Human-induced versus historical habitat shifts: identifying the
processes that shaped the genetic structure of the threatened grassland
legless lizard, *Delma impar*

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Abstract

Historical and contemporary events are known to affect the genetic structure and diversity of species. Thus, in order to design effective conservation management strategies for threatened species, it is important to identify the processes that shaped their genetic patterns. The Striped Legless Lizard *Delma impar*, is currently listed as a threatened species, and is restricted to the temperate grasslands of south eastern Australia. This habitat has undergone both historical climatic and recent anthropogenic distributional changes. We used mitochondrial DNA and microsatellite markers to examine the phylogeography and population genetic structure of *D. impar*. Analysis of mtDNA revealed four distinct historically isolated lineages with high levels of genetic divergence that have been isolated for more than one million years. These lineages should be considered separate Evolutionarily Significant Units (ESU) for management purposes. Analysis of microsatellite genotypes did not reveal evidence of strong population sub-structuring despite recent anthropogenic fragmentation within the south west Victorian ESU. However, two populations were identified by the program Structure, which coincide with the transition of two regions with distinct soil and vegetation characteristics. Spatial autocorrelation analyses indicate that *D. impar* do not disperse long distances as they are genetically structured at distances less than 400m.

Keywords: phylogeography, population genetics, Pygopodidae, temperate grasslands

Introduction

A common goal of conservation is to maintain and protect genetic diversity (McNeely *et al.* 1990). Species with high levels of genetic diversity may have a greater potential to adapt to environmental change and fight disease and, therefore, are at a lower risk of extinction (Lacy 1997). A number of historical and contemporary processes are known to significantly impact the genetic diversity and structure of species (Avise 1992; Olivieri *et al.* 2008). The management strategies implemented for the protection and maintenance of a species' genetic diversity depend on the processes that have affected it. For instance, human-induced habitat fragmentation often impedes dispersal between habitat patches and consequently reduces gene flow between remnant populations (Saunders *et al.* 1991). In such cases the management strategy would be to re-establish gene flow between these recently fragmented populations in an effort to maintain genetic diversity within populations. This may, in turn, decrease the risk of inbreeding (Frankham *et al.* 2002) and increase the long-term survival of a species (Storfer 1999). Naturally occurring historic fragmentation events, such as periods of glaciation or marine incursions, may isolate populations for long periods (Chapple and Keogh 2004; Schneider *et al.* 1998; Vandergast *et al.* 2007). This may lead to high levels of genetic differentiation between populations due to drift and local adaptation (Frankham *et al.* 2002). Re-establishing gene flow between these historically isolated populations could drastically alter the genetic structure of populations and alter the evolutionary potential of a species (Mortiz 2002). These historically isolated populations may be designated as Evolutionarily Significant Units (ESU) by conservation managers as a way to maintain and protect genetic diversity (Fraser and Bernatchez 2001). In these cases ESUs should be managed and protected independently from each other in order to maintain the unique genetic composition of each historical lineage (Moritz 2002). Thus, it is important to have an understanding of the different processes, both natural historic and current

anthropogenic, that have shaped the genetic structure of a species in order to make informed conservation management decisions.

The Striped Legless Lizard, *Delma impar*, is an ideal candidate to illustrate the different fragmentation processes that impact the genetic diversity and structure of a species. *Delma impar* is a habitat specialist restricted to the temperate grasslands of south eastern Australia, a habitat that has undergone both ancient and modern habitat fragmentation. Although this ecosystem underwent distributional changes during the glacial-interglacial cycles of the late Pliocene-Pleistocene (Kershaw *et al.* 1994), large expanses of temperate grasslands were present in south eastern Australia prior to European settlement (Kirkpatrick *et al.* 1995; Lunt 1991) (Fig. 1). Since then, these areas have been highly fragmented and severely modified due to urbanisation and agriculture, and it is estimated that only 0.5% of these landscapes remain (Kirkpatrick *et al.* 1995).

Owing to severe habitat modifications and reductions, *D. impar* is now patchily distributed in remnant temperate grasslands of New South Wales (NSW), the Australian Capital Territory (ACT), Victoria (VIC) and South Australia (SA), and is listed as threatened in jurisdictions across its range (NSW Threatened Species Conservation Act 1995, ACT Nature Conservation Act 1980, VIC Flora and Fauna Guarantee Act 1988, SA National Parks and Wildlife Act 1972) (Smith and Robertson 1999). One of the management objectives for *D. impar* has been to maintain genetic variation across its geographic range to ensure the evolutionary potential of the species (Smith and Robertson 1999). Working groups and agencies that are responsible for co-ordinating all management actions relating to *D. impar* have developed a population management cluster approach for the conservation of this species (ARAZPA 1996). Populations, or groups of populations, have been tentatively arranged into 17 management clusters (Fig.1), which have been determined according to the habitat characteristic of the surrounding bioregion and the notion that they may represent

different ESUs (ARAZPA 1996). This management approach was chosen because it assumes that populations in each region are adapted to the local habitat and may contain a unique genetic make-up distinctive to that region. Currently, there is information on genetic variation for only three of these management clusters, with analysis of allozyme frequency data indicating little variation either within or among clusters (ARAZPA 1996; Osmond 1994). A better understanding of the population genetic structure of this species and the historical phylogeographic structure between populations is needed in order to improve the conservation management strategies of *D. impar*. Both historical and modern fragmentation events are known to have affected the genetic structure and diversity of other grassland specialists (modern fragmentation: Eastern Barred Bandicoot *Perameles gunnii*, Golden Sun Moth *Synemon plana* – Clarke and O’Dwyer 2000; Clarke and Whyte 2003; Robinson *et al.* 1993; historic fragmentation: *Perameles gunnii*, *Synemon plana*, Grassland Earless Dragon *Tympanocryptis pinguicolla* – Clarke and Whyte 2003; Melville *et al.* 2007; Robinson 1995; Scott and Keogh 2000), and it is likely that *D. impar* presents similar patterns.

