

Restricted gene flow in the endangered pygmy bluetongue lizard (*Tiliqua adelaidensis*) in a fragmented agricultural landscape

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Abstract. Habitat fragmentation can have several adverse genetic impacts on populations. Assessing the extent of these threatening processes is essential in conservation management. In the present study, we investigated the genetic population structure of the endangered pygmy bluetongue lizard, *Tiliqua adelaidensis*, which is now restricted to a few small fragments of its previously more extensive grassland habitat. The aim of our study was to investigate genetic diversity and gene flow both among and within sample sites. The information will assist in making recommendations for habitat conservation and translocation programs. We collected DNA from 229 individuals from six isolated sample sites and genotyped them for 16 polymorphic microsatellite loci. Across all six sample sites, observed heterozygosity ranged from 0.75 to 0.82. There was no evidence of population bottlenecks and little evidence of inbreeding due to consanguineous mating. Genetic differentiation was low to moderate although significant for all pairs of sample sites ($F_{ST} = 0.021\text{--}0.091$). Results from Bayesian clustering analyses revealed distinct clusters in the overall sample and suggested restricted gene flow between sample sites separated by distances ranging from 1.7 to 71.6 km. By using spatial autocorrelation, we also found a significant genetic structure within sample sites at distances up to 30 m, suggesting restricted gene flow even in small patches of continuous habitat. It will be important to preserve this finely clustered population structure in captive breeding and translocation programs. Increasing opportunities for gene flow through habitat corridors or population augmentation may help maintain genetic diversity and prevent an increase in differentiation. Although endangered species do not always present model systems for studying fragmentation, our approach shows how important genetic information can be acquired to aid conservation in highly fragmented ecosystems.

Introduction

Large-scale habitat fragmentation by humans has been identified as one of the greatest threatening processes to global biodiversity (Saunders *et al.* 1991) because small, isolated populations have an increased risk of extinction from demographic processes (MacArthur and Wilson 1967). Fragmentation also has adverse genetic impacts on populations. For example, small populations lose genetic diversity at a faster rate than do large ones because genetic drift increases as population size is reduced (Lacy 1987). Loss of genetic variation equates to a loss of evolutionary potential, leaving species less able to adapt to environmental change and disease (Templeton *et al.* 2001). Fragmentation also disrupts gene flow, leading to an increase in genetic differentiation among populations (Wright 1931). Furthermore, genetic factors such as inbreeding depression (Saccheri *et al.* 1998) and mutational meltdown (Lynch *et al.* 1995) can put fragmented populations at a greater risk of extinction. Genetic studies are thus essential in conservation management to identify the extent of these threatening processes and help make decisions about habitat-restoration, connectivity, land-protection and translocation programs.

Because genetic variation can be lost through drift if populations are isolated, genetic diversity can be maintained by translocating individuals to augment small or inbred populations (Mills and Allendorf 1996; Moritz 1999). Although this has been

a successful strategy for some species (Spielman and Frankham 1992; Madsen *et al.* 1999; Pimm *et al.* 2006; Trinkel *et al.* 2008), translocations can lead to outbreeding depression by reducing local adaptation of functional genes (Storfer 1999; Allendorf *et al.* 2001). Outbreeding depression will be a problem only where there is substantial genetic divergence among populations (Allendorf *et al.* 2001); thus, assessing the amount of genetic divergence among sites is essential when translocation is part of a conservation plan. Similarly, when translocation is used to reintroduce a population into protected land, or sites of local extinction (e.g. Taylor and Jamieson 2008; Witzemberger and Hochkirch 2008), understanding spatial genetic structure is necessary to maintain natural genetic relationships within a species (Reinert 1991; Sigg *et al.* 2005).

Studies of fragmented animal populations have been strongly biased towards birds and mammals (Mac Nally and Brown 2001), even though reptiles may experience greater impacts because of their limited mobility (Driscoll 2004). Reptiles that have been studied have shown reduced genetic diversity and increased population differentiation in fragmented landscapes compared with more continuous habitat (Sarre *et al.* 1990; Sarre 1995; Gullberg *et al.* 1998; Sumner *et al.* 2004). The impact was high for species where fragmentation has reduced dispersal (Stow and Sunnucks 2004a), and less pronounced for those reptiles that can disperse among patches following fragmentation (Driscoll and

Hardy 2005). Studies from a broader range of taxa are required to understand better the response of reptiles to habitat fragmentation (Stow and Sunnucks 2004a) and, from a conservation perspective, data from endangered reptile species are especially valuable. In the present study, we investigated the genetic diversity and population structure of the endangered (IUCN 2009) pygmy bluetongue lizard, *Tiliqua adelaidensis*, which is now restricted to a few small fragments of its previously more extensive grassland habitat. Although fragmentation can also alter within-population processes, including inbreeding avoidance (Stow and Sunnucks 2004b) and kin relationships (Walker *et al.* 2008), in the present study we focussed mainly on gene flow within and among study sites. Relatedness and mating systems will be dealt with in more detail in a follow-up study.

Tiliqua adelaidensis is a long-lived (up to 9 years) skink that gives birth to 2–4 live young in late summer (Milne *et al.* 2002). For over 30 years *T. adelaidensis* was considered extinct; however, since its rediscovery in 1992 (Armstrong *et al.* 1993) it has been found inhabiting remnant patches of degraded

native grassland in the mid-northern agricultural region of South Australia (Hutchinson *et al.* 1994; Fig. 1a). This region was completely developed for agriculture by 1880, within 50 years of European settlement of South Australia (Harris 1976). The small, isolated patches of grassland that remain are predominantly used for sheep grazing and are surrounded by cultivated land. Cultivated land is unsuitable for *T. adelaidensis* because ploughing disrupts the behaviour of the spiders that construct the burrows in which *T. adelaidensis* lives (Souter 2003; Souter *et al.* 2007). Changes in land use from sheep to wheat production have continued since early agricultural development (130 years ago) and the exact timing of fragmentation among remnant patches is unknown.

