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Current and historical patterns of drainage connectivity in eastern Australia inferred from population genetic structuring in a widespread freshwater fish *Pseudomugil signifer* (Pseudomugilidae)

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Abstract

Dispersal can play an important role in the genetic structuring of natural populations. In this regard, freshwater fishes often exhibit extensive population genetic subdivision and are ideal subjects for investigating current and historical patterns of connection and dissociation between drainages. We set out to generate a comprehensive molecular phylogeny for a widespread freshwater fish from eastern Australia, the Pacific blue-eye *Pseudomugil signifer*. Although movement via flood events may be important in the southern end of the species' range, genetic structuring revealed the importance of historical drainage connections and dissociations in mediating or disrupting dispersal. A dominant feature of our phylogeny is a split between northern and southern populations, which appears to be congruent with a biogeographical barrier recently implicated as important for the connectivity of freshwater organisms in eastern Australia. The extent of the split also has taxonomic implications consistent with suggestions that the Pacific blue-eye may represent more than a single species.

Keywords: ATP synthase six, biogeography, dispersal, mitochondrial DNA, phylogeny, river

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Introduction

Dispersal can play a pivotal role in the genetic structuring of natural populations (Slatkin 1987; Waters *et al*. 2000). Highly vagile species, unencumbered by physical barriers to dispersal, tend to show minimal phylogeographical structuring across wide areas (e.g. Chapco *et al*. 1992). In contrast, stronger genetic subdivision among populations is expected in situations in which dispersal is limited. This genetic structuring could be a reflection of life history as seen, for example, in certain insects where the primary dispersal mechanism may be restricted to a particular stage of their life cycle (Schultheis *et al*. 2002). The physical nature of the environment may also be important. Unsuitable or impenetrable habitat may encumber movement and result in population subdivision as demonstrated in several species of rainforest-restricted birds and reptiles (Joseph *et al*. 1995; Schneider *et al*. 1998).

For many freshwater fishes, both the terrestrial environment and the sea represent formidable barriers to dispersal between drainages. Freshwater species tend to exhibit higher levels of genetic structuring than those inhabiting estuarine or marine environments (Gyllensten 1985; Ward *et al*. 1994). In some cases, freshwater species that display saltwater tolerance may be capable of marine dispersal and therefore show lower levels of genetic structuring, as in New Zealand populations of the common jollytail *Galaxias maculatus* (Waters *et al*. 2000). However, in general, movement between drainages by freshwater species is dependent upon the connectivity of fresh water (Unmack 2001). Connections between drainages may occur over geological time through changes in sea level (e.g. Bermingham &

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Avise 1986; McGuigan *et al*. 2000) or rearrangement of drainages and river courses (Rowland 1993; Hurwood & Hughes 1998; McGlashan & Hughes 2000). Over shorter temporal scales, floods may also connect waterways by inundating low divides (Jerry & Woodland 1997) or releasing traversable plumes of freshwater into the ocean (Grimes & Kingsford 1996; Pusey & Kennard 1996). The relative importance of these processes can leave distinct phylogenetic 'signatures' useful in reconciling species' biology with population connectivity and earth history (McGlashan & Hughes 2000; Waters *et al*. 2000). We attempt such an enquiry using, as a model system, a widespread species of Australian freshwater fish.

The Pacific blue-eye *Pseudomugil signifer* Kner is a small, colourful freshwater fish found in coastal drainages across much of eastern Australia. The species is one of the most readily identifiable and ubiquitous members of the Australasian ichthyofauna, occurring in a range of habitats from rainforest streams to swamps, tidal mangrove creeks, estuaries and offshore marine islands (Allen *et al*. 2002). The widespread distribution of *P. signifer*, and its tolerance to saline conditions, suggest that the species may have a high proclivity for dispersal across the landscape (Unmack 2001), a notion supported by apparent clinal variation along the species' range (Hadfield *et al*. 1979). This, however, is inconsistent with recent molecular work indicating extensive genetic subdivision among populations (McGlashan & Hughes 2002).

Apparent evidence of extensive genetic subdivision in a widespread species also makes *P. signifer* an ideal study subject for improving our understanding of the biogeography of Australia's freshwater fishes. Australia provides a unique situation for investigating freshwater biogeography. Unmack (2001) has recently provided, using distributional and geological information, an unprecedented suite of testable predictions concerning the distribution of freshwater fishes in Australia. In the context of the Pacific blue-eye, one of the most striking patterns observed in a recent mitochondrial DNA (mtDNA) study was the identification of two major genetic groupings (McGlashan & Hughes 2002). A possible dichotomy between northern and southern populations of *P. signifer* may provide important insights into an apparent biogeographical barrier disrupting the connectivity of freshwater organisms in eastern Australia (Pusey *et al*. 1998; Unmack 2001; McGlashan & Hughes 2002). The 'Burdekin Gap', a dry corridor in mid coastal Queensland, has long been identified as a biogeographical barrier in terrestrial systems (Keast 1961; Simpson 1961; Cracraft 1986). However, it has only recently been implicated as important in delimiting the range of several Australian fish groups as the boundary between two aquatic biogeographical provinces (northern Australia and eastern Australia) (Unmack 2001). The genetic divergence between northern and southern blue-eyes identified by McGlashan & Hughes (2002) are consistent with the Burdekin Gap. However, the large geographical gap in sampling between the northern and southern clades (650 km coastline distance) needed to be filled in order to more accurately identify where the break in *P. signifer* occurs.

