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Critically low levels of genetic diversity in fragmented populations of the endangered Glenelg spiny freshwater crayfish *Euastacus bispinosus*

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ABSTRACT: The Glenelg spiny freshwater crayfish *Euastacus bispinosus* is a large endangered freshwater invertebrate of southeastern Australia that has suffered major population declines over the last century. Disjunct populations in the state of South Australia are in a particularly critical condition, restricted to a few isolated rising-spring habitats and in an ongoing state of decline. We assessed genetic diversity and gene flow within *E. bispinosus* across its current range using allele frequencies from 11 nuclear microsatellite loci and DNA sequence data from a single mitochondrial locus (cytochrome oxidase subunit I). Populations were characterized by low levels of genetic diversity and found to be highly structured, with gene flow restricted both within and across catchments, highlighting the species' vulnerability to further habitat fragmentation and the importance of managing environmental threats on local scales across its current natural range. South Australian populations were characterized by critically low levels of genetic diversity generally, highlighting their potential vulnerability to localized extinction. Holistic conservation efforts are necessary to conserve populations, including local habitat management and, potentially, translocations to increase genetic diversity and evolutionary potential, and reduce possible inbreeding effects and the threat of extinction.

KEY WORDS: Population genetics · Gene flow · Genetic diversity · Species conservation · Habitat protection/restoration · Translocation · Inbreeding

INTRODUCTION

Parastacid freshwater crayfish of the genus *Euastacus* are endemic to Australia and comprise one of the largest, most diverse and threatened groups of freshwater crayfish in the world (Furse & Coughran 2011b). This group of crayfish is of considerable conservation concern, with 39 of the 50 *Euastacus* species currently listed under International Union for Conservation of Nature (IUCN) threat categories (17 Critically Endangered, 17 Endangered and 5 Vulnerable; Furse & Coughran 2011a). Yet conservation management of many threatened *Euastacus* species remains limited by a lack of information concerning critical aspects of their life history (Furse & Coughran 2011b). Increasingly, genetic studies are becoming a

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major component of conservation planning in threatened species programs. Genetic markers can provide information on evolutionary history, contemporary estimates of genetic diversity and gene flow and key factors contributing to the fitness of populations (Frankham et al. 2009, Hedrick 2011). This can help in the identification of critical populations for protection, understanding of life-history traits (e.g. dispersal), development of programs to facilitate and maintain gene flow, and the design of captive breeding programs (Weeks et al. 2011, Coleman et al. 2013).

The Glenelg spiny freshwater crayfish *E. bispinosus* Clark, 1936 is an iconic long-lived species endemic to the Glenelg River catchment in southwestern Victoria (VIC), and karst rising-spring habitats in southeastern South Australia (SA) (Zeidler 1982, Morgan 1986, Hammer & Roberts 2008). Populations are also know to occur in 2 isolated sinkholes to the northwest and southwest of Mount Gambier in South Australia (Whiterod et al. 2014), which are suspected to be the result of translocations (Fig. 1). Across its range, the species has undergone severe fragmentation and population decline over the past century as a result of overfishing, hydrological alteration, and habitat degradation (Coughran & Furse 2010, DSEWPC 2012). Recent assessment has elevated the species to 'endangered' nationally under the Environment Protection and Biodiversity Conservation Act 1999, and 'Vulnerable' globally under IUCN criteria, resulting in the closure of the recreational fishery. The disjunct South Australian populations are in a particularly critical condition, persisting in 7 isolated karst spring-fed coastal ponds and streams and 2 nearby sinkholes across a 2 km^2 area of occupancy (Eardley 1943, Hammer & Roberts 2008, Whiterod et al. 2014). Historically, these habitats were connected and embedded within a peat swamp system, which has been extensively drained from the 1940s to the present day for agricultural purposes (Eardley 1943, Armstrong 1974, SEWCBD 1993). These fragmented populations are characterized by low abundances and limited recruitment, and most appear to be in slow decline (Whiterod et al. 2014).

In dispersal-limited species such as freshwater crayfish, habitat fragmentation is expected to reduce levels of gene flow among populations (Frankham et al. 2009, Coleman et al. 2013). Small isolated populations are particularly prone to the loss of genetic diversity due to random genetic drift, making them more susceptible to fitness reductions (e.g. inbreeding depression), stochastic environmental change, and localized extinction (Frankham et al. 2009). Hence there are concerns for the genetic health of the fragmented South Australian *E. bispinosus* populations. While direct evidence is lacking (Nielsen et al. 2012), several lines of evidence suggest that the South Australian populations may be suffering inbreeding depression. Low numbers of berried females (those carrying eggs) have been observed during the breeding season, and recruitment levels are typically low (Honan & Mitchell 1995a, Whiterod et al. 2014). Also, exceptionally high frequencies of aberrant individuals (possessing both male and female gonopores) occur in South Australian populations (>50% of individuals at some sites) (Whiterod et al. 2014). Whilst gonopore aberrations are common in freshwater crayfish taxa including *Euastacus* species (Morgan 1997, Horwitz & Adams 2000, Parnes