In this study, we used mitochondrial DNA (mtDNA) sequences and multilocus microsatellite genotypes to examine the population genetic structure and phylogeographic patterns of *D. impar*. This information will allow a comprehensive assessment of historical biogeographic versus current anthropogenic impacts on this threatened species. By obtaining information on the phylogeography of this species, ESUs may be identified and, if so, managed appropriately. Information on population genetic structure and dispersal ability will help us determine the extent of gene flow across the landscape in this species, and where gene flow may need to be re-established between fragmented populations.

Materials and Methods

Tissue sampling

133 tissue samples (liver, tail tips and shed skins) were obtained from 12 of the 17 *Delma impar* management clusters (Fig. 1). These samples covered a broad geographic area: South Australia (SA), the Australian Capital Territory (ACT), two clusters in New South Wales (NSW), and eight in Victoria (VIC). Samples were provided by the Victorian Department of Sustainability and Environment (DSE), the South Australian Department of Environment and Heritage (DEH), and from tissue collections at the Australian National Wildlife Collection and Museum Victoria (Appendix A). The largest numbers of samples were obtained from south western VIC, collected by the DSE as part of an ongoing survey program which has been running since 2004. In this survey *D. impar* has been recorded at 73 sites, with the majority located within management clusters 3A and 3B (G. Peterson and C. Grant pers. comm.). Each survey site consists of a grid laid out with roof tiles that are used as artificial refuges by *D. impar* (Rohr and Peterson 2003) and which facilitate capture. Tissue samples were collected from captured lizards by removing the tail tip and storing it in 75% ethanol. In addition, *D. impar* skin sloughs were found under the tiles and these were also collected and used as genetic samples.

DNA extraction and mtDNA sequencing

Genomic DNA was isolated from tissues using the Easy DNA High Speed Extraction Tissue Kit (Saturn Biotech), following the manufacturer's protocol. The mtDNA NADH dehydrogenase subunit 2 (ND2) gene was sequenced to determine the phylogeographic structure of *D. impar*. Amplification primers used were: forward 5' AAGCTTTCGGGCCCATACC 3' (Macey *et al.* 1997) and reverse 5' GTCTGAGTTGCA TTCAGAGGA 3' (this study). DNA amplifications were performed in a 20µL total volume containing 2µL DNA (diluted to 1:100 or 1:200 in TE buffer), 10µL Gotaq (Promega), 0.8µL of each primer (10µM) and 6.4µL H₂O. The polymerase chain reaction (PCR) amplification

was performed on a Corbett Research Palm Pilot Thermal Cycler using a touchdown program with the following parameters: initial denaturation 94°C for 5 minutes, followed by two cycles of 94°C for 30 seconds, an annealing step of 65°C for 30 seconds and 72°C for 1 minute and 30 seconds; this cycle was repeated with annealing temperatures of 60°C, 55°C and 50°C (2 cycles each) and a final annealing temperature of 48°C for 30 cycles. This was followed by a final extension step of 5 minutes at 72°C. Negative controls were included in all amplifications. In some cases, particularly with shed skin samples, the first amplifications were weak and a reamplification was necessary, using 1 µl of the initial PCR product (diluted to 1:100 in TE buffer) and the same primers and PCR conditions. Amplified products were purified with Exo Sap IT (USB) following manufacturer's instructions and the purified products were sent to Macrogen (Korea) for sequencing. DNA sequences were assembled and edited in Geneious version 4.5 (Drummond *et al.* 2008) and then manually aligned in PAUP* beta version 4.0b10 (Swofford 2000). Translated amino acids were assessed in MEGA version 4.1 (Tamura *et al.* 2001) to verify the alignment and to check for internal stop codons.

Phylogeographic analysis and estimates of divergence times

Where available, up to six tissue samples from each management cluster were included in the analyses of mtDNA (Appendix A). Also, one previously published sequence of *D. impar* from the ACT management cluster (Jennings *et al.* 2003) was included, and ten previously published sequences were used as outgroups (Jennings *et al.* 2003).

Phylogeographic relationships within *D. impar* were estimated with Bayesian criterion using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). jModelTest version 0.1.1 (Posada 2008) was used to find the optimal model of molecular evolution for the mitochondrial data, using the Akaike Information Criterion (AIC). The parameters of the best fitting model were used in the Bayesian analysis. Four Markov chains were used in each

of two simultaneous runs starting from different random trees. Analyses were run for 10 million generations, sampling every 100 generations, resulting in 100,000 sampled trees. The first 10,000 trees were discarded as “burn-in”, and the remaining 90,000 trees were used to estimate Bayesian posterior probabilities.

A relaxed molecular clock method was used within the program BEAST version 1.4.5 (Drummond and Rambaut 2007) to estimate the historical divergence times between populations of *D. impar*. A pygopodid fossil, *Pygopus hortulanus*, dating from the early Miocene (20-23 million years ago) (Hutchinson, 1997) was used as a calibration point for the analysis. This fossil provides a minimum age estimate for the group that includes *Pygopus lepidopodus* and *P. negriceps* (Jennings *et al.* 2003). As fossils are a minimum estimate of age, a lognormal distribution was used at the calibration point, with a Zero Off-set of 20 and standard deviation of 1.3. The node of *P. lepidopodus* and *P. negriceps*, and the node of all *D. impar* sampled were constrained to be monophyletic compared with other taxa. A GTR+I+ Γ model of evolution was employed, using an uncorrelated lognormal relaxed molecular clock without a fixed substitution rate across the tree. A Speciation: Yule Process prior was used and the analysis was run for 10 million generations, sampling every 1000 generations. The output was input into Tracer version 1.4.1 (Drummond and Rambaut 2007) to check that stationarity had been reached and to assess the autocorrelation of rates from ancestral to descendant lineages (Drummond *et al.* 2006).