The extent of the natural range of *T. adelaidensis* is unknown, because only 20 specimens were collected before 1992. Most of these had imprecise or no location information (Armstrong *et al.* 1993). The original distribution probably extended as far south as the Adelaide plains (Ehmann 1982; Fig. 1a). The total number of *T. adelaidensis* remaining in the wild is unknown;

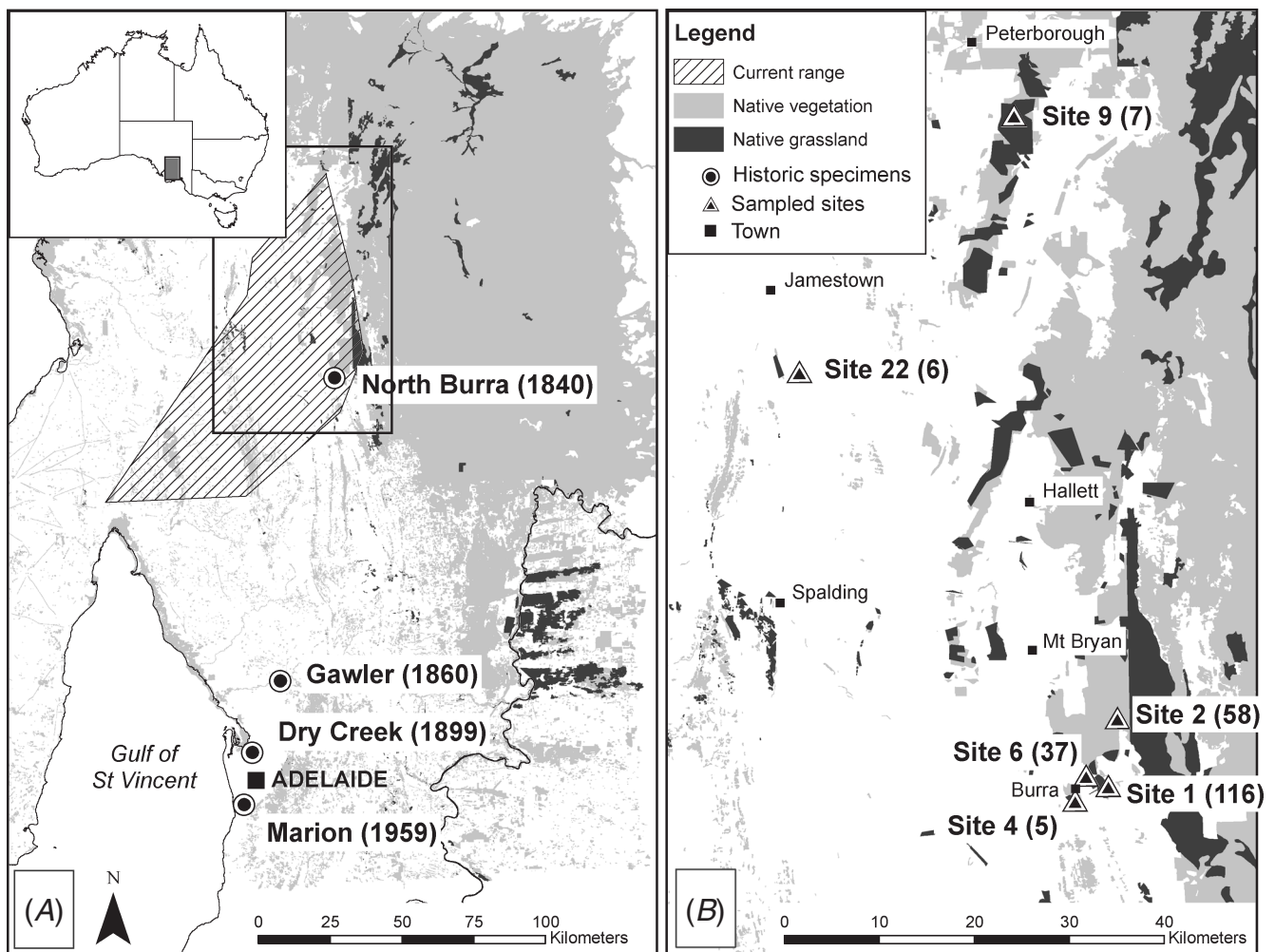


Fig. 1. (A) Locations of the 24 known *Tiliqua adelaidensis* sites in the mid-northern agricultural region of South Australia and locations of four samples collected before 1960. (B) Locations of the six *T. adelaidensis* sites sampled in the study, with sample sizes shown in parentheses. Native grasslands shown are not necessarily suitable habitat for *T. adelaidensis*.

however, a preliminary study of 10 sites by Milne (1999) estimated the total population size as >5700. Although the number of known sites has increased since this estimate, total population size has decreased substantially during recent years of low rainfall (J. Schofield, pers. comm., 2008). Captive-breeding programs in *T. adelaidensis* have had little success so far. However, the establishment of a breeding colony on protected land remains an important goal because all known populations occur on privately owned farmland.

The aims of the present study were to (1) determine whether populations showed evidence of low genetic diversity or population bottlenecks, (2) examine the level of gene flow among sites at a broad scale (up to 72 km) and (3) examine the level of gene flow within sites on a smaller scale (up to 400 m) to determine the local population structure in this species. This will help determine whether isolated populations would benefit from greater habitat connectivity to increase gene flow and overcome any consequences of reduced genetic variation. Developing translocation programs for *T. adelaidensis* will be assisted by understanding the population structure at a local scale, to mimic the natural genetic structure and prevent outbreeding or inbreeding depression in artificially founded or augmented populations.

A common approach in genetic studies of fragmented populations is to compare traits of a species in undisturbed, continuous habitats with those in fragmented habitats (e.g. Sumner *et al.* 2004; Driscoll and Hardy 2005). Unfortunately, the range of *T. adelaidensis* has been so extensively altered by farming that no populations remain in continuous habitats. The lack of an unfragmented control site is therefore an inherent limitation in our study system. Our approach took advantage of a few well sampled populations to examine gene flow within habitat patches, as a surrogate for a continuous population. We discuss how this may relate to the broader pattern among populations.

Materials and methods

Sample collection

We sampled 229 adult pygmy bluetongue lizards from six isolated sites around the town of Burra (33°40'57"S, 138°56'18"E), South Australia (Fig. 1b), between September 2005 and March 2006. Distances among sample sites ranged from 1.7 to 71.6 km. We lured lizards from their burrows with a baited fishing rod (Milne and Bull 2000) and clipped toes for individual identification. Blood from the toe clip was stored on FTA paper (Whatman, Maidstone) to provide a DNA sample. The location of each lizard was recorded with a geographic positioning system. The limited overall population size, the shy nature of the lizards (they retreat to burrows at the first sign of movement), and the time-consuming 'fishing' method of capture meant that larger sample sizes of *T. adelaidensis* were difficult to obtain. We focussed sampling effort on the three sites closest to Burra (Sites 1, 2 and 6) to obtain large enough samples for detailed spatial genetic analysis. We collected fewer samples from three sites that were further afield (Sites 4, 9 and 22). Sample sizes from each site (5–116 individuals) thus reflected the sampling effort rather than the population size.