Determining the phylogenetic relationships among populations of the Pacific blue-eye also has important conservation implications. Molecular techniques are increasingly being used to investigate taxonomic and conservation issues in a range of taxa including fish (McCusker *et al*. 2000). In the case of the Pacific blue-eye, considerable taxonomic uncertainty has centred on whether *P. signifer* may, in fact, represent a complex of species (reviewed in Saeed *et al*. 1989). Although it is currently recognized as a single taxon, recent mtDNA sequencing work suggests that more detailed investigation is warranted (McGlashan & Hughes 2002). Taxonomic uncertainty also plagues the closely related *P. mellis* (Allen & Ivantsoff 1982). Previously considered to be just a variety of *P. signifer*, *P. mellis* is now recognized as a separate species listed as endangered by the IUCN (Allen *et al*. 2002). Its taxonomic status, however, may be equivocal and its relationship with *P. signifer* warrants further investigation particularly in light of its current conservation status (W. Ivantsoff, personal communication).

Here we generate a comprehensive molecular phylogeny of *P. signifer* from throughout the species' range. We use the resulting patterns of genetic subdivision to infer historical and current patterns of connectivity between drainages in eastern Australia. In particular, we determine whether the major genetic subdivision identified by McGlashan & Hughes (2002) is, indeed, concordant with the Burdekin Gap in light of its possible importance in the biogeography of Australia's freshwater fishes (Unmack 2001). The inclusion of samples of *P. signifer* from throughout its range, as well as samples of *P. mellis*, also allows us to explore taxonomic relationships among the putative taxa.

Materials and methods

Samples

For our molecular phylogeny of *Pseudomugil signifer*, we amassed a comprehensive data set of 56 samples from 25 populations across the species' range (Table 1). In particular, we focused on closing the 650 km geographical sampling gap in the study of McGlashan & Hughes (2002) and extending the sampling to include more populations from the southern end of the species' range. Additional samples from two other drainages were obtained from the Australian Museum. We also obtained tissue samples of *P. mellis* from two populations. Samples of two other species (*P. tenellus* and *P. gertrudae*) were used as outgroups.

Table 1 Summary of specimens sampled, their locality information and GenBank Accession nos. Sampling sites, other than the main drainage channel, are given in brackets

No.	Species	Drainage	Tissue code	Accession no.	Sample no.
1	*P. signifer*	Cape Melville	MEL1.Q1	AF272096	1
2		Cape Melville	MEL2.Q1	AF272096	2
3		Low Isles	LOW1.Q2.5	AF272099	1
4		Low Isles	LOW2.Q2.5	AF272099	2
5		Daintree (Stewart Ck)	STW5.Q2	AF272097	1
6		Daintree (Stewart Ck)	STW6.Q2	AF272098	2
7		Mossman (South Mossman R)	MOS1.Q3	AY305876	1
8		Mossman (South Mossman R)	MOS2.Q3	AY305877	2
9		Barron (Stoney Ck)	STB7.Q4	AF272100	1
10		Barron (Stoney Ck)	STB9.Q4	AF272100	2
11		Trinity Inlet (Middle Ck)	MDL4.Q5	AF272101	1
12		Trinity Inlet (Middle Ck)	MDL5.Q5	AF272101	2
13		Mulgrave-Russell (Mulgrave R)	MUL9.Q5	AY305878	1
14		Mulgrave-Russell (Mulgrave R)	MUL10.Q5	AY305879	2
15		Mulgrave-Russell (No Name Ck)	LMB54.Q5	AF272102	1
16		Mulgrave-Russell (No Name Ck)	LMB56.Q5	AF272103	2
17		Mulgrave-Russell (Behana Ck)	BHB2.Q5	AF272101	1
18		Mulgrave-Russell (Behana Ck)	BHB4.Q5	AF272104	2
19		Mulgrave-Russell (Woopen Ck)	WCT43.Q5	AF272101	1
20		Mulgrave-Russell (Woopen Ck)	WCT49.Q5	AF272105	2
21		Johnstone (Culla Ck)	CLC34.Q6	AF272106	1
22		Johnstone (Culla Ck)	CLC35.Q6	AF272106	2
23		Johnstone (Tregonthanaan Ck)	TRE8.Q6	AY305880	1
24		Johnstone (South Johnstone R)	SJR148.Q6	AF272107	1
25		Johnstone (South Johnstone R)	SJR149.Q6	AF272107	2
26		Johnstone (Miskin Ck)	MSC67.Q6	AF272108	1
27		Johnstone (Miskin Ck)	MSC68.Q6	AF272109	2
28		Tully (Banyan Ck)	BYC23.Q7	AF272110	1
29		Tully (Banyan Ck)	BYC24.Q7	AF272111	2
30		Herbert (Stone R)	STR3.Q8	AY305881	1
31		Herbert (Stone R)	STR5.Q8	AF272112	2
32		Herbert (Stone R)	STR21.Q8	AF272113	3
33		Ross (Ross Ck)	ROS6.Q9	AY305882	1
34		Don	DON11.Q10	AY305893	1
35		Cedar Falls (No name Ck)	PRO5.Q11	AF272114	1
36		Pioneer (Cattle Ck)	CTC9.Q12	AF272115	1
37		Pioneer (Cattle Ck)	CTC15.Q12	AY305883	2
38		Pioneer (Cattle Ck)	CTC11.Q12	AF272116	3
39		Calliope	CAL14.Q13	AY305884	1
40		Kolan (Gin Gin Ck)	GIN12.Q14	AY305885	1
41		Burnett	BUR6.Q15	AF272117	1
42		Burnett	BUR9.Q15	AF272118	2
43		Burnett	BUR15.Q15	AY334549	3
44		Mary (Amamoor Ck)	AMA5.Q16	AF272119	1
45		Mary (Amamoor Ck)	AMA18.Q16	AF272120	2
46		Pine (North Pine R)	NPR21.Q17	AY305886	1
47		Pine (North Pine R)	NPR22.Q17	AY305887	2
48		Pine (North Pine R)	NPR23.Q17	AY305888	3
49		Clarence (Orara R)	GRA25.N18	AF272121	1
50		Clarence (Orara R)	GRA30.N18	AF272121	2
51		Manning (Dingo Ck)	TAR35.N19	AF272122	1
52		Manning (Dingo Ck)	TAR36.N19	AF272123	2
53		Myall Lake	MYA31.N20	AY305889	1
54		Wamberal Lagoon	WAM19.N21	AY305890	1
55		Willinga Lake	WIL16.N22	AY305891	1
56		Clyde (Nelligen Ck)	NCK18.N23	AY305892	1