Microsatellite amplification

Eight polymorphic microsatellite loci, developed for *D. impar* as part of this study (Genetic Identification Services USA), were used to identify the population genetic structure of south western VIC populations obtained from the DSE survey program and to assess dispersal ability using molecular methods (Table 1). DNA was extracted using the methods

described above. DNA amplifications were performed in a 10 μ L total volume containing 1 μ L DNA (diluted to 1:10 in TE buffer), 5 μ L Gotaq (Promega), 3.5 μ L H₂O, 0.2 μ L of reverse primer, 0.2 μ L of a forward primer modified with the additional 5' sequence 5'GCCTCCCTCGCGCCATCAG3' and 0.1 μ L of a fluorescently labelled primer with the reverse complement of this linking sequence adapted from Schuelke *et al.* (2000) (Blacket *et al.* 2012). PCR amplifications were performed on a Corbett Palm Pilot Thermal Cycler using the following steps: initial denaturation 95°C for 5 minutes; followed by ten cycles of; a denaturation step of 94°C for 30 seconds, an annealing step of 57°C for 1 minute and an extension step of 72°C for 30 seconds, which was repeated for a further 25 cycles but with an annealing temperature of 55°C; and a final extension step of 10 minutes at 72°C. The PCR products were sent to the Australian Genome Research Facility for fragment analysis and genotyping, using an AB3730xl capillary sequencer and AB GeneMapper (Applied Biosystems) respectively.

Population genetic analyses

The population genetic analyses used genotypes of 107 individuals from 13 sites within the two main south western VIC management clusters (3A and 3B - Fig. 1). The program Microsatellite Toolkit version 3.1.1 (Park 2001) was used to check for samples that had identical genotypes. Population structure was examined using two Bayesian clustering software programs: Structure version 2.3.2 (Pritchard *et al.* 2000) and Geneland version 3.1.5 (Guillot *et al.* 2005a,b). Both of these programs probabilistically cluster individuals based on their genotypes into K clusters (or populations) while minimising Hardy-Weinberg and linkage disequilibrium. The major difference between the two programs is that Geneland takes into account the geographical location of samples; Structure can use information on putative population clusters to assist in determining the number of populations (K).

In Structure, we ran the model 10 times with under a parameter set of no admixture of ancestry and correlation of allele frequencies. A LOCPRIOR model was used with individuals assigned to one of 5 location clusters according to sampling locality. The number of populations (K) was estimated for 1 to 7, for 10,000 burn-in, followed by 1,000,000 MCMC iterations. To determine the number of populations (K) we plotted the mean posterior probability, $\ln \Pr(X|K)$, from the 10 runs against K to determine where the values reached a plateau (Pritchard *et al.* 2009). The number of clusters was also inferred by calculating ΔK from the rate of change between successive simulations (Evanno *et al.* 2005).

For Geneland, we used the spatial model with no correlation of allele frequencies. This model is appropriate if a pattern of isolation by distance is suspected (Guillot 2009). Ten independent runs with 100,000 MCMC iterations (thinning=100) were performed allowing K to vary from 1 to 7. The potential error for spatial coordinates was set to 100m; other parameters were left at default values. The outputs were processed with a burn-in length of 200. The results (number of populations, probability of membership and posterior densities) were checked for consistency across the ten runs.

Genotypes at all loci and populations (as determined by Structure) were analysed with the program ML-NullFreq (Kalinowski and Taper 2006) to test for the presence of null alleles, using 10,000 randomizations and adjusting the significance levels ($P < 0.05$) with Bonferroni corrections for multiple tests. Linkage disequilibrium (LD) between pairs of microsatellite loci and departures from Hardy-Weinberg equilibrium (HWE) within each population and locus were evaluated using a Markov Chain Monte Carlo (MCMC) method (10,000 dememorizations, 200 batches, 5000 iterations) in GenePop web version (Rousset 2008), and significance levels were adjusted with Bonferroni corrections for multiple tests at $P < 0.05$. To evaluate genetic diversity within identified populations, the number of total and private alleles (A, A_p), and observed and expected heterozygosity (H_o, H_e), was calculated in

GenAIEx version 6.2 (Peakall and Smouse 2006). Additionally, estimates of allelic richness (A_E), derived from rarefaction and corrected for variable sample sizes were calculated in HP-Rare (Kalinowski 2004). We calculated the inbreeding coefficient (F_{IS}) in Arlequin version 3.11 (Excoffier *et al.* 2005), to determine if there was evidence of inbreeding in any of the identified populations.

We calculated pairwise F_{ST} values among the populations inferred by Structure using Arlequin version 3.11 (Excoffier *et al.* 2005). The program Bottleneck version 1.2.02 (Cornuet and Luikart 1996) was used to test if any of the three populations presented an excess of heterozygosity, indicative of a recent reduction in population size. This was tested by using a one-tailed Wilcoxon test for a two-phase mutation model with 95% single step and 5% multiple-step mutations, as recommended for microsatellite data (Piry *et al.* 1999).

Isolation by distance (IBD) patterns between pairs of individuals were also determined by testing the correlation between individual genetic distance (represented by a_i (Rousset 2000) and e_i statistics (Leblois *et al.* 2003; Watts *et al.* 2007)) and the logarithm of geographical distances using Genepop version 4.0 (Rousset 2007). In continuous populations, an IBD pattern will occur if genetic differentiation among individuals increases with their geographic distance (Wright 1943). Therefore, a significant positive IBD pattern will be observed if dispersal in *D. impar* is limited by distance.

The spatial pattern of genetic variation was investigated by using spatial autocorrelation analysis which examines the genetic similarity between pairs of individuals at different distance classes, thus providing results on the scale at which spatial pattern occurs. This was done in GenAIEx version 6.2 (Peakall and Smouse 2006) by running the analysis using distance size classes of 50m, 100m, and 150m over 6, 7, and 8 size classes. Individuals were permuted among spatial locations 999 times and 95% confidence intervals were

obtained from the permuted data. The results of the autocorrelation analysis are presented graphically in correlograms showing changes in genetic correlation values (r) over distance classes. A significant positive genetic structure at any distance class was inferred when the standard errors of the r -values fell outside the 95% confidence intervals.