DNA extraction and PCR amplification

DNA was extracted from FTA paper according to the procedure for nucleated erythrocytes in Smith and Burgoyne (2004). Individual lizards were genotyped for 16 polymorphic microsatellite loci: one isolated from *Egernia stokesii*, Est12 (Gardner *et al.* 1999), and 15 isolated from *Tiliqua rugosa*, Tr3.2 (Cooper *et al.* 1997), TrL9, TrL12, TrL14, TrL15, TrL16, TrL19, TrL21, TrL27, TrL28, TrL29, TrL32, TrL34, TrL35 and TrL37 (Gardner *et al.* 2008). Multiplex PCR conditions followed Gardner *et al.* (2008).

Samples were genotyped on an ABI 3730 capillary electrophoresis DNA analyser (Applied Biosystems, Foster City, CA). A fluorescently labelled size standard (GS500 (-250) LIZ) was run with the samples and alleles were scored by using GeneMapper software version 3.0 (Applied Biosystems).

Disequilibrium and null alleles

We checked our dataset for evidence of Hardy–Weinberg (HW) and genotypic disequilibrium by using GENEPOP version 3.4 (Raymond and Rousset 1995). There were 462 comparisons of genotypic disequilibrium and 96 tests for HW disequilibrium; thus, *P*-values were adjusted for multiple testing by the sequential Bonferroni method (Hochberg 1988). For genotypic disequilibrium tests, pairs of loci were grouped by population and the Bonferroni adjustment was applied to each population separately. We report significant deviations from disequilibrium both before and after the adjustment for multiple tests. Each locus was checked for the presence of null alleles by using the program MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004). Only locus TrL37 showed evidence of HW disequilibrium at sample Site 2 after the Bonferroni adjustment ($P < 0.001$). Locus TrL21 showed evidence of HW disequilibrium without the Bonferroni adjustment at Sites 1 ($P = 0.005$) and 2 ($P = 0.002$). Only two of the 462 pairwise comparisons showed evidence of genotypic disequilibrium after the adjustment. These were between TrL21 and TrL28 ($P < 0.001$) and TrL19 and TrL37 ($P < 0.001$) at sample Site 1. Without the adjustment TrL16 and TrL27 at Site 2 ($P = 0.006$) and TrL21 and TrL12 at Site 6 ($P = 0.002$) also showed evidence of linkage. The locus TrL21 displayed evidence of null alleles at sample Sites 1 and 2. Because patterns of disequilibrium and null alleles were not consistent across sample sites, we retained all 16 loci for our analyses except where indicated below.

Genetic diversity

Mean observed (H_O) and expected (H_E) heterozygosities over all loci were estimated by using Nei's (1978) unbiased estimate of heterozygosity for each sample site in POPGENE version 1.32 (Yeh *et al.* 1997). We investigated local inbreeding in *T. adelaidensis* by calculating F_{IS} for each site separately (Weir and Cockerham 1984) by using FSTAT version 2.9.3.2 (Goudet 2002). Significant values of F_{IS} can indicate a departure from random mating, and may identify populations that show signs of consanguineous mating (Keller and Waller 2002). Values of F_{IS} were tested for significant departures from zero in each population by randomising alleles among individuals. Significance at the 0.05 and the adjusted α levels

are reported. The adjusted P -value based on the number of comparisons was 0.00052 for our dataset.

Genetic-diversity statistics such as the number of alleles per locus depend on the sample size and thus were difficult to compare among our study sites. We calculated allelic richness with FSTAT as an estimate of genetic diversity because it provides a value standardised for sample size (El Mousadik and Petit 1996). This statistic is based on the size of the smallest sample. We therefore report allelic richness based on all six sample sites (smallest $n=5$) and on the three sites with larger samples (smallest $n=37$).

The program BOTTLENECK version 1.2.02 (Cornuet and Luikart 1996) was used to determine whether any population of *T. adelaidensis* had suffered a recent bottleneck. This program performs tests to determine the probability of a heterozygosity excess at a site. Rare alleles do not contribute substantially to heterozygosity and are lost rapidly during a bottleneck (Luikart and Cornuet 1998). Thus, populations that have suffered a recent bottleneck may show higher levels of heterozygosity in relation to the number of alleles than would be shown in a population at mutation-drift equilibrium (Luikart and Cornuet 1998). The analysis was performed only on the three sites with the largest sample sizes as a minimum of 30 individuals is required for a reliable result (Piry *et al.* 1999). Because these tests are sensitive to deviations from Hardy–Weinberg equilibrium (Luikart and Cornuet 1998), we removed locus TrL37 from the dataset because it did not meet this assumption. This left a panel of 15 loci for each site, providing sufficient power to detect a recent bottleneck (Luikart and Cornuet 1998). The Wilcoxon's test was selected to determine the significance of heterozygosity excess (Piry *et al.* 1999) with 10 000 permutations. We calculated probability values based on the stepwise-mutation model (SMM), the infinite-alleles model (IAM) and the two-phase-mutation model (TPM). The TPM is considered the best descriptor for genetic data derived from microsatellite alleles (Di Rienzo *et al.* 1994). Following recommendations of Piry *et al.* (1999) we weighted the TPM with 5% IAM and 95% SMM. We also examined the allele frequency distributions in each population for evidence of a mode shift from the normal L-shape (Luikart *et al.* 1998).

Population structure and gene flow

We examined population structure (i.e. how the overall sample was subdivided) in *T. adelaidensis* to estimate genetic differentiation and the extent of gene flow among sample sites. Population structure was examined first with allele frequency-based statistics, and second with Bayesian clustering software. A disadvantage of using only allele frequency-based statistics to study population structure is that these methods require prior and subjective definition of population boundaries, which may not correspond to actual genetic populations (Pritchard *et al.* 2000). Bayesian clustering methods use individual genotypes to determine population structure with no prior information of population boundaries (Pritchard *et al.* 2000). Genotypic methods can also be applied at the individual, rather than the population level, giving more detailed information and on a more recent time scale (Sunnucks 2000). This approach is ideal for our unbalanced dataset, because all individuals are drawn from the

same pool and boundaries are not forced around those sites by smaller sample sizes.

