.*, 2009), and with all genera except *Engaewa* and *Tenuibranchiurus* present in SEA it is widely accepted as the region of origin of freshwater crayfish in Australia (i.e. Crandall & Buhay, 2008; Crandall *et al.*, 2000b; Riek, 1972; Schultz *et al.*, 2009). Furthermore, the burrowing clade appears to sit within the phylogeny of Australian crayfish, making a south-western origin for *Engaewa* unlikely. Based on these assumptions the question arises; does *Engaewa* represent a single introduction of burrowing freshwater crayfish into SWA that have later speciated, or have there actually been a number of invasions by already distinct species?

At least as early as Hooker (1860) the climatic and taxonomic affinities between SWA and SEA were noted (and subsequently reinforced by numerous authors, e.g. Burbidge, 1960; Diels, 1906). South-western Australia and SEA together have been considered to represent the temperate Bassian element of the continent, which is bisected by the more arid Eyrean element (Burbidge, 1960; Schodde, 1989; Spencer, 1896). The affinity between SWA and SEA is shown by the presence of many genera, or related genera, in both regions, which has been interpreted as representing a historical pattern of connectedness (Morgan, Roberts & Keogh, 2007). Although the two regions share genera there are very few species that are found in both areas; therefore, any model attempting to explain the biodiversity in SWA must account for both the similarities and differences between SWA and SEA (Morgan *et al.*, 2007).

South-western Australia has long been seen as a biogeographical enigma. It lacks obvious geographical barriers (arising from events such as glaciation and mountain building) that would promote speciation yet, as has been stressed numerous times throughout this thesis, it is recognised as a region of elevated richness and diversity for many taxa. The lack of obvious dispersal barriers led to the theory that endemic speciation in SWA is implausible. This notion has been postulated at least

since Main, Lee & Littlejohn (1958), who explained the diversity of frog fauna in the south-west via a multiple invasion hypothesis (MIH). The MIH suggests that the biodiversity in SWA is the result of multiple east-west dispersal events (especially throughout the Pleistocene) and found much support within the literature on frog diversity (e.g. Lee, 1967; Littlejohn, 1967; Main, 1968). The MIH also found some support in the literature on birds (e.g. *Malurus* Vieillot, *Eopsaltria* Swainson and *Calyptorhynchus* Desmarest (Keast, 1981 and references therein)) and spiders (Main, 1962).

The MIH was later criticised by White (1977) who suggested that clades have radiated *in situ* in SWA and proposed the alternative endemic speciation hypothesis (ESH). White's ESH has received considerable support recently via molecular phylogenetic studies that have shown many of the clades in SWA are monophyletic to the exclusion of their eastern relatives (e.g., frogs: *Crinia* Tschudi (Barendse, 1984), *Heleioporous* Gray (Maxson and Roberts, 1984; Morgan *et al*., 2007), *Litoria* Tschudi (Burns & Crayn, 2006); lizards: Pygopodidae Boulenger (Jennings, Pianka & Donnellan, 2003); spiders: *Raveniella* Rix and Harvey (Rix *et al*., 2010); crayfish: *Cherax* (Munasinghe *et al*., 2004b; Schultz *et al*., 2009)). For example, the main argument against *in situ* speciation, namely a lack of required barriers, has been shown not to hold for the frog genus *Geocrinia* Blake, as genetic data strongly suggests that the *Geocrinia rosea* complex is the result of *in situ* speciation in SWA (Driscoll, 1998a; Roberts & Maxson, 1985a; Roberts & Wardell-Johnson, 1995; Wardell-Johnson & Roberts, 1993).

Geocrinia possesses high intra-specific genetic divergence between contemporary populations, which demonstrates the potential for genetic sub-division and population isolation in the region. Recently Driscoll and Roberts (2008) have further supported this notion and suggested the magnitude of allozyme divergence between northern and southern *G. rosea* likely represents further species level differentiation. These two 'candidate species' were separated geographically by a 12 km disjunction prior to the study by Driscoll and Roberts (2008), who sampled the intervening region and found that whilst there does appear to be a species level

distinction between the two major genetic clades, there is a single population that represents a hybrid zone. This situation may be evidence that complex genetic boundaries can arise even in a relatively subdued landscape and that endemic speciation is possible, within these frogs at least, in SWA.

Further to the genetic data, there have also been criticisms of the MIH based on geological evidence. The MIH was originally formulated on the basis that the last glacial maximum (LGM) would have provided a dispersal corridor along the southern coast, due to lower sea-level and increased rainfall (Main *et al.*, 1958). However, there is much evidence to suggest that the LGM, and all glacial periods for that matter, actually correspond with substantial aridity in southern Australia (Bowler, 1982; Galloway & Kemp, 1981; Kershaw, Moss & Van Der Kaars, 2003). Furthermore, during the wetter Pleistocene interglacial periods there was unlikely to have been connection between the faunas of SEA and SWA, as the Nullabor Plain is formed from porous limestone that does not allow for the accumulation of significant surface waters (Lowry & Jennings, 1974).

Molecular dating of splits within moisture dependent taxa that span SEA and SWA estimate a cessation of gene flow within the Oligocene/Miocene/Pliocene (e.g., *Crinia* frogs - Barendse, 1984; *Heleioporous* frogs - Maxson and Roberts, 1984; Morgan *et al.*, 2007, *Cherax* crayfish – Munasinghe *et al.*, 2004b; Schultz *et al.*, 2009). Thus, it is reasonable to conclude that any migrations across the southern margin of the continent must have occurred in, or before, the late Miocene/early Pliocene (Roberts & Maxson, 1985b). Whilst this does not preclude the possibility of multiple invasions occurring, it does add an important caveat (i.e. that the invasions must have occurred earlier than often previously assumed).

Despite the accumulation of evidence that makes endemic speciation a likely scenario for *Engaewa*, there is no reason to presuppose this will hold for all taxa and thus it warrants being tested. It is possible to test the assumption of endemic speciation, as an alternative to multiple invasions, in a phylogenetic framework. A hypothetical view of the MIH would see two or more introductions into SWA (species 1 and 3 in

Figure 5.2). Multiple invasions versus endemic speciation are not, however, entirely mutually exclusive, thus multiple invasions may be followed by speciation either by further dispersal (species 1-2) or by vicariance (species 3-4 in Figure 5.2).

If the genus *Engaewa* is found to be polyphyletic then the MIH would be the most likely explanation (Figure 5.2). However, if this was the case then the taxonomy of *Engaewa* would need to be reconsidered, and the hypotheses altered to reflect this new understanding. Even if the genus *Engaewa* is monophyletic it may be possible that multiple invasions still occurred (assuming the ancestor is no longer present in their centre of origin). In this scenario, a portion of an ancestral species migrated westward and then, at a later stage, a second wave of immigrants from the same parental stock again migrated west. This second wave would either have been the remainder of the parent species, or those that remained later died out. This would be a more problematic proposition to test, however, it is likely that the divide between the first and second wave of immigration would be very deep (representing a lengthy period of time without gene flow). It is also possible that a sudden burst of speciation may follow dispersal into a new region as new niches are available and new selection pressures exert their influence. If, as in the example represented below, a deep divide forms two lineages which then further speciate at vastly different times, it may represent separate invasions by the two lineages. There is also the (perhaps unlikely) possibility, that there could be 'back colonisation' (as noted by Emerson (2002) for island assemblages), whereby the east-west migration was reversed for a portion of the species, which would be evidenced by *Engaewa* being paraphyletic. In this case the hypotheses being tested may still be applicable to the portion of the genus present in SWA, however the taxonomy of *Engaewa* would need to be changed to reflect this situation.
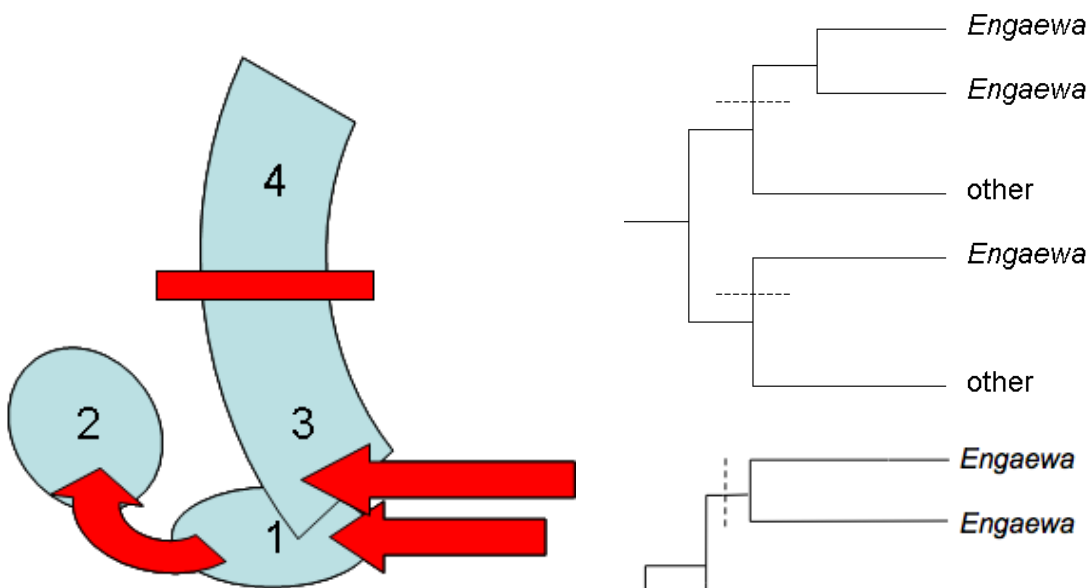
Figure 5.2 A possible scenario resulting from the multiple invasion hypothesis.
LEFT: A diagrammatic representation of one possible example, with the arrow leading to species 1 representing an initial dispersal event into the region with a second species (2) forming via secondary dispersal. A second major dispersal event forms species 3, which in this case is divided by a vicariance event (red bar) to form two species 4. RIGHT: Two possible phylogenetic trees (polyphyletic above and monophyletic below) resulting from such a scenario, with invasions represented by dotted lines.

Hypothesis

The MIH can be falsified if; (1) the genus *Engaewa* is found to be monophyletic to the exclusion of eastern Australian crayfish, and (2) if there is not at least one deep divide within the genus, probably with two (or more) different episodes of speciation bursts within the genus that may represent the scenario of multiple east-west migrations, followed by extinction of the parent species. As the MIH and ESH are the only two alternatives, a refutation of one will be seen as an acceptance of the other.

Evidence

The phylogenetic trees presented in Chapter 3 show *Engaewa* to be monophyletic, which concurs with the findings of Crandall *et al*. (1999), Horwitz and Adams (2000), Schultz *et al*. (2009) and Toon *et al*. (2010). Monophyly of the genus can be seen as an initial line of evidence falsifying the MIH. Furthermore, there is no evidence consistent with the alternative form of MIH proposed above (whereby multiple waves of immigration were followed by extirpation of the parental taxa in the

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area of origin), which could account for multiple invasions of a monophyletic taxon. This is because there is no suggestion of deep divides within the genus between subsequent waves of immigrants, or sudden bursts of speciation at significantly different times, as predicted by this hypothesis. The dating presented in this study also suggests that if multiple invasions actually had occurred they must have been (significantly) earlier than often previously considered in the MIH. The evidence available clearly rejects the MIH and suggests that the most parsimonious explanation is provided by the ESH. Thus it is assumed that *Engaewa* entered Western Australia as a single ancestral lineage and speciated *in situ*. The complex distribution pattern of genetic lineages presented in this study further supports the notion that endemic speciation has occurred within the genus.

Conclusion

The MIH is rejected, and the alternative ESH proposed, for the genus *Engaewa*.

5.2.2 Stepping stone versus simultaneous vicariance
Outline

Once the outcome of the multiple invasion versus endemic speciation debate has been settled, attention can turn to the factors that have affected the genetic structuring of the genus within SWA. These hypotheses could be equally related to the divergences of lineages at any level (it is, after all, at the population level where speciation actually occurs). The distribution of *Engaewa* populations is highly disjunct, suggesting that there are seemingly significant barriers dividing regions of suitable habitat, isolating many populations. There are two scenarios by which such a situation could arise. Firstly, if the current isolated nature of patches of suitable habitat is typical throughout the period of *Engaewa*'s presence in SWA, then it suggests these crayfish must have occasionally traversed the intervening regions between patches. The alternative to this is that, during a previous period, suitable habitat was much more widespread, and so too were these crayfish, however, the habitat receded, resulting in the current disjunct distribution seen. From a biogeographical perspective these two scenarios clearly represent classic dispersal and vicariance, respectively.

Dispersal in biogeography generally refers to 'jump' or 'random' dispersal, where individual species traverse a geographic barrier that would usually restrict their distribution (Sanmartin, 2012). As the term random dispersal suggests, it occurs in an unpredictable fashion when a single species overcomes what is generally a significant barrier (e.g. a chance ocean crossing). However, dispersal can also be thought of as 'dispersion' or 'range expansion', where species expand their distribution due to the removal of a barrier (Sanmartin, 2012). Dispersion is often not lineage-specific as it occurs due to geologic or climatic events that open up habitat for species to expand into; this has also been referred to as "geodispersal" (Lieberman & Eldredge, 1996) or "predicted dispersal" (Ronquist, 1998). Whereas some biogeographers debate whether jump dispersal occurs and/or should be included in biogeographic analyses (e.g. reviews in Cox & Moore, 2010; Lomolino *et al.*, 2006), dispersion is accepted as the process that allows ancestors to gain widespread distributions prior to vicariance events (Humphries & Parenti, 1999). In this study both of these are viewed as possible scenarios, with the term 'dispersal' being used to signify the crossing of barrier that usually would restrict distribution and 'dispersion' used for the situation of range expansion into newly available habitat.

Dispersal events are expected to happen rarely, due to the difficulties of surviving in the intervening regions of unsuitable habitat. However, if a series of dispersal events occurred new species could be formed in each isolated population. This scenario was one model considered by Ponniah and Hughes (2004) for the crayfish genus *Euastacus*. Ponniah and Hughes (2004) noted that *Euastacus* has a linear distribution along the east coast of Australia and, with SEA believed to be a centre of crayfish origin (Riek, 1969), an ancestral *Euastacus* species may have progressed up the east coast, via dispersal, in a stepping stone like manner. If speciation occurred between steps then this stepping stone process could have produced a series of isolated species. *Engaewa*, like *Euastacus*, has an essentially linear distribution, with a number of isolated species/populations, thus this model may account for the speciation processes that have occurred in the genus.

In the stepping stone hypothesis (SSH), a series of dispersal events following a roughly linear sequence create a series of isolated populations, which then speciate and form a matching sequence of species (Figure 5.3). A phylogenetic tree of such a situation would mirror this step-like progression where the most recent 'steps' are most closely related (Figure 5.3). Regardless of whether stepping stone dispersal happens in a single direction (as in Figure 5.3), or if it actually commences in a central region and occurs outwards in two directions (it is, by its very nature linear, hence these are the two possibilities) the resulting phylogeny would still appear 'ladderised' due to the multiple dispersal events.
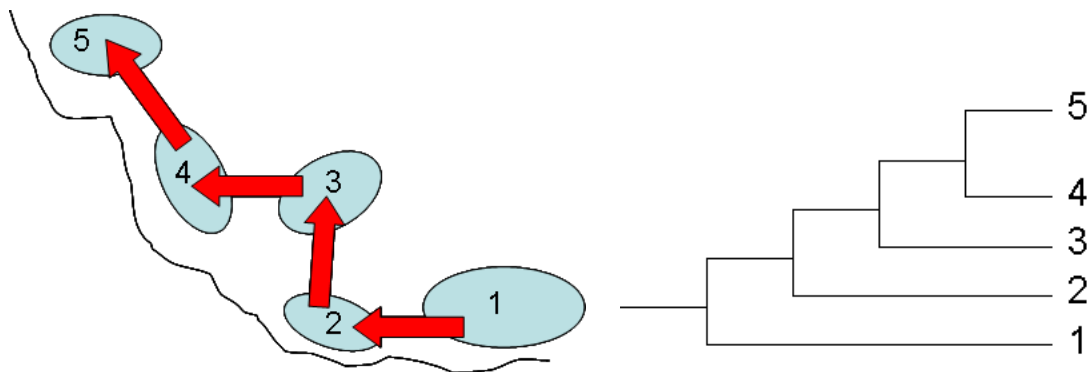
Figure 5.3 A possible scenario resulting from the stepping stone hypothesis. LEFT: A diagrammatic representation of the stepping stone hypothesis, with arrows representing dispersal events commencing from species 1 and continuing sequentially to form additional populations (which lead to distinct species, represented by different numbers). RIGHT: The expected topology of a phylogenetic tree resulting from a stepping stone scenario (although the branch lengths may vary), with the numbers matching the species in the diagram.

As previously mentioned, the SSH is not the only scenario that could result in many isolated and restricted species. Climatic conditions varied substantially throughout the Cenozoic, with habitat potentially suitable for *Engaewa* likely to have expanded and receded in response. If an ancestral *Engaewa* species was present in SWA during a period that was suited to its dispersal (i.e. if there were extensive regions of swamp-like habitat), this may have allowed it to become widely distributed throughout the region. During such conditions, gene flow may have been largely continuous throughout the entire range of the genus. A shift in climate may have caused the range of the genus to contract into a number of small 'islands' of suitable habitat, thus isolating populations (Figure 5.4). These isolated refugial populations would form

discrete gene pools and, over time, the accumulation of mutations would result in speciation. Ponniah and Hughes (2004) provided evidence that suggests that *Euastacus* has speciated in such a manner along the eastern coast of Australia when hotter and drier conditions forced the ancestral lineage to recede to the cooler mountains.
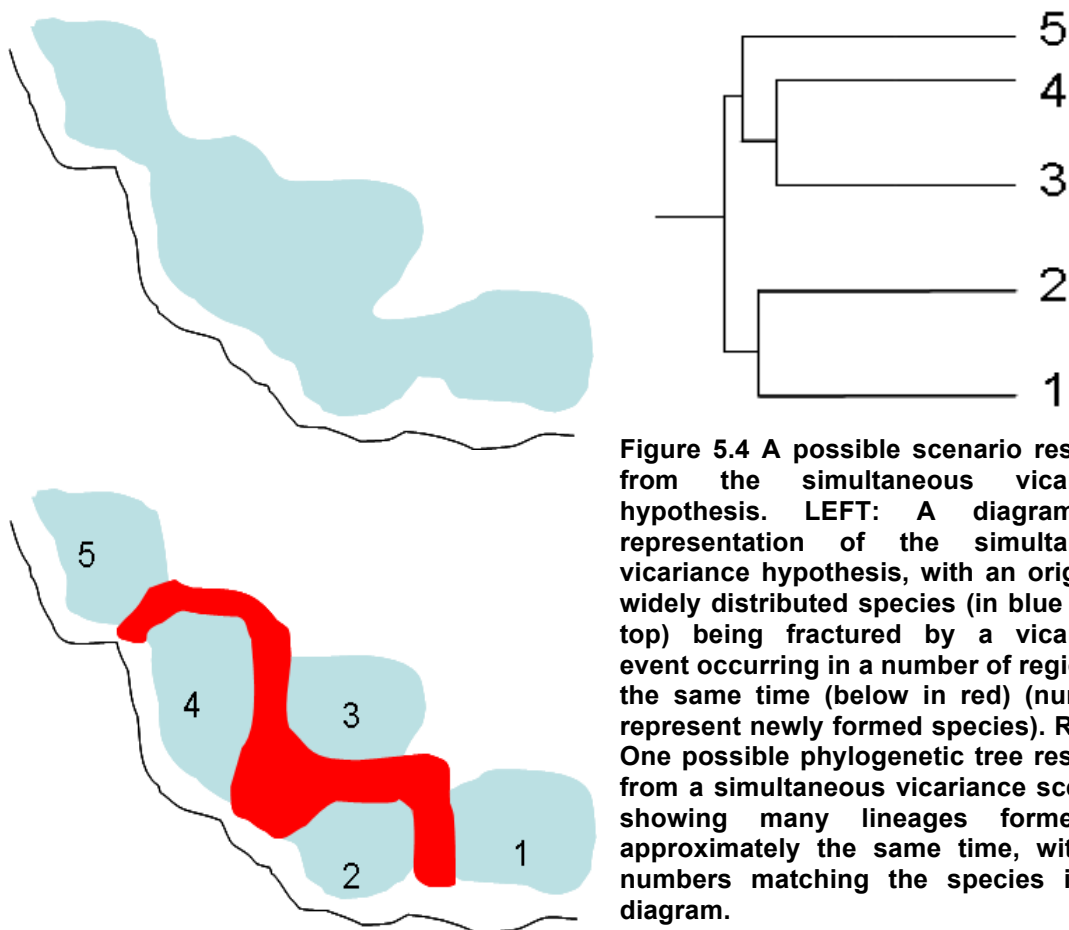
Figure 5.4 A possible scenario resulting from the simultaneous vicariance hypothesis. LEFT: A diagrammatic representation of the simultaneous vicariance hypothesis, with an originally widely distributed species (in blue at the top) being fractured by a vicariance event occurring in a number of regions at the same time (below in red) (numbers represent newly formed species). RIGHT: One possible phylogenetic tree resulting from a simultaneous vicariance scenario showing many lineages formed at approximately the same time, with the numbers matching the species in the diagram.

The simultaneous vicariance hypothesis (SVH) suggests that an event, which splintered the widely distributed ancestral lineage, occurred in multiple places throughout the ancestral species' range at (geologically speaking) essentially the same time. If the ancestral population at the time was truly panmictic then we would expect all current taxa to be equally distantly related to each other (Ponniah & Hughes, 2004). A similar assumption was made by Knowles (2000) who tested a SVH for montane grasshoppers and suggested that the resultant tree would be poorly resolved, thus forming a star phylogeny. If, however, there was some structuring of the ancestral gene pool, due to isolation by distance, at the time of the event then current neighbouring

taxa should appear more closely related to each other than to other species (Ponniah & Hughes, 2004). However, each of these neighbouring pairs should show similar genetic distances to all other neighbouring pairs (Ponniah & Hughes, 2004).

The SVH, as promoted by Ponniah and Hughes (2004), shows a clear similarity to the Turnover Pulse Hypothesis of Vrba (1980, 1992, 1993) who recognised that during intervals of climate change species may become widespread, then as temperatures change their ranges would retract, forcing them into isolated refugia. The contraction into refugia would cause some species to go extinct (the Turnover phase), whilst a number of the populations that became isolated would persist and speciate (the Pulse phase). Following the Turnover Pulse Hypothesis (which is essentially interchangeable in this study with the SVH) it would be expected that this would occur to many species at the same time, as it relies on a significant change to the climate. In contrast, the SSH relies, to a large degree, on more stochastic dispersal events and would not be expected to affect numerous different taxa at the same time.

