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Abstract

Species occurring in fragmented urban habitats often exhibit low genetic diversity which can be attributed to restricted gene flow and elevated levels of inbreeding. This can have serious implications for the survival of species especially when faced with additional pressures caused by urbanisation. The population genetic structure of the generalist skink *Ctenotus fallens* was examined within and among three urban vegetation remnants in the Perth metropolitan area in Western Australia, using both microsatellite and AFLP markers. Historic genetic connectivity between the populations of each remnant was shown as well as weak patterns of genetic differentiation which appeared to suggest isolation by distance. The results presented two possible scenarios; that genetic connectivity is maintained between populations via dispersal between remnants through the urban matrix or, that the urban matrix is a barrier to gene flow for *C. fallens* and contemporary levels of gene flow cannot be detected due to large, randomly mating populations persisting within the remnants which may be acting as a buffer for any genetic change associated with isolation.

Introduction

Habitat Fragmentation

The study of habitat fragmentation has become one of the central themes of conservation biology. Although the term is often ambiguous with different meanings within between disciplines (Haila, 2002; Fahrig, 2003) it is generally understood to mean; a landscape-scale process whereby habitats are transformed into a number of smaller fragments, isolated from one another by a habitat or matrix of habitats quite unlike the original (Wilcove et al. 1986; Fahrig, 2003). Although this process occurs naturally in most habitats to some degree (usually referred to as habitat mosaicism), anthropomorphically induced habitat fragmentation is of greater concern to the conservationist due to its severity (Whittaker & Levin, 1977; Haig et al. 2000). Through processes such as deforestation, urbanisation, agriculture and the construction of transport infrastructure, habitat fragmentation is considered the driving force behind the global loss of biodiversity (Wilcove et al. 1998; Fahrig, 2003; Foley et al. 2005; Krauss et al. 2010).

The importance of habitat fragmentation was bought to the forefront of ecological thinking via MacArther & Wilson's (1967) seminal book 'The Theory of Island Biogeography'. The theory states that rates of species immigration and extinction are determined by island size and distance from a source of colonists. According to the theory, biodiversity and abundance is higher on large islands with good connectivity to other islands or the mainland and lower on small isolated islands (MacArther & Wilson, 1969; Laurance, 2008). 'Islands' do not have to be islands of land surrounded by ocean but can mean any patch of habitable environment surrounded by inhospitable areas such as natural reserves surrounded by agricultural land (Laurance, 2008). Island biogeography theory also stimulated thinking relating to reserve design and emphasised the importance of conserving large, well connected habitat remnants in order to maintain as many species as possible (Laurance, 2008).

While island biography theory is still largely relevant and has laid a strong foundation of understanding, habitat fragmentation is now thought to have superseded island biogeography theory since the advent of disciplines such as metapopulation dynamics, landscape ecology and conservation genetics (Haila, 2002; Laurance, 2008). It is now appreciated that the space between the islands is not, in many cases, as homogenously hostile as an ocean but as a heterogeneous matrix of varying levels of impermeability depending on the species in question (Haila, 2002). Traversable corridors may exist between habitat fragments for some species that do not exist for others (Laurance, 2008). Indeed, it is also appreciated that the effects of fragmentation of species within a habitat remnant may be more subtle than simply a reduction in abundance or species richness (Laurance, 2008).

With the rapid expanse of urban areas during the second half of the 20th century, especially in developing nations, habitat fragmentation as a result of urbanisation is becoming an increasingly important issue for conservationists (McKinney, 2007). Habitat fragments in urban areas are usually set aside as parks or reserves (McKinney, 2007). These are recognised not only for their aesthetic and recreational value but also for their role in educating urbanised communities in the importance of nature and conservation (Miller & Hobbs, 2002). Urban fragmentation is analogous to fragmentation caused by agriculture or deforestation (Miller & Hobbs, 2002). However, the nature of the urban matrix is unique in its complexity, which may offer novel ecological niches for some species and act as a strong barrier to dispersal for others (McKinney, 2007; Noel et al. 2007).

The genetic impact of habitat fragmentation

As a result of fragmentation, dispersal between remnant habitats is often reduced or prevented as per island biogeography theory (Allendorf & Luikart, 2007). Additionally, population size is usually reduced (MacArther & Wilson, 1967; Laurance, 2008). These factors increase the occurrence of inbreeding within a population (the mating of individuals with common ancestry) as mating becomes non random (Keyghobadi, 2007). This leads to a loss of genetic diversity as genetic drift causes allele frequencies

to become fixed at homozygotes (Allendorf & Luikart, 2007). Accumulation of deleterious recessive alleles in homozygotes leads to a general decrease in fitness known as an inbreeding depression (Lynch & Walsh, 1998). This has been linked in the wild to both population decline and extinction (Saccheri et al. 1998). The frequency of heterozygotes in a population can be used as an indicator for inbreeding (Wright, 1931, 1951). This is because inbred populations typically have fewer heterozygotes than outbred populations. Another measure of inbreeding is allelic frequency, as rarer alleles tend to be lost in inbred populations (Allendorf & Luikart, 2007).

Interpreting the genetic impact of fragmentation

One of the most commonly used mathematical frameworks to describe levels of heterozygosity in a population is *F* statistics (Wright, 1931, 1951). In this framework, *F* represents a reduction in heterozygosity relative to the heterozygosity in random mating populations with the same allelic frequencies. *F* is described as;

$$F = 1 - (H_O/H_E)$$

where H_O is the observed proportion of heterozygotes and H_E is the expected proportion of heterozygotes, if the populations were at Hardy-Weinberg equilibrium (Allendorf & Luikart, 2007). *F* therefore, can not only be used as an indicator for inbreeding but also as a measure of how genetically connected populations are from a measure of their relative heterozygosity (Allendorf & Luikart, 2007). Wright (1931, 1951) developed a series of coefficients, F_{IS}, F_{ST} and F_{IT}, that can be used to describe genetic variation within a species. F_{IT} gives a measure of inbreeding for an individual relative to the total population (Wright, 1931, 1951). F_{IS} is the inbreeding coefficient of an individual relative to a subpopulation and F_{ST} is a measure of the divergence of allelic frequencies among subpopulations. These coefficients can be expressed as:

$$F_{IT} = F_{IS} + F_{ST} = (F_{IS})(F_{ST})$$

F_{ST} is therefore, commonly used as a measure of genetic differentiation in the study of populations occurring in fragmented habitats. F_{ST} ranges from zero, where all populations have equal allelic frequencies to 1, where all populations have a different assortment of fixed alleles (Allendorf & Luikart, 2007).

Measures of genetic variation

Before genetic differentiation between populations can be estimated, the genetic variation within individuals must first be measured. Two of the most common markers used to generate population genetic data for statistical analysis are microsatellites and AFLP.

Microsatellites (or short sequence repeats) are short (usually 1-4 bp) sequences of nDNA or mtDNA that are repeated consecutively, usually 10 – 100 times (Goldstein & Schlötterer, 1999). They are useful as markers in population genetic research as they exhibit high mutation rates and genotypes are relatively simple to score as co-dominant markers (Lowe et al. 2004). The initial identification of microsatellites is costly and protracted. An alternative method is cross species amplification whereby microsatellites characterised for related species, may be used (Lowe et al. 2004). Success rates are associated with the relatedness of the two species (Primmer et al. 1996; Primmer & Merilä, 2002).

AFLP markers (developed by Vos et al. 1995) are an arbitrary subset of fragmented DNA selected by pairs of primers and amplified by Polymerase Chain Reaction (PCR) (see methods for a detailed description). Unlike microsatellites, they can be generated quickly, at a lower cost and without prior knowledge of the genome (Lowe, et al. 2004). However, the markers generated are dominant and therefore are informationally poor (Mariette et al. 2002). Numerous markers therefore, must be generated to give an accurate estimate of genetic differentiation (Mariette et al. 2002).

Genetic impact of urban habitat fragmentation

The effect of urban habitat fragmentation on the population genetic structure of species has to shown to be similar to that of other forms of habitat fragmentation. Species inhabiting fragmented urban habitats are often characterised by strong genetic differentiation between populations as the urban matrix can act as a strong barrier to

gene flow (Magel et al. 2010; Delany et al. 2010; Noel et al. 2007; Vandergast et al. 2009). Roads, ubiquitous in urban areas, have been shown to be a strong barrier to dispersal, especially for reptiles (Andrews et al. 2008; Shepard et al. 2008; Clark et al, 2010). An example of the effect of roads on population genetic structure was shown in a study by Riley et al (2006). This study identified genetically differentiated populations of bobcats and coyotes on either side of a road in an otherwise unfragmented habitat.

For other more vagile species the urban matrix is less of a barrier to dispersal and fragmented populations are less genetically differentiated (Takami et al. 2004). For example, butterflies have been shown to be relatively unaffected genetically by urban fragmentation as they are able to easily disperse through the urban landscape (Takami et al. 2004). Similarly, some plant species have also been shown to be unaffected by urban fragmentation where there is an abundance of insect pollinators dispersing through the urban matrix to facilitate gene flow (Culley et al. 2007). The level of genetic differentiation between urban fragmented populations depends on how strong a barrier the intervening landscape is to gene flow of the particular species in question.

Aims

Few studies have investigated the genetic effects of urban habitat fragmentation on lizards. This question is of particular relevance to the urban habitat remnants of the Perth metropolitan area which has some of the richest assemblages of lizards of any urban area in the world (How & Dell, 1994; 2000). It is the aim of this study to determine the effects of urban habitat fragmentation on the population genetic structure of the scincid lizard *Ctenotus fallens*. This will be achieved by determining the level of genetic differentiation between populations occurring in three urban habitat remnants. From the level of genetic differentiation observed, it may be possible to identify whether *C. fallens* is able to disperse through the urban matrix or if it remains isolated within urban habitat fragments. Additionally, populations within a habitat remnant will be compared to determine the level of genetic structure that occurs within that particular remnant.

Materials and Methods

Study organism

The genus *Ctenotus* is the most speciose vertebrate genera in Australia represented by 102 species (Rabosky et al. 2009; Wilson & Swan, 2010). Species are mostly diurnal, insectivorous and agile, typically occupying a home range of less than 60m (Pianka, 1969; James, 1991; Read, 1998; Jennings & Thompson, 1999). Adults become sexually mature within a year but do not breed until their second spring, thus each generation represents two years (Bamford 1986; Jennings and Thompson, 1999). Most *Ctenotus* species studied lay two eggs which typically hatch between January and June (Read, 1992). The ecology of *Ctenotus* has previously received attention as species often occur sympatrically, partitioning available food resources within a single habitat (Pianka, 1969; James, 1991). In areas where they are abundant several species of *Ctenotus* are increasingly being used as environmental indicators due to their trapability, abundance and preference for a range of habitats (Read 1992; Read, 1998).

C. fallens is abundant throughout the Swan Coastal Plane and is arguably the most readily encountered reptile in Perth's urban vegetation remnants (How & Dell, 1994, 2000; How, 1998). It favours low coastal vegetation on dunes and limestone outcrops but can be found in any habitat which supports a shrub-dominated understory, including disturbed areas with introduced grasses (Bush et al. 2010). It is also known to occur on granite outcrops in the Darling Range (Bush et al. 2010). The range of *C. fallens* extends throughout coastal Western Australia from Cape Cuvier to Waroona and inland to Koolanooka Hills (Bush et al. 2010). It is has a maximum total length of 300mm and a snout-vent-length of 95mm (Bush et al. 2010). It is thought to be ecologically similar to other species of *Ctenotus*, i.e. agile, diurnal and largely insectivorous (Jennings and Thompson 1999; Bush et al 2010). It has been experimentally shown to display intraspecific aggression and may be territorial (Reid, 1998; Jennings & Thompson, 1999). Individuals maintain site fidelity and individuals have been found to travel no further than 30 – 40m in a period of 120 days (Jennings & Thompson, 1999).

Study sites

Site description

Populations were sampled from three urban vegetation remnants on the Swan Coastal Plain near to the city of Perth, on the west coast of Western Australia. The area has a Mediterranean climate with wet mild winters and hot dry summers. The vegetation remnants were Bold Park (-31.948°, 115.772°), Shenton Bushland (-31.961°, 115.799) and Kings Park (-31.964°, 115.831°) (Figure 1). Vegetation across all sites historically belonged to the widespread Cottesloe, Quindalup and Karrakatta complexes consisting of open forest of Tuart (*Eucalyptus gomphocephala*), Jarrah (*E. marginata*), Marri (*Corymbia calophylla*) and low woodland of *Banksia* and *Allocasurina* species (Anon, 2000). Urban development occurred throughout the area in the early 1900's and further rapid expansion in the second half of the century has strongly fragmented the natural vegetation. As of 2000, 18% of this original vegetation complex remained in the Perth Metropolitan Area (Anon, 2000). Bold Park and Kings Park are completely bounded by roads and all three study sites are completely surrounded by development with the expectation of the southwest edge of Bold Park. The sites are also internally fragmented by tracks and pathways. Kings Park is intersected by roads which at times receive light to moderate amounts of traffic.

Kings Park (KP) contains 267ha of remnant vegetation alongside recreational areas and botanic gardens (Bennett, 1995). Most of the site occurs on the plateau of Mt. Eliza which gives way to a steep escarpment on the eastern margin of the site leading down to the Swan River. The original vegetation complex of *E. gomphocephala, E. marginata* and *C. calophylla* is thought to be undergoing a transition into the *Banksia* and *Allocasurina* woodland that now dominates the site, as a result of human disturbance (Bennett, 1995). Weed invasion has also been severe in many parts of the site and is under management (Keighery et al. 1990). The urban areas surrounding KP are some of the oldest in the region (Jarvis, 1979). KP was the first of the three vegetation remnants to become fragmented and was separated from the areas that were to become Shenton Bushland (SB) and Bold Park (BP) by the construction of the Perth – Fremantle railway in 1881 (Jarvis, 1979). The site was partially surrounded by urban

development in 1916 and became completely surrounded in the 1940s (Jarvis, 1979). Notable features within the urban matrix between KP and Shenton Bushland include; Thomas Street on the sites immediate western boundary which is a major thoroughfare and is in places six lanes wide and the aforementioned Perth - Fremantle train line, which is fenced and flanked by roads on both sides.

