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ORIGINAL ARTICLE

Clarifying an ambiguous evolutionary history: range-wide phylogeography of an Australian freshwater fish, the golden perch (*Macquaria ambigua*)

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ABSTRACT

Aim We conducted a range-wide phylogeographic study of a common Australian freshwater fish, the golden perch (*Macquaria ambigua*), to investigate the relationship between environmental processes and evolutionary history in drainage basins.

Location Inland [Lake Eyre (LEB), Murray–Darling (MDB) and Bulloo (BULL)] and coastal basins [Fitzroy (FITZ)] of eastern Australia.

Methods A total of 590 samples were collected from across the entire species' distribution and a section of the mitochondrial DNA control region was sequenced. In order to reconstruct the evolutionary history of *M. ambigua* a comprehensive suite of phylogeographic analyses was conducted, including nested clade phylogeographic analysis, mismatch analysis and isolation-with-migration model simulations.

Results Three major lineages corresponding to the major drainage basins, FITZ, MDB and LEB/BULL, were identified (Φ_{ST} = 0.92). Lineages from the coastal basin (FITZ) were highly divergent from those of the inland basins (up to 6%). Levels of genetic diversity in the inland basins were relatively low and our analyses indicate that these populations experienced both demographic and range expansions during the Pleistocene.

Main conclusions Investigation of the range-wide phylogeography of *M. ambigua* has revealed new insights into the biogeography of the Australian arid zone, particularly with regard to evolutionary events chronologically associated with cyclical moist and dry conditions. We propose that *M. ambigua* originated on the east coast (FITZ) and crossed a major geographic barrier, the Great Dividing Range (GDR), to colonize the inland basins (MDB, LEB and BULL). We infer a series of demographic and range expansion events for *M. ambigua* consistent with a scenario of moister Pleistocene conditions and increased connectivity of freshwater environments, both within and among drainage basins. Major lineages then diversified following isolation of freshwater environments under increasingly arid climate conditions. We suggest that management priorities for *M. ambigua* should include the resolution of taxonomic uncertainties and the maintenance of genetic diversity of both stocked populations in the MDB and native populations of the LEB that may be at risk of further isolation and reduced gene flow due to increased aridification under future climate change scenarios.

Keywords
Australia, biogeography, Bulloo Basin, climate change, fisheries management, Fitzroy Basin, Lake Eyre Basin, *Macquaria ambigua*, Murray–Darling Basin.

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INTRODUCTION

The Australian arid zone occupies *c.* 70% of the continent's land area and is one of the largest desert ecosystems in the world (Byrne *et al.*, 2008). However, during most of the history of the continent, the environmental conditions were quite different, with the climate being warm and moist and inland seas and rain forests widespread (Bowler, 1990; Byrne *et al.*, 2008). It was only during the late Miocene (*c.* 6–10 Ma) that the continent began to experience a gradual transition towards more arid and fluctuating climatic conditions. By the Pleistocene (*c.* 2 Ma) the climate was characterized by cyclical warm/moist and cool/dry conditions and by *c.* 400 ka the extremes of these cycles were at their peak (Bowler, 1990; Byrne *et al.*, 2008). During the late Pleistocene, inland Australia became increasingly dry, resulting in the loss of large inland freshwater lakes and a reduction in connectivity of riverine systems. Although some freshwater ecosystems in the southern part [southern Murray–Darling Basin (MDB)] of the Australian arid zone remain relatively well connected, many others are now intermittent, persisting as isolated waterholes connected only during flood events [north-west MDB, Lake Eyre Basin (LEB) and Bulloo (BULL)]. Overall, the freshwater environments of the Australian arid zone have experienced significant changes over a relatively recent time period. A deeper understanding of the history of these environments can be achieved through the study of the biogeography of the organisms which inhabit them, particularly freshwater fishes (Bermingham & Avise, 1986; Bernatchez & Wilson, 1998; Soltis *et al.*, 2006; Beheregaray, 2008; Hughes *et al.*, 2009).

A review of the biogeography of Australian freshwater fishes (Unmack, 2001) highlighted the endemic nature of fish assemblages in different regions of the continent and stated that these patterns were the result of isolation due to aridity and drainage divides. However, a number of species are found across biogeographic provinces and explaining patterns of individual species distribution, particularly how and when fishes crossed drainage divides, is difficult. Although recent studies have provided some insight into this issue (Hughes & Hillyer, 2006; Faulks *et al.*, 2008; Huey *et al.*, 2008; Jerry, 2008), our understanding of the effect of aridification on the biogeographic relationships among the largest drainage basins in central and eastern Australia remains uncertain. One species that might help fill this knowledge gap is the golden perch, *Macquaria ambigua*.

Macquaria ambigua is a member of the Percichthyidae, a family considered to be secondarily of freshwater habit and derived from marine protopercoid ancestors (Jerry *et al.*, 2001). The species is naturally distributed in the MDB, LEB, BULL and Fitzroy (FITZ) basins and translocated populations exist in various coastal catchments including the Hunter (Glenbawn Dam), Clarence, Glenelg, Yarra, Barwon, Millicent Coast, Corangamite, Werribee and Marybyrnong (Allen *et al.*, 2002; Joy Sloan, DPI Victoria, pers. comm.) (Fig. 1). The native range of *M. ambigua* covers approximately 2,250,000 km^2 and includes all of the inland basins and the largest coastal basin of eastern Australia. This distribution exposes *M. ambigua* to a wide variety of climatic and habitat conditions, ranging from isolated groundwater-fed waterholes in the arid zone, to broad lowland river channels and streams. The species is relatively common and is found most often in pools with cover from timber, rocks or undercut banks, features that are abundant throughout most of its distribution (Crook & Robertson, 1999). Their diet is opportunistic (Pusey *et al.*, 2004). In addition, *M. ambigua* possesses good dispersal abilities (Reynolds, 1983), allowing the species to rapidly respond to environmental triggers for spawning, or to retreat from adverse conditions. These characteristics make *M. ambigua* resistant to a broad range of disturbance regimes and therefore an ideal species with which to investigate the relationship between landscape and freshwater fish evolution across a large continental area and multiple hydrological basins. Previous studies, based on allozyme electrophoresis and morphology (Musyl, 1990; Musyl & Keenan, 1992; Keenan *et al.*, 1995), have demonstrated that populations of *M. ambigua* both among and within basins are distinct from one another. These data suggested the recognition of a separate subspecies, *Macquaria ambigua oriens*, from the coastal FITZ and they also indicated that fish from the LEB could be of a separate species, with its own subspecies in the BULL. However, none of these putative taxa have been formally described.

