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The role of continental shelf width in determining freshwater phylogeographic patterns in south-eastern Australian pygmy perches (Teleostei: Percichthyidae)

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Abstract

Biogeographic patterns displayed by obligate freshwater organisms are intimately related to the nature and extent of connectivity between suitable habitats. Two of the more significant barriers to freshwater connections are seawater and major drainage divides. South-eastern Australia provides a contrast between these barriers as it has discrete areas that are likely influenced to a greater or lesser extent by each barrier type. We use continental shelf width as a proxy for the potential degree of river coalescence during low sea levels. Our specific hypothesis is that the degree of phylogeographic divergence between coastal river basins should correspond to the continental shelf width of each region. This predicts that genetic divergences between river basins should be lowest in regions with a wider continental shelf and that regions with similar continental shelf width should have similar genetic divergences. Pygmy perches (*Nannoperca australis* and *Nannoperca* 'flindersi') in south-eastern Australia provide an ideal opportunity to test these biogeographic hypotheses. Phylogeographic patterns were examined based on range-wide sampling of 82 populations for cytochrome *b* and 23 polymorphic allozyme loci. Our results recovered only limited support for our continental shelf width hypothesis, although patterns within Bass clade were largely congruent with reconstructed low sea-level drainage patterns. In addition, we identified several instances of drainage divide crossings, typically associated with low elevational differences. Our results demonstrate high levels of genetic heterogeneity with important conservation implications, especially for declining populations in the Murray–Darling Basin and a highly restricted disjunct population in Ansons River, Tasmania.

Keywords: conservation, drainage divides, ESU, introgression, phylogeography sea-level changes

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Introduction

Biogeographic studies often focus on freshwater organisms. These taxa are particularly valuable for examining the effect of earth history events on the distribution of biotic diversity because opportunities for movement are limited to freshwater connections among drainage basins (Myers 1938; Banarescu 1990; Unmack 2001). As a result, river basins are considered a fundamental unit in aquatic biogeography (Matthews 1998; Rahel 2007), and they often predict patterns of population genetic

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structure (Hughes 2007). Most freshwater connections between basins occur by one of two means, either breaching drainage divides or overcoming sea water barriers. Drainage divides usually change over long time periods, depending upon local geological processes (Bishop 1995; Unmack 2001). In contrast, major changes in sea water barriers occur quite frequently over 100 000–150 000 year cycles, governed by Milankovich cycles and global climate, with the most recent major change occurring around 20 000 years ago (Bennett 1990; Burckle 1995). These climatic changes cause a lowering of sea levels by up to 130–140 m, depending on the amount of water accumulated in glaciers during each glacial cycle (Yokoyama *et al.* 2001; Clark & Mix 2002; Bintanja *et al.* 2005). Other factors can also influence coastal connections between river basins. For instance, over longer time scales, the positions of river mouths can change (Nott *et al.* 1991; Spry *et al.* 1999) and coastal geomorphic processes can modify the topography of the continental shelf.

Although most rivers terminate at a sea water barrier, and despite many papers referring to sea-level changes as being important relative to phylogenetic or phylogeographic patterns (e.g. Perdices *et al.* 2002; Huyse *et al.* 2004; Yang & He 2008), few studies have specifically addressed hypotheses relating to sea-level changes. In most cases, the hypotheses tested relate to predicting phylogeographic relatedness based on reconstructed drainage patterns at low sea level (e.g. Voris 2000; Schultz *et al.* 2008; Lohman *et al.* 2011; Cook *et al.* 2012), with few discussing how characteristics of the continental shelf might influence patterns of connectivity during low sea levels, except in passing (e.g. Wong *et al.* 2004; Thacker *et al.* 2007, 2008; Unmack *et al.* 2012). Here, we specifically assess the influence of continental shelf width on a group of freshwater fishes in south-eastern Australia.

