Human-induced vs. historical habitat shifts:

Identifying the processes that shaped the genetic structure of the Striped Legless Lizard, *Delma impar*

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To Tito.

Someone once said to me,

"I've never seen a dad care so much for his daughters".

I must say while blushing that I haven't either.

Gracias, pa.

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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 underwent both historical and recent distributional changes, which could have affected the lizard's genetic structure. I 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 more than one million years, indicating that these lineages should be considered as separate Evolutionarily Significant Units for management purposes. The microsatellite analyses did not reveal strong evidence of population sub-structuring, due to recent anthropogenic fragmentation within the south-western Victoria lineage, but the analyses suggest that *D. impar* may not be capable of dispersing over long distances. This study not only provides useful information for the conservation of this threatened species, but also emphasizes that in order to develop effective conservation management strategies an understanding of the processes that have shaped the genetic structure of species is required.

INTRODUCTION

The loss of species diversity in Australia over the last two centuries, resulting from human-induced changes in the environment (Healey 2007), has led to an increase in the importance of conservation-based research. Historically, conservation biology has focussed mainly on protecting biological diversity at the species level. More recently, however, the protection of diversity at a genetic level has been recognised as a primary conservation priority (McNeely et al. 1990). The preservation of genetic diversity is believed to be of particular importance because species with high levels of genetic diversity may have a greater potential to adapt to threats, such as disease and environmental change, and are at a lower risk of extinction (Lande & Shannon 1996; Lacy 1997).

Patterns of genetic structure and diversity in species can be influenced by various factors, both ancient and modern, including natural historic events and recent anthropogenic disturbance (Avise 1992; Osborne et al. 2000; Epps et al. 2005; Olivieri et al. 2008). Conservation case studies involving anthropogenic habitat fragmentation are usually given high conservation priority, as it has been found that such fragmentation has the most significant negative impact on the genetic diversity of populations (DiBattista 2008). Human-induced habitat fragmentation often disrupts dispersal between habitat patches and consequently reduces gene flow between remnant populations (Saunders et al. 1991; Dayanandan et al. 1999). By reducing dispersal and gene flow, habitat fragmentation is likely to decrease genetic diversity in populations and increase inbreeding, reducing the likelihood of long-term survival (Frankham et al. 2002; Reed & Frankham 2003).

Alternatively, naturally occurring historic fragmentation events, such as periods of glaciation or marine incursions, may isolate populations for long periods (Chapple & Keogh 2004; Kasapidis et al. 2005; Vandergast et al. 2007). These long periods of isolation may have caused high levels of genetic differentiation and/or the adaptation of populations to different ecological niches. These historically isolated populations contain independent evolutionary potential and may even be in the process of speciation (Frankham et al. 2002). Populations with such deep historical divergence provide important contributions to intraspecific genetic diversity,

and their separate management and protection will increase the probability of maintaining present and future genetic diversity (Moritz 1994).

Historically isolated populations are often managed using the concept of evolutionarily significant units (ESUs) as a way to maintain and protect diversity (Fraser & Bernatchez 2001). This method was developed to identify separate management units and to prioritize areas for protection (Ryder 1986). The interpretation and derivation of an ESU has been an area of continuous debate, and numerous definitions have been developed (Waples 1991; Dizon et al. 1992; Vogler & DeSalle 1993; Crandall et al. 2000). Moritz (1994) suggested that in order to determine ESUs, historically isolated populations should be identified based on molecular genetic data, where an ESU should have reciprocally monophyletic mitochondrial haplotypes and exhibit significant genetic divergence of allele frequency at nuclear loci. This definition of an ESU is the most widely used. However, it has been recently proposed that an ESU should be identified by obtaining information on both recent adaptive divergence (identified through the analysis of phenotypes or ecology) and long term historical isolation (different historical lineages identified through molecular phylogeography) (Fraser & Bernatchez 2001; Moritz 2002; Green 2005; Cronin 2006; de Guia & Saitoh 2007). In cases where information on recent adaptive divergence cannot be obtained, the identification of historical genetic divergence alone should provide enough evidence to determine ESUs (Fraser & Bernatchez 2001; Abellan et al. 2007). Even though it is suggested that ecological traits should be analysed whenever possible to identify ESUs, in practice, genetic divergence is often the only or main criterion used (Monsen & Blouin 2003). Despite the various ways in which an ESU is determined, every definition tries to identify populations that have a long and ongoing history of restricted gene flow between each other (Fraser & Bernatchez 2001).

As a consequence, two very different isolating processes (natural historic and current anthropogenic fragmentation) can alter the genetic diversity and structure of a species through fragmentation and the reduction of gene flow between populations. Even though these processes are acting in a similar way, they are occurring at different rates, and therefore the response of a species to each event may be different (Keyghobadi et al. 2005). Consequently, management strategies implemented for the protection and maintenance of a species' genetic diversity would be different for each of these isolating events. On one hand, historically isolated populations

(ESUs) should be managed independently from each other in order to maintain the unique genetic composition of each historical lineage (Moritz 2002). On the other hand, gene flow should be re-established between populations that have been recently isolated by anthropogenic fragmentation to help maintain genetic diversity within populations and to decrease the risk of inbreeding (Frankham et al. 2002). Such measures could increase the long-term survival of a species (Storfer 1999). Therefore, in order to optimise conservation management strategies, it is important to have an understanding of the processes (natural historic and current anthropogenic) that have shaped the genetic structure of a species.

Very few conservation genetics studies have incorporated genetic markers to investigate the effects of both historical and recent habitat changes (Johnson et al. 2003; Vandergast et al. 2007; Wilson et al. 2008), despite the importance of this information in the effective management of threatened species in highly fragmented habitats. Conservation genetic studies that include both population genetics and phylogeographic analyses have the greatest probability of identifying historical isolation of populations and/or current anthropogenic events. Mitochondrial DNA (mtDNA) is a molecular marker that has been widely used for phylogeographic analyses (Firestone et al. 1999; Johnson 2005; Brown et al. 2006) and can be used to determine the spatial arrangement of genetic lineages within species (Avise 2004). This phylogeographic data can be used in conjunction with information on the historical biogeography of habitats to determine what processes may have influenced the spatial distribution of genetic lineages (Garrick et al. 2007). Alternatively, microsatellite markers, with a mutation rate much faster than mtDNA (Hedrick 2001; DeSalle & Amato 2004), provide a useful tool to detect how recent environmental changes have influenced the current population structure of species (Sunnocks 2000). Because of their different properties, it is advisable to employ a range of molecular markers in order to distinguish between the processes that have shaped the genetic structure and diversity of threatened species whose habitat have been affected by both historical fragmentation and more recent anthropogenic events.

In this study, I use both mtDNA and microsatellite markers to investigate the genetic structure of a federally threatened reptile species, which occurs in a habitat that has undergone both ancient and modern habitat fragmentation. My study will provide an integrated investigation, incorporating phylogeography and population genetics, to provide a comprehensive

assessment of the complex processes that have shaped the genetic structure of a species. It is essential to obtain such information in order to determine which management strategies would be most appropriate for the conservation of this threatened species.

Study species – distribution, biology and habitat preferences

The Striped Legless Lizard, *Delma impar*, belongs to the family Pygopodidae and is the only pygopodid that is restricted to the native temperate grasslands of south-eastern Australia (Smith & Robertson 1999). These grasslands have undergone dramatic distributional changes, both before and after European settlement. 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 (Lunt 1991; Kirkpatrick et al. 1995) (Fig. 1). Since then, these areas have been highly fragmented due to urbanisation and agriculture, and it is estimated that only 0.5% of these landscapes remain (Kirkpatrick et al. 1995).

D. impar is patchily distributed in remnant temperate grasslands of New South Wales, the Australian Capital Territory, Victoria and South Australia, and is listed as threatened under numerous acts of legislation across its range (Smith & Robertson 1999). One of the management objectives for *D. impar* has been to maintain genetic variation across its geographic range, in order to ensure the evolutionary viability of the species (Smith & 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, Table 1), which have been determined depending on the habitat characteristic of the surrounding bioregion and may represent different ESUs (ARAZPA 1996). This management approach was chosen because it assumes that populations in each region are adapted to the local habitats and may comprise a unique genetic make-up that is distinctive to that region. Currently, there is information on the genetic composition of only three management clusters. This research indicated that there is little variation in allozyme frequency between populations within a geographic region (Osmond 1994; ARAZPA 1996). A better understanding of the population genetic structure of this species and the historical phylogeographic structure between populations is urgently needed to allow the development of more effective conservation

management strategies. Both historical and modern fragmentation events are known to have affected the genetic structure and diversity of other grassland specialists (modern fragmentation: *Perameles gunnii*, *Synemon plana* – Robinson et al. 1993; Clarke & O'Dwyer 2000; Clarke & Whyte 2003; historic: *Perameles gunnii*, *Synemon plana*, *Tympanocryptis pinguicolla* – Robinson 1995; Scott & Keogh 2000; Clarke & Whyte 2003; Melville et al. 2007), and it is likely that *D. impar* presents similar patterns.

The primary aim of my study was to obtain a detailed understanding of both the population genetic structure and the phylogeographic patterns of *D. impar* to 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 can be identified and managed independently. Information on the population genetic structure may also determine if and where gene flow may need to be re-established between fragmented populations. These management strategies may be essential for the long-term survival of this threatened species.

The specific aims of this study are to:

1. Identify the phylogeographic structure of *D. impar* and to estimate the age of each independent historical lineage.

2. Determine if each of the current management clusters represents an independent evolutionarily lineage, in order to establish and designate ESUs.

3. Estimate the current levels of genetic connectivity and the effects of anthropogenic fragmentation within the main management clusters of south-western Victoria (clusters 3A and 3B).