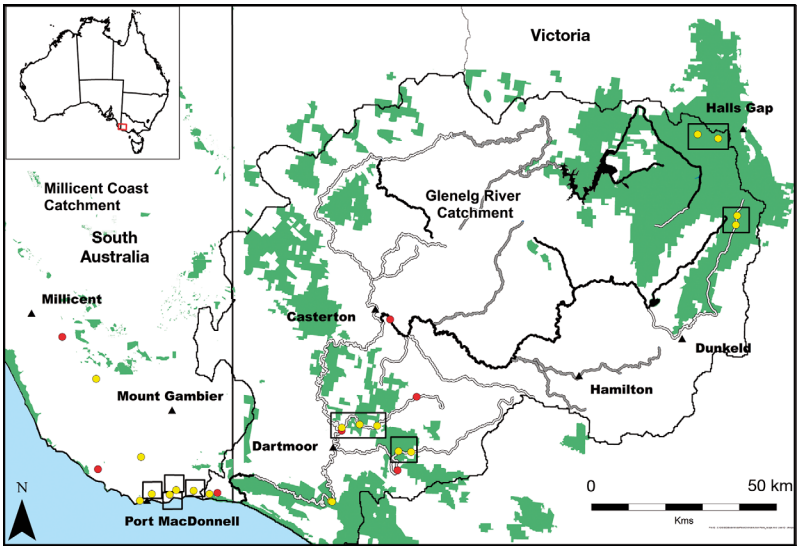

Fig. 1. Glenelg River Catchment (thin black line) in Victoria and the Millicent Coast Catchment in South Australia. Thick black lines indicate stretches of the Glenelg River where *Euastacus bispinosus* was thought not to be present, dark grey lines indicate stretches where *E. bispinosus* was thought to potentially occur and light grey lines indicate areas where *E. bispinosus* was thought likely to occur. Yellow circles indicate sample sites where *E. bispinosus* was captured; red circles indicate sample sites where *E. bispinosus* was not recorded. Black polygons indicate site groups with accompanying site codes as described in Table 1 (except for cluster of sites in SE South Australia). Green polygons represent native vegetation; black triangles represent population centres. Note: due to scale restrictions, the 4 polygons in the South Australian study area do not precisely represent the locations of site groups and are indicative only

et al. 2003, Ford 2012), higher incidences have been linked to genetic abnormalities (Parnes et al. 2003) and environmental pollution (Ford 2012), and may adversely impact reproductive function and success. Finally, recent preliminary genetic research indicated that a South Australian population had critically low levels of genetic diversity and was highly differentiated from 2 populations sampled in the state of Victoria (Miller et al. 2013).

The South Australian populations are of significant conservation importance for various reasons. First, they occupy habitats that are ecologically unique across the species' range (relatively constant discharge rates and temperature, low salinity, and high dissolved oxygen) (Hammer & Roberts 2008). Second, the depletion of the species is expected to have detrimental ecological impacts on the transformation of energy through aquatic food webs (Reynolds & Souty-Grosset 2011), with potential knock-on effects on other species in these unique ecosystems. Third, these populations occur at the western edge of the species range; fringe populations can play a disproportionately large role in the persistence of species through adaptation to changing environmental conditions such as climate change (Willi et al. 2006). Finally, the fact that *E. bispinosus* now persists in only a limited number of fragmented populations emphasizes the importance of preserving as many remnant populations as possible as security for the species into the future.

Given this information, there is an urgent need for a comprehensive population genetic assessment of *E. bispinosus* throughout their current distribution (South Australia and Victoria) to identify current and historic patterns of gene flow, population structure, and genetic diversity. This will help identify genetically resilient and potentially vulnerable populations and guide future conservation strategies for the species. The present study describes patterns of genetic variation in *E. bispinosus* using nuclear microsatellite loci and mitochondrial DNA sequence data to assess patterns of genetic structure, genetic diversity, and gene flow at regional (i.e. South Australia and Victoria) and local (i.e. waterway) scales. Using this information, we explore genetic management options that will help maintain and/or restore the genetic diversity, adaptability, and evolutionary potential of *E. bispinosus* populations. These results are also used to provide a spatial framework for informing on-ground habitat restoration works and environmental flow management regimes that will assist the long-term preservation of *E. bispinosus* populations across the species' current range.

MATERIALS AND METHODS

Species and sample collection

The Glenelg spiny freshwater crayfish *Euastacus bispinosus* is a slow-growing, long-lived (>25 yr), late-maturing (8–11 yr) species with low fecundity (63–812 eggs) (Honan & Mitchell 1995b,c). Twenty-seven sites in 17 waterways were surveyed across the discontinuous distribution of the species. These individuals were obtained through targeted trapping in areas where the species had previously been recorded (Honan 2004, Johnston et al. 2008, Hammer & Roberts 2008) and in other stretches of river that possessed optimal riparian habitat, such as state forests and national parks, to maximize the probability of obtaining sufficient crayfish for analysis (Table 1, Fig. 1). Crayfish were surveyed using a combination of methods, including Munyana, opera, and bait nets (baited with ox liver and set overnight), hoop nets (used in Victoria to avoid negative impacts on the platypus *Ornithorhynchus anatinus*, and checked hourly), and dip netting and backpack electrofishing (LR-24, Smith-Root), which was employed in shallower stream reaches. From all captured individuals, a small (approximately 5 mm^2) clip of the uropod was obtained and stored in 90% ethanol. This clip also acted as an identifier and precluded the possibility of sampling the same individual twice during and between surveys. All trapping was undertaken from July 2011 to December 2012. Sample sizes in some cases were constrained by low catch rates, and catches ranged from 3 (Jerusalem Creek, SA) to 36 (Spencers Pond, SA).

DNA extraction

Total genomic DNA was extracted using a modified Chelex extraction protocol (Walsh et al. 1991). Using a 0.5 ml Eppendorf tube, approximately 10 mg of tissue was taken from uropod clips, macerated with a scalpel, combined with 150 µl of 5% Chelex (Roche) solution and 3 µl Proteinase K (10 mg ml^{-1}), and mixed briefly by vortex. Samples were incubated at 56°C for 2 h with periodic vortexing, followed by further digestion at 95°C for 15 min. Tissue extractions were cooled on ice for 20 min and stored at –20°C until required for analysis. Prior to PCR, Chelex extractions were homogenized by inversion and centrifuged at 13 000 rpm for 2 min. Supernatant was subsequently taken for PCR from the bottom half of the supernatant, above the Chelex resin precipitate.