This taxonomic uncertainty is of particular concern due to the fairly intensive stocking activities undertaken to support the recreational fishery of *M. ambigua*. The genetic risks associated with supplementing wild populations with hatchery-reared fish are well recognized (Hindar *et al.*, 1991; Ward, 2006) and have been highlighted as a major issue for *M. ambigua* (NSW Fisheries, 2003). Although current regulations prevent the exchange of fish across drainage basins within the species' natural range, this has occurred in the past (Wager & Unmack, 2000). The majority of stocking occurs in the MDB, where since 2003 broodstock collection and fingerling release zones [northern (Darling), central (Lachlan) and southern (Murray/Murrumbidgee)] have been arbitrarily designated for releases undertaken in New South Wales (NSW) (NSW Department of Primary Industries, 2005). In addition to these zones, NSW developed a Hatchery Quality Assurance Scheme (Rowland & Tully, 2004; NSW Department of Primary Industries, 2008) outlining guidelines for fish production and release and specific procedures for minimizing risks to the genetic integrity of populations. Similar strategies are under development in Queensland and Victoria.

Here we assess the range-wide phylogeography of *M. ambigua* to reconstruct evolutionary events associated with climatic conditions in a large section of the arid zone of Australia and to contribute to the management of this iconic species. In particular we aimed to do the following:

1. Reconstruct *M. ambigua*'s evolutionary history and use the species as a model for assessing the historical hydrological relationship among drainage basins where the species is distributed. We tested the hypothesis that inland basins