The partitioning of overall genetic variation within and among sample sites of *T. adelaidensis* was examined with an analysis of molecular variance (AMOVA). The AMOVA was conducted in GENALEX version 6 (Peakall and Smouse 2006) with 9999 permutations for statistical testing. F_{ST} -values (Wright 1951) were calculated as a measure of genetic differentiation between each pair of sample sites by using the program MICROSATELLITE ANALYSER (Dieringer and Schlötterer 2003). Significant genetic differentiation between sample-site pairs was determined by 10 000 permutations of alleles and P -values were adjusted by the sequential Bonferroni method (Hochberg 1988). We explored isolation by distance (Wright 1943) among sample sites by using a Mantel test in GENALEX to examine the correlation between pairwise $F_{ST}/(1 - F_{ST})$ and the geographic distances among sites. We used 9999 permutations for statistical testing of the Mantel test. For visual assessment of the genetic relationships among sample sites, we also conducted a principal coordinates analysis (PCoA) based on F_{ST} with GENALEX.

We employed the following two commonly used Bayesian clustering packages to study population structure: STRUCTURE version 2.2 (Pritchard *et al.* 2000) and GENELAND version 3.1 (Guillot *et al.* 2005b) operated through the statistical program R (Ihaka and Gentleman 1996; R Development Core Team 2005). Both of these methods use a Markov chain Monte Carlo (MCMC) algorithm to define the number of clusters (K) in a sample that are at Hardy–Weinberg and linkage equilibrium (Pritchard *et al.* 2000; Guillot *et al.* 2005a). Each individual is given a probability of membership to one of the identified clusters (Q), allowing the user to identify the extent of gene flow, and potential migration among sample sites. The major difference between the two packages is that GENELAND uses spatial data in the model on the assumption that individual membership will be spatially dependent to some degree (Guillot *et al.* 2005a), whereas STRUCTURE is based only on genotypes, with no assumptions that clusters are related to geographical proximity.

For both STRUCTURE and GENELAND we used the correlated allele-frequencies model which assumes that all individuals are derived from the same ancestral population, and that allele frequencies from one population are not independent of those from other populations (Falush *et al.* 2003). This model is appropriate for datasets such as ours, where populations are closely related and only low differentiation is detected (Falush *et al.* 2003).

STRUCTURE was run with the following parameters: the admixture model with no prior population information, a burnin length of 50 000 and a MCMC length of 1 million iterations. We simulated the number of clusters from $K=1$ to $K=12$ and performed 10 independent simulations of each K -value to check for consistency across runs. We plotted the mean posterior probability, $\text{Ln Pr}(X|K)$, from the 10 runs against K to determine where the values reached a plateau as an indication of the most likely number of clusters (Pritchard *et al.* 2007). The number of clusters was also inferred by calculating ΔK from the rate of change between successive simulations (Evanno *et al.* 2005). We assigned individuals to a cluster on the basis of the highest probability of membership and checked the consistency

of assignments across the 10 runs at the most likely number of clusters.

GENELAND was run using the spatial model, maximum $K=12$, and thinning=100. Other parameters were left at default levels. We ran 10 independent simulations, each with 1 million MCMC iterations. The outputs were post-processed with a burnin length of 1500 after checking the posterior density of the model from preliminary analyses to determine an appropriate length. We then checked the results (number of clusters, probability of membership and posterior densities) for consistency across the 10 runs.

Fine-scale population structure

Population structure was examined within sites by spatial autocorrelation, a statistical method to examine the distribution of a variable, in our case relatedness among individuals, through space (Hardy and Vekemans 1999; Smouse and Peakall 1999). We implemented the analysis in the program SPAGED version 1.2 (Hardy and Vekemans 2002) which allowed us to pool data from multiple sample sites while restricting pairwise comparisons to within sites. This increased the sample size, and thus the power of the analysis. Relatedness (Li *et al.* 1993) was estimated and Euclidean distances were calculated between all pairs of individuals within each of the six sample sites. Spatial distances were divided into classes of 0–15, 16–30, 31–60 and 61–100 m to maximise the number of pairs in each class yet still providing fine-scale resolution. Above 100 m, additional distance classes were increased by 50 m. Individuals were permuted among spatial locations 10 000 times and 95% confidence intervals were obtained from the permuted data. Standard errors of mean observed relatedness estimates were generated by jackknifing over loci (Hardy and Vekemans 2002). Significant positive genetic structure was inferred if the standard errors of observed relatedness fell outside the confidence intervals of the permuted data.

Results

Genetic diversity

Heterozygosity estimates in *T. adelaidensis* were similar across all six sample sites (Table 1). Mean observed heterozygosity ranged from 0.750 (± 0.237 s.d.) to 0.823 (± 0.197 s.d.) and mean expected heterozygosity ranged from 0.778 (± 0.182 s.d.) to 0.826 (± 0.139 s.d.). At the 0.05 α level F_{IS} was significantly greater than zero at Sites 1 ($P=0.034$) and 2 ($P=0.022$). This

indicates some level of consanguineous matings at these sites. At the adjusted α level F_{IS} did not differ significantly from zero at any of the sample sites (Table 1). Allelic richness ranged from 5.17 to 5.96 when based on a sample size of 5, and from 12.13 to 13.89 when based on a sample size of 37 for the three larger sampled populations (Table 1).

At the three extensively sampled sites, there was no evidence of a recent bottleneck under either the TPM or the SMM ($P>0.5$ and $P>0.7$, respectively, at each site). Although there were significant values derived under the assumptions of the IAM ($P<0.001$ at each site), this is probably a less realistic model for microsatellites (Luikart and Cornuet 1998) and we placed less weight on that result. We found no significant mode shifts at any sample site, with allele frequencies showing normal L-shaped distributions.

Population structure and gene flow

Overall there was significant genetic differentiation among the six sample sites of *T. adelaidensis*, although it accounted for only 4% of the total genetic variation, whereas 96% was attributed to within-site variation (AMOVA: F_{ST} among sample sites = 0.038, $P<0.001$). Pairwise F_{ST} -values were low to moderate (range 0.021–0.091; Table 2) although significant for all 15 sample-site pairs following a sequential Bonferroni adjustment for multiple tests. For the three sites with large sample sizes, pairwise F_{ST} -values were all significant despite being within 7.5 km of each other (Table 2). Pairwise $F_{ST}/(1 - F_{ST})$ and the geographic distance between pairs of sample sites were weakly, although positively, correlated (Mantel test: $y=0.0005x+0.0494$, $R^2=0.332$, $P=0.098$), indicating non-significant trend of isolation by distance over the 72-km scale of the study (Fig. 2).