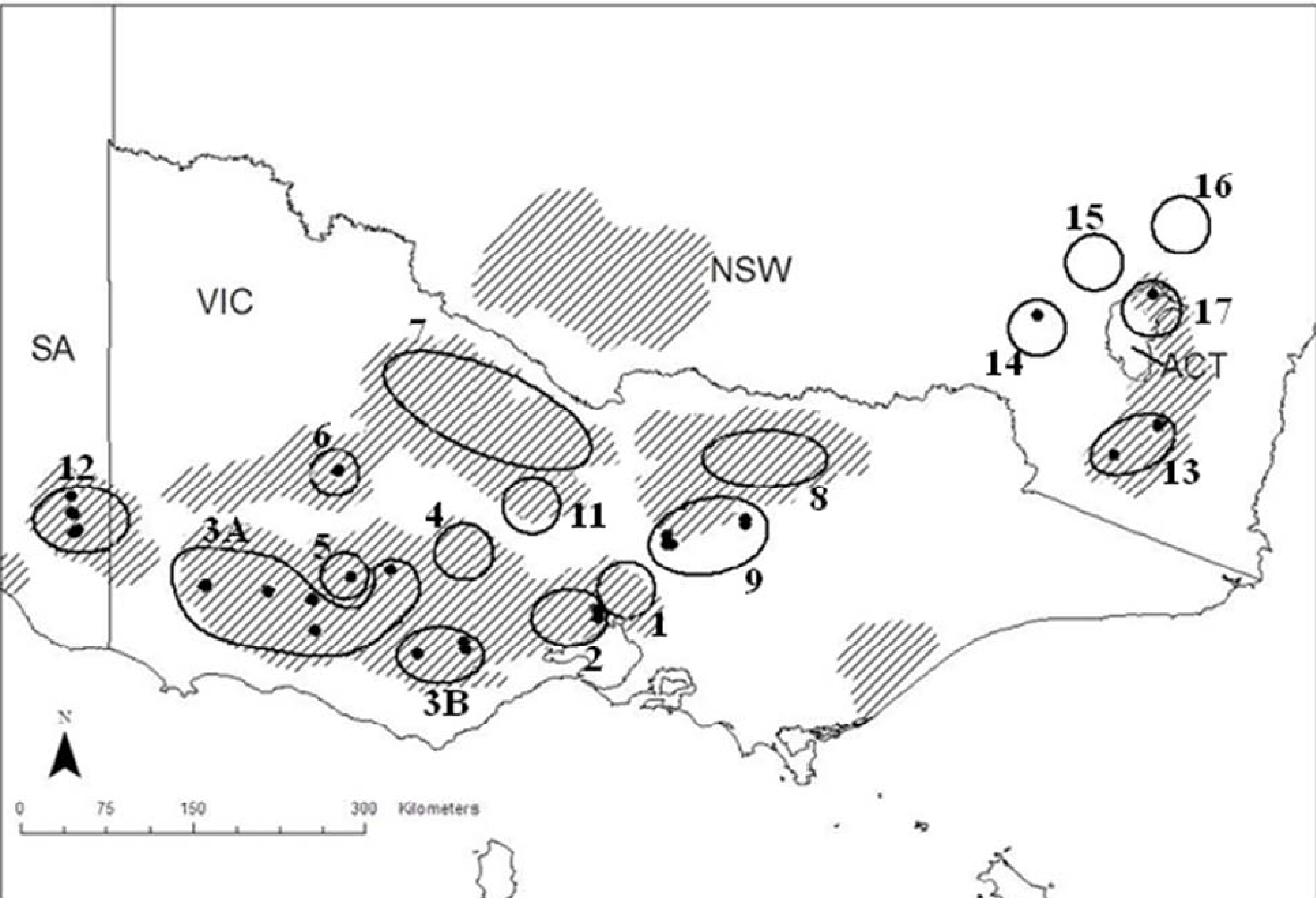


Figure 2. Map of south-eastern Australia indicating the estimated distribution of native temperate grasslands prior to European settlement circa. 1770 (hashed areas; see Lunt 1991; Kirkpatrick et al. 1995). *Delma impar* managment clusters with their respective numbers (see Table 1 for details) are indicated by circled areas. Management cluster 10, in the Gippsland plains, predicted by climate and landscape analysis has no *D. impar* records and is not included in the map. Locations of samples included in the phylogeographic analysis are indicated by black dots.

Table 1. *Delma impar* management clusters.

Management cluster	Location
1	North Melbourne, Craigieburn Volcanic Plains, Victoria
2	West Melbourne, Keilor, Werribee Plains, Victoria
3A	Western Victorian (west) Volcanic Plains, Victoria
3B	Western Victorian (east) Volcanic Plains, Victoria
4	North Ballarat, isolated volcanic areas in central Victoria
5	East Grampians, Victoria
6	Horsham, Wimmera grey cracking clays, Victoria
7	Northern alluvial plains, Victoria
8	North eastern slopes, Benalla, Victoria
9	North eastern slopes, Alexandra, Victoria
10	Gippsland Plains, Victoria (BIOCLIM prediction, no *D. impar* records)
11	South-east Bendigo, Mitiamo volcanic belt, Victoria
12	Bool Lagoon, South Australia
13	Cooma-Monaro Plains Treeless basalt plains, New South Wales
14	Gilmore, Batlow and Tumut; New South Wales
15	Yass and Young, New South Wales
16	Goulburn and Windellama Road, New South Wales
17	Gungahlin, Majura Valley, Jerrabombera Valley and Yarramundi Reach, Australian Capital Territory; Suton, New South Wales

MATERIALS AND METHODS

Data sampling

Tissue samples were obtained from ten out of the 17 *Delma impar* management clusters (Fig. 1). These sampled management clusters covered a broad geographic area: South Australia (SA), the Australian Capital Territory (ACT), two clusters in New South Wales (NSW) and five in Victoria. 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 (Table 2).

The largest numbers of samples were obtained from south-western Victoria, collected by the DSE as part of an ongoing survey program, which has been running since 2004. The specific aim of this DSE survey has been to detect new populations of *D. impar* and monitor existing ones. *D. impar* has been recorded at 73 sites in this survey, with the majority located within management clusters 3A and 3B (G. Peterson & C. Grant *pers. comm.*). Each survey site consists of a grid made up of roof tiles that are used as artificial refuges by *D. impar* (Rohr & Peterson 2003), which facilitates 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 also found under the tiles, which were collected and used as genetic samples.

Phylogeographic structure – mitochondrial DNA

DNA extraction and mitochondrial DNA sequencing

A region of approximately 1300 base pairs (bp) of mitochondrial DNA (mtDNA), incorporating the whole of the NADH dehydrogenase subunit 2 (ND2) gene, was used to determine phylogeographic structure in *D. impar*. MtDNA is a powerful tool that has been widely used to identify intraspecific genetic structure (Avise 2004). The relatively high mutation-fixation rate of this marker, which evolves faster than the nuclear genome, makes it an appropriate marker for assessing within species genetic divergence and identifying historically isolated populations (Avise 2004; Chenuil 2006; Avise 2009). This specific region of the mtDNA was chosen as it has

been used successfully in a molecular systematics study of the pygopodids (Jennings et al. 2003) and studies of the closely related diplodactyline geckos, which showed phylogenetically informative levels of variability (Melville et al. 2004; Oliver et al. 2007).

Genomic DNA was extracted from ethanol preserved or frozen liver, tail tips and shed skins using an Easy DNA High-Speed Extraction Tissue Kit (Saturn Biotech), following the manufacturer's protocol. 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 at 1:100 or 1:200 in TE buffer), 10μL Gotaq (Promega), 0.8μL of each primer (10μM) and 6.4μL H_2O. The polymerase chain reaction (PCR) amplification was performed on a Peltier Thermal Cycler using a touchdown thermal cycle 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. PCR amplifications were visualised with 1.2% agarose gel electrophoresis. In some cases, particularly with shed skin samples, the first amplifications were weak and a reamplification was necessary, using 1μl 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, using the amplification primers 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 (Swoford 2000). Translated amino acids were assessed in MacClade 4.0 (Maddison & Maddison 2000) to verify the alignment and to check for internal stop codons.

Table 2. Management clusters (as defined by ARAZPA 1996), sampling locality, identification number and haplotype of individuals included in the phylogeographic analysis. The management cluster identifier corresponds to those in Fig. 1 and Table 1. Haplotype letter refers to the mtDNA clade to which the individual belongs, as indicated in Fig. 3. Museum and government agency acronyms are MV= Museum Victoria, ANWC= Australian National Wildlife Collection, DSE= Department of Sustainability and Environment and DEH= Department of Environment and Heritage.

D. impar management cluster	Location	Latitude	Longitude	Specimen	Haplotype
2	Grantham Green Estate, St.Albans, Victoria	-37.61	144.4	MV VIC 21	C
2	Deer Park, Victoria	-37.76	144.38	MV VIC22	D
2	Derrimut Grasslands Reserve, Victoria	-37.81	144.79	MV VIC23	E
2	Deer Park, Victoria	-37.76	144.38	MV VIC27	A
2	Derrimut Grasslands Reserve, Victoria	-37.81	144.79	MV VIC210	F
3A	Cavendish-Dunkeld Rd, corner of Old Adelaide Rd, Victoria	-37.6	142.21	DSE VIC10	A
3A	Caramut Chatsworth Rd , Victoria	-37.9	142.58	DSE VIC58	A
3A	Goodwood, Pattisons La, Nareeb, Victoria	-37.66	142.56	DSE VIC62	A
3A	Warralong, Coleraine-Balmoral Road, Coleraine, Victoria	-37.55	141.72	DSE VIC113	B
3A	Challicum Park, Old Geelong Road, Crossroads, Victoria	-37.43	143.16	DSE VIC127	A
3A	Six Chain Rd, Victoria	-37.64	141.45	DSE VIC143	B
3B	Camperdown-Foxhow Rd, Victoria	-38.09	143.38	DSE VIC4	A
3B	Camperdown-Foxhow Rd, Victoria	-38.09	143.38	DSE VIC5	A
3B	Carters Rd, Lesly Manor, Western side Lake Corangamite, Victoria	-38.13	143.36	DSE VIC9	A
3B	Geggies Rd, Cressy, Victoria	-37.99	143.74	DSE VIC33	G
3B	North Poorneet Rd, Cressy, Victoria	-38.05	143.75	DSE VIC43	A
5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.86	DSE VIC51	A
5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.86	DSE VIC52	A
5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.87	DSE VIC56	A
5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.87	DSE VIC59	A
5	Rossbridge Flora Reserve, Grange Rd, Victoria	-37.49	142.87	DSE VIC511	A

Table 2. Continued

D. impar management cluster	Location	Latitude	Longitude	Specimen	Haplotype
6	Val Mathews Rd, Burrum, Victoria	-36.63	142.76	DSE VIC61	H
6	Val Mathews Rd, Burrum, Victoria	-36.63	142.76	DSE VIC62	H
6	Val Mathews Rd, Burrum, Victoria	-36.63	142.76	DSE VIC63	I
6	Burrum Banyena Rd/Rail Reserve, Victoria	-36.62	142.77	DSE VIC66	J
6	Burrum Banyena Rd/Rail Reserve, Victoria	-36.62	142.77	DSE VIC67	K
9	"Caithness" property 10km NW of Yea	-37.15	145.33	DSE VIC92	L
9	Old Yea Cemetery, Sth side of Yea township, Victoria	-37.22	145.33	DSE VIC93	L
9	Hamilton farm, 3km SW of YEA, Victoria	-37.22	145.37	DSE VIC94	M
9	Maindample, Victoria	-37.06	145.95	DSE VIC95	L
9	Maindample, Victoria	-37.02	145.95	DSE VIC96	N
12	Riddock Highway North of Naracoorte, SA	-36.84	140.68	DEH SA1	O
12	Hacks Lagoon, SA	-37.1	140.74	DEH SA21	O
12	Riddock Highway North of Naracoorte, SA	-36.84	140.68	DEH SA22	O
12	Lake Ormerod, SA	-36.97	140.68	DEH SA32	O
12	Big Hill / Bool Lagoon, SA	-37.13	140.69	DEH SA36	P
13	3.4 km SE of Cooma, NSW	-36.37	149.17	ANWC R5898	Q
13	Dalgety, NSW	-36.5	148.83	ANWC R6768	R
14	The Pines Camping Ground, Blowering Reservoir, NSW	-35.38	148.23	ANWC R6674	S
17	Crace, Canberra	-35.22	149.13	MV ACT1	T

Phylogeographic analyses and estimates of divergence times

Whenever possible, five to six tissue samples from each management cluster were included in the analyses (Table 2). Also, one previously published sequence of *D. impar* from the ACT management cluster (Jennings et al. 2003) was included. Ten previously published sequences were used as outgroups (Jennings et al. 2003): *Aprasia smithi*, *Pletholax gracilis*, *Pygopus orientalis*, *P. lepidopodus*, *P. nigriceps*, *Delma labialis*, *D. borea*, *D. inornata*, *D. mitella* and *D. molleri*.