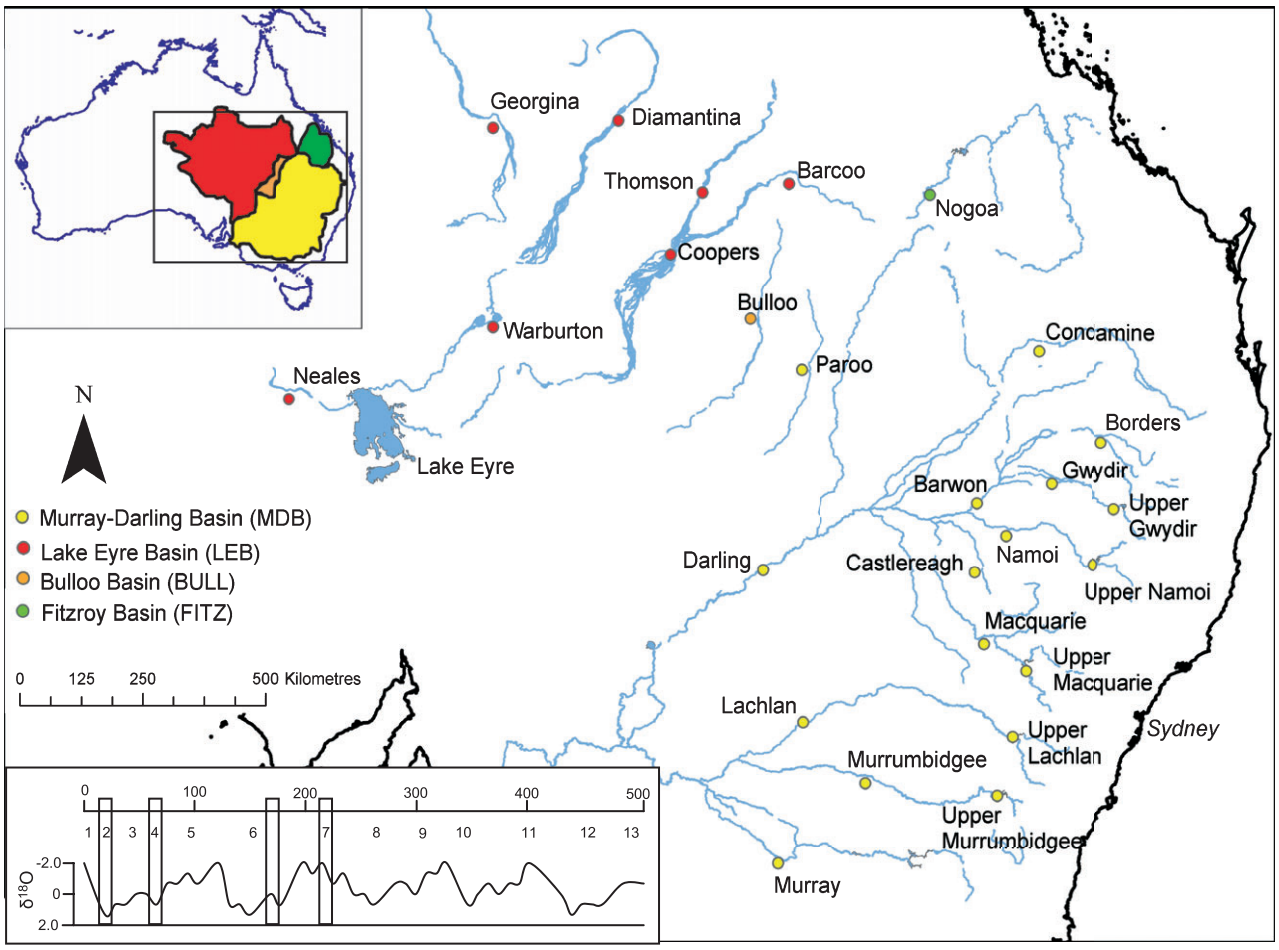

Figure 1 Map of eastern Australia showing the natural distribution of *Macquaria ambigua* and the four river basins studied. The main map displays details of basin hydrology and the 26 sampling locations for this study. A second inset shows the oxygen isotope record, even numbers are glacial phases and odd numbers are interglacial phases (Imbrie *et al.*, 1984). A time-scale in thousands of years ago is indicated above the graph. Rectangular bars indicate significant events in the evolutionary history of *M. ambigua* referred to in the text.

(MDB, LEB and BULL) represented the region of origin of freshwater fish species, a scenario followed by colonization of east coast drainages (FITZ) (Musyl & Keenan, 1992). We also aimed to clarify the temporal progression of the major lineage splits inferred among the inland basins (MDB, LEB and BULL).

2. Assess matrilineal population structure across the species' range. We predicted that the genetic structure of populations would reflect current drainage basin boundaries. In addition, some structure was expected within basins, particularly where hydrological connectivity among catchments is limited, e.g. Paroo, Lachlan, Neales and Georgina.

3. Address management questions surrounding ongoing stocking programmes and help resolve current taxonomy.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 590 samples were obtained from across the species' range in eastern Australia (Fig. 1). Twenty-six locations, including 21 major catchments and five upper catchments (above major impoundments), were sampled. Samples were collected using electrofishing and netting. 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 & Hales, 1996). Voucher specimens from the Nogoa, Bulloo, Thomson, Diamantina and Georgina catchments have been deposited at the Australian Museum (I.44841–I.44845).

Single-stranded conformation polymorphism and sequencing

Approximately 400 base pairs (bp) of the control region of mitochondrial DNA (mtDNA) were amplified using primers A (5′-TTCCACCTCTAACTCCCAAAGCTAG-3′) and E (5′-CCT GAAGTAGGAACCAGATG-3′) (Lee *et al.*, 1995). This region is highly variable in fishes and is an informative marker for investigating intraspecific genealogical relationships (Lee *et al.*, 1995). Variation at this region was screened using single-stranded conformation polymorphism (SSCP; Sunnucks *et al.*,

2000). This method provides a relatively fast and inexpensive way of determining differences in samples so that only a subset needs to be sequenced. Reactions consisted of: 1 μL template DNA, 1.7 pmol of each primer, 0.4 mM of each deoxyribonucleotide triphosphate (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, Madison, WI, USA), 0.1 μL α^{33}P deoxyadenosine triphosphate (dATP) at 1000 Ci mmol^{-1}, and distilled (d)H_2O to a volume of 10 μL. The region was amplified using a step-up polymerase chain reaction (PCR) program (Beheregaray & Sunnucks, 2001): 94 °C for 3 min, then five cycles at 94 °C for 30 s, 45 °C for 1 min, 72 °C for 1 min, followed by 34 cycles at 94 °C for 30 s, 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, CA, USA)] and run in 0.5 × TRIS/borate/EDTA (TBE) at 12 W for 4 h at a constant temperature of 4 °C. Gels were dried, autoradiographed, then 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 and minimize the chance of missing rare unique haplotypes we also sequenced multiple samples of the same haplotype (Sunnucks *et al.*, 2000). The region was amplified using the PCR conditions described above except that the reaction volume was increased to 30 μL and no α^{33}P dATP was incorporated. The products were purified using a GENECLEAN® III Kit (Qbiogene, Inc., Carlsbad, CA, USA). DNA sequencing was carried out by Macrogen (Korea) on an automated sequencing system (Applied Biosystems Instrument 3730xl, Applied Biosystems, Foster City, CA, USA).

Data analysis

Sequences were cleaned and aligned using SEQUENCHER 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and submitted to GenBank (EU682091–682113). Before running subsequent analyses the most appropriate model of sequence evolution was assessed using Akaike's information criterion (AIC) in MODELTEST 3.7 (Posada & Crandall, 1998). The selected model, or the closest possible when that was not implemented in the required software (see Results), was used in all subsequent analyses. Levels of genetic diversity were determined by calculating both haplotype and nucleotide diversities and levels of population subdivision were assessed using the Φ_{ST}-based hierarchical partitioning of molecular variance (AMOVA) implemented in ARLEQUIN 2.0 (Schneider *et al.*, 2000). The following hierarchical levels of subdivision were investigated: (1) among all basins, (2) among locations in the MDB and (3) among locations in the LEB.