An additional caveat of the SVH adopted in this thesis (which was not specified by Ponniah and Hughes (2004) as their analysis was purely based on molecular data) is that the species formed by this process will likely show little ecological specialisation. The reason for this proviso is that the SVH assumes that populations contract into refugia that continue to represent the 'normal' habitat of the species, thus these species should be isolated in similar patches of habitat and maintain their plesiomorphic state. It is noted, however, that should these events have occurred far enough in the past some ecological specialisation may have subsequently developed. Thus, whilst this is a potential line of evidence that can add collaboration to the conclusions of this analysis, it is not essential for this hypothesis to be generally accepted.

Hypotheses

The SSH can be falsified if the phylogenetic tree for the genus *Engaewa* does not display a ladderised topology. The SVH can be falsified if: (1) the phylogenetic tree for the genus *Engaewa* does not display a number of species forming at essentially the same time, with all current taxa either equally distantly related to each other or with neighbouring pairs of taxa showing similar genetic distances to all other neighbouring pairs, or (2) if species show considerable ecological specialisation.

Evidence

The trees presented in Chapter 3 do not support the SSH hypothesis as they generally present groupings of species pairs and do not follow a pattern of daughter species branching in a ladderised manner. The evidence from the phylogenetic tree clearly rejects the SSH as a major factor in the speciation of *Engaewa*.

Under the SVH it would be expected that the tree would show evidence of species level diversification in different lineages at a contemporaneous time (or at a number of contemporaneous times, if it were a series of simultaneous vicariance events), due to a widespread alteration to the environment. This expectation is largely met in the tree presented, as there is evidence of two major periods of splitting within the genus. These splits occur at ~45 and ~30 MYA. Furthermore, this assumption was statistically supported by the lineage through time plot, which showed a rapid increase in lineage diversification, suggesting that there was a relatively sudden burst of speciation. In the mitochondrial markers the species groups were well resolved, however, the branching pattern between them was not, suggesting that the diversification that occurred happened relatively quickly and recently (i.e. it resembles a star phylogeny).

The second proviso of the SVH (i.e. all current taxa are equally distantly related to each other or in neighbouring pairs of taxa with similar genetic distances to all other neighbouring pairs) is also generally supported, as the divergences between most species were roughly equivalent, with a slight bias towards the northern species showing smaller divergences. This would be expected as the diverging of lineages in the

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northern species represents a more recent episode of speciation. Geographically close species showing smaller divergences (as opposed to all taxa being equally related) is not surprising as, even with highly favourable conditions, *Engaewa*'s dispersal rate will potentially be low due to their burrowing habit and reduced locomotive capabilities and, as such, it is highly likely that the gene pool would be affected by isolation by distance.

There is one further line of evidence that needs to be considered before the SVH could be accepted; whether species show significant ecological specialisation. The data provided in Chapter 4 suggest that the splits occurring ~45 MYA produced four species that would have largely maintained the plesiomorphic burrowing characteristics of the genus (the species in question being *E. subcoerulea*, *E. clade B*, ancestral *E. similis/clade A*, and ancestral *E. reducta/pseudoreducta*). In comparison, the splits occurring at ~30 MYA appear to have resulted in some specialisation, as evidenced by the different ecological niches occupied by *E. reducta* and *E. pseudoreducta*. Admittedly, this one episode of specialisation may have occurred later than the initial speciation event and, as there are recognisable contemporaneous splits in multiple taxa at this time, this hypothesis is not dismissed entirely; rather it may, in an altered form, contribute to a more complex hypothesis that still needs to be explored.

Conclusion

The SSH is rejected. The SVH is generally accepted as a significant factor in the biogeography of the genus *Engaewa*; however, it cannot be accepted as the explanation for the speciation events leading to all extant taxa.

5.2.3 Taxon pulse
Outline

It has been widely acknowledged that the cyclical nature of the climate, and the associated changes in sea-level, would have resulted in a pattern of marine regression in SWA (which would open new habitat and encourage dispersal), alternating with marine intrusion (which would close previously available habitat), potentially creating vicariance events. Erwin's (1979; 1981) taxon pulse hypothesis (TPH) provides the

foundation for a model that can incorporate these fluctuations in a more sophisticated manner than the previously explored hypotheses.

Erwin's (1979; 1981) 'taxon pulse' model stemmed from the 'taxon cycle' concept that was originally proposed by Darlington (1943) and later named by Wilson (1961). The concepts of taxon cycling and taxon pulses feature prominently in island biogeography, as they were each formulated to explain speciation and diversification of these biota (e.g. Darlington, 1943; Erwin, 1981; Liebherr & Hajek, 1990; Wilson, 1959; Wilson, 1961). The TPH assumes that species initially occur in a stable, continuously occupied 'centre of diversification', from which their distributional ranges periodically fluctuate (Halas, Zamparo & Brooks, 2005). This hypothesis describes an adaptive shift from one habitat to another, along deterministic pathways, from a core habitat to suboptimal habitats (Erwin, 1981). Erwin (1981) proposed that this process is driven by ecological factors, including habitat change and competition, and is irreversible, as it is accompanied by increasing specialisation to more extreme conditions, thus resulting in speciation.

Under a TPH scenario, species may disperse along a broad front when suitable habitat becomes available. This phase of dispersion is closely associated with changing climate conditions, as species shift to remain within their climatic optima, which has seen some (e.g. Brooks & McLennan, 2010) suggest that this model may describe a general response to climate change. As species disperse they encounter geographical and environmental heterogeneity, which can cause uneven dispersion and result in peripheral isolates (Halas *et al.*, 2005). These isolates may experience restricted gene flow with populations remaining in the ancestral region, becoming effectively isolated and allowing speciation to occur (Halas *et al.*, 2005). This process would result in new species that are adapted to the habitat found on the margins of the species' range (an apotype) and species that persist in the main range/habitat optima (a plesiotype) (Erwin, 1981). If habitat opens and closes in a cyclic pattern this process of expanding distributions becoming fractured may be repeated numerous times.

Unlike a simple vicariance event, which may result in populations accumulating genetic differences due to random drift, the nature of the TPH suggests an important role for selective adaptation due to ecological differentiation. Populations in this model will experience very different biotic and abiotic stresses as they attempt to disperse into new habitat and then are segregated by vicariance. This is not to say that speciation will only occur due to selection as, depending on the nature and extent of the fluctuations, the separation of small isolates may also promote peripatric speciation due to the founder effect or genetic bottlenecks in addition to random drift (Fazalova *et al.*, 2010). As this process occurs due to a series of expansion and retraction phases, there are two possible outcomes when species' ranges come into contact, and these outcomes will depend on the degree and type of diversification that has occurred between the two species in question. The first possibility is that, when expanding populations of different species come into contact, there may be extinction of one species resulting from competition, whereas the second alternative is both species may be able to persist due to habitat partitioning (Liebherr & Hajek, 1990).

Speciation resulting from a process that closely mirrors the TPH has been proposed by Horwitz (1988a) for species within the freshwater crayfish genus *Engaeus*, although he did not refer to it as such. Horwitz (1988a) proposed a model of speciation whereby, during a period of falling sea-level, coastal-adapted species dispersed following the shifting coastline but left behind isolated populations. As sea-level rose again the coastal populations shifted in response, until they came into contact with the populations that had remained *in situ*. This process may have been repeated several times and, if sufficient divergence had occurred, Horwitz (1988a) hypothesised that it would result in two species formed from the coastal population (which would now be a 'lowland adapted' species) and the resident population (which would now be a 'highland adapted' species). The appearance of closely related lowland and highland species groups, which separate via longitudinal zonation along a drainage in both *Engaeus* (Horwitz, 1988a; Horwitz & Richardson, 1986) and *Euastacus* (Morgan, 1986) could possibly be explained by such a hypothesis.

There is, however, one significant difference between Erwin's taxon pulse and the model described by Horwitz. The TPH suggests that the majority of populations remain *in situ* and that expanded peripheral isolates will adapt to differences in habitat, thus becoming more specialised. Under the scenario described by Horwitz, the main species' range shifts to remain in their climatic optima, and it is the populations that remain *in situ* that are likely to develop ecological specialisation as their habitat changes (e.g. Figure 5.5). In this sense, whilst the TPH being tested in this study is largely derived from Erwin's, it does follow the variation seen in the model of Horwitz.
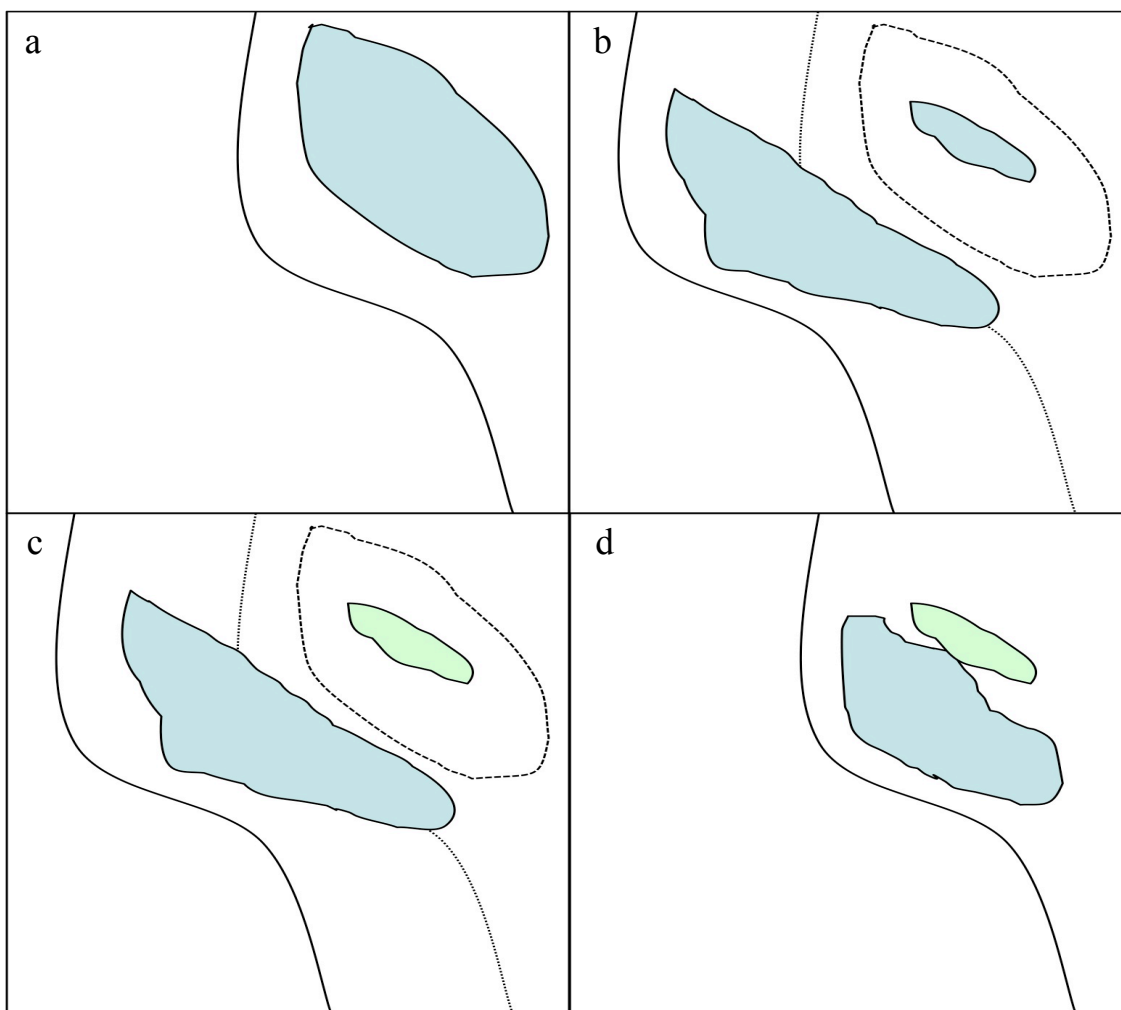
Figure 5.5 A possible scenario highlighting the speciation pattern associated with the taxon pulse hypothesis. Initial distribution and coastline is shown in (a). In (b) the coastline has shifted and the species' distribution split into two (solid lines, with the previous coastline and distribution shown in hashed lines), which later forms two species (c). The coastline and distribution of the original species shifts again in (d), resulting in two closely related species with significant differences in the size of the distributional range, and that are ecologically divergent (i.e. a more widespread and plesiomorphic species and a relatively restricted and more apomorphic species).

Hypothesis

The TPH can be falsified if: (1) there is not a pattern of restricted species displaying more apomorphic states in relation to their nearest genetic relatives which will be relatively widespread.

Evidence

The TPH predicts that there should be widespread species, which produce small peripheral isolates in suboptimal habitat during expansion phases. Some of these peripheral isolates may manage to persist after becoming separated from the main range of the species and evolve in isolation to form a unique species that is specifically adapted to the previously 'suboptimal' habitat. Thus there should be a pattern of ecologically divergent species occurring in narrow distributions (apotypes) with more widespread species that more closely resembles the ancestral species (plesiotypes).

This prediction can be reasonably met for species found within the genus *Engaewa*. The plesiotype for *Engaewa* is assumed to be a strongly burrowing, coastally adapted species. Thus, within the genus *Engaewa* there are three highly restricted species (*E. walpolea, E. pseudoreducta, E. clade A*), of which at least two are ecologically divergent (more data are needed for *E. clade A*), as well as four relatively more widespread 'generalist' species (*E. reducta, E. similis, E. subcoerulea, E. clade B*) (Table 4.3). The divergent forms are also generally smaller in their overall body dimensions; a propensity that has been previously noted in relation to the TPH (Losos, 1992). Furthermore, there is also no evidence that a restricted species has given rise to a more widespread species, which satisfies the unidirectional nature of Erwin's taxon pulse (i.e. the resulting habitat specialisation is irreversible).

Conclusion

The data from this study cannot falsify the TPH, as expounded in this thesis; thus, it is considered to be of significance when explaining the speciation processes within the genus *Engaewa*.

5.2.4 Summary

The testing of various biogeographic hypotheses in this study suggests that no single model can explain all events that have occurred in the evolution of the genus *Engaewa*. However, what can be deduced is that an ancestral *Engaewa* entered Western Australia just once and then underwent a number of occurrences of simultaneous vicariance (based on a rapid increase in the number of lineages) that largely aligned with the taxon pulse hypothesis (based on the uneven geographic partitioning of species' ranges combined with ecological specialisation) (Table 5.1).

Table 5.1 Biogeographic hypothesis-testing conclusions for the genus *Engaewa*.

Hypothesis	Outcome	Reason
Multiple Invasion/ Endemic Speciation	The MIH is rejected in favour of ESH	*Engaewa* was found to be monophyletic and without bursts of speciation occurring at vastly different times in different lineages, thus falsifying the MIH.
Stepping Stone/ Simultaneous Vicariance	The SSH is rejected. The SVH is partially accepted.	The lack of 'ladderisation' within the phylogeny of *Engaewa* falsifies the SSH. Conversely, the molecular dating, LTT plot, and lack of resolution within the mtDNA phylogeny suggest at least one occurrence of a rapid and sudden increase in the number of species.
Taxon Pulse	The TPH is accepted	The occurrence of a number of ecologically divergent and narrowly distributed species and more plesiomorphic widespread species conforms to the TPH.

5.3 Discussion

5.3.1 Origin of the genus *Engaewa*

That *Engaewa* is monophyletic and has speciated *in situ* seems almost incontrovertible. It also seems certain that the genus is sister to *Engaeus,* or *Engaeus sensu stricto/Engaeus lyelli* if the supposition of Schultz *et al.* (2009) that the currently defined genus *Engaeus* is actually composed of two distinct genera is accepted. What is

unclear, however, is when the separation between these lineages occurred. The use of molecular dating in phylogenetic analyses gives us a tool with which we can interpret the evolutionary history of taxa (Ladiges *et al.*, 2011). Even with the uncertainty surrounding the validity of various molecular clock methods, and the large errors often reported, the vastly different estimates of divergence between *Engaewa* and *Engaeus* by Schultz *et al.* (2009) (~50-20 MYA) and Toon *et al.* (2010) (~150-100 MYA) are intriguing and warrant further consideration.

Schultz *et al.* (2009) did not provide an explicit date for the time to the most recent common ancestor (TMRCA) of *Engaewa*, as the dating of the node representing this was not statistically supported. Furthermore, the tree produced by the dating method employed neither suggested the same branching pattern between taxa, nor provided statistical support for the node from which *Engaewa* split. Based solely on the dated tree provided by Schultz *et al.* (2009) the next most recent split after that involving *Engaewa* occurred ~37 MYA (though with error bars spanning from 55-20 MYA) and from this tree it could be assumed that TMRCA is around 40 MYA. Schultz (2009) presented the same data but proposed that all generic level splits within the burrowing clade probably occurred between 37-20 MYA.

Schultz *et al.* (2009) found support for their method of dating in their estimate of the divide between eastern and western *Cherax* species, which they suggested occurred in the late Oligocene to early Miocene. This is reasonably close to the estimate of Munasinghe *et al.* (2004b) who loosely dated the east/west disjunction in *Cherax* at the mid to late Miocene. Further support for the importance of this period in relation to the biogeography of SWA and SEA can be found in Morgan *et al.* (2007), who dated the divergence between south-eastern and south-western frogs of the genus *Heleioporus* to ~25 MYA (late Oligocene). Studies of other anurans, in which a molecular clock was applied based on allozyme and immunological data, suggested the major east-west divergence events occurred perhaps slightly earlier in the Miocene or Pliocene (Barendse, 1984; Roberts & Maxson, 1985a). For obligate freshwater fishes Unmack (2001) found that the 'South-western Province' had generic level relationships with SEA but no species in common with any other region and, based on these relationships

combined with climate data, concluded that the Miocene is most likely the minimum age that freshwater fishes could migrate east-west across southern Australia. Unmack (2001) also suggested that a number of marine transgressions from the Eocene to mid-Miocene might have facilitated the dispersal of species along the coastline during this time.

The timing of divergences between eastern and western species of many genera at ~25±10 MYA is essentially coeval with a palaeoclimatic transition from wet to arid conditions (Martin, 2006) and the formation of the Nullabor Plain (Benbow, 1990; Van de Graaff, Crowe, Bunting & Jackson, 1977). Prior to this time, conditions across southern Australia would, presumably, have been favourable to freshwater species, as there appeared to be a temperate climate with rivers supplying sediments and major dunes forming (Benbow, 1990). Thus, this period appears to be significant to the biogeography of the region. However, the aforementioned studies are all dating splits within genera, whereas the divergence between *Engaewa* and *Engaeus* is a split between genera. This may suggest the split between these two genera is much older than the ~25 MYA date, however, there may be other explanations. It may result from incongruence between what is considered to be a species/genus in different groups, as it has been argued that divergences between different taxonomic levels are not the same in different groups, with higher levels being essentially artificial and somewhat arbitrary (Avise & Johns, 1999). A generic level split where other groups have specific level splits may also result from some characteristic unique to *Engaewa* and/or *Engaeus*.

A possibility is that the proto *Engaewa*/*Engaeus* was highly geographically structured (due to their burrowing habit) prior to the division, hence becoming more highly differentiated compared to other taxa with higher gene flow over the same time period. However, this may also not be valid as other fauna, such as the frog *Geocrinia*, appear to be both similarly restricted and highly genetically structured, yet only differentiated between SEA and SWA at the species level (although within these two regions there are species complexes, as outlined in 5.2.1 in relation to the ESH). A further possibility is that for some unknown reason the ancestral forms diverged from each other more rapidly than any of the other taxa that have been compared. A final

possibility that needs to be considered is that it is actually a combination of any, or all, of the above factors. A possible explanation may be *that Engaewa* spp. do actually differ by more than the eastern/western *Cherax* split, due to genetic structuring resulting from their burrowing habit, and that they actually represent a similar degree of divergence as seen in other taxa such as *Geocrinia*, but in freshwater crayfish this level of divergence has been elevated to generic level, rather than species level.

Whilst the date of TMRCA for *Engaewa* suggested by Toon *et al.* (2010) is substantially different from that of Schultz *et al.* (2009) it is not without support. The date of Toon *et al.* (2010) falls within the Cretaceous period, during which there was a substantial marine subdivision of Australia (the Eromanga Sea), which essentially separated eastern and western Australia (Twidale, 1994) (Figure 5.6). This has been suggested as being the period during which there were east/west separations of numerous genera, including some older invertebrate groups (Howden, 1981; Main, 1981a, 1981b). Breinholt *et al.* (2009) used the fossil data of Martin *et al.* (2008) to calibrate their timing of the origin of the Parastacidae, which they estimate at ~161 MYA. The TMRCA for *Engaeus* and *Geocharax* arrived at by Breinholt *et al.* (2009) was 144 MYA, which suggests that the separation between *Engaeus* and *Engaewa* may be consistent with that suggested by Toon *et al.* (2010) (~150-100 MYA) as it would be expected that it should be (relatively speaking) slightly closer to the present.