The PCoA (Fig. 3) showed a trend for genetic relationships to reflect the spatial relationships among sites. The geographically clustered sites (1, 2 and 6) were clustered on the PCoA, whereas the geographically more separated sites (9 and 22) were also separated by genetic distance. The trend was not supported by Site 4, geographically close to Sites 1, 2 and 6, but separated from those sites on the PCoA. However, larger samples are needed from Sites 4, 9 and 22 to draw firm conclusions about their genetic relationships.

To determine the number of clusters from the STRUCTURE results, Pritchard *et al.* (2007) suggested that when successive values of K give similar estimates of $\text{Ln Pr}(X|K)$, the smallest of these is most likely the true number of clusters. On the basis of this recommendation our data indicated four distinct clusters in the

Table 1. Genetic diversity statistics for *Tiliqua adelaidensis* at six sample sites with varying sample sizes (n)
Mean observed (H_O) and expected (H_E) heterozygosities (\pm s.d.) over 16 loci, the inbreeding coefficient (F_{IS}), and mean allelic richness for a sample size of 5 ($R_{(5)}$) and a sample size of 37 ($R_{(37)}$) are shown. *Significantly greater than zero, $\alpha=0.05$. No F_{IS} values were significantly different from zero at the 0.00052 adjusted level

Sample site	n	H_O	H_E	F_{IS}	Mean $R_{(5)}$	Mean $R_{(37)}$
1	116	0.813 (0.150)	0.826 (0.139)	0.016*	5.92	13.25
2	58	0.801 (0.166)	0.821 (0.164)	0.025*	5.96	13.89
4	5	0.750 (0.237)	0.799 (0.163)	0.068	5.31	–
6	37	0.799 (0.163)	0.812 (0.153)	0.016	5.71	12.13
9	7	0.813 (0.213)	0.787 (0.182)	–0.035	5.48	–
22	6	0.823 (0.197)	0.778 (0.182)	–0.065	5.17	–

Table 2. F_{ST} for each pair of *Tiliqua adelaidensis* sample sites are shown below the diagonal line

All pairwise values of F_{ST} were significant. Geographic distances between sample-site pairs are shown in kilometres above the diagonal line. Values for the extensively sampled sites (Sites 1, 2 and 6) are shown in bold

	1	2	4	6	9	22
1		7.30	2.89	1.71	70.40	53.72
2	0.030		9.59	6.77	63.74	49.45
4	0.061	0.066		2.83	71.61	53.56
6	0.021	0.028	0.069		69.16	52.06
9	0.065	0.060	0.089	0.069		34.35
22	0.059	0.082	0.078	0.069	0.091	

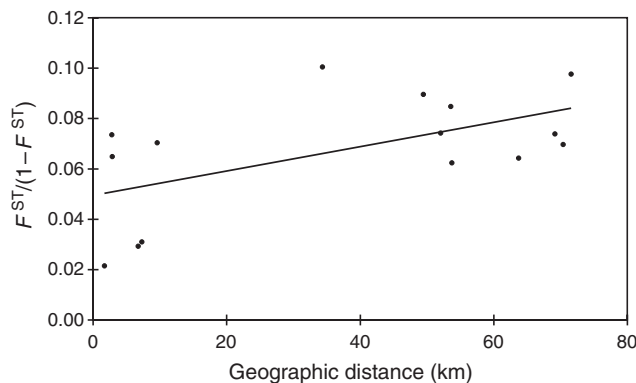


Fig. 2. A non-significant trend of isolation by distance (Mantel test: $y=0.0005x+0.0494$, $R^2=0.332$, $P=0.098$) across six sample sites of *Tiliqua adelaidensis*.

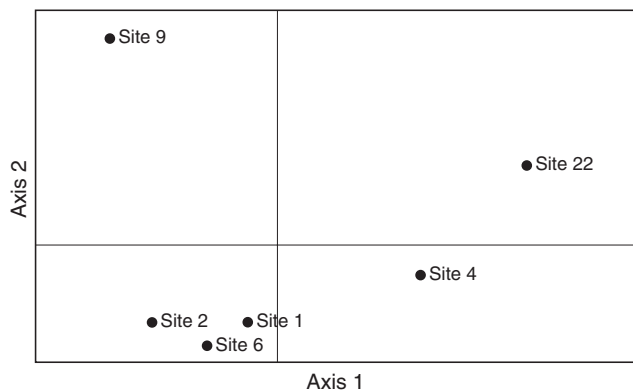


Fig. 3. Principal coordinates analysis for six sample sites of *Tiliqua adelaidensis*. Percentage of variation explained by each axis: Axis 1 = 32.39, Axis 2 = 26.97.

overall sample. However, as this is a conservative estimate of the number of clusters, we also examined the results where the number of clusters was six, which had the highest probability. The ΔK method (Evanno *et al.* 2005) also suggested that the number of clusters from the STRUCTURE result was four. Individuals were consistently assigned to the same clusters across the 10 runs. Assignments from the $K=4$ results showed that individuals from sample Sites 1, 2 and 6 formed distinct

clusters corresponding to their sampled area, whereas the sites with smaller sample sizes (4, 9 and 22) clustered in a fourth group (Fig. 4a). This was despite Site 4 being geographically closer to the larger sampled sites. Twelve individuals from Sites 1 ($n=7$), 2 ($n=2$) and 6 ($n=3$) were consistently assigned to clusters not corresponding to their sampled areas, although the probability of membership (Q) for these 'misassigned' individuals was generally marginal (mean $Q=0.65 \pm 0.13$ s.d.) (Fig. 4a). When the assignment results for $K=6$ were examined the three smaller sample sizes continued to cluster together, with the extra genetic structure being detected within the samples from Site 1 (Fig. 4b).

GENELAND identified six distinct clusters within the overall sample of *T. adelaidensis* and this result was consistent across the 10 independent simulations. In 8 of the 10 runs, individuals were consistently assigned to the same clusters, and these corresponded precisely to their sample locations. This was in contrast to the STRUCTURE results in which Sites 4, 9 and 22 clustered together when $K=6$.

Fine-scale population structure

We found significant positive spatial genetic structure within sample sites, with pairs of individuals within the 0–15- and 16–30-m distance being more highly related than by chance (Fig. 5). Relatedness in the distance classes ≥ 60 m did not differ significantly from random.