To elucidate the evolutionary history of *M. ambigua*, a phylogenetic reconstruction was carried out in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). Monte Carlo Markov chains were run for 1×10^6 generations and sampled every 100th generation with 25% burn-in. The most closely related species, *Macquaria australasica* (Jerry *et al.*, 2001), was used as the outgroup and is presented with an abbreviated branch length representing a corrected genetic distance of 25%. We dated the divergence of lineages to enable us to interpret our data together with geographic and palaeoclimatic events. The divergence times of lineages were estimated using a variable molecular clock, where sequence divergence (%) = $-2.2e^{(-9t)} + 2.5t + 2.2$, with t being the time in thousands of years (Craw *et al.*, 2008). This clock was calibrated using data from galaxiid fish lineage divergences and geological events in New Zealand and has been proposed as a potential dating tool for river drainage changes in the late Quaternary in the Southern Hemisphere (Craw *et al.*, 2008). The isolation-with-migration (IM) model is able to estimate dates of divergence under the scenario of continued gene flow between populations during the process of isolation (Hey & Nielsen, 2004). We used IM to estimate the divergence time of the LEB and BULL, as these two basins have previously been shown to have a very close biogeographic association with the potential for ongoing migration (Unmack, 2001). Several runs were performed, with each run lasting until all parameters had an effective sample size (ESS) of at least 50 to ensure mixing and consistency. The HKY model was chosen as the most appropriate as it is the only model that takes into account multiple mutations at each site, differences in nucleotide frequencies and the presence of transition/transversion (Tr/Tv) bias. The following input parameters were used: $Q_{max} = 10$, $M_{max} = 10$, $T_{max} = 10$ and a burn-in of 100,000. A mutation rate of $\mu = 6.84 \times 10^{-6}$ over the 380 bp was calculated based on a sequence divergence rate of 3.6% Myr^{-1} (Donaldson & Wilson, 1999). This rate is considered the best available as it is from the control region of a fish within the same suborder as *M. ambigua* (Percoidei). This rate is also very similar to the average rate derived from the variable molecular clock of Craw *et al.* (2008) that was used for our other analyses. However, we do acknowledge the large variation in molecular clocks for the control region of fishes, and as such we also calculate all of our divergence and expansion time estimates using an alternative clock of 10% Myr^{-1} (*sensu* Bowen *et al.*, 2006).

Further inferences about the population history of *M. ambigua* were obtained through nested clade phylogeographic analysis (NCPA), which has the power to distinguish between patterns caused by processes such as restricted gene flow, past fragmentation and range expansion (Templeton, 1998). A haplotype network was constructed using the statistical parsimony method implemented in TCS 1.21 (Clement *et al.*, 2000). This method links haplotypes with the smallest number of sequence differences first, providing high resolution for inferring relationships among recently diverged populations (Posada & Crandall, 2001). Loops in the haplotype network were resolved using predictions based on coalescent theory (Posada & Crandall, 2001) and clades were nested manually. Riverine distances were incorporated using GEODIS (Posada

et al., 2000). The revised inference key (Posada & Templeton, 2005) was used to interpret significant geographic associations of haplotypes or clades. Although NCPA has recently been criticized (e.g. Petit, 2008), its use in this field of study is still warranted. This is particularly true when NCPA is interpreted and validated in combination with a suite of coalescent-based methods and geomorphological data (Garrick *et al.*, 2008; Templeton, 2009). To interpret and test our NCPA inferences we used information from isolation by distance (IBD), mismatch analyses and palaeoclimatic data. In particular we referred to the oxygen isotope record, which represents fluctuations in climate between glacial and interglacial conditions (Fig. 1) (Imbrie *et al.*, 1984). Oxygen isotope stages (OIS) are numbered: peaks with odd numbers indicate interglacial phases, while troughs with even numbers indicate glacial phases. To test for IBD among locations within basins we ran Mantel tests (ARLEQUIN). Riverine distances were measured along the defined main river channel using ARCMAP. Mismatch analysis (ARLEQUIN) was used to assess the scenario that colonization was followed by population expansion within the MDB and LEB. In situations where historical expansion was statistically supported, the time of expansion was estimated based on demographic parameters derived from the mismatch distribution (i.e. estimator of time to the expansion and the mutation parameter) (Rogers & Harpending, 1992).

RESULTS

Genetic diversity and structure

We found a relatively low level of genetic diversity in the populations analysed, with 23 haplotypes reported in the 26 localities (Table 1) and haplotype and nucleotide diversities of 0.060 ± 0.021 and 0.010 ± 0.006, respectively. MODELTEST indicated that the most appropriate model for the dataset was K81 uf + I, which accounts for equal base frequencies with three substitution types (Kimura, 1981). There was marked population structure across the species' range (Table 2), with an overall Φ_{ST} of 0.92 ($P < 0.001$) and the majority of structure due to differences among basins, $\Phi_{CT} = 0.91$ ($P < 0.001$). There was also significant structure among populations in the LEB, $\Phi_{ST} = 0.71$ ($P < 0.001$), but not in the MDB, $\Phi_{ST} = 0.007$ ($P = 0.139$).

Evolutionary history

Our phylogenetic reconstruction revealed two major clades (Fig. 2): one composed of the coastal lineages (FITZ) and a second including all of the inland lineages (MDB, LEB and BULL). The FITZ lineages are basal in this phylogeny, indicating a coastal origin of the species. With one exception these lineages are also reciprocally monophyletic and up to 6% divergent from inland lineages, suggesting that they have been isolated for a significant period of time. The variable molecular clock estimated that the divergence time between these two clades was approximately 1.5 Ma (*c.* 1.7 Ma under the 3.6% Myr^{-1} clock, and *c.* 600 ka under the 10% Myr^{-1} clock). There was one haplotype (11) found in the FITZ clade that was sampled from one fish in the Border Rivers of the MDB. This sample was resequenced with the same result, and we conclude that this anomaly could be due to illegal stocking practices. There are two very well supported groups within the inland clade: MDB and LEB/BULL. Haplotypes within the MDB group were not isolated to disparate areas, but were dispersed throughout catchments in both the northern and southern portions of the basin. Although there is a split within the group of MDB lineages, this node has a relatively low Bayesian posterior probability and may indicate uncertainty and/or recency of the relationships among lineages in the MDB. The variable molecular clock estimated the divergence of the MDB and LEB lineages at *c.* 58 ka during OIS 4, the penultimate glacial phase (*c.* 280 ka under the 3.6% Myr^{-1} clock and *c.* 100 ka under the 10% Myr^{-1} clock; both interglacial phases). An intricate pattern of relationships among lineages was identified within the LEB/BULL group. The BULL lineages were nested within those from the LEB, whereas some lineages from catchments within the LEB (Diamantina, Warburton, Georgina and Neales) were divergent (node at the tip of the tree). These results suggest a relatively recent connection between the LEB and BULL, despite isolation of some catchments within the LEB. Indeed, the IM model estimated that the divergence of LEB and BULL lineages could have occurred as recently as 28 ka, coinciding with the last glacial phase (OIS 2) (95% confidence interval (CI) *c.* 22 ka–1.5 Ma) [*c.* 10 (CI *c.* 8–520) under the 10% Myr^{-1} clock].