In an attempt to add clarity to the situation, molecular dating was undertaken in this study. The data and method used was similar to that of Schultz *et al.* (2009) but used a combined 16S and GAPDH dataset (rather than just 16S) and the program *BEAST (which is a modified version of BEAST as used by Schultz *et al.* (2009)). As with the method of Schultz *et al.* (2009), the *BEAST species tree obtained in this study had a slightly different (and non-supported) topology in comparison to the 'accepted' phylogeny. Despite this, the dates obtained in this study clearly align more closely to those of Toon *et al.* (2010). Whilst there is still considerable error involved and the dates are far from certain, they refute the hypothesis of Schultz *et al.* (2009) and suggest the origin of the genus *Engaewa* lies within the Cretaceous.
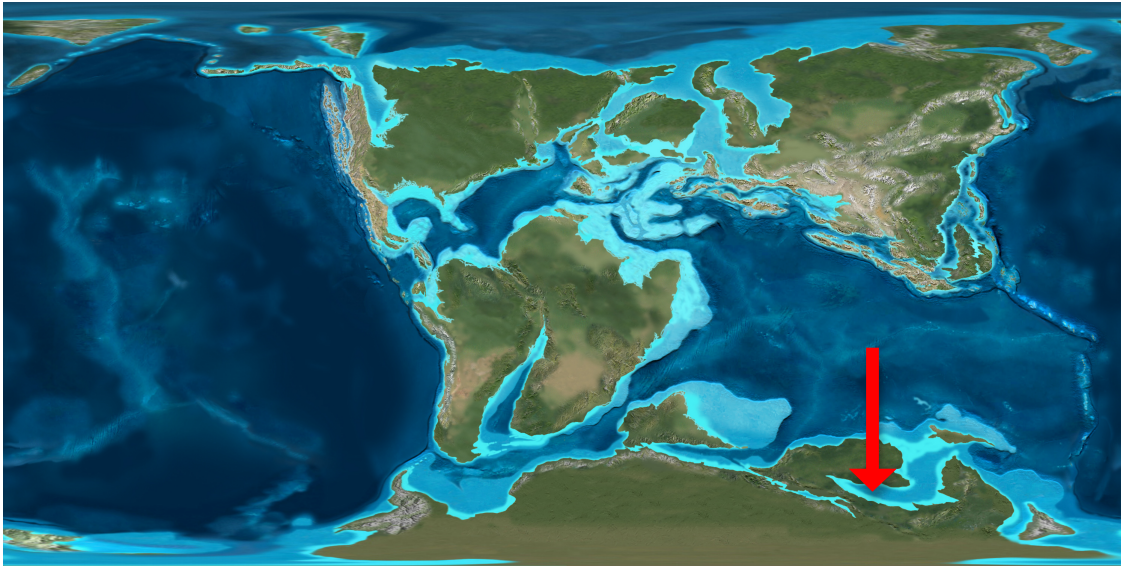
Figure 5.6 Global sea-levels and continental arrangements ~120 MYA, with the presence of the Eromanga Sea in the centre of Australia evident (highlighted by the red arrow) (Blakey, 2008).

The failure of the molecular data available to produce a definitive pattern of diversification between *Engaewa*, *Engaeus sensu stricto* and *Engaeus lyelli* hints at the separation between the three 'genera' being largely synchronous. If this is an accurate assumption then, based on the fact that two of the genera are found in eastern Australia and only one on the western seaboard, it suggests that the ancestral species may have been found in eastern Australia (an assumption supported by the general recognition of SEA as being the centre of origin for the entire family). However, if the dating of Toon *et al.* (2010) is accepted then the ancestral form may actually have spanned both SEA and the landmass that is now Antarctica. Crayfish fossils were believed to have been found in Antarctica (Babcock *et al.*, 1998) though this view was later revised (Hasiotis, 2002). Despite this, the Gondwanan distribution of parastacids strongly suggests that crayfish would have been present in Antarctica during the Cretaceous and it is possible that many of the major lineages (and perhaps even the ancestral *Engaewa*/*Engaeus*) originated there.

5.3.2 Speciation in the genus *Engaewa*

A logical interpretation of the phylogeny presented herein following the 'rules' predicted by the *a priori* hypotheses tested suggests that no single model can explain the patterns of speciation and distribution within the genus. Rather, a combination of the

models needs to be employed. Considering the time-scale involved and the complexity of biological evolution, it is not surprising that this is the case. Edwards, Roberts and Keogh (2008, p. 1812) arrived at a somewhat similar conclusion for the frog fauna of SWA as they noted that, "There are no simple, common patterns in the biogeography of south-western Australian frogs, as might have been expected given the history of adaptation to gross climate change (e.g. from summer to winter rainfall patterns)". Thus, there are likely to be many intricate factors pertinent to this investigation.

Several considerations must be kept in mind before attempting to explain the proposed biogeographic history of the genus *Engaewa*. This study has both removed and added examples of overlapping distributions within the genus, but highlighted only one known case of sympatry (and it is proposed to be a very recent, human-mediated, occurrence). This, combined with the highly restricted and disjunct distribution of populations and allopatry being widely accepted as the most common first step in the process of speciation (Coyne & Allen Orr, 2004), suggests that speciation within *Engaewa* has most likely occurred solely via allopatric mechanisms. Furthermore, whilst dispersal and vicariance have long been seen as competing theories in biogeography (Sanmartin & Ronquist, 2004) most biogeographers now accept the occurrence of both (Yoder & Nowak, 2006), and it has been more recently acknowledged that they are not mutually exclusive hypotheses for explaining the distribution of species (e.g. Toon *et al.*, 2010 and examples therein). Thus, a model that can reconcile the relative importance of both dispersal and vicariance may provide the best explanation of the current pattern of *Engaewa* in SWA. In order to most clearly synthesise the biogeographic history of the genus *Engaewa*, firstly some concepts that apply to the genus generally will be expounded. Once this has occurred, the processes that have led to lineage splitting and diversification within the genus will be explored by following a sequence that is approximately linear (both in a spatial and temporal sense).

As *Engaewa* species are predominantly coastal-adapted and reliant on sufficient access to water to persist, populations following a fluctuating sea-level are also generally following the shifting high rainfall zone, and remaining in the 'optimal' habitat for the genus. As was shown in Chapter 4, *Engaewa* can exploit a wide range of

habitats, including the sandy and coastal habitat that would be exposed during periods of low sea-level. Whilst rainfall would often be low during these periods (due to the association between low sea-level and dry climate), it would generally be elevated closer to the coast, and there would feasibly be much larger and swampier coastal plains, as any water flowing from the inland plateau would leave the well defined drainages on the plateau margins and lose momentum across sandy coastal plains. Thus, periods of low sea-level could actually experience less directed flow, increased size and number of swampy regions, and significantly higher groundwater levels.

Although it could be argued that the coastal habitat made available during periods of lowered sea-level would be highly saline, and therefore not favourable to inhabitation, it has been shown that the salinity tolerance of numerous freshwater crayfish would suggest that short-term, mildly saline environments are not likely to block dispersal (Schultz *et al.*, 2008), minimising the barrier that salt water intrusion may provide. Therefore, despite living in what may be considered somewhat marginal coastal habitat, by following a fluctuating HRZ throughout periods of climatic fluctuation these crayfish have been able to persist. This is significant as *Engaewa* show many characteristics typical of adversity selected species (first proposed by Greenslade (1972), later named by Whittaker (1975), and further developed by Southwood (1988) (amongst others)). Adversity selected species are expected to be found in habitat that has low 'favourableness' but high 'predictability' (Greenslade, 1983), which in the case of *Engaewa* is provided by accessing groundwater within coastal habitats.

Engaewa species are largely sedentary, thus the longer periods of time during glacial periods, combined with increased availability of habitat, would facilitate their dispersion. It is hypothesised that during a period of falling sea-level, populations will disperse into vacant habitat, following the shifting coastline, but in doing so will leave behind isolated populations. These populations (rarely) may be able to persist, due to the effect of local microclimates (i.e. creating refugia for these populations). As sea-level rose again, the coastal populations would shift in response, until they potentially came into contact with the populations that had remained *in situ*. This process could result in two species, formed from the coastal populations and the resident population,

which would most likely remain parapatric, as each species will be better adapted for slight differences in the habitat occupied. This is the basic model of speciation within the genus *Engaewa*.

Repeated periods of separation between populations due to climate change is not necessarily enough to ensure speciation, as one of the critical factors is the duration of separation. It has been argued (e.g. Jansson & Dynesius, 2002) that fluctuating climate may not result in speciation, as the periods of separation may be too short for full reproductive isolation to occur before the populations' ranges expand and they once again come into contact. However, it has also been suggested that speciation can occur as a result of the accumulation of differences from repeated periods of geographic isolation (Avise & Walker, 1998). Furthermore, the lineages most likely to speciate due to a period of climate change are those that have low-dispersal ability as, during favourable periods, their expansion will be much slower than other species; essentially elongating the duration of isolation (Waldron, 2010). Thus, the frequency of cycles combined with the rate of species' expansion may be as significant as the duration of a single cycle (Waldron, 2010).

A general interpretation of the data produced in this study is that shifting habitat zones (driven by an overall drying trend of the Australian continent) have resulted in a pattern of lineage splitting events that created, at each split, a widespread species and a relatively more restricted species (Table 5.2). The single exception to this rule is the split between *E. clade B*/*E. subcoerulea* and the northern species, which can roughly be seen as dividing the distribution of the genus in two. This uneven partitioning of species range, when combined with relative specialisation, creates a dichotomy within *Engaewa* and the evolutionary paths of the various species. The most restricted species (*E. walpolea* and quite possibly *E. pseudoreducta*) show a higher affinity with standing water than other *Engaewa* species and, associated with this, a less obvious morphological specialisation towards the typical characteristics of strongly burrowing crayfish. By not burrowing so deeply, and having a lesser reduction of the abdomen and less inflated chelae, they appear more adept at dispersing via surface waters when the opportunity arises, compared to the strongly burrowing species. The contradiction in this situation, however, arises due to the drying nature of the climate in SWA. Whilst *E.*

walpolea may be able to disperse widely *given favourable conditions*, the lack of substantial surface waters in SWA appear to have limited this species to a very discrete region, whereas the strongly burrowing species have spread (albeit presumably at a slow rate) through the coastal plains along the south coast of SWA. Thus, it could be argued that *Engaewa* species exist that have the potential, yet no opportunity, to disperse fairly rapidly existing in isolated habitat (which is likely to become, if anything, further restricted), and species that have the potential to persist in a drying climate, yet will feasibly experience considerable difficulty keeping up with shifting climate zones.

Table 5.2 Division of *Engaewa* lineages following speciation events, showing the uneven geographic partitioning resulting from the fracturing of peripheral isolates from the main species' range.

Peripheral isolate	Widespread lineage
E. walpolea	the ancestor of the other species
E. clade B	*E. subcoerulea*
ancestral *E. reducta/E. pseudoreducta*	ancestral *E. similis/E. clade A*
E. clade A	*E. similis*
E. pseudoreducta	*E. reducta*

5.3.3 Diversification within the southern group

The initial separation within this genus appears to be between the ancestral *E. walpolea* and the ancestor of the rest of the genus. This division clearly corresponds with an ecological separation, with *E. walpolea* existing in one of the more topographically diverse areas of the south coast, whereas the neighbouring species (*E. subcoerulea/E. clade B*) are found on larger coastal plains. The origin of this ecological differentiation can best be explained by incorporating shifting climate zones corresponding with sea-level fluctuations.

Based on the dates estimated in this study, the initial diversification within the genus may have been driven by the general cooling of the global climate since the early Eocene followed by the initial formation and expansion of the Southern Ocean resulting from the rifting of Australia and Antarctica. This process would have created expanded

coastal-type swamp systems, radically altering the distribution of these crayfish. Conversely (considering the uncertainty in dating), this event could just as easily be attributed to a more recent glacial period, which would have had a similar effect. Regardless of the timing of this event, the scenario is essentially the same. The creation of an expanded coastal plain along the south coast of what is now continental Australia would have resulted in a gradual shift of many populations to this newly exposed and expanded habitat. It is generally accepted that the zone of highest rainfall shifts in conjunction with shifting sea-level, so that a rainfall gradient (decreasing with distance from the coastline) would have persisted. Due to the rainfall gradient, any inland *Engaewa* populations that were unable to migrate rapidly enough to remain in the preferred rainfall zone (due to environmental heterogeneity) would have faced extreme survival pressure. However, it has previously been recognised that some small pockets of topographically complex landforms may have allowed for the persistence of a range of species in SWA (e.g. Main, 1996b; Moir *et al.*, 2009). Thus, an ancestral population of *Engaewa* would have been maintained in the topographically diverse habitat around what is now the Nornalup Inlet and become specifically adapted to the local conditions (forming *E. walpolea*).

The hypothesis that *E. walpolea* was formed from a small peripheral isolate is supported by the very low genetic diversity found in the species (i.e. representing a genetic bottleneck). This population would have undergone ecological specialisation combined with genetic drift, driving diversification and eventually resulting in speciation. This is now evident on the basis of morphological, molecular and ecological characteristics. As discussed in Chapter 3, the plesiomorphic state for *Engaewa* is believed to be strongly burrowing, suggesting *E. subcoerulea/E. clade B* maintain the plesiomorphic character, whilst *E. walpolea* represents a more derived form.

As the distribution of the closely related species *E. subcoerulea* and *E. clade B* is divided by the occurrence of *E. walpolea*, and these two species are morphologically and ecologically similar, it is assumed that the initial diversification was due solely to allopatric speciation (i.e. due to geography not ecology). Thus, there are two possible alternatives to explain their distribution; one based on dispersal and one on vicariance.

The dispersal scenario is that there was an ancestral species present on one side of the range of *E. walpolea* and that there was a dispersal event to the other side. It is possible this occurred by either directly 'jumping' *E. walpolea* or, for a period, co-existing with *E. walpolea*. The scenario that is more likely, and fits with the general hypothesis supported in this thesis (that shifting climate/sea-level is the predominant driving force behind speciation) is based on vicariance. It seems likely that along the south coast the presence of a broad and relatively flat coastal plain during a period of lower sea-level would have facilitated dispersion. In contrast, when the climate was generally wetter and dispersal across headwaters is predicted to be more prevalent (i.e. during a typical interglacial period) sea-levels would generally be higher, and the topography of the southern coast of SWA would have been much more diverse, with greatly reduced coastal plains and many of the drainages originating high in the landscape (Figure 5.7). This scenario would have seen an ancestral *E. subcoerulea/E. clade B* occupying a large coastal strip that has since become unavailable due to rising sea-level. The rising sea-level would have driven populations of this ancestral species further inland, creating a vicariance event, whereby the ancestral species was divided by the complex geography of the Walpole region (which may have been further reinforced by competitive exclusion by *E. walpolea*).

Many populations within both *E. subcoerulea* and *E. clade B* have remained genetically connected, at least until fairly recently, suggesting that they have continued to occupy a relatively well-connected environment throughout their history. This is in contrast with the situation for the northern species that generally show extremely high population differentiation. The pattern of haplotypes in both *E. subcoerulea* and *E. clade B* is characterised by two common features: closely related haplotypes across geographically disparate populations, as well as other geographically proximate populations that do not even connect via a 90% parsimony limit haplotype network (see Figures 3.24 and 3.26 in Section 3.3.5). This pattern has likely resulted from repeated range fluctuations, whereby lineages were split long enough to begin forming unique haplotypes (in at least rapidly evolving portions of the genome) but populations were later forced back into close contact, resulting in odd mixing of ancestral and newly derived haplotypes. This is further evidence for the occurrence, and significance, of

repeated range expansions and contractions over vast periods of time, and that they continue up until the present day.
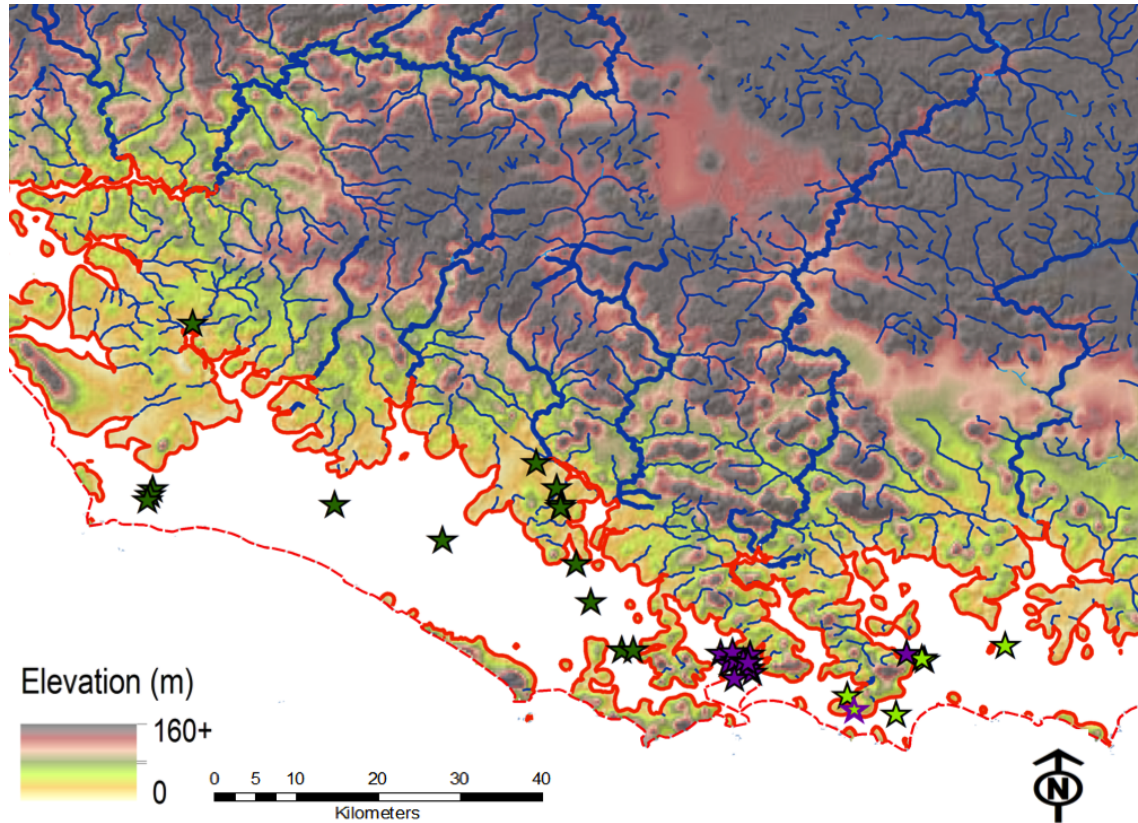
Figure 5.7 South coast of SWA showing the current distribution of *Engaewa subcoerulea*, *Engaewa walpolea* and *Engaewa clade B* (dark green, purple and light green, respectively), based on the sampling for this study. Present day sea-level is show by the dashed red line, whilst a proposed elevated sea-level (+60 m) is shown by the solid red line. For sources of GIS data see Section 2.5.

5.3.4 Separation between southern and northern groups

The genetic split between the southern and northern species within the genus *Engaewa* corresponds to a current geographic division. The shortest distance between sampled populations of *E. subcoerulea* and *E. similis* is currently 40 km, which represents the largest gap between species of *Engaewa*[*]. Whether this is a true void within the distribution of the genus or an artefact of sampling (this area is the most inaccessible within the range of the genus) is uncertain. Part of the area between these two species may be unsuitable for *Engaewa* (approximately corresponding to the

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[*] There was a gap of 50 km within the sampled range of *E. similis* in this study, although this is believed to be purely due to sampling.

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Warren River Catchment) as it contains a stretch of coastline where the Southern Dunes landscape unit contacts the central and northern Karri landscape units: Figure 5.8 shows a clear discontinuity in the swampy coastal plain systems favoured by these crayfish.

Whether there is a modern day geographic discontinuity or not, the genetic division between the northern and southern species provides evidence that at some point in the past there must have been a boundary to gene flow. There are two alternative scenarios that could explain this (which are essentially the same as those considered in the preceding section discussing the split between *E. subcoerulea* and *E. clade B.*). Firstly, it is possible that there was a dispersal event across a historically inhospitable region allowing *Engaewa* populations to expand further north and speciate in isolation from the southern species. Alternatively, an ancestral species of the northern and southern species groups may have occurred widely along the south coast and had its distribution fractured by rising sea-level. Following the general model proposed, the second alternative is considered more likely.
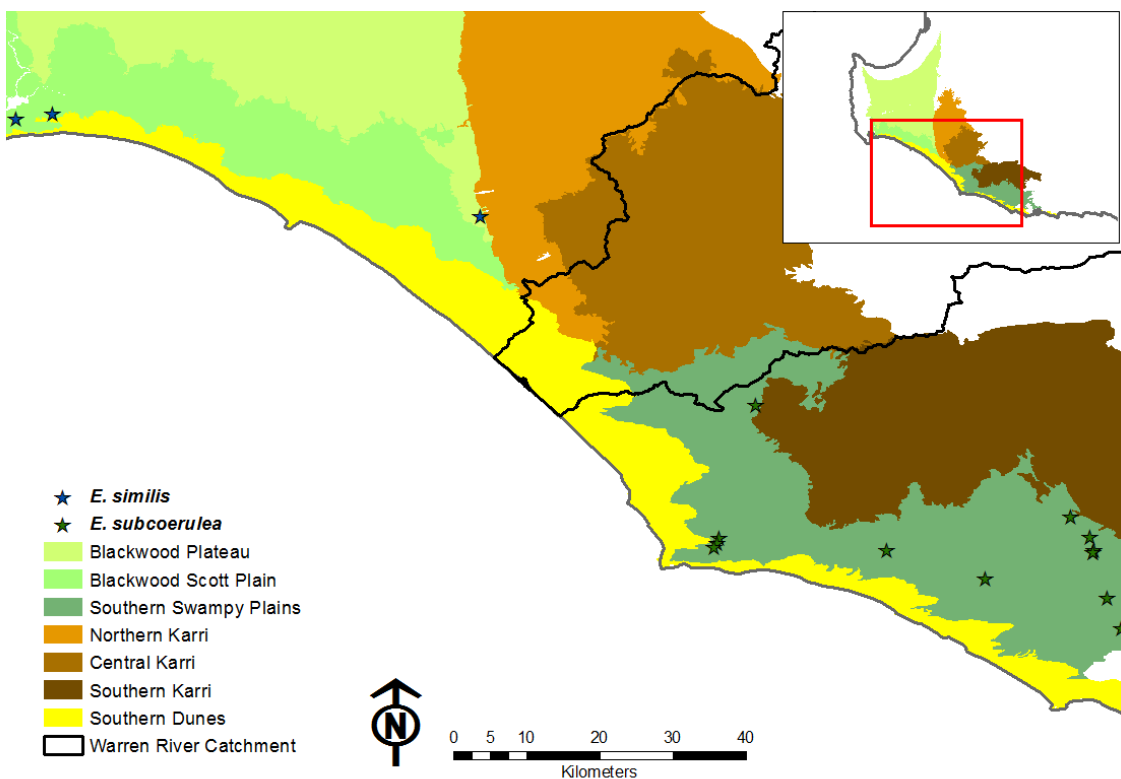
Figure 5.8 Landscape units of the Warren River Catchment region, highlighting the boundary between the distribution of *Engaewa similis* and *Engaewa subcoerulea*. For sources of GIS data see Section 2.5.

