

Evolution and maintenance of divergent lineages in an endangered freshwater fish, *Macquaria australasica*

Leanne K. Faulks · Dean M. Gilligan ·
Luciano B. Beheregaray

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Abstract Variable hydrological regimes and habitat availability are factors that affect the distribution of freshwater dependent species and are expected to influence their levels of genetic diversity. Although geologically relatively stable, the south eastern region of Australia has experienced significant changes in hydrological conditions during the Quaternary. This area has also been recently affected by anthropogenic activities, resulting in dramatic population declines of Macquarie Perch (*Macquaria australasica*). We conducted a range-wide phylogeographic study of this endangered fish to assess the relationship between landscape and freshwater fish evolution in south eastern Australia and infer levels of genetic diversity and population structure. Surprisingly, we detected high genetic diversity, with 46 mtDNA control region haplotypes found across 37 sampling locations. Some lineages were remarkably divergent; one represents a putative undescribed species that probably went extinct during the period of this study. Our reconstruction of population history using a combination of coalescent and phylogenetic methods indicates that the species originated on the coast, east of the Great Dividing Range (GDR), with subsequent colonisation of the Murray-Darling basin, west of the GDR.

Nested clade and IM analyses inferred a series of range expansions and fragmentations across the species range consistent with the history of climatic oscillations in south eastern Australia during the Pleistocene. We conclude that the unexpected high levels of diversity and divergence observed in *M. australasica* may be due to specific habitat requirements, localised recruitment, and Pleistocene climate fluctuations. Under expectations of a drier climate and increased sea levels due to global warming, populations of this and other freshwater species may be expected to experience increased habitat fragmentation and loss of genetic diversity. Conservation management should focus on habitat protection, the maintenance of genetic diversity and taxonomic review.

Keywords Conservation biology · Phylogeography · Habitat specialist · mtDNA · South eastern Australia · Climate change

Introduction

The maintenance of genetic diversity is one of the foundations of conservation genetics. It enables species to tolerate a range of environmental conditions and ensures their evolutionary potential. However, there are processes, such as reduction in population size, which decrease genetic diversity, a situation commonly observed in threatened species (Frankham 1996; Montgomery et al. 2000; Wright et al. 2008). During the past decade we have observed an increasing incidence of declines in population numbers and almost 17 000 species are now listed as threatened (IUCN Red list 2008). Habitat destruction is responsible for many of these recent population declines. However, the availability of preferred habitat can also change over much

L. K. Faulks · L. B. Beheregaray (✉)
Molecular Ecology Laboratory, Department of Biological
Sciences, Macquarie University, Sydney, NSW 2109, Australia
e-mail: Luciano.Beheregaray@bio.mq.edu.au

D. M. Gilligan
Batemans Bay Fisheries Centre, New South Wales Department
of Primary Industries, Batemans Bay, NSW, Australia

L. B. Beheregaray
School of Biological Sciences, Flinders University, Adelaide,
SA 5001, Australia

longer timescales and likely plays an important role in the evolutionary history of species. Investigation of the phylogeography of threatened species can help by assessing the role of natural processes in creating and maintaining genetic diversity, and ultimately contribute to conservation management programs.

We used this approach to contribute to the conservation of *Macquaria australasica* (Percichthyidae), an endangered (Australian Environment Protection and Biodiversity Conservation Act 1999) Australian freshwater fish. Originally described from the Macquarie River, the species was historically distributed across both sides of the Great Dividing Range (GDR) (Fig. 1). The species is now restricted in range and abundance due to habitat degradation, barriers to dispersal, increased competition from native species due to stocking (*M. novemaculeata*), introduced species (*Perca fluviatilis* and Salmonids), overfishing, and susceptibility to epizootic haematopoietic necrosis virus (EHNV) (Cadwallader 1978; McDowall 1996; Allen et al. 2002; Lintermans 2007). *M. australasica* is presumed extinct in the Macquarie River catchment as it has not been collected from there since its original description, nor been reported ethnographically for many decades (W.T. Trueman personal communication). The species is still present

in the headwaters of the Lachlan, Murrumbidgee, Murray, Kiewa, Ovens, and Goulburn Rivers in the Murray-Darling basin (MDB) to the west of the GDR; as well as the Hawkesbury–Nepean (HN), Georges River, and Shoalhaven basins on the east of the GDR (NSW Department of Primary Industries Freshwater Fish Research Database; Lintermans 2007; Fig. 1). However, one Shoalhaven (Kangaroo River) population declined rapidly during the late 1990s, and despite intensive sampling effort (118 sampling occasions) no individuals have been observed in the wild since 1998. In this study the Kangaroo River was represented by a tissue sample from a single captive fish. The death of this individual in 2008 could mean the extinction of this population. The only other population of *M. australasica* in the Shoalhaven basin (Mongarlowe River) is presumed to have been translocated from the MDB (McDowall 1996). Translocation from the MDB has also occurred into the Yarra River and Cataract Dam. However, these stocking activities no longer occur and the species is protected throughout its natural range.

The GDR formed ~90 mya (Wellman 1979) and is the most prominent geographical barrier within *M. australasica*'s distribution. Morphological and allozyme differentiation has been observed in populations of *M. australasica*