Table 1 *Continued*

No.	Species	Drainage	Tissue code	Accession no.	Sample no.
57	*P. mellis*	Tin Can Bay	P.mell.24	AY305894	1
58		Tin Can Bay	P.mell.25	AY305895	2
59		Tin Can Bay	P.mell.26	AY305896	3
60		Fraser Island	P.mell.FI	AY334550	1
61	*P. tenellus*	Adelaide (Marrakai Plains)	P.tene.27	AY305897	1
62		Adelaide (Marrakai Plains)	P.tene.28	AY305898	2
63	*P. gertrudae*	Blyth (Cadell R)	P.gert.29	AY305899	1
64		Blyth (Cadell R)	P.gert.30	AY305900	2

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from caudal muscle tissue using a modified CTAB protocol. For each sample we targeted a 633-bp DNA fragment of the ATP synthase six mitochondrial gene. This fragment was targeted by McGlashan & Hughes (2002) in their study on *P. signifer*. The results of their work, and that done at comparable taxonomic levels in other freshwater fishes, indicate useful levels of variability (Bermingham *et al*. 1997; Bermingham & Martin 1998; Hurwood & Hughes 1998; McGlashan & Hughes 2000). As in McGlashan & Hughes (2002), samples were polymerase chain reaction (PCR) amplified using the primers ATP8.2L and COIII.2. Sequencing was then carried out using the internal primers PS3L and PSH (McGlashan & Hughes 2002). PCRs were 40 μL in volume and contained ~100 ng template DNA, 4 μL 10× reaction buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 10 pmol of each amplification primer and 1 unit of platinum *Taq*-polymerase (GibcoBRL, Life Technologies). Each reaction, contained in an individual PCR tube, was overlaid with 10 μL of mineral oil. PCR amplification of double-stranded product was completed using a Corbet PC-960C cooled thermal cycler using a step-down cycling profile. Reactions were run at 94 °C for 5 min, 60 °C for 20 s and 72 °C for 1.5 min for two cycles. This pattern was repeated for four more cycles with the annealing temperature decreasing by 5 °C each cycle. The next 35 cycles were run with the annealing temperature at 40 °C. A final extension step at 72 °C was carried out for 4 min.

PCR products were gel purified using the UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories Inc.) following the manufacturer's instructions. After purification, products were sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amershan Pharmacia Biotech). Sequencing reactions were performed using 4 μL of ET Terminator Sequencing Mix and 2 pmol of each sequencing primer. We used ~10–15 ng of purified PCR product as template. Cycle sequencing was performed using the following profile for 50 cycles: 95 °C for 30 s, 44 °C for 20 s, 60 °C for 80 s. Ramping was set for 1 °C/ s. Reactions were brought to 4 °C on completion of cycle 50. We then removed extension products from under the oil and placed them in 1.5 mL tubes. The volume was then brought to 20 μL with deionized water. Dried extension products were resuspended in 1.5 μL of formamide loading dye. Sequences were electrophoresed on 5.2% denaturing polyacrylamide (Thermo-PAGE™, Amresco) gels (36 cm well-to-read) and analysed on a ABI377XL™ automated DNA sequencer. Sequence data was edited using SEQUENCHER v. 3.0 (Genes Code Corp.).

Phylogenetic analysis and hypothesis testing

Sequences were aligned using CLUSTALX (Thompson *et al*. 1997) and the alignment was refined by eye. We used maximum-likelihood (ML) and Bayesian approaches to analyse the data. We used the objective criteria provided by the computer program MODELTEST v. 3.06 (Posada & Crandall 1998) with both the Hierarchical Likelihood Ratio Test (hLRT) and the Akaike Information Criterion (AIC) to select the most appropriate model of molecular evolution for our data. We used the MODELTEST estimates of the empirical nucleotide frequencies, substitution rates, gamma distribution, and proportion of invariant sites in our ML analyses implemented in PAUP* (Swofford 2002).