5.3.5 Diversification within the northern group

Whereas the pattern seen in the southern portion of the range of the genus *Engaewa* strongly suggests diversification was driven by dispersion (via a coastal route during periods of lower sea-level) combined with vicariance (resulting from periods of elevated sea-level), the pattern in the north is more complex. The occurrence of populations of both *E. reducta* and *E. similis* in separate drainages that flow to the north and south out of the area between Cape Naturaliste and Cape Leeuwin (which shall herein be referred to as the Cape-to-Cape region), as well as *E. similis* in a westerly flowing drainage, suggests there are factors other than coastal dispersion involved (Figure 5.9). As the distance between the various drainages is significantly shorter via an inland route, compared to the coastal distances or palaeodrainage distances (Figure 5.9), an explanation of the relationship between populations of these species may also need to incorporate inland dispersal.

Within the northern group there are two species pairs – *E. reducta/E. pseudoreducta* and *E. similis/E. clade A*. The molecular dating of this study suggests the splitting event between these was largely concurrent with that of *E. subcoerulea* and *E. clade B* in the south. This suggests that the event that drove the initial speciation in the north likewise resulted from a period of rising sea-level. It is hypothesised the ancestral species of these northern species occurred widely throughout an expanded coastal plain (Figure 5.10a). Rising sea-level would have driven these coastal populations into more topographically diverse areas creating lineage diversification via a vicariance event, resulting in the ancestral *E. reducta/E. pseudoreducta* and *E. similis/E. clade A* lineages (Figure 5.10b).
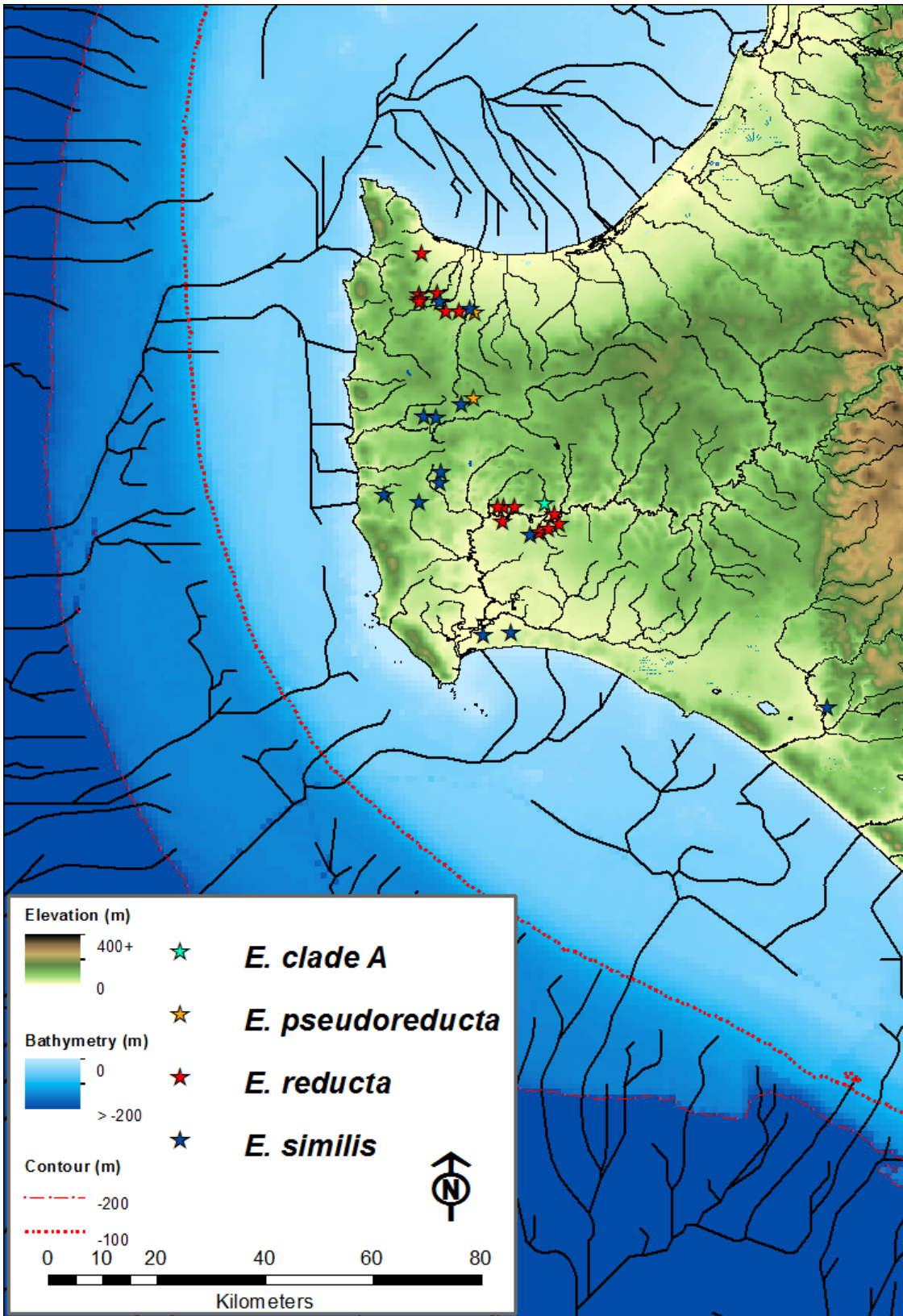
Figure 5.9 Modern coastline, waterways and elevation of the coastal corner of south-western Australia, as well as reconstructed palaeodrainages and shoreline under scenarios of sea-levels of -100 m and -200 m below modern sea-level. For sources of GIS data see Section 2.5.

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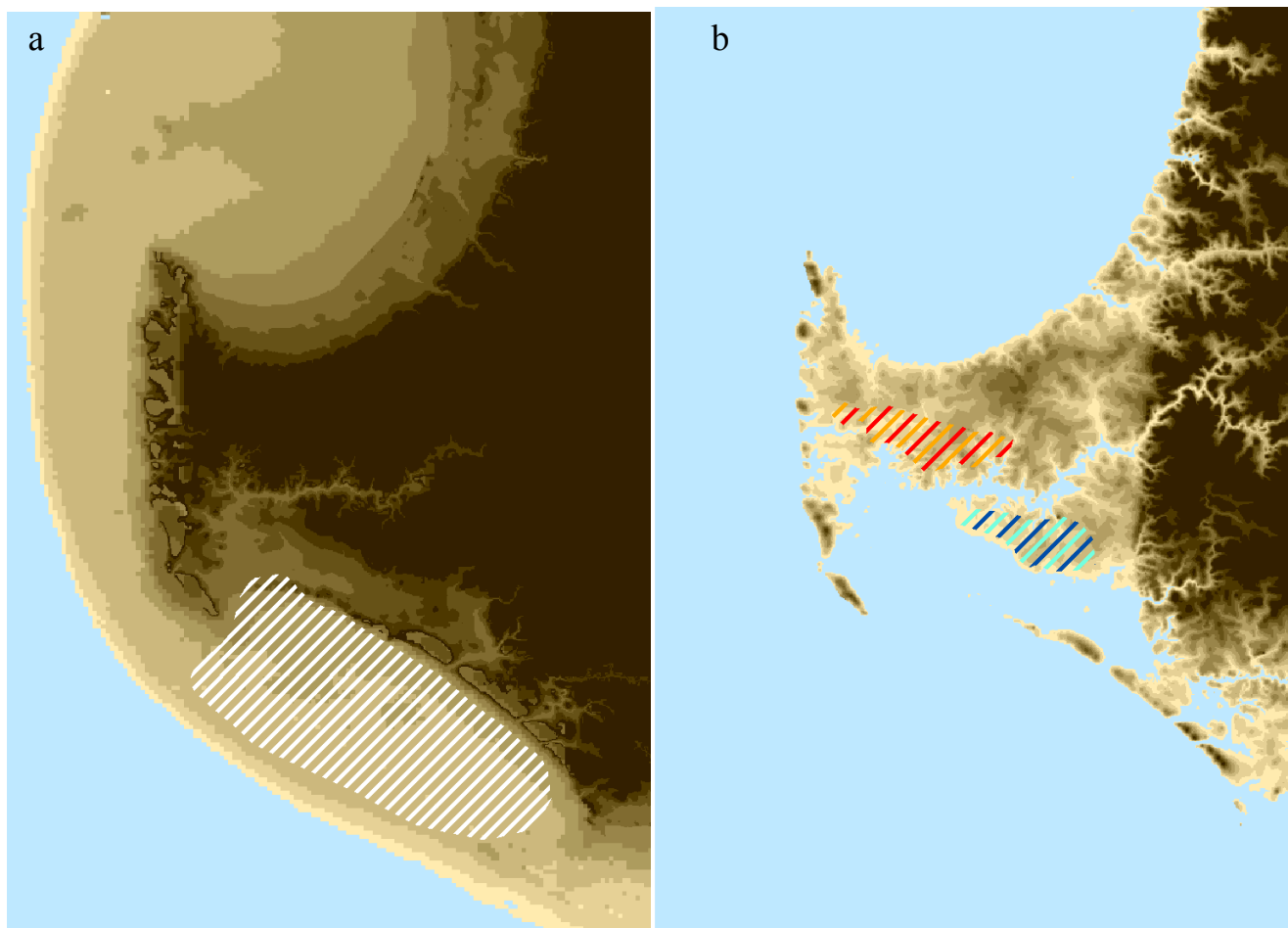
Figure 5.10 Initial diversification in the northern range of the genus *Engaewa*, showing (a) the hypothesised distribution of a single ancestral form with a sea-level 100 m below present, and (b) the hypothesised distribution of ancestral *similis/clade A* and *reducta/pseudoreducta* lineages (dark & light blue, and red & gold, respectively) during a period of elevated sea-level (60 m above present). Shading represents elevation from each depicted sea-level. For sources of GIS data see Section 2.5.

As sea-level once again receded, populations would have shifted coastally following the high rainfall zone. Dispersion would have occurred predominantly to the north and south out of this region, due to the presence of the Leeuwin-Naturaliste Ridge (a granitic, fault-bounded horst of Precambrian antiquity (Myers, 1990)), which forms a north-south trending ridge, 15 km wide and 100 km long. This process would have created isolated populations in suitable microhabitats further inland (following the TPH). Two of these isolated populations appear to have persisted to the present day and formed *E. pseudoreducta* and *E. clade A* (and possibly a third represented by the population at Payne Road). Thus, this process can explain the splitting of both of the lineages in this region, producing the two extant species-pairs.

Based on the present distribution of *E. reducta* and *E. pseudoreducta*, it seems that the ancestor of these species had a distribution to the north of the present day Blackwood River (Figure 5.10b). It is hypothesised that during a lowering of the sea-level most populations of the ancestral species shifted to the north-west, in order to follow the receding coastline (Figure 5.11a). A small number of populations would have remained in isolated pockets of habitat that acted as refugia. As discussed in Chapter 4, *E. pseudoreducta* appears to have a somewhat unique ecology/biology when compared to its sister species and exists in unusual habitat. This habitat is unusual in large part due to the clay soil present, which provides significant water holding capabilities. It also occurs on a south-facing slope, which would help maintain moisture due to reduced insolation. A southerly aspect has been noted for supporting relict species (Main, 1996b), whilst the significance of aspect and slope to microclimate variation has been shown in studies in Britain where differences of up to 12°C were recorded between north- and south-facing slopes (Ackerly *et al.*, 2010; Rorison, Sutton & Hunt, 1986). Thus, the soil type, combined with the southerly aspect, would have allowed populations to persist within this isolated refugium.
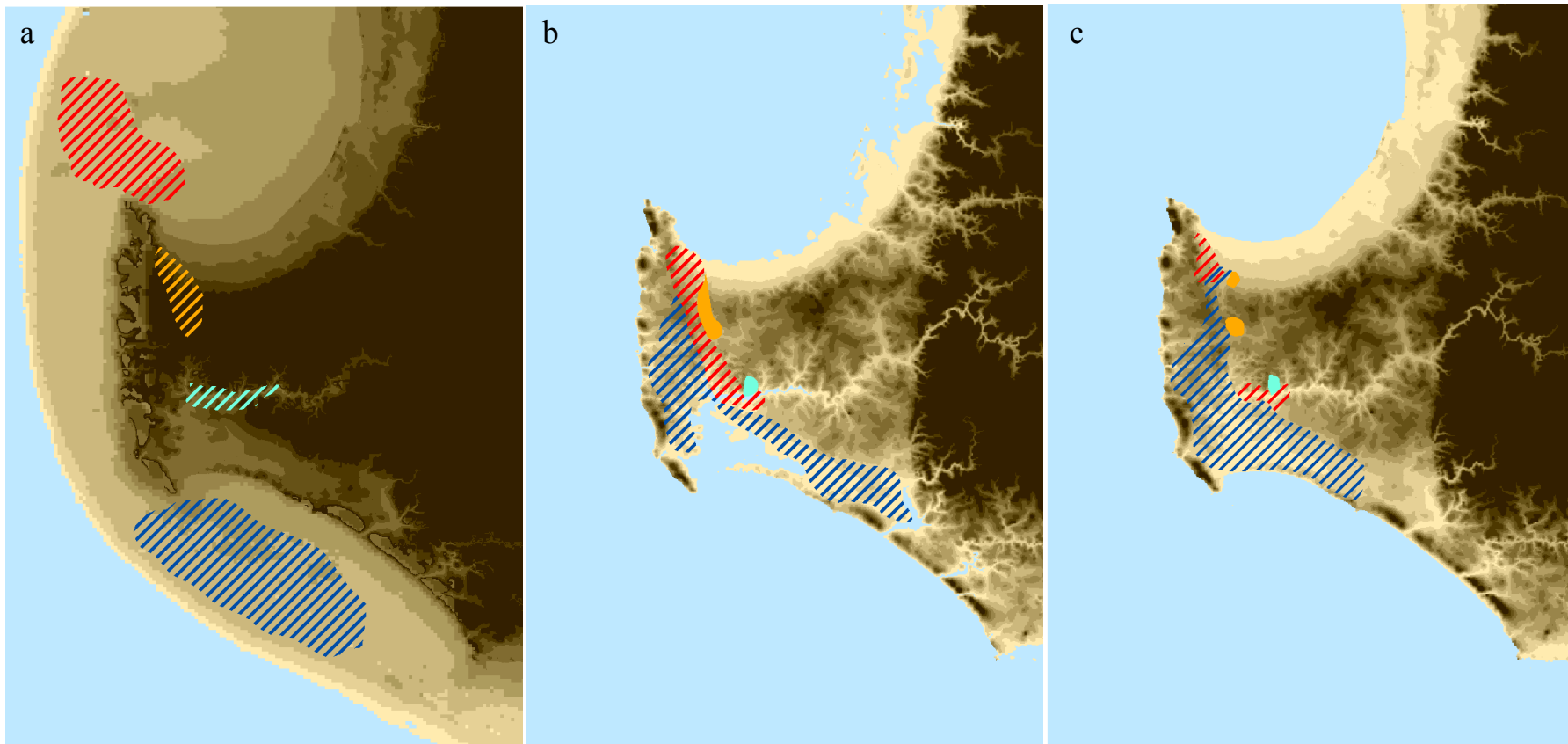
Figure 5.11 Diversification in the northern range of the genus *Engaewa*, showing (a) the hypothesised splitting of the *similis/clade A* and *reducta/pseudoreducta* lineages (dark & light blue, and red & gold, respectively) with a sea-level 100 m below present, (b) the hypothesised distribution of these lineages in a subsequent period of elevated sea-level (20 m above present), and (c) recent distributions of the four species with current sea-level. Shading represents elevation from each depicted sea-level. For sources of GIS data see Section 2.5.

The presence of the nearest relative of *E. similis* further up the Blackwood River catchment suggests that the ancestral form of this species previously occupied a larger proportion of this drainage, therefore, the distribution pattern of species around the Blackwood River requires explanation (i.e. three species being found within ~5 km; including one entire species in a single site surrounded by a species to which it is not most closely related (Figure 5.12)).
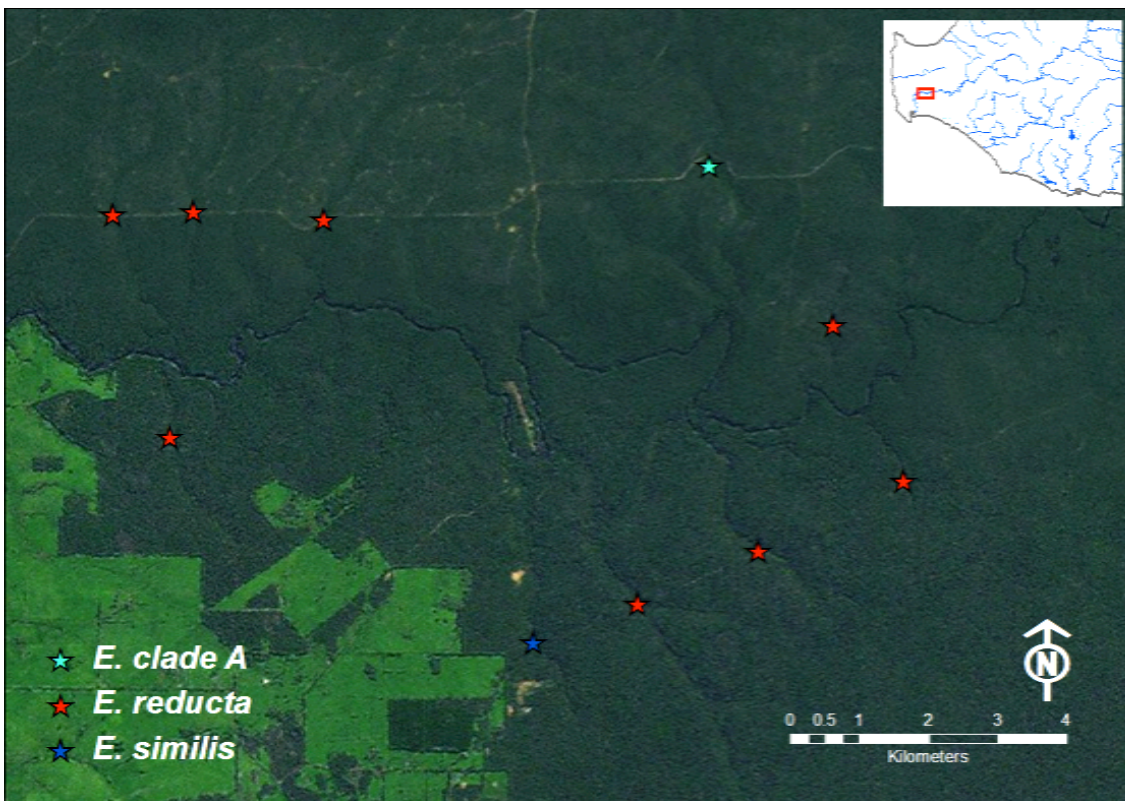
Figure 5.12 Distribution of *Engaewa* species in the Spearwood Creek region.

This scenario commences with a relatively widespread *E. clade A/E. similis* ancestor occurring throughout the drainages along the Blackwood River. A period of drier climate may have seen a number of the smaller tributaries of the river dry up entirely, causing extirpations of the *Engaewa* populations present. It is entirely possible that this may have occurred in all (or nearly all) of the drainages in this region *except* Spearwood Creek, where *E. clade A* now resides exclusively. This is because Spearwood Creek derives water directly from the Leederville aquifer, which ensures that it remains wetter than many of the surrounding creek lines even during periods of extended drought. There are, however, drainage lines on the southern side of the

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Blackwood River that also derive water from this source. These drainages are now inhabited by *E. reducta*, so several questions need to be discussed: why did the ancestral *E. clade A* not manage to persist in nearby creek systems, why are they now occupied by *E. reducta*, and why is *E. similis* only found further down this drainage?

It is possible that *E. clade A* is the only species that has ever occurred within Spearwood Creek, and that it is not genetically closest to the species surrounding it due to some chance dispersal event of an ancestral species, though this explanation seems intellectually unsatisfying and biologically unfeasible. Furthermore, if there were a scenario whereby Spearwood Creek became disconnected from the other surrounding drainage lines (i.e. there was a vicariance event that isolated this drainage from the others) and it remained an isolated 'island' within a 'sea' of connected drainages then we could project the population in this particular creek to speciate independently of its neighbours. Had this happened, however, the species present would be most closely related to those geographically nearest to it (i.e. *E. reducta*), which it clearly is not (it is phylogenetically closest *E. similis*). If there is a mechanism that causes one species to outcompete another (as may be suggested by the near complete lack of sympatry for the entire genus) it is possible that either an ancestor of *E. clade A* by chance arrived in this single drainage, which was within an *E. reducta* 'stronghold' and outcompeted the resident *E. reducta* to the point it was eradicated. Alternatively, it is also possible that *E. reducta* entered either one, or a number of, drainage lines along the Blackwood River and, either due to a unique factor in Spearwood Creek or some purely stochastic reason, was able to outcompete *E. clade A* (or an *E. clade A/E. similis* ancestor) in all but this one drainage line. The most plausible explanation, however, is complex, as outlined below.

Spearwood Creek maintains a moist microclimate that would be suitable for *Engaewa* species as it is the only one of the aquifer-fed drainages that lies on a south facing aspect. Thus, it is proposed that within Spearwood Creek the *E. similis/E. clade A* ancestor became isolated, managed to persist, and evolved in isolation, forming *E. clade A*. The extreme pressures faced by this population reduced genetic diversity (as

highlighted in Chapter 3) and promoted ecological specialisation (as highlighted in Chapter 4).

Following the suggested scenario, a later change of climate would have 're-opened' the habitat in the surrounding drainage lines and *E. reducta* was, this time, the species that entered these drainage lines rather than *E. similis*, by utilising an inland route (Figure 5.11b). It could be argued that *E. clade A* should have been able to spread into these adjoining drainage lines when the conditions were right for *E. reducta* to expand to its current range, however, its absence could be explained by an ecological restraint (i.e. it being wedded to the ecological conditions specific to Spearwood Creek). Even if *E. clade A* were able to expand into adjoining drainages then *E. reducta* might have been able to outcompete *E. clade A* in all but Spearwood Creek, due to morphological and physiological adaptations. *Engaewa reducta* has a larger body size and more obvious dimorphism of the chelae than *E. clade A*; both of these characteristics provide advantages in aggressive interactions between a range of crayfish species (e.g. Nakata & Goshima, 2003; Pavey & Fielder, 1996; Ranta & Lindström, 1993; Rutherford, Dunham & Allison, 1995).