Results

Sequence alignment and phylogeographic inference

Forty three new sequences of 1093 bp of the mitochondrial genome are reported for *Delma impar*. These sequences were aligned for phylogeographic analysis with previously published sequences: one *D. impar* and ten outgroup sequences of *Aprasia smithi*, *Pletholax gracilis*, *Pygopus orientalis*, *P. lepidopodus*, *P. nigriceps*, *Delma labialis*, *D. borea*, *D. inornata*, *D. mitella* and *D. mollerii* (Jennings *et al.* 2003). All base positions in the protein-coding gene ND2 were alignable. Twenty-five complete ND2 sequences (two from cluster 6, three from cluster 9, four from cluster 12, two from cluster 3A and fourteen from clusters 2, 3A, 3B and 5) shared identical haplotypes (Appendix A). In such cases, only one representative sequence was incorporated in the phylogeographic analyses to optimise the ability of analyses to determine phylogenetic relationships between haplotypes. Of the 1093 aligned sites, 159 were variable and 359 were parsimony informative. A GTR+I+ Γ model was selected as the best-fitting model for likelihood analysis using AIC criteria. Model parameters are: $\alpha=1.8030$; proportion of invariant sites=0.46; substitution rates $R(a)=2.1707$, $R(b)=10.8309$, $R(c)=2.1707$, $R(d)=1.0$, $R(e)=10.8309$, $R(f)=1.0$; and empirical base frequencies $A=0.3644$, $C=0.3524$, $G=0.0757$, $T=0.2075$. The two runs in the Bayesian analysis converged on similar mean ln-likelihoods (-7022.57, -7019.16).

The phylogram recovered from the Bayesian analysis (Fig. 2) indicated the presence of four highly supported monophyletic lineages within the sampled populations of *D. impar*

($\geq 99\%$ posterior probabilities). The lineage that comprises all of the sampled management clusters from NSW and the ACT is the sister clade to all other lineages. Another lineage (SA/Horsham) is composed of the SA management cluster (cluster 12) and the south west VIC management cluster near Horsham (cluster 6). The other south west VIC management clusters (clusters 2, 3A, 3B, 4, 5 and 11) form a clade (south west VIC), which is sister to the lineage composed of the east VIC management cluster (cluster 9). These last two lineages (south west VIC and east VIC) form a clade that is sister to the SA/Horsham lineage.

Estimates of divergence times

Examination of the BEAST log file in Tracer indicated that the dataset had a small degree of autocorrelation, with a mean estimate of the covariance of parent and child branches of -0.003. However, this was not considered significant because zero was included in the 95% HPD (-0.386-0.340) (Drummond *et al.* 2006). The co-efficient of rate of variation was estimated to be 0.373 (95% HPD: 0.190-0.583), indicating that the data set is not strictly clock-like and that a lognormal relaxed clock is appropriate.

The divergence dating analysis indicates that the common ancestor of the *D. impar* lineages date to the mid Miocene, approximately 18.9 million years ago (Ma) (95% CI: 6.9-24.9). It is estimated that the NSW/ACT lineage originated in the Pliocene, approximately 3.1 Ma (95% CI: 2.7-12.3). Within the VIC/SA cluster, the split between the SA/Horsham lineage and the rest of VIC was approximately 1.6 Ma (95% CI: 0.9-4.0). The last two lineages, south west VIC and east VIC, diverged during the Pleistocene approximately 0.8 Ma (95% CI: 0.3-1.9).

Population genetic structure and dispersal

Four pairs of samples had identical microsatellite alleles at all loci. The sample collection data indicated that all eight samples were shed skins that were collected 9-11 months apart on the same study grid. It is likely that these shed skins were from the same individuals, thus the four duplicate samples were excluded from all analyses. This left a total of 103 samples for which 97.3% of alleles were scored at eight microsatellite loci.

We estimated the number of clusters as occurring at the maximum $\ln Pr(X|K)$ score generated by Structure, $K=3$ (Fig. 3). The Evanno *et al.* (2005) calculation confirmed $K=3$ as the estimated number of clusters. Cluster A consisted of individuals from sample sites west of Hamilton. Individuals sampled from sites east of Hamilton up to Geelong formed Cluster C, but at two sites north of Warrnambool, indicated by the dashed line in Fig. 3, some individuals were allocated to Cluster C and some individuals from the same sites were allocated to a distinct cluster, Cluster B.

Geneland inferred the presence of two distinct clusters; this result was consistent across the ten independent simulations, with all individuals assigned to the same clusters. One cluster included all individuals sampled in the site west of Hamilton, and the second cluster includes all individuals sampled from the sites east of Hamilton (Fig. 3).

We looked at genetic diversity within and among the three identified population clusters identified using Structure. All eight loci in the three population clusters were polymorphic, and heterozygosity estimates in *D. impar* were similar across all the sampling localities (Table 2). Mean observed heterozygosity (H_O) ranged from 0.52 (± 0.22 s.d.) to 0.75 (± 0.19 s.d.) and mean expected heterozygosity (H_E) ranged from 0.56 (± 0.22 s.d.) to 0.76 (± 0.15 s.d.). Allelic richness (A_E) ranged from 1.99 to 16.79 when based on a sample size of 12, and private alleles (A_P) were detected in all sampled sites (Table 2). Departures from Hardy-Weinberg equilibrium were found in Cluster C for loci DiB102 and DiD104 (Table 2) after adjusting the significance (α) level for the pairwise comparisons of population and

loci ($n=24$; $\alpha=0.002$). There was no evidence of linkage disequilibrium after adjusting the significance level for multiple comparisons. Locus DiC5 had a significant probability of null alleles being present at Cluster C and locus DiD104 had a significant probability of null alleles being present at Clusters A and C ($P<0.002$ after Bonferroni adjustment). As null alleles were not present at these loci across all clusters, we retained all eight loci for subsequent analyses. There is no evidence of inbreeding in any of the identified clusters (A: $F_{IS}=0.01$, $P>0.05$; B: $F_{IS}=0.02$, $P>0.05$; C: $F_{IS}=0.03$, $P>0.05$). Analyses in Bottleneck did not show evidence of a recent reduction in population size, as there was no significant excess of heterozygosity in any of the three populations. Pairwise estimates of F_{ST} revealed significant differentiation between all three population pairs (Table 3).