Shenton Bushland covers an area of 19.7 ha, approximately half way between KP and BP. Prior to becoming a class A reserve in 1996 localised areas of the remnant became severely degraded due to rubbish dumping and weed invasion, (Berry & Berry, 2008). However more than 50% of the vegetation is in very good condition and significant rehabilitation has occurred in recent years (Anon, 2000; Berry & Berry, 2008). Although the majority of urban development separating SB and BP did not appear until the 1980s some fragmentation of the connecting vegetation did occurred in the decades prior to urban development as the construction of roads and clearing of land (Jarvis, 1979).

Bold Park is one of the largest urban vegetation remnants on the Swan Coastal Plain covering 437 ha. Significant weed invasion has occurred but the original vegetation structure remains largely intact and is well managed (Keighery et al. 1990). It is the only study site to have natural free standing water and is geologically different from SB and KP as parts occur on the Quindalup dune system whereas the latter sites occur on the older Spearwood system.

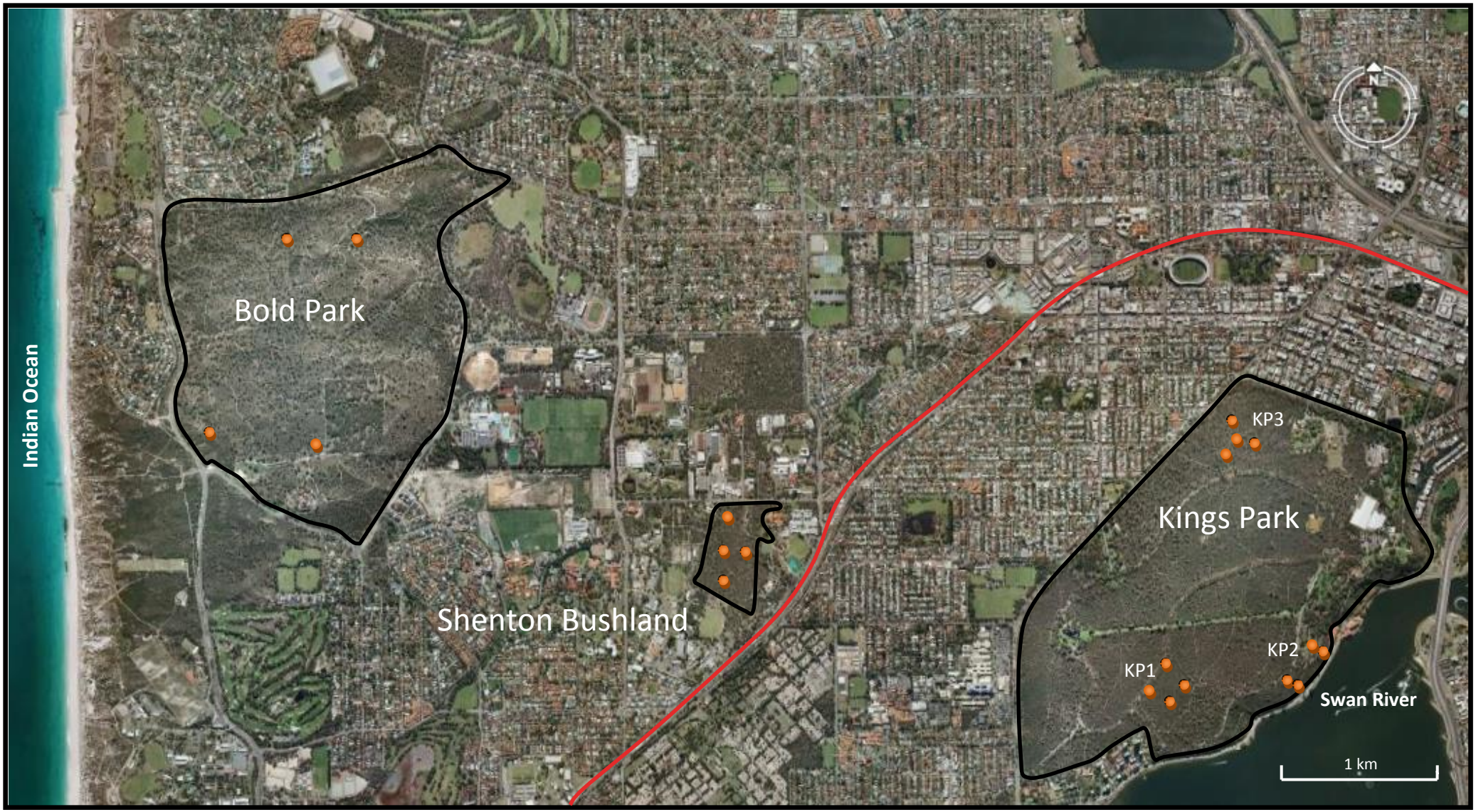


Figure 1 Modified satellite image of study area with an eye level 8km above sea level. The study sites are outlined and trap-sites represented by orange markers. The Perth – Fremantle railway is shown in red. The Perth CBD is located approximately 1km east of the area shown (Modified from satellite imagery obtained from Google Earth v5.0, Google Inc. 2009)

Site selection

In order to detect possible genetic structuring within KP due to internal fragmentation by roads and pathways, populations were sampled at three separate locations which were treated as independent sites. These were labelled KP1 (the plateau of Mt. Eliza), KP2 (the limestone escarpment) and KP3 (the northernmost section of the park – Figure 1). Populations at each site were sampled at four trap-sites. While trap-sites at KP1, KP2, KP3 and SB were a similar distance apart and sampled a similar sized area, the trap-sites a BP were further apart to give a better representation of the entire population of the site. While a comparison of genetic differentiation within habitat fragments between KP and BP would be of interest, due to the number of individuals needed to provide a statistically powerful analysis of a population (approximately 30 – Berg & Hamrick 1995) this would have required a tripling of trapping effort at BP which was beyond the scope of this project.

The tissue samples used for genetic analysis in this study were donated from herptofaunal monitoring projects conducted by Dr. Ric How (Museum of Western Australia) and Dr. Robert Davis (Edith Cowan University). Trap-sites at KP1 and KP2 include sites that were burnt in 2009 as these sites were set-up to determine the effect of a fire on the herpetofauna of KP (Davis, unpublished) while trap-sites at BP have been used by How (1998) in long term monitoring of herpetofaunal assemblages.

Sample collection

At each trap-site at KP1 and KP2 were nine pitfall traps arranged in 3x3 grids with each trap 10m apart. The pits were PVC buckets 40cm deep and 30cm in diameter dug into the ground. Each trap was located in the centre of a 7m long, 30cm tall flywire drift fence running in a random direction and dug 5cm into the substrate. Five double ended funnel traps were also used that were placed along one side of the drift fence.

Pitfall traps at BP were 60cm deep, 17.5cm diameter PVC pipes dug into the ground and sealed at the bottom with flywire mesh. Six pits were arranged in a line at each

trap site 7-8m apart. Fifty metres of 30cm high flywire drift fence dug 5cm into the substrate crossed each of the six pits.

At SB and KP3 between 5-10m of drift fence was used at each trap-site dug set up as described for KP1 & 2. Sections were cut out of the fence and funnel traps were positioned in-between the cut out sections so that animals following both sides of the fence were directed into the trap. This doubled the effectiveness of the funnel traps as compared to those at KP1 and KP2.

Trapping took place between November and February 2011. Tissue samples obtained were in most cases 2cm tail clippings. Tail clipping is a standard technique for collecting genetic material from lizards and has been used in nearly all comparable studies (Stow & Briscoe, 2005; Hoehn et al. 2007; Richmond et al. 2009; Delany et al. 2010). Although non invasive buccal swabbing has been proposed as an alternative method for DNA sampling of squamate reptiles (Miller, 2006; Beebee, 2008) the technique was trialled here to no effect, as the lizards simply could not be induced open their mouths. Additionally buccal swabs have much shorter shelf lives than tail tips (Miller, 2006) and tail clipping has the added advantage of being an indicator that an individual has been previously sampled. Samples collected from KP and SB were stored on ice for up to two hours before being transferred to a freezer (-18°C). Samples collected from BP were stored in 100% ethanol at room temperature for up to eight weeks. Once tissue had been collected individuals were marked on the throat with a xylene free paint pen to prevent resampling and released. Resampling is unlikely to have occurred as sampled individuals with missing tail tips were obvious even after regrowth of the tail had begun. In cases where there was contention over whether an individual had been previously sampled or not, the individual was released without collection of tissue.

DNA extraction

Genomic DNA was extracted using the standard salt protocol of Sunnucks & Hales (1996). Tail tips were placed in 300µl TNES buffer (Sunnucks & Hales, 1996) with 1.66µl Proteinase K (Invitrogen™, CA USA) and crushed slightly to facilitate the introduction of Proteinase K into the tissue. Samples were then incubated for 12-18hrs at 37°C. Following incubation 85µl NaCl (5M) was added and the samples were shaken for 15s to precipitate out proteins from the solution. Samples were then centrifuged at 13,000 rpm for 5min and the supernatant was transferred to new tubes. 385µl of 100% ethanol at -18°C was then added and samples were rocked gently precipitate out DNA before being centrifuged again at 13,000 for 10min. The ethanol was poured off to leave DNA pellets at the base of the tubes. These pellets were then washed in 500µl of 70% ethanol by centrifuging for another 10min at 13,000 rpm and pouring off the ethanol. The DNA pellets were left for 1hr to allow excess ethanol to evaporate before being dissolved in 30µl of TE buffer. The concentration of DNA in solution was determined using a NanoDrop 1000 spectrophotometer (Thermo-Scientific, DE, USA (now Thermo Fisher Scientific). DNA was then diluted with dH_2O to the specific requirements of each protocol.

Microsatellite amplification

For all procedures a blank sample was used per column of eight, where DNA was replaced with the corresponding amount of dH_2O in order to detect possible contamination of samples. We attempted to cross amplify eight microsatellite loci (*Ct1* - *Ct8*) characterised for *Ctenotus taeniolatus* (Smith & Stow, 2008) in *Ctenotus fallens*. PCRs were carried out using a Veriti® 96-Well Fast Thermal Cycler (Model 9902) (Applied Biosystems, CA, USE) with the following concentrations: 2µl DNA (5ng/µl), 2µl 5x polymerisation buffer (Fisher Biotech, WA, Australia), 0.8 µl $MgCl_2$ (25mMm Fisher Biotech), 0.8 µl of both forward and reverse unlabeled primer (2mM) and 0.08 µl *Taq* polymerase (Fisher Biotech) made up to 10 µl with dH_2O. PCR conditions were as follows: Denaturisation at 94°C for 3min followed by cycling of 40s at 94°C, annealing for 40s at 52°C and extension for 30s at 72°C. Thirty-five cycles were performed

followed by a final extension of 7min at 72°C. Conditions were the same for all loci except *Ct1* and *Ct3* which had annealing temperatures of 55°C and 54°C respectively. PCR products were separated via electrophoresis by running 4µl sample and 1µl of loading buffer (0.125g Bromophenol blue, 25ml glycogen, 50ml dH_2O) through a 1% agarose gel at 240v. The gel was then stained with SYBR® Safe DNA gel stain (Invitrogen) for 30min and products were visualised under UV light.

The optimisations of PCR conditions consisted of altering the annealing temperature and concentration of $MgCl_2$ used in the PRC mix for each primer. All other conditions remained the same with the exception of the unlabelled forward primers which were replaced by fluorescently labelled primers (2mM). Seven randomly selected samples were used for optimisation and annealing temperature of 52, 55, 57 and 58°C were trialled initially with concentrations of $MgCl_2$ at 1.5 and 2mM.

For each sample 1µl of PCR product was added to 41 µl of formamide (Amresco OH USA) [de-ionised using AG 501-X8 mixed bed resin (BioRad Laboratories CA USA), syringe filtered into aliquots] and 0.3µl of size standard 400 (Beckman, Coulter). This then underwent capillary electrophoresis through a denaturing polyacrylamide gel on a CEQ 8800 Genetic Analysis System (Beckman Coulter). The presence of alleles was scored as peaks on a chromatograph (Figure 2-4) against the size standard using Beckman Coulter CEQ software (2004). For loci that peaks could not be confidently scored for following PCR at any of the initially trialled conditions (Figure 3) further changes in PCR conditions were trialled using annealing temperatures of 61° and 65°C (where higher annealing temperature appeared to be required) and $MgCl_2$ at concentrations of 1mM, 1.25mM and 1.75mM. All samples then underwent PCR and were scored as described for loci for which PCR conditions could be successfully optimised to give scorable peaks and were also polymorphic.

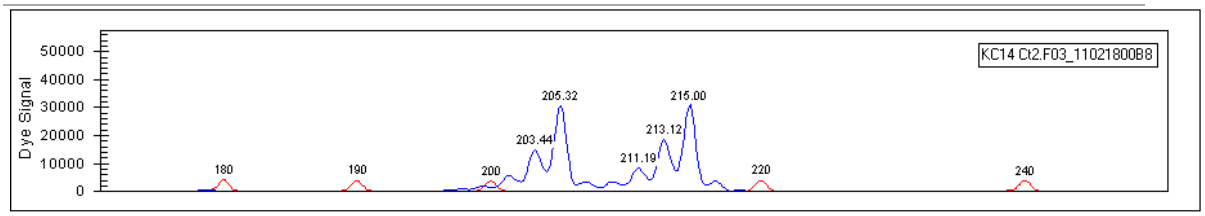


Figure 2 Chromatograph of the microsatellite locus *Ct2* amplified in a heterozygotic *Ctenotus fallens* possessing alleles 205 and 215 (blue) alongside size standard 400 (red). Although stutter peaks are present they do not effect the scoring of this sample as they are considerably weaker than the true peaks.

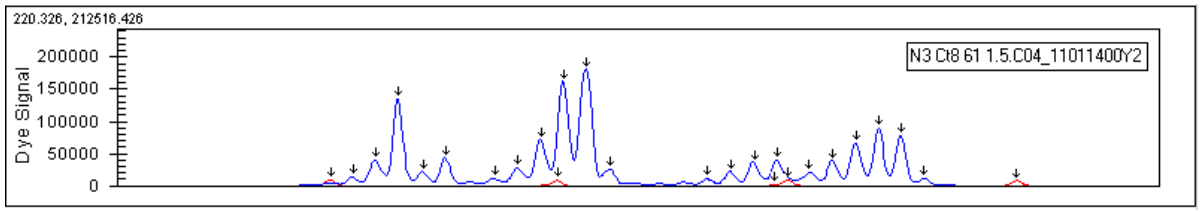


Figure 3 Chromatograph of the microsatellite locus *Ct8* amplified in *Ctenotus fallens*. At this locus there is considerable stutter as well as other amplified artefacts. As a result alleles for this locus could not be scored with certainty.