Table 1. Site location details and corresponding codes for 17 collection sites of *Euastacus bispinosus*. Multiple GPS coordinates are provided for pooled sites from common waterways. Numbers of individuals collected from each site are provided in parentheses

Catchment/river system	Waterway	Site code	GPS coordinates	Total samples
Victoria				
Glenelg	Crawford River	CR	–37.9332, 141.5150 (19); –37.9315, 141.4760 (7)	26
Glenelg	Lower Glenelg River	LG	–38.0542, 141.2686 (22)	23
Glenelg	Stokes River	ST	–37.8747, 141.3016 (3); –37.8698, 141.4099 (4)	7
Glenelg	Wannon River	WA	–37.3487, 142.5067 (10); –37.3716, 142.5031 (12)	22
Glenelg	Scrubby Creek	SC	–37.1613, 142.4449 (11)	11
South Australia				
Eight Mile Creek	Ewens Ponds	EW	–38.0261, 140.7906 (30); –38.0261, 140.7906 (1); –38.0279, 140.7929 (28)	30
Eight Mile Creek	Blacks Pond	BL	–38.0275, 140.7823 (21)	21
Eight Mile Creek	Spencer Pond	SP	–38.0306, 140.8069 (30)	36
Eight Mile Creek	Lower section	EM	–38.0428, 140.7968 (5)	7
Cress Creek	Cress Creek	CC	–38.0380, 140.7143 (15); –38.0386, 140.7154 (10)	26
Deep Creek	Western tributary	DE	–38.0382, 140.7713 (11); –38.0432, 140.7776 (12); –38.0440, 140.7812 (1)	24
Deep Creek	54 ft Pond	FF	–38.0383, 140.7834 (7)	7
Hitchcox Drain	Bones Pond	BO	–38.0222, 140.8230 (10)	10
Isolated	Gouldens Waterhole	GO	–37.9453, 140.6836 (23)	23
Isolated	The Pines Sinkhole	PI	–37.7551, 140.5465 (25)	25
Jerusalem Creek	Jerusalem Creek	PC	–38.0454, 140.7274 (3)	3
Piccaninnie Ponds	Donovan's Drain	DO	–38.0368, 140.8941 (7)	7

Mitochondrial DNA analysis

DNA sequence data from the mitochondrial cytochrome oxidase I (*COI*) gene were analysed primarily to assess the species' status, but also to provide insights into the phylogeographic history of *E. bispinosus*. Approximately 650 bp of the *COI* gene was amplified for 5 and 2 individuals from each Victorian and South Australian site, respectively, by PCR using primers HCO and LCO (Folmer et al. 1994). PCRs were prepared in 25 µl volumes each containing 10.25 µl ddH2O, 1× reaction buffer, 5.0 µl dNTPs (1 mM), 0.4 µM HCO primer (10 µM), 0.4 µM LCO primer (10 µM), 0.25 units NEB taq (Biosciences, New England), and 5 µl DNA extract. PCRs were conducted using an Eppendorf Gradient *S* Master Cycler with cycling conditions consisting of an initial denaturing at 94°C for 2 min; 35 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 50 s; and a final extension step of 72°C for 5 min. PCR products were directly sequenced in both directions using the primers described above on an ABI 3730 capillary DNA analyser. Forward and reverse sequences were aligned and manually edited in Geneious version 5.6.5 (Biomatters 2012). Genealogical relationships between mitochondrial haplotypes were inferred from a haplotype network (Templeton et al. 1992). Unrooted networks were generated with TCS version 1.21 (Clement et al. 2000), using maximum parsimony to connect haplotypes with a 95% confidence interval.

Microsatellite analysis

Individual samples of *E. bispinosus* were genotyped at 11 microsatellite loci using procedures described in Miller et al. (2013). Individuals from different sample sites within the 17 waterways were grouped for microsatellite analyses to maximize sample sizes and improve the statistical rigour associated with estimates of gene flow and population structure. These poolings were justified by Hardy Weinberg equilibrium (HWE) estimates and Bayesian analyses that indicate each pooled sample represents a randomly breeding panmictic population (see Table 2 and Fig. 4). This pooling regime applied to the Crawford River, Stokes River, Wannon River, Scrubby Creek, Ewens ponds, Eight Mile Creek, Cress Creek, and Western Tributary waterways (Table 1, Fig. 1).

The pooled samples provided a data set consisting of 17 sample groups referred to henceforth as 'sites'.

The software MICRO-CHECKER (Van Oosterhout et al. 2004) was used to assess microsatellite loci for null alleles and scoring errors using formula 1 outlined by Brookfield (1996), as evidence of null homozygotes was not apparent. Descriptive statistics were calculated for the microsatellite data using FSTAT, version 2.9.3 (Goudet 1995), including allelic richness per population averaged over loci, Weir and Cockerham's inbreeding coefficient (F_{IS}: the deficiency of heterozygotes relative to the level expected with random mating), a global estimate of population differentiation (F_{ST}) with 95% confidence limits (Weir & Cockerham 1984). In addition, population pair-wise measures of F_{ST} and their significance were determined using permutation (10 000), and pairs of loci were tested for linkage disequilibrium using a log-likelihood ratio test. Mean allelic richness and observed heterozygosity were compared among groups (South Australia and Victoria) using a 2-sided permutation test (10 000 permutations) also implemented in FSTAT. In order to overcome potential limitations of F_{ST} calculations using multiallelic loci (Jost 2008), additional estimates of population differentiation, global D_{est}, and population pair-wise measures of D_{est} (significance determined using 10 000 permutations) were generated using GenAlEx 6.5 (Peakall & Smouse 2006). The False Discovery Rate (FDR) procedure (Benjamini & Hochberg 1995) was used to adjust significance levels when performing multiple simultaneous comparisons. POWSIM version 4.0 (Ryman & Palm 2006) was used for evaluation of the alpha error and statistical power of the microsatellite loci for accurately detecting different levels of F_{ST}.