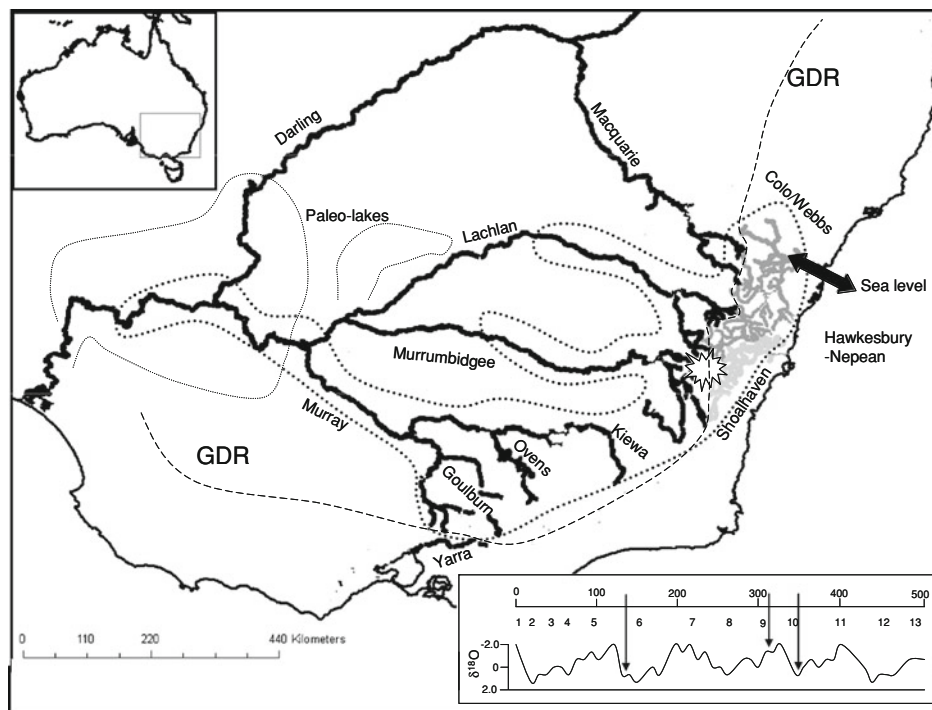


Fig. 1 Map of Australia indicating the study region. Major features referred to in the text are shown. Murray-Darling basin catchments are black, Hawkesbury–Nepean dark grey, and the Shoalhaven light grey. The previous distribution of *M. australasica* is surrounded by the dark dotted line. The Great Dividing Range (GDR) is the dashed line. The boundary of historical inland lakes is the fine dotted line. The double arrow signifies the influence of fluctuating sea levels. The

Lake George, Lake Bathurst and Bredalbane Plain region are indicated by a star. The inset shows the oxygen isotope record and stages (Imbrie et al. 1984). Even numbers are glacial phases and odd numbers are interglacial phases. A time scale in thousands of years is indicated above the graph. Arrows refer to significant events in the evolutionary history of *M. australasica*

from either side of the GDR (Dufty 1986). This barrier has also been implicated as a major factor in shaping the evolution of many other freshwater fish lineages (McGlashan and Hughes 2001; Rowland 1993; Musyl and Keenan 1992; Crowley et al. 1986; Faulks et al. 2008; Musyl and Keenan 1996; Jerry 2008). However, the degree of lineage divergence within these species is variable, and the inferred dates of divergences are always more recent than the formation of the GDR. Although there are some arguments for the connectivity of catchments across the GDR (Ollier 1978), the bulk of geological evidence suggests that the major rivers of eastern Australia have remained stable for the last 30 my (Bishop 1982). Therefore, more recent and geographically small scale processes must be responsible for shaping the evolutionary history of freshwater taxa distributed on either side of the GDR. For *M. australasica* we consider the role of habitat requirements, life history strategy, and Pleistocene climate fluctuations.

Macquaria australasica has specific habitat preferences and appears sensitive to habitat alteration (Bruce et al. 2007). The species is most often associated with rocky pools and riffles in the upper reaches of catchments. Therefore, dispersal to surrounding drainages is limited to long distance dispersal through sub-optimal lowland habitats (including through estuaries in coastal catchments), or rare occasions of headwater connectivity during flooding. In addition to restricted adult dispersal, *M. australasica* lays demersal eggs within its preferred habitat where they remain until they hatch (Cadwallader and Rogan 1977). Limited dispersal due to localised recruitment and habitat requirements has the potential to severely restrict gene flow among populations. We hypothesise that this has resulted in a high degree of genetic structure across the species distribution, and may also have facilitated the divergence of lineages in isolated populations.

The evolutionary history of fauna and flora around the world has been significantly influenced by Pleistocene climate fluctuations (reviewed in Beheregaray 2008; Hewitt 2000), with many examples in fishes (Bernatchez and Wilson 1998; Beheregaray et al. 2002; Durand et al. 2005; Curry 2007; Makinen and Merila 2008). In Australia, changes in climate have had a significant affect on the landscape, particularly freshwater environments. Up until the early Pliocene (5 mya), Australia experienced very moist and warm conditions. Much of the inland was inundated by large freshwater lakes and the eastern Australian coastline was inland of its current position. The climate then developed more extreme and frequent periods of aridity, and during the Pleistocene (particularly from 500 kya) the climate was fluctuating between glacial and interglacial conditions (Bowler 1990). These fluctuations are evident in the oxygen isotope record (Fig. 1). Although periglacial activity was restricted to altitudes over 1,000 m,

lower altitudes experienced alternating cycles of cool and dry conditions followed by warm and moist conditions (Kershaw et al. 2003). The outcome for river drainages was periods of drying and isolation followed by extensive flooding and connectivity. In addition, coastal rivers were exposed to sea level fluctuations. We predict that these climate and related environmental changes have shaped the evolutionary history of *M. australasica*.

In particular, we hypothesise that fish were able to disperse across drainage boundaries via continuous wet divides during moist interglacial phases. In this scenario a low relief swampy area fed by surface or ground water has channels leading to both sides of a divide (Craw et al. 2007). Although swampy areas are not the preferred habitat of *M. australasica*, if suitable habitat was available in catchments surrounding the continuous wet divide, dispersal by this method is plausible. Locations in south eastern Australia that could have been continuous wet divides during moister environmental conditions include Lake George, Lake Bathurst and the Bredalbane Plain (Paul Hesse and Paul Bishop pers comm.) (Fig. 1). The area around Lake Bathurst and the Bredalbane Plain is in very close proximity to the Wollondilly River flowing to the coast, the Lachlan River flowing to the MDB, and to the Shoalhaven. Nearby Lake George is also in close proximity to the Shoalhaven, and catchments of the MDB (Murrumbidgee and Lachlan), and is known to have overflowed during the Pleistocene (Galloway 1965). Either of these locations could have enabled dispersal of *M. australasica* across drainage boundaries.