AFLP amplification

As with microsatellite amplification, for all procedures a blank sample was used per column of eight. The protocol used was developed by Vos et al (1995) and consists of four main components; restriction digestion, adapter ligation, preselective amplification and selective amplification by PCR.

Restriction digestion

During restriction genomic DNA is digested by two enzymes, one frequently cutting (MseI) and one rarely cutting (both EcoRI and PstI were tested here for suitability) (Vos et al. 1995). These restriction enzymes, produced by bacteria, recognise 4 (frequent cutter) to 6 (rare cutter) bp sequences of nDNA called restriction sites (Lodish et al. 2008). The enzymes make a staggered cut in the DNA leaving each fragment with a short 4-6bp single stranded overhang known as a sticky end (Lodish et al. 2008). This

process produces a population of fragments which have either similar or dissimilar ends (Vos et al. 1995). Concentrations for restriction are as follows; 2µl NE buffer (New England Biolabs Inc. MA, USA) 2µl BSA (0.5%, New England Biolabs Inc.), 0.25µl MseI (10U/µl, New England Biolabs Inc.), 0.26µl EcoRI (Roche Applied Science, Germany) or PstI (10U/µl, New England Biolabs Inc.), 250ng DNA (volume determined by the known concentration of each sample) and topped up with dH_2O to 20µl. Samples were incubated at 37°C for 2hrs.

Adaptor ligation

Due to the action of the rarely cutting enzyme, fragments with dissimilar ends after digestion will be uncommon (Vos et al. 1995). These fragments are selected by the ligation of adaptors to the sticky ends of the fragments. Two adaptors are used, one ligates to the end cut by the rarely cutting enzyme the other to the end cut by the frequently cutting enzyme. The adaptors provide the binding site for primers used in amplification (Vos et al. 1995). During ligation the following reagents were added to the restriction solution; 0.5µl 10X ligations buffer (Roche Applied Science), 0.5µl T4 ligase (Invitrogen), 4µl adaptor solution. The solution was then incubated for 12-18hr at 20°C. Following ligation samples underwent gel electrophoresis as described to determine whether digestion was successful and whether samples required further dilution (Figure 4). All samples were diluted 1:10 with dH_2O or more as indicated by the gel.

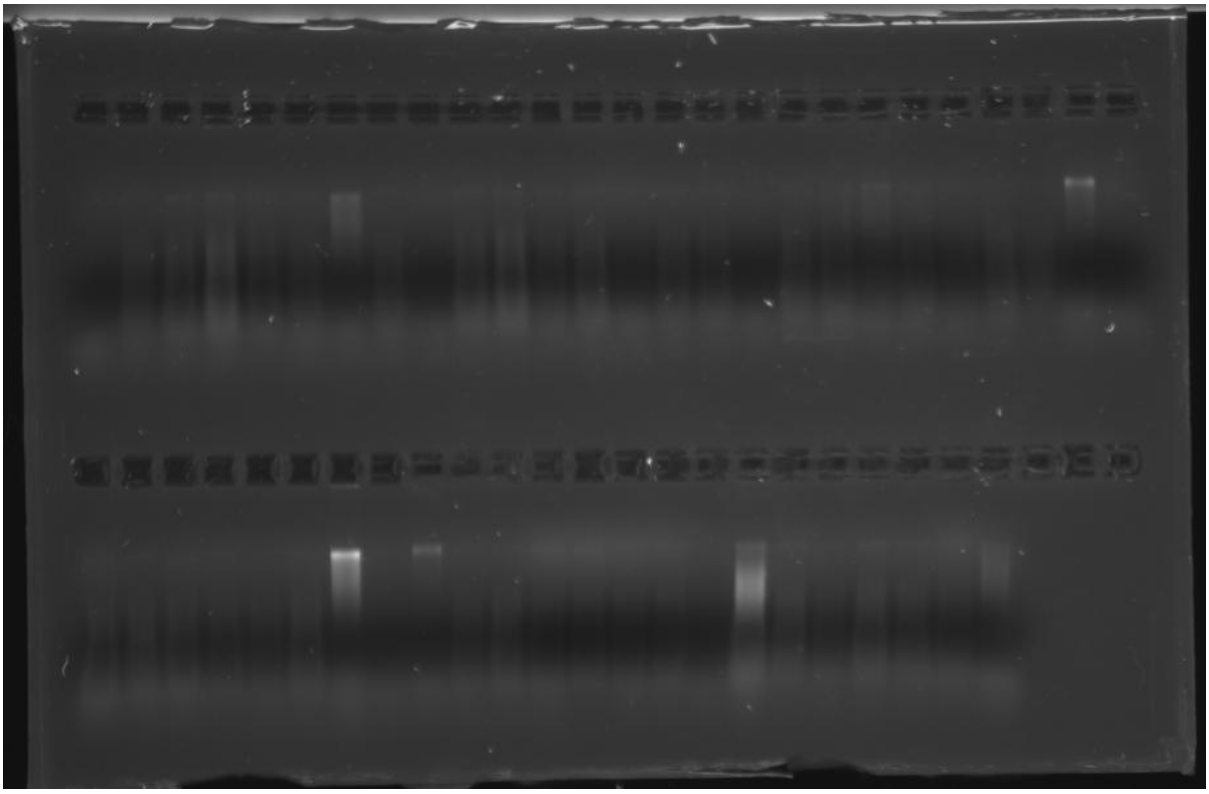


Figure 4 Restriction-Ligation products of *Ctenotus fallens* samples separated on a 1% agarose gel. The lack of the formation bars at each sample indicates that digestion of the DNA was successful. Samples producing dull products were diluted 1:10, brighter products 1:20 and the two very bright samples on the bottom row were diluted 1:30.

Preselective amplification

The primers used at this stage are specific to the adaptors ligated to the restricted fragments. However at the end of the primer sequence there is a single arbitrary selective base pair that will bind to the DNA. This reduces the number of fragments that can be amplified through PCR 16 fold (Vos et al. 1995). The concentrations for PCR used here were; 4µl diluted restriction-ligation template, 4µl 5X polymerisation buffer, 1.2µl $MgCl_2$ (25mM), 0.5µl Mse1 primer (5µM), 0.5µl EcoR1 primer (5µM) or Pst1 primer (5µM), 0.15µl *Taq* DNA polymerase, 9.65µl dH_2O. PCR conditions were; 72°C for 2 minutes followed by 25 cycles of 94°C for 20s, 56°C for 30s, and 72°C for 4 minutes followed by a final extension at 60°C for 30 minutes.

Selective amplification

Preselective samples were diluted 1:10. The primers used in the selective amplification have two, three or four arbitrary selective base pairs. This reduces again the number of fragments amplified. For example if both primers used have three selective bases 1/65,536 of the original fragments will be amplified. Thirty-six primer pairs (Table 1) were trialled for seven randomly chosen samples. Concentrations for PCR were; 2.5µl diluted preselective template, 2µl 5X polymerisation buffer, 0.6µl $MgCl_2$ (25mM), 0.5µl MseI primer (5µM), 0.25µl EcoRI primer or Pst1 fluorescently labelled primer (1µM, Sigma Aldrich, Well-Red Oligos), 0.08µl *Taq* DNA polymerase, 4.07µl dH_2O. PCR conditions were; denaturising at 94°C for 20 seconds followed by 50 cycles of 94°C for 20 seconds, 66-56°C for 30 seconds (the first cycle is at 66° and decreases by 1 degree every cycle until 56°, the rest of the cycles were performed at 56°C), and 72°C for 2 minutes followed by a final extension at 60°C for 30 minutes. Selective PCR products underwent capillary electrophoresis as described for the microsatellite amplification. The selective primer pairs Eco –ACT / Mse –CACT and Eco – ACC / Mse – CACT were found to produce good quantities of scorable and reproducible fragments. These primer pairs were used to develop AFLP markers for all samples as described. Presence and absence of markers between 50 and 300 bp were scored manually from the resulting chromatographs (Figure 5).

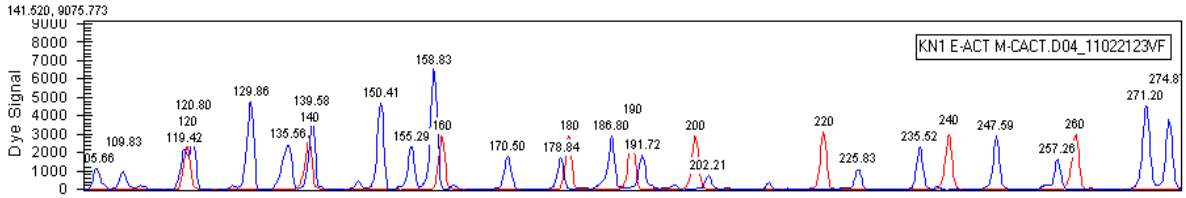


Figure 5 Chromatograph of AFLP markers (blue) amplified in *Ctenotus fallens* using selective primers E-ACT and M-CACT alongside size standard 400 (red).

Table 1 Primer pairs trialled for selective amplification of AFLP markers in *Ctenotus fallens*

Pst/Mse pairs	**Eco/Mse pairs**	
Pst -AC / Mse -CAC	Eco -ACT / Mse -CAC	Eco -AGG / Mse -CACG
Pst -AC / Mse -CAG	Eco -ACT / Mse -CTG	Eco -AGG / Mse -CACC
Pst -AC / Mse -CTG	Eco -ACT / Mse -CAG	Eco -AGG / Mse -CACT
Pst -AC / Mse -CTT	Eco -ACT / Mse -CTTA	Eco -AGG / Mse -CTGA
Pst -CA / Mse -CAC	Eco -ACT / Mse- CACG	Eco -ACC / Mse -CAC
Pst -CA / Mse -CAG	Eco -ACT / Mse -CACC	Eco -ACC / Mse -CTG
Pst -CA / Mse -CTG	Eco -ACT / Mse -CACT	Eco -ACC / Mse -CAG
Pst -CA / Mse -CTT	Eco -ACT / Mse -CTGA	Eco -ACC / Mse -CTTA
Pst -CG / Mse -CAC	Eco -AGG / Mse -CAC	Eco -ACC / Mse -CACG
Pst -CG / Mse -CAG	Eco -AGG / Mse -CTG	Eco -ACC / Mse -CACC
Pst -CG / Mse -CTG	Eco -AGG / Mse -CAG	Eco -ACC / Mse -CACT
Pst -CG / Mse -CTT	Eco -AGG / Mse -CTTA	Eco -ACC / Mse -CTGA

Data analysis

Microsatellite analysis

The microsatellite datasets were tested for the presence of null alleles, large allele dropout and scoring errors as a result of stutter using the software Micro-checker (Oosterhaut et al. 2004). Each locus was analysed individually using GENALEX 6.41 software ad-in for Microsoft Excel (Peakall & Smouse, 2006). Descriptive statistics including number of alleles per locus, number of private alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) were generated. Wright's (1951) *F* statistics, including inbreeding coefficient (F_{IT}), divergence in the frequency of alleles among populations (F_{ST}) and the degree of inbreeding within populations (F_{IS}) were calculated across all five populations. Principle component analyse (PCA) was performed for pairwise F_{ST} values to determine similarities between populations. Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) was calculated for both loci to determine the proportion of variance within and among populations. Pairwise values for Nei's (1973, 1978) genetic distance (*D*) between populations were also calculated. The correlation of these values was tested against pairwise geographic

distances between populations using a Mantel (1967) randomisation test. The geographic location of each population was given as a point estimated to be equal distance from all trap-sites for that population. This was estimated by eye from satellite imagery (Figure 1). AMOVA, pairwise F_{ST}, pairwise *D* and Mantel test were calculated with 999 permutations.

AFLP analysis

Descriptive statistics including number of polymorphic loci per population, proportion of polymorphic loci with an allelic frequency >5%, H_J (analogous to H_E), pair-wise φ_{PT} (analogous to F_{ST}) and pairwise *D* were calculated using the software AFLP-SURV 1.0 (Veckemans et al. 2002). AMOVA was performed to determine the percentage of total variation partitioned into, among and within population components. Principle component analyses (PCA) were performed to examine similarities between populations based on pairwise φ_{PT} values between populations and also for genetic distance between individual samples calculated as a straight count of differences of each AFLP marker. The relationship between the geographic distance between populations and *D* were explored using a Mantel test. AMOVA, PCA, Mantel tests and genetic distance between individual samples were performed using GENALEX 6.41 with 999 permutations.

Microsatellite data were also included into the AFLP dataset with each microsatellite allele used as a binary marker. Presence or absence of alleles was recorded in the same way as AFLP markers. The analyses performed for the AFLP dataset were then repeated as described.

Results

A total of 160 tail tips were obtained and DNA was successfully extracted from all samples. The number of individuals sampled from each population were; KP1 - 35, KP2 - 31, KP3 - 31, SB - 30, BP - 33.

All eight microsatellite loci cross amplified successfully. However two of these, *Ct6* and *Ct7* were monomorphic in all populations while PCR conditions for *Ct1*, *Ct3*, *Ct4* and *Ct8* could not be optimised to give results that could be scored with confidence. Micro-checker (Oosterhaut et al. 2004) results indicated no evidence for null alleles, large allele dropout or scoring errors due to stuttering for *Ct2* and *Ct5*. Microsatellite data was generated for 135 samples at *Ct2* and 117 samples at *Ct5*. A total of ten alleles were identified at *Ct2* and six alleles at *Ct5*, with an average number of alleles per population of 8.8 at *Ct2* and 3.8 at *Ct5*. The total number of alleles among populations at both loci was similar with the exception of KP1 at *Ct5*. This population had just two alleles whereas all other populations had four or five (Table 2). The two alleles present at KP1 however were those that were most common in the other populations and *Ct5* lacks only the rarer alleles (Figure 6). If not for a single heterozygotic individual possessing two rare alleles at KP2, this population would have an almost identical assortment of alleles as KP1. A similar pattern is seen at *Ct2,* where there was a core group of similar sized alleles common through all five populations (Figure 6). At *Ct2* all populations were at Hardy-Weinberg equilibrium (HWE) except KP3, whereas at *Ct5* all of the King Park populations deviated away from HWE (Table 2).