Discussion

Habitat fragmentation has been cited as a major threat to the persistence of *T. adelaidensis* (Milne 1999), as it is for most species that now occupy only a fraction of their previous range (Saunders *et al.* 1991). The present study took an important step in assessing the impact of this threat on *T. adelaidensis*. Although we had limited ability to compare the results with those from a naturally more continuous habitat, we documented several patterns of contemporary gene flow and genetic diversity in *T. adelaidensis*, which will be important for the conservation management of this species. First, we found no evidence of population bottlenecks in *T. adelaidensis* and none of the sample sites showed particularly low levels of microsatellite genetic diversity. Second, all sample-site pairs had low to moderate, although significant, levels of F_{ST} and there was a distinct genetic structure among samples sites separated by only a few kilometres. Third, genetic structure was detected within small patches of continuous habitat, indicating a fine-scale pattern of isolation by distance in this species.

Genetic diversity in an endangered lizard

In frogs and reptiles, restricted gene flow and small population size in fragmented landscapes have been shown to lead to inbreeding depression (Andersen *et al.* 2004) and substantial decreases in genetic diversity (Sarre 1995; Ciofi and Bruford 1999; Gullberg *et al.* 1999). In threatened lizard species occupying habitat fragments, Reid *et al.* (2004) and Ciofi and Bruford (1999) both reported mean observed heterozygosities at microsatellite loci of <0.50 . In contrast, an endangered grassland skink from New Zealand, *Oligosoma grande*, was found to have a relatively high microsatellite genetic diversity (mean $H_O=0.79$)

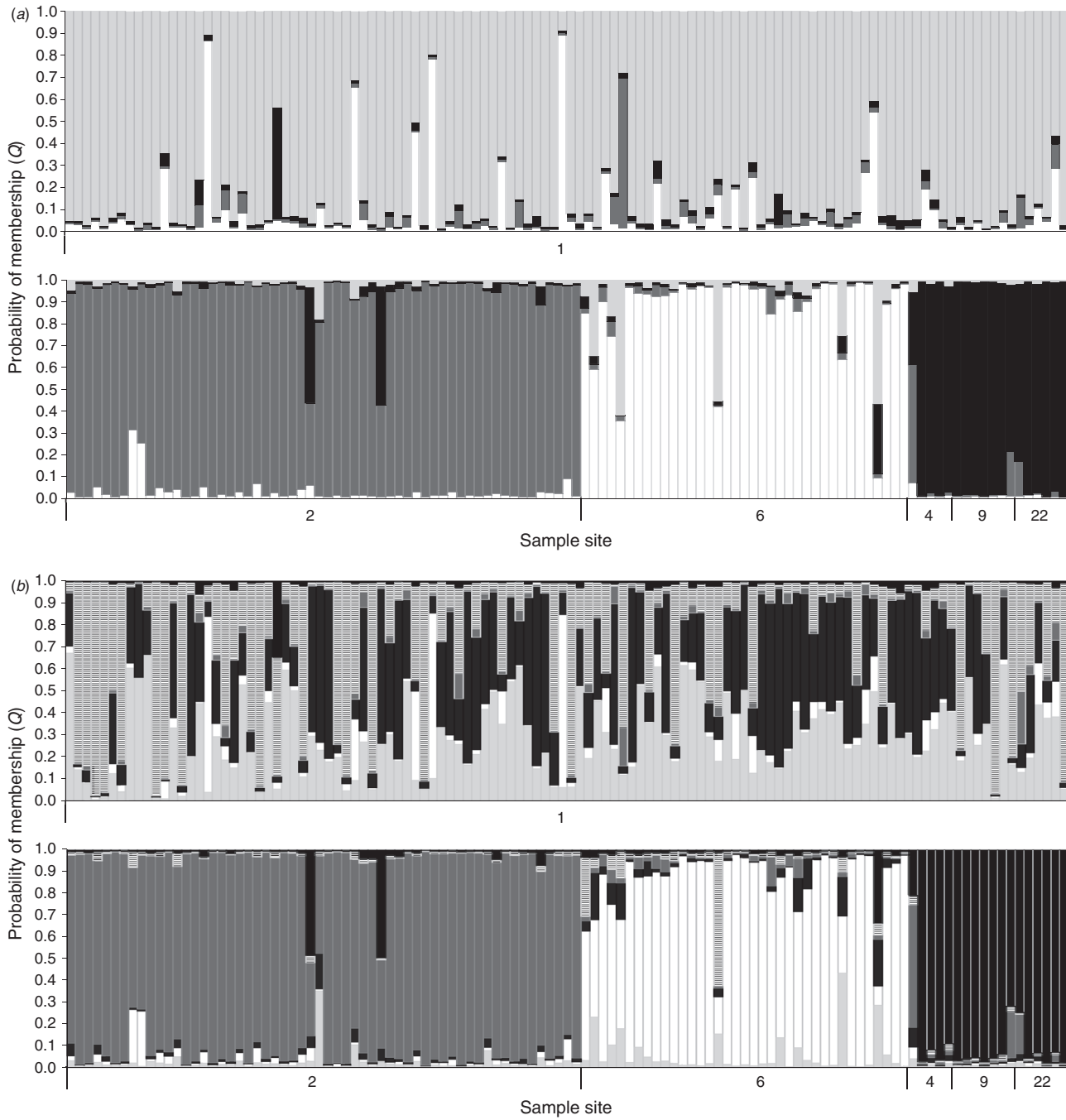


Fig. 4. Probability of assignment to a cluster (Q) for all sampled *Tiliqua adelaidensis* individuals where the number of clusters is (A) $K=4$ and (B) $K=6$. Each bar represents one individual and individuals are grouped by sample site. The different shades represent the clusters identified by STRUCTURE.

(Berry *et al.* 2005). In *T. adelaidensis*, we found a similarly high microsatellite diversity, with the mean observed heterozygosity ranging from 0.75 to 0.82. Although our data showed little evidence of inbreeding due to consanguineous mating, a related skink species has been shown to behaviourally increase inbreeding avoidance in small fragments (Stow and Sunnucks 2004b). Two sites in the present study showed negative (although non-significant) values of F_{IS} (Sites 9 and 22) and these sites had the lowest levels of H_E and lower than average allelic richness.

Conversely, Sites 1 and 2 showed some evidence of mating with relatives and the highest levels of H_E . This result could indicate increased inbreeding avoidance at sites where genetic diversity is reduced and warrants a more detailed investigation of the mating system in *T. adelaidensis*.