The continental shelf corresponds to the portion of the sea floor that has a relatively gentle slope and extends to ocean depths of around 140 m. During low sea levels, virtually the entire shelf of each continent is exposed and its margin is essentially the low sea-level coastline. Beyond the continental shelf is the continental slope, which has a much steeper gradient and extends into greater depths. Characteristics of the continental shelf could play an important role in determining connectivity between river basins. For example, during low sea levels, a narrow continental shelf should restrict movement between rivers to a greater extent than a broad continental shelf. This is because on a wide shelf, there is a greater chance of rivers being able to coalesce together as they flow for a longer distance before ending at a sea water barrier. In addition, broader shelves should on average have a lower gradient, which also

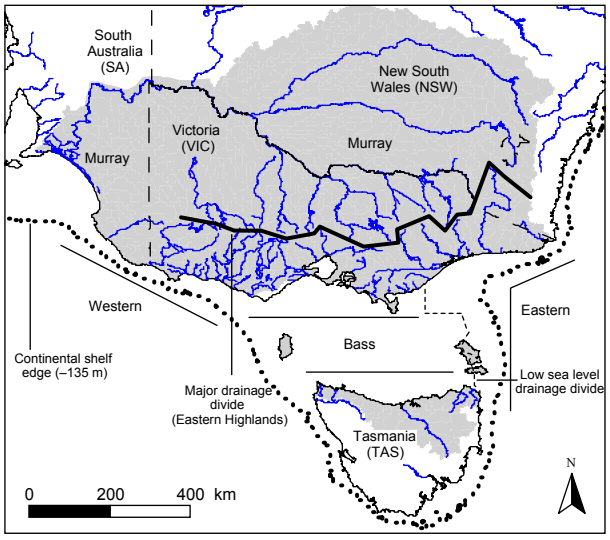

Fig. 1 Overview map of south-eastern Australia showing the Western, Bass, Eastern and Murray regions, the shoreline/continental shelf edge at the last glacial maximum and major drainage divides.

increases the chance that fishes may be able to disperse 'over land' during high rainfall events. Consequently, in the absence of other influences, fish that occur in river basins draining onto a narrow continental shelf should show higher levels of between-basin genetic divergence than they do for river basins draining onto a wide continental shelf. A second expectation is that we should observe similar levels of genetic divergence in different regions with similar comparable continental shelf widths (assuming rates of evolution and selective forces are consistent across regions). Obviously this characteristic will not uniformly structure aquatic communities, as differences in ecology (e.g. dispersal ability, habitat use) and geomorphology among river basins can also play important roles (Unmack 2001). Nonetheless, continental shelf width should be one primary predictor of phylogeographic structure in many freshwater systems.

South-eastern Australia is an ideal area for studying the influence of continental shelf width on phylogeographic patterns, as its many rivers drain regions where continental shelf width varies considerably (Fig. 1). On mainland Australia, numerous isolated coastal rivers flow east or south from the major drainage divide formed by the Eastern Highlands, while north or west flowing rivers coalesce in Murray–Darling Basin to form Murray River, whose outlet is to the west (Fig. 2). To the south is the island of Tasmania, which has many rivers draining north from its central mountains. Where these rivers meet the ocean, there is substantial variation in continental shelf width. Essentially the entire continental shelf between Tasmania and mainland Australia is exposed during low sea level; moreover,