The two AFLP primer pairs generated 117 markers for 130 samples all of which were polymorphic across all populations. The number of polymorphic markers within each population ranged from 95 - 77 (Table 3). However a number of these were only slightly polymorphic and this range dropped to 83 - 72 markers when only those markers with a frequency of >0.05 were considered (95% criterion, Table 3). The mean number of markers per individual across all populations was 39.2.

Expected heterozygosity (H_J) ranged from 0.21 to 0.24 across all populations for the AFLP marker dataset as well as for the combined dataset (AFLP dataset of 117 markers with the 16 alleles from both microsatellite datasets included as binary markers) (Table 3). Similarly at both microsatellite loci H_E was comparable across all populations (Table 2). At *Ct2,* all H_O values were slightly lower than H_E with the exception of KP2, which had a higher H_O than H_E resulting in a negative value for *F.* At *Ct5,* H_O was higher than H_E at all populations except KP1. Average expected heterozygosity was highest at *Ct2* and lowest for AFLP and combined data. Values for F_{ST} were consistently low across AFLP, combined and *Ct2* datasets (0.026, 0.024 and 0.020 respectively) and slightly higher at *Ct5* (0.045) (Tables 2 & 3). F_{IS} and F_{IT} values were also low for *Ct2* and negative at *Ct5* (Table 3). Pairwise F_{ST} and φ_{PT} values were typically low (Tables 4, 5). Mean pairwise F_{ST} values were 0.007 and 0.036 for *Ct2* and *Ct5* respectively (Table 4) and mean pairwise φ_{PT} values were 0.046 and 0.045 for the AFLP and combine data respectively (Table 5). Pairwise values for *D* were also low with no outstanding values (Tables 6, 7).

Table 2 Statistics of genetic diversity within five populations and of *Ctenotus fallens* for two microsatellite loci *Ct2* (above) and *Ct5* (below), global *F* statistics (Wright, 1931, 1951) are also included for each locus.

	n	*A*	*Pr*	H_O	H_E	*F*	F_{IS}	F_{IT}	F_{ST}
KP1	29	9	0	0.793	0.832	0.047			
KP2	27	8	0	0.926	0.804	-0.152			
KP3	23	10	0	0.783*	0.870	0.100	0.041	0.060	0.020
SB	26	8	0	0.692	0.805	0.140			
BP	30	9	0	0.733	0.783	0.063			
KP1	25	2	0	0.2*	0.385	0.480			
KP2	21	4	1	0.524*	0.432	-0.213			
KP3	24	4	0	0.708*	0.563	-0.259	-0.104	-0.054	0.045
SB	28	5	1	0.679	0.58	-0.169			
BP	19	4	0	0.632	0.525	-0.203			

n number of individuals, *A* number of alleles, *Pr* number of private alleles, H_O observed heterozygosity, H_E expected heterozygosity, *F* Wright's fixation index = $1 - H_O/H_E$, * denotes deviation away from Hardy-Weinberg equilibrium.

Table 3 Statistics of genetic diversity within five populations of *Ctenotus fallens* for 117 AFLP markers (above) and for a combined dataset of 133 markers (below) where each allele of two microsatellite loci was added as a binary marker to the AFLP data set, global F_{ST} values for each dataset are also included.

	n	NPL (no minimum criterion)	PPL (no minimum criterion)	NPL (95% criterion)	PPL (95% criterion)	H_J	F_{ST}
KP1	30	93	79.5	72	61.5	0.243 ± 0.016	
KP2	25	95	81.2	83	70.9	0.231 ± 0.015	
KP3	24	93	79.5	73	62.4	0.228 ± 0.016	0.0262
SB	24	89	76.1	74	63.2	0.232 ± 0.016	
BP	27	77	65.8	75	64.1	0.221 ± 0.015	
KP1	30	104	78.2	83	62.4	0.236 ± 0.015	
KP2	25	106	79.7	94	70.7	0.227 ± 0.014	
KP3	24	106	79.7	86	64.7	0.226 ± 0.015	0.0238
SB	24	102	76.7	84	63.2	0.228 ± 0.015	
BP	27	90	67.7	85	63.9	0.207 ± 0.014	

n number of individuals, NPL number of polymorphic loci, PPL percentage of polymorphic loci, the 95% criterion is all loci with allele frequencies >0.05, H_J is analogous to H_E for AFLP markers.

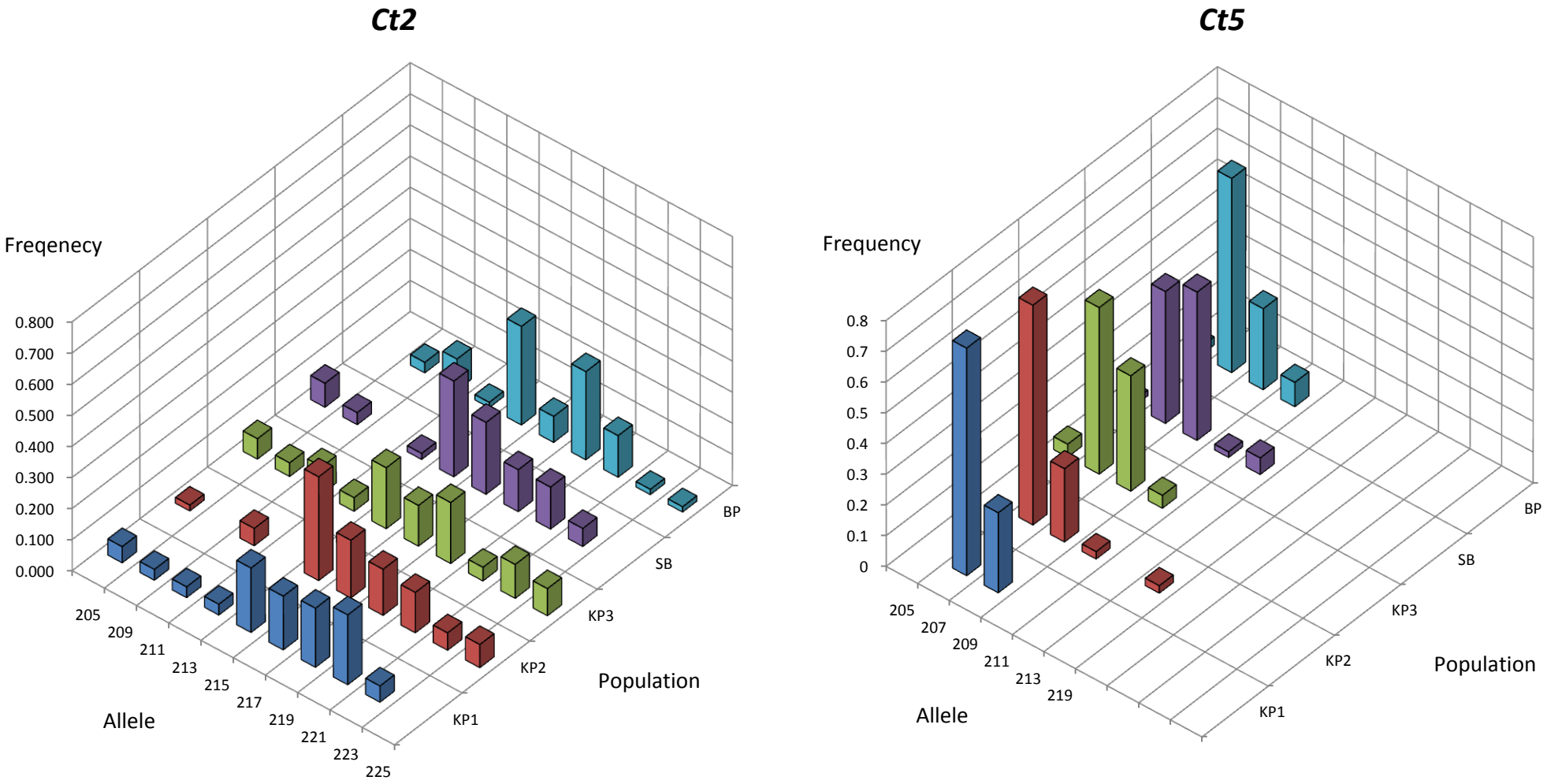


Figure 6 Allele frequencies for five populations of *Ctenotus fallens* at two microsatellite loci.

Table 4 Pairwise F_{ST} values for five populations of *Ctenotus fallens* based microsatellite loci *Ct2* (below diagonal) and *Ct5* (above diagonal)

	KP1	KP2	KP3	SB	BP
KP1	*	0.000	0.036	0.116	0.000
KP2	0.004	*	0.027	0.104	0.000
KP3	0.008	0.004	*	0.005	0.000
SB	0.000	0.000	0.011	*	0.058
BP	0.011	0.006	0.011	0.020	*

Table 5 Pairwise φ_{PT} values (analogous to F_{ST}) for five populations of *Ctenotus fallens* based on AFLP 117 markers (below diagonal) a combined dataset of 133 markers (above diagonal).

	KP1	KP2	KP3	SB	BP
KP1	*	0.051	0.057	0.069	0.085
KP2	0.060	*	0.022	0.057	0.040
KP3	0.059	0.011	*	0.041	0.042
SB	0.085	0.058	0.038	*	0.029
BP	0.098	0.056	0.039	0.012	*

Table 6 Pairwise values for Nei's (1973, 1978) genetic distance (*D*) between five populations of *Ctenotus fallens*, based on 117 AFLP markers (below diagonal) a combined dataset of 133 markers (above diagonal).

	KP1	KP2	KP3	SB	BP
KP1	*	0.007	0.007	0.009	0.013
KP2	0.007	*	0.001	0.008	0.012
KP3	0.009	0.001	*	0.006	0.007
SB	0.010	0.008	0.007	*	0.002
BP	0.014	0.014	0.009	0.002	*

Table 7 values for Nei's (1973, 1978) genetic distance (*D*) between five populations of *Ctenotus fallens*, based on microsatellite loci *Ct2* (below diagonal) and *Ct5* (above diagonal).

	KP1	KP2	KP3	SB	BP
KP1	*	0.001	0.040	0.138	0.009
KP2	0.102	*	0.044	0.147	0.007
KP3	0.170	0.115	*	0.033	0.024
SB	0.074	0.044	0.158	*	0.111
BP	0.118	0.094	0.127	0.161	*

The Analysis of Molecular variance (AMOVA) for both microsatellite datasets and AFLP and combined datasets showed nearly all of the total variation (99% at *Ct2*, 96% at *Ct5* and 95% AFLP and combined data) was partitioned within populations not among them (Table 8). PCA plots of genetic distance between all samples based on the AFLP and combined datasets show very weak clustering with all populations largely overlaying each other (Figures 7 & 8). PCA plots and Mantel tests of these data revealed some relative similarities among pairs of populations which was somewhat related to geographic distance.

The PCA plot of the *Ct2* dataset revealed no strong associations between populations and set KP3 and BP apart from the other populations (Figure 9) while a PCA plot for *Ct5* sets SB apart from the other populations while grouping KP1 and KP2, and also KP3 and BP (Figure 10). Conversely PCA plots of pairwise φ_{PT} values for the AFLP and combined datasets both set KP1 apart from the other populations while indicating relative similarity between KP2 and KP3 (Figure 11). The plot derived from the AFLP dataset also indicated relative similarity between SB and BP; however these two points were driven further apart with the inclusion of the microsatellite data (Figure 12). No significant trends were detected by Mantel tests correlating *D* with geographic distance based on the microsatellite data (Figure 13). The same test of the AFLP and combined datasets revealed a significantly positive association (Figure 14). However, where *D* for microsatellite data ranged from 0 to 0.17 the range for AFLP and combined data of 0.001 to 0.013 was an order of magnitude smaller.

Table 8 Analysis of molecular variance of five populations of *Ctenotus fallens* based on two microsatellite loci, 117 AFLP markers and a combined dataset of 133 markers.

Source	*df*	*SS*	*MS*	*Est. Var.*	Percentage of total variance
Among Pops					
Ct2	4	2.252	0.563	0.003	1%
Ct5	4	2.944	0.736	0.010	4%
AFLP (117)	4	121.642	30.410	0.703	5%
Combined (133)	4	150.061	37.515	0.841	5%
Within Pops					
Ct2	265	110.248	0.416	0.416	99%
Ct5	229	58.415	0.255	0.255	96%
AFLP (117)	125	1521.312	12.170	12.170	95%
Combined (133)	125	1962.709	15.702	15.702	95%

SS sum of squared deviations, *MS* variance components estimates, *Est. Var.* variance components estimates.