We used the computer program MRBAYES (v. 3.0b4; Huelsenbeck & Ronquist 2001) for our Bayesian analyses. Using the identical data set as our ML analyses, the General Time Reversable (GTR) + gamma distribution + proportion of invariant sites parameters were all estimated from the data during the run. We used the default value of four Markov chains per run and also ran the full analysis five times to make sure overall tree-space was well sampled and to avoid getting trapped in local optima. We ran each analysis for a total of 500 000 generations and sampled the chain every 100 generations, resulting in 5000 sampled trees. Log-likelihood values reached a plateau after ~50 000 generations (500 sampled trees), so we discarded the first 500 trees as the burn-in phase and used the last 4500 trees to estimate Bayesian posterior probabilities.

We used the bootstrap and Bayesian posterior probabilities to assess branch support. Our data set was too large to

perform ML bootstraps, so we performed a weighted parsimony bootstrap using the observed ti/tv ratio with 1000 pseudoreplicates. In addition to this, Bayesian analysis provided posterior probabilities for branches. The use of posterior probabilities to access branch support is still rather new (Holder & Lewis 2003) and some questions have been raised with regard to how they compare to bootstrap values (Suzuki *et al*. 2002; Alfaro *et al*. 2003; Douady *et al*. 2003). Nonetheless, they serve as an additional source of information on branch support and may represent a better estimate of phylogenetic accuracy (Wilcox *et al*. 2002; Reeder 2003). As a rough guide, we consider branches supported by bootstrap values ≥ 70% (Hillis & Bull 1993) and posterior probability values ≥ 95% (Wilcox *et al*. 2002) to be significantly supported by our data.

We tested the significance of log-likelihood differences between our optimal ML/Basyesian tree (using the ML –ln L) and topologies representing various alternative hypotheses with the Shimodaira–Hasegawa test in PAUP* (Shimodaira & Hasegawa 1999; see also Goldman *et al*. 2000) using full optimization and 1000 replicates.

Results

With all taxa and individuals included, the edited alignment comprised 633 bp, of which 289 were variable. Of these, 276 were informative under parsimony. Excluding the outgroups, 184 sites were variable and 167 informative under parsimony. For comparison with other studies, we present Jukes-Cantor (1969) genetic distances among taxa in Table 2.

Both the hLRT and the AIC from MODELTEST supported the general time reversible (GTR) plus invariant sites (+ I) plus gamma shape (+ G) model as the best-fit substitution model. The estimated parameters were as follows: nucleotide frequencies (A = 0.25380 C = 0.32660 G = 0.11840 T = 0.3012); substitution rates A↔C 1.000, A↔G 11.9115, A↔T 1.000, C↔G 1.000, C↔T 4.8324, G↔T 1.0000; proportion of invariant sites = 0.4630; gamma shape parameter = 2.1517. The Bayesian analysis produced very similar parameter estimates to those of MODELTEST. The ML analysis in PAUP* using the above parameters and the Bayesian analysis both yielded the same optimal tree (ML –ln L = 3797.54280 in the ML analysis; Fig. 1).

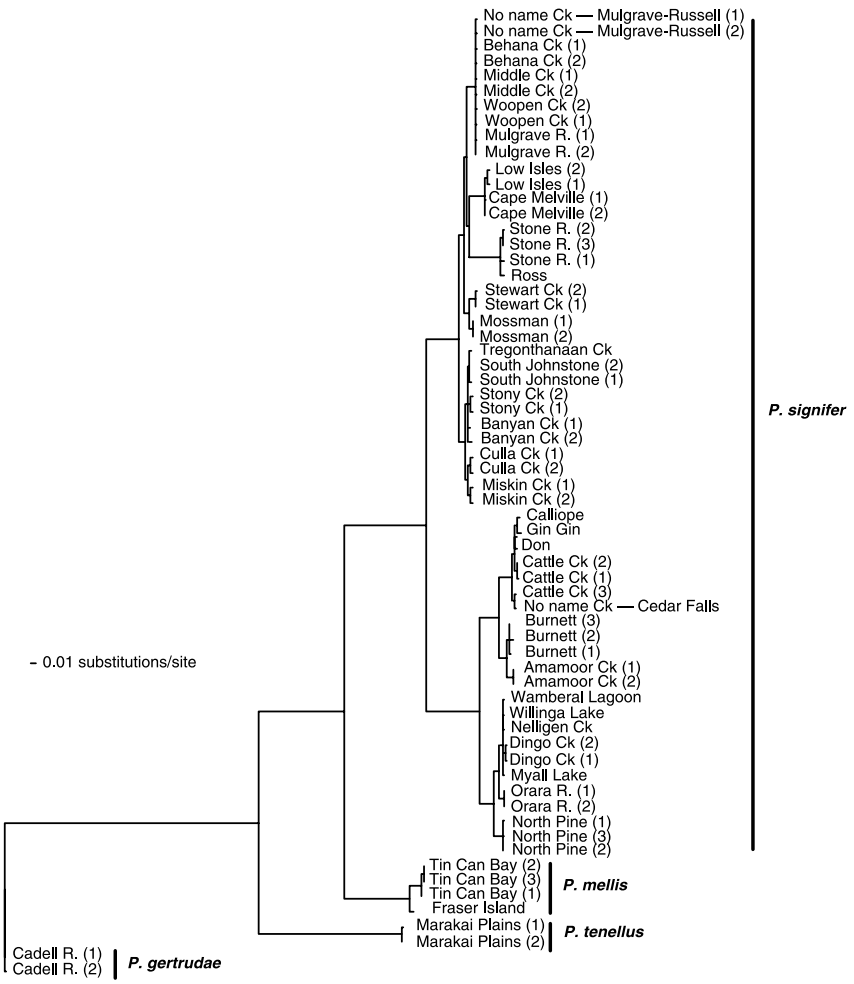

Fig. 1 Phylogram for *Pseudomugil signifer* based on 633 bp of the ATP synthase six mitochondrial gene.