Estimates of observed (H_o) and expected (H_e) heterozygosity were determined using the Excel Microsatellite Toolkit (Park 2001) and deviations from HWE were determined using Genepop version 3.4 (Raymond & Rousset 1995). An analysis of molecular variation (AMOVA) was performed in GenAlEx 6.5 (Peakall & Smouse 2006) using pairwise F_{ST} as the distance measure, with 10 000 permutations and missing data for loci set at 10%. The model for analysis partitioned variation among regions (South Australia and Victoria), among sample sites within regions, and within sample sites. A factorial correspondence analysis (FCA), implemented in GENETIX version 4.05 (Belkhir et al. 2004), was used to summarize patterns of genetic differentiation between sample sites. The first 2 underlying factors that explained the majority of variation in multi-locus genotypes across loci were plotted.

Bayesian analyses were conducted to estimate the number of populations within the sample data using 2 software packages. First, STRUCTURE (Pritchard et al. 2000) was used to identify the number of distinct population clusters, to assign individuals to clusters, and to identify migrants and admixed individuals using genetic data only. To determine the number of populations (K), 10 independent simulations for $K = 1–17$ with 100 000 burn-in and 1 000 000 data iterations were run. Analyses were performed using the admixture model of population structure (i.e. each individual draws some fraction of their genome from each of K populations) and allele frequencies were set as independent among populations. The most likely K was estimated using Evanno's ΔK (Evanno et al. 2005) in Structure Harvester (Earl & vonHoldt 2012).

A second Bayesian analysis of population genetic structure was performed with the R package software GENELAND (Guillot & Santos 2009). The method makes use of a geographically constrained Bayesian model that explicitly takes into account the spatial position of sampled multilocus genotypes without any prior information on the number of populations and degree of differentiation between them. Like STRUCTURE, K can be treated as variable, enabling the determination of the modal (i.e. most likely) value. The inference algorithm was launched with a single step approach (Guillot 2008) using the Dirichlet distribution as prior for allele frequencies and where K was allowed to vary from 1 to 17. Ten independent runs of 500 000 Markov chain Monte Carlo iterations were performed with a maximum of 300 nuclei and a burn-in phase of 50 000 iterations consistent with recommendations (Guillot 2008, 2009, Guillot & Santos 2009).

Evidence of population bottlenecks was investigated using the software BOTTLENECK 1.2.02 (Cornuet & Luikart 1996). BOTTLENECK tests for the departure from mutation drift equilibrium based on heterozygosity excess or deficiency. In a population at mutation-drift equilibrium, there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit.

The significance of any observed excess was tested using a Wilcoxon's sign-rank test based on the stepwise mutation (SMM) and two-phase mutation (TPM; 70% SMM, 30% infinite alleles model [IMM]) models (Dirienzo et al. 1994) following 1000 iterations. A qualitative descriptor of the allele frequency distribution ('mode-shift' indicator), which discriminates bottlenecked populations from stable populations, was also calculated in BOTTLENECK (Luikart et al.

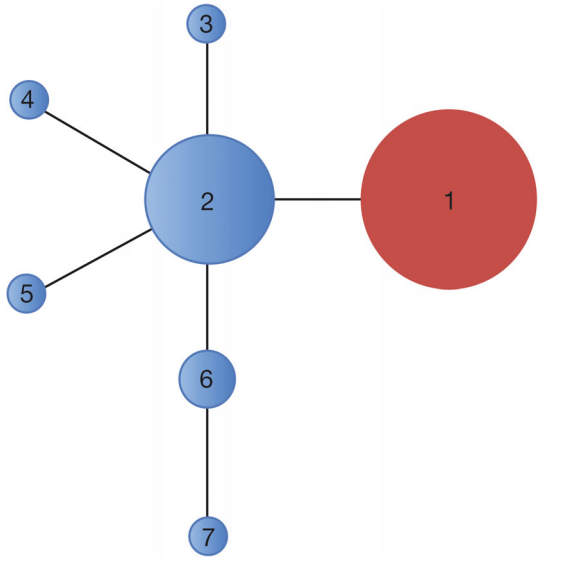

Fig. 2. Haplotype network produced by maximum parsimony for *Euastacus bispinosus* across its range. Each circle represents a unique haplotype and sizes are roughly proportional to haplotype frequency within the data set. Each connecting branch between haplotypes indicates a single base mutation. Colours indicate region where haplotypes were detected (blue: Victoria; red: South Australia). Assignment of haplotype numbers to sample site is provided in Table 2

Table 2. Site locations and corresponding codes for 17 sample sites and summary statistics screened with 11 polymorphic microsatellite loci for *Euastacus bispinosus*. n: Number of individuals genotyped per site. Mean values over loci are presented for number of alleles (*a*), allelic richness (*r*), expected (H_e) and observed (H_o) heterozygosities, Hardy-Weinberg equilibrium p-values (HWE), and inbreeding (F_{IS}). Statistical significance ($\alpha = 0.05$) after correction for multiple comparisons is indicated by **bold** text. Some statistics could not be calculated at all sites due to inadequate sampling (–) or complete homozygosity (NA). Mitochondrial haplotypes and their relative frequencies per sample site are also provided (mtDNA). Site codes are derived from Table 1