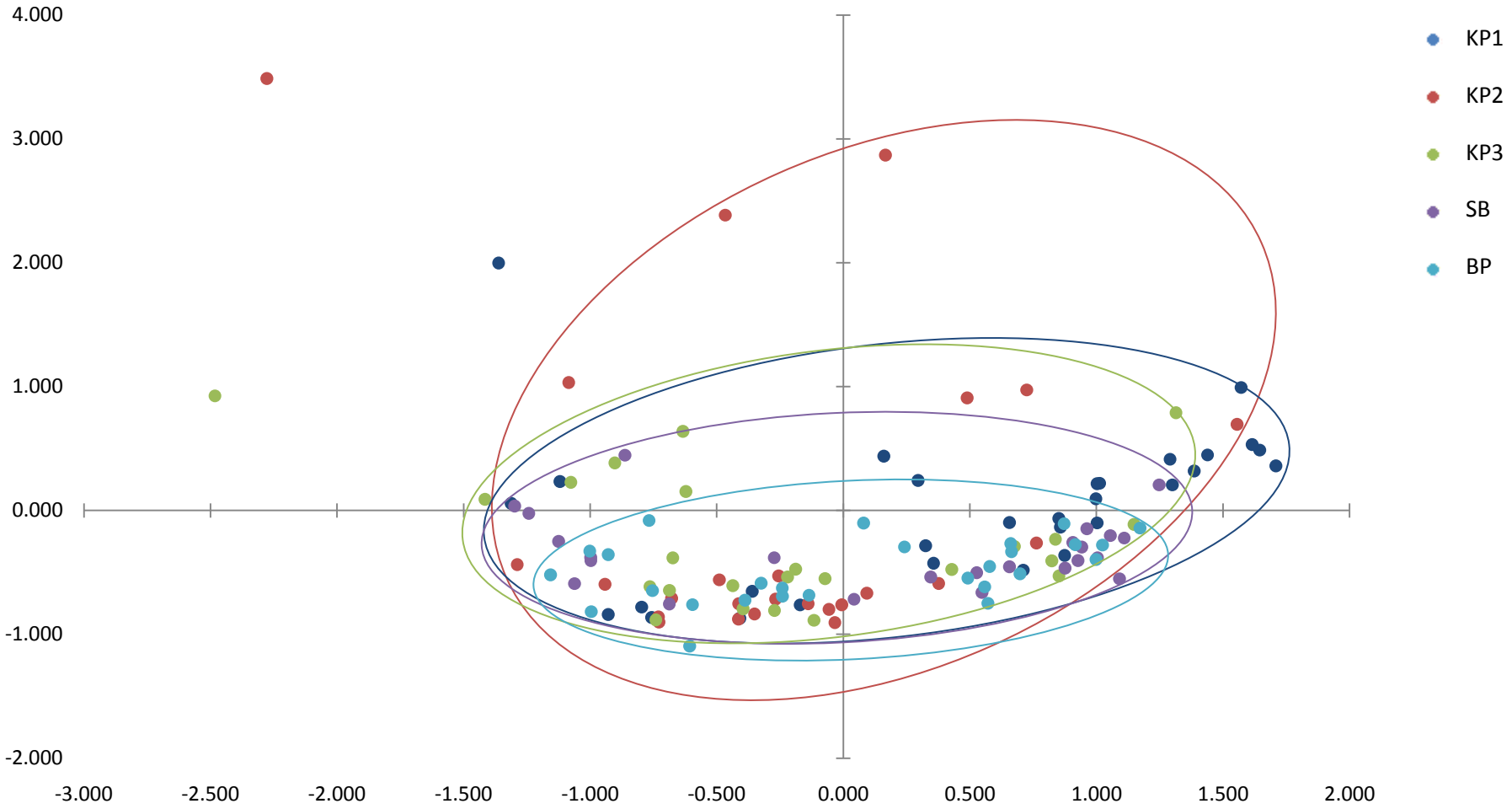


Figure 7 Plot of the first two components of a principle coordinate analysis of the genetic distance between 130 *Ctenotus fallens* individuals from five populations based on 117 AFLP markers. The *x* axis accounts for 38.37% of variation and the y axis 26.29%. Genetic distance was calculated in GENALEX (REF, 0000) and ellipses drawn by hand excluding extreme outlying data points.

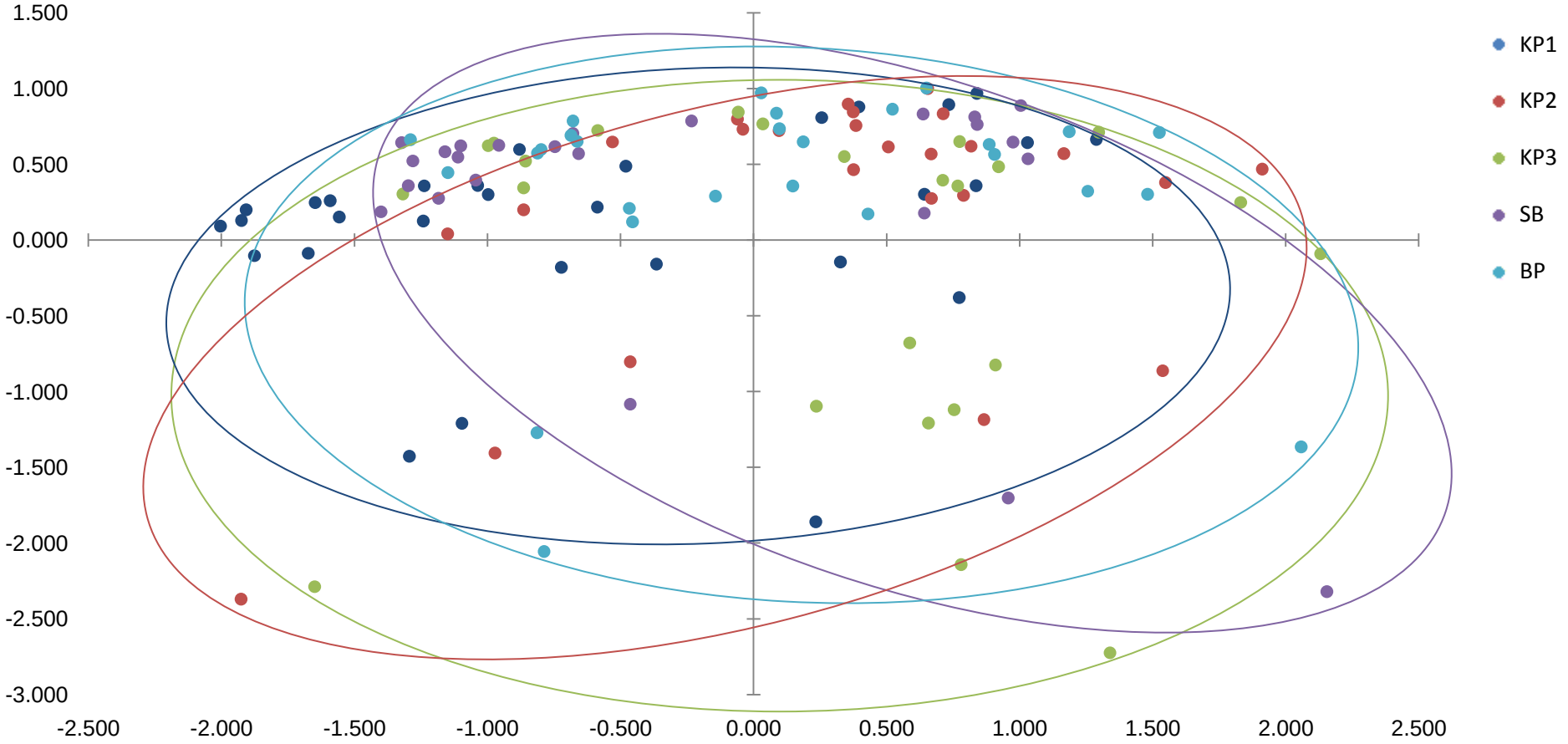


Figure 8 Plot of the first two components of a principle coordinate analysis of the genetic distance between 130 *Ctenotus fallens* individuals from five populations based a combined dataset of 133 markers. The *x* axis accounts for 38.37% of variation and the y axis 26.29%. Genetic distance was calculated in GENALEX (REF, 0000) and ellipses drawn by hand.

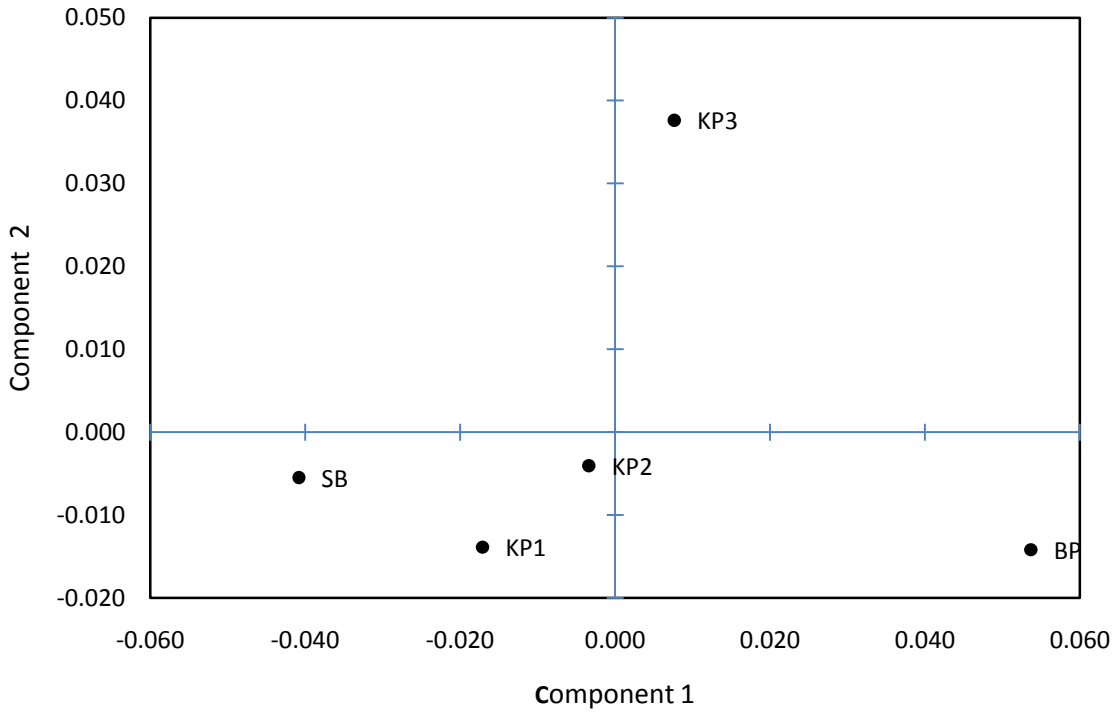


Figure 9 Plot of the first two components of a principle coordinate analysis of pairwise F_{ST} values between five populations of *Ctenotus fallens* based on one microsatellite loci, *Ct2*. The first component accounts for 63.14% of variation, the second 23.86%.

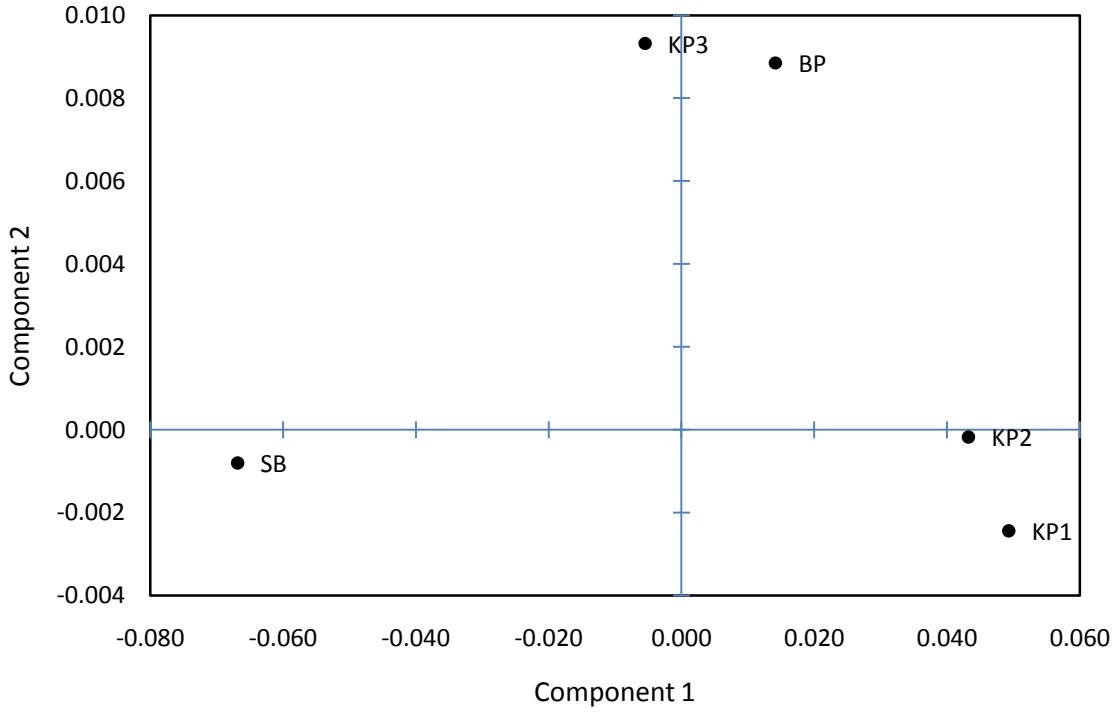


Figure 10 Plot of the first two components of a principle coordinate analysis of pairwise F_{ST} values between five populations of *Ctenotus fallens* based on one microsatellite loci, *Ct5*. The first component accounts for 98.13% of variation, the second 1.87%.

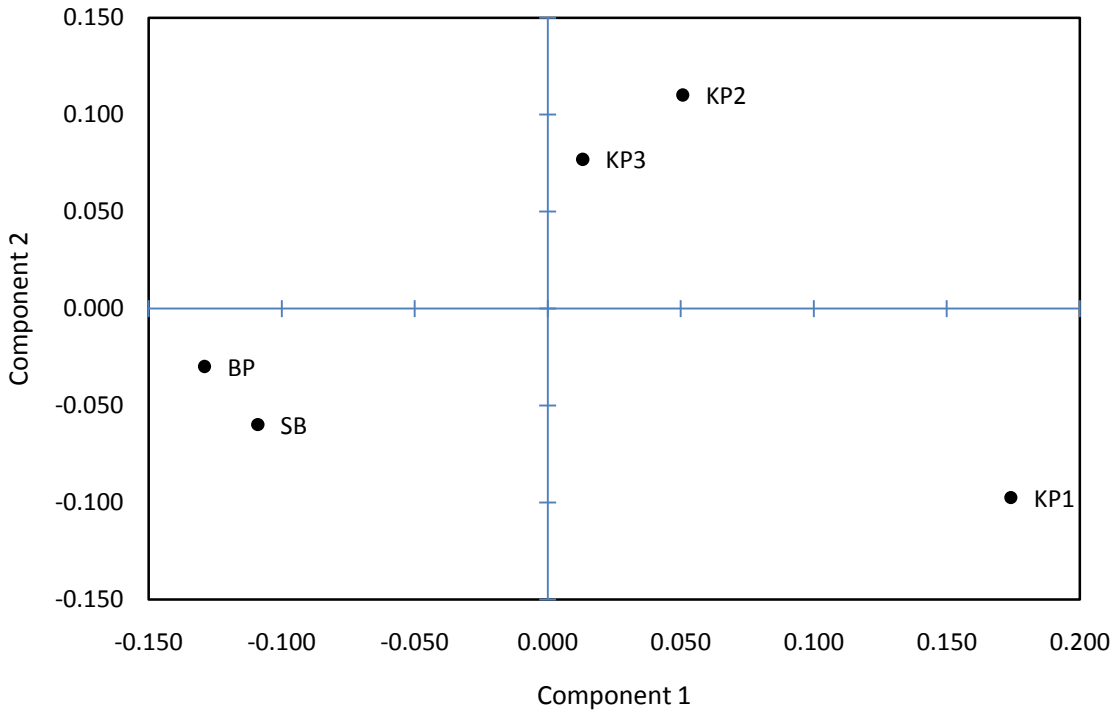


Figure 11 Plot of the first two components of a principle coordinate analysis of pairwise φ_{PT} values between five populations of *Ctenotus fallens* based a dataset of 117 AFLP markers. The first component accounts for 59.51% of variation and the second 30.93%.