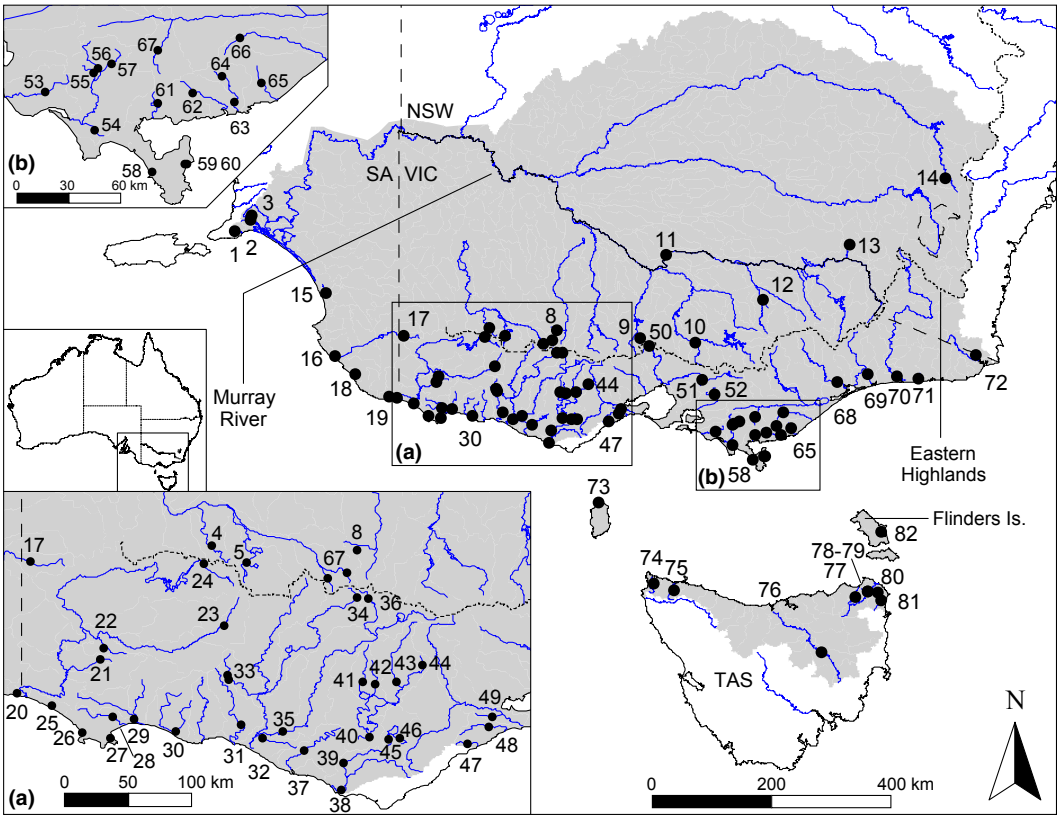

Fig. 2 Locality data for all *Nannoperca* samples examined. Refer to Table 1 for the corresponding locality details and species abbreviations. The shaded area identifies the known distribution of *Nannoperca* species at the river basin scale in southern Australia.

most river basins in this area are predicted by geographic information system (GIS) analysis of bathymetric data to coalesce into one large river basin, which we refer to as the Bass drainage (Unmack 2001; Fig. 3). Areas of continental shelf east and west of Bass drainage in mainland Australia quickly narrow, but are of similar width. To the west in South Australia, the continental shelf rapidly broadens again (Fig. 1).

Obligate freshwater fishes that lack any special ability to transverse terrestrial or marine habitats (e.g. species that can cross land-like eels or diadromous fishes that use marine habitats) are thus entrapped in the drainage network. These represent ideal target study organisms for studying aquatic biogeography (Avise 2000). The southern pygmy perch, *Nannoperca australis* Günther 1861 meets these criteria as a small (up to ~100 mm) sedentary species broadly distributed across south-eastern Australia (Fig. 2) from Genoa River in eastern Victoria west to Inman River, South Australia, including southern Murray–Darling Basin and northern Tasmania (Llewellyn 1974; Kuiter & Allen 1986; Koehn & O'Connor 1990; Hammer & Walker 2004). Within this range, it was historically widespread and abundant, especially in low-gradient creeks and rivers and floodplain habitats.

The contrast between rivers draining onto the continental shelf with varying widths in south-eastern Australia provides an excellent model to test biogeographic hypotheses. We divided the coastline into three regions; Eastern, Bass and Western, with a referential fourth category including the inland Murray–Darling Basin that spans and abuts the first three (Fig. 1). Eastern and Western regions have narrow shelf widths, while Bass region, which occurs between the Eastern and Western regions, has an extremely broad shelf width. The Murray region is based on the Murray–Darling Basin whose populations are hydrologically interconnected today, independent of continental shelf width (Fig. 1). Based on the degree of isolation, we predict that populations within regions with the highest potential degree of river coalescence (i.e. during times of lowered sea level) or actual river coalescence (i.e. the Murray) will display lower average levels of genetic divergence when compared to populations in regions where catchments remained more isolated. Thus, regions would be ranked, in order of decreasing within-region hydrological connectivity (Fig. 1), as Murray (most similar due to current connectivity), Bass (intermediate, only connected at lowest sea levels) and Eastern/Western (least