Evidence for an isolation by distance (IBD) pattern was observed (Fig. 4), as indicated by a significant correlation between pairwise individual genetic distance (a_r and e_r values) and the log of geographical distance (Mantel test, $P<0.001$ for a_r and e_r statistics). The regression lines illustrated in Fig. 4 indicate that in both analyses genetic differentiation increased with increasing geographical distance. The spatial autocorrelation analysis also shows a significant r -value at the first three distance classes, 100m ($r=0.088$, $P=0.001$), 200m ($r=0.120$, $P=0.001$), and 300m ($r=0.100$, $P=0.001$) (Fig. 5); indicating that a pattern of small scale dispersal is present in *D. impar*. We chose a distance size class of 100m over 7 size classes as these distances are biologically relevant to this species (O'Shea 2005).

Discussion

Phylogeographic relationships and historical biogeography

The mtDNA sequence analyses revealed high levels of genetic differentiation between geographically isolated populations of *Delma impar*, indicating isolation over long

evolutionary time frames. Sequence divergence between *D. impar* populations in SA and VIC ranged from 0.8 to 2.1%, and between the NSW/ACT and SA/VIC lineages sequence divergence ranged from 5.2 to 6.2%. These latter values are comparable to subspecies levels of divergence found in other south eastern Australian lizard species (Chapple and Keogh 2004; Scott and Keogh 2000).

Our results indicated that the divergence between *D. impar* and its most common ancestor, *D. malleri*, occurred during the mid Miocene; these results are comparable to the ones obtained by Jennings *et al.* (2003). The phylogeographic analysis also showed that the first isolation event within *D. impar* occurred during the Miocene to mid Pliocene (12.3-2.7 Ma), where the NSW/ACT lineage became isolated from the VIC and SA populations. A phylogenetic study on another grassland specialist, *Tympanocryptis pinguicolla*, also found deep genetic divergences (3.9-5.2%) between these two areas (Melville *et al.* 2007). A review of recognised biogeographic breaks in eastern Australia (Chapple *et al.* 2011) identified Southern NSW, and the Murray Basin as a barrier to gene flow in multiple species. Genetic breaks in these regions, estimated to have occurred from the late Miocene to early Pliocene, have been found in the common froglet *Crinia signifera* (Symula *et al.* 2008), White's skink *Liopholis whitii* (Chapple *et al.* 2005), and the garden skink *Lampropholis guichenoti* (Chapple *et al.* 2011). The inclusion of samples from East Gippsland in these studies provide support for the possibility that the phylogeographic break occurred in eastern VIC, possibly due to a marine incursion in the Gippsland Basin that occurred during the late Miocene (Dickinson *et al.* 2002; Gallagher *et al.* 2003). Although this marine incursion may have led to the isolation of *D. impar* populations and to the subsequent genetic divergence of the NSW/ACT populations, there are no records of this species in East Gippsland, therefore phylogeographic methods cannot be used to determine the geographic location of this divergence event.

The historical isolation of *D. impar* during the mid Miocene to mid Pliocene between the NSW/ACT and VIC/SA lineage probably did not result from glaciation, as glaciation in this region did not commence until the late Pliocene (Martin 2006; Wagstaff et al. 2001). Instead shifts to warm/wet climates may have played a role. During the late Miocene and throughout the Pliocene there was a trend towards a cooler and dryer climate, which led to a shift in vegetation in south eastern Australia where rainforests were being replaced by open woodlands and grasslands (Kershaw et al. 1994). However, it is believed that during the early Pliocene the climate became warmer and wetter for a brief period (Gallagher et al. 2003; Macphail 1997), which led to the reappearance of rainforest in the river valleys of the western slopes in south eastern Australia (Martin 2006). Thus, this change in vegetation may have isolated the NSW/ACT lineage of *D. impar* as the grasslands retracted during the early Pliocene.

The divergence between the three lineages of *D. impar* within VIC and SA were estimated to have occurred during the Plio-Pleistocene (0.9-4.0 Ma). The molecular studies of *E. whitii* (Chapple et al. 2005) and *C. signifera* (Symula et al. 2008) also found genetic breaks in this geographic region dating to the Plio-Pleistocene (*E. whitii*) and late Miocene and Pliocene (*C. signifera*). The Plio-Pleistocene period coincides with major climatic and vegetational changes in south eastern Australia (Bowler 1982). Climates during the late Pliocene (approximately 2.5 Ma) oscillated between short, warm periods and longer, cool periods (Bowler 1982). These oscillations were the glacial-interglacial cycles of the Pliocene-Pleistocene. It is believed that woodlands and grasslands expanded and contracted during these glacial oscillations (Kershaw et al. 2004; Martin 2006) and it is probable that these climatic fluctuations and vegetation changes affected the distribution of *D. impar* populations. As a grassland specialist, the distribution of *D. impar* most likely tracked the expansion and contraction of its habitat. Thus, as grasslands fragmented, so would the lizard

populations. The repeated changes in vegetational distribution could have isolated populations, which would have reduced gene flow and may have caused the observed genetic differentiation between the three clades in south west VIC and SA.

In addition, volcanic activity in western VIC during the late Pliocene and Holocene (Joyce 1973; Rosengren 1994) could have shaped genetic structure of *D. impar*. This volcanic activity created the young basalt units known as the Newer Volcanic Province, which extends from central VIC, including Melbourne, through the south west to SA (Rosengren 1999). Extensive lava beds allowed the formation of suitable soil for the spread of grasslands in the region (Jones, 1999), which in turn could have lead to the expansion of the south western VIC lineage.

These natural events could have played an important role in the genetic structure of *D. impar* by isolating populations and preventing or reducing gene flow between areas over long periods of time. The long history of restricted gene flow between these lineages has significant implications for conservation management.