Site code	n	*a*	*r*	H_e	H_o	HWE	F_{IS}	mtDNA
Victoria								
CR	25	2.360	1.764	0.328	0.315	0.513	0.039	6(4), 7(1)
LG	23	2.640	1.847	0.333	0.352	0.508	–0.006	2(4), 3(1)
ST	6	2.360	2.078	0.433	0.424	–	–	2(3), 4(1), 5(1)
WA	22	2.910	1.907	0.336	0.301	0.126	0.098	2(5)
SC	12	1.270	1.152	0.068	0.038	0.309	0.288	2(5)
South Australia								
EW	30	1.000	1.000	0.000	0.000	NA	NA	1(2)
BL	21	1.000	1.000	0.000	0.000	NA	NA	1(2)
SP	36	1.360	1.038	0.013	0.013	0.523	0.147	1(2)
EM	7	1.090	1.055	0.013	0.013	–	–	1(2)
CC	26	1.270	1.121	0.046	0.052	0.978	–0.146	1(1)
DE	24	1.180	1.058	0.032	0.038	1.000	–0.16	1(2)
FF	7	1.000	1.000	0.000	0.000	NA	NA	
BO	10	1.820	1.385	0.142	0.109	**0.025**	0.242	1(2)
GO	23	1.090	1.012	0.004	0.004	NA	NA	1(2)
PI	25	1.090	1.011	0.004	0.004	NA	NA	1(2)
PC	3	1.090	1.091	0.049	0.061	–	–	1(2)
DO	7	1.090	1.039	0.013	0.013	–	–	1(2)

1998). However, type I error rate varies with sample size, and estimates from samples of fewer than 30 individuals should be treated cautiously. To account for multiple comparisons, we applied the FDR procedure (Benjamini & Hochberg 1995).

RESULTS

Mitochondrial DNA analysis

An approximately 650 bp fragment of the mitochondrial *COI* gene was amplified and sequenced for 45 individuals representing 17 *Euastacus bispinosus* sample sites (Table 1). Following editing, the DNA sequence alignment yielded a 623 bp fragment for analysis. Subsequent genetic analyses revealed 7 haplotypes, with all individuals from South Australian sites carrying a single unique haplotype (haplotype 1). Six haplotypes were identified from Victorian sample sites, with a single dominant haplotype found at each site (haplotype 2), except the Crawford River, where individuals were found to carry 2 unique haplotypes (haplotypes 6 and 7). The extent of differentiation between haplotypes was minimal (<0.5%) (Fig. 2). Haplotype sequences are available on GenBank under accession numbers KJ713384–KJ713390.

Microsatellite analysis

A total of 307 *E. bispinosus* individual specimens from 17 sample sites were successfully genotyped at 11 microsatellite loci (Table 2). Fifty-one alleles were detected (one of which was exclusive to South Australia), with a mean of 1.50 alleles per locus over all sites. Allelic richness over all loci ranged between 1.00 and 2.08 (Table 2). Allele frequencies for all sites per locus are provided in the supplementary material (see Table S1 in the Supplement at www.int-res.com/articles/suppl/n025p043_supp.pdf). Marker independence was confirmed across all sample sites, with linkage disequilibrium analysis indicating no significant linkage between loci, and MICRO-CHECKER found no evidence of null alleles across sites and loci. Estimates for total num-

ber of alleles and allelic richness were lower at South Australian sites (mean values of 1.00 and 1.073, respectively) than Victorian sites (mean values of 2.30 and 1.75, respectively). Similarly, expected heterozygosities were lower at South Australian sites (range 0.00–0.14; mean H_e = 0.03) than Victorian sites (range 0.33–0.43; mean H_e = 0.36). Individuals from a single Victorian site (SC) had very low heterozygosity (H_e = 0.07), comparable with estimates from South Australian sites (Table 2). Permutation tests indicate that mean allelic richness and H_o across South Australian sites are significantly lower than estimates observed across Victorian sites ($p < 0.005$).

All sites were found to conform to Hardy-Weinberg expectations where calculations were possible (small sample sizes and homozygosity across all loci precluded calculations for some sites; Table 2). Similarly, no F_{IS} estimates were found to be significant, although high estimates were observed at sites SC and BO but may be due to small sample sizes (Table 2). Global estimates of F_{ST} and D_{est} across all loci were significantly different from zero (F_{ST} = 0.63, 95% CI = 0.55–0.69; D_{est} = 0.25, 95% CI = 0.16–0.34), indicating limited gene flow and genetic structuring between sampling sites. Pairwise population comparisons of F_{ST} indicate restricted gene flow between Victorian sites, but larger genetic differentiation between Victorian and South Australian sites (Table 3). Of the 28 pairwise estimates among South Australian populations, 21 were found to be significant, indicating restricted gene flow between most sites. Pairwise population comparisons of D_{est} were found to be largely consistent (Table 3). POWSIM analyses confirmed that the microsatellite marker set is capable of detecting a true F_{ST} of 0.01 or larger with a probability of 100%. The alpha error (i.e. the probability of obtaining false significances when the true F_{ST} = 0) was approximately 5%.

AMOVA analyses indicated that the majority of the variation in microsatellite loci was explained by between region differences (61%, $p < 0.01$), highlighting genetic differentiation between Victorian and South Australian populations. Within-site variation explained 25% ($p < 0.01$) of the total variation, while high between-site variation within regions (14%, $p < 0.01$) emphasizes the genetic differentiation between sites. The relationships between sites are best depicted by the 2-dimensional FCA of the microsatellite variation (Fig. 3). When the 2 factors explaining the highest percentage of the microsatellite variation (factor 1 = 26.12%, factor 2 = 13.98%) are plotted against each other, Victorian and South Australian sample regions are clearly separated. Victorian sites separate out further, with individuals from the Wannon River and Scrubby Creek clustering separately. These results are largely consistent with the independent measures of population differentiation above, highlighting the degree of differentiation between South Australian and Victorian populations and significant structuring of Victorian sample sites.