Combining these reasons, a difference in habitat between Spearwood Creek and the other drainages (which would have allowed *E. clade A* to persist in only this single drainage line in the first place) favours one species over the other. Due to the larger size of *E. reducta* (potentially along with other adaptations) it may be able to better utilise the comparatively drier and less peaty soil of the surrounding drainage lines (the differences in soils and vegetation occupied by these two species was highlighted in Chapter 4). In comparison, the smaller and (based on the observation of possibly shallower burrow depths and the occurrence of this species being found via the spotlighting method) less burrowing adapted *E. clade A* may be favoured in Spearwood Creek, where water is more plentiful and soil more amenable to digging. Potential differences between these two species may also include an improved reproductive success in these conditions for *E. clade A* as they can more readily find mates out of the burrow, and expend less energy on burrowing.

The situation described above suggests that an ancestral form of *E. similis* would have previously occupied the region of the Blackwood River now occupied by *E. reducta*. If *E. similis* represents a generally coastal adapted species (as suggested by it being widespread throughout the Scott Coastal Plain), its current distribution may be explained by it shifting out of this region during a period of lower sea-level and, following rising sea-level, subsequently recolonising up to the edge of *E. reducta*'s range. If this species occupied a broad coastal front at the last glacial maximum, its presence in the Blackwood and Margaret River catchments would be readily explained, however, there is yet another anomaly. Two populations were identified in the northerly draining Carbanup catchment to the north of the Margaret River. These appear likely to be the result of a later inland dispersal from the Margaret River catchment, rather than via coastal dispersion (Figure 5.11c). If this were not the case it would be difficult to explain why *E. similis* was found higher up the Carbanup catchment, whereas *E. reducta* is found higher up the Blackwood catchment. A strictly coastal model would favour one or the other of these species being in the upper reaches of the catchment and the other representing a more coastal form.

5.3.6 Summary

Although glaciation is often cited as having driven speciation (e.g. Hewitt, 2001; Knowles & Richards, 2005), it has been noted that the cyclical nature of glacial and interglacial periods themselves can have similar effects on flora and fauna, without the impact of direct glaciation (Avise, 2000; Hewitt, 2004; Schneider, Cunningham & Moritz, 1998). For example, latitudinal and altitudinal range shifts in response to a changing climate can drive demographic changes and provide opportunities for adaptation to occur, which will have stochastic and selective effects on gene pools (Hewitt, 1999; Hewitt, 2000; Hewitt, 2004). Due to the isolated nature of the mesic portion of SWA and the muted topography, neither latitudinal or altitudinal distributional shifts are viable options for many taxa, instead it is proposed that these taxa have contracted into refugia in response to changing climatic conditions.

Refugia can promote diversification and lead to speciation (Davison & Chiba, 2008), as contractions in range generally reflect reductions in abundance and, by extension, are likely to lead to genetic bottlenecks and increased effects of genetic drift (due to small effective population sizes) (Bennett & Provan, 2008). Furthermore, whilst climate driven adaptation and range alterations may lead to speciation of taxa while they are restricted to refugia, there is also the possibility that when conditions become more favourable and these species expand their range they can undergo an adaptive radiation as they disperse into newly available habitat. Thus, speciation can actually be promoted through both the contraction into, and expansion out of, refugia.

Whilst the contraction of species into refugia can cause rapid divergence between allopatric populations, it also can result in low within-population genetic variation (Hampe & Petit, 2005) and increase the likelihood of extinctions due to demographic or environmental stochasticity (Cowlishaw, 1999; Diamond, 1984; Harcourt & Schwartz, 2001; Lande, 1993; Lawton, Daily, Newton & Lawton, 1994; MacArthur & Wilson, 1967). Thus, while refugial remnants have often been suggested to provide relief against extinction for the species occupying them (Haffer, 1997) it is also clear that species in small refugia will generally exist in only a few isolated populations and, therefore, must face a high extinction risk (Waldron, 2010).

For *Engaewa*, it is clear that shifts in climate have caused phases of contraction into refugia, resulting in vicariance between isolated demes. The populations within these refugia are more closely related to adjoining refugia than more distant ones, due to the geographic-based genetic structuring prior to the retraction period. If retraction proceeds to the point where genetic bottlenecks occur at the population level, rare alleles will likely be removed from these populations. However, genetic drift in a relatively small population may result in new alleles arising and rapidly becoming fixed in the local population. Therefore, through bottlenecks, combined with genetic drift and/or selective sweeps, each of these populations in the major refugial areas have ended up with a small number of predominant haplotypes for that geographic region.

This process has resulted in a situation where there are isolated pockets of populations in refugia that are only distantly related to other populations in similar refugia, closely related though still differentiated from populations within their refugium, and containing little diversity within populations (i.e. high inter-population diversity, low intra-population diversity). This can be seen in the haplotype networks, as the networks do not connect refugia but do often connect populations within a refugium, albeit with both very few shared haplotypes between populations and a small number of haplotypes within populations. The areas that still contain connected networks are most likely to represent the areas in which populations previously persisted, whereas the isolated populations are more derived populations and more likely to be lost.

It is likely that *Engaewa* are best able to disperse during periods of low sea-level and/or wetter climates, which suggests that the current period that is warm and dry with relatively high sea-level represents a period of contraction. Prior to this, the climate must have been more amenable to *Engaewa*, which would have seen both higher numbers, and connectivity, of populations, though likely with highly geographically structured genetic diversity (i.e. extreme isolation by distance rather than panmixia). The genetic data suggest that the species level lineages within the genus arose approximately concurrent with the formation of the Antarctic ice sheets (around the Eocene/Oligocene boundary), and the climatic fluctuations that have occurred since are responsible for the highly differentiated populations currently seen (Figure 5.13).

Speciation within the genus *Engaewa* generally concurs with the predictions of the TPH. It appears that lineages have repeatedly undergone periods of expansion during which they would have encountered environmental heterogeneity, which acted to create isolated populations and resulted in them speciating. Through this process many unique lineages would have formed in isolation (which links it to the DVM), of which only a small number have persisted. This speciation model suggests there would have been the formation, and subsequent loss or reabsorption, of many peripheral isolate populations (as per the Ephemeral Speciation Model of Rosenblum *et al.* (2012)). Through this process, and considering that *Engaewa* is an ancient group (based on molecular dating and phylogenetic affinities), it is likely that the genus would have, at

times, experienced extreme losses in genetic diversity, with many lineages being removed from the tree. This would have been followed by a period of expansion into newly available habitat, which in turn was followed by the recent trend of extreme and extended simultaneous vicariance, driving diversification and resulting in an increase in the number of lineages within this genus. This process explains why *Engaewa* contains both genetically diverse, as well as genetically depauperate, species.
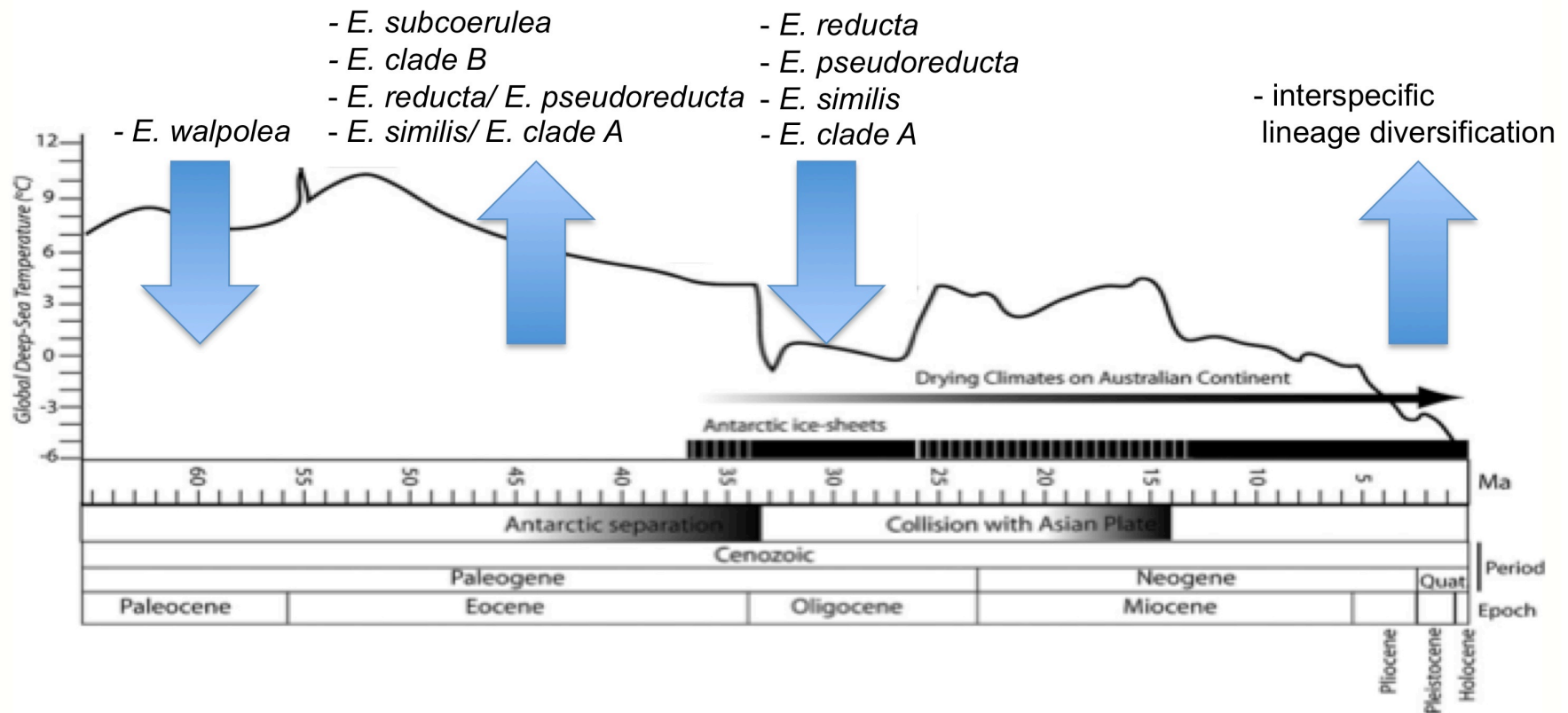
Figure 5.13 Relationship between climate (temperature), sea-level shifts and periods of diversification within the genus *Engaewa*. Hypothesised periods of shifting distributions resulting in lineage diversification within the genus are indicated by arrows. Downward arrows suggest expansion into extended coastal plains resulting in lineage diversification through refugial populations being left behind in upland areas, whilst upward arrows suggesting a general contraction resulting in vicariance and increasing the number of lineages within the genus. (Adapted from Byrne *et al*., 2011 and Hou *et al*., 2008)

6) BIOGEOGRAPHY OF THE COASTAL CORNER OF SWA: PAST AND FUTURE

6.1 Introduction

In this chapter the biogeography of SWA is explored by comparing insights gained from investigating *Engaewa* with patterns derived from other taxa. Seeking congruence between taxa is a basic tenet of biogeographic studies as it can provide evidence of historical events that have shaped regional biota (Ladiges *et al.*, 2011). Comparative approaches can help elucidate the relative roles of environmental change, vicariance, and dispersal in the distribution and diversity of taxa, and where shared discontinuities in population connectivity are detected, barriers can be inferred (Riddle *et al.*, 2008). Whilst correlation does not equate to causation it can be used to propose reasonable hypotheses, which can then be further tested and refined with the addition of more data (Riddle *et al.*, 2008), and if predictions based on biogeographic hypotheses are accurate they can be seen as positive tests of the validity of the hypothesis (Ball, 1978).

Complex histories involving multiple events affecting different taxa over long periods of time mean that congruence would not always be expected (Crisp *et al.*, 1995), and even if congruence is demonstrated confounding factors mean that no single causal explanation can necessarily be assumed (Crisp *et al.*, 1995; Page, 1988). Differing ecological tolerances between taxa mean that a vicariance event for one taxon may not be so for another (Crisp *et al.*, 1995), and whilst genetic breaks within species often correspond with geographical barriers, computer simulations have demonstrated that they can also emerge as stochastic by-products of the spatial coalescent process (Irwin, 2002; Kuo & Avise, 2005). It has been recognised in SWA that there can be speciation at very small geographic scales, with deep lineage splits in areas that are not necessarily concordant across species (Edwards *et al.*, 2008). Thus, it has been noted (e.g. Riddle *et al.*, 2008) that patterns of associations between genetic breaks and geographical barriers should be interpreted with caution and, wherever possible,

verified both by spatial concordance across loci within species (Kuo & Avise, 2005) and across co-distributed species (Avise *et al.*, 1987).

Despite the caveats noted above, an approach of testing for congruence, both spatially and temporally, across co-distributed taxa is one of the foundations of biogeography (Avise, 2000; Lessios, 1998; Wen, 1999). Further to this, there is both good theoretical and empirical evidence that congruent area patterns do actually exist (see examples in Crisp, West & Linder, 1999) and, as was noted by Crisp *et al.* (1999, p. 332), "general patterns and general explanations will never be found if they are not sought". With this in mind, the next section will discuss whether the refugia identified in the previous chapter for *Engaewa* have been so for other taxa too.

6.2 Refugia within SWA

One approach to analysing the biogeography of the coastal corner of SWA is to contrast the distribution of taxa with the scheme produced for the Interim Biogeographic Regionalisation for Australia (IBRA) (Thackway & Cresswell, 1995), as it would be expected that a biogeographic scheme such as this should be meaningful across a wide-range of taxa. The boundaries of the IBRA Warren Bioregion concur closely with the distribution of *Engaewa*; with the exception of an extension of the distribution of these crayfish into the adjacent Jarrah Forest Bioregion on the Blackwood Plateau and the northern portion of the Leeuwin-Naturaliste Ridge, where the correlation between the northern limit of the distribution of *Engaewa* and the boundary of the Swan Coastal Bioregion is particularly striking (Figure 6.1). The combined distributions of *Cherax crassimanus* and *C. glaber* (Austin, 1986), and the combined distribution of the *Geocrinia* species found in SWA (Driscoll, 1998b), each also concur quite closely with the boundaries of the Warren Bioregion (these observations were also noted by Judd (2004)).
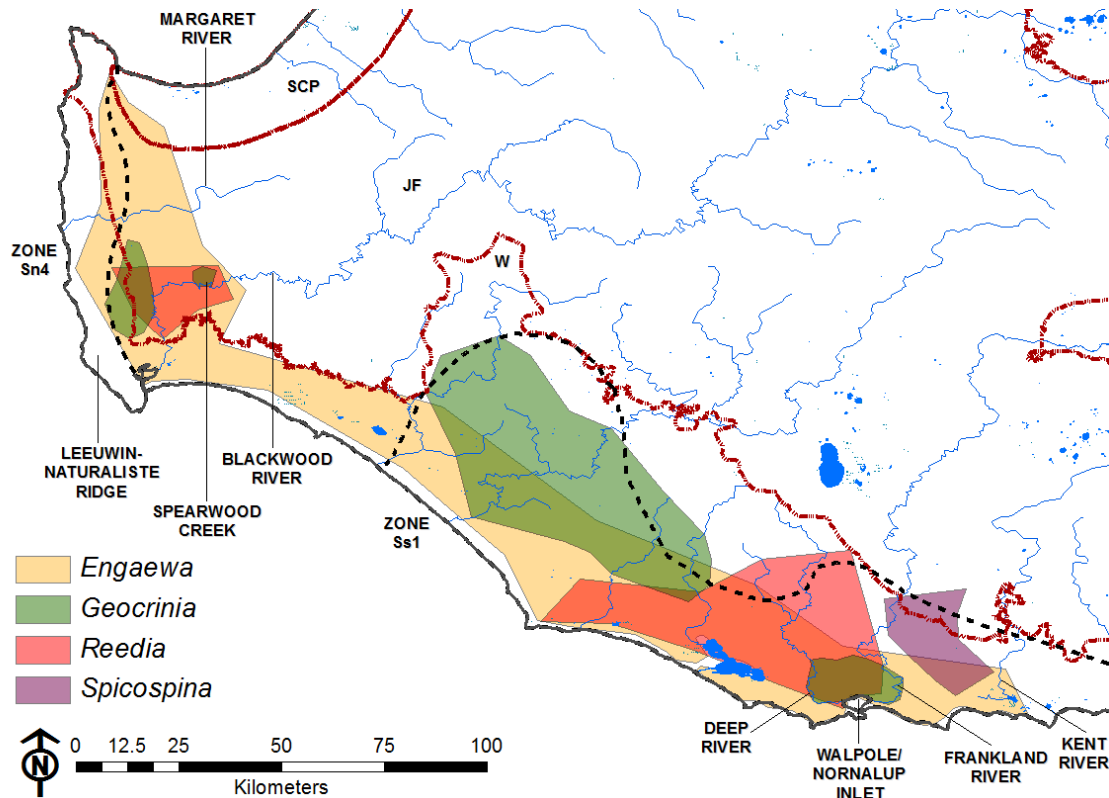
Figure 6.1 Boundaries of Judd's (2004) zones Ss1 and Sn4 are demarcated by dashed black lines. Boundaries of the Swan Coastal Plain, Jarrah Forest, and Warren IBRA bioregions are shown in red and labelled as SCP, JF, and W, respectively. For sources of GIS data see Section 2.5. Sources of distributions are as follows – *Geocrinia* (Driscoll & Roberts, 2008), *Reedia* (Tauss, pers. comm.), *Spicospina* (Edwards & Roberts, 2011).

As the IBRA regions in Western Australia largely follow Beard's (1980) phytogeographical regionalisation of the state (which was based on data from the Vegetation Survey of Western Australia) the Warren Bioregion is largely delineated by the occurrence of Karri forest (*Eucalyptus diversicolor* F.Muell.), although less so on the Scott Coastal Plain and within the Cape-to-Cape region. However, the above examples highlight that, whilst a tall forest tree is generally considered to define the Warren Bioregion, it could have equally been based on freshwater and/or moisture dependent taxa with minimal changes to the current boundary.

The IBRA provides a useful framework; however, it has been noted that significant diversity and heterogeneity occurs within the Warren Bioregion (e.g. Trayler *et al.*, 1996; Wardell-Johnson & Horwitz, 1996), which is overlooked by such broad regionalisation schemes. For example, Judd (2004) outlined the biogeography of SWA based on the diversity and distribution of terrestrial isopods and recognised the region

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along the south coast as a distinct zone in his scheme (Zone Ss1, which was largely defined by what is known as the Nicholls Line), and with only two exceptions found little to connect this area to the western part of the Warren Bioregion (Zone Sn4, essentially the Leeuwin-Naturaliste Ridge) (Figure 6.1). Judd's (2004) findings suggest that within the bioregions of the coastal corner of SWA there are nodes of high diversity and endemicity (which can indicate the presence of refugia in the landscape (Fjeldsa & Lovett, 1997; Lawes, Eeley, Findlay & Forbes, 2007; Médail & Diadema, 2009)) that may be overlooked by broad-scale schemes such as the IBRA; this study aims to further identify such nodes.

The refugia identified for *Engaewa* in the previous chapter formed from contractions within the species' range, and can therefore be considered as internal (or *in situ*) refugia (Keppel *et al.*, 2012; Shoo *et al.*, 2013). These refugia contrast with external (or *ex situ*) refugia that result from large-scale geographic shifts in response to unfavourable conditions (Keppel *et al.*, 2012; Shoo *et al.*, 2013), such as the continental-scale migrations that have occurred in the Northern Hemisphere. Such wide-scale shifts appear not to have occurred in Australia specifically, nor the Southern Hemisphere generally (Huntley, 1993; Huntley & Webb III, 1989; Markgraf *et al.*, 1995), rather species have generally persisted by contracting into isolated refugia during unfavourable periods, before expanding out again when conditions change.

The contraction into internal refugia is evident within the genetic structure of *Engaewa*. *Engaewa* species are characterised by low intra-population diversity, suggesting each population has at some stage been greatly reduced in number and isolated from all other populations. A reduction in the population size would be expected when taxa contract into internal refugia, correlating with a reduction of habitat size. The reduction in range size that would occur as species contract into internal refugia provides an obvious link between external and internal refugia and macro- and micro- refugia, respectively. The concept of macrorefugia can be seen in the glacial refugial hypothesis, which is widely accepted as an explanation of persistence through time via wide-scale shifts in the distribution of populations (in the Northern Hemisphere at least). There is an obvious link between macrorefugia and external refugia, as rather

than being greatly reduced in number whole populations migrate. In comparison, the microrefugial hypothesis suggests taxa often persist largely *in situ*, in small isolated pockets (and therefore they will often be internal refugia) (Rull, 2009). It will be argued that it is such microrefugia (and primarily internal microrefugia) that are highly significant for much of the biota of the coastal regions of SWA. The general lack of shared haplotypes between populations of *Engaewa* and the presence of highly restricted species highlights how long these isolated populations have been disconnected from each other (substantial periods of time would be required for unique haplotypes have formed whilst these crayfish are in allopatry), and that these refugia must allow for the long term persistence of at least a small number of populations through cycles of climatic change.

Hampe and Jump (2011, p. 317) proposed two nonexclusive environmental scenarios that would result in the 'long-term persistence of populations approximately *in situ*': (a) low climatic variation, or (b) the buffering of climatic variation by a heterogeneous landscape with patchy habitats and steep microclimatic gradients. In situations where these two scenarios are combined it is likely that there will be a large concentration of both climate relicts and endemics (Denk, Frotzler & Davitashvili, 2001; Fjeldsa & Lovett, 1997; Qian & Ricklefs, 2000; Rodríguez-Sánchez *et al.*, 2008). Areas matching this scenario have been hypothesised to be significant in the generation of biodiversity and can be considered to represent conservation hotspots (Fjeldsa & Lovett, 1997; Hampe & Petit, 2005; Jansson & Davies, 2008). South-western Australia can be seen as providing the type of buffering described above, both on a broad scale (i.e. it represents the remnants of a more mesic environment now surrounded by desert) and at much finer scale (i.e. microrefugia within SWA).

Internal microrefugia can form when the distributions of species retract in response to a changing climate, resulting in a small number of populations persisting in isolated enclaves that provide suitable environmental conditions within an inhospitable regional climate. These populations will become 'climate relicts' (Hampe & Jump, 2011). The climate of these isolated enclaves must be largely decoupled from the wider regional climate for climate relicts to persist within them (Keppel *et al.*, 2012), which

may occur through the influence of topography (Ackerly *et al.*, 2010; Dobrowski, 2011; Weiss, Murphy & White, 1988), smaller-scale terrain effects (Pepin & Lundquist, 2008), edaphic particularities (Spitzer & Danks, 2006), or vegetation structure and physiognomy (Suggitt *et al.*, 2011), which can all act to buffer climatic variability (Hampe & Jump, 2011).