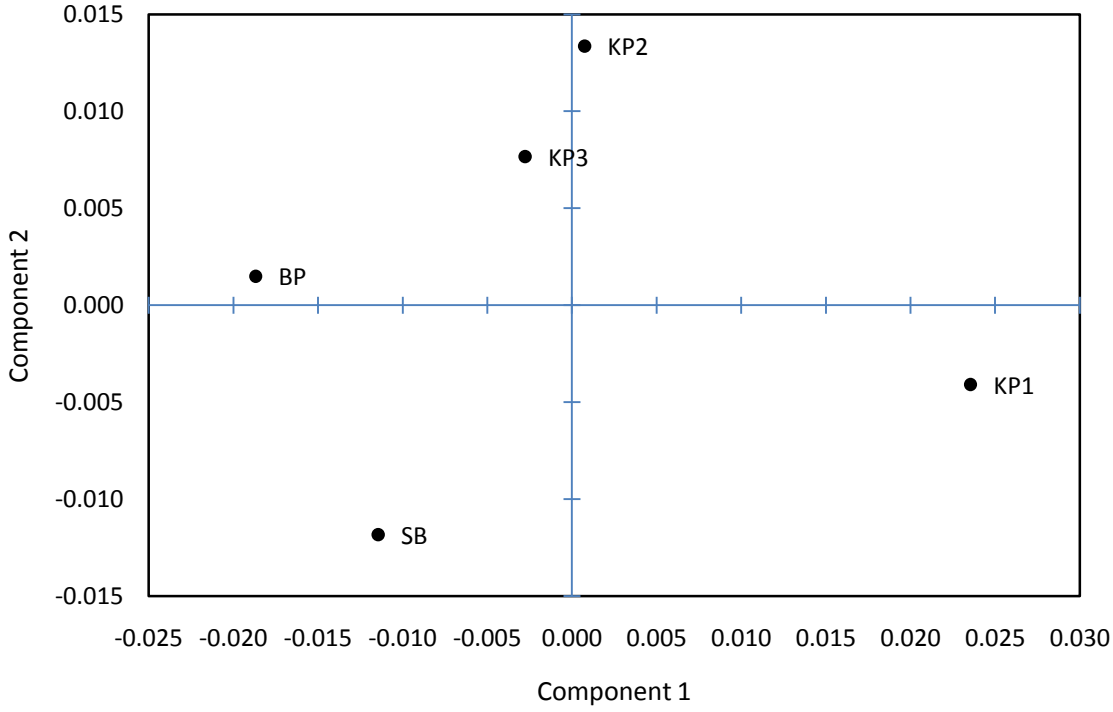
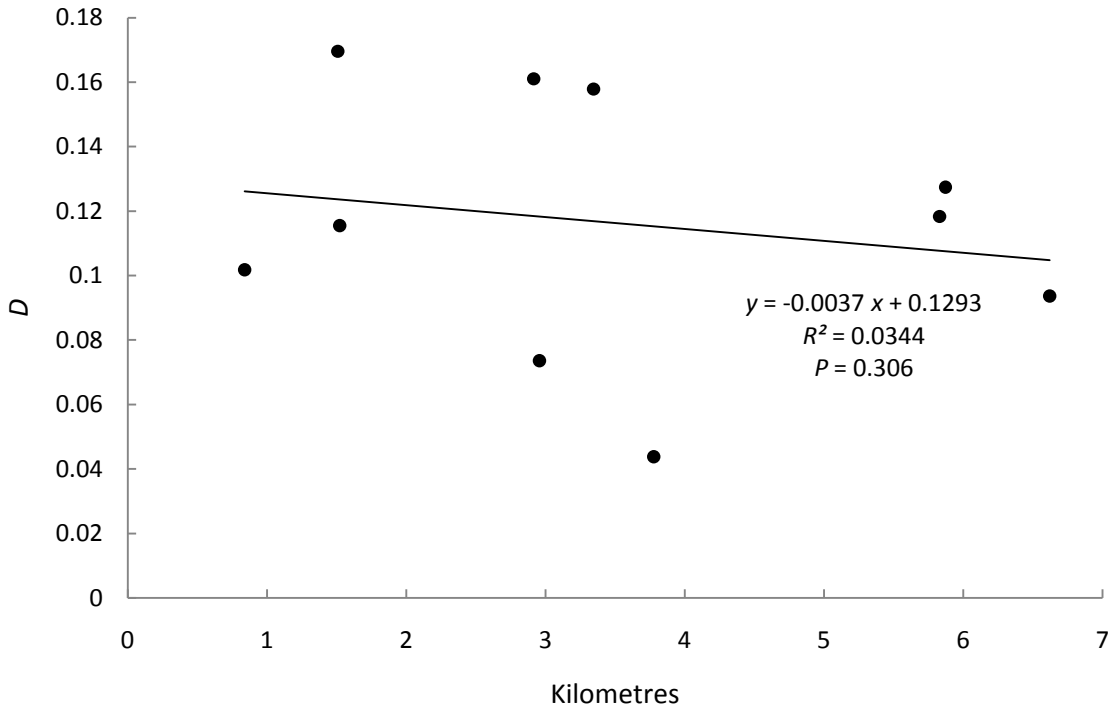


Figure 12 Plot of the first two components of a principle coordinate analysis of pairwise φ_{PT} values between five populations of *Ctenotus fallens* based on a combined dataset of 133 markers. The first component accounts for 66.55% of variation and the second 25.27%.



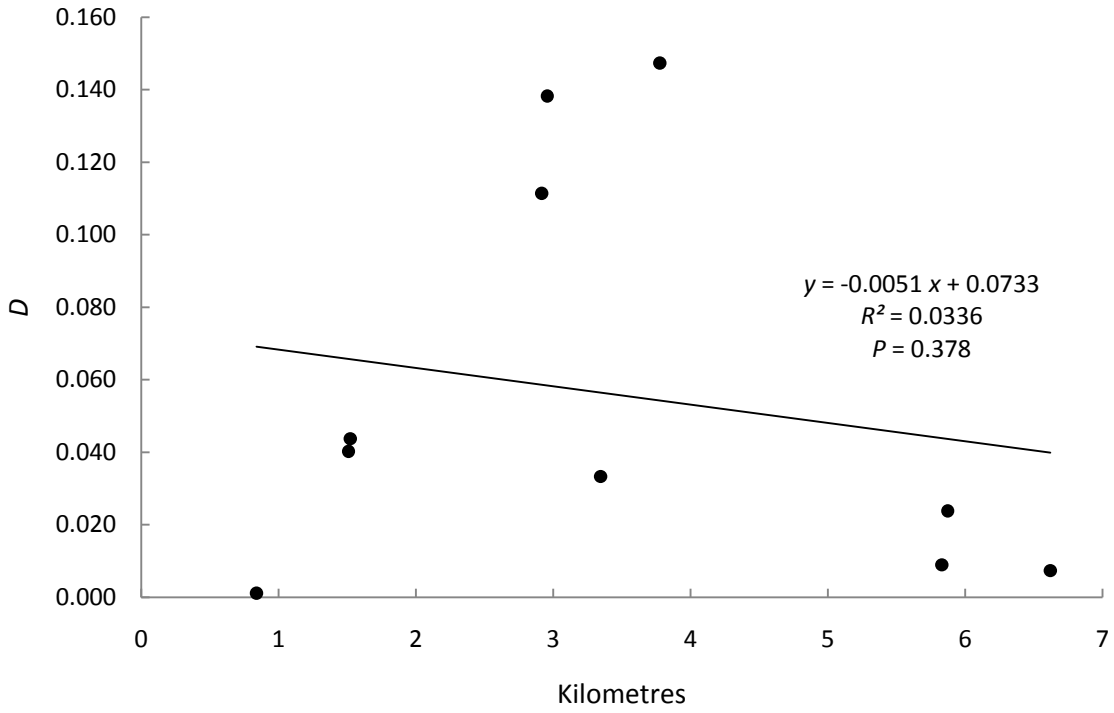
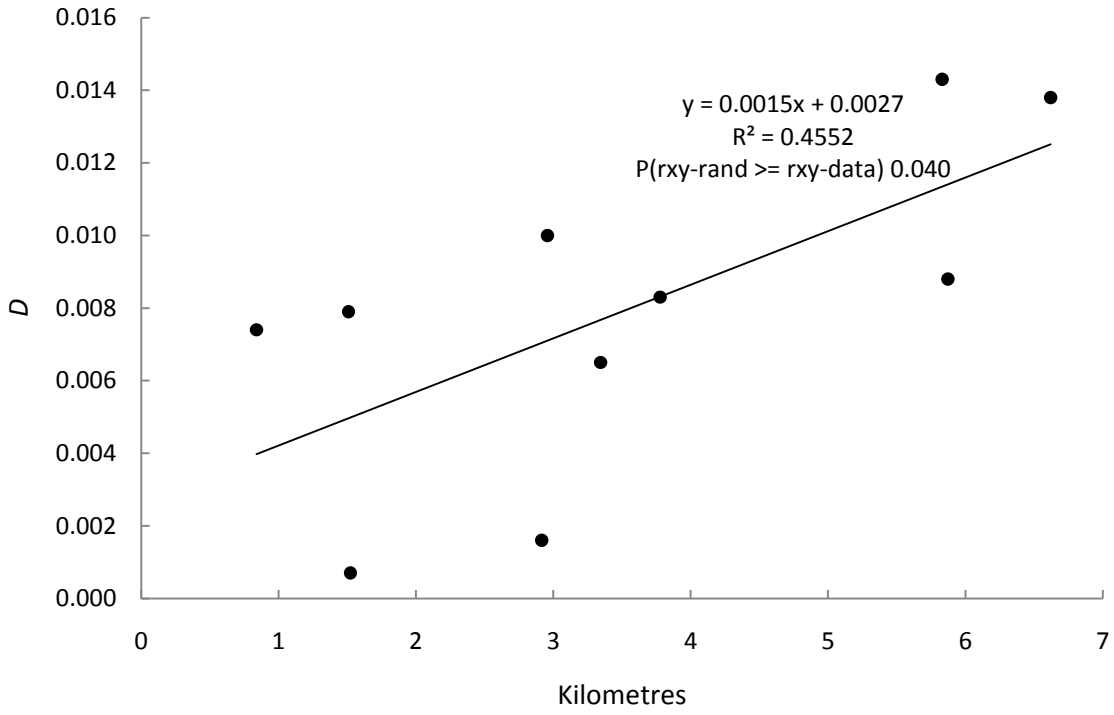


Figure 13 Nei's (1973, 1978) genetic distance verses geographic distance for all possible pairwise combinations for five populations of *Ctenotus fallens* based on two microsatellite loci, *Ct2* (top) and *Ct5* (bottom). Significance was tested using a Mantel (1967) randomisation test (999 permutations). R^2 fit of regression line, *P* probability of positive autocorrelation (one tailed).



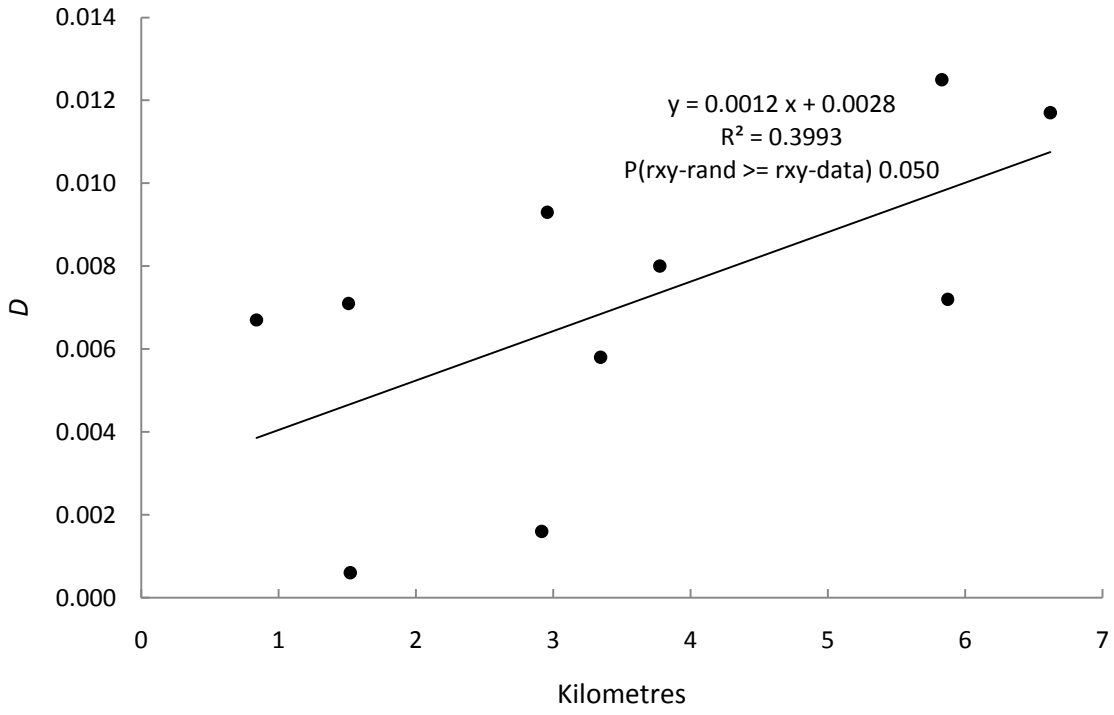


Figure 14 Nei's (1973, 1978) genetic distance verses geographic distance for all possible pairwise combinations for five populations of *Ctenotus fallens* based on 117 AFLP markers (top) and a combined dataset of 133 markers (bottom). Significance was tested using a Mantel (1967) randomisation test (999 permutations). R^2 fit of regression line, *P* probability of positive autocorrelation (one tailed).

Discussion

The results obtained from both microsatellite and AFLP analyses overall tell a similar story with regards to spatial genetic structure among populations. Estimates of genetic differentiation among populations as shown by F_{ST} and AMOVA were similarly low for the AFLP and microsatellite datasets. The application of these two independent marker techniques is a strength of the current study and the concordance in results for genetic differentiation generates confidence as to the accuracy of the conclusions drawn.