Both Bayesian clustering analyses were consistent in identifying significant population differentiation between the South Australian and Victorian sites despite the different approaches used to identify the true number of populations (K) within the data. Initially, STRUCTURE identified 2 populations (K = 2; corresponding to populations in South Australia and populations in Victoria clustering separately), although

Table 3. Pairwise estimates of F_{ST} (lower diagonal) and D_{est} (upper diagonal) between 12 *Eusatacus bispinosus* collection sites. Values shown in **bold** are significant ($p < 0.001$) after 10 000 permutations and correction for multiple comparisons. Sites ST, EM, FF, PC and DO were excluded from analysis of global and pairwise F_{ST} and D_{est} due to low sample sizes. Site codes are derived from Table 1

	CR	LG	WA	SC	EW	BL	SP	CC	DE	BO	GO	PI
CR		**0.071**	**0.381**	**0.386**	**0.317**	**0.317**	**0.315**	**0.299**	**0.294**	**0.245**	**0.315**	**0.314**
LG	**0.121**		**0.392**	**0.361**	**0.356**	**0.355**	**0.353**	**0.357**	**0.352**	**0.286**	**0.353**	**0.355**
WA	**0.400**	**0.399**		**0.634**	**0.663**	**0.662**	**0.659**	**0.645**	**0.637**	**0.609**	**0.660**	**0.660**
SC	**0.528**	**0.518**	**0.645**		**0.569**	**0.569**	**0.564**	**0.586**	**0.579**	**0.521**	**0.566**	**0.570**
EW	**0.594**	**0.645**	**0.765**	**0.938**		0.000	0.000	**0.003**	**0.003**	**0.007**	0.000	0.000
BL	**0.567**	**0.620**	**0.743**	**0.948**	**0.081**		0.000	**0.003**	**0.003**	**0.007**	0.000	0.000
SP	**0.529**	**0.595**	**0.718**	**0.896**	**0.080**	**0.102**		**0.003**	**0.003**	**0.006**	0.000	0.000
CC	**0.574**	**0.638**	**0.755**	**0.929**	**0.087**	**0.095**	**0.012**		0.000	**0.005**	**0.003**	**0.003**
DE	**0.608**	**0.659**	**0.777**	**0.933**	0.000	0.022	**0.075**	**0.066**		**0.005**	**0.003**	**0.002**
BO	**0.364**	**0.422**	**0.594**	**0.796**	**0.131**	**0.143**	**0.066**	**0.097**	**0.119**		0.007	**0.007**
GO	**0.575**	**0.628**	**0.751**	**0.947**	**0.078**	0.000	**0.103**	**0.093**	0.024	**0.144**		0.000
PI	**0.585**	**0.639**	**0.760**	**0.950**	**0.082**	0.000	**0.092**	**0.071**	0.026	**0.151**	0.000	

evidence of further structure was apparent due to a secondary peak in the plot of ΔK against K in Structure Harvester ($K = 4$). GENELAND identified additional population structure within the Glenelg River catchment ($K = 4$). Sites from the lower reaches of this Victorian catchment clustered as 1 distinct population (sites CR, LG, and ST), while the Wannon River (WA) and Scrubby Creek sites (SC) in the upper reaches clustered independently (Fig. 4). A second STRUCTURE analysis was repeated using the Victorian sites only, this time revealing a pattern of genetic structure identical to that of GENELAND. Evidently lower levels of genetic structure were obscured by the higher levels of structure observed in the original analysis (see Evanno et al. 2005). These findings are largely consistent with pairwise estimates of F_{ST} and D_{est} and the FCA (Table 3, Fig. 3).

BOTTLENECK analysis found evidence of recent bottlenecks across all sample localities. Under each mutation model, Wilcoxon's sign-rank test for heterozygote excess was significant after correction for multiple comparisons for all populations, except for the SMM estimate for population BO. These results were further supported by evidence of mode shifts at each site. Combined, these results suggest that populations at each site are not in mutation-drift equilibrium and have undergone severe reductions in effective population size in the recent past.

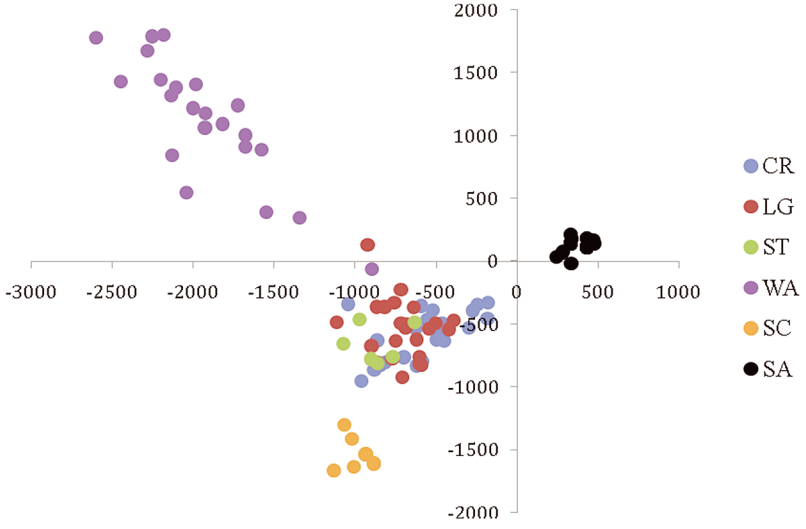

Fig. 3. Two-dimensional scatter plot showing the relationships among *Euastacus bispinosus* sample locales based on a factorial correspondence analysis of 11 microsatellite loci for 17 sites. The first factor (x-axis) explains 26.12% of the variance, whilst the second factor (y-axis) explains 13.98%. All South Australian sites are presented under code SA. Site codes are derived from Table 1