Frankham (1996) showed that genetic variation is positively related to population size. Thus, threatened species that have not experienced substantial declines following fragmentation or that have naturally low population sizes may not show reduced

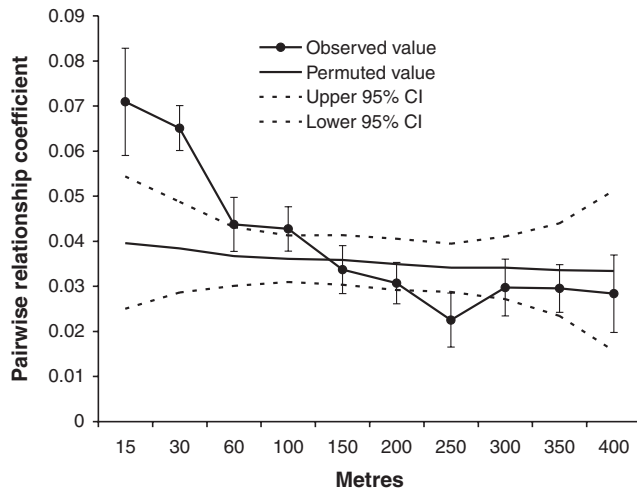


Fig. 5. Positive spatial genetic structure within sample sites of *Tiliqua adelaidensis*. Mean pairwise relationship coefficients and standard errors are shown.

genetic variation within habitat fragments. The surviving populations of *T. adelaidensis* may have remained large enough to sustain genetic diversity through random mating or by selecting unrelated mates, because there was no evidence for a population bottleneck. However, undetected population bottlenecks may have occurred, because the signal of excess heterozygosity can be restored in as little as three generations after a bottleneck (Keller *et al.* 2001). Alternatively, the long-lived nature of *T. adelaidensis* (up to 9 years) may have protected it from rapid loss of diversity following fragmentation. Without knowing the historic levels of microsatellite variation we cannot tell whether habitat fragmentation has caused a decline. Regardless, maintaining high genetic diversity should be a priority for conservation management of *T. adelaidensis* to ensure that its evolutionary potential is protected (Moritz 2002).

Gene flow in fragmented landscapes

Several skink species have reduced gene flow in fragmented compared with continuous habitats (Stow *et al.* 2001; Sumner *et al.* 2004; Berry *et al.* 2005). The significant F_{ST} values among the sample sites of *T. adelaidensis* and the distinct genetic clusters in the overall sample indicated restricted gene flow among sample sites. Without information on gene-flow rates before fragmentation, we cannot tell whether the gene flow is naturally low in this species, or whether it has been reduced because of recent fragmentation. However, results from our spatial autocorrelation analysis revealed a significant genetic structure within habitat patches at a fine scale (<400 m); thus, genetic structure over several kilometres would be expected with or without fragmentation. Lada *et al.* (2008) combined genetic analysis with simulation modelling to distinguish between the current and historic gene flow among populations of a small, carnivorous marsupial. Such an approach could be useful in future studies of *T. adelaidensis*, provided that reliable estimates of population sizes can be obtained (Lada *et al.* 2008).

In a 3-year mark-recapture study of *T. adelaidensis*, most lizards within a monitoring hectare showed high site fidelity, and

usually dispersed <20 m and never >70 m (Milne 1999). Although long-distance dispersal events were not monitored, such low mobility might lead to spatial genetic structure even without habitat fragmentation. However, the dramatic decline in native-grassland habitat within the distribution of the species has certainly reduced the opportunity for gene flow. Previously connected grassland habitats in South Australia (Specht 1972) are now separated by roads and cultivated fields. Even roads can decrease dispersal in small terrestrial animals, leading to increased levels of genetic structure (Gerlach and Musolf 2000; Keller and Largiadèr 2003). Results from our spatial autocorrelation analysis supported Milne's (1999) findings of low dispersal by showing that related individuals are finely clustered within sites. Actual dispersal distances cannot be inferred from our spatial-autocorrelation results because the x -intercept (i.e. the distance at which observed and random relatedness are equal) depends strongly on the distance classes chosen for analysis (Fenster *et al.* 2003). Quantifying dispersal rates and distances and sex bias in dispersal will be an important goal in future studies on this species.

We found low F_{ST} values among sites separated by up to 70 km, suggesting that gene flow between these locations was a natural feature of genetic structure in *T. adelaidensis* (mean pairwise $F_{ST} \pm$ s.d. = 0.062 ± 0.021). Sumner *et al.* (2004) recorded similar F_{ST} values in a small skink among five sites in continuous forest habitat (mean pairwise $F_{ST} \pm$ s.d. = 0.059 ± 0.032). By using STRUCTURE, we detected 'migrants' among sites; however, these may reflect ancestral gene flow, rather than current migration events (Waser and Strobeck 1998). The GENELAND results did not reveal any misassigned individuals. Fully Bayesian models such as STRUCTURE assume that all candidate populations have been sampled and force individuals into one of the identified clusters (Berry *et al.* 2004). Berry *et al.* (2004) recommended that a higher level of stringency (e.g. probability of membership = 95 or 99 for fully Bayesian models) should be applied if the accuracy of assignment is critical. We based assignments only on the highest probability of membership values, which were generally low for misassigned individuals (range = 0.50–0.89, mean = 0.65 ± 0.13 s.d., Fig. 4).

There was a slight discrepancy in the results from the two Bayesian clustering methods. GENELAND identified six clusters, which corresponded precisely with the six sample sites, whereas STRUCTURE indicated four clusters, with the smaller sampled sites aggregated into one group. There was a slightly higher probability for six clusters in the STRUCTURE results, although the smaller sampled sites were still aggregated and the extra genetic structure was detected within the Site 1 sample. The three sites with small sample sizes may not contain enough information to delineate population boundaries accurately, thus forming one cluster. Discrepancies between GENELAND and STRUCTURE results were also found in studies by Dudaniec *et al.* (2008) and Coulon *et al.* (2006). This suggests that including spatial data in the GENELAND model makes it more sensitive to a weak genetic structure (Coulon *et al.* 2006). Determining the true number of clusters (four or six?) from our data is probably irrelevant in terms of conservation management. The important conclusion is that there are distinct

genetic clusters in *T. adelaidensis* that probably result from low gene flow, even among sites in close geographic proximity.