In contrast, the drier conditions of glacial phases may have prevented dispersal, especially in the lower Darling, Lachlan, Murrumbidgee and Murray Rivers where the effects of aridification were the strongest. Finally, sea level rises coupled with increasing upstream tidal influence during interglacial phases may have isolated populations in the lower reaches of coastal drainages like the Colo. These periodic connections among catchments are predicted to have facilitated expansion and subsequent isolation of populations in new habitats. We also predict that isolated populations will now host divergent genetic lineages. Finally, we anticipate that overall levels of genetic diversity within *M. australasica* will be low due to recent population declines.

By exploring the evolutionary history of *M. australasica* we aim to identify the processes responsible for the evolution and maintenance of genetic diversity and divergence in this threatened species. Considering recent population declines there is an urgent need for improving our understanding of these processes. Addressing this knowledge gap will guide the development of appropriate management actions to conserve the evolutionary potential of the species. The approach used here, incorporating knowledge of

the species life history and habitat requirements with past climate, geomorphological and molecular data, will be a valuable contribution to such conservation management strategies.

Methods

Sampling and tissue extraction

A total of 316 samples from 37 locations ($n = 1-41$) (Table 1) were obtained from across the species range (Fig. 2). Fish were collected using non-destructive methods, and small pieces of caudal fin tissue were taken, placed in 100% ethanol and stored at -20°C in the laboratory. Total DNA was extracted from the tissue using a modified salting out method (Sunnucks and Hales 1996). One sample from the South Australian Museum and a few other samples that yielded low quality DNA with the salting out method were extracted using a QIAGEN DNeasy kit.

SSCP and sequencing

Four hundred base pairs (bp) of the control region of the mitochondrial DNA (mtDNA) were amplified using primers A (5' TTCCACCTCTAACTCCCAAAGCTAG) and E (5' CCTGAAGTAGGAACCAGATG) (Lee et al. 1995), and variation at the region was screened using single stranded conformation polymorphism (SSCP) (Sunnucks et al. 2000). Reactions consisted of: 1 μl template DNA, 1.7 pmol of each primer, 0.4 mM each dNTP, 60 mM KCl, 12 mM Tris HCl pH 9.0, 0.12% Triton-X, 2 mmol MgCl_2 , 0.5 U Taq DNA polymerase (Promega), 0.1 μl $\alpha^{33}\text{P}$ dATP at 1,000 Ci/mmol, and dH_2O to a volume of 10 μl . The region was amplified using a step up PCR program (Beheregaray and Sunnucks 2001): 94°C for 3 min, then 5 cycles at 94°C for 30 sec, 45°C for 1 min, 72°C for 1 min, followed by 34 cycles at 94°C for 30 sec, 58°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min. At completion of cycling 10 μl of formamide dye was added to each product, then denatured at 94°C for 5 min, and placed on ice. Products were placed in a non-denaturing polyacrylamide gel [37.5:1 acrylamide: bis-acrylamide (Biorad, Hercules, California)] and run in $0.5\times$ TBE at 12 W for 4 h at a constant temperature of 4°C . Gels were dried and autoradiographed. Autoradiographs were developed and examined after 3–4 days. Autoradiographs were scored by eye, meaning that haplotypes were assigned to samples based on banding patterns. Unique haplotypes were sequenced, and to confirm the reliability of our scoring we also sequenced five samples of the same haplotype (Sunnucks et al. 2000). The region was amplified

using the conditions described above except that the reaction volume was increased to 30 μl and no $\alpha^{33}\text{P}$ dATP was incorporated. The products were purified using a GENE-CLEAN[®] III Kit (Qbiogene, Inc., Carlsbad, California). DNA sequencing was carried out by Macrogen (Korea) on an automated sequencing system (ABI 3730xl).

Data analysis

Sequences were edited and aligned using SEQUENCHER 4.1 (Gene Codes Corporation, Ann Arbor, MI) and deposited in GenBank (EU527921-66). The most appropriate model of sequence evolution was assessed in MODELTEST3.7 (Posada and Crandall 1998). The model selected using Akaike's information criterion was TVM + I + G with a gamma distribution of shape parameter 0.7595. The closest available model [Tamura (Tamura and Nei 1993)] was used in ARLEQUIN (Excoffier et al. 2006). The nature of sequence differences was identified by recording the presence of indels and diagnostic sites. To determine levels of genetic diversity both haplotype and nucleotide diversity were calculated (ARLEQUIN).

The origin and evolutionary history of *M. australasica* lineages was investigated by reconstructing a Bayesian phylogeny in MrBayes (Ronquist and Huelsenbeck 2003). The GTR model was implemented, and incorporated the gamma distribution of shape parameter 0.7595. Chains were run for 1,000,000 generations and sampled every 100th generation with 25% burnin. In a phylogenetic review of the Australian Percichthyidae (Jerry et al. 2001), the Bloomfield River Cod (*Guyu wajulwajulensis*) was identified as being the most closely related taxon to *M. australasica*. As such, this species was used as outgroup and is presented with an abbreviated branch length representing a corrected genetic distance of 25%.