However the microsatellite data also produced some results that did not match those of the AFLP analysis and in particular with regard to the pattern of isolation by distance detected by AFLP but not either microsatellite. Given that the microsatellites represent just two points on the genome whereas AFLP represents 117 it is likely that the AFLP analysis had more power to resolve patterns in genetic differentiation than the microsatellites. As the addition of the microsatellite data to the AFLP data made little difference to the AFLP results the two data sets will for the most part be discussed concurrently, referred to as the AFLP data.

Firstly genetic variation within populations will be discussed, focusing on some of the more unexpected results arising from the microsatellite analysis. This will be followed by a discussion of genetic differentiation among populations. While this discussion draws on a few core examples from the literature, it should be noted that few studies have examined the genetic consequences of habitat fragmentation on lizards with most molecular genetic work focusing on resolving patterns of dispersal and the phylogeographic structure over wide areas (Stow et al. 2001; Branch et al. 2003; Urquhart et al. 2009). The relative advantages and disadvantages of the two methods will be discussed ending with some suggestions for future research.

Genetic differentiation within populations

The most direct comparison to make for interpreting estimates of heterozygosity at the microsatellite loci is between *C. fallens* and *C. taeniolatus* (the species for which the microsatellites were characterised)(Smith & Stow, 2008). At *Ct2* H_E was slightly lower for all populations than that reported for *Ct2* in *C. taeniolatus* during characterisation of the microsatellite loci. However H_O in *C. fallens* was slightly higher than that reported for *C. taeniolatus* (H_E 0.892, H_O 0.714 vs. H_E 0.819, H_O 0.785) (Smith & Stow, 2008). In small genetically isolated populations high levels of inbreeding (as indicated by high F_{IS} values caused by an excess of homozygotes in comparison to HWE expectations) are predicted (Lowe et al. 2004; Allendorf & Luikart, 2007). *Ct2* however suggests the opposite.

Heterozygosity at *Ct5* however was lower than that of *C. taeniolatus* (H_E 0.866, H_O 0.786 vs. H_E 0.549, H_O 0.497). Interestingly three populations showed a significant excess in heterozygotes at *Ct5* when compared to HWE which resulted in the negative values observed for F, F_{IS} and F_{IT}. Small effective population sizes can often account for an excess of heterozygotes, especially if the population has recently gone through a genetic bottleneck or experienced a founder effect so that all individuals sampled are the offspring of a small number of parents (Allendorf & Luikart, 2007). Although the size of populations of *C. fallens* at each remnant is unknown, 30 adults were trapped at each site with a relatively small trapping effort (approx. 250 trap nights at Shenton Bushland) so it is unlikely that the population sizes are small. Recent fires (Jan. 2009) at KP1 and KP2 may have caused local extinctions and genetic bottlenecks, but there are no obvious reasons why a bottleneck may have also occurred at KP3, SB and BP.

An excess in heterozygotes can also occur if natural selection favours heterozygotes over homozygotes. The selective advantage of heterozygotes however must be substantial for any changes to be detected in genotypic proportions (Allendorf & Luikart, 2007). While microsatellites predominantly occur in non-coding regions of DNA and are usually non selective (Lowe et al. 2009), studies have shown that non random distribution of microsatellite alleles can occur between populations with alleles becoming associated with different habitats (Li et al. 2000). Evidence suggests

that the distribution of microsatellites throughout the genome is also non random and that microsatellites have been shown to have roles in chromatin organisation, gene expression and DNA replication (Li et al. 2000). If *Ct5* is affected by selection pressures this may account for the observed excess in heterozygotes. This idea is also supported by the low allelic diversity observed at *Ct5* in comparison to *Ct2* as selection pressures would maintain a high number of similar sized alleles by selecting against detrimental alleles. Indeed if *Ct5* is subject to selection pressures, selection seems to favour individual with allele sizes of 207 and 209 which would explain the lack of alleles among all five populations and specifically at KP1. In contrast the greater allelic diversity at *Ct2* may well be due to higher mutation rates at this locus. However, this does not appear to be the case for these loci in *C. taeniolatus* which were shown to have 8 alleles at *Ct5* and 12 at *Ct2* (Smith & Stow, 2008).

Another possibility is that *Ct5* occurs on a sex chromosome. Sex linked markers may also produce an excess of heterozygotes if alleles numbers are different between the sex chromosomes (Clarke 1988; Allendorf et al. 1994). When this is the case genotypic probabilities become influenced by sex which throws out HWE (Allendorf & Luikart, 2007). If this were the true however it would be expected that the same excess in heterozygosity should have been observed in *C. taeniolatus* on characterisation of the microsatellite, which again was not the case (Smith & Stow, 2008).

It is also interesting that KP1 did not follow the same pattern as the other four populations at *Ct5*. The lack of rare alleles at KP1 may be further evidence that this population recently went through a genetic bottle neck and this idea would account for KP1's position as an outlier in the PCA plot of pairwise φ_{PT} values from the AFLP analysis. The majority of samples from KP1 were collected from areas that were recently burnt and again this may account for a loss of alleles in the population. However this appears unlikely as the effects of a bottleneck should also be observed in the AFLP data and for *Ct2* which was not the case. Another possible explanation for the *Ct5* at KP1 result is resampling of the same homozygotes at that site. However this is unlikely given the sampling process. Although recaptures were frequent and paint marks did wear off individuals that had been sampled were easily recognisable by the shape of the tail tip. Recently sampled individuals had blunt tips and while tail

regrowth occurred within the trapping period, regrown tails were rounded and the regenerated tissue was grey. Only individuals with tapered tails showing no signs of regrowth were sampled, making resampling very unlikely.

Genetic differentiation among populations

Genetic differentiation of neutral markers is determined by restrictions in gene flow (Lowe et al. 2004). In species where panmictic gene flow occurs there is likely to be little to no genetic structure. Conversely, in species where gene flow is limited by either low vagility, barriers or geographic distance, genetic differentiation will be higher (Lowe et al. 2004; Allendorf & Luikart, 2007). Genetic differentiation between populations of *C. fallens* has been shown here as represented by positive F_{ST}. The question that poses however is whether or not this genetic differentiation is natural due to a limited ability of *C. fallen* to disperse through the landscape, or is a product of recently reduced gene flow due to urban fragmentation. While F_{ST} values are significantly greater than zero, they are also low, indicating a historical capacity for genetic connectivity between populations. This also raises questions as to whether this is a historic pattern which has not yet been effected by recent habitat fragmentation or whether genetic connectivity is real and *C. fallen* is able to disperse between the fragments studied. These issues will be discussed with reference to the relevant literature.

Lizards have been shown to exhibit high genetic connectivity over large areas. In the Gobi Desert, populations of *Phrynochephalus prezwalski* that were hundreds of kilometres apart had much greater genetic connectivity than the populations studied here (Urquhart et al. 2009). This was demonstrated by extremely low pairwise F_{ST} values of 0.0062-0.0266. On a similar scale to this study, pairwise F_{ST} values of 0.0024-0.0213 were reported for the lizard *Anolis roquet* along a 4km transect in continuous habitat on the island of Martinique (Johansson, et al 2008). Three species of lizard in continuous habitat in the Santa Monica Mountains in California were found to have average pairwise F_{ST} values of 0.02 (*Uta stansburiana*), 0.0016 (*Plestiodon skiltonianus*) and 0.013 (*Chamaea fasciata*) (Delany et al. 2010). In north-eastern Queensland

populations of the rainforest skink *Gnypetoscincus queenslandiae* sampled in continuous habitat over an area of approximately 10km were found to have a global F_{ST} of 0.057 (Sumner et al. 2004). The results obtained by these studies indicate that estimates of genetic differentiation for lizard populations are generally low reflecting populations with historically high rates of gene flow.

However dispersal (and therefore genetic connectivity) between populations has also been shown differ significantly in closely related species. Populations of two species of gecko *Oedura reticulata* and *Gehyra variegata* were studied in continuous habitat reserves in the Western Australian wheat belt. Due to the different vagilities of the two species genetic differentiation between populations of each species was significantly different as demonstrated by F_{ST} values which were different by an order of magnitude (0.044 and 0.004) (Hoehn et al. 2007).

Habitat can also play a major role in differentiation of populations within a species. Populations of the alpine lizard *Eulamprus leuraensis* in south-eastern Australia had pairwise F_{ST} values of 0.028-0.32. In this instance genetic differentiation between populations was caused by restricted gene flow with elevation (Dubey & Shine, 2010). Some of the highest F_{ST} results for lizards were recorded for the *Microlophus albemarlensis* complex, which inhabit the Galapagos Islands (Jordan & Snell, 2008). Genetic differentiation was highest between populations on different islands and global F_{ST} was 0.44 as a result of populations being naturally fragmented by the sea.

Few studies have addressed the effects of habitat fragmentation in lizards. Sumner et al (2004) found that while populations of *G. queenslandiae* in recently (less than 100 years) fragmented populations had similar F_{ST} values to those in continuous habitat (0.046 vs. 0.057), populations in fragmented sites were shown to have fewer alleles per locus and the relationship between geographical distance and genetic distance was greater than in continuous habitats. These results were consistent with a disruption in gene flow predicted to occur among the fragmented sites. Similarly populations of the geckos *O. reticulata* and *G. variegata* in habitat fragmented for approximately 100 years were shown to have lower genetic diversity and higher global F_{ST} values than

those in continuous reserves (0.102 vs. 0.044 and 0.004 vs. 0.003) (Hoehn et al. 2007). Hoehn et al (2007) concluded that, as in continuous habitat, genetic connectivity was higher in *G. variegata* which disperse more readily and more frequently than *O. reticulata.* Sumner et al (2004) concluded that there is an inherent difficulty in detecting disruption in genetic connectivity due to fragmentation in species such as *G. queenslandiae* where dispersal rates are naturally low.

Where few studies have addressed the genetic consequences of habitat fragmentation in lizards, even fewer have explored the effects of urban habitat fragmentation. The few studies that are available for comparison present differing results. Weak genetic differentiation was detected in populations of the skink *Plestiodon reynoldsi* in fragmented urban habitats in Florida (Richmond et al. 2009). Here the authors made similar conclusions to Sumner et al (2004) that as the fragmentation had only occurred 60 years prior, not enough time had passed for detectable changes to occur within the populations and that their results were biased by "genetic inertia" caused by natural demographic traits (Richmond et al. 2009). As a result, any reduction in genetic connectivity due to urbanisation was undetectable (Richmond et al. 2009). Perhaps the most comparable work to that conducted here is a recent study which examined the genetic impacts of urban fragmentation on three lizards (*U. stansburiana, P. skiltonianus* and *C. fasciata*) in southern California (Delaney et al. 2010). This study found that changes in the genetic structure between habitats fragmented by urban development occurred in some cases within a matter of decades. The small isolates examined were of a similar size to those used here, were a similar distance from one another and had been isolated between 13 and 43 years, again comparable to those investigated here. The average pairwise F_{ST} values for the three species (0.073, 0.04 and 0.04) were similar to the values obtained here for *C. fallens* and were significantly higher than the values obtained for the same species in continuous habitat (0.02, 0.016 and 0.013). Here the authors concluded that the urban matrix between habitat fragments was a much stronger barrier to gene flow than natural continuous habitat of the three species. Once again, the authors did acknowledge that significant genetic differentiation occurred between populations in continuous habitat due to low rates dispersal. However in this case, urbanisation was such a strong barrier to gene flow

that differences in the genetic structure of the fragmented populations could be detected.

The populations of *C. fallens* studied here are similarly genetically differentiated to populations of the three species studied by Delany et al (2010) in urban fragmented habitat. It may therefore, be concluded that as for *U. stansburiana P. skiltonianus* and *C. fasciata* the urban matrix between the habitat remnants studied here is an unnatural barrier to dispersal and therefore restricts gene flow in *C. fallens.* However, it should also be taken into account that greater genetic differentiation has been shown between populations for *E. leuraensis* (Dubey & Shine, 2010) in a natural habitat and therefore the differentiation shown here may well reflect natural patterns that are a result of natural low vagility or habitat preference. Yet a third possibility is that the genetic differentiation shown here is natural and historic and that the urban matrix presents yet another barrier to gene flow between populations. However as was hypothesized by Sumner et al (2004) and Richmond et al (2009) it may be that the true effects of urban habitat fragmentation are not yet detectable over the natural genetic differentiation between populations due to "genetic inertia" (Richmond et al. 2009.

The results obtained here suggest that a historical level of gene flow connected the populations studied. If as in the study by Delany et al (2010) fragmentation of the populations had caused a rapid and strong genetic effect it would be expected that more variation would have been shown in the AMOVA and more clustering of samples from the same population in the PCA of genetic distance. That given, it is therefore unlikely that the F_{ST} observed here is a result of habitat fragmentation. There are therefore two possible explanations for the genetic differentiation observed here between populations of *C. fallens*:

1. That the genetic differentiation between the populations is largely natural and urbanisation has had little effect on the population genetics of the species due to its ability to disperse through the urban matrix: *Urban dispersal hypothesis.*

2. That the urban matrix is a barrier to dispersal and therefore gene flow, however not enough time has past to detect the effects due to historic patterns genetic differentiation: *Recent isolation hypothesis.*

These two hypotheses will be discussed in turn.

Urban dispersal hypothesis

It is possible that *C. fallens* is able to disperse through the urban matrix and find mates in adjacent urban habitat remnants. The results of the AMOVA, which showed weak differentiation among populations, supports this hypothesis as does the very weak pattern of clustering, observed in the PCA of genetic distance. Urban areas however, have been shown to act as very strong barriers to dispersal leading to genetic differentiation of populations with examples in nearly every animal taxa: Reptiles – Delany et al. 2010; Clark et al 2010; Mammals – Magel et al. 2010; Riley et al. 2006; Birds - Delany et al. 2010; Amphibians – Noel et al. 2007; Invertebrates – Vandergast et al. 2009; Field et al. 2006). Roads in particular are known to be a very strong barrier to dispersal especially for reptiles (Andrews et al. 2008; Clark et al, 2010). Direct mortality from road crossing may be exacerbated by immobilisation behaviour in response to traffic noise, or by roads actively attracting reptiles for thermoregulatory purposes (Andrews et al. 2008). Urban areas also expose reptile to introduced predators such as cats and foxes and dogs (Barrat, 1997; Koenig et al. 2002). Additionally reptiles are at increased risk of death from environmental factors in urban areas due to lack of refugia (Koenig et al. 2002).