Table 2 Pair-wise Jukes-Cantor (1969) genetic distances between representative samples of *Pseudomugil signifer* from each of the 25 drainages

		OG	*OG*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
OG	P.gert.29	—													
OG	P.mell.FI	0.3327	—												
1	Ps.MEL1.q1	0.3229	0.2228	—											
2	Ps.STW6.q2	0.3302	0.2122	0.0523	—										
3	Ps.LOW2.q2.5	0.3180	0.2207	0.0032	0.0523	—									
4	Ps.MOS1.q3	0.3253	0.2122	0.0422	0.0176	0.0456	—								
5	Ps.STB9.q4	0.3108	0.2185	0.0523	0.0456	0.0557	0.0406	—							
6	Ps.LMB54.q5	0.3326	0.2164	0.0456	0.0372	0.0490	0.0356	0.0456	—						
7	Ps.TRE8.q6	0.3180	0.2164	0.0507	0.0439	0.0540	0.0389	0.0079	0.0439	—					
8	Ps.CLC35.q6	0.3253	0.2164	0.0523	0.0456	0.0557	0.0406	0.0128	0.0456	0.0111	—				
9	Ps.BYC23.q7	0.3156	0.2143	0.0473	0.0406	0.0507	0.0356	0.0048	0.0406	0.0032	0.0079	—			
10	Ps.STR5.q8	0.3277	0.2185	0.0799	0.0730	0.0782	0.0747	0.0870	0.0695	0.0852	0.0835	0.0817	—		
11	Ps.ROS6.q9	0.3351	0.2207	0.0817	0.0764	0.0799	0.0747	0.0888	0.0730	0.0870	0.0853	0.0835	0.0095	—	
12	Ps.DON11.q10	0.3401	0.2356	0.1734	0.1655	0.1754	0.1518	0.1499	0.1616	0.1499	0.1557	0.1518	0.1694	0.1714	—
13	Ps.PRO5.q11	0.3476	0.2356	0.1794	0.1675	0.1774	0.1577	0.1557	0.1675	0.1557	0.1596	0.1577	0.1734	0.1754	0.0128
14	Ps.CTC15.q12	0.3426	0.2270	0.1754	0.1635	0.1734	0.1538	0.1518	0.1635	0.1518	0.1577	0.1538	0.1694	0.1714	0.0095
15	Ps.CAL14.q13	0.3501	0.2313	0.1734	0.1655	0.1754	0.1518	0.1538	0.1616	0.1499	0.1557	0.1518	0.1714	0.1734	0.0111
16	Ps.GIN12.q14	0.3501	0.2335	0.1794	0.1714	0.1814	0.1577	0.1596	0.1675	0.1557	0.1616	0.1577	0.1714	0.1774	0.0144
17	Ps.BUR15.q15	0.3401	0.2378	0.1754	0.1596	0.1734	0.1499	0.1557	0.1655	0.1557	0.1538	0.1577	0.1635	0.1655	0.0439
18	Ps.AMA5.q16	0.3451	0.2378	0.1774	0.1616	0.1754	0.1518	0.1557	0.1675	0.1557	0.1538	0.1577	0.1694	0.1714	0.0523
19	Ps.NPR21.q17	0.3612	0.2335	0.1700	0.1719	0.1720	0.1579	0.1638	0.1658	0.1598	0.1597	0.1618	0.1781	0.1803	0.0892
20	Ps.GRA25.n18	0.3628	0.2378	0.1557	0.1557	0.1577	0.1441	0.1460	0.1499	0.1422	0.1441	0.1441	0.1655	0.1694	0.0888
21	Ps.TAR36.n19	0.3577	0.2356	0.1596	0.1596	0.1616	0.1480	0.1538	0.1518	0.1499	0.1518	0.1518	0.1694	0.1754	0.0835
22	Ps.MYA31.n20	0.3501	0.2356	0.1596	0.1596	0.1616	0.1480	0.1538	0.1518	0.1499	0.1518	0.1518	0.1694	0.1754	0.0817
23	Ps.WAM19.n21	0.3551	0.2400	0.1557	0.1557	0.1577	0.1441	0.1499	0.1480	0.1460	0.1480	0.1480	0.1655	0.1714	0.0852
24	Ps.WIL16.n22	0.3526	0.2378	0.1577	0.1577	0.1596	0.1460	0.1518	0.1499	0.1480	0.1499	0.1499	0.1675	0.1734	0.0835
25	Ps.NCK18.n23	0.3551	0.2399	0.1577	0.1596	0.1596	0.1441	0.1538	0.1518	0.1499	0.1518	0.1518	0.1694	0.1714	0.0852