Current population structure and levels of genetic connectivity

Understanding both historical and current levels of gene flow and population connectivity are important in conservation and management planning for threatened species. We looked at population structure among sites in south western VIC for which there were sufficient samples for population level analyses as a result of a long term ecological study on this species. Analyses indicated limited genetic structure in the sampled area, identifying the presence of either two (Geneland analysis) or three populations (Structure analysis) and low but significant F_{ST} values among all three populations identified by Structure. The three population clusters identified by Structure showed similar levels of genetic diversity (mean number of alleles and heterozygosity), with no indication that microsatellite diversity in *D.*

impar is particularly low (H_o : 0.61 - 0.75). We did not find evidence of a recent bottleneck or inbreeding in any of the population clusters. We did, however, find a significant isolation by distance pattern across the sampled region and positive spatial autocorrelation up to approximately 400 metres. Both these statistics indicate that dispersal over this area is non random with individuals more closely related to those animals closer to them geographically. The first x-intercept of this graph can provide an estimate the extent of non-random genetic structure, which occurs over about 500m. Overall these results indicate low, but significant population structure across the sampled area, but the structure does not reflect current habitat fragmentation patterns.

Low genetic differentiation between recently fragmented populations could be a result of two main processes: the first is high levels of vagility, with individuals moving among habitat fragments (Hickerson and Wolf 1998; Ramirez and Haakonsen 1999), and the second is that the observed pattern of genetic structure reflects historical rather than current gene flow (Bossart and Prowell 1998; Monaghan *et al.* 2001). In this case, human-induced habitat changes might be too recent to result in marked genetic effects (Sumner *et al.* 2004) and population densities may be high enough to maintain genetic diversity in the short term without dispersal among fragmented areas. Estimates of population size are hard to obtain for this species, making field verification of this difficult (Smith and Robertson 1999). We suggest that the lack of genetic structure in *D. impar* is a reflection of historical levels of gene flow across the landscape. Field-based studies indicate that *D. impar* is not a vagile species (Coulson 1990; O'Shea 1996). Little is known about its dispersal patterns, the longest movement distance recorded in *D. impar* is 60 m (O'Shea 2005). These data, along with the observed autocorrelation analysis, the isolation by distance pattern obtained in this study and the previous allozyme study (Osmond 1994), suggest that *D. impar* may not be capable of dispersing over long distances, but may disperse frequently over shorter distances. There still

exist remnant patches of native grasslands between the sampled sites that could support populations of *D. impar*. These areas could not be sampled during this study, but it is unlikely that these unsampled populations are contributing to gene flow, as the long distances between the patches (>20 km) and the presence of unsuitable habitat in between the patches would make it very difficult for individuals to move between populations. Therefore, the lack of genetic differentiation observed may be indicative of historical patterns of frequent small scale gene flow across previously continuous habitat rather than current long distance movement of *D. impar* among the isolated habitat patches.

In south western VIC, the transformation of native grassland areas into exotic pasture began in the 1940s and 1950s (Conn 1993; Gibbons and Rowan 1993); and the alteration of grazing properties into crop production areas has been increasing since 1990 (Institute of Land and Food Resources 2000). *D. impar* has been detected in grasslands dominated by exotic perennial tussock grasses (Coulson 1990; Smith and Robertson 1999) and it has been suggested that this species may be able to survive in such areas (Rohr and Peterson 2003; Smith and Robertson 1999). Thus, it is likely *D. impar* populations have only been isolated for approximately 60-70 years. Female *D. impar* start reproducing at 3-4 years of age, have a small clutch size (two eggs maximum), and the maximum recorded longevity is seven years, although researchers estimate a maximum age of 10 years or longer (ARAZPA 1996; Smith and Robertson 1999). Consequently, the fragmentation of populations as a result of significant land clearing may only have occurred ~10 generations ago. Thus, this fragmentation may be too recent to detect in the population genetic data, as more generations are probably required to show detectable genetic signatures (Hartl and Clark 1989).

The boundary between the two populations inferred by Geneland coincides with a transition between the Dundas Tablelands and Victorian Volcanic Plain bioregions. Structure provided similar results to the Geneland analysis, but with further substructure in sites within

the Victorian Volcanic Plain bioregion in close proximity to the Dundas Tablelands (Fig. 3). Although there were discrepancies between Structure and Geneland results, these findings and the significant levels of F_{ST} found between the three populations identified by Structure, indicate that some degree of substructure is present. These two bioregions have different geological and vegetational characteristics. The Dundas Tableland consists of basement rocks that are Cambrian in origin with overlaying Tertiary deposits, while the vegetation is a mixture of grasslands and woodlands (Paine and Phang 2003). In contrast, the Victorian Volcanic Plains are dominated by the younger basalt of the Newer Volcanic Province and much of the area was grassland prior to European settlement (Joyce 2003). It would be advisable to obtain further samples from populations in each of the bioregions in order to determine whether the observed genetic substructure is due to the different ecological characteristics of each of the bioregions.

This study provides support to previous findings that *D. impar* has relatively low dispersal abilities. These findings will be useful for the implementation of new management strategies for the conservation of *D. impar*, as the low vagility of this species and the great distances between remaining habitat patches could eventually lead to the loss of genetic diversity (Frankham *et al.* 2002).

Management implications

The high levels of mtDNA variation between the four historical lineages indicate that each lineage should be considered as a unique Evolutionarily Significant Units (ESUs) (NSW/ACT, SA/Horsham, south west VIC and east VIC). The ESUs reported meet part of Moritz's (1994) definition for ESUs, which requires the identification of historically isolated populations that have reciprocally monophyletic haplotypes. It remains to be determined whether this significant subdivision also applies to nuclear genes. However, even if the four

divergent clades are only present in the mtDNA, each independent historical lineage would still require independent management to maintain the current phylogeographic structure. Thus, a management strategy aimed at protecting and maintaining current genetic diversity would preclude the translocation of individuals between the four geographic regions.

The results from the microsatellite data indicated a potential division within the south west VIC ESU due to a transition between two bioregions. Further sampling is required at the boundary of these two bioregions and in the remaining bioregions within this ESU in order to verify these findings. Direct approaches used to evaluate dispersal levels, such as mark recapture techniques, tend to determine movement over one or two generations; while indirect approaches, such as genetic studies, usually provide evidence of movement patterns that occurred over tens to hundreds of generations (Allendorf and Luikard 2007).