In light of these facts, is it then possible that *C. fallens* is currently able to disperse through an urban environment? How & Dell (1994) reported that seven species of lizard are known to persist to in the inner urban gardens of the Perth Metropolitan area and *C. fallens* is not among them. All of these urban persisting species are small, less than 10cm snout-vent-length (barring *Tiliqua rugosa*, the persistence of which according to How & Dell (1994) is uncertain). One possibility is that small reptiles have a survival advantage over larger species in urban environments. Although patterns of dispersal in *C. fallens* are largely unknown, one theory is that dispersal is natal and

occurs in the first year. It is possible that juveniles are able to cross the urban matrix into other habitat fragments due to their small size. On the single occasion that the author has encountered *C. fallens* within an urban area the individual was a juvenile and had presumably crossed a busy intersection from the nearest habitat. Although mortality rates would presumably be high for *C. fallens* dispersing through urban areas, population genetic theory shows that only a single migrant per generation is required between remnants to prevent genetic differentiation among populations by drift alone (Wright, 1931; Slatkin, 1987). However this number is thought to become high as 20 in populations that experience large temporal fluctuations in size (Vucetich & Waite, 2000).

A defining feature of the urban matrix is that it presents a wealth of novel microhabitats and ecological niches (Bolund & Hunhammar, 1999).) In Switzerland it was found that urban populations of the wall skinks (*Podarcis muralis*) connected by train lines showed higher levels of genetic connectivity than unconnected fragments that were geographically closer together (Altherr, 2007). Through the application of further GIS techniques the author concluded that train lines act as a dispersal corridor in that species (Altherr, 2007). While the only train line relevant to this study (Fig 1) is more likely to act as a barrier than a corridor, it is possible that other unconventional dispersal corridors are present between the habitat fragments studied.

If not the effects of urbanisation, what then could be causing the genetic differentiation observed between the populations? As previously mentioned a number of studies have shown patterns of isolation by distance among reptile communities in continuous habitat (Sumner et al. 2004; Hoehn et al. 2007; Dubey & Shine, 2010). Isolation by distance occurs both within and among populations where gene flow is restricted by distance alone (Wright, 1943, 1946; Lowe et al. 2009). It is characterised by organisms with low vagility that are more likely to find a mate a short distance away creating a cline in the assortment of alleles across the landscape or in the case of *E. leuraensis* up the side of a mountain (Lowe et al. 2004; Dubey & Shine, 2010). The significant association between genetic distance and geographic distance observed here is a strong indicator of isolation by distance (Wright, 1943, 1946). Current understanding of the ecology of *C. fallens* supports this idea. Both males and females

maintain site fidelity, possibly defending a home range and individuals have been experimentally shown to move less than 50m in a 120 day period (Jennings & Thompson, 1999). Interestingly studies that have shown significant genetic differentiation between populations of lizards due to h`abitat fragmentation have shown no or reduced correlation between geographic distance and genetic distance (Sumner et al. 2004; Delany et al. 2010). It should also be noted that the pattern of isolation by distance was only detected by the AFLP analysis and not for the microsatellites. This is probably due to the different powers associated which each analysis which will be discussed further below.

Recent isolation hypothesis

On finding evidence for weak genetic structuring, a number of studies of reptiles in fragmented habitats have concluded that fragmentation was so recent that a reduction in gene flow was undetectable (Sumner et al. 2004; Richmond et al. 2009). Richmond et al. (2009) put forward three hypotheses to explain the lack of differentiation observed between populations of the sand skink *P. reynoldsi* in fragmented urban habitats. The first hypothesis, that contemporary migration prevents inbreeding and genetic differentiation has already been discussed and the conclusions Richmond et al (2009) are not relevant here as they relate to the specific ecology of *P. reynoldsi*.

The second hypothesis put forward was that populations of *P. reynoldsi* within fragments were large enough to allow random mating and therefore prevent a loss of genetic diversity, even within small fragments. This idea is supported by research which suggests that in some instances the abundance and species richness of reptile assemblages in fragmented urban habitat is dependant on the quality of the remnant vegetation complexes as opposed to fragment size and degree of isolation as suggested by island biogeography theory (Jellinek et al. 2004; MacAther & Wilson, 1967). As previously discussed the population size of *C. fallens* in each fragment is unknown but is assumed to be large given the ease of trapping. Additionally each site is managed and weed species are controlled to some degree which has slowed the degradation of the native vegetation complex. This, according to Jellinek et al (2004),

may maintain the abundances of reptile species within the fragments. Additionally following fragmentation ecological specialists are usually the first species to begin declining (Fahrih, 2003). As an ecological generalist (Read, 1998) *C. fallens* would presumably be one of the last species to decline following fragmentation. Further support for this idea comes from the high heterozygosity and allelic diversity at *Ct2* and low inbreeding coefficient.

The third hypothesis put forward by Richmond et al (2009) is somewhat an extension of the second which proposes that a time lag exists where urban habitat fragmentation is having an effect on the genetic structure of the populations but aspects of the species life history and demography, including large population size, buffer the effects. Large populations could buffer the genetic effects of fragmentation until abundance had dropped below a certain threshold. Similarly long generation times would have a similar buffering effect. In *G. queenslandiae* (Sumner et al. 2004) genetic differentiation was not detected in populations fragmented for 50 to 80 year even with greatly reduced population densities. This was attributed to life history strategies and long generation time (Sumner et al. 2004). Based on simulations, a reduction in genetic drift should be detectable after 10 generations (Noel et al. 2007). In *C. fallens* this equates to 20 years as individuals breed in their second spring. While BP and SB only became isolated from each other in the past 30 years, KP has been completely isolated by urban development since at least 1953 and may have been isolated by land clearing earlier still. In light of this, if this hypothesis were true it is more likely that high abundance in *C. fallens* is causing a time-lag and this is preventing the genetic impact of fragmentation from being detected above the inertia of the historical patterns of isolation by distance.

Comparison of methods

Diversity estimates made using different genetic markers often differ (Mariette et al. 2002; Lowe, 2004). Therefore the use of different types of marker is desirable in conservation genetics and this is a strength of the current study. As they are under different selection pressures, different mutation rates and represent different parts of

the genome, results obtained for different sets of markers can better inform on current and historical processes occuring with and among populations (Alacs et al. 2011)

Microsatellites are widely used in the study of population genetics (Lowe, et al. 2004). As they are multialleleic and co-dominant at a single locus, they can be highly informative and can therefore be used to detect very recent (<50yrs) changes in populations structure due to anthropomorphic barriers to gene flow (Keller & Largiadèr, 2003; Riley et al. 2006; Delany et al. 2010). While cross species amplification of microsatellites is often attainable (e.g. Riley et al. 2006), it can also be problematic. This can be due to due to the persistence of stutter bars (Fig 3) (thought to be caused by the slip-strand misrepair during PCR) (Litt et al. 1993) poor PCR amplification, null alleles and large allele drop-out (Urquhart et al. 2009). These problems that can render markers useless, even in the analysis of species for which they were characterised (e.g. Urquhart, 2009). In this study while microsatellites for *C. taeniolatus* could be amplified in *C. fallens* poor PCR amplification prevented any usable data from being generated from most loci. This was a hindrance to the study as a lot of time had been spent attempting to optimise the PCR conditions for the amplification of the microsatellites.

AFLPs on the other hand have a relatively quick and cost effective start up time as markers are not specific to any organism (Lowe et al. 2004). Typically numerous markers (>1000) can be generated relatively easily with moderate cost (Lowe, 2004; Bensch & Åkesson, 2005). The individual markers however are dominant and biallelic and it is therefore impossible to distinguish between homozygous and heterozygous genotypes (Mariette et al. 2002; Bensch & Åkesson, 2005). This makes estimates of genetic variation subject to bias (Meriette et al 2002). This was observed here in the lower estimates of heterozygosity for the AFLP markers than microsatellites. Given that each AFLP marker is informationally poor more of them are needed than co dominant markers such as microsatellites. Mariette et al (2002) estimated that four times as many dominate markers (such as AFLPs) were required than co-dominant markers to give results with the same efficiency.

As each different marker set has different properties one type marker may provide information that another cannot. Mutations at microsatellite markers in the form of addition and deletion of repeat sequences occur relatively frequently. Microsatellites are therefore useful for detecting recent changing in genetic structure (Lowe et al. 2004). AFLPs represent randomly amplified points on the genome therefore contain markers that exhibit both fast and slow mutation rates (Lowe et al. 2004). AFLP markers are therefore useful as they are able to detect historical patterns of genetic structuring due to their conservative estimates of differentiation (Alacs et al. 2011). With this in mind it is therefore advantageous to use as many different marker systems as possible. For example Alacs et al. (2011) used both AFLP and microsatellites to determine the population genetic structure of quokkas in Western Australia. Data generated from AFLP detected historic pattern of connectivity between mainland populations which were differentiated from an island population. Microsatellites revealed more recent structuring of mainland populations (Alacs et al. 2011). The different evolution rates between the two sets of markers may also explain why AFLP detected a pattern of isolation by distance here, when the microsatellites did not. Similarly the fast mutation rates of the microsatellites may account for the unusual grouping of populations observed in the PCA for pairwise F_{ST} as these results did not correlated with each other or with the results obtained for AFLP.

The use of AFLPs has been dominated by botanical studies since their development by Vos et al in 1995. Studies initially focused on agricultural species and their pathogens (Bensch & Åkesson, 2005). In 2003 only 115 studies had applied AFLP technology studies of animals, compared with 1223 plant studies (Bensch & Åkesson, 2005). It is thought that the spread of the application of AFLP from plants to animals may have been held back as botanists and zoologists often work in different institutions and publish in different journals (Bensch & Åkesson, 2005). However it should be noted that there is no fundamental reason why AFLPs should not be used in the analysis of the population genetic structure of animals provided a sufficient number of markers are generated (Mariette et al. 2002; Bensch & Åkesson, 2005).

Further research

With sufficient time and resources, the ability to compare populations within a fragmented habitat to an equivalent continuous habitat is required to understand the effect of habitat fragmentation (Sumner et al. 2004; Hoehn et al. 2007; Noel et al. 2007; Delany et al. 2010). Due to the scope of the current research, sampling of continuous habitat here was unfeasible. If, as for *U. stansburiana, P. skiltonianus* and *C. fasciata* (Delany et al. 2010), populations of *C. fallens* in continuous habitat were shown to be significantly less genetically differentiated than those in the fragmented habitat, then it could be concluded that the differentiation between populations observed here was a result of urban habitat fragmentation. Comparison of results with populations in continuous habitat should also confirm whether isolation by distance on the scale observed here is a common genetic feature in populations of *C. fallens*. If however this was found not to be the case, it could be concluded that the patterns of genetic differentiation could be due to habitat fragmentation. Another option would be to sample a population separated from those studied here by a strong natural barrier such as the Swan River (Fig 1). From this we would expect to see much greater differentiation between populations.

Many comparable studies have further resolved current levels of long distance dispersal between populations by using assignment tests (Riley et al. 2006; Noel et al. 2007; Delany et al. 2010). Population assignment testing can be used to identify genetically similar groups of individual and can also be used to infer individual instances of gene flow. Riley et al (2006) used this method effectively to identify coyotes and bobcats that had crossed a highway by assigning them to populations originating on the opposite side. The method could be used here to determine whether individuals sampled at one site are assigned to another, thus potentially confirming current dispersal through the urban matrix. Given sufficient markers, populations assignment testing can be performed using either AFLP or microsatellite data (Lowe et al. 2004).

In the course of this research, knowledge of dispersal in *C. fallens* was found to be

lacking. This information would offer insight as to the likelihood of dispersal between habitat fragments. Radiotelemetry could be used to determine dispersal in *C. fallens* as well as providing direct measures of gene flow. This was achieved successfully for the Siberian flying squirrel (Selonen et al. 2010). Transmitters are now available that weight just 0.47g and have been used to track animals as small as shrews (Rychlik et al. 2010). It would therefore be feasible to track even juvenile *C. fallens* via radio telemetry to determine dispersal and perhaps also whether they enter the urban areas or do not approach edges as is thought to be the case for *P. reynoldsi* (Richmond et al. 2009).

As the occurrence of recaptures was high and the marking scheme to prevent resampling was successful, one missed opportunity of this study was to collect mark-recapture-release data. This could have been used to estimate effective population size and even contribute some information on dispersal (as per Jennings & Thompson, 1999). The trapability and recapture rate for this species does however indicate future success for similar mark-recapture-release studies of this species. As mark-recapture-release studies can be used to give estimates of population size, this information could be used to confirm or refute Richmond et al.'s (2009) hypothesis that large populations are able to maintain random mating within habitat fragments and forestall genetic differentiation. Additionally the hypothesis that habitat fragmentation has not affected the population genetic structure of *C. fallens* due to large population size could be tested by performing a similar experiment on a less abundant species of lizard.

A complementary non-genetic method used alongside genetic analysis by some other studies is a comparison of morphology between populations (Noel et al. 2007; Skidmore et al. 2010). Patterns in morphology could be used to support patterns observed in the analysis of genetic features. One morphological variance noted for *C. fallens* during sample collection was arrangement of its nasal scales; some were fused and others separate. More simply length and weight could have been recorded for all samples and compared across populations.

Conclusions

This study produced insights into the population genetic structure of *C. fallens* within a fragmented urban landscape, which was interpreted in the light of other population genetic studies on lizards and from which levels of historical gene flow were inferred. Throughout this study numerous avenues for further research relating to this prevalent and for the most part poorly studied species have been identified. The Perth metropolitan area has one of the richest inner city assemblage of herpetofauna of anywhere in the world (How & Dell, 1994; 2000). Present knowledge suggests that reptile diversity remains high even in small remnants (How & Dell, 1994). Despite this however, previously common species are disappearing from these habitats. For example, in a survey of SB in 2008, one *C. australis* was trapped for every five *C. fallens* (Berry & Berry). None were encountered during the trapping associated with this study, which targeted *Ctenotus* specifically in SB. While it is unlikely that such a fate awaits *C. fallens*, it is important that an understanding is obtained of how fragmentation is affecting reptiles on a genetic level and if genetic isolation is a factor contributing to the local extinction of species. This study demonstrated high historic levels of genetic connectivity between populations of *C. fallens* up to 8km. What remains unclear is whether these same levels of genetic connectivity are currently occurring within the fragmented urban matrix and whether these populations are at risk of a genetic decline. Further studies are required to resolve these outstanding issues so that appropriate management can be implemented.

Drawing by Vincent Tran – taken from Jennings & Thompson 1999

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