		13	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*
OG	P.gert.29													
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5	Ps.STB9.q4													
6	Ps.LMB54.q5													
7	Ps.TRE8.q6													
8	Ps.CLC35.q6													
9	Ps.BYC23.q7													
10	Ps.STR5.q8													
11	Ps.ROS6.q9													
12	Ps.DON11.q10													
13	Ps.PRO5.q11	—												
14	Ps.CTC15.q12	0.0095	—											
15	Ps.CAL14.q13	0.0144	0.0111	—										
16	Ps.GIN12.q14	0.0176	0.0144	0.0128	—									
17	Ps.BUR15.q15	0.0406	0.0422	0.0473	0.0507	—								
18	Ps.AMA5.q16	0.0490	0.0507	0.0557	0.0591	0.0144	—							
19	Ps.NPR21.q17	0.0892	0.0947	0.0929	0.0910	0.0819	0.0873	—						
20	Ps.GRA25.n18	0.0906	0.0924	0.0906	0.0924	0.0852	0.0870	0.0329	—					
21	Ps.TAR36.n19	0.0870	0.0870	0.0852	0.0870	0.0817	0.0870	0.0347	0.0176	—				
22	Ps.MYA31.n20	0.0835	0.0852	0.0835	0.0852	0.0782	0.0835	0.0330	0.0144	0.0095	—			
23	Ps.WAM19.n21	0.0870	0.0888	0.0870	0.0888	0.0817	0.0870	0.0330	0.0144	0.0095	0.0064	—		
24	Ps.WIL16.n22	0.0852	0.0870	0.0852	0.0870	0.0799	0.0852	0.0313	0.0128	0.0079	0.0048	0.0016	—	
25	Ps.NCK18.n23	0.0870	0.0888	0.0870	0.0888	0.0817	0.0870	0.0314	0.0144	0.0096	0.0064	0.0032	0.0016	—

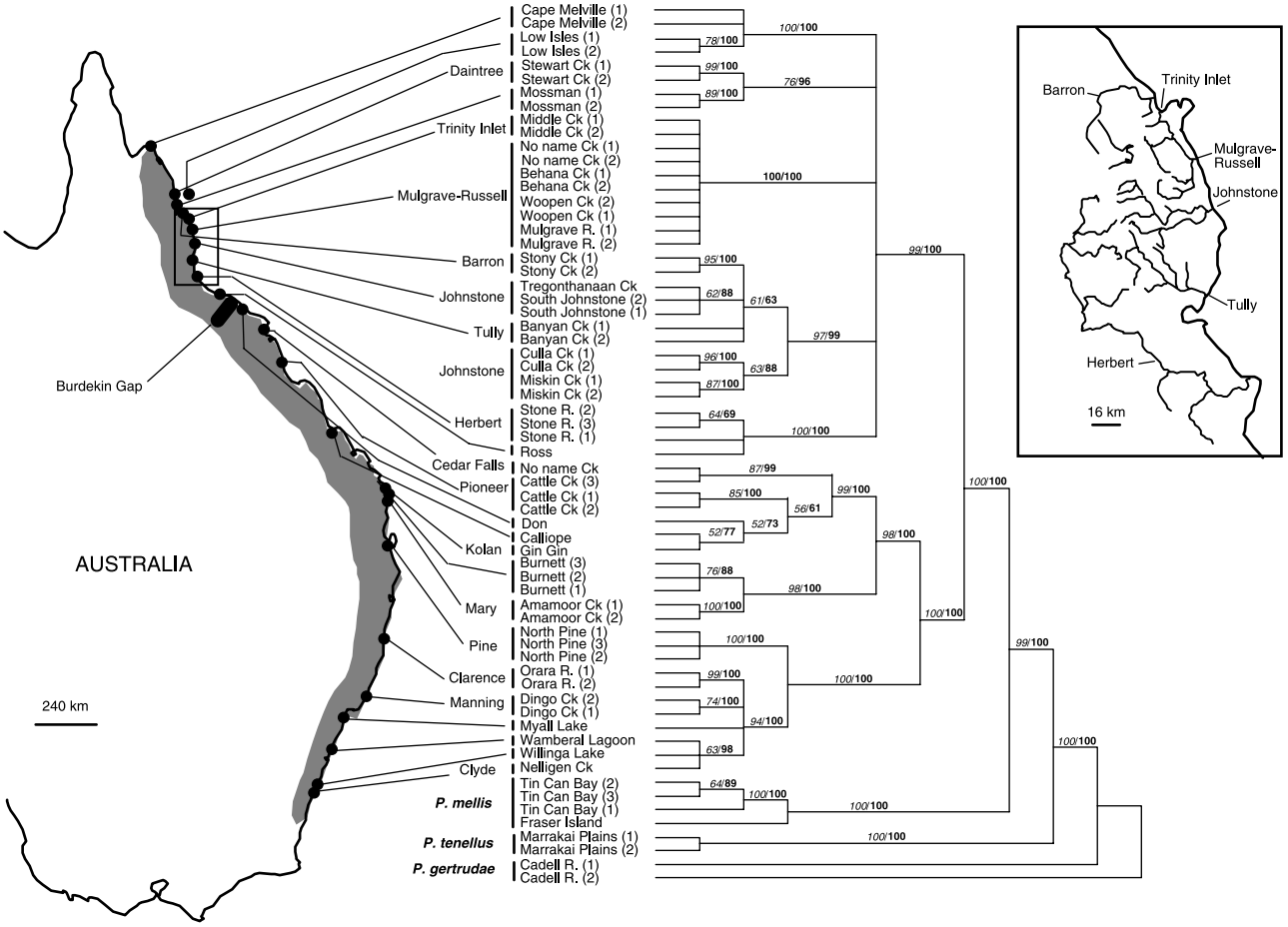

Fig. 2 Conservative phylogenetic tree only showing branches that were supported by both bootstrap values and Bayesian posterior probabilites. Parsimony bootstrap values are shown in italics and Bayesian posterior probabilities are shown in bold. Shading indicates species distribution.