Consequently, it is suggested that a combination of both direct and indirect approaches should be used in taxa with moderate to long generation times and high levels of historic gene flow in order to test for fragmentation-induced limitations to contemporary movement (Howeth et al. 2008). Thus, long-term studies that include direct approaches to test the movement patterns in *D. impar* may be essential in order to determine how the current levels of habitat fragmentation have affected the connectivity of these populations.

By combining phylogeographic and population genetic data we have been able to assess the effects that both historic and contemporary factors have had on the genetic diversity and structure of *D. impar*, which provided insights on this species' past and present population connectivity. This study has demonstrated the utility of employing multiple molecular techniques to assess the genetic structure of populations at different spatial and temporal scales. Such an approach provides detailed information for management authorities on gene flow between current and historical habitat fragments and can help in decisions of whether fragments should be maintained or connections re-established.

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772 **Appendix A.** Specimen ID, management clusters (as defined by ARAZPA 1996), sampling
773 locality and haplotypes of individuals included in the phylogeographic analysis. The
774 management cluster identifier corresponds to those in Fig. 1A. Haplotypes letters refers to the
775 mtDNA clade to which the individual belongs to, as indicated in Fig. 2. Museum and
776 government acronyms are MV, Museum Victoria; ANWC, Australian National Wildlife
777 Collection; DSE, Department of Sustainability and Environment; DEH, Department of
778 Environment and Heritage. Note: GenBank accession numbers will be provided after paper is
779 accepted for publication

780

Specimen	<i>D. impar</i> management cluster	Locality	Latitude	Longitude	Haplotype	Genbank accession no.
MVVIC21	2	Grantham Green Estate, St.Albans, Victoria	-37.74	144.79	C	
MVVIC22	2	Deer Park, Victoria	-37.78	144.77	D	
MVVIC23	2	Derrimut Grasslands Reserve, Victoria	-37.81	144.75	E	

MVVIC27	2	Deer Park, Victoria	-37.78	144.77	A	
MVVIC210	2	Derrimut Grasslands Reserve, Victoria	-37.81	144.75	F	
DSEVIC3A10	3A	Cavendish-Dunkeld Rd, corner of Old Adelaide Rd, Victoria	-37.60	142.21	A	
DSEVIC3A58	3A	Caramut Chatsworth Rd , Victoria	-37.91	142.58	A	
DSEVIC3A62	3A	Goodwood, Pattisons La, Nareeb, Victoria	-37.66	142.56	A	
DSEVIC3A113	3A	Warralong, Coleraine-Balmoral Road, Coleraine, Victoria	-37.55	141.72	B	
DSEVIC3A127	3A	Challicum Park, Old Geelong Road, Crossroads, Victoria	-37.43	143.16	A	
DSEVIC3A143	3A	Six Chain Rd, Victoria	-37.64	141.45	B	
DSEVIC3B4	3B	Camperdown-Foxhow Rd, Victoria	-38.09	143.38	A	
DSEVIC3B5	3B	Camperdown-Foxhow Rd, Victoria	-38.09	143.38	A	
DSEVIC3B9	3B	Carters Rd, Lesly Manor, Western side Lake Corangamite, Victoria	-38.13	143.36	A	
DSEVIC3B33	3B	Geggies Rd, Cressy, Victoria	-37.99	143.74	G	
DSEVIC3B43	3B	North Poorneet Rd, Cressy, Victoria	-38.05	143.75	A	
DSEVIC41	4	Majorca Rd, Talbot, Victoria	-37.18	143.73	U	
DSEVIC43	4	Majorca Rd, Talbot, Victoria	-37.18	143.73	V	
DSEVIC44	4	Majorca Rd, Talbot, Victoria	-37.18	143.73	W	
DSEVIC51	5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.86	A	
DSEVIC52	5	Rossbridge Flora Reserve,	-37.49	142.86	A	

		Grange Rd, Victoria				
DSEVIC56	5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.87	A	
DSEVIC59	5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.87	A	
DSEVIC511	5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.50	142.77	A	
DSEVIC61	6	Val Mathews Rd, Burrum, Victoria	-36.63	142.76	H	
DSEVIC62	6	Val Mathews Rd, Burrum, Victoria	-36.63	142.76	H	
DSEVIC63	6	Val Mathews Rd, Burrum, Victoria	-36.63	142.76	I	
DSEVIC66	6	Burrum Banyena Rd/Rail Reserve, Victoria	-36.62	142.77	J	
DSEVIC67	6	Burrum Banyena Rd/Rail Reserve, Victoria	-36.62	142.77	K	
DSEVIC92	9	"Caithness" property 10km NW of Yea	-37.15	145.33	L	
DSEVIC93	9	Old Yea Cemetery, Sth side of Yea township, Victoria	-37.22	145.33	L	
DSEVIC94	9	Hamilton farm, 3km SW of Yea, Victoria	-37.22	145.37	M	
DSEVIC95	9	Maindample, Victoria	-37.02	145.95	L	
DSEVIC96	9	Maindample, Victoria	-37.02	145.95	N	
DSEVIC111	11	Stone Jug Lane, Mia Mia, Victoria	-37.04	144.60	X	
DEHSA1	12	Riddock Highway North of Naracoorte, South Australia	-36.84	140.68	O	
DEHSA21	12	Hacks Lagoon, South Australia	-37.10	140.74	O	
DEHSA22	12	Riddock Highway North of Naracoorte, South Australia	-36.84	140.68	O	

DEHSA32	12	Lake Ormerod, South Austrlia	-36.97	140.68	O	
DEHSA36	12	Big Hill / Bool Lagoon, South Australia	-37.13	140.69	P	
ANWCR5898	13	3.4 km SE of Cooma, New South Wales	-36.27	149.17	Q	
ANWCR6768	13	Dalgety, New South Wales	-36.50	148.83	R	
ANWCR6674	14	The Pines Camping Ground, Blowering Reservoir, New South Wales	-35.38	148.23	S	
MVACT1	17	Crace, Canberra, Australia Capital Territory	-35.22	149.13	T	