Phylogeographic relationships within *D. impar* were estimated with maximum likelihood (ML) criterion, using PAUP* beta version 4.0b410a (Swoford 2000), and Bayesian criterion using BEAST version 1.4.5 (Drummond & Rambaut 2007). MoldelTest version 3.06 (Posada & Crandall 1998) was used to find the optimal model of molecular evolution for the mitochondrial data; 56 alternative models of molecular evolution were evaluated for their goodness of fit using the Akaike Information Criterion (AIC). The parameters of the best-fitting model were used in a ML analysis of 10 heuristic searches with random addition of taxa to determine the best likelihood tree topology and a Neighbour-Joining starting tree. A bootstrap analysis, with 100 replicates, was conducted to assess the reliability of the tree topology.

Bayesian analysis, in BEAST version 1.4.5 (Drummond & Rambaut 2007), was also used to estimate both phylgeographic relationships in *Delma impar* and historical divergence times between populations. BEAST version 1.4.5 (Drummond & Rambaut 2007) infers time-measured phylogenies using a relaxed molecular clock, which can be used to estimate the divergence times between clades. Therefore, I estimated a phylogenetic tree under Bayesian criteria and the divergence time between each of the *D. impar* management clusters using a relaxed molecular clock method, in a single analysis. A pygopodid fossil, *Pygopus hortulanos*, 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. negirceps* (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 1.1. 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 & 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). The searches resulted in 10000 saved trees; the first 1000 trees were discarded as burn-in, and the remaining 9000 trees were used to construct a maximum clade credibility tree with the posterior probability limit set at 0.5. The percentage of trees that had a particular clade represented that clade's posterior probability.

Population genetics – microsatellites

Microsatellite amplification

Seven polymorphic microsatellite loci were used to identify the population genetic structure of the south-western Victoria populations of *Delma impar* (Table 3). Microsatellite markers have been of great utility in conservation (Goldstein & Schlötterer 1999). Their high mutation rate and the high probabilities of finding loci with 6-10 alleles within a small population of sexually reproducing individual, make microsatellites an ideal marker to identify the effects of recent habitat changes on the population structure and genetic diversity of species (Wan et al. 2004). The disadvantage of these markers is that they tend to be species specific, and often new microsatellites need to be developed for a study species (Zane et al. 2002). Four microsatellite loci developed for Australian geckos have been successfully amplified in two species of pygopodids (Hoehn & Sarre 2006). However, the use of a limited number of loci would probably not provide sufficient information for a population genetic study (Cooper et al. 1999; Zane et al. 2002). Thus, as part of this study, a microsatellite library and seven polymorphic microsatellite loci were developed for *D. impar* by Genetic Identification Services (USA).

Genomic DNA was extracted using the methods described above for mtDNA analysis. DNA amplifications were performed in a 10µL total volume containing 1µL DNA (diluted at 1:10 in TE buffer), 5µL Gotaq (Promega), 3.5µL H_2O, 0.2µL of each primer (10µM) and 0.1µL of fluorescently labelled primer (see Table 3). 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 genotyping, using capillary electrophoresis.

Table 3. Details of *Delma impar* microsatellite loci used in this study.

Loci name	**Primer sequence (5' to 3')**	**Label colour**	**Repeat motif**	**No. of alleles N=110**	**Size range**
DiA105	GGGCTGTCTAATCCAGTTCA AGGGCATACCAGGCATTC	NED	$(TCCA)_{12}$	4	153-156
DiA2	CAACCCAATATGCCTACTTAGC GTCATGTGTTCATGCAGTCAC	VIC	$(GTT)_{11}$	7	291-312
DiB102	CACTCAAAGGTGGCTCAGT AATGGTGAAATGCTGTAATAGC	NED	$(TCA)_{13}$ $(AC)_{10}$	19	287-308
DiB111	GCCCTGAATGTTGAGATTTG CGGCTGTTAATTTCATCCTC	FAM	$(CGA)_{3}$ $(TGACGA)_{2}$ $(TGA)_{7}$	9	148-160
DiC101	TCTCCTAGTTAGCTTCCATGTG TACCTCACAGGGTTGTTATGAG	VIC	$(TCCA)_{12}$	7	209-221
DiC5	CTTGGCTCAAAGGGATAGTTC CTCCACTGACAGAAAGACCAC	FAM	$(GGAT)_{7}$	8	246-268
DiD104	TGCATTACTACACAGGTGAGC AGTGGTTTCTGGGAACTTATG	PET	$(ATCT)_{15}$ $(GTCT)_{11}$ $ACC(TATC)_{2}$ $ATC(TATC)_{3}$ $AT(CTTT)_{9}$	26	225-348

Population genetics analyses

The population genetic analyses included 110 individuals from 13 sites within the two main south-western Victoria management clusters (3A and 3B - Fig. 2). Sites that were in close proximity to each other were clustered into one group, giving a total of five sampling localities (Fig. 2). These sites were chosen and further clustered together in order to provide a large enough sample size for each sampling locality, which would be suitable for a microsatellite analysis.

The program Microsatellite Toolkit version 3.1.1 (Park 2001) was used to check for samples that had identical genotypes, which were then removed from subsequent analyses. Genotypes at all loci and all sampling localities were then analysed with the program Micro-Checker version 2.2.3 (Oosterhout et al. 2004) to test for the presence of null alleles, stuttering and small allele dominance. Linkage disequilibrium (LD) between pairs of microsatellite loci and departures from Hardy-Weinberg equilibrium within each sampling locality and locus were evaluated using a Markov Chain Monte Carlo (MCMC) method (1000 dememorizations, 100 batches, 1000 iterations) in GenePop version 4.0 (Rousset 2007). Associated probability values were adjusted for multiple comparisons to correct for type I error (false positive results) using sequential Bonferroni corrections (Rice 1989). To evaluate genetic diversity within sampled localities, the number of total and private alleles (A, A_{p}), and observed and expected heterozygosity (H_{O}, H_{E}), was calculated in GenAlEx version 6.2 (Peakall & Smouse 2006). The inbreeding coefficient (F_{IS}) was calculated for each sampling locality using GenePop version 4.0 (Rousset 2007).

Two Bayesian model-based clustering algorithms were applied to the microsatellite data set to investigate the genetic structure of the two main management clusters of south-western Victoria. The first approach was executed in Structure version 2.2 (Pritchard et al. 2000). This program probabilistically clusters individuals based on their genotypes into K groups (or populations) while minimizing Hardy-Weinberg and linkage disequilibrium between loci within groups (Pritchard et al. 2000; Falush et al. 2003). Genotypes alone are used. The number of clusters, K, is a fixed parameter of the model; the method consists of running several MCMC chains with different values for K (several runs for each K in order to verify the consistency of the results) and inferring which K is the most likely from the approximation of their probabilities. Five independent runs of a model with admixture of ancestry (letting the program infer the degree of admixture (α) from the data) and correlation of allele frequencies was run for each value of K (K=1-7), with a burn-in period of 1000, followed by 1000000 MCMC iterations. The estimated number of clusters (K) was taken to be the value of K with the highest probability (Pritchard et al. 2000).

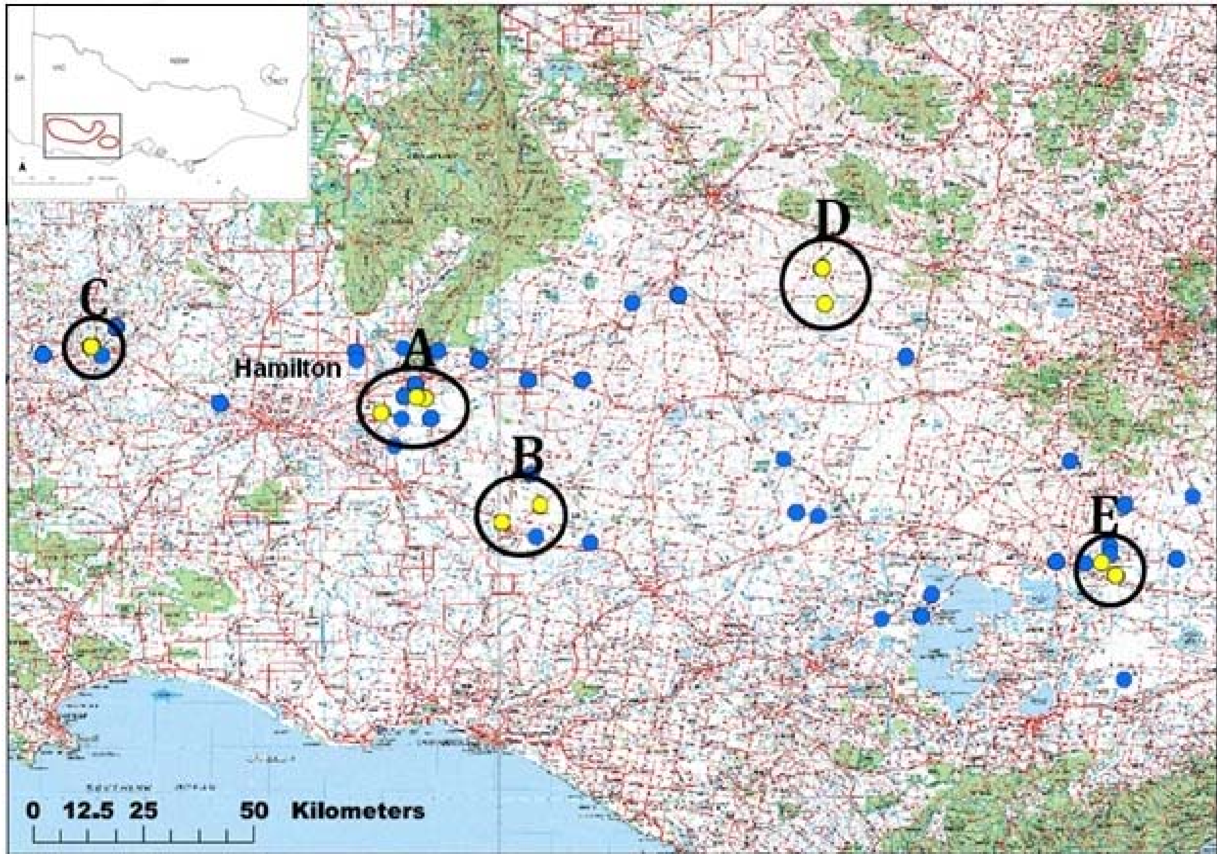


Figure 2. Map of south-west Victoria. *Delma impar* sites within the management clusters 3A and 3B are represented by the blue and yellow circles. Localities of samples included in the population genetics analyses are represented by yellow circles. Samples from sites that were clustered for analyses purposes are circled and labelled with a unique letter.

The second technique, employed in Geneland version 2.0.6 (Guillot et al. 2005a; Guillot et al. 2005b), also groups genotypes by minimizing Hardy-Weinberg and linkage disequilibria. However, this approach also takes into account the geographic location of individuals, which may provide a better definition of spatial genetic units (Coulon et al. 2006). First, the possible number of populations (K) was inferred by setting the priors for minimum, maximum and initial population numbers to 1, 7 and 1 respectively. Spatial coordinates were actual coordinates, maximum rate of Poisson process = (number of individuals), and the number of nuclei in the Poisson-Veroni tessellation = 3 x (number of individuals) as recommended by Guillot et al. (2005a). The Dirich model was used as the model for allelic frequencies as it has been demonstrated to perform better than the alternative model (Guillot et al. 2005a). The model was

run five times for 100000 MCMC iterations, with a burn-in period of 4000. Once the number of populations (K) was determined (inferred from the modal K of the five runs), the model was run five more times with K fixed to the inferred number of populations. Other parameters remained similar to those of the runs with variable K. Maps of posterior probability of population membership were obtained and compared among the five independent runs.