Our molecular phylogeny revealed that *Pseudomugil signifer* forms two distinct genetic clades supported by a bootstrap value of 100%. Haplotypes north of, and including, Ross Creek formed a clade separate to those south of, and including, the Don River (Fig. 2). The genetic distances separating populations between the two clades ranged from 14.41 to 17.94%. The split between northern and southern populations appears to be consistent with the terrestrial Burdekin Gap (Fig. 2). The geographical location of the genetic split is supported by the rejection of an alternative topology grouping fish from the Don River, located just south of the Burdekin Gap, with those from the Ross and Herbert river drainages to the north (–ln L 236.92780 units greater, $P < 0.001$).

Our molecular phylogeny revealed five minor clades among populations of northern Pacific blue-eyes (Fig. 2). All five clades were supported by high bootstrap values and high posterior probabilities. One clade, comprising fish from the Ross and Herbert river drainages, exhibits high levels of divergence from the other northern populations. The relationships among the remaining clades are poorly resolved and are characterized by short internodes and long tips. One of these clades comprises fish from the Johnstone, Barron and Tully rivers. The results suggest that Johnstone River fish may be paraphyletic (Fig. 2). However, the branch lengths are short and so we cannot reject the alternative hypothesis that they may, in fact, be monophyletic (–ln L 1.89420 greater, $P = 0.890$). Furthermore, although the mouth of the Barron is only 10 km from the mouth of Trinity Inlet, fish from the latter do not group with those from the Barron. Instead, Trinity Inlet fish form a separate clade with those from the Mulgrave-Russell the mouth of which is some 50 km to the south. Alternative topologies grouping either Trinity Inlet or Mulgrave River fish with the Barron–Johnstone–Tully clade were significantly longer than the shortest tree, lending support to the phylogenetic pattern observed (–ln L 39.74140 greater, $P = 0.031$). Daintree and Mossman fish formed a clade separate from the geographically proximate offshore population at Low Isles. Indeed, fish from Low Isles appear to be more

closely related to fish from the more distant Cape Melville further to the north. An alternative topology, grouping Low Isles fish with those from the geographically proximate Mossman and Daintree drainages, was rejected because the alternative tree was significantly longer than the shortest tree (–ln L 84.4551 units greater, $P < 0.001$).

Our results indicate that southern populations of Pacific blue-eye comprise four minor clades (Fig. 2). One clade contains fish from Don, Cedar Falls, Pioneer, Calliope and Gin Gin. Within this clade, fish from the most northerly drainage, the Don River, grouped with geographically more distant southerly populations (Calliope and Gin Gin) instead of those that were geographically more proximate (Cedar Falls and Pioneer). The branch lengths among haplotypes, however, were short. Fish from the Burnett and Mary drainages in southeastern Queensland grouped together with a bootstrap value of 98%. Samples from the Pine River, supported by a bootstrap of 100%, form a sister group to a clade that contains the remaining populations from the southern most distribution of the species' range.

Populations of the closely related *P. mellis* form a sister group to *P. signifer* (Fig. 2). The two taxa are separated by a genetic distance of 21.12–24.00%.

Discussion

Levels of drainage connectivity and the influence of historical processes can leave important signatures in the phylogeographical structuring of freshwater fishes. In this regard, three scenarios are possible (McGlashan & Hughes 2001): (i) negligible gene flow among drainages over long periods resulting in strong genetic structuring; (ii) some gene flow among drainages mediated by events such as freshwater plumes that connect closely adjacent drainages; (iii) extensive gene flow reflecting a high dispersal potential that can overcome the isolation imposed by drainage structure. The current data provided a test of these different scenarios over the entire distributional range of a widespread freshwater species from eastern Australia, the Pacific blue-eye, *Pseudomugil signifer*.

P. signifer is expected to have a high proclivity for dispersal (Unmack 2001; McGlashan & Hughes 2002). The species appears to be tolerant of a wide range of salinity levels and is found in habitats as diverse as freshwater streams, tidal creeks, coastal lagoons and marine offshore islands (Allen *et al*. 2002). It is conceivable, therefore, that fish may be capable of moving between adjacent drainages during flood events. This may, indeed, be the case along the southern end of the species' range based on the genetic similarities revealed in our phylogeny among southern populations. The flood plumes of many rivers in this region frequently merge during pulse events (Grimes & Kingsford 1996). These plumes may be important in facilitating contemporary movement of fish between neighbouring drainages in southeastern Australia as suggested for another species, *Macquaria novemaculeata* (Jerry & Cairns 1998). In contrast, population structuring among the northern grouping suggests that movement between neighbouring rivers during flood events may, in fact, be limited in the northern end of the species' range. For example, fish from Johnstone and Mulgrave-Russell were found to be genetically divergent despite occurring in adjacent drainages.

The genetic structuring evident in our molecular phylogeny reflects some important historical connections and dissociations. A dominant feature of the phylogeny is the split between northern and southern populations of *P. signifer*. Specifically, we found an extensive genetic break between the Ross River near Townsville and the Don River near Bowen. The mouths of the two rivers are separated by a distance of ~200 km. This phylogenetic break is geographically congruent with the terrestrial Burdekin Gap, a dry corridor that dates back to before the Pliocene (Joseph *et al*. 1993; Joseph & Moritz 1994). The importance of the terrestrial Burdekin Gap as a biogeographical barrier has been demonstrated in phylogeographical studies of several terrestrial vertebrate taxa including frogs (James & Moritz 2000; Schauble & Moritz 2001) and birds (Joseph & Moritz 1994).