Currently *Engaewa* is experiencing a period of contraction into refugia in response to a generally unfavourable climate, which means it was previously more widespread. This highlights an important characteristic of 'climate relicts', namely that they are, to a degree, arbitrary, as ranges contract and expand over time and whether their distribution is considered reduced due to climate depends on the reference point against which it is compared (Rodríguez-Sánchez & Arroyo, 2008). Current conditions suggest that the climate will likely continue to dry in SWA (Pittock, 2009), reducing moist refugia (Moir *et al.*, 2009). Further drying of the climate in SWA will likely lead to *Engaewa*, and other taxa adapted to mesic habitat, being displaced by dry-adapted species (Moir *et al.*, 2009). To assess the adaptive potential of *Engaewa* species under a scenario of ongoing future climate change, the types of data collected in this study (e.g. genetic diversity, and habitat preferences and suitability) need to be collated and analysed (Behrman & Kirkpatrick, 2011; Eckert, Samis & Lougheed, 2008; Sexton, McIntyre, Angert & Rice, 2009; Sgrò, Lowe & Hoffmann, 2011). Not only do the intrinsic characteristics of the relevant taxa need to be considered, but also other interacting factors, such as changing land use (Bomhard *et al.*, 2005) and climate change related impacts on fitness and species performance (Clusella-Trullas, Blackburn & Chown, 2011), need be considered.

Whilst this discussion of refugia is based specifically upon those related to climate (incorporating sea-level), refugia from other factors, such as disturbance, may exist. It has been noted that habitats that protect taxa from disturbance may be considered as refuges initially, yet if they repeatedly act as such over evolutionary timescales they may become refugia (Keppel *et al.*, 2012). A particularly significant example of this, and one that has been much discussed in the context of SWA, is refugia from fire (for example see reviews in Abbott & Burrows, 2003a). Refugia from fire are

only indirectly considered in this study, however, it should be noted that changes in fire regime are likely to be linked to shifts in climate, and that climate refugia for taxa that are adapted to mesic habitats will also often be so from fire (as noted in Abbott & Burrows, 2003b).

Refugia for *Engaewa* have been identified in the previous chapter on the basis of the presence of restricted lineages and areas of high haplotype connectivity. These refugia are focused on the eastern margin of the Leeuwin-Naturaliste Ridge and on the adjoining Blackwood Plateau (specifically around both the Margaret River and Spearwood Creek) within the Cape-to-Cape region, and on the south coast, particularly around Walpole. These areas possess geomorphological features that have allowed them to act as refugia for mesic adapted taxa, including (any or all of) being under the influence of rain-bearing westerly winds, providing a variety of habitats, elevated areas that provide sanctuary from high sea-level, a southerly aspect, and local hydrogeological factors such as being aquifer fed or the presence of water-holding soils. Not surprisingly the features that have allowed these areas to act as refugia for *Engaewa* appear to have allowed them to act as such for numerous other taxa too, as these areas contain high species richness and/or the presence of rare taxa, and have been referred to as refugia for relictual taxa (e.g. Main, 1996b).

Both Main (1996b) and Main (1981a) considered parts of the Cape-to-Cape region to represent refugial areas, based on patterns within terrestrial invertebrates. Freshwater snails are one group that provide evidence of the importance of these habitats, as the monotypic *Austroassiminea letha* Solem, Girardi, Slack-Smith and Kendall occurs only in seepage and splash zones of freshwater streams at five localities near the coast in this region (Solem *et al.*, 1982), whilst most populations of *Westrapyrgus westralis* Ponder, Clark, and Miller are also found in the freshwater coastal springs of the Cape-to-Cape region (with additional disjunct populations at Windy Harbour and Broke Inlet) (Ponder, Clark & Miller, 1999). Within this region the Margaret River catchment appears to represent a significant biogeographic feature and is the only location of the freshwater crayfish *Cherax tenuimanus* (Austin & Ryan, 2002).

Spearwood Creek has been recognised as containing a significant ecological community (Department of Environment and Conservation, 2012) and Koenders and Horwitz (2010) highlighted the presence of a unique invertebrate assemblage, noting both elevated endemism and taxa richness. The significance of Spearwood Creek (and the surrounding drainages) as a refuge is highlighted by the presence of the restricted and relictual frog *Geocrinia vitellina* Wardell-Johnson and Roberts and sedge *Reedia spathacea* (Mueller). *Geocrinia* and *Reedia*, like *Engaewa*, require the maintenance of moisture (and little disturbance) to persist and these three taxa share many similarities in their distribution (Figure 6.1) (as shall be discussed in detail below).

Main (1996b) also highlighted the Walpole/Nornalup topographically diverse region (where there is high rainfall on geologically old terrain) and rivers such as the Deep River (which are wet due to a combination of old erosional phenomena and orientation) as being significant in terms of identifying refugia along the south coast of SWA. The region around Walpole has been recognised as a centre of endemism for numerous groups including millipedes (Moir *et al.*, 2009), aquatic invertebrates (Horwitz, 1997) and frogs (particularly Myobatrachidae Schlegel, 1850) (Slatyer, Rosauer & Lemckert, 2007).

The distribution of the monotypic and relictual frog *Spicospina flammocaerulea* Roberts, Horwitz, Wardell-Johnson, Maxson and Mahony encompasses the region between Frankland and Kent Rivers (Edwards & Roberts, 2011), which coincides with the distribution of *E. clade B*, though extending further inland up the catchments (Figure 6.1). Furthermore, the Kent River, which represents the eastern most extension of *Engaewa* uncovered during sampling for this project (Figure 6.1), also corresponds with the boundary proposed by Morrissy (1978) for the crayfish *Cherax cainii*. Morrissy (1978) suggested that beyond (east of) this boundary there is a lack of suitable deep pools and summer flow, resulting from lower average rainfall, and that the distribution has been extended over the last century due to translocations associated with aquaculture.

For terrestrial isopods, Judd (2004) reported high local diversity in the localities of Deep River and Mount Frankland. Judd (2004) highlighted that his Zone Ss1 has particularly high isopod richness, including all families examined, and noted its significant Gondwanan heritage (i.e. mygalomorph spiders from Walpole/Nornalup). Judd's Zone Ss1 encompasses the entire distribution of *Geocrinia rosea* and, to a large extent, the relictual Gondwanan monotypic salamanderfish *Lepidogalaxias salamandroides* Mees (Christensen, 1982). Eight of the nine native species of fish in the Karri forest streams (equivalent to Judd's Zone Ss1) are also endemic to the region (Christensen, 1982). This recognition of the unique aspects of the far south coast, particularly the region around Walpole, suggests that conditions in this region have allowed freshwater and moisture dependent species to persist and/or speciate in these particular drainages. This conclusion is supported by this study as evidenced by the presence of the more aquatic, and highly restricted, species *E. walpolea*.

The same areas discussed above are also significant for groups within the flora of SWA. As SWA has lost its rainforest, where Gondwanan plants do persist in the HRZ they are generally associated with wetlands and damplands (Hopper, Keighery & Wardell-Johnson, 1992). For example, orchids are a predominantly mesic family (Cribb, Kell, Dixon & Barrett, 2003) with particularly high richness within the HRZ (Phillips, Brown, Dixon & Hopper, 2011). This high richness is believed to result from their presence in areas with relatively high rainfall and a diversity of edaphic environments; particularly forests (on varying soils), swamps and coastal dunes (Phillips *et al.*, 2011). Rare orchid taxa (in terms of low abundance and/or restricted distributions) have nodes of particularly high richness reflecting those previously identified, such as the Leeuwin-Naturaliste Ridge, and the south coast between Walpole and Albany (Phillips *et al.*, 2011). These same areas are also recognised as being nodes of high endemism within the vascular plant flora generally (Lyons, Keighery, Gibson & Wardell-Johnson, 2000), and in *Eucalyptus* specifically (reviewed in Wardell-Johnson & Horwitz, 1996).

Hopper (2009) developed the concept of OCBILs (old, climatically buffered, infertile landscapes) as an explanation for the high diversity in the Southwest Australian Floristic Region (along with the Greater Cape in Southern Africa), which in his words: "continue to confound attempts to understand the origins of species richness and are usually ignored, overlooked or regarded as minor exceptions by global modellers" (Hopper, 2009 p. 50) (see also Fiedler (2009), Mucina and Wardell-Johnson (2011), and Standish and Hobbs (2010), for further discussion on, and refinement of, this concept). Whilst OCBILs are of obvious significance when studying the biodiversity of SWA, they are predominantly found inland of the Meckering Line (Mulcahy, 1967), which distinguishes the drainage divide between active westward-flowing river systems and inland uncoordinated drainage (Hopper, 2009) (Figure 6.2).
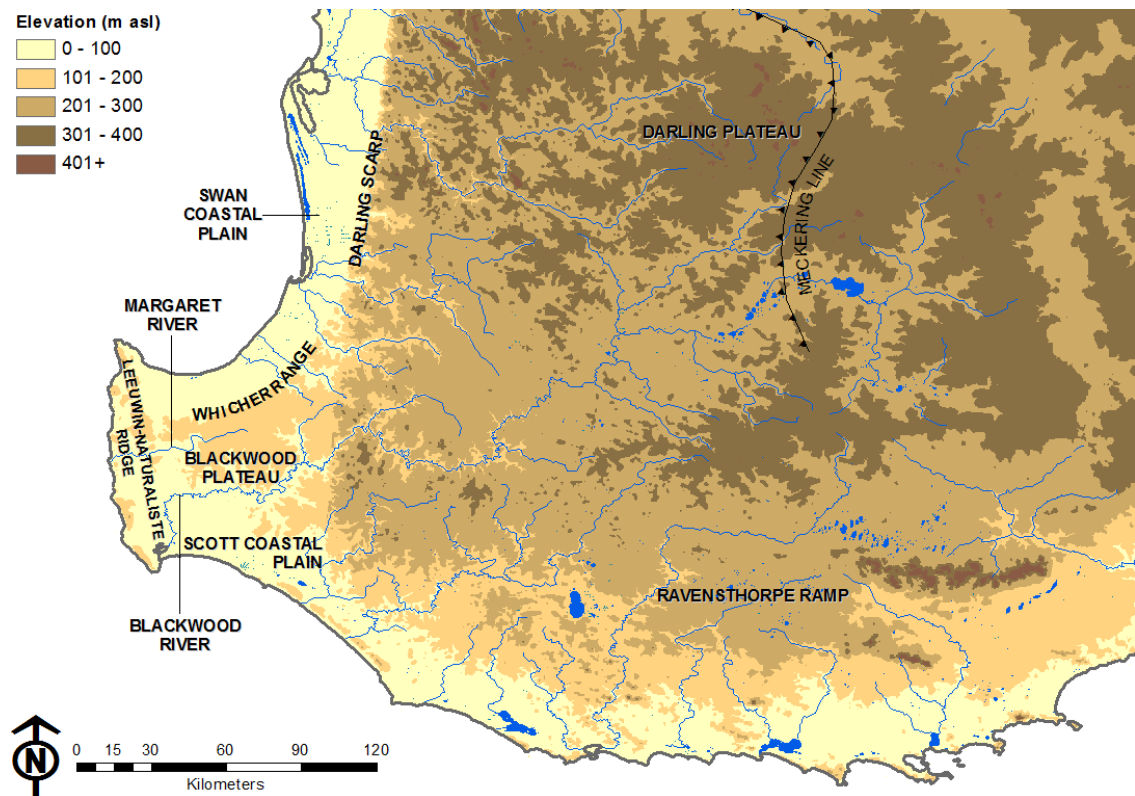
Figure 6.2 Major geologic features and elevation of south-western Australia. For sources of GIS data see Section 2.5.

Where OCBILs occur closer to the coast they are found in the lateritic hills and sandplains of the Darling Plateau, as well as granite outcrops; whereas areas influenced by significant hydrology (i.e. habitats where *Engaewa* are likely to be found), or more recent marine inundations, are considered rejuvenated and, therefore, not OCBILs

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(rather they are YODFELs (young, often disturbed, fertile landscapes)) (Hopper, 2009). Whilst OCBILs were linked by Keppel *et al*. (2012) to the concept of climatic refugia, this study has identified refugia in habitats that are not characteristic of OCBILs in SWA. The increasing awareness of high diversity within particular groups (with ancient affinities) in the HRZ suggests that OCBILs and refugia cannot be linked to the exclusion of areas of refugial nature within YODFELs, and that OCBILs cannot explain the distribution and diversity of many mesic adapted taxa found in SWA.

Whilst the areas discussed above as being refugia for numerous taxa are congruent with refugia described for *Engaewa* in this study, concordance of distributions as a defining characteristic of refugia for similarly adapted taxa is too simplistic, and there may be other reasons why restricted taxa occur together. Whilst refugia have been characterised in this study primarily on the basis of abiotic factors, the biological and community aspects of these refugial areas also need to be considered. Thus, for ancient components of the biota of the coastal regions of SWA that are similarly adapted to mesic environments, similarities between their distributions and the habitat they are adapted to provide good reason to assume that the association between them has a long history. With their shared history in mind, it is possible (and perhaps even likely) that the association between these taxa goes beyond simply requiring similar habitat, and that one may actually create habitat for the other, or that they may both create habitat for each other.

Another example of such an association (and as outlined in Chapter 4) is found between species from the two freshwater crayfish genera found in SWA. *Engaewa* species have been found co-occurring with four of the six Western Australian *Cherax* species (the two species of marron (*C. cainii* and *C. tenuimanus*) are both only found in relatively large bodies of water that would not be inhabited by *Engaewa* spp.), however, the closest and most common association is with *C. crassimanus* and *C. glaber* (Horwitz & Adams, 2000; Riek, 1967, 1969). As noted when discussing the boundaries of the Warren Bioregion at the start of this section, the distributions of these two *Cherax* species, and the distribution of *Engaewa* species, are highly concordant. All of these crayfish species burrow to varying degrees, and it is evident from field observations that

they will utilise each other's burrows as smaller, or more weakly burrowing, crayfish are found occupying the burrows of other crayfish, often by creating small cavities off the main burrow. Such behaviour has been noted in other freshwater crayfish species from the eastern states of Australia (Johnston and Robson, 2009).

As a further example, regions within the Blackwood Plateau (i.e. the Spearwood Creek area) and the area around Walpole have been recognised as representing the two foci of the distribution of *Reedia* (Department of the Environment, Water, Heritage and the Arts, 2008), due to hydrological and geological, and topographical and stratigraphical factors, respectively (Department of Environment, 2014). The conspicuous presence, and unique ecological aspects, of *Reedia* have seen the communities in which they occur described as '*Reedia* communities' (which due to conservation concern are a proposed threatened ecological community (Department of Environment and Conservation, 2012)). Whilst they are described as *Reedia* communities, an association between this plant and the frog *Geocrinia* (e.g. Department of Sustainability, Environment, Water, Population and Communities, 2008), though surprisingly not between it and *Engaewa*, has been noted previously. In fact, *Reedia* is rarely, if ever, found without *Engaewa* (all *Reedia* sites encountered during surveying for this project were found to possess *Engaewa*), although not vice versa.

The significance of the burrowing habit of *Engaewa* on its surrounding environment was described in Chapter 1, where it was stated that this crayfish is likely an ecosystem engineer, and potentially a keystone species. *Reedia* grow in highly anoxic soil conditions and it has been suggested that its stem-borne roots form horizontal branches below the surface that have upwardly-growing rootlets which protrude above the ground and oxygenate the root zone (Department of the Environment, Water, Heritage and the Arts, 2008). Therefore, the burrowing habit of *Engaewa* may be important for *Reedia*, due to the additional aeration of the soil that would occur via the presence of these burrows. Whilst the activities of *Engaewa* modify their environment in a way that is likely to be beneficial to other taxa such as *Reedia*, and undoubtedly create habitat for pholeteros; whether *Engaewa* are reliant upon any other taxa is unclear. Thus, when describing refugia, it needs to be considered that they

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may not just form where abiotic factors allow, but refugia may be, to some degree, created by the taxa that populate the area, and that concordance may be created by taxa that are ecologically-coupled.

Based on similarities in environmental requirements between *Engaewa* and *Reedia* (and the above discussed possibility that they belong to a co-evolved community) it could be expected that refugia for one would be refugia for the other, and their distributions should be entirely concordant. Whist they are highly correlated, they are not exactly the same; hence an explanation of the discrepancies is required. Such an explanation can be derived from considering the 'resilience' of these two taxa. In order for internal refugia to allow a taxon to avoid extinction through cycles of climatic change, the taxon in question must have high resilience. Resilience in this context refers to the ability of a taxon to persist in small, isolated populations of potentially sub-optimal habitat (i.e. within refugia), combined with the ability to rapidly disperse when conditions are favourable (e.g. Markgraf *et al*., 1995). The present distribution of relict taxa can be considered in terms of these characteristics, as resilience will directly influence the number of refugial populations that manage to persist through inhospitable periods and how rapidly they can expand and become more widespread when conditions are favourable. These characteristics will also (in combination with stochastic factors) determine how many refugia are occupied in each subsequent cycle of climate change, driving range fluctuations across cycles. Range changes across cycles will occur as the number of populations that persist through a particular period of contraction, combined with how quickly they can disperse during the following period of expansion, will dictate how many refugia are reached, and consequently occupied, during the next unfavourable period.

The burrowing habit of *Engaewa* provides an advantage in terms of resilience over *Reedia*, as it will allow these crayfish to persist in habitat that is sub-optimal for longer periods of time. Thus, they may occupy a greater number of refugia during any particular climate cycle, allowing them to disperse into more habitats during the next favourable period. This advantage for *Engaewa*, resulting from their burrowing habit, would be further compounded by their decreased sensitivity to fire, as a single fire

could potentially remove *Reedia* from a refuge in which it may otherwise have persisted (Department of the Environment, Water, Heritage and the Arts, 2008; Department of Sustainability, Environment, Water, Population and Communities, 2008), whereas it is unlikely to do so for *Engaewa*. Furthermore, as well as having an advantage over *Reedia* in terms of persistence, *Engaewa* also have an advantage in terms of dispersion. Although *Engaewa* are considered to have relatively poor dispersal abilities, during favourable periods they would be able to disperse more rapidly than *Reedia*, which relies primarily on clonal reproduction (Department of the Environment, Water, Heritage and the Arts, 2008; Department of Sustainability, Environment, Water, Population and Communities, 2008); again allowing *Engaewa* to occupy a larger range during expansion phases, and more refugia during the following retraction phase.

Understanding the significance of resilience gives us a tool by which the present distribution of relictual taxa can be explained. For example, *Spicospina flammocaerulea* (the sunset frog) has been recognised as a relict within SWA and has a highly restricted distribution (EOO ~300 km^2, with an AOO probably <20 km^2) (Edwards & Roberts, 2011), which is largely dictated by rainfall (Swan, 2007). This species has been able to persist in only very few sites where microhabitats, in the form of permanently wet peat swamps, allow (Roberts *et al*., 1997). Furthermore, it has maintained an ancestral summer breeding pattern (Edwards & Roberts, 2011), which may further reduce suitable habitat as it must possess sufficient surface waters during the dry summer months to allow for breeding. These characteristics constrain the resilience of this taxon as their persistence would be relatively low, which can be seen in its genetic structure as all populations are connected in haplotype networks (based on the ND2 marker) by a maximum of three mutational steps from the ancestral haplotype (Edwards & Roberts, 2011). However, these frogs do have the ability to reproduce explosively (following disturbance such as fire), and genetic data suggests females will move up to 10 km (Edwards & Roberts, 2011), which may contribute to improving dispersal during favourable conditions and increase their resilience.

In comparison to the highly restricted sunset frog, *Geocrinia* spp., which are another direct-developing relictual frog lineage in SWA, are able to utilise a wider variety of habitats (Wardell-Johnson & Roberts, 1993). Thus, it is reasonable to assume that the ability to occupy additional habitat types affords *Geocrinia* higher persistence than *Spicospina*. However, the relative strength of dispersal ability of individuals within these two species shows the opposite pattern, as sunset frogs will disperse up to 10 km (Edwards & Roberts, 2011), whereas *Geocrinia* rarely disperse more than 20 m (Driscoll, 1997). Therefore, the characteristics of *Geocrinia* that have resulted in higher persistence than *Spicospina* will have allowed populations of this frog to persist in a relatively greater number of refugia, however, the limited dispersal ability of *Geocrinia* spp. means that they will not be able to expand rapidly out of refugia during favourable periods. High persistence but low dispersion will likely result in long-lasting disjunct distributions of populations, and taxa with genetic relationships that are highly geographically structured – as is seen in *Geocrinia* species (Driscoll, 1998b). Populations in this situation are likely to speciate due to classic allopatric processes, so that independent refugia will promote the formation and persistence of multiple species, which is evident in *Geocrinia*, but not the monotypic genus *Spicospina*.

The concept of resilience can also be linked to the biogeographic models considered in the previous chapter, which will help further explain how the extant biota in SWA has formed. The overarching model that was proposed as the predominant explanation of the biogeography of the genus *Engaewa* was based largely on the Taxon Pulse Hypothesis (TPH). Based on the TPH, uneven splitting of lineages is expected as a result of the vicariance events that occur between the main species' range and peripheral isolate populations, which will form species pairs comprised of a more widespread species and a comparatively restricted species. This process of lineage splitting is not only evident from the *Engaewa* phylogeny presented in Chapter 3, but can also be seen in the distribution of species within the genera *Cherax* and *Geocrinia*.

The genus *Cherax* in SWA contains six species, composed of two phylogenetic species pairs: the smooth and hairy marron (*C. cainii* and *C. tenuimanus*, respectively), and the koonac and glossy koonac (*C. preissii* and *C. glaber*, respectively), whereas the gilgie and restricted gilgie (*C. quinquecarinatus* and *C. crassimanus*, respectively) are a pair by common name only as they are not supported phylogenetic sister species (Munasinghe, Burridge & Austin, 2004a). Each of these species pairs is composed of a widespread and relatively more restricted species. Species of the genus *Geocrinia* in SWA comprise two species pairs: (1) the white-bellied and orange-bellied frog – *G. alba* Wardell-Johnson and Roberts and *G. vitellina*, respectively, and (2) the roseate and Walpole frog – *G. rosea* and *G. lutea* Main, respectively (Driscoll & Roberts, 2008). Again, each of these species pairs is composed of a widespread and relatively more restricted species. With the exception of the marron species, which are likely to have experienced extensive human mediated translocation (and therefore are of lesser value when considering biogeographic patterns), the relative distribution of these species pairs can be considered in terms of their resilience.