In addition to these clustering methods, a frequency-based population assignment test was employed using GenAlEx version 6.2 (Peakall & Smouse 2006), where the data was grouped by the five sampled localities. This test provides the probability that a given genotype originated in the population where it was sampled relative to other populations (Paetkau et al. 1995). The expected frequency of each individual's genotype at each locus is calculated, this is multiplied across all loci and log transformed to provide a log-likelihood value. This calculation assumes random mating and linkage equilibrium within each population. For each individual a log-likelihood value is estimated for each population, and an individual is assigned to the population with the highest log-likelihood value. In cases where an allele was absent from a population (allele frequency value of zero) a value of 0.01 was used.

Isolation by distance (IBD) patterns between pairs of individuals were also determined by testing the correlation between individual genetic distance (represented by a_r (Rousset 2000) and e_r 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 the dispersal of *D. impar* is limited by distance.

To further examine the fine-scale genetic patterns among the five sampled localities, two additional tests were employed on the microsatellite data set using GenAlEx version 6.2 (Peakall & Smouse 2006). Analysis of Molecular Variance (AMOVA) was used to perform the analysis of genetic differentiation following the methods in Excoffier et al. (1992). AMOVA provides estimates of traditional F-statistics and their analogues (R_{ST} and Φ_{PT}). F_{ST} was calculated to estimate the genetic variation among the five groups. The spatial pattern of genetic variation was investigated using a microspatial correlation technique developed by Smouse & Peakall (1999)

for multiallelic codominant loci that examines the genetic similarity between pairs of individuals at different distant classes, thus providing results on the scale at which spatial patterns occur. The results of the autocorrelation analysis are presented graphically in correlograms showing changes in genetic correlation values (r) over distance classes. A significant genetic structure at any distance class is obtained when a positive r-value falls outside the 95% CI. The correlogram provides the average r-value within each distance class (distance size class of 100 m over 7 size classes used in this study).

RESULTS

Phylogeographic structure – mitochondrial DNA

Sequence alignment

Thirty nine new sequences representing 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 from *Aprasia smithi*, *Pletholax gracilis*, *Pygopus orientalis*, *P. lepidopodus*, *P. nigriceps*, *Delma labialis*, *D. borea*, *D. inornata*, *D. mitella* and *D. molleri* (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 3A, 3B and 5) shared identical haplotypes (Fig. 3, Table 1). 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.

Phylogeographic inferences using maximum-likelihood and Bayesian analyses

Among the 56 likelihood models examined for maximum likelihood (ML) analysis, the TVM+I+Γ model had the highest likelihood and was significantly favoured over alternatives by the AIC. The model parameters were: alpha shape parameter=1.9232; proportion of invariant sites=0.4664; substitution rates R(a)=1.4509, R(b)=7.4762, R(c)=1.4578, R(d)=0.5253, R(e)=7.4762; and empirical base frequencies A=0.3695, C=0.3524, G=0.0746 and T=0.2035. The ML analysis yielded a single optimal tree with ln-likelihood of -6943.58 (Fig. 3). Bayesian analysis performed using the GTR+I+Γmodel of sequence evolution and the relaxed molecular clock model produced a single optimal tree with ln-likelihood of -6988.84. Analysis of the data using ML and Bayesian methods resulted in two trees with identical relationships between the management clusters of *D. impar* and the outgroups belonging to the genus *Delma*. Therefore, the ML phylogram is presented with Bayesian posterior probabilities on Fig. 3. However, tree topologies were incongruent in the phylogenetic relationships between outgroups, but differences between topologies only occurred on poorly supported braches.

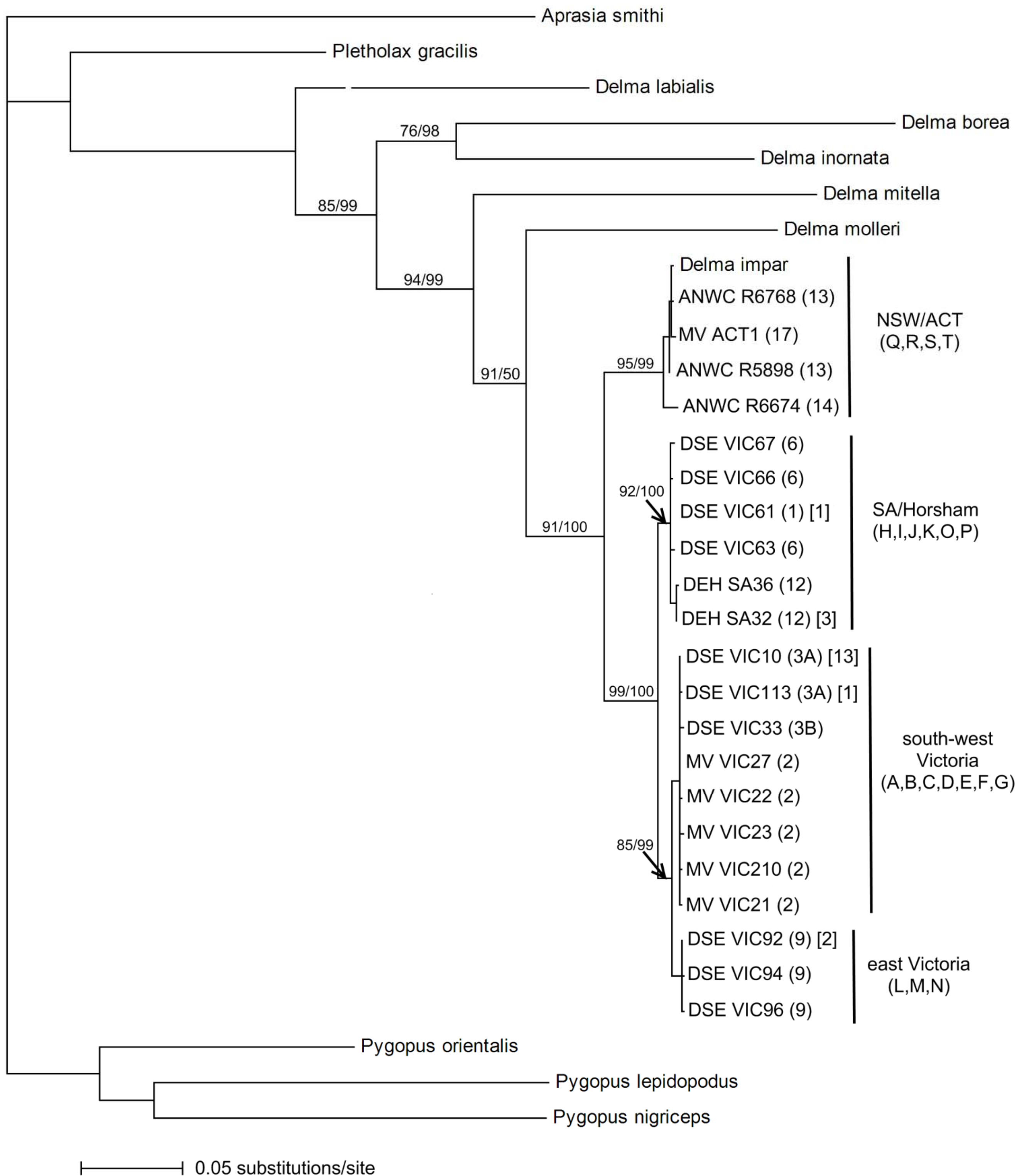


Figure 3. Maximum likelihood phylogram representing ten *Delma impar* management clusters, written in brackets after sample name. Values represented above the branches are as follows: maximum likelihood bootstrap values are presented first and Bayesian posterior probability values are presented second. Numbers in square brackets represent the number of individuals with identical haplotypes. Each clade is indicated next to samples with their respective haplotype letters in brackets, as indicated in Table 2.

The phylogram recovered from the ML and Bayesian analysis indicates the presence of four highly supported monophyletic lineages within the sampled populations of *D. impar* (bootstrap value 85-99%, posterior probability 99-100%). The phylogenetic relationships between the management clusters are shown in Fig. 3 and the geographic location of each lineage is displayed in Fig. 4. The lineage that comprises all of the sampled management clusters from NSW and the ACT is sister to a clade composed of all other lineages. Another lineage (SA/Horsham) is composed of the South Australian management cluster (cluster 12) and the south-west Victorian management cluster near Horsham (cluster 6). The other south-west Victorian management clusters (clusters 2, 3A, 3B and 5) form a clade (south-west Victoria), which is sister to the lineage composed of the east Victorian management cluster (cluster 9). These last two lineages (south-west Victoria and east Victoria) form a clade that is sister to the SA/Horsham lineage.

Estimates of divergence times

Examination of the BEAST version 1.4.5 (Drummond & Rambaut 2007) log file in Tracer version 1.4.1 indicated that the dataset had a small degree of autocorrelation; with a mean estimate of the covariance of parent and child branches of 0.029. However, this was not considered significant because zero was included in the 95% HPD (-0.249-0.308) (Drummond et al. 2006). The co-efficient of rate of variation was estimated to be 0.530 (95% HPD: 0.171-1.178), indicating that the data set is not strictly clock-like and that a lognormal relaxed clock is appropriate.

The divergence dating analysis indicates that *D. impar* diverged from its sister taxon, *D. mitella*, during the Miocene, approximately 13.9 million years ago (Ma) (95% CI: 8.7-19.6). It is estimated that the NSW/ACT lineage diverged from the Victoria/SA cluster approximately 7.6 Ma (95% CI: 3.8-12.8). Within the Victoria/SA cluster, the split between the SA/Horsham lineage and the rest of Victoria was approximately 2.9 Ma (95% CI: 1.1-6.0). The last two lineages, south-west Victoria and east Victoria, diverged during the Pleistocene approximately 1.6 Ma (0.5-3.6).