Further inferences about the evolutionary history of the species were obtained from examination of the haplotype network (Fig. 3). The five coastal haplotypes (11–15) are of relatively equal frequency and form a pattern indicative of ancient lineages in a stable population. In contrast, the relationships among inland haplotypes are indicative of recent population expansion, with the high frequency of haplotype 1 in the MDB (found in 356 fish) and its star-like radiation of rare, unique haplotypes. In comparison, moderate phylogeographic structure is evident in the LEB with the divergence of haplotypes 20 and 23 found in the western and northern catchments of the basin.

Nested clade phylogeographic analysis indicated that the geographic association of haplotypes was significant in four clades; this enabled the identification of a combination of contemporary and historical processes that might have affected the phylogeography of the species (Table 3). The process inferred for the geographic association of lineages across the species range (total cladogram) was allopatric fragmentation. Contiguous range expansion with long distance colonization, followed by fragmentation, was the inferred process for the inland haplotypes, clade 3-2. Contiguous range expansion was also inferred for the western and northern LEB, clade 1-14. Mismatch analysis provided additional evidence of historical demographic expansion of the inland populations. The mismatch distributions within both the MDB (raggedness index = 0.648, $P = 0.753$; sum of square deviations (SSD) =

Table 1 Distribution of haplotypes across major catchments of the natural range of *Macquaria ambigua* in eastern Australia.

Basin	Fitzroy	Murray–Darling																	Bulloo	Lake Eyre							
	Catchment Nogoa	Borders	Barwon	Castlereagh	Condamine	Darling	Gwydir	Lachlan	Macquarie	Murray	Murrumbidgee	Namoi	Paroo	Upper Murrumbidgee	Upper Gwydir	Upper Namoi	Upper Macquarie	Upper Lachlan	Bulloo	Barcoo	Coopers	Diamantina	Georgina	Neales	Thomson	Warburton	Total
n	15	32	24	24	24	23	21	18	15	19	19	23	23	20	23	24	24	24	21	23	34	24	24	16	25	28	590
Haplotype																											
1		29	23	24	23	20	18	14	14	19	17	22	23	20	23	23	21	23									356
2								1																			1
3					1	2									1		1										5
4																1											1
5			1				1																				2
6						1																					1
7		1							1		2					2											6
8	1																										1
9							1	3																			4
10							1					1															2
11		1																									1
12	1																										1
13	1	1																									1
14	8																										8
15	4																										4
16																			19	21	11		3		17	1	72
17																			1								1
18																			1								1
19																				2					3		5
20																						5		2			7
21																					20				3		23
22																					3				2		5
23																						19	21	14		27	81

Table 2 Hierarchical analysis of molecular variation (AMOVA) based on 380 bp of mitochondrial (mt)DNA control region haplotypes in *Macquaria ambigua* among river basins in eastern Australia.

Level of partitioning	Phi-value	*P*-value
Among basins	Φ_{CT} = 0.91	<0.01
Among populations within basins	Φ_{SC} = 0.50	<0.01
Within all populations	Φ_{ST} = 0.92	<0.01
Among populations in the MDB	Φ_{ST} = 0.01	0.14
Among populations in the LEB	Φ_{ST} = 0.71	<0.01

MDB, Murray–Darling Basin; LEB, Lake Eyre Basin.
Φ_{CT}, test statistic among basins; Φ_{SC}, test statistic among populations within basins; Φ_{ST}, test statistic among populations among basins.

0.001, P = 0.224) and LEB (raggedness index = 0.101, P = 0.304; SSD = 0.030, P = 0.138) were not significantly different from those expected under a model of historical population expansion (Fig. 4). Population expansion in the MDB was estimated to have occurred *c.* 220 ka (CI *c.* 36–310) during an interglacial phase (OIS 7) [*c.* 60 ka (CI *c.* 7–180) under the 10% Myr^{-1} clock]. Population expansion in the LEB occurred *c.* 170 ka (CI *c.* 20–500), during a glacial phase (OIS 6) [*c.* 80 ka (CI *c.* 13–110) under the 10% Myr^{-1} clock]. Restricted gene flow with isolation by distance was inferred as the significant process acting on the LEB and BULL haplotypes in clade 1-12. This inference was supported by our Mantel test, which indicated significant isolation by distance among the LEB populations (R^2 = 0.79, P = 0.023).