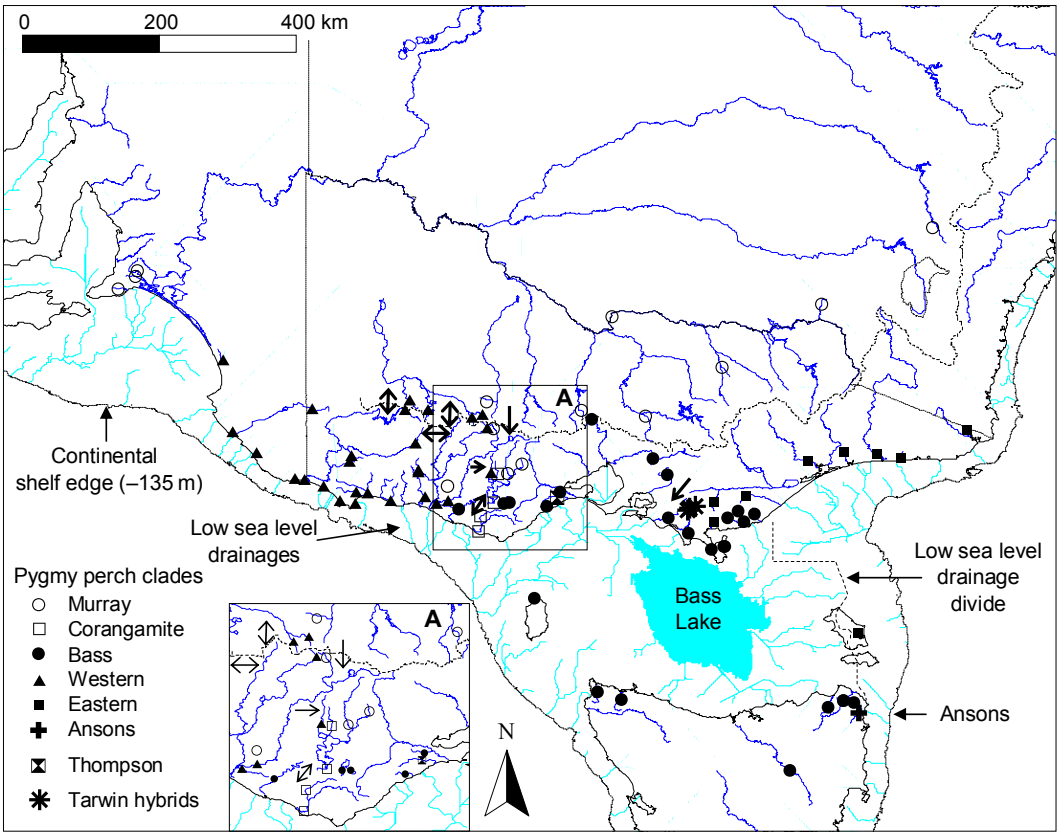

Fig. 3 South-eastern Australia showing the distribution of each mitochondrial lineage (as shown in Fig. 4) and the low sea-level drainage patterns to 135 m below sea level. Bass Lake forms when sea levels drop below 67 m. Arrows show the direction and approximate location of exchange across drainage divides. Double-headed arrows indicate the direction of movement is unclear.

similar due to narrow continental shelf which limits connectivity).

Aside from testing hypotheses related to continental shelf width, our study also examines the role of the major drainage divide, the Eastern Highlands, separating the short coastal drainages from the inland Murray–Darling Basin (Fig. 1). The Eastern Highlands have been a major barrier to the movement of fishes, although some appear to have crossed this mountain range in several places (Unmack 2001). Overall faunal similarity across this divide in Victoria is low, with five shared fish species (Unmack 2001). However, it remains unclear whether these species have crossed the drainage divide, or if they have been exchanged via coastal routes.

Materials and methods

Study taxa and sampling

Three subspecies, *Nannoperca a. australis*, *Nannoperca a. tasmaniae* (Johnston 1883) and *Nannoperca a. flindersi* Scott 1971 have been recognized (e.g. Llewellyn 1974; Cadwallader & Backhouse 1983); however, they remain poorly characterized, and the subspecific designations are not in common usage. Unmack *et al.* (2011) examined species relationships within pygmy perches (*Nannatherina* and *Nannoperca*) and coarsely examined molecular variation within *Nannoperca australis* across its range (but from only 12 populations) and suggested that some eastern populations of *N. australis* should be elevated to full species status, based on a substantial number of diagnostic genetic markers in broad parapatry. Based on the previously described subspecies name from this region, we informally refer to this eastern species as *Nannoperca* 'flindersi' and the western species as *N. australis* (*sensu stricto*).