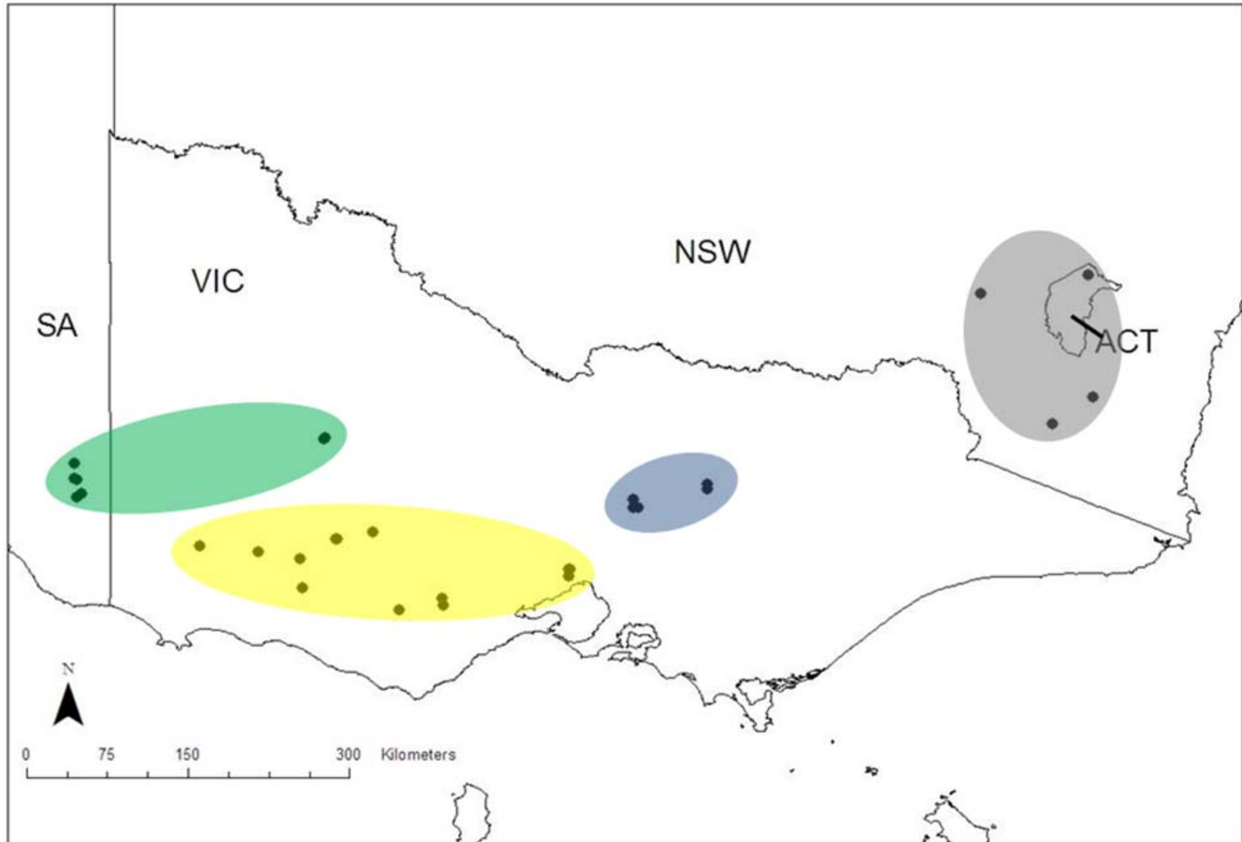


Figure 4. Map of south-east Australia. Colour shading on the map represents the four historical lineages: gray= NSW/ACT, green= SA/Horsham, yellow= south-west Victoria and blue= east Victoria. Samples included in the phylogeographic analysis are represented by the black dots.

Population genetics – microsatellites

Genetic diversity

Two pairs of samples had identical microsatellite alleles at all loci. The sample collection data indicated that all four samples were shed skins that were collected 9 or 11 months apart on the same row in the same study grid. Therefore, it is likely that two of these skin sheds were from the same individuals. The two duplicate samples were excluded from all analyses. This left a total of 108 samples for which 86.5% of alleles were scored at seven microsatellite loci. The seven microsatellite loci analysed displayed variable levels of genetic diversity within the sampling localities, and different levels of expected and observed heterozygosity (H_E, H_O) between the areas sampled (Table 4). Sampled site C supported the greatest number of alleles (A), while site

Table 4. Microsatellite genetic diversity of *Delma impar.* The number of alleles (A), private alleles (A_p), expected heterozygosity (H_E) and observed heterozygosity (H_O) of the seven microsatellite loci amplified. * indicates loci for which null alleles may be present.

	Group A (n=22)				Group B (n=23)				Group C (n=27)				Group D (n=16)				Group E (n=20)			
Locus	A	A_p	H_E	H_O	A	A_p	H_E	H_O	A	A_p	H_E	H_O	A	A_p	H_E	H_O	A	A_p	H_E	H_O
DiA105	4	1	0.46	0.59	4	1	0.54	0.39	2	0	0.38	0.35	2	0	0.4	0.56	3	0	0.49	0.35
DiA2*	5	0	0.49	0.5	7	1	0.63	0.42	7	1	0.67	0.67	4	0	0.67	0.69	5	0	0.71	0.75
DiB102	12	2	0.89	0.75	11	1	0.85	0.82	19	8	0.93	1	15	4	0.89	0.93	9	0	0.85	0.89
DiB111	7	0	0.74	0.89	7	1	0.57	0.39	9	2	0.77	0.85	5	0	0.7	0.79	4	0	0.71	0.74
DiC101	6	0	0.54	0.63	5	0	0.54	0.62	7	0	0.8	0.9	7	1	0.8	1	6	0	0.54	0.46
DiC5	8	1	0.75	0.62	4	0	0.64	0.71	6	0	0.77	0.77	5	0	0.62	0.5	4	0	0.74	0.77
DiD104*	19	1	0.92	0.84	19	3	0.92	0.86	26	5	0.94	0.83	20	2	0.93	0.86	21	2	0.94	0.79
Mean ± SE	8.71 ±1.97	0.71 ±0.76	0.68 ±0.07	0.68 ±0.05	8.14 ±2.03	1 ±1.00	0.67 ±0.06	0.6 ±0.08	10.86 ±3.20	2.29 ±3.09	0.75 ±0.07	0.77 ±0.08	8.29 ±2.50	1 ±1.53	0.71 ±0.07	0.76 ±0.07	7.43 ±2.38	0.29 ±0.76	0.71 ±0.06	0.68 ±0.07

E supported the fewest. Private alleles (A_p), a measure of population differentiation, which includes alleles unique to a sample site and an individual, were detected in all sampled sites. After Bonferroni corrections for multiple tests, no significant linkage disequilibria were detected ($P<0.002$) but significant departures from Hardy-Weinberg equilibrium were observed for DiD104 at two sampling localities (A and E). Micro-Checker did not detect the presence of stuttering or small allele dominance but loci DiA2 and DiD104 were found to have a significant probability of presenting null alleles. Thus, all subsequent analyses were performed twice, with and without these two loci. F_{IS} values ranged from below zero, indicating no evidence of inbreeding to 0.153, indicating non-random breeding (Table 5). The F_{IS} value obtained for sampling locality B was relatively high in comparison with the rest of the sampled areas indicating that inbreeding might be occurring in this population.

Table 5. Pairwise population F_{IS} calculated using all seven loci and using the five loci with no evidence of null alleles.

	7 loci	**5 loci**
Group A	0.023	0.003
Group B	0.153	0.131
Group C	0.005	0.035
Group D	-0.030	-0.076
Group E	0.082	0.069

Population genetic structure and gene flow

Results from the Bayesian clustering analyses are summarized in Table 6. The estimated logarithm of likelihood for the data analysed with Structure was highest for K=1 in both analyses run with and without the loci that may have null alleles. Geneland inferred the presence of two populations only when all seven loci were included in the analysis: the first cluster gathers all individuals from sampling localities A, B, D and E (all individuals having 90% posterior probability of belonging to cluster one, represented by the yellow colour in Fig. 5), and the second includes all individuals in sampling locality C (where individuals only had a 10% posterior probability of belonging to cluster one, cluster two is represented by the red colour in

Fig. 5). However, the analysis run without the loci that may have null alleles inferred only one population.

The frequency-based population assignment test employed with all seven loci correctly assigned 61% of individuals to their sampled locality. The same test performed without the loci that may have null alleles only assigned 45% of individuals to the group where they were sampled from. Furthermore, the log-likelihood values estimated for each individual were very similar across all populations in both tests.

Table 6. Results of individual-based Bayesian clustering analyses. For Geneland, the five runs were ranked by decreasing value of likelihood (in the log scale) and the K value estimated for each run is presented. In Structure, K is fixed from 1 to 7, the highest likelihood value out of the five runs for each K are presented and ranked by decreasing value.

Geneland				**Structure**			
7 loci		5 loci		7 loci		5 loci	
Likelihood	K	Likelihood	K	Likelihood	K	Likelihood	K
-2359.9	2	-1729.0	1	-2591.9	1	-1571.0	1
-2417.8	2	-1849.6	1	-2709.5	4	-1691.6	2
-2481.2	2	-1885.7	1	-2803.1	5	-1736.1	3
-2577.6	2	-1907.5	1	-2865.9	3	-1830.8	7
-2677.9	2	-1974.6	1	-2871.1	6	-1840.9	4
				-2978.5	7	-1848.0	6
				-2998.1	2	-2040.5	5

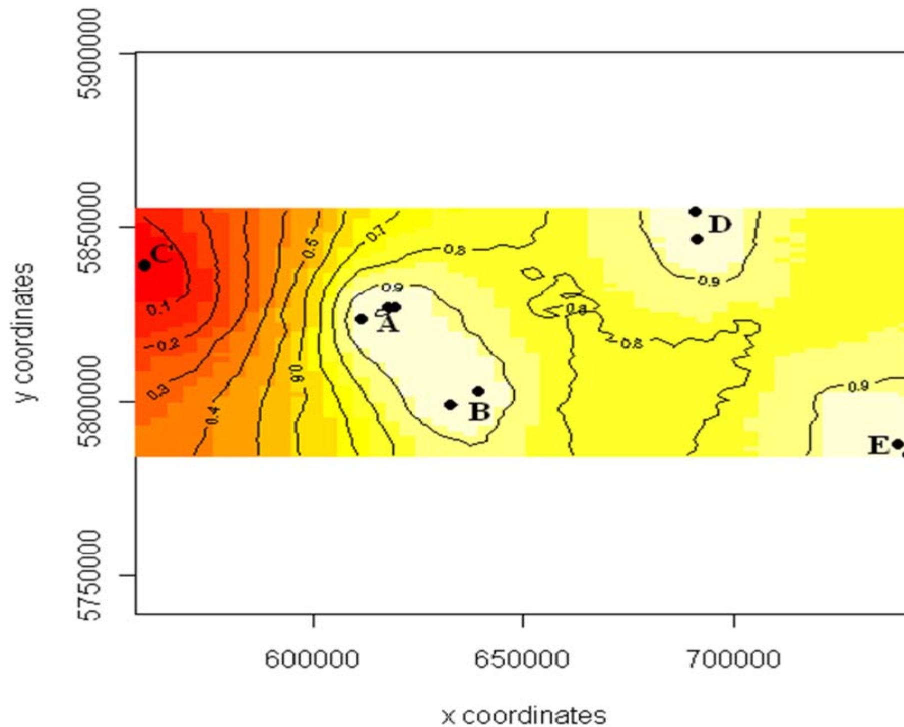
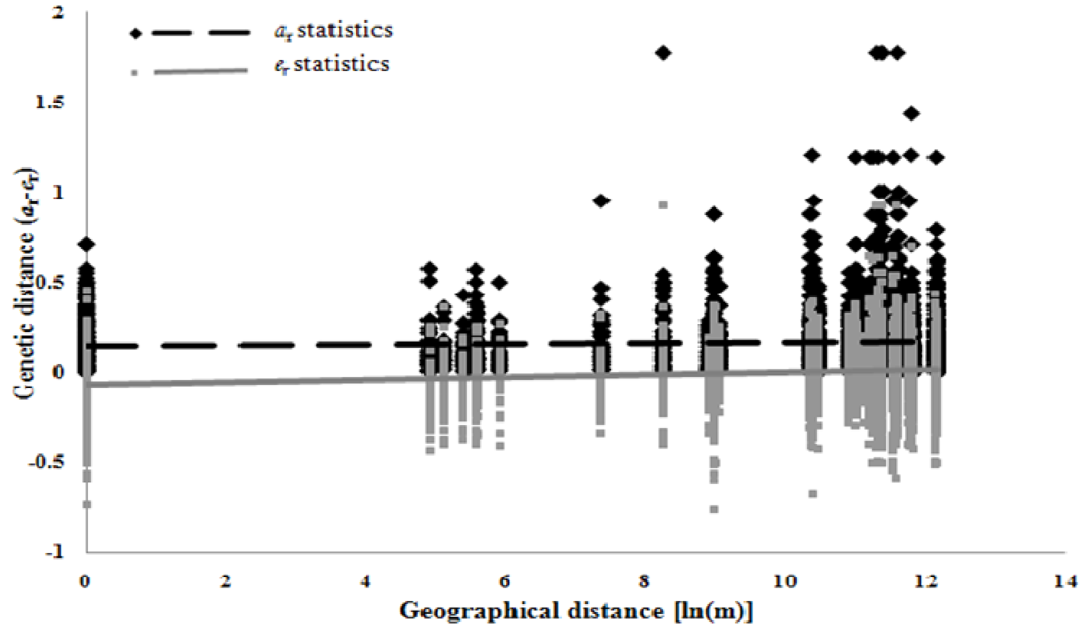