To test our hypothesis that the habitat requirements and life history strategy of *M. australasica* have produced a high degree of genetic structure among populations, the hierarchical partitioning of molecular variance (AMOVA) was used (ARLEQUIN) on the following groups: (1) among all locations, (2) among locations in the MDB, (3) among locations on the coast, and (4) among the two groups (MDB and coast). This method indicates the degree of genetic structure among populations, but provides no information about genealogical relationships. Therefore, a haplotype network was constructed using the statistical parsimony method implemented in TCS (Clement et al. 2000). Our hypotheses of population history of *M. australasica* were then assessed by performing nested clade analysis (NCA) on our haplotype network. NCA has the power to distinguish between phylogeographic patterns caused by processes like contemporary restricted gene

Table 1 Frequencies of haplotypes per sampling location of *Macquaria australasica* based on 380 bp mtDNA control region

Location (n) haplotype	WL 18	CP 1	BOC 6	WC 11	WO 1	CL 1	WB 4	GB 12	EC 7	KD 12	CX 12	KW 23	KN 1	LT 12	GF 1	WOC 8	CR 15	COR 6	KR 1	PP 1	
(a) Coastal sampling locations																					
1																				1	
3	17	1		10		1															
4						1															
5																				1	
6			1																		
7														7							
8			5											1							
9																					
10										12	7										
11																					
12											1										
13									3			1									
15																			3		
27																			1		
33									1	1											
34									4	4		2									
35												3									
36																				1	
37																			4		
38															1						
39																					
40									4	6	12	2	1	1	8		3		3	5	
41																					
42																				1	
43																				1	
44											3										
45											1										
46																				1	
(b) Murray-Darling basin sampling locations and translocated populations																					
Location (n) haplotype	CO 10	KPC 9	YEA 3	HU 9	BR 6	SEV 2	MB 20	BUF 6	QR 3	AB 41	LN 14	DD 7	YR ^a 12	MON ^a 6	LD ^a 8	CD ^a 6	SA ^b 1				
2																				1	
13																				8	
14																			2		

Table 1 continued

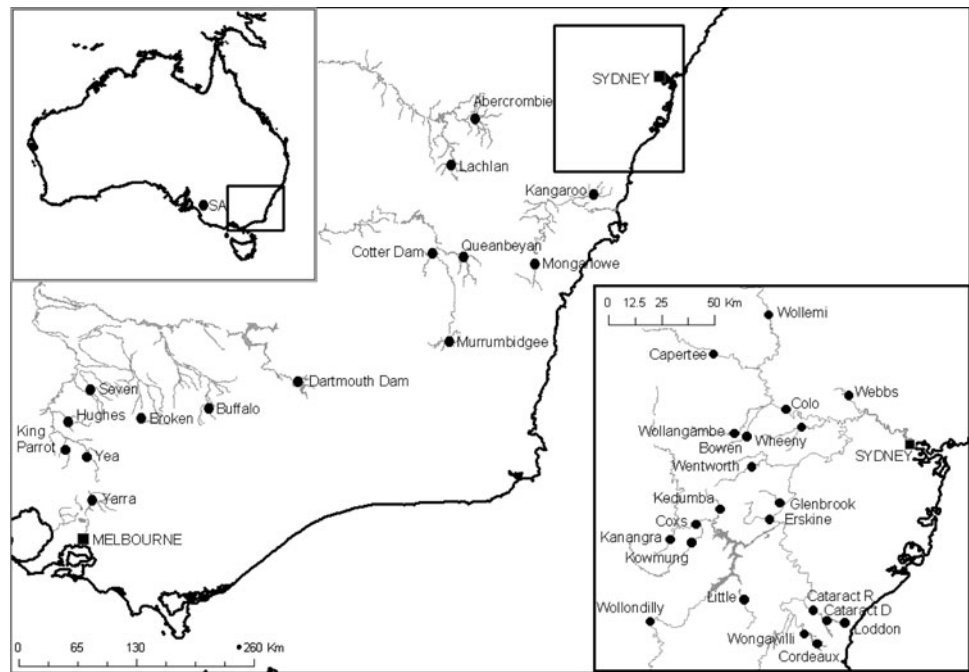
Location (n) haplotype	CO 10	KPC 9	YEA 3	HU 9	BR 6	SEV 2	MB 20	BUF 6	QR 3	AB 41	LN 14	DD 7	YR ^a 12	MON ^a 6	LD ^a 8	CD ^a 6	SA ^b 1
15	10				2	2	8	1				3	3			4	
16																	1
17				1	1			5				2					
18				2													
19																	
20													1				
21		3	2	4		1											
22							11						1				
23														5			
24														1			
25				1													
26										41	14						
28		1			2				3				4			1	
29				1													
30																	
31		5	1														
32																	1

WL Wollemi Ck, CP Capertee R, BOC Bowens Ck, WC Wheeny Ck, WO Wollangambe R, CL Colo R, WB Webb's Ck, GB Glenbrook Ck, EC Erskine Ck, KD Kedumba R, CX Coxs R, KW Kowmung R, KN Kanangra R, LT Little R, GF Wollondilly R, WOC Wongawilli Ck, CR Cataract R, COR Cordeaux Dam, KR Kangaroo R, PP Wentworth Ck, CO Cotter Dam, KPC King Parrot Ck, YEA Yea R, HU Hughes Ck, BR Broken R, SEV Seven Creeks, MB Murrumbidgee R, BUF Buffalo R, QR Queanbeyan R, AB Abercrombie R, LN Lachlan R, DD Dartmouth Dam, YR Yarra R, MON Mongarlowe R, LD Loddon Ck, CD Cataract Dam, SA Murray R South Australia

^a From the MDB to coast

^b From the coast to MDB

Fig. 2 Map of Australia indicating the study region. The first inset shows details of sampling locations of *Macquaria australasica*. The second inset provides further details of sampling locations in the Hawkesbury–Nepean Basin



flow, past fragmentation, and range expansion (Templeton 1998). Although NCA has recently been criticised for having a high rate of type I error (Petit 2008), this method provides a means of exploring complex scenarios of evolutionary history. When NCA is interpreted and validated in combination with a suite of coalescent-based methods and Earth history data, the error rates can be reduced substantially (Garrick et al. 2008). As such we used tests of isolation by distance (IBD), mismatch analysis, Fu's test of neutrality and the isolation with migration model (IM) to complement our NCA inferences, and interpreted the results in conjunction with past climate information.

In order to prevent false inferences due to translocation events, sampling locations and haplotypes from known stocked populations (see "Introduction"; 4 locations, 33 individuals, 8 haplotypes) were removed from our NCA. Clades were nested manually, riverine distances were incorporated using GeoDis (Posada et al. 2000), and the revised inference key (Posada and Templeton 2005) used to interpret significant geographical associations of haplotypes or clades.