Our sampling strategy was designed to cover the entire range of *N. australis* and *N.* 'flindersi', with the goal of sampling as many separate drainages as possible. A total of 278 individuals from 82 populations were sampled for variation in the mitochondrial cytochrome *b* (cyt*b*) gene with an average of 3.4 individuals per population (Fig. 2, Table 1). For allozymes, a total of 72 populations were examined (some populations lacked frozen material, had small sample sizes or were geographically close to other examined populations), with

Table 1 Locality data for all *Nannoperca* populations examined

pop	Locality	Field codes	cyt*b*	pop.	Clade	Basin
1	Inman R, Victor Harbor, SA	FISH84:ML-77+	6	9	Murray	Murray
2	Tookayerta Ck trib, SA	FISH84:ML-48+	5	6	Murray	Murray
3	Finniss R, Ashbourne, SA	FISH84:Fin1+; F-E49:PP-1+	5	9	Murray	Murray
4	McKenzie R, Tatlocks Bridge, VIC	FISH99:S38	1	7	Western	Wimmera
5	Fyans Ck diversion, Fyans Ck, VIC	PU00-08	1	10	Western	Wimmera
6	Mount Cole Ck, Warrak, VIC	PU00-06	1	—	Western	Wimmera
7	Wimmera R, VIC	FISH83:HS-38+	4	6	Western	Wimmera
8	Middle Ck trib, Warrenmang, VIC	PU99-33	5	10	Murray	Murray
9	Jews Harp Ck, Sidonia, VIC	PU00-01	4	10	Murray	Murray
10	Murray R, Barmah, VIC	n/a	5	—	Murray	Murray
11	Yea R, Yea, VIC	PU92-08	5	10	Murray	Murray
12	Meadow Ck, Moyhu, VIC	PU99-79	5	10	Murray	Murray
13	Coppabella Ck, Coppabella, NSW	PU99-82	5	10	Murray	Murray
14	Lachlan R trib., Dalton, NSW	FISH98:LPP-1+	5	8	Murray	Murray
15	Henry Ck, Salt Creek, SA	FISH98:MT-95+	4	10	Western	SE SA
16	Drain L, Robe, SA	FISH21:C18+	1	8	Western	SE SA
17	Mosquito Ck, Langkoop, VIC	PU00-16; F-FISH83:HS-8+	1	9	Western	SE SA
18	Lake Bonney, Millicent, SA	FISH83:HS-101+; FISH99:SE-13+	1	7	Western	SE SA
19	Ewens Ponds, Port McDonnell, SA	FISH83:HS-75+	2	9	Western	SE SA
20	Piccaninnie Pond, Port McDonnell, SA	FISH83:HS-47+	2	10	Western	SE SA
21	Stokes R, Digby, VIC	PU00-19	1	—	Western	Glenelg
22	Merino Ck, Merino, VIC	PU00-17	1	8	Western	Glenelg
23	Wannon R, VIC	PU02-117	3	7	Western	Glenelg
24	Glenelg R, Glenisla Crossing, VIC	PU00-14	4	10	Western	Glenelg
25	Lake Monibeong, VIC	PU09-128	2	6	Western	Glenelg
26	Bridgewater Lake, main lake, Tarragal, VIC	PU09-122	2	10	Western	Glenelg
27	Wattle Hill Ck, Portland, VIC	PU09-124	2	10	Western	Wattle Hill
28	Surrey R, Heathmere, VIC	PU00-20	4	10	Western	Surrey
29	Fitzroy R, Tyrendarra, VIC	PU09-131	1	8	Western	Fitzroy
30	Shaw R, Yambuk, VIC	PU00-21	4	10	Western	Shaw
31	Merri R, Grassmere, VIC	PU00-22	4	10	Western	Merri
32	Brucknell Ck, Cudgee, VIC	PU09-134	2	10	Western	Hopkins
33	Mustons Ck, Purdeet, VIC	TR02-363B; PU09-121	3	12	Western	Hopkins
34	Fiery Ck, Raglan, VIC	PU08-15	3	10	Western	Hopkins
35	Mount Emu Ck, Panmure, VIC	PU00-23	6	10	Western (4), Murray (2)	Mt Emu
36	Trewalla Ck, Waterloo, VIC	PU08-16	3	10	Murray	Mt Emu
37	Curdies R, Curdie, VIC	PU00-24	7	10	Bass	Curdies
38	Gellibrand R floodplain, VIC	PU02-92	6	10	Corangamite	Gellibrand
39	Kennedy Ck, Kennedy Creek, VIC	PU00-25	6	10	Corangamite	Gellibrand
40	Pirron Yaloak Ck, Pirron Yaloak, VIC	PU00-26	6	10	Corangamite	Corangamite
41	Mundy Gully, Lismore, VIC	PU08-11	5	10	Western	Corangamite
42	Gnarkeet Ck, Lismore, VIC	PU00-27; FISHY2:GB-4+	5	7	Corangamite	Corangamite
43	Woady Yallock R, Werneth, VIC	TR-1768	1	—	Murray	Corangamite
44	Kuruc A Ruc Ck, Dereel, VIC	PU08-10	3	10	Murray	Corangamite
45	Deanes Ck, Colac, VIC	TR02-438	2	2	Bass	Colac
46	Barongarook Ck, Colac, VIC	PU08-13	5	10	Bass	Colac
47	Anglesea R, VIC	PU02-94	3	7	Bass	Angelsea
48	Thompson Ck, VIC	PU02-107	7	7	Bass (3), Corang. (2), YPP (2)	Thompson
49	Waurn Ponds Ck, Geelong, VIC	PU00-29	4	8	Bass	Barwon
50	Deep Ck, Lancefield, VIC	PU00-03	6	10	Bass	Marybrnong