Figure 5. Map of Geneland individual assignment to clusters for K=2 (scale units in m). Cluster one is represented in yellow and cluster two in red. Each sampling locality is labeled with the same letter given in Fig. 2. Contour lines of the figure represent the posterior probability of individuals belonging to cluster one, where individuals in groups A, B, D and E have 90% probability of belonging to cluster one and individuals in group C only present a 10% probability. The plot is based on the highest-probability run at K=2.

Evidence for an isolation by distance (IBD) pattern was observed (Fig 6.), indicated by a significant correlation between pairwise individual genetic distance (a_r and e_r values) and geographical distance (7 loci: Mantel test, P<0.01 for a_r statistics and P<0.001 for e_r statistics; 5 loci: Mantel test, P<0.05 for a_r statistics and P<0.001 for e_r statistics). The regression lines displayed in Fig. 6 illustrates that in both analyses genetic differentiation increased with increasing geographical distance.



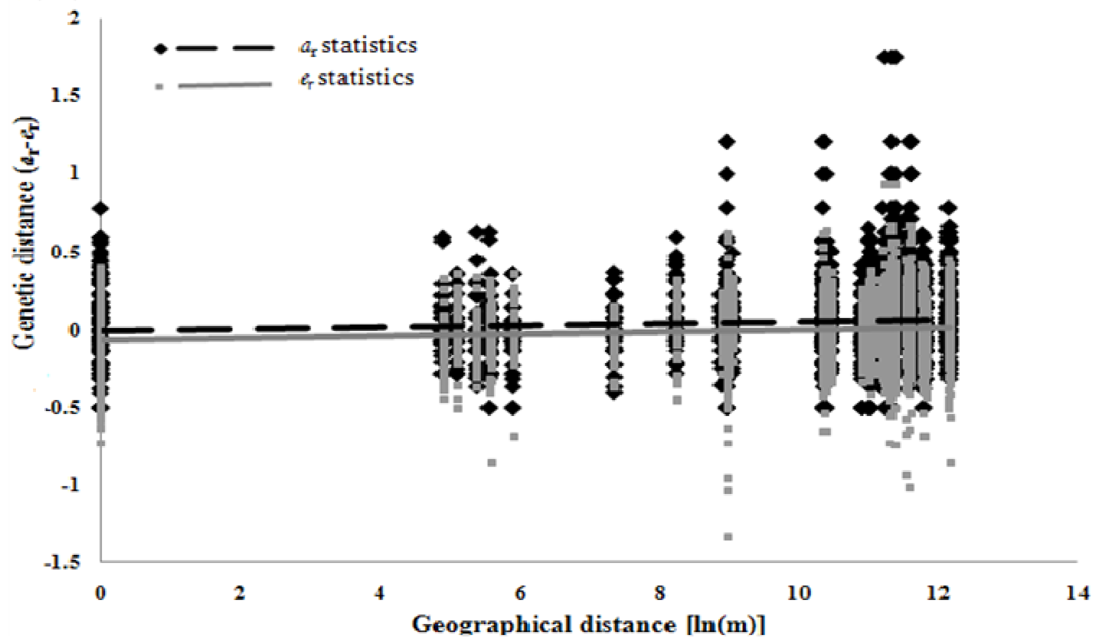


Figure 6. Genetic differentiation in *Delma impar,* shown as the regression of pairwise differentiation between individuals against the logarithm of distance. Analysis with 7 loci (A) a_r = -0.016 + 0.0108 ln(distance), e_r = -0.119 + 0.0115 ln(distance); with 5 loci (B) a_r = -0.031 + 0.0084 ln(distance), e_r = -0.034 + 0.0084 ln(distance).

The AMOVA with data grouped as five populations showed low but significant levels of genetic differentiation (7 loci: F_{ST}=0.035, P=0.001; 5 loci: F_{ST}=0.025, P=0.001), with only 4% (7 loci) and 2% (5 loci) of the total genetic variation due to differences among subpopulations. Values for pairwise F_{ST} calculated between sampled locations were generally small and some did not vary significantly from zero (Table 7). The highest F_{ST} values obtained with all seven loci were between group C, which was identified as a single population in the Geneland analysis with seven loci, and all the other sampled localities. However, this high F_{ST} was not observed when five loci were analysed.

Table 7. Pairwise population F_{ST} for all seven loci (above diagonal) and five loci (below diagonal). ns indicates no significant difference, *P<0.05, **P<0.01, ***P<0.001.

	Group A	Group B	Group C	Group D	Group E
Group A	-	0.030**	0.045***	0.010ns	0.021**
Group B	0.035**	-	0.056***	0.041***	0.035***
Group C	0.020**	0.048***	-	0.027**	0.037***
Group D	0.005ns	0.047**	0.002ns	-	0.032**
Group E	0.002ns	0.039**	0.020*	0.016ns	-

The outcome of the microspatial autocorrelation analyses, performed with and without the loci that presented null alleles, are shown in Fig. 7. The correlogram produced with all seven loci indicates a significant positive correlation in the first three distance classes, 100m (r=0.059, P=0.001), 200m (r=0.087, P=0.001) and 300m (r=0.067, P=0.001), with an intercept of 363. The analysis performed with only five loci revealed a significant positive genetic structure up to a distance of 235, with positive r in the distance classes, 100m (r=0.047, P=0.001) and 200m (r=0.058, P=0.02).

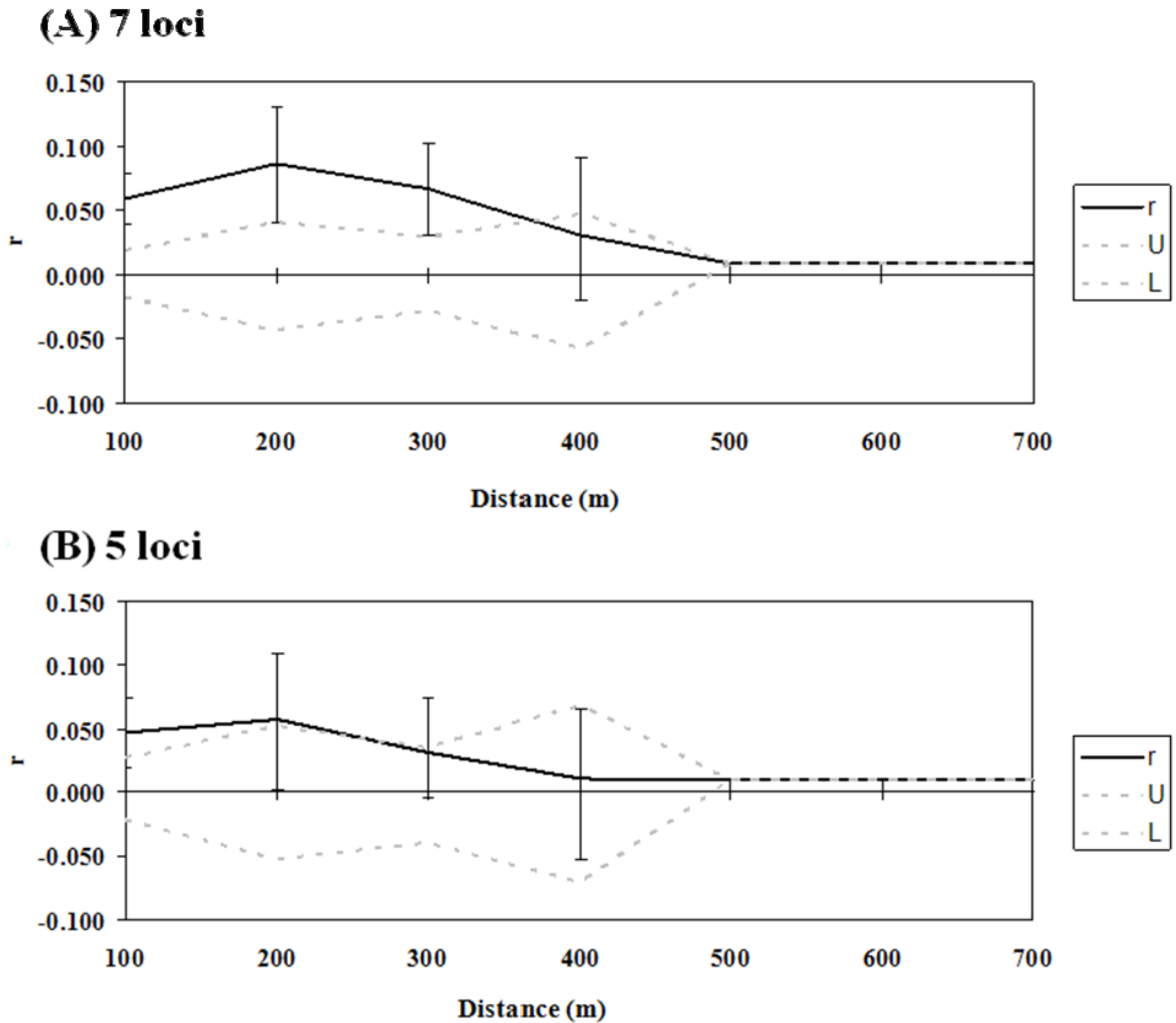


Figure 7. Spatial autocorrelation correlograms for *Delma impar,* including all 7 loci (A) and 5 loci (B). The permuted 95% confidence intervals (gray dashed lines) and the 95% confidence error bars are shown. The autocorrelation coefficient, r, provides a measure of the genetic similarity between pairs of individuals and significant positive autocorrelation implies that individuals within a particular distance class are more genetically similar than expected by chance.