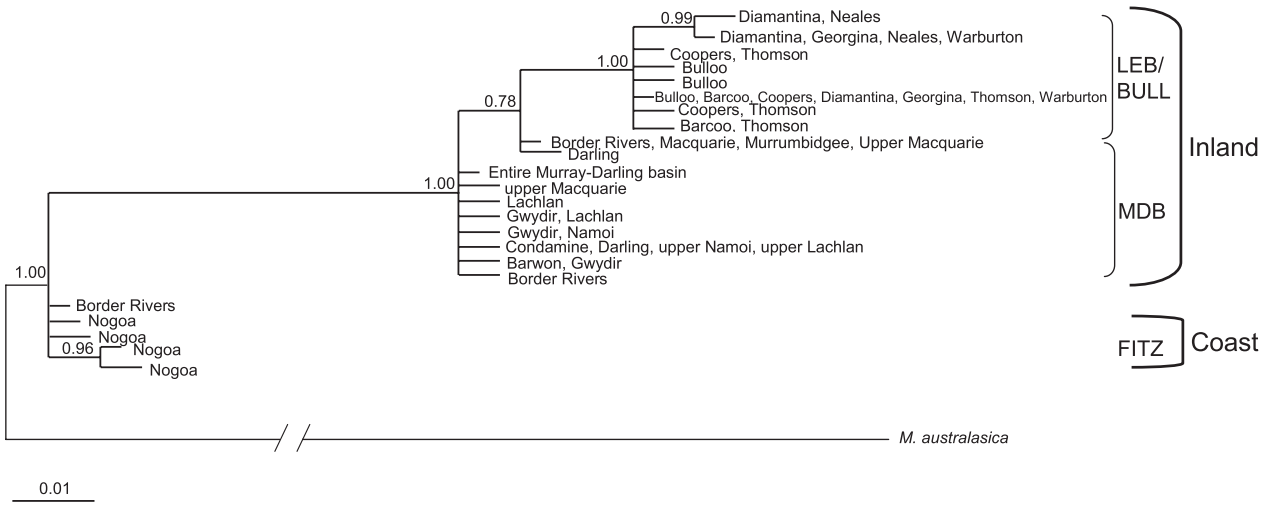

Figure 2 Phylogenetic reconstruction of *Macquaria ambigua* based on the 380-bp mitochondrial (mt)DNA control region. Labels on the nodes indicate Bayesian probabilities. The scale bar represents expected changes per site. The outgroup is *M. australasica*. Two clades are indicated: one from the Fitzroy basin (FITZ), and one from the Murray–Darling (MDB), Lake Eyre (LEB) and Bulloo (BULL) basins.

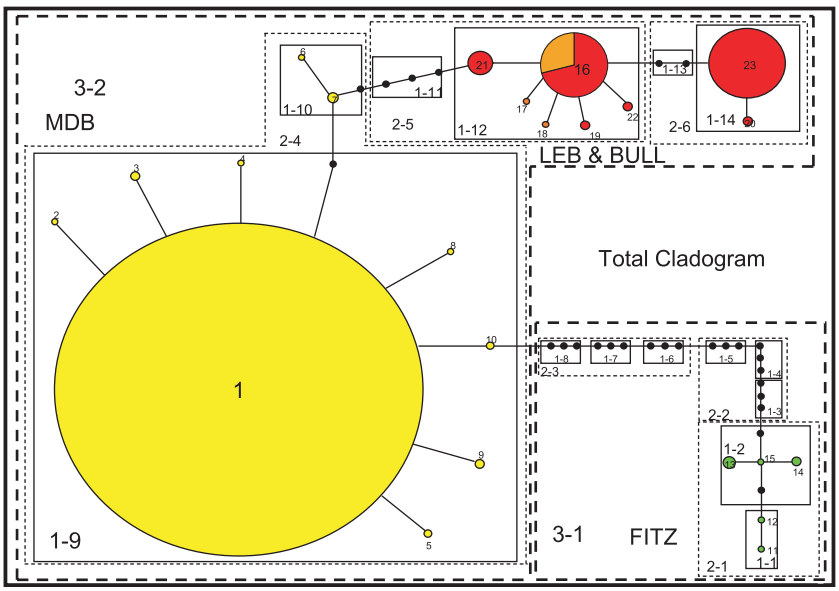

Figure 3 Network and nested clade design of the genealogical relationships in *Macquaria ambigua* based on 380-bp mitochondrial (mt)DNA control region haplotypes. The size of the circles indicates the frequency of that haplotype, lines joining haplotypes represent one base pair mutation and dots along the lines are missing haplotypes (not sampled or extinct). The minimum number of steps between the Fitzroy Basin (FITZ) lineages (haplotypes 11–15) and the remainder of the network is 19 (not supported by statistical parsimony). MDB, Murray–Darling Basin; LEB, Lake Eyre Basin; BULL, Bulloo Basin.

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

Significant level of cladogram	*P*-value	Inference chain	Outcome
Clade 1-12	0.031	1–2–3–4	RGF with IBD
Clade 1-14	0.015	1–2–11–12	Contiguous range expansion
Clade 3-2	<0.001	1–2–11–12	Contiguous range expansion LDC subs. F
Total cladogram	<0.001	1–19	Allopatric fragmentation

RGF, restricted gene flow; IBD, isolation by distance; LDC, long-distance colonization; F, fragmentation.