DISCUSSION

Genetic structure and diversity across the species' distribution

Microsatellite analyses indicate limited contemporary gene flow between the South Australian and Victorian regions, and between each of the 5 Victorian sample sites from the Glenelg River catchment. These findings indicate limited dispersal and structuring of populations within and between catchments. This is consistent with previous findings of limited dispersal and small home ranges for *Euastacus bispinosus* and other freshwater crayfish species (Honan & Mitchell 1995a, Chaplin & Ayre 1997, Gervasio et al. 2004, Verovnik et al. 2004, Nguyen et

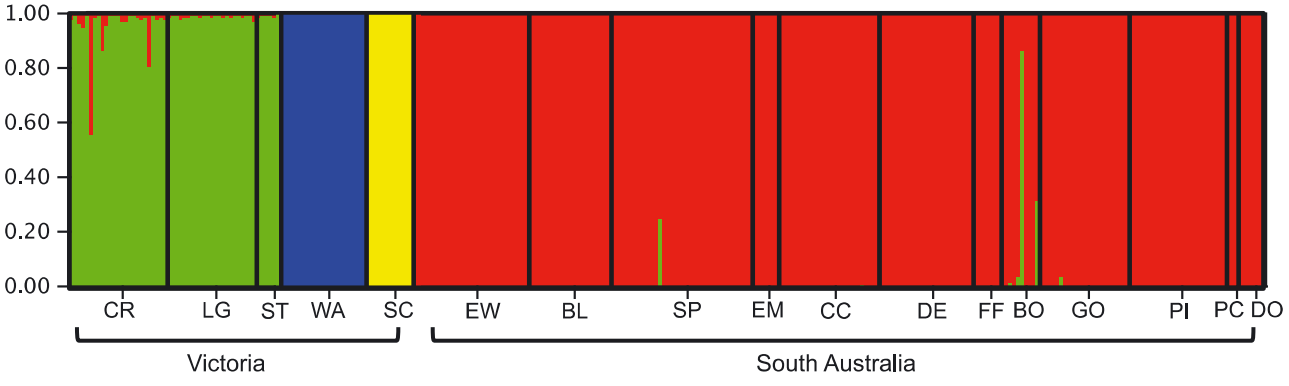

Fig. 4. Summary plots of the estimated membership coefficient (y-axis) for each *Euastacus bispinosus* individual in each colour-differentiated population cluster by STRUCTURE and GENELAND analyses. Single vertical lines broken into segments reflect each individual in the STRUCTURE summary plot, where segments are proportional to the membership coefficient for each of the population clusters. Individuals are arranged into sites from which they were sampled following the order given in Table 1 (site codes are also derived from Table 1), and sites are pooled into regions (Victoria and South Australia)

al. 2005, Baric et al. 2006, Gouin et al. 2011). The mitochondrial DNA analysis indicates that the high level of contemporary structuring is likely to be a relatively recent demographic feature, with 4 of the 5 Victorian sites sharing a single dominant haplotype due to historical gene flow and recent common ancestry. Conversely, the Crawford River in Victoria and South Australian sites harbor unique haplotypes, suggesting comparatively longer-term isolation. Further sampling within the Glenelg catchment and use of more rapidly evolving mitochondrial genomic regions (i.e. mitochondrial control region) would provide more comprehensive insights into the species' phylogeographic history, including the timing of diversification events (Unmack et al. 2012).

Estimates of contemporary structure across South Australian sites should be treated cautiously as measures of gene flow are likely to be confounded by the extremely low levels of genetic diversity found across all sites. However, using estimates of gene flow and genetic structuring from Victoria as a guide, it is likely that current levels of gene flow in South Australia are restricted to connected habitats. The lack of genetic differentiation across South Australian sites thus more likely reflects a pattern of recent common ancestry rather than high connectivity. Given that the karst rising-spring habitats of southeastern South Australia were historically connected (Eardley 1943, Armstrong 1974, SEWCBD 1993), contemporary fragmented populations would likely have constituted a larger, and possibly panmictic, population prior to hydrological disruption. However, the lack of genetic diversity across extant South Australian populations suggests that genetic diversity was low in the ancestral population, a likely consequence of demographic processes such as founder or bottleneck events, and exacerbated by subsequent inbreeding, small population size, and random genetic drift. Other studies of freshwater fauna including river blackfish *Gadopsis marmoratus* (Miller et al. 2004), dwarf galaxias *Galaxiella pusilla* (Coleman et al. 2013), and pygmy perches *Nannoperca australis* and *N. obscura* (Hammer et al. 2010, Brauer et al. 2013, Unmack et al. 2013) also indicate recent common ancestry between populations in the rising-spring habitats and the Glenelg River catchment, though the mechanism behind this is not yet clearly understood.

While estimates of genetic diversity are particularly low in South Australia, Victorian sites also exhibit limited genetic diversity. This is consistent with other freshwater crayfish (Fetzner & Crandall 2001, Baric et al. 2006, Gouin et al. 2011) and may be attributed to an inherent tendency to inbreed due to poor dispersal ability and small population size. Other factors such as male dominance hierarchies, commonly observed in freshwater crayfish and other crustacean groups (Moore & Bergman 2005), will also act to reduce effective population sizes (N_e) and exacerbate the severity of inbreeding. Population fragmentation and decline, observed across the range of *E. bispinosus*, is also likely to be contributing to genetic erosion; small isolated populations resulting from habitat loss and disturbance (e.g. drought, fire and flood events, and intensive fishing pressure) are susceptible to random genetic drift processes, leading to local depletions of genetic diversity. There are a number of other Australian freshwater species that appear prone to similar forces, with some populations showing very low levels of genetic diversity, including *G. pusilla* (Coleman et al. 2013), *N. australis* and *N. obscura* (Cook et al. 2007, Brauer et al. 2013), golden perch *Macquaria ambigua* (Faulks et al. 2010), and the eel-tailed catfish *Neosilurus hyrtlii* (Huey et al. 2006).