DISCUSSION

Phylogeographic relationships and historical biogeography

The ND2 mtDNA sequence analyses revealed high levels of genetic differentiation between geographically isolated populations of *Delma impar*, indicating isolation over long evolutionary time-frames. The mtDNA sequence divergence between *D. impar* populations in SA and Victoria ranged from 0.8 to 2.1%, and between the NSW/ACT and SA/Victorian lineages the 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 (Scott & Keogh 2000; Chapple & Keogh 2004). Genetic divergences such as these, can be used, in conjunction with an estimated mutation rate of the mtDNA, to approximate the time intervals at which lineages became isolated. However, the use of a constant mutation rate for all species has been described as biologically unrealistic, as it is clear that rates of evolution can, and do vary considerably among species (Bromham & Penny 2003; Kumar 2005). The relaxed molecular clock approach used in this study, which was calibrated with a fossil record, has been suggested as a more appropriate method to estimate divergence times, as it allows the mutation rate to vary among groups of organisms and across the phylogeny (Drummond et al. 2006; Pybus 2006). Even though this method was employed, the divergence time calculations should still be treated as an approximation, as it is recommended to calibrate the molecular clock from multiple genes in order to avoid having a gene dependent molecular clock rather than one dependent upon a lineage (Pamilio & Nei 1988).

Results of the phylogeographic analyses showed that the first isolation event occurred during the mid Miocene to mid Pliocene (3.8-12.8 Ma), where the NSW/ACT lineage became isolated from the Victorian and South Australian (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). Additionally, similar genetic breaks, estimated to have occurred from the late Miocene to early Pliocene between these regions, have been found in two habitat generalists: the common froglet *Crinia signifera* (Symula et al. 2008) and the skink *Egernia whitii* (Chapple et al. 2005). The inclusion of samples from East Gippsland in both these studies provided support to the possibility that the phylogeographical break

occurred in eastern Victoria, possibly due to a marine incursion 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.

Symula et al. (2008) found that the location of the genetic break between NSW/ACT and Victorian and SA populations in *C. signifera* differed to that in *D. impar*. The *D. impar* sample obtained from cluster 14 (near Yass, NSW) formed a distinct lineage with the individuals sampled in other areas of NSW and the ACT, whereas the *C. signifera* sampled from near Yass formed a clade with individuals sampled in Victoria and SA. Symula et al. (2008) proposed that populations of *C. signifera* may have been isolated during the late Miocene to early Pliocene by the glaciation of the Eastern Highlands. However, glaciation in this region did not commence until the late Pliocene (Wagstaff et al. 2001; Martin 2006). Therefore, it is unlikely that glacial events lead to the divergence of *C. signifera* in the late Miocene to early Pliocene. Similarly, the historical isolation of *D. impar* during the mid Miocene to mid Pliocene between the NSW/ACT and Victorian/SA lineage probably did not result from glaciation, 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 (Macphail 1997; Gallagher et al. 2003), which led to the reappearance of rainforest in the river valleys of the Western Slopes in southeast 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 Victoria and SA were estimated to have occurred during from the late Miocene, into the Pleistocene (6.0-0.5 Ma). Molecular studies of other south-eastern herpetofauna (skink: *E. whitii* Chapple et al. 2005; frog: *C. signifera* Symula et al. 2008) also found genetic breaks in this geographic region. Samples from the geographic region of the SA/Horsham lineage formed a distinct clade in *C. signifera*,

with similar phylogeographic patterns of clade differentiation to *D. impar* (Symula et al. 2008). Although the sampling in the *E. whitii* study does not match the areas sampled in my study, there is a clear genetic break between the eastern and western Victorian samples (Chapple et al. 2005). It was estimated that the divergence of eastern and western populations in these studies, as with *D. impar,* occurred during the Plio-Pleistocene for *E.whitii* and late Miocene and Pliocene for *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. 1994; 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 Victoria and SA.

The impacts of the Plio-Pleistocene glaciations on the phylogeography of *D. impar* are likely to be significant. However, there are a number of other geomorphological events that could have significantly impacted the phylogeographic patterns observed in *D. impar.* For example, during the late Miocene (6 Ma) the western Murray Basin was subjected to a major marine incursion (Bowler et al. 2006). Water levels reached Lake Tandou and Lake Mungo in the north, and Kerang and Horsham in the east (Bowler et al. 2006) (Fig. 8). After maximum transgressions (6 Ma) the sea gradually retreated, reaching Naracoorte in SA around 1 Ma (Bowler et al. 2006). This marine incursion could have caused the isolation of the SA/Horsham lineage from the remainder of the Victorian populations, as this event is concordant with the phylogeographical break between these lineages. In addition, volcanic activity in western Victoria during the late Pliocene and Holocene (Joyce 1973; Rosengren 1994), could have significantly changed the landscapes of the region. Within this area, 355 volcanoes have been identified (Rosengren 1994).

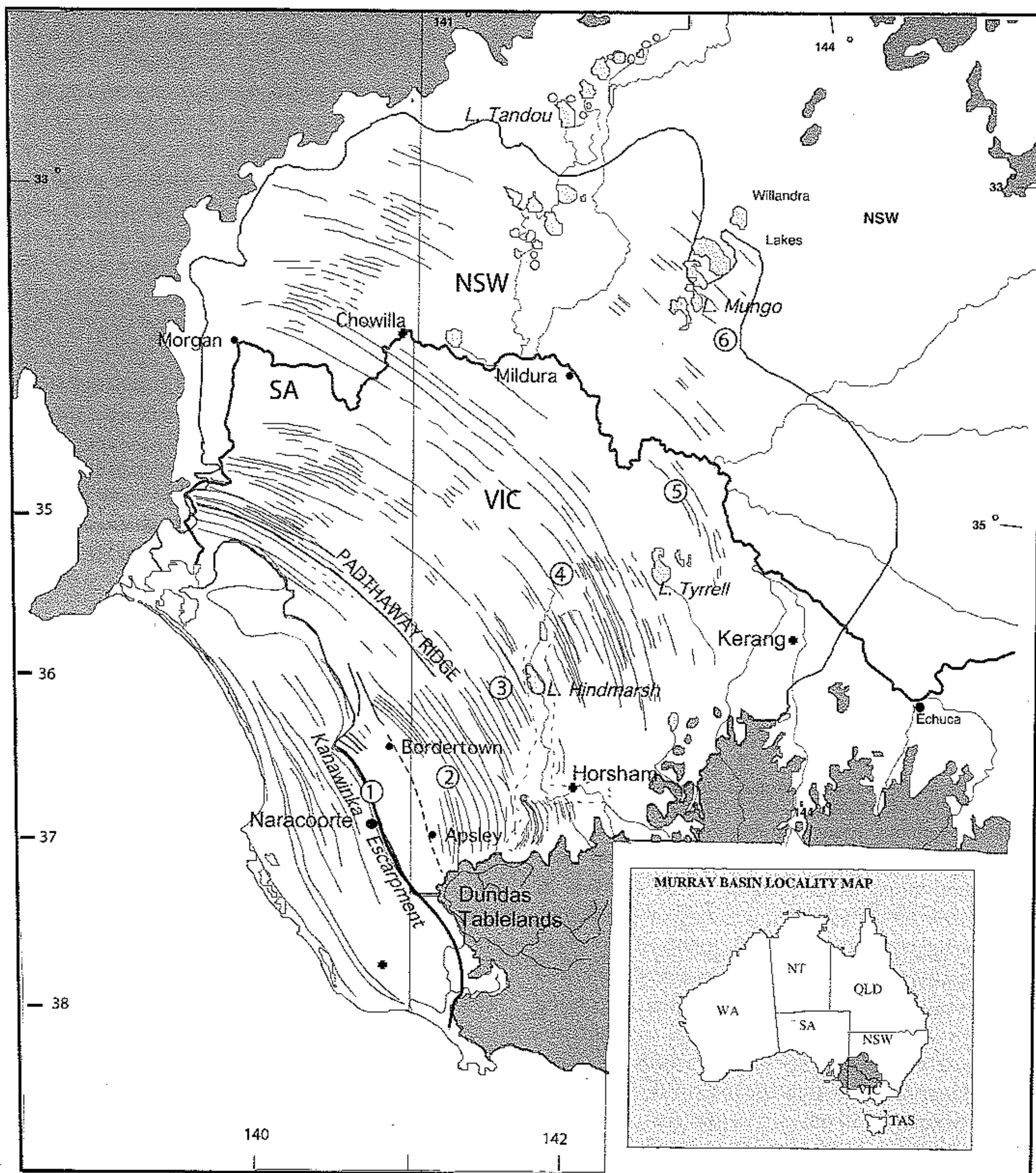


Figure 8. Extent of late Miocene to Pliocene marine invasion of the western Murray Basin showing. Numbered sequence 1-6 denotes shoreline positions, 1 to 6 million years ago (after Bowler et al. 2006).

These created the young basalt units known as the Newer Volcanic Province, a region that extends from central Victoria, including Melbourne, through the southwest to SA (Rosengren 1999). The majority of the volcanoes erupted during the late Pleistocene and Holocene (Wellman 1974 in Rosengren 1994). The resulting lava flows, along with the glacial-interglacial oscillations could have caused the more recent division of the south-west Victorian and east Victorian lineages. The soil type of the Newer Volcanic Province, which originated from the region's volcanic activity, has influenced the distribution of the grasslands in western Victoria (Jones 1999). Extensive lava beds allowed the formation of suitable soil for the spread of grasslands in the region, which in turn could have lead to the expansion of the south-western Victorian lineage.

These natural events could have played an important role in the distribution 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; each historical lineage should be considered as a unique ESU.

Current population structure and levels of genetic connectivity

The distribution of the native temperate grasslands has not only been affected by historic events. Recent anthropogenic activities, such as urbanisation and agriculture, have also fragmented this habitat (Kirkpatrick et al. 1995). Phylogeographic studies can identify the effects that historic events had on populations of species but in order to determine the effects of current anthropogenic disturbance on population connectivity, a population genetic analysis with molecular markers that present high mutation rates and high levels of variability, such as microsatellite markers, is necessary. The five sampling localities analysed with microsatellite markers 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_E 0.67 - 0.75). The sampling locality which presented the highest level of inbreeding was "B", which may be due to a smaller effective population size in this sampling locality in comparison to the others. Information on population size has been hard to obtain for this species (Smith & Robertson 1999), so it is hard to ascertain whether a reduction in population size, or other factors, such as

relatives not dispersing far, is causing the higher levels F_{IS} observed. Nonetheless, it would be useful to obtain an estimation of population size in order to verify this.

Most of the analyses indicated that there is limited genetic structure in the sampled area, identifying the presence of only one population (Geneland analysis with 5 loci and Structure analyses with 7 and 5 loci). In the population assignment tests, only a small percentage of the individuals were assigned to the group from which they were sampled, which can be indicative of low population sub-structuring. However, due to the lack of sufficient samples in most sites from management clusters 3A and 3B, only a small number of sites from this area were included in this study. It is advisable to incorporate in an assignment test samples from all the source populations of a recently fragmented area (where a single population used to occur) (Vazquez-Dominguez et al. 2001). It will be necessary to acquire more samples from the remaining sites the area in order to obtain more accurate results.