To test for IBD that may have resulted following colonization across drainage boundaries, a mantel test was conducted using riverine distances measured in ArcMap. Hypotheses of past connectivity were used for locations no longer connected by water [Wollondilly–Lachlan, and Shoalhaven–Wollondilly (Bishop 1982)]. Although there is an alternative colonisation route from the HN into the MDB via the Macquarie catchment, the absence of samples from the extinct Macquarie population precluded this possibility from any further analysis.

We assessed the scenario that colonization of new drainage basins (HN and MDB) was followed by population expansion using mismatch analysis (ARLEQUIN). The time of expansion can be estimated based on demographic parameters derived from the mismatch distribution (Rogers and Harpending 1992) and then compared to the climate record to determine if there is a correlation between expansion events and high connectivity conditions of interglacial phases. Evidence for population expansion was also obtained by calculating Fu's F_s (Fu 1997) test of selective neutrality. The null hypothesis in this test is that the population is in equilibrium; hence a significant result may support demographic expansion.

Finally, we used IM (Hey and Nielsen 2004) to estimate three divergence times: (1) colonisation of the MDB (divergence of the MDB from the coast), (2) the hypothesis of divergence of the Lachlan from the Murray/Murrumbidgee due to aridification during glacial phases, and (3) the hypothesis of divergence of Colo/Webbs from the HN due to increasing sea level during interglacial phases. Several runs were performed, each starting with a burn-in of 100,000 steps and lasting until all parameters had an ESS of 50. This ensured that the Markov chain was exploring the entire parameter space and providing reliable values. The following input parameters were used: the HKY model, $Q_{\max} = 10$, $M_{\max} = 10$ and $T_{\max} = 10$. We used a generation time of 3 years (Appleford et al. 1998) and a molecular clock of 3.6%/my for the control region of a species from the same Order as *M. australasica* (Donaldson and Wilson 1999). This resulted in a mutation rate per year over the locus, $u = 6.84 \times 10^{-6}$. Given the

existing range of published mtDNA mutation rates (Berminham et al. 1997; Ho et al. 2007), and the absence of a specific rate for our study species, we acknowledge the error that may be associated with our interpretations of dates of divergence.

Results

Genetic diversity and structure

Despite recent population declines, we found surprisingly high levels of genetic diversity. There were 46 haplotypes in 37 localities (Table 1), and haplotype and nucleotide diversities were 0.9164 ± 0.007 and 0.0188 ± 0.0098 , respectively. As we had predicted from the habitat preferences and life history strategy of *M. australasica*, there was marked population genetic structure across the species range (Table 2). Significant structure was detected among all populations ($\Phi_{ST} = 0.85$, $P < 0.001$), populations on the coast ($\Phi_{ST} = 0.68$, $P < 0.001$) and populations in the MDB ($\Phi_{ST} = 0.84$, $P < 0.001$). There was also significant structure between the coast and MDB ($\Phi_{CT} = 0.51$, $P < 0.001$) confirming the GDR is a significant barrier to gene flow. Isolation among populations was also evident in the nature of the sequence differences. One insertion distinguished the Kangaroo River lineage and there were three sites with fixed differences between the coastal and MDB lineages.

Phylogenetic reconstruction and phylogeographic structure

The most basal lineages in the phylogeny were from coastal samples, including a highly divergent lineage (7.4–9.3%) from the Kangaroo River. The accuracy of this sequence was confirmed by re-sequencing the sample twice (in our lab and another at Macquarie University), with no discrepancies. Two major groups can be identified; one of coastal samples and one of MDB samples (Fig. 3). The tree is a series of lineage splits with the MDB group the most recently derived. As we expected from our records of

translocation, some coastal locations such as Cataract Dam and the Mongarlowe and Yarra Rivers, are genetically grouped within the MDB samples, and samples from the Cataract River display a mixture of both coastal and MDB haplotypes. In addition to the two groups identified in the phylogeny, the haplotype network displays moderate phylogeographic patterns within the MDB (shaded) and coast (clear) (Fig. 4). In the MDB the Lachlan (26) haplotype is separated from the remaining haplotypes found throughout the Murrumbidgee and Murray catchments. Similarly on the coast, the Colo (3, 4, 6 and 41) and Webb's (44 and 45) catchments are separated from the remainder of the HN.

Demographic history and divergence estimates

In support of our hypothesis of fish dispersal across drainage divides, NCA inferred that range expansion with long distance colonization has influenced phylogeographic structure across the species distribution (total cladogram; Table 3). Further demographic analyses including the divergent lineage from the Kangaroo River weren't possible because it is represented by just one sample. However, a molecular clock formula (Divergence (%) = $-2.2e^{(-9t)} + 2.5t + 2.2$ (Craw et al. 2008) estimated the divergence time of this lineage to be ~ 2 mya. A signal of IBD was inferred by NCA for the clade spanning the GDR, although gene flow within this clade is now restricted (clade 4-3), and the Mantel test found no strong evidence of IBD ($R^2 = 0.00006$, $P < 0.001$), suggesting a more complex scenario of colonisation. The divergence of the east coast and MDB lineages was estimated by IM to have occurred 657 kya.