Catchment-level habitat management implications

To increase the chances of *E. bispinosus* recovery across its entire range, it is essential that ecological issues are managed effectively and integrated with genetic management strategies. The high levels of genetic structuring observed in this study illustrate the importance of maximizing the quality of local habitats within and across catchments to enhance connectivity and facilitate dispersal. Riparian vegetation plays a critical role in providing suitable habitat for *E. bispinosus* through bank stabilization, which permits burrowing and prevents siltation, while also providing shade and organic debris that is used for cover. In-stream physical habitat (rocks, snags and aquatic vegetation) is also important as it provides shelter and access to food resources, while the species also has specific water quality tolerances (Honan 2004). Therefore, the preservation and restoration of riparian vegetation and in-stream habitat and maintenance of suitable water quality will be critically important for limiting further fragmentation as well as maintaining and enhancing population size.

Flow management regimes that promote habitat connectivity will also be vital for long-term species persistence. Although *E. bispinosus* appears to be a poor disperser with small home ranges, the species will likely become increasingly reliant on dispersal to adapt to climate change (Hoffmann & Willi 2008).

Habitat connectivity will therefore provide opportunities for populations to migrate to more favourable habitats beyond their current range under a changing environment. Also, historical habitat connectivity is likely to have played a major role in maintaining population sizes and genetic diversity. The future management of environmental flows in the Glenelg River catchment and southeastern South Australia should therefore look to promote habitat connectivity to promote gene flow and outcrossing among resident *E. bispinosus* populations.

Genetic rescue of South Australian populations

The South Australian populations of *E. bispinosus* have critically low levels of genetic diversity, and evidence suggests that these populations are suffering declining fitness, possibly due to inbreeding depression. Consequently, intervention activities such as translocations aimed at the genetic rescue of these South Australian populations should be considered as a part of a holistic conservation effort (Weeks et al. 2011, Coleman et al. 2013) to ensure the persistence of these potentially important fringe populations. Translocations are being increasingly proposed as a way of conserving biodiversity, particularly in the management of threatened species, and management frameworks for assessing the risks and benefits have been well documented (e.g. Frankham et al. 2011, Weeks et al. 2011). Genetic rescue through translocation provides an opportunity to increase genetic fitness, resilience and evolvability (Weeks et al. 2011); however, precautions need to be taken to preserve potentially uniquely adapted traits in recipient populations, and risks associated with outbreeding depression need to be considered (Frankham et al. 2011).

The results from the present study indicate that risks associated with the mixing of South Australian and Victorian gene pools are likely to be minimal compared with the imminent risks of ongoing inbreeding. South Australian populations occupy unique habitats, suggesting that environmental conditions may have historically influenced the accumulation of local adaptations. However, evidence of historical inbreeding and small population size suggests that South Australian populations are likely to be inherently prone to genetic drift and potential maladaptation, and therefore translocations are unlikely to have adverse impacts on locally adapted traits (Lopez et al. 2009). The presence of only a single unique allele in South Australia and the low levels of mitochondrial variation between South Australian and Victorian populations indicate that these populations are clearly conspecific and derived from a recent common ancestor. This suggests that outbreeding risks are likely to be minimal, although determining haplotype diversity at more variable mitochondrial markers would help clarify the timing of ancestry and potential outbreeding risks (Frankham et al. 2011). Nevertheless, outbreeding risks can be mitigated through the adoption of current genetic rescue protocols. Undertaken in a calculated fashion (e.g. 20% gene flow), the detrimental genetic load (and inbreeding depression if present) can be reduced and genetic variation increased, while uniquely adapted alleles are preserved and negative fitness effects associated with outbreeding depression are minimized (Hedrick 1995, Lopez et al. 2009, Hedrick & Fredrickson 2010, Frankham et al. 2011, Weeks et al. 2011).

Ideally, the ability to perform controlled crosses in captivity provides a way of assessing risks of outbreeding depression in F_2 or F_3 generations. However, given the lengthy generation time of the species (8–11 yr; Honan & Mitchell 1995b) and the urgent need for intervention, wild translocations offer the most practical option for increasing genetic variation and reducing the risk of localized extinction. Victorian populations of *E. bispinosus* with comparatively higher diversity are likely to be good candidates as source populations for translocations to South Australian habitats. However, additional surveys to further characterize the extent of genetic diversity across the Glenelg catchment would be beneficial for developing effective composite mixing strategies. Likewise, additional information on population sizes across the Glenelg catchment would be needed to determine the number of individuals that can be sourced from any one location for translocation without jeopardizing the integrity of local populations.

We also provide evidence of vulnerable populations persisting in the Glenelg River catchment. Four of the 5 Victorian sites appear to be in comparatively better genetic condition than those from South Australia, although 1 site appears to be in a similar genetic state (Scrubby Creek). Field surveys indicate that this population has declined considerably in recent years, possibly due to the severe fires and drought conditions that have affected the Grampians National Park in the last decade (K. Johnston, pers. comm.). However, the low levels of genetic diversity indicate a history of isolation and inbreeding pre-dating the recent disturbances at this site. More compre-

hensive genetic sampling of *E. bispinosus* across the Glenelg River catchment will provide a more effective spatial framework for conserving the species into the future, identifying genetically resilient and at-risk populations and prioritizing conservation investments such as habitat restoration works and translocations where required.

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