Gene flow in continuous habitats

Within small patches of continuous grassland, we observed a significant fine-scale genetic structure in *T. adelaidensis*, indicating a natural pattern of isolation by distance (Wright 1943). Peakall *et al.* (2003) suggested that the fine-scale positive spatial genetic structure could result from (1) habitat heterogeneity, (2) social organisation and (3) restricted gene flow. Habitat heterogeneity is unlikely to explain the fine-scale structure in *T. adelaidensis*. Their grassland habitat occurs on uniform, undulating low hills, with no major natural barriers within or among sites. The lizards depend on mygalomorph and lycosid spiders to construct their burrows (Hutchinson *et al.* 1994; Souter *et al.* 2007); thus, a patchy distribution of spiders could produce a naturally fragmented distribution of lizards. However, within our study areas the burrows did not show any obvious clustering.

Social organisation can affect genetic structure within populations if related individuals aggregate (Lawes *et al.* 2000), as shown in several sister taxa to *T. adelaidensis* (Chapple 2003; Fuller *et al.* 2005; Gardner *et al.* 2007). Unlike those species, *T. adelaidensis* is solitary, with individuals living alone in their burrows and interacting only briefly for mating (Milne *et al.* 2003). *T. adelaidensis* may have a less overt social organisation where individuals tolerate proximity of relatives more than non-related individuals. Studies of social interaction in this species may identify its relative contribution to the observed positive genetic structure.

Restricted gene flow can produce a positive spatial genetic structure even in continuous habitats (Wright 1943; Turner *et al.* 1982). Restricted gene flow within *T. adelaidensis* arising from limited dispersal is probably the main process for generating the fine-scale positive genetic structure we observed within sites. By extrapolation this might also explain a historical origin for the broader-pattern genetic structure among sites and the trend of isolation by distance. Given the significant genetic structure observed at a local scale, a stronger pattern of isolation by distance might be expected at a larger scale than what we have reported here. However, the three populations with small sample sizes may not provide enough power to detect such a signal. The weak correlation between F_{ST} and the geographic distance across the whole study area may become significant with larger samples.

Our findings from the analysis of gene flow within sites suggested that the population structure of *T. adelaidensis* is shaped by naturally low dispersal distances and a consequent clustering of related individuals. This implies that the genetic effects of recent fragmentation may not be severe because long-distance dispersal may have rarely occurred. Species that naturally live in small, clustered populations may not be affected by fragmentation if the scale of fragmentation is too coarse to be perceived (Henle *et al.* 2004). Because of the limitations faced in the present study, continued investigation of the impacts of fragmentation is necessary to address this issue.

Conservation implications

The lack of evidence for population bottlenecks, and the high microsatellite diversity relative to some other endangered species

are a positive result for *T. adelaidensis*. Although we are currently unsure whether there has been a decline in microsatellite diversity following habitat fragmentation, management of this species should focus on maintaining the high level of genetic diversity observed in the study. Habitat preservation, restoration and population monitoring will be essential to ensure that populations do not suffer further declines in numbers. Because grasslands are one of the most threatened ecosystems in southern Australia (Harris 1976; Lunt *et al.* 1998) habitat-conservation projects for *T. adelaidensis* will ultimately have major benefits for biodiversity. Habitat conservation should focus not only on sites with existing *T. adelaidensis* populations, but also on restoring links among sites. Establishing habitat corridors can be an effective conservation tool for maintaining genetic diversity and evolutionary potential in species threatened by fragmentation (Beier and Noss 1998; Gillies and St. Clair 2008). Although we are unsure whether restricted gene flow among sites reflects natural or altered genetic structure, habitat corridors would help restore the natural connectivity among sites that have been fragmented by roads and agricultural practices. Artificial burrows have successfully increased population densities in *T. adelaidensis* (Souter *et al.* 2004) and would assist in establishing links among isolated sites. Management of *T. adelaidensis* would greatly benefit from a detailed study of the effect of connectivity and isolation on dispersal to assess the extent of connectivity required.

A strategy complimentary to building habitat corridors is to augment populations with individuals from other sites, thus artificially increasing gene flow (Moritz 1999). A study of adders, *Vipera berus*, in Sweden found that the effects of inbreeding depression were reduced in an isolated population following the introduction of male snakes from non-isolated populations (Madsen *et al.* 1999). Although such translocations carry the risk of causing outbreeding depression by reducing local adaptation (Storfer 1999), the risk is low where genetic divergence among sites is small (Allendorf *et al.* 2001). Considering the low, albeit significant genetic differentiation that we observed among sites in the present study, the risk of outbreeding depression for *T. adelaidensis* is probably low and translocations may prove useful in maintaining genetic diversity and preventing inbreeding depression. Careful consideration of variation in habitat, selection pressure and population history among sites is essential when assessing the number of migrants and source sites of translocated individuals (Mills and Allendorf 1996; Storfer 1999). Movement of individuals among isolated populations of *T. adelaidensis* should thus be restricted to sites that were connected before agricultural development and have similar local environmental conditions. Continued work to distinguish between historic and contemporary gene flow could help identify previously connected populations (Hansen and Taylor 2008; Lada *et al.* 2008). Furthermore, combining the present genetic study with further studies of ecological similarity or divergence would help determine which local populations are exchangeable (Rader *et al.* 2005).

One of the greatest threats to *T. adelaidensis* is the potential change in land use, as all known sites are on privately owned farmland. It is probable that some of the populations will suffer further habitat loss despite the best efforts of conservation managers. Establishing populations on protected land is

therefore an important goal for the conservation of this species. Preserving the finely clustered population structure observed in the present study will be important if translocation attempts are to be successful (Reinert 1991). This could be achieved by ensuring that the spatial relationships among individuals in the relocated population are similar to those in the source population. In establishing a new population, the majority of individuals should be sourced from the same site to maintain a finely structured population and avoid potential outbreeding depression. Miller *et al.* (2009) suggested an adaptive management approach for another threatened skink species where both pure and hybrid populations are maintained to determine the fitness of individuals from different management strategies. Such an approach would help determine the most successful mix of individuals in translocated populations of *T. adelaidensis*. Preservation of mating systems and social structure are also essential for translocation and captive-breeding programs (Sigg *et al.* 2005) and will be the focus of continuing studies on *T. adelaidensis*.

Although endangered species do not always present model systems for studying genetic effects of fragmentation because of small sample sizes and restricted distributions, understanding their population genetic structure can greatly assist conservation decision making. By studying genetic structure at different spatial scales, the present study has expanded the current knowledge of endangered species in fragmented ecosystems. It has also added to the growing literature on the genetic structure in reptiles and will benefit research on this taxon which has previously been under-represented in fragmentation studies.

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