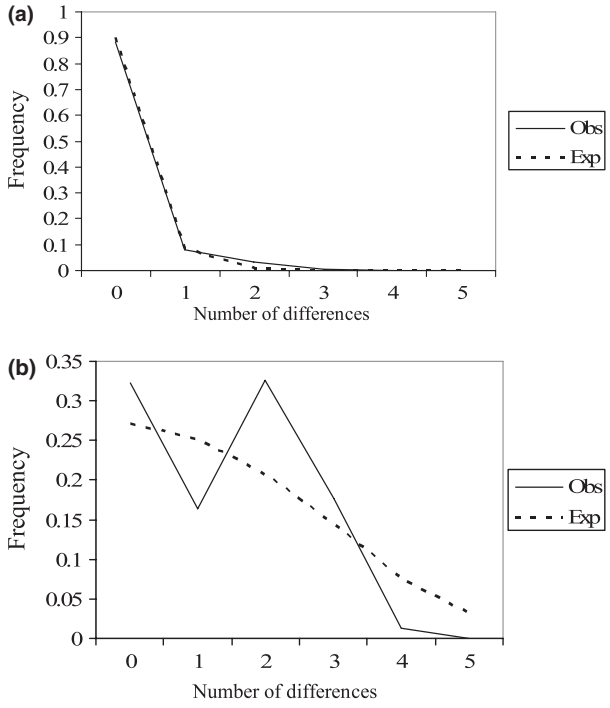

Figure 4 Mismatch distributions displaying the observed distribution (obs = full lines) and the distribution expected under a model of demographic population expansion (exp = dashed lines) in *Macquaria ambigua* populations: (a) Murray–Darling Basin (MDB), (b) Lake Eyre Basin (LEB).

DISCUSSION

We have found significant biogeographic structure in *M. ambigua*, with major phylogenetic lineages corresponding to the major drainage basins of the species' distribution. This has enabled us to infer relationships among the major drainage basins of the Australian arid zone and propose an alternative hypothesis for the evolutionary origin and subsequent expansion of the species' range. Our data indicate that major events in *M. ambigua*'s evolutionary history occurred during the Pleistocene, suggesting that conditions in the Australian arid zone were significantly different at that time, with heightened connectivity of freshwater environments, even across drainage divides.

Coastal origin and inland expansion – an alternative hypothesis

The high levels of divergence, basal position in the phylogenetic reconstruction and shape of the haplotype network all indicate that the ancestral lineages are those from the coastal FITZ. Therefore, we propose that *M. ambigua* originated on the coast. Further, a coastal origin of the species can be supported by: (1) recent findings of coastal origins in other Australian freshwater species (Thacker *et al.*, 2007; Faulks *et al.*, 2008; Jerry, 2008); (2) the presence of Tertiary age fossil fishes on the Queensland coast that resemble present-day Percichthyidae (Hills, 1934; Unmack, 2001); and (3) the current coastal distribution of the other Percichthyidae species *Macquaria colonorum*, *Macquaria novemaculata*, *Maccullochella* sp., *Guyu wujalwujalensis* (Allen *et al.*, 2002). Nonetheless, these findings contradict the hypothesis of Musyl & Keenan (1992), who suggested an inland origin for the species. This discrepancy could be due to differences in analytical treatment and the resolving power of the markers used. Musyl & Keenan (1992) used midpoint rooting to construct UPGMA (unpaired pair group method with arithmetic mean) and Wagner dendrograms based on allozyme distance matrices; this strategy tends to produce poor phylogenetic signal compared with phylogenetic reconstruction based on DNA sequences. In contrast, our phylogeny was constructed based on mtDNA sequences using a Bayesian framework with an evolutionary model appropriate for our data (Kimura, 1981) and it was rooted with the most closely related species known, *M. australasica*, as the outgroup. In addition, the distance measure (Nei's *D*; Nei, 1978) used in the allozyme study can be strongly influenced by the presence of fixed alleles (Musyl & Keenan, 1992). There were no fixed allozyme alleles observed between the MDB and FITZ populations, but there were several between the MDB and LEB populations. This resulted in a higher value of *D* between the MDB and LEB, compared with MDB and FITZ. These fixed differences in the LEB could be due to different selection pressures and recurrent bottlenecks in the more arid environmental conditions of that basin, resulting in a poor evolutionary signal in the allozyme data.

Evolutionary history

Following the establishment of *M. ambigua* populations on the coast, the species must have crossed the Great Dividing Range (GDR) in order to colonize the inland drainages. There is very strong evidence for colonization followed by population expansion in the inland basins, including low levels of genetic diversity associated with colonization events, star-like haplotype networks with little or no phylogeographic structure and NCPA inferences of contiguous range expansion. Our mismatch analyses also support this scenario, indicating that demographic

expansions within the inland basins occurred during the last 500 kyr. Although these expansion events occurred well after the divergence of the coastal and inland lineages (*c.* 1.5 Ma); they could represent the most recent demographic events that have masked the signal of any earlier expansions. This would also imply that *M. ambigua* has not experienced large demographic expansions in inland Australia since the onset of aridity in inland Australia *c.* 400 ka (Byrne *et al.*, 2008).

Several colonization paths among the basins are possible, e.g. FITZ → MDB → BULL → LEB; FITZ → simultaneous colonization of the MDB and LEB → BULL; or FITZ → LEB → MDB → BULL. The phylogenetic reconstruction indicates that the FITZ has a closer association with the MDB (5%) than the LEB (6%), suggesting the MDB was colonized first, followed by further divergence following colonization of the LEB. The contact area between the FITZ and MDB (*c.* 710 km) is much larger than that of the FITZ and LEB (*c.* 40 km) (Geoscience Australia, 1997) (Fig. 1) also favouring the MDB first colonization route. The divergence of lineages in these two basins was estimated at *c.* 1.5 Ma (600 ka–1.7 Ma), a period in Australia's history that was geologically relatively stable but had climatic conditions that were considerably moister than at present (Byrne *et al.*, 2008). The most probable modes of connectivity between the coastal and inland drainage basins of *M. ambigua*'s range are episodic tributary connections and continuous wet divides during moister climatic conditions (Craw *et al.*, 2007). In addition, dispersal among the inland basins could have been facilitated by terminal floodplain connections in the surrounding low-relief areas (Unmack, 2001). However, determining precise locations and mechanisms of dispersal across drainage divides requires further investigation, preferably using a multi-species comparative phylogeographic approach.