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Table 1 Characteristics of the eight microsatellites developed for *Delma impar*

Locus name & Sequence for forward (F) and reverse (R) primers	Repeat sequence	All samples (n=103)	
		No. of alleles	Size range (bp)
DiA2 F:CAACCCAATATGCCTACTTAGC R:GTCATGTGTTTCATGCAGTCAC	(GTT) ₁₁	8	289-313
DiA105 F:GGGCTGTCTAATCCAGTTCA R:AGGGCATACCAGGCATTC	(AAC) ₈ AAT(AAC) ₃	4	129-153
DiB5 F:GAAGCATTCAGACCCTCCAC R:TGCCTTGAGTCCCAGTGAG	(CAT) ₅ (CCT) ₂ (CAT) ₆	14	251-283
DiB102 F:CACTCAAAGGTGGCTCAGT R:AATGGTGAAATGCTGTAATAGC	(TCA) ₁₃ (AC) ₁₀	29	278-333
DiB111 F:GCCCTGAATGTTGAGATTTG R:CGGCTGTTAATTTTCATCCTC	(CGA) ₂ (TGACGA) ₂ (TGA) ₇	11	137-181
DiC5 F:CTTGGCTCAAAGGGATAGTTC R:CTCCACTGACAGAAAGACCAC	(GGAT) ₇	13	242-326
DiC101 F:TCTCCTAGTTAGCTTCCATGTG R:TACCTCACAGGGTTGTTATGAG	(TCCA) ₁₂	9	193-229
DiD104 F:TGCATTACTACACAGGTGAGC R:AGTGGTTTCTGGGAAGTTATG	(ATCT) ₁₅ (GTCT) ₁₁ AAC(TATC) ₂ ATC(TATC) ₃ AT(CTTT) ₉	47	211-365

Table 2 Microsatellite molecular diversity of *Delma impar*. The total number of alleles (A), estimates of allelic richness (A_E), number of private alleles (A_P), expected heterozygosity (H_E) and observed heterozygosity (H_O) of each of the three populations identified by Structure

Group		Locus								Mean	± SD
		DiA2	DiA105	DiB5	DiB102	DiB111	DiC5	DiC101	DiD104		
A (n=25)	A	8	2	8	19	9	7	7	26	10.75	±7.78
	A_E	6.08	1.99	6.66	13.56	6.65	5.67	6.28	15.71	7.83	±4.50
	A_P	1	0	1	8	2	1	0	5	2.25	±2.82
	H_E	0.70	0.37	0.75	0.92	0.78	0.77	0.80	0.94	0.75	±0.18
	H_O	0.68	0.32	0.86	0.96	0.84	0.72	0.80	0.83	0.75	±0.19
B (n=12)	A	4	4	7	6	4	4	3	8	5.00	±1.77
	A_E	4.00	3.91	7.00	6.00	3.75	3.83	3.00	8.00	4.94	±1.82
	A_P	0	1	0	1	1	1	1	3	1.00	±0.93
	H_E	0.32	0.50	0.78	0.76	0.23	0.49	0.56	0.81	0.56	±0.22
	H_O	0.18	0.42	0.64	0.82	0.25	0.58	0.55	0.73	0.52	±0.22
C (n=66)	A	6	3	13	20	8	11	8	39	13.50	±11.50
	A_E	4.86	2.73	7.55	10.10	5.73	6.29	5.50	16.79	7.44	±4.33
	A_P	0	0	5	7	1	5	1	15	4.25	±5.09
	H_E	0.69	0.48	0.84	0.90	0.77	0.77	0.67	0.97	0.76	±0.15
	H_O	0.70	0.47	0.77	0.82*	0.79	0.65	0.70	0.82*	0.68	±0.11

* Significant departure from Hardy-Weinberg equilibrium ($P < 0.002$) using “exact HW test”

Table 3 Pairwise population F_{ST} values for the three identified populations of *Delma impar*

Group	B	C	ADE
B	-		
C	0.1624 *	-	
ADE	0.0555 *	0.0996 *	-

* $P > 0.001$

Fig 1 Sampled localities of *Delma impar*. Map of south eastern Australia indicating the estimated distribution of native temperate grasslands prior to European settlement, circa.1770 (grey areas; after Lunt 1991; Kirkpatrick et al. 1995). *Delma impar* management clusters with their respective numbers are indicated by bold lines. Collection localities included in the mitochondrial and microsatellite dataset are indicated by closed and open circles respectively. Gippsland Basin is indicated by the dashed line. State and territory names are abbreviated: ACT, Australian Capital Territory; NSW, New South Wales; VIC, Victoria; SA, South Australia.

Fig 2 Phylogeographic relationships among the 12 management clusters of *Delma impar* and the geographic distribution of clades. Bayesian tree based on the GTR+I+Γ model is shown. Major clades are indicated by different shades, which correspond to shading on the map. Management cluster from which the sample came is written in brackets after the sample name. Numbers in square brackets represent the number of individuals with identical haplotypes. Abbreviations on the map are: ACT, Australian Capital Territory; NSW, New South Wales; SA, South Australia; VIC, Victoria

Fig 3 Map of south west Victoria Bioregions. Collection localities included in the microsatellite dataset are indicated by closed circles. The three clusters estimated by Structure are indicated by circled areas. Individuals within the dashed line formed part of Clusters B and C.

Fig 4 Genetic differentiation in *Delma impar*, shown as the regression of pairwise genetic differentiation between individuals against the logarithm of distance (m). $a_r = 0.114 + 0.0092 \ln(\text{distance})$; $a_t = -0.121 + 0.0117 \ln(\text{distance})$

Fig 5 Correlogram showing the results of the spatial autocorrelation analysis where genetic correlation values (r) are plotted as a function of distance. 95% confidence error bars are

shown and dashed lines represent the upper and lower 95% confident intervals around the null hypothesis of no structure. The plot shows that there is significant positive genetic structure at the first three distance classes.

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