The overall F_{ST} of 0.035 and 0.025 for the AMOVA with 7 and 5 loci respectively, also suggest a low differentiation among sampling sites. A previous allozyme study on *D. impar* found low levels of genetic differentiation between populations in the ACT over a similar geographic scale (Osmond 1994). Studies usually justify low genetic differentiation between recently fragmented populations with two explanations: the first is that the study species has high levels of vagility (Hickerson & Wolf 1998; Ramirez & Haakonsen 1999) and the second is that the observed pattern of genetic structure may reflect historical rather than current gene flow (Bossart & Prowell 1998; Monaghan et al. 2001), where human-induced habitat changes might be too recent to notice marked genetic effects (Sumner et al. 2004; Glechet et al. 2005). It is probable that the lack of genetic structure in *D. impar* is a result of historical gene flow. I found evidence of isolation by distance in *D. impar*, where a significant positive correlation was obtained between pairwise individual genetic distance and geographical distance (Fig. 6). The spatial autocorrelation analysis also confirms small-scale dispersal, where the genetic similarity between individuals is significantly positive up to the 300 m distance class (200 m distance class for 5 loci). Field-based studies also indicate that *D. impar* is not a vagile species (Coulson 1990; Kutt 1992; O'Shea 1996). Although little is known about its movement patterns, the longest movement distance recorded in *D. impar* is 60 m (Kukolic et al. in 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. Genetic connectivity between these populations prior to European settlement was observed in the mtDNA analysis, where populations in these areas formed an independent historical lineage. Therefore, the lack of genetic differentiation observed may be indicative of historical gene flow patterns rather than the current movement of *D. impar* between the isolated habitat patches.

In central-western Victoria, grazing activities began in the late 1830s on the native perennial grasses and herbaceous vegetation, without significant land-clearing activities (Lang 2000). Then in the 1940s and 1950s, farmers started transforming their properties from areas of native perennial grasses and herbaceous vegetation into areas of exotic annual and perennial pasture grasses (Conn 1993; Gibbons & Rowan 1993). The transformation 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; O'Shea 1996; Smith & Robertson 1999) and it has been suggested that this species may be able to survive in such areas (Smith & Robertson 1999; Rohr & Peterson 2003). Thus it is likely *D. impar* populations have only been isolated for approximately 60-70 years. It has been estimated that female *D. impar* start reproducing at 3-4 years of age and the maximum age recorded for an individual was 7 years, though researchers estimate a maximum age of 10 years or longer (ARAZPA 1996; Smith & 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 a detectable genetic signatures (Hartl & Clark 1989).

One analysis that indicated the presence of two populations in this study area; the Geneland and AMOVA analyses on 7 loci. In these analyses individuals sampled at locality "C" were considered a unique population (Fig. 5). Furthermore, the highest pairwise Fst values (results with 7 loci only) between the sampling locations was recorded between site "C" and all the other sampling localities. Site "C" also presented the highest number of private alleles, which can be interpreted as a greater population differentiation in this group. This last observation has to be interpreted with caution, as the high sample size in this group compared to others may have resulted in the higher detection of rare alleles. Nonetheless, the boundary between the two

inferred populations coincides with a transition between the Dundas Tablelands and Victorian Volcanic Plain bioregions. (Fig. 9). Also, the two samples from the Dundas Tableland bioregion included in the phylogeographic analyses presented a unique haplotype (haplotype B) compared to the samples from the same ESU, which were sampled from the Victorian Volcanic Plain bioregion (Table 2). Bioregions are determined depending on the patterns of ecological characteristic in the region (Thackway & Cresswell 1995). 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 mainly woodlands, with three major species of gum trees (Paine & Phang 2003). In contrast, the Victorian Volcanic Plains is mainly dominated by the young basalt of the Newer Volcanic Province and much of the area was dominated by grassland prior to European settlement, although some areas supported dry sclerophyll forest and savannah woodland (Joyce 2003).

Although this boundary between bioregions provides strong evidence for the possible reasons that might have lead to population divergence in this area, these results need to be treated with caution. A study showed similar results when genetic structure was examined with both Structure and Geneland (Coulon et al. 2006), where only Geneland was able to detect weak spatial genetic structure (Coulon et al. 2006). This study suggested that the main reason for the observed results was due to methodological difference in the two software packages. Geneland includes in its analyses the spatial information of the samples, whereas Structure does not, this could explain why the Geneland analyses provide a better definition of spatial genetic units (Coulon et al. 2006). However, the presence of the two loci with null alleles in the 7 loci dataset cannot be ignored, even though testing of models in Geneland have shown that this software is robust to the presence of null alleles (Guillot unpublished in Coulon et al. 2006). Consequently, future analyses including greater sampling localities, more individuals and added polymorphic markers are needed in order to determine whether there is a genetic break between the two bioregions located east of site “C”.

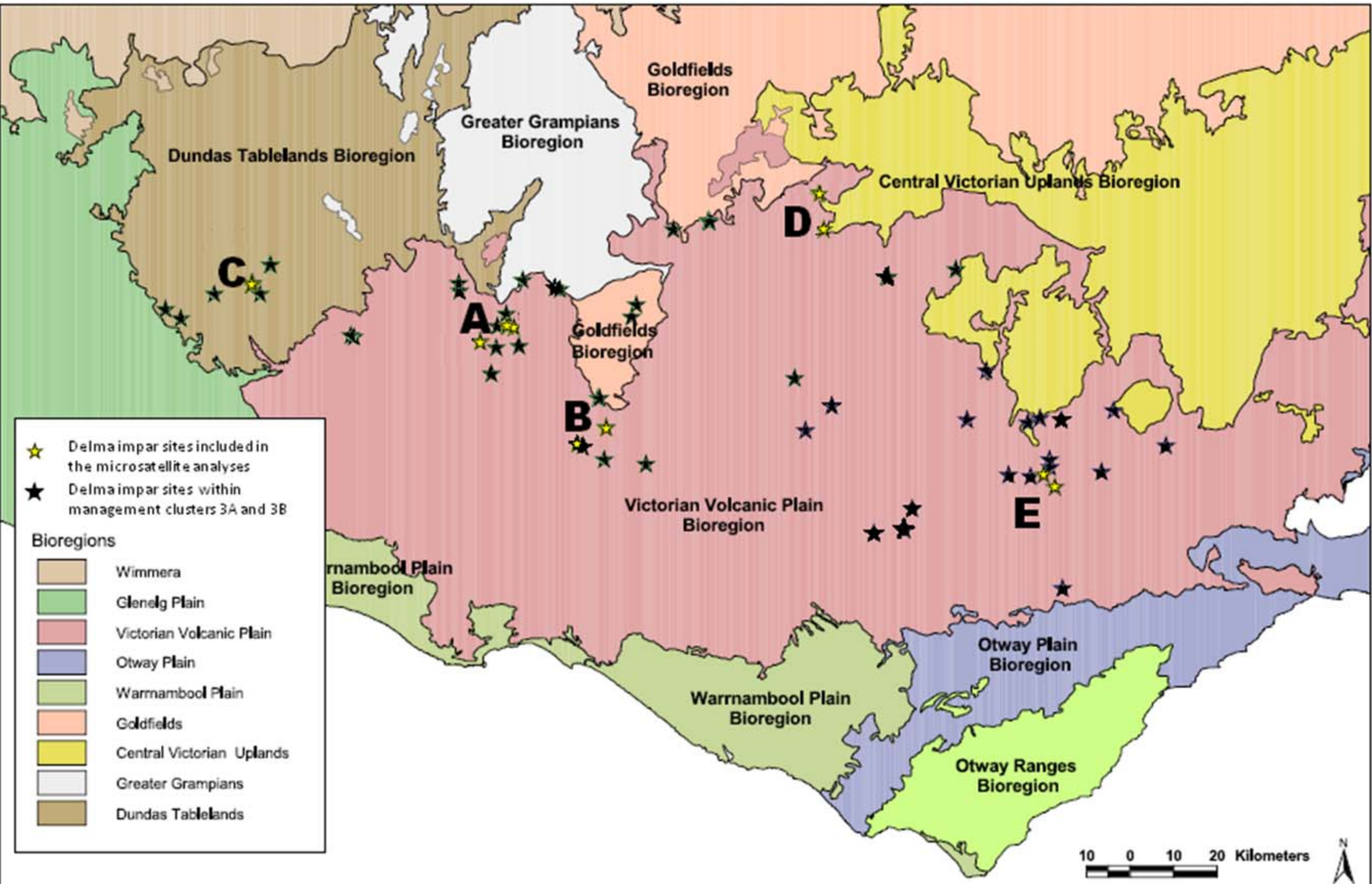


Figure 9. Map of south-west Victorian bioregion. *Delma impar* sites within the management clusters 3A and 3B are represented by the black and yellow stars. Localities of samples included in the population genetics analyses are represented by yellow circles. Each sampling locality is labelled with the same letter given in Fig. 2

Even though the analyses could not provide a strong evidence of population sub-structuring due to recent anthropogenic fragmentation; it does provide support to previous findings that *D. impar* has relatively low dispersal abilities. Furthermore, my study provides a base for future research, where new microsatellite loci can be used in order to determine the significance of the population structure observed in the different bioregions. These current finding and future directions 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 habitat patches could eventually lead to the loss of genetic diversity (Frankham et al. 2002).

Management implications

The arrangement of *D. impar* populations into management clusters has been used by working groups and agencies responsible for the conservation of *D. impar* as a way to manage areas that could present unique genetic identities (ARAZPA 1996; Smith & Robertson 1999). This approach was taken in order to protect and maintain the genetic diversity of the species across its geographical range. It has been suggested that each of the management clusters may represent a unique Evolutionarily Significant Unit (ESU) but this could not be determined due to the lack of detailed information on the genetics of this species.

My study included ten of the 17 management clusters and phylogeographic analyses identified four divergent clades, which should be considered as separate ESUs. The most widely used definition of an ESU relies largely on identifying historically isolated populations that have reciprocally monophyletic haplotypes and exhibit significant genetic divergence of allele frequencies at nuclear loci (Moritz 1994). The lineages identified in this study meet part of this definition but further information on divergence levels at nuclear loci is required in order to confirm that the deep genetic divergence are not restricted to the mtDNA, which is a wholly maternally inherited marker (Avise 2000). 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.

It would be advisable to obtain samples from the remaining management clusters, especially cluster 1, in order to determine if they also constitute unique ESUs. The area of management clusters 1 and 2 is the region of Victoria that is being heavily impacted by urbanisation, with the rapid expansion of Melbourne's outer suburbs. Housing developments are dramatically altering the remnant native grassland habitats in these areas and individuals of *D. impar* will have to be relocated to suitable habitats (Smith & Robertson 1999). Therefore, it is necessary to determine if cluster 1 should be considered as a unique ESU, as the introduction of individuals into a population with a different evolutionary history can result in the loss of unique genetic characteristics of a region (Dobzhansky 1970; Rhymer & Simberloff 1996).

Additionally, the Geneland and AMOVA analyses performed with 7 loci indicated that there are possibly two separate populations in central-western Victoria, occurring at the boundary between two bioregions. Further sampling and polymorphic microsatellite loci are needed to determine whether there are two populations in this area. The results from the microsatellite data also supported previous observations that *D. impar* has low dispersal capabilities. 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 & 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 using a combination of molecular markers (mtDNA and microsatellites) I was able to estimate the effects that natural historic and current anthropogenic events had on the genetic diversity and structure of D. impar, which provided insights on this species' past and present population connectivity. Additionally, my study has demonstrated the utility of employing multiple molecular markers to assess the genetic structure of populations at different spatial and

temporal scales. Such an approach provides detailed information for management authorities on when a lack of gene flow between habitat fragments should be maintained versus re-established. I believe that it would be beneficial for more conservation genetic research to use a similar approach when investigating the population connectivity of endangered species that inhabit highly fragmented landscapes.

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