The MDB, LEB and BULL occupy a very large area (*c.* 2,000,000 km^2) of inland Australia and populations of *M. ambigua* may be over 4000 km apart, explaining the NCPA inferences for long-distance colonization and isolation by distance within the inland clades. Despite covering such an extensive and often arid area only three major groups were identified: MDB, eastern LEB/BULL (Bulloo, Barcoo, Cooper Ck, Thomson) and western LEB (Diamantina, Georgina, Neales, Warburton). Using the variable molecular clock the divergence of the MDB from the LEB/BULL was estimated to have occurred during a dry glacial phase (*c.* 58 ka) when connectivity among freshwater environments may have been limited. However, our alternative molecular clocks provide dates of divergence up to *c.* 280 ka in conjunction with both glacial and interglacial phases – this makes it difficult to discern the role of specific climate phases. Indeed, divergence dates for freshwater taxa distributed across the MDB and LEB/BULL are highly variable, from *c.* 73 ka in catfish to *c.* 1.8 Ma in mussels (reviewed in Hughes *et al.*, 2009). These dates suggest that in addition to variation in molecular clocks, there may be species-specific responses to changes in climate and environmental conditions and drainage divides may be breached by species at different times and places. In addition, our divergence dates are often much later than the dates of demographic expansion within the inland basins, suggesting the possibility of gene flow among basins prior to eventual isolation.

Interestingly, there was a very close genetic relationship between the LEB and BULL populations. Although nested between the LEB and MDB, the BULL has previously been shown to have a strong geographic and faunal association with the LEB (Unmack, 2001). We suggest that in the past the LEB and BULL have behaved as a single basin. That basin was colonized by *M. ambigua* from the MDB, possibly via the 'BULL'. Connectivity between the LEB and BULL may have been facilitated by episodic moist periods, right up until the extremely dry last glacial phase. The phylogeographic relationship among the MDB, LEB and BULL has been extensively investigated in the freshwater taxa of the region. In most species there is a much closer genetic affinity between populations from the LEB and BULL than the MDB and BULL (e.g. crayfish (Hughes & Hillyer, 2003), mussels (Hughes *et al.*, 2004), smelt and bony bream (Hughes & Hillyer, 2006) and prawns (Cook *et al.*, 2002; Carini & Hughes, 2004; Murphy & Austin, 2004).

Also of note is the isolation of *M. ambigua* populations in the western LEB catchments. A similar phylogeographic pattern has been observed in mussels (Hughes *et al.*, 2004), snails (Carini & Hughes, 2004, 2006) and prawns (Cook *et al.*, 2002; Murphy & Austin, 2004), but not in other species that may be considered to have a high dispersal potential (e.g. bony bream; Masci *et al.*, 2008). Under current climatic conditions these populations are isolated from the remainder of the LEB by large and often dry riverine distances and at best by the massive Lake Eyre itself. In summary, there are many similarities in the evolutionary history and biogeographic patterns within the freshwater taxa of the inland basins of Australia. Although dates of divergence can be variable and details of locations and mechanisms of drainage basin crossings require further investigation, evidence for the influence of climatic and environmental changes during the Pleistocene is strong and should be considered in the management of *M. ambigua* and other freshwater taxa of the Australian arid zone.

Taxonomic and management implications

It has been proposed that the LEB and MDB lineages be recognized as separate species and the FITZ and BULL lineages as subspecies (Musyl & Keenan, 1992). However, our data suggest that the FITZ/MDB lineage divergence is the most distinct, not the MDB/LEB and there is no evidence for the separation of the BULL lineage. Potential reasons for the discrepancies between the two datasets were discussed earlier. However, given that morphological differentiation of the FITZ lineages has also been observed (Musyl, 1990) we recommend that they be designated as a new species. The LEB and BULL lineages combined could then be designated as an evolutionarily significant unit (ESU; Moritz, 1994; Bernatchez, 1995; Crandall *et al.*, 2000). Although there is strong evidence based

on allozymes (Musyl & Keenan, 1992), mtDNA (this study) and microsatellite data (Faulks, 2010) for a taxonomic revision of *M. ambigua*, an investigation into the morphological differentiation of the LEB/BULL lineages and formal description of all the resulting taxa is still needed.

Because of the absence of matrilineal genetic structure within the MDB, *M. ambigua* could perhaps be managed at the basin scale in this system. However, given that significant population structure was detected in the LEB, we do not recommend the commencement of a broad-scale stocking programme in that basin. We are not able to provide advice on the management implications for the BULL or FITZ as our study incorporated only a single sampling locality from each. Additional studies for *M. ambigua* based on multi-locus nuclear markers (Faulks, 2010) are required to infer levels of variability and connectivity that can be used to provide management considerations at a finer, catchment-level scale.

To conclude, we highlight the significance of past climate change in shaping the evolutionary history of *M. ambigua*, and note the potential use of the phylogeographic framework to predict the response of species to future climate change scenarios. Reduced rainfall coupled with increased temperatures and evaporation will result in more widespread aridification of central Australia (Christensen *et al.*, 2007). These conditions will further isolate freshwater environments, and unless the frequency and magnitude of flood events are maintained, populations of *M. ambigua* will experience reduced gene flow. This may be of particular concern for the maintenance of genetic diversity and evolutionary potential in the already isolated and divergent populations from the Georgina, Diamantina and Neales catchments of the LEB.

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BIOSKETCH

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