Our scenario of population expansion following colonisation was also supported. Within the MDB, NCA inferred range expansion with long distance colonization into the Lachlan catchment (clade 2-11). This expansion event was also detected by our mismatch analysis, as the mismatch distribution of the MDB populations was not significantly different from that expected under the model of expansion (Ragedness Index = 0.066, $P = 0.149$; SSD = 0.0242, $P = 0.210$). Expansion was estimated to have occurred 536 kya. Gene flow among the MDB populations is now restricted, although some long distance dispersal through areas not suitable for the species, like the lower reaches, may occur (NCA inference 3-6). On the east coast, range expansion with long distance colonization followed by fragmentation has also shaped phylogeographic structure, particularly the isolation of the Colo and Webbs catchments (NCA inference 3-4 & 4-2). Once again the NCA inferences for range expansion were supported by our tests of demographic expansion. The mismatch distribution of the coastal populations was not significantly different from that expected under the model of expansion

Table 2 Results of hierarchical analysis of molecular variation (AMOVA) based on 380 bp mtDNA control region haplotypes in *Macquaria australasica*

Level of partitioning	Phi-value	P value
Among all populations in the MDB	$\Phi_{ST} = 0.84$	$P < 0.001$
Among all populations on the coast	$\Phi_{ST} = 0.68$	$P < 0.001$
Among all populations	$\Phi_{ST} = 0.85$	$P < 0.001$
Between the MDB and coast	$\Phi_{CT} = 0.51$	$P < 0.001$

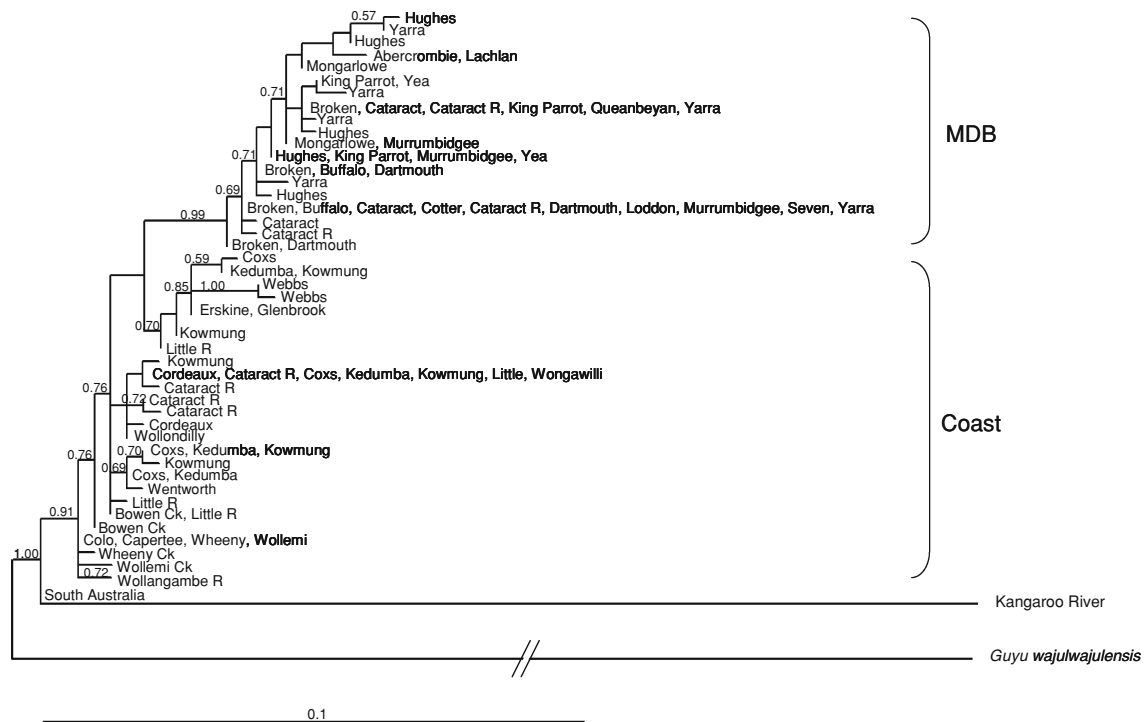


Fig. 3 Bayesian phylogenetic reconstruction of *Macquaria australasica* based on 380 bp of the mtDNA control region. Labels on the nodes indicate Bayesian probabilities. *Guyu wajulwajulensis* is the outgroup and is shown with an abbreviated branch length representing

a corrected genetic distance of 25%. Two groups are indicated: lineages from the Murray-Darling Basin, and lineages from the coast. Also of note is the divergent lineage from the Kangaroo River

(Raggedness Index = 0.045, $P = 0.069$; SSD = 0.0184, $P = 0.055$), and Fu’s test of neutrality for the coastal populations was significant ($F_s = -8.2$, $P = 0.021$). The estimated time of population expansion on the coast was 349 kya, during a glacial period identified as OIS 10. Our other two tests of divergence also support the hypothesis of climate mediated dispersal and isolation. The divergence of the Lachlan from the remaining MDB lineages was estimated by IM to have occurred 310 kya, during an interglacial period identified as OIS 9. Finally, IM estimated that the Colo and Webbs catchments diverged from the remainder of the HN 130 kya, towards the end of glacial period OIS 6.

Discussion

Our data demonstrate clear correlations between palaeoclimatic conditions and evolutionary history in *M. australasica*. The suite of analytical tools used here support our hypotheses about the influence of Pleistocene climate fluctuations, geomorphology, specific habitat requirements, and life history on the phylogeographic structure of *M. australasica*. As discussed below, we infer that populations of *M. australasica* expanded in response to climate changes and habitat availability during the Pleistocene,

promoting the evolution of genetically diverse and divergent lineages that were subsequently isolated in the upper reaches of catchments. However, the phylogeographic method has shortcomings, as seems to be the case for any discipline with a historical dimension, and it is possible that processes not inferred here might have contributed to the evolutionary history of the species. Nevertheless, it is clear that diverse lineages have persisted over time but are now threatened and being lost due to human activities. Conservation efforts should prioritise the protection and restoration of habitat, and continued research on this endangered species.