The distribution of species in both *Cherax* and *Geocrinia* is restricted by access to sufficient moisture; however, the dispersal/dispersion ability of *Cherax* species will generally exceed that of *Geocrinia* species, meaning that we would expect that the distribution of *Cherax* species to be more extensive than that of the *Geocrinia* species. This expected pattern is seen within these taxa in SWA, except for the aforementioned caveat that the translocation of marron species likely has obscured the original distribution of these taxa, so that there is now one species (*C. tenuimanus*) that is restricted to the Margaret River whilst the other member of this pair is widespread throughout the south-west corner of Western Australia (Austin & Ryan, 2002).

For the *Cherax* species, the distribution of two of the restricted species (*C. crassimanus* and *C. glaber*) coincides tightly with that of *Engaewa*, whilst the third restricted species (*C. tenuimanus*) sits within the distribution of *Engaewa*. Not only is the distribution of this species within the range of *Engaewa*, but it also appears to represent a significant biogeographic boundary and is closely associated with one of the refugia identified for *Engaewa* (the creeks in which *E. pseudoreducta* is found drain

into the Margaret River). Whilst there is concordance between the distribution of *Cherax* and *Engaewa* in SWA, the distributions of all six species of *Cherax* overlap (Morgan *et al.*, 2011). These wider, overlapping distributions can be attributed to greater dispersal ability of these crayfish (as they will utilise both open water and walk overland when conditions allow, plus they will (to varying degrees) burrow to avoid temporarily unfavourable conditions.

The two species pairs found within the genus *Geocrinia* in SWA show even greater concordance with the distribution of *Engaewa* species than do the *Cherax* species, as they are separated into a northern species pair (*G. alba* and *G. vitellina*) and a southern species pair (*G. rosea* and *G. lutea*) and share refugia with *Engaewa*. The more restricted species in the northern part of the distribution of *Geocrinia* in SWA (*G. vitellina*) is located only in and around Spearwood Creek (Driscoll, 1998b; Driscoll & Roberts, 2008), where *Engaewa clade A* is likewise restricted. The more restricted species in the southern part of the distribution of *Geocrinia* in SWA (*G. lutea*) is located only in and around Walpole (Driscoll, 1998b; Driscoll & Roberts, 2008), where the highly restricted species *Engaewa walpolea* is found. Thus, the distribution pattern seen within these *Geocrinia* species further supports the recognition of the refugial areas that were identified by analysing *Engaewa* species, and suggests that the TPH may represent a generalised model of speciation for taxa that are adapted to mesic habitat in SWA.

It is clear from this discussion that, at least in the study region and likely throughout much of the Southern Hemisphere generally (based on the prevalence of internal refugia), knowledge of the biological and ecological characteristics of taxa may allow us to: (1) describe the characteristics of areas within the landscape that are likely to act as refugia (and with knowledge of the landscape locate the specific areas themselves), (2) explain differences in distributions of various taxa and predict possible components of ecological communities, (3) anticipate genetic structure of poorly known groups with a reasonable degree of certainty, and (4) combine these data to 'describe' a taxon (or an ecological community) including both its past and a prediction of its future.

This study has helped refine the characteristics of areas that allow them to function as refugia for climate relicts, and the processes that occur as taxa retract into, and expand out of, such refugia. These processes are, in large part, responsible for the high species richness and endemicity seen within certain taxa in the coastal region of the south-west corner of Western Australia and help explain how, in an isolated, topographically muted, and tectonically stable region with generally low productivity, a hotspot of biodiversity can form. Particular nodes of diversity have been recognised for *Engaewa* in the Cape-to-Cape region and the Walpole/Nornalup region, both of which have been found to be concordant with diversity in numerous other taxa. These areas contain not only high diversity generally but also a high proportion of restricted and relictual endemics, and represent regions in which microhabitats exist that act as refugia for taxa adapted to mesic habitats in SWA. Not only has concordance been demonstrated but it is also suggested that there will, in fact, be many more taxa for which the significance of these refugial areas has not yet been noted (as the taxa themselves have not been sufficiently studied) but will further reinforce the pattern observed. It is also proposed that with further surveying and characterisation of these habitats many of these taxa (particularly invertebrates such as burrow pholeteros) will likely be identified as components of a specific relictual community restricted to the coastal corner of SWA.

6.3 Conclusions

The unusual burrowing habit of *Engaewa* is both a blessing and curse for researchers seeking to use it as a model organism, not to mention for the crayfish themselves (as it both improves resilience, by increasing the likelihood of persistence, yet reduces it, by decreasing dispersal ability). The questions that can be addressed by looking at this animal are nearly endless, yet one considerable difficulty is that any form of sampling is intensive and destructive. This suggests that large-scale or repeated sampling likely cannot occur. However, now that the systematics of this genus have been reviewed, and proposed biogeographic models have been explored, there is a foundation for undertaking more targeted studies, including exploring the influence of various biological and ecological determinants of distribution, as well as the

significance of this crayfish to community function. Such studies would significantly advance the endeavour of conservation of these species, as well as further refine the biogeographical aspects dealt with in this thesis. Despite the difficulties associated with sampling, it has become clear throughout this thesis that the choice of *Engaewa* as a model organism has provided a number of benefits when attempting to unravel the biogeography of the coastal corner of SWA.

Significant findings of this study include the recognition of a genetic structure that is largely unique, and which provides many insights into the historical processes that have occurred within this biodiversity hotspot. Based on the dates placed on the various nodes within *Engaewa* these species appear to be an ancient component of the biota of SWA. The unique genetic diversity contained within virtually every population suggests that there is no possibility of metapopulation processes occurring for this species in the foreseeable future, and the predicted continuing drying trend in the region suggests this will be further exacerbated. This unusual genetic structure is of particular significance for a number of reasons, not least of which is the fact that each unique genetic group within a species represents a repository of genetic diversity, which may hold the potential for the species to adapt under a scenario of changing climate (Hampe & Petit, 2005; Sepulveda-Villet & Stepien, 2012). The fine-scale sampling and associated extensive genetic profiling undertaken in this study together raise the question of whether similar patterns of distribution and diversity may be found in other taxa. Where studies have been undertaken of taxa in this region they have been focussed either upon taxa that are much more widely distributed throughout SWA (e.g. *Eucalyptus* spp. (Wheeler & Byrne, 2006), *Metacrinia* frogs (Edwards *et al*., 2008), and *Cherax* crayfish (Nguyen, Meewan, Ryan & Austin, 2002) or are monotypic and highly restricted (e.g. *Spicospina flammocaerulea* (Edwards & Roberts, 2011)), or less resolved techniques have been used (e.g. allozymes in *Geocrinia* spp. (Driscoll, 1998b; Driscoll & Roberts, 2008)). For many taxa such studies simply have not been undertaken.

The increasing awareness of short-range endemism in SWA has been a significant step forward in our understanding of the long-term dynamics of the biota of this region. This study has highlighted the importance of trying to more narrowly define biogeographic regions, specifically as they relate to invertebrates (due to the often restricted distributions of these taxa). Through the fine-scale sampling undertaken in this study three species have had their range expanded (*E. pseudoreducta*, *E. reducta*, *E. similis*), one wide-spread species (*E. subcoerulea*) has had its range significantly reduced to reflect it being divided into two species, and a second additional unrecognised species was detected from a single population. Observations such as this provide weight to the argument that, in order to better understand a region, there may well be a need to recognise microareas (Giribet & Edgecombe, 2006). Furthermore, it highlights that discovering relictual taxa within the landscape will require surveying at a scale minimally set by the known distribution of species, and will be greatly improved by incorporating recognised associations between taxa (as noted by Roberts *et al*., 1997).

Whilst the concept of climate relicts is widespread within the literature (whether explicitly stated or not) (Rull, 2009), and the concept of refugia as a haven for climate relicts during periods of unfavourable conditions is not new, this study has contributed to recent efforts to update these concepts (as undertaken by authors such as Bennett & Provan, 2008; Keppel *et al.*, 2012; Médail & Diadema, 2009; Rull, 2009). Characteristics used to identify refugia in this study include a number of those considered by Keppel *et al*. (2012) to represent both pattern (e.g. Ecology (via biogeography), Genetics (via phylogeography), and to a lesser extent Palaeobiology (via geology)) and process (e.g. Climatic conditions (via meteorology, geography, and vegetation), Resource Availability (via hydrology, pedology, and vegetation cover), and to a lesser extent Disturbance (via geology)), thus providing a well-rounded and detailed analysis of refugia in SWA. This study has particular significance in the context of identifying and describing refugia in regions generally considered to represent YODFELs.

Not only has this study further clarified and consolidated recognised concordant patterns within components of the biota in SWA (e.g. between *Engaewa*, *Geocrinia* and *Reedia*) but it has (to the best of my knowledge) been the first work to suggest that these taxa do not show concordance simply due their shared habitat preferences, rather they should be considered an ecological community that actually creates habitat. The creation of habitat by these co-evolved taxa may mean that refugia do not exist simply because of abiotic characteristics of the landscape; rather relictual taxa such as these may actually help create the refugia they depend upon. Where exceptions to the anticipated concordance between these taxa occur this study has provided a framework through which such exceptions can be understood. The inherent characteristics of taxa that represent climate relicts determine their resilience (i.e. their ability to persist and disperse), which (in combination with the influence of stochasticity) can be used to explain discrepancies in the distribution of similarly adapted taxa.

It has been proposed that past refugia are likely to act as refugia again in the future (e.g. Leroy & Arpe, 2007) and that species in SWA which survived the Pleistocene aridification might be able to again persist through a period of drying *in situ*, whereas more recently derived taxa may not (Moir, Brennan & Harvey, 2009). However, the combination of habitat fragmentation and climate change may well drive taxa with limited dispersal ability that are adapted to mesic habitat (like *Engaewa*) to extinction (see Travis, 2003) if there is not relatively intact refugia available for them to contract into during times of unsuitable climate. Relatively intact ecosystems (i.e. those that possess a full complement of species) maintain functional redundancy and are more likely to be buffered from the effects of climate change, when compared to degraded systems that are prone to trophic cascades (Walther, 2010). The extreme modification of natural environments that have occurred in SWA make it impossible to know what components of the freshwater biota have already been lost through changed hydrological regimes, increased eutrophication and salinisation, particularly in light of the high proportion of both locally and regionally restricted endemics in the region (Trayler *et al*., 1996). Thus, the link between *Engaewa* and community function and, by extension, the role these crayfish play in protecting relictual taxa within refugia may be vital and need be recognised.

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APPENDICES

Appendix 1 Specimens, collection data and samples used for the various molecular analyses in this study.

SPECIES	CODE	SITE	LATITUDE	LONGITUDE	DATE	SEX	16S	COI	LSU	GAP	mt	all
pseudoreducta	PRD01	Payne Rd	33°45'12.93"S	115°11'45.55"E	08/10/2007	I	✓	✓	✓	✓	✓	✓
	PRD02	Payne Rd	33°45'12.93"S	115°11'45.55"E	09/10/2007	F	✓		✓	✓		
	TT201	Treeton Reserve	33°53'45.03"S	115°11'38.86"E	11/10/2007	M	✓	✓	✓	✓	✓	✓
reducta	ACK01	Adelaide Ck	34° 5'14.15"S	115°19'41.73"E	18/07/2007	M	✓	✓	✓		✓	
	ACK02	Adelaide Ck	34° 5'14.15"S	115°19'41.73"E	18/07/2007	M						
	ACK03	Adelaide Ck	34° 5'14.15"S	115°19'41.73"E	18/07/2007	M	✓	✓	✓		✓	
	BGR01	Wylarah Way	33°39'16.13"S	115° 6'25.49"E	10/10/2007	F	✓	✓	✓	✓	✓	✓
	BL101	Blythe Rd	33°43'25.14"S	115° 6'13.03"E	07/09/2007	F		✓				
	BL102	Blythe Rd	33°43'25.14"S	115° 6'13.03"E	07/09/2007	M		✓				
	BL103	Blythe Rd	33°43'25.14"S	115° 6'13.03"E	07/09/2007	M	✓	✓	✓		✓	
	BL201	Blythe Rd	33°44'8.00"S	115° 6'12.00"E	07/09/2007	M	✓	✓	✓	✓	✓	✓
	BL202	Blythe Rd	33°44'8.00"S	115° 6'12.00"E	07/09/2007	M						
	BL301	Blythe Rd	33°43'49.45"S	115° 6'19.83"E	05/09/2007	M						
	BL302	Blythe Rd	33°43'49.45"S	115° 6'19.83"E	05/09/2007	M	✓	✓	✓		✓	
	BW201	Blackwood Rd	34° 7'2.08"S	115°18'9.71"E	09/07/2006	M	✓	✓	✓		✓	
	BW202	Blackwood Rd	34° 7'2.08"S	115°18'9.71"E	09/07/2006	M						
	BW203	Blackwood Rd	34° 7'2.08"S	115°18'9.71"E	03/10/2006	F						
	BW204	Blackwood Rd	34° 7'2.08"S	115°18'9.71"E	03/10/2006	F						
	BW205	Blackwood Rd	34° 7'2.08"S	115°18'9.71"E	18/07/2007	F		✓				
	BW206	Blackwood Rd	34° 7'2.08"S	115°18'9.71"E	18/07/2007	M						
	BW301	Blackwood Rd	34° 6'41.92"S	115°19'6.13"E	09/07/2006	M		✓				
	BW302	Blackwood Rd	34° 6'41.92"S	115°19'6.13"E	09/07/2006	F						
	BW303	Blackwood Rd	34° 6'41.92"S	115°19'6.13"E	02/10/2006	F		✓				
	BW304	Blackwood Rd	34° 6'41.92"S	115°19'6.13"E	17/07/2007	M		✓				
	BW305	Blackwood Rd	34° 6'41.92"S	115°19'6.13"E	17/07/2007	M	✓		✓			
	BW401	Blackwood Rd	34° 6'14.59"S	115°20'14.20"E	02/10/2006	F						
	BW402	Blackwood Rd	34° 6'14.59"S	115°20'14.20"E	02/10/2006	M	✓		✓			

SPECIES	CODE	SITE	LATITUDE	LONGITUDE	DATE	SEX	16S	COI	LSU	GAP	mt	all
reducta	BW403	Blackwood Rd	34° 6'14.59"S	115°20'14.20"E	17/07/2007	M						
	BW404	Blackwood Rd	34° 6'14.59"S	115°20'14.20"E	17/07/2007	F						
	DNY01	Denny Rd	34° 4'31.00"S	115°14'4.00"E	20/07/2007	M	✓	✓		✓	✓	
	DNY02	Denny Rd	34° 4'31.00"S	115°14'4.00"E	20/07/2007	M			✓			
	DNY03	Denny Rd	34° 4'31.00"S	115°14'4.00"E	20/07/2007	M	✓	✓	✓	✓	✓	✓
	ENS01	Roy Rd	33°45'0.00"S	115°10'14.00"E	08/10/2007	M	✓	✓	✓	✓	✓	✓
	FOR01	Forrest Rise	33°45'0.00"S	115° 8'52.00"E	10/09/2007	M						
	FOR02	Forrest Rise	33°45'0.00"S	115° 8'52.00"E	10/09/2007	M	✓	✓	✓	✓	✓	✓
	HAG01	Haag	33°43'15.51"S	115° 7'59.47"E	05/10/2006							
	HAG02	Haag	33°43'15.51"S	115° 7'59.47"E	05/10/2006	M	✓					
	HAG03	Haag	33°43'15.51"S	115° 7'59.47"E	05/10/2006	M	✓		✓			
	HAG04	Haag	33°43'15.51"S	115° 7'59.47"E	05/10/2006	M	✓	✓	✓	✓	✓	✓
	HAG05	Haag	33°43'15.51"S	115° 7'59.47"E	05/10/2006	F						
	HAG06	Haag	33°43'15.51"S	115° 7'59.47"E	05/10/2006	F						
	HRD01	Denny Rd	34° 4'30.00"S	115°14'42.00"E	20/07/2007	F						
	HRD02	Denny Rd	34° 4'30.00"S	115°14'42.00"E	20/07/2007	I			✓			
	HRD03	Denny Rd	34° 4'30.00"S	115°14'42.00"E	20/07/2007	M						
	MLR01	Miles Rd	34° 5'57.71"S	115°14'30.99"E	06/09/2007	F	✓	✓	✓	✓	✓	✓
	MLR02	Miles Rd	34° 5'57.71"S	115°14'30.99"E	06/09/2007	M						
	PIG01	Denny Rd	34° 4'33.00"S	115°15'43.00"E	20/07/2007	F						
	PIG02	Denny Rd	34° 4'33.00"S	115°15'43.00"E	20/07/2007	F	✓	✓	✓	✓	✓	✓
	PIG03	Denny Rd	34° 4'33.00"S	115°15'43.00"E	20/07/2007	M		✓				
similis	ALL01	Roy Rd	33°44'50.46"S	115°11'17.89"E	08/10/2007	F	✓	✓	✓		✓	
	ALL02	Roy Rd	33°44'50.46"S	115°11'17.89"E	08/10/2007	M	✓	✓	✓	✓	✓	✓
	BP101	Bramley Reserve 1	33°55'41.00"S	115° 7'57.00"E	21/07/2007	F	✓	✓	✓	✓	✓	✓
	BP102	Bramley Reserve 1	33°55'41.00"S	115° 7'57.00"E	21/07/2007	M		✓				
	BP103	Bramley Reserve 1	33°55'41.00"S	115° 7'57.00"E	21/07/2007	F		✓				
	BP201	Bramley Reserve 2	33°55'33.55"S	115° 6'44.68"E	30/09/2007	F	✓	✓	✓	✓	✓	✓
	BP202	Bramley Reserve 2	33°55'33.55"S	115° 6'44.68"E	30/09/2007	M	✓	✓			✓	
	BP203	Bramley Reserve 2	33°55'33.55"S	115° 6'44.68"E	02/10/2007	F	✓	✓			✓	
	BW101	Blackwood Rd	34° 7'17.25"S	115°17'20.76"E	03/10/2006	M	✓	✓	✓	✓	✓	✓

SPECIES	CODE	SITE	LATITUDE	LONGITUDE	DATE	SEX	16S	COI	LSU	GAP	mt	all
similis	BW102	Blackwood Rd	34° 7'17.25"S	115°17'20.76"E	03/10/2006	F		✓				
	BW103	Blackwood Rd	34° 7'17.25"S	115°17'20.76"E	03/10/2006	F	✓	✓			✓	
	CRE01	Yelverton Rd	33°44'6.87"S	115° 8'16.78"E	09/10/2007	F	✓	✓	✓		✓	
	DVR01	Davis Rd	34° 2'7.18"S	115° 8'14.12"E	02/10/2006	M						
	MAM01	Calgardup	34° 3'17.70"S	115° 2'43.05"E	09/03/2007	M	✓	✓	✓	✓	✓	✓
	MAM02	Calgardup	34° 3'17.70"S	115° 2'43.05"E	09/03/2007	F		✓				
	NYS01	Nyundimurra School	34° 4'1.07"S	115° 6'14.72"E	04/09/2007	F	✓	✓	✓	✓	✓	✓
	OSM01	Osmington Rd	33°54'18.29"S	115°10'23.06"E	06/10/2006	M						
	OSM02	Osmington Rd	33°54'18.29"S	115°10'23.06"E	06/10/2006	M	✓	✓	✓	✓	✓	✓
	OSM03	Osmington Rd	33°54'18.29"S	115°10'23.06"E	06/10/2006	F	✓	✓	✓	✓	✓	✓
	SC101	Scott River Rd 1	34°17'21.62"S	115°12'35.94"E	01/10/2007	F	✓	✓	✓	✓	✓	✓
	SC102	Scott River Rd 1	34°17'21.62"S	115°12'35.94"E	01/10/2007	M	✓	✓			✓	
	SC103	Scott River Rd 1	34°17'21.62"S	115°12'35.94"E	01/10/2007	F	✓	✓			✓	
	SC104	Scott River Rd 1	34°17'21.62"S	115°12'35.94"E	01/10/2007	M	✓	✓			✓	
	SC201	Scott River Rd 2	34°17'1.61"S	115°15'20.74"E	01/10/2007	F	✓	✓	✓		✓	
	SCR01	Scott Rd	34°24'35.21"S	115°46'51.50"E	18/04/2007	M						
	SCR02	Scott Rd	34°24'35.21"S	115°46'51.50"E	18/04/2007	I			✓			
	SCR03	Scott Rd	34°24'35.21"S	115°46'51.50"E	18/04/2007	F	✓	✓			✓	
	SCR04	Scott Rd	34°24'35.21"S	115°46'51.50"E	18/04/2007	M	✓	✓	✓		✓	
	WTC01	McLean Rd	34° 1'6.93"S	115° 8'21.27"E	02/10/2007	M	✓	✓	✓		✓	
	WTC02	McLean Rd	34° 1'6.93"S	115° 8'21.27"E	02/10/2007	F	✓	✓			✓	
subcoerulea	BIN01	South Western Hwy	34°52'44.05"S	116°33'5.71"E	24/09/2006	I	✓	✓	✓	✓	✓	✓
	BIN02	South Western Hwy	34°52'44.05"S	116°33'5.71"E	24/09/2006	F						
	CH101	Chesapeake Rd	34°49'13.67"S	116°16'47.30"E	06/10/2007	F	✓					
	CH201	Chesapeake Rd east	34°51'19.85"S	116°24'3.33"E	06/10/2007	M	✓	✓			✓	
	CH202	Chesapeake Rd east	34°51'19.85"S	116°24'3.33"E	06/10/2007	M						
	CH203	Chesapeake Rd east	34°51'19.85"S	116°24'3.33"E	06/10/2007	F						
	CH204	Chesapeake Rd east	34°51'19.85"S	116°24'3.33"E	06/10/2007	F						
	NSA01	South Western Hwy	34°46'5.00"S	116°30'7.00"E	13/07/2007	F						
	NSA02	South Western Hwy	34°46'5.00"S	116°30'7.00"E	13/07/2007	M						
	NSB01	South Western Hwy	34°48'14.00"S	116°31'46.00"E	13/07/2007	F						
	NSB02	South Western Hwy	34°48'14.00"S	116°31'46.00"E	13/07/2007	M						
	NSB03	South Western Hwy	34°48'14.00"S	116°31'46.00"E	13/07/2007	F						
	NSC01	South Western Hwy	34°49'12.00"S	116°32'5.00"E	13/07/2007	M		✓				