It has recently been suggested that the Burdekin Gap may also be important for the connectivity of freshwater organisms. For example, the area marks the southern-most limit in the range of at least 13 freshwater fish species from northern Australia, the greatest difference in species ranges between adjacent regions anywhere around the coastline of Australia (Unmack 2001). Indeed, in a comprehensive synthesis on the biogeography of Australia's freshwater fishes, Unmack (2001) proposed that areas north and south of the Burdekin River, which flows through the dry corridor, represented two separate biogeographical regions. McGlashan & Hughes (2002) hypothesized that the Burdekin Gap probably also played an important role in the long-term isolation of northern and southern groupings of *P. signifer*. They were, however, cautious in their interpretation due to a large geographical gap in their sampling, which spanned a coastal distance of some 650 km between the two major clades in their data set. We were unable to obtain samples from the Burdekin River itself but, in closing the sampling gap of McGlashan & Hughes (2002), it appears that the phylogeographical break observed in our results is at least geographically consistent with the terrestrial Burdekin Gap.

Other historical dissociations may also have been important. For example, we found a phylogeographical break between the Mary and Pine rivers in southeastern Queensland. This break is consistent with the results of recent genetic studies on Oxylean pygmy perch *Nannoperca oxyleyana* (Hughes *et al*. 1999) and a species of freshwater shrimp *Caridinia* sp. (Woolschot *et al*. 1999). In this regard,

a ridge south of the Mary River may have been an important barrier to movement (Hughes *et al*. 1999).

Drainage rearrangements may also have left conspicuous signatures in the phylogenetic structuring of *P. signifer*. It has been suggested, for example, that the Mulgrave River previously flowed north into Trinity Inlet instead of its current southerly course (Willmott *et al*. 1988). The greater genetic similarity between fish from Trinity Inlet and the Mulgrave River, compared with the Barron River, is consistent with a recent Trinity Inlet exit for the Mulgrave River (McGlashan & Hughes 2002).

Connectivity of drainages may also be influenced by changes to the width and depth of the continental shelf with changing sea levels. The continental shelf is narrow along much of southeastern Australia. Historically, this means that drainages in this region were long isolated, even during periods when sea levels were low. This historical dissociation of waterways, however, does not appear to have significantly sundered contemporary movement of *P. signifer* between drainages in the southern end of the species' range. In contrast, although precise historical connections are difficult to reconstruct, lower sea levels probably led to the coalescence of some of the northern drainages during the Pleistocene, especially where the continental shelf is wide. Our results, and those of McGlashan & Hughes (2002) show, for instance, that the northern group had short internode distances between most of the clades but long tips. Such a pattern is consistent with the concomitant fragmentation of a common population (see also Bermingham & Martin 1998).

Eustatic changes leading to a complex pattern of genetic diversity may also explain the relationships observed in the clade comprising fish from the Don, Cedar Falls, Pioneer, Calliope and Gin Gin. Here, fish from Cedar Falls and the Pioneer River form a sister clade to a group comprising populations to the north and south of both drainages. However, the branch lengths are short and we cannot rule out an alternative topology based on a north to south geographical arrangement of populations in this clade (–ln L 25.97597 units greater, $P = 0.168$). Finer scale sampling may prove illuminating and could provide greater insights into the structure of this clade.

The spectacular morphological variation that exists between populations has led to considerable taxonomic confusion. In this regard, debate has centred largely on whether northern and southern populations represent a single species (reviewed in Saeed *et al*. 1989). The results of allozyme and morphological analyses by Hadfield *et al*. (1979) suggested that *P. signifer* is a single species and that differences among geographical locations were largely clinal. However, the split between northern and southern groupings, provides a good reason for suggesting that *P. signifer* may represent at least two distinct species. Support for this contention is not only consistent with some of the previous taxonomic designations (e.g. Whitley 1932) recognizing a separate northern species, *P. signatus* (Gunther), but is also consonant with recent behavioural work. In mate choice trials, Wong *et al*. (2004) demonstrated that female blue-eyes were significantly more attracted to males belonging to the same major clade than those from another. Moreover, results of a breeding experiment showed that fish from opposite ends of the species range (i.e. between the two major genetic groupings) would not interbreed (Semple 1986). These results suggest possible premating reproductive isolation between the two major clades.

Our study supports the taxonomic designation of *P. mellis* as a species distinct from *P. signifer*. *P. mellis* was previously considered to be just a variety of *P. signifer* but is currently recognized as a separate species and is listed as endangered by the IUCN (Allen *et al*. 2002). Our study showed that the two species are separated by a genetic distance of 21.12–24.00%, a level of divergence which justifies the designation of *P. mellis* as a separate species.

An overriding biogeographical scenario for Australia's unique freshwater ichthyofauna remains a challenge. A recent treatize by Unmack (2001), based on distributional and geological information, has laid an important foundation by providing a suite of testable hypotheses pertaining to Australian freshwater biogeography. More broadly, as the current study demonstrates, phylogenetic studies on individual species, may offer illuminating insights into current and historical patterns of connections and dissociations affecting dispersal between drainages. More phylogenetic studies across a suite of species should provide patterns of genetic structuring that can then be reconciled to provide a common biogeographical framework for Australia's freshwater fishes.

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