Habitat requirements and life history traits

Levels of genetic diversity were unexpectedly high given the observed recent population declines. However, the long life span (20 years Mark Lintermans pers. comm.) and generation interval of *M. australasica* (age at sexual maturity >3 years) means that only a modest number of generations have contributed to a loss of genetic diversity through drift since populations started to decline (Lake 1978). There were also many haplotypes specific to locations or catchments (Table 1), and estimates of Φ_{ST} were large, indicating a high degree of population genetic structure across the species range. The present distribution

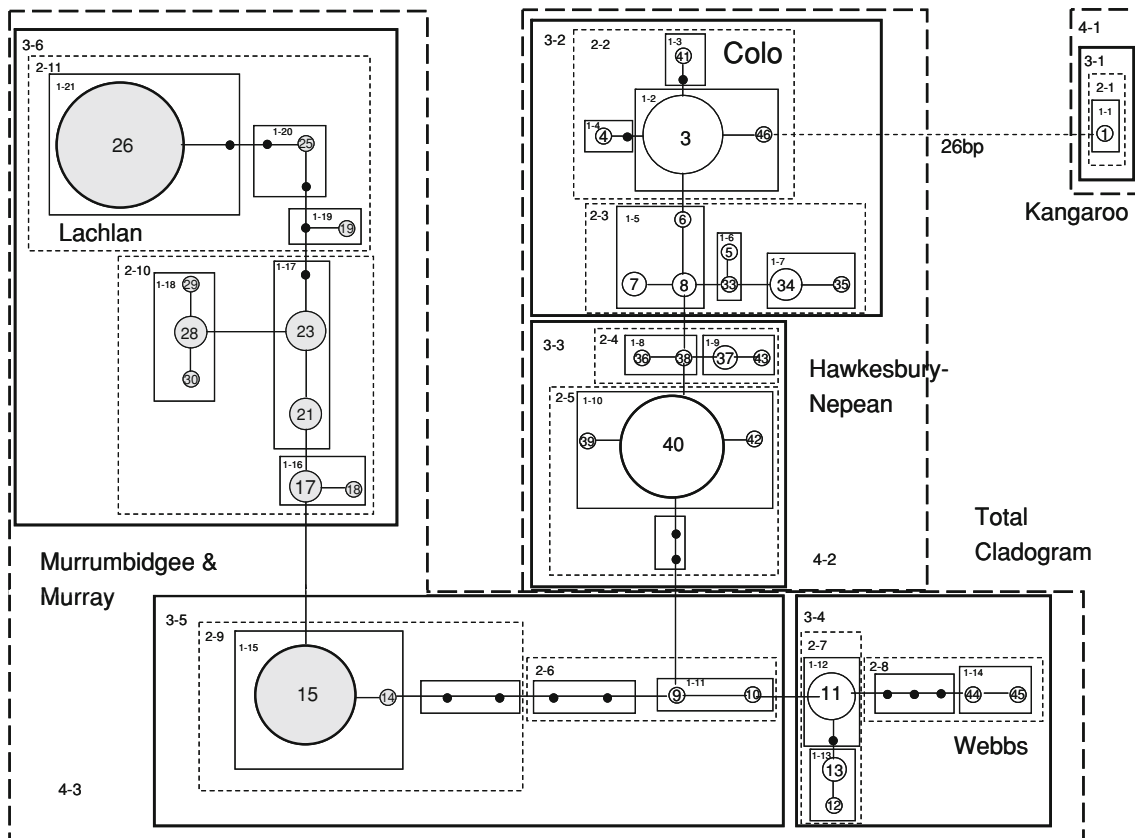


Fig. 4 Network and nested design of the genealogical relationships in *Macquaria australasica* based on 380 bp mtDNA control region haplotypes (translocated individuals removed). The size of the circles indicates the frequency of that haplotype, lines joining haplotypes represent one base pair mutation, and dots along the lines are missing haplotypes (not sampled or extinct). Clear circles are haplotypes found on the coast and shaded circles are haplotypes found in the Murray-Darling Basin. Numbers inside the circles correspond to the

haplotypes listed in Table 1. Numbers in the top left hand corner of clades correspond to those used in Table 3 and throughout the text. The first digit refers to the nested level of a clade and the second digit is a unique number for a clade at a particular nested level. Labels describe the geographic locations mentioned in the text. The minimum number of steps between the Kangaroo River (haplotype 1) and the remainder of the network is 26 (not supported by statistical parsimony)

of *M. australasica* in the upper reaches of catchments, coupled with the species tendency to recruit in local areas, restricts gene flow among populations. Although *M. australasica*'s historical distribution was greater, the species habitat preferences and life history strategy are unlikely to have changed. Therefore, populations have been isolated from one another throughout time, creating the genetic structure we have observed. Strong genetic structure has also been documented in Oxleyan pygmy perch, another Australian freshwater fish with specific habitat preferences (Hughes et al. 1999). In addition to generating high levels of genetic structure, restricted gene flow also promotes the divergence of lineages. This is clearly evident in the levels of divergence observed between lineages of *M. australasica* from the three drainage basins, particularly the Kangaroo River, a lineage that may represent a previously unrecognised species. Moderate levels of divergence are also seen within the basins, most notably in the Webbs and Lachlan catchments. High levels of genetic divergence

within species were also discovered in a study of freshwater macroinvertebrate assemblages in the Sydney water supply catchment (encompassing the geographic range of coastal *M. australasica* populations; Baker et al. 2004). Further studies may continue to reveal divergent lineages and highlight the potential importance of this region for the conservation of freshwater taxa.

Geomorphology and Pleistocene climate fluctuations

Our dates of lineage divergence indicate that dispersal from the Shoalhaven into the HN occurred ~2 mya, and from the HN into the MDB ~657 kya. These events predate the onset of aridity in Australia. During this early part of the Pleistocene the continent experienced higher amounts of rainfall, and increased runoff to freshwater environments. These were favourable conditions for creating continuous wet divides. Although further inferences about the Shoalhaven basin are difficult without additional samples, the

Table 3 Biological inferences of processes influencing the geographical distribution of genetic variation in *Macquaria australasica* based on significant clades of the nested design in Fig. 3