SPECIES	CODE	SITE	LATITUDE	LONGITUDE	DATE	SEX	16S	COI	LSU	GAP	mt	all
subcoerulea	NSC02	South Western Hwy	34°49'12.00"S	116°32'5.00"E	13/07/2007	F		✓				
	NSC03	South Western Hwy	34°49'12.00"S	116°32'5.00"E	13/07/2007	F						
	NSD01	South Western Hwy	34°49'22.00"S	116°32'3.00"E	13/07/2007	F						
	NSD02	South Western Hwy	34°49'22.00"S	116°32'3.00"E	13/07/2007	F		✓				
	NSD03	South Western Hwy	34°49'22.00"S	116°32'3.00"E	24/09/2006	M		✓				
	NSE01	South Western Hwy	34°54'59.37"S	116°34'5.55"E	24/09/2006	F		✓				
	NSE02	South Western Hwy	34°54'59.37"S	116°34'5.55"E	24/09/2006	F						
	NSE03	South Western Hwy	34°54'59.37"S	116°34'5.55"E	24/09/2006							
	RWP01	South Western Hwy	34°57'50.10"S	116°36'10.86"E	24/09/2006	F	✓	✓	✓		✓	
	RWP02	South Western Hwy	34°57'50.10"S	116°36'10.86"E	24/09/2006	M						
	TWO01	Two Rd	34°57'50.38"S	116°36'56.66"E	10/07/2007	F		✓				
	TWO02	Two Rd	34°57'50.38"S	116°36'56.66"E	10/07/2007	F	✓	✓	✓		✓	
	TWO03	Two Rd	34°57'50.38"S	116°36'56.66"E	10/07/2007	F						
	WCR01	Wheatley Coast Rd	34°38'29.63"S	116° 7'11.77"E	15/08/2007	F			✓			
	WCR02	Wheatley Coast Rd	34°38'29.63"S	116° 7'11.77"E	15/08/2007	F						
	WCR03	Wheatley Coast Rd	34°38'29.63"S	116° 7'11.77"E	15/08/2007	M	✓		✓			
	WH101	Windy Harbour Rd	34°48'18.54"S	116° 4'29.73"E	16/08/2007	F	✓	✓	✓	✓	✓	✓
	WH201	Windy Harbour Rd 2	34°48'38.64"S	116° 4'19.44"E	14/08/2007	M						
	WH202	Windy Harbour Rd 2	34°48'38.64"S	116° 4'19.44"E	14/08/2007	F						
	WH203	Windy Harbour Rd 2	34°48'38.64"S	116° 4'19.44"E	14/08/2007	I						
	WH301	Windy Harbour Rd 3	34°48'57.72"S	116° 4'7.22"E	16/08/2007	F	✓	✓	✓		✓	
	WH302	Windy Harbour Rd 3	34°48'57.72"S	116° 4'7.22"E	16/08/2007	F						
walpolea	BCK01	Butler Creek	34°58'27.41"S	116°43'48.97"E	27/09/2006	F						
	BCK02	Butler Creek	34°58'27.41"S	116°43'48.97"E	27/09/2006	D						
	BOR01	Boronia Ridge	34°58'37.74"S	116°43'22.44"E	10/07/2007	F	✓		✓			
	BOR02	Boronia Ridge	34°58'37.74"S	116°43'22.44"E	10/07/2007	M						
	BRD02	Beach Rd	35° 1'24.23"S	116°51'52.24"E	10/07/2007	M	✓	✓	✓	✓	✓	✓
	BRD03	Beach Rd	35° 1'24.23"S	116°51'52.24"E	10/07/2007	M	✓	✓	✓		✓	
	BRD04	Beach Rd	35° 1'24.23"S	116°51'52.24"E	10/07/2007	F	✓	✓	✓	✓	✓	✓
	BRD05	Beach Rd	35° 1'24.23"S	116°51'52.24"E	10/07/2007	M	✓		✓			
	CCC01	Cemetery Creek	34°58'34.00"S	116°44'43.00"E	25/09/2006	F	✓					

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walpolea	CCC02	Cemetery Creek	34°58'34.00"S	116°44'43.00"E	25/09/2006	F						
	CCF01	Cemetery Creek	34°58'5.00"S	116°44'50.00"E	25/09/2006	M	✓	✓	✓	✓	✓	✓
	CCF02	Cemetery Creek	34°58'5.00"S	116°44'50.00"E	25/09/2006	M	✓	✓			✓	
	CHG01	Chugg St	34°58'28.09"S	116°44'22.99"E	25/09/2006	F						
	CHG02	Chugg St	34°58'28.09"S	116°44'22.99"E	25/09/2006	M						
	CHG03	Chugg St	34°58'28.09"S	116°44'22.99"E	25/09/2006	F						
	CHG04	Chugg St	34°58'28.09"S	116°44'22.99"E	25/09/2006	M						
	CHG05	Chugg St	34°58'28.09"S	116°44'22.99"E	25/09/2006	M						
	COC01	Collier Creek	34°59'10.00"S	116°44'59.00"E	11/04/2007	M	✓					
	COC02	Collier Creek	34°59'10.00"S	116°44'59.00"E	07/07/2007	F	✓					
	COT01	Collier Ck tributary	34°58'55.00"S	116°44'46.00"E	10/04/2007	M						
	COT02	Collier Ck tributary	34°58'55.00"S	116°44'46.00"E	10/04/2007	M						
	COT03	Collier Ck tributary	34°58'55.00"S	116°44'46.00"E	10/04/2007	M						
	COT04	Collier Ck tributary	34°58'55.00"S	116°44'46.00"E	07/07/2007	M	✓					
	COT05	Collier Ck tributary	34°58'55.00"S	116°44'46.00"E	07/07/2007	I	✓		✓			
	EB301	Ebbett Rd 3	34°58'3.00"S	116°55'24.00"E	26/09/2006	M	✓		✓			
	EB302	Ebbett Rd 3	34°58'3.00"S	116°55'24.00"E	26/09/2006	M	✓	✓	✓		✓	
	EB303	Ebbett Rd 3	34°58'3.00"S	116°55'24.00"E	26/09/2006	M						
	LRT01	Nth Walpole Rd	34°57'57.00"S	116°43'39.00"E	27/09/2006	F	✓	✓	✓	✓	✓	✓
	LRT02	Nth Walpole Rd	34°57'57.00"S	116°43'39.00"E	27/09/2006	F						
	LRT03	Nth Walpole Rd	34°57'57.00"S	116°43'39.00"E	08/07/2007	M						
	TKN01	The Knoll	34°59'31.00"S	116°43'47.00"E	23/09/2006	M			✓			
	TKN02	The Knoll	34°59'31.00"S	116°43'47.00"E	24/09/2006	F	✓					
	TKN03	The Knoll	34°59'31.00"S	116°43'47.00"E	24/09/2006	M	✓		✓			
	WAC01	Plain Rd	34°58'1.63"S	116°42'51.36"E	13/07/2007	M		✓				
	WAC02	Plain Rd	34°58'1.63"S	116°42'51.36"E	13/07/2007	F						
	WAC03	Plain Rd	34°58'1.63"S	116°42'51.36"E	13/07/2007	F	✓	✓	✓	✓	✓	✓
clade A	SWC01	Spearwood Ck	34° 4'12.19"S	115°18'43.30"E	19/08/2006	F	✓	✓	✓	✓	✓	✓
	SWC02	Spearwood Ck	34° 4'12.19"S	115°18'43.30"E	19/08/2006	F	✓	✓			✓	
	SWC03	Spearwood Ck	34° 4'12.19"S	115°18'43.30"E	19/08/2006	F	✓	✓			✓	
	SWC04	Spearwood Ck	34° 4'12.19"S	115°18'43.30"E	19/08/2006	M	✓	✓			✓	
	SWC05	Spearwood Ck	34° 4'12.19"S	115°18'43.30"E	19/08/2006	F	✓	✓	✓	✓	✓	✓
	SWC06	Spearwood Ck	34° 4'12.19"S	115°18'43.30"E	19/08/2006	F		✓				
clade B	BRD01	Beach Rd	35° 1'24.23"S	116°51'52.24"E	07/09/2007	M	✓		✓			

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clade B	BRD06	Beach Rd	35° 1'24.23"S	116°51'52.24"E	11/07/2007	M	✓					
	BRD07	Beach Rd	35° 1'24.23"S	116°51'52.24"E	11/07/2007	M	✓		✓			
	EB101	Ebbett Rd 1	34°58'25.02"S	116°56'36.00"E	23/09/2006	I						
	EB102	Ebbett Rd 1	34°58'25.02"S	116°56'36.00"E	11/07/2007	F	✓	✓	✓		✓	
	EB103	Ebbett Rd 1	34°58'25.02"S	116°56'36.00"E	11/07/2007	M		✓				
	EB201	Ebbett Rd 2	34°58'20.41"S	116°56'26.38"E	23/09/2006	M						
	EB202	Ebbett Rd 2	34°58'20.41"S	116°56'26.38"E	23/09/2006	M	✓		✓			
	FFR01	Ficifolia Rd	35° 1'31.00"S	116°55'3.00"E	11/07/2007	F	✓	✓	✓	✓	✓	✓
	KTR01	Kent River	34°57'34.33"S	117° 2'3.73"E	04/10/2007	M	✓	✓	✓		✓	
	STR01	Station Rd	35° 0'32.53"S	116°51'22.79"E	09/07/2007	M						
	STR02	Station Rd	35° 0'32.53"S	116°51'22.79"E	09/07/2007	F	✓		✓			
	STR03	Station Rd	35° 0'32.53"S	116°51'22.79"E	09/07/2007	F						
	STR04	Station Rd	35° 0'32.53"S	116°51'22.79"E	09/07/2007	D						

Appendix 2 Sequences (Genus, Species, Sample ID and GenBank accession numbers) retrieved from GenBank and included in this study.

Genus	Species	Sample ID	GenBank accession No. 16S	GenBank accession No.GAPDH
Cherax	*albidus*	DWY	AF500610	EU977401
Cherax	*cainii*	BEB28	EF493070	EU977402
Cherax	*crassimanus*	DON219	AF492806	EU977403
Cherax	*crassimanus*	TUB215	AF492805	EU977404
Cherax	*cuspidatus*	BEL2.3	EU977342	EU977405
Cherax	*destructor*	FIN	AF500601	EU977406
Cherax	*dispar*	MAR	AY191772	EU977407
Cherax	*preissii*	CAN (H133)	AF492807	EU977408
Cherax	*quadricarinatus*	GRG1.1	EF493080	EU977409
Cherax	*quinquecarinatus*	REG(H146)	AF492801	EU977410
Cherax	*robustus*	BRB1.4	EU977343	EU977411
Cherax	*robustus*	BRI	AY191759	EU977412
Cherax	*rotundus*	BAR	AF500617	EU977413
Cherax	*setosus*	KAR	AY191769	EU977414
Cherax	*setosus*	UFF	AY191770	EU977415
Cherax	*cf peknyl*	NEW	AY191775	EU977416
Engaeus	*affinis*	CCE1.2	EU977344	n/a
Engaeus	*affinis*	CCE1.3	EU977345	n/a
Engaeus	*affinis*	CCE1.4	EU977346	n/a
Engaeus	*affinis*	Mus Vic J3840; MV1	EU977347	n/a
Engaeus	*australis*	Mus Vic J3909; MV6	EU977348	n/a
Engaeus	*cisternarius*	Mus Vic J45407; MV7	EF493110	n/a
Engaeus	*cisternarius*	Mus Vic J45408; MV9	EU977349	n/a
Engaeus	*cisternarius*	Qld Mus W23980; Q75	EU977350	n/a
Engaeus	*curvisuturus*	Mus Vic J45502; MV43	EU977351	n/a
Engaeus	*cymus*	Aus Mus P53211; A29 (K3?)	EU977352	EU977417
Engaeus	*cymus*	K4	AY223709	EU977418
Engaeus	*disjuncticus*	Mus Vic J45405; MV51	EU977353	n/a
Engaeus	*disjuncticus*	Mus Vic J45405; MV52	EF493102	n/a
Engaeus	*fossor*	Mus Vic J45510; MV53	EF493103	n/a
Engaeus	*fossor*	KC620	AF135979	n/a
Engaeus	*fultoni*	AIR2.1	EF493042	EU977419
Engaeus	*fultoni*	PDS1.1	EU977354	n/a
Engaeus	*fultoni*	ELZ1.1	EU977355	n/a
Engaeus	*fultoni*	Mus Vic J45703; MV56	EU977356	n/a
Engaeus	*fultoni*	STV1.1	EU977357	n/a
Engaeus	*fultoni*	GEL1.1	EU977358	EU977420
Engaeus	*granulatus*	Mus Vic J14702; MV98	EU977359	n/a
Engaeus	*hemicirratulus*	Mus Vic J14750; MV58	EF493104	n/a
Engaeus	*karnanga*	Mus Vic J56537; MV10	EU977360	EU977421
Engaeus	*karnanga*	Mus Vic J45692; MV59	EF493105	n/a
Engaeus	*laevis*	LEL1.1	EF493088	n/a
Engaeus	*laevis*	Mus Vic J39380; MV63	EF493106	n/a
Engaeus	*lengana*	Mus Vic J45515; MV67	EU977361	n/a

Engaeus	*lengana*	Mus Vic J45516; MV224	EU977362	n/a
Engaeus	*lengana*	Mus Vic J45516; MV66	EU977363	n/a
Engaeus	*leptorhynchus*	Mus Vic J39364; MV69	EU977364	n/a
Engaeus	*lyelli*	DUN1.1	EU977365	EU977422
Engaeus	*lyelli*	ENF1.2	EF493073	EU977423
Engaeus	*lyelli*	MOY2.1	EU977366	n/a
Engaeus	*lyelli*	MOY3.1	EU977367	n/a
Engaeus	*lyelli*	MOY5.1	EU977368	n/a
Engaeus	*lyelli*	MOY5.4	EU977369	n/a
Engaeus	*lyelli*	Mus Vic J14710; MV71	EF493107	n/a
Engaeus	*lyelli*	Mus Vic J14711; MV72	EF493108	n/a
Engaeus	*lyelli*	NRN1.1	EU977370	EU977424
Engaeus	*lyelli*	NRN2.1	EF493121	n/a
Engaeus	*lyelli*	RED2.1	EU977371	n/a
Engaeus	*lyelli*	STA1.1	EU977372	n/a
Engaeus	*lyelli*	?	AY223711	n/a
Engaeus	*mairener*	Mus Vic J45680; MV78	EF493109	n/a
Engaeus	*mallacoota*	Mus Vic J14713; MV107	EF493096	n/a
Engaeus	*martigener*	Mus Vic J45432; MV80	EF493111	n/a
Engaeus	*merosetosus*	BAR1.1	EU313347	n/a
Engaeus	*merosetosus*	WIN1.1	EU313417	n/a
Engaeus	*merosetosus*	WPC2.1	EF493153	n/a
Engaeus	*nulloporius*	Mus Vic J4106; MV84	EF493112	n/a
Engaeus	*orientalis*	Mus Vic J14725; MV85	EF493113	n/a
Engaeus	*phyllocercus*	Aus Mus P67188; A27	EF493041	EU977425
Engaeus	*quadrimanus*	MOR1.2	EU977373	n/a
Engaeus	*quadrimanus*	MOR1.3	EU977374	n/a
Engaeus	*quadrimanus*	MOR2.2	EU977375	n/a
Engaeus	*quadrimanus*	WAN1.1	EU977376	n/a
Engaeus	*quadrimanus*	Mus Vic J42306; MV187	EU977377	n/a
Engaeus	*rostrogaleatus*	Mus Vic J14734; MV99	EU977378	n/a
Engaeus	*sericatus*	AIR3.1	EU313346	EU977426
Engaeus	*sericatus*	CUR2.1	EU313368	EU977427
Engaeus	*sericatus*	GenBank	AY223713	n/a
Engaeus	*sericatus*	PAN1.3	EU313402	EU977428
Engaeus	*sericatus*	PEN1.4	EF493125	n/a
Engaeus	*sericatus*	WAL1.1	EU313411	EU977429
Engaeus	*spinicaudatus*	Mus Vic J456981; MV198	EU977379	n/a
Engaeus	*spinicaudatus*	Mus Vic J45696; MV89	EF493114	n/a
Engaeus	*sternalis*	Mus Vic J456980; MV90	EU977380	n/a
Engaeus	*strictifrons*	CRP1.1	EU977381	n/a
Engaeus	*strictifrons*	DTS1.1	EU977382	n/a
Engaeus	*strictifrons*	FIT1.3	EU977383	n/a
Engaeus	*strictifrons*	KRK2.1	EU977384	n/a
Engaeus	*strictifrons*	MCP1.1	EU977385	n/a
Engaeus	*strictifrons*	MYA2.7	EU977386	n/a
Engaeus	*strictifrons*	TWH1.1	EF493149	n/a
Engaeus	*strictifrons*	WOO1.1	EU977387	n/a
Engaeus	*tayatea*	Mus Vic J45690; MV92	EU977388	n/a
Engaeus	*tuberculatus*	MCS1.2	EU977389	EU977430
Engaeus	*tuberculatus*	MON1.4	EU977390	n/a

Engaeus	*urostrictus*	HAR1.1	EU977391	EU977431
Engaeus	*urostrictus*	Mus Vic J45681; MV94	EF493115	n/a
Engaeus	*victoriensis*	BAY1.2	EU977392	n/a
Engaeus	*victoriensis*	YRG1.1	EU977393	n/a
Engaeus	*yabbimunna*	Mus Vic J34475; MV116	EF493101	n/a
Engaewa	*similis*	?	AF135982	n/a
Engaewa	*subcoerulea*	805 2L	EU977394	EU977432
Engaewa	*subcoerulea*	RPD2	EU977395	EU977433
Engaewa	*walpolea*	Mus Vic J39560; MV179	EU977396	n/a
Euastacus	*bispinosus*	H101	AF044244	EU977434
Geocharax	*falcata*	FWC1.1	EF493076	EU977435
Geocharax	*falcata*	RED1.1	EF493134	EU977436
Geocharax	*falcata*	SWC1.1	EF493144	EU977437
Geocharax	*gracilis*	BRY1.2	EF493052	EU977438
Geocharax	*gracilis*	MSQ1.3	EF493095	EU977439
Geocharax	*gracilis*	TMC1.1	EF493146	EU977440
Geocharax	*sp.*	EEL1.1	EF493068	EU977441
Geocharax	*sp.*	KNB1.1	EF493085	EU977442
Geocharax	*sp.*	KNC1.1	EF493086	EU977443
Geocharax	*sp.*	SER2.3	EF493139	EU977444
Gramastacus	*insolitus*	3BX1.1	EF493040	EU977445
Gramastacus	*insolitus*	DWY3.1	EF493066	EU977446
Gramastacus	*sp.*	MYL1.1	EF493118	EU977447
Gramastacus	*sp.*	MYL1.3	EF493120	EU977448
Paranephrops	*planifrons*	H89	AF135995	EU977449
Tenuibranchiurus	*glypticus*	BEL1.2	EU977397	EU977450
Tenuibranchiurus	*glypticus*	BEL2.2	EU977398	EU977451
Tenuibranchiurus	*glypticus*	BRB1.1	EU977399	EU977452
Tenuibranchiurus	*glypticus*	TEW1.1	EU977400	EU977453

Appendix 3 Taxonomic key to *Engaewa* species.

1. Cervical groove broadly U-shaped at meson; LP 1st P and 2nd P usually with closed or open slit- or pit-like pores ………………….. **2**

    Cervical groove broadly V-shaped at meson; LP 1st P and 2nd P without closed slit- or pit-like pores **3**

2. Chelae: small dimorphs with three rows of tufts of long setae on dorsal surface of dactyl continuing as a single row on dorsal surface of propodus anteriorly, and with three rows of tufts of long setae on ventral edge of propodal palm and finger; tubercles on dorsal edge of propodus of large dimorph extending along entire edge but faint and sparse; tubercles on dorsal edge of dactyl of large dimorph small and numerous, not forming two distinct rows. LP 2nd P with an open ovoid pore ………………………………………………………………………….. ***E. clade B***

    Chelae: without long tufts of setae; tubercles on dorsal edge of propodus markedly reduced and extending at most to halfway along edge; tubercles on dorsal edge of dactyl of large dimorph large and sparse, forming two distinct rows. Pore in the LP 2nd P ranging from (rarely) absent to a closed slit/slit-like pore to an open slit-like pore …………………………………………………………….... ***E. subcoerulea***

3. Rostral carinae present but low and short, or if not present: inflated portion of keel at 3rd P rounded anteriorly, or if not: ventral carina of propodal palm smooth along edge; antennal flagella extending to AS3, 4 or 5; antennules with inner flagellum 0.4–0.7× as long as outer ………………………………………..…….... **4**

    Rostral carinae absent; inflated portion of keel at 3rd P pointed anteriorly; ventral carina of propodal palm granulate minutely; antennal flagella extending to AS 5 or 6; antennules with inner flagellum 0.7–0.8× as long as outer .... ***E. walpolea***

4. LP 3rd P without pit or pore; dorsal surface of dactyl without dense patch of setae …………………………………………………………………………………….... **5**

    LP 3rd P with pit or pore; chelae: isomorphs and small dimorphs (on adult individuals only) with dense patch of setae on dorsal surface of dactyl ….. ***E. reducta***

5. Chelae: most chelae on most individuals with patches of setae on lateral sides of cutting edges only; sternal keel usually terminating at LP 4th P, and LP 4th P usually sloping inwards; rostral carinae usually present on anterior part of rostrum; caudolateral corners of telson each usually with spine (but may be present as a notch only) **6**

    Chelae: adult individuals with patches of setae on ventral surface of merus, ventrally and distally on carpus, laterally adjacent to cutting edges, and occasionally on propodal palm as well; sternal keel not terminating at LP 4th P, and LP 4th P sloping in posterior direction; rostral carinae absent; telson with caudolateral corners spineless (but they may be present as a notch) .... ***E. pseudoreducta***

6. Chelae: isomorphic and small dimorphic chelae (rarely large dimorphic) with dense patches of short bristle setae on lateral sides of cutting edges and long setae on ventral edge of propodus; large dimorphs and large isomorphs with dorsal surface of dactyl bearing two rows of large granulations, where granulations are paired transversely ……………………………………………………………….………… ***E. similis***

    Chelae: isomorphic and large dimorphic chelae with long but sparse patches of setae on lateral sides of cutting edges; all chelae with dorsal surface of dactyl bearing large granulations over entire surface (not arranged in distinct rows) ….. ***E. clade A***