	Significant level of cladogram	P value	Inference chain	Outcome
<p><i>AF</i> allopatric fragmentation, <i>CRE</i> contiguous range expansion, <i>F</i> fragmentation, <i>IBD</i> isolation by distance, <i>LDC</i> long distance colonisation, <i>LDD</i> long distance dispersal, <i>PF</i> past fragmentation, <i>PGRE</i> past gradual range expansion, <i>RE</i> range expansion, <i>RGF</i> restricted gene flow</p>	Clade 1-5	0.003	1-2-3-4-9	AF
	Clade 1-7	0.057	1-2-3-4	RGF with IBD
	Clade 1-17	0.001	1-2-11-12	CRE
	Clade 1-18	0.003	1-2-3-4-9	AF
	Clade 2-3	<0.001	1-2-3-5-6-7	RGF some LDD
	Clade 2-7	<0.001	1-19	AF
	Clade 2-10	<0.001	1-2-11-12	CRE
	Clade 2-11	<0.001	1-2-3-5-15-21	LDC, PGRE followed by F
	Clade 3-2	<0.001	1-19-20-2-3-4-9	AF
	Clade 3-3	<0.001	1-2-3-5-6-7	RGF some LDD
	Clade 3-4	<0.001	1-19-20-2-11-12-13-21	RE, LDC with subsequent F
	Clade 3-5	<0.001	1-19	AF
	Clade 3-6	<0.001	1-2-3-5-6-7-8	RGF some LDD over intermediate areas not occupied by species
	Clade 4-2	<0.001	1-2-11-12	RE, LDC with subsequent F or PF followed by RE
	Clade 4-3	<0.001	1-2-3-4	RGF with IBD
	Total Cladogram	<0.001	1-2-11-12-13-21	RE, LDC with subsequent F or PF followed by RE

high level of divergence suggests that conditions facilitating connectivity across this basin boundary were temporary and rare.

Following colonisation, populations within the HN and MDB basins expanded and were subsequently isolated. These events coincided with changes in climate and river hydrology (Fig. 1). The MDB expansion event (536 kya) occurred shortly after the colonisation of the basin, and prior to the onset of widespread arid climate conditions. The ancestral rivers of the MDB had greater flow and large inland lakes inundated the lower reaches of many catchments (Pels 1964). These conditions created a high level of connectivity among catchments, facilitating expansion into new habitats and promoting population growth. Colonisation of the most distant catchments like the Lachlan and Macquarie (population now extinct) may have occurred at this time. However, these conditions soon deteriorated and the climate became more arid and variable due to El Niño Southern Oscillations (Kershaw et al. 2003). Freshwater environments of the MDB were then isolated from one another (Bowler and Wasson 1983), as evidenced by our divergence date of the Lachlan catchment (310 kya).

Around this same time (349 kya) the coastal HN populations were expanding. Although this date is later than expected given divergence from the Shoalhaven lineage occurred 2 mya, earlier signals of expansion may have been masked by this most recent event. Unstable climate conditions ~350 kya (as described above) may have necessitated HN populations to colonise new habitats. Drier climate and less runoff resulted in shallower rivers,

and shifted riffle habitats downstream. In addition, sea levels were lower moving the tidal limit further down river valleys. These were ideal conditions for *M. australasica* to colonise catchments like the Colo and Webbs. However, sea levels rose, and fluvial activity was heightened as the climate became increasingly warm and moist at the end of the penultimate glacial cycle 130 kya (Nott et al. 2002). These changes would have prevented further dispersal between the Colo and Webbs, and remaining HN populations, and coincide with our estimated date of divergence of these lineages.

Not only have differences in landscape and climate conditions contributed to the divergence of mtDNA lineages in *M. australasica*, they may have resulted in morphological differentiation of populations (Dufty 1986). *M. australasica* from the MDB are substantially larger than on the coast, and vary in several morphometric and meristic characteristics (Dufty 1986). These different morphological forms are likely to be associated with ecological conditions. MDB lineages may have experienced phenotypic evolution under conditions of high ecological opportunity like new habitat, and different fish communities (Bernatchez and Wilson 1998; Orr and Smith 1998). A similar process has been observed in dwarf and normal smelt (Taylor and Bentzen 1993).

Conservation implications

Maintenance of the long term processes that aid the evolution of a species are a challenge for conservation

managers (Driscoll 1998; Moritz 2002). We demonstrate that a phylogeographic approach can help by identifying how these processes have affected populations in the past, and inferring how they may shape those in the future. *M. australasica*'s high levels of genetic diversity and divergence have been promoted by the species' specific habitat requirements and life history strategy, as well as Pleistocene climate fluctuations. Therefore, management strategies should consider habitat availability under future climate change scenarios to conserve current levels of diversity.

South eastern Australia is expected to have a drier climate and higher sea levels under climate change (Meehl et al. 2007). This could result in increased habitat loss and fragmentation of *M. australasica* populations. Habitat will be lost in smaller order streams as they dry out, and lower flows in higher order streams may lead to a change in distribution of riffle-pool sequences. Sea level rises will result in an upstream migration of the tidal influence, also causing loss of suitable habitat. However, Penrith Weir should prevent saline intrusion of catchments upstream such as Glenbrook and Erskine Creeks. Continued loss of remnant habitat could lead to further population declines and result in reduced genetic variability through drift. We recommend habitat protection and restoration of populations within each major lineage group observed: Kangaroo River, Webbs Creek, Colo River, Nepean River, Murray-Murrumbidgee Rivers, and Lachlan River. This approach will help maintain genetic diversity and evolutionary potential under future climate change scenarios [*sensu* the Phylogenetic Diversity measure (Faith 1992)].

In addition to maintaining genetic diversity, the genetic integrity of lineages requires protection. The MDB and eastern lineages of *M. australasica* are genetically and morphologically distinct; however, their taxonomic status remains in question. The presence of both lineages in the Cataract River is of particular concern. Fish from the MDB were stocked into Cataract Dam in 1915 (NSW State Fisheries 1916), and our data indicates that some of these fish have escaped downstream into the lower Cataract River. The degree of hybridisation between these two lineages in the river is unknown, but has the potential to result in the extinction of lineages (Rhymer and Simberloff 1996; Allendorf et al. 2001; McClelland and Naish 2007). This situation exemplifies the importance of understanding the phylogeography of species prior to undertaking translocation or stocking activities. Finally, the highly divergent Kangaroo River lineage may represent a separate (albeit likely extinct) species, and warrants further taxonomic investigation.

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