Table 1 *Continued*

pop	Locality	Field codes	cyt*b*	pop.	Clade	Basin
51	Woori Yallock Ck, VIC	PU92-07	1	—	Bass	Yarra
52	Diamond Ck, Tonimbuk, VIC	PU99-89	1	10	Bass	Bunyip
53	Powlett R, VIC	PU99-88	1	10	Bass	Powlett
54	Bald Hills Ck, VIC	PU02-75	5	7	Bass	Tarwin
55	Coalition Ck, Leongathata, VIC	PU08-05	7	7	Eastern (6), Bass (1)	Tarwin
56	Wilkur Ck, Leongathata, VIC	PU08-06	1	—	Bass	Tarwin
57	Berrys Ck, Berrys Creek, VIC	PU99-77	10	10	Eastern	Tarwin
58	Darby R, Wilsons Prom, VIC	PU02-73	1	7	Bass	Darby
59	Miranda Ck, Wilsons Prom, VIC	PU02-69	1	7	Bass	Miranda
60	Freshwater L Ck, Wilsons Prom, VIC	PU02-70	1	7	Bass	Miranda
61	Pebble Ck, Forster, VIC	PU99-75	5	8	Eastern	Franklin
62	Billy Ck, VIC	PU02-98	3	7	Bass	Albert
63	Tarra R, VIC	PU02-96	3	7	Bass	Tarra
64	Macks Ck, Calrossie, VIC	PU99-74	4	10	Bass	Tarra
65	Bruthen Ck, Woodside, VIC	PU08-08	5	10	Bass	Bruthen
66	Merrimans Ck, Hiamdale, VIC	PU99-72	4	10	Eastern	Merrimans
67	Morwell R, Yinnar, VIC	PU99-78	4	10	Eastern	La Trobe
68	Prospect Ck, VIC	PU99-87	4	10	Eastern	Mitchell
69	Stony Ck, VIC	TR03-170	2	—	Eastern	Tyers
70	Snowy R lagoon, Orbost, VIC	PU99-85	4	10	Eastern	Snowy
71	West Branch Yeerung R, VIC	TR03-165	2	—	Eastern	Yeerung
72	Maramingo Ck, Genoa, VIC	PU99-84	4	10	Eastern	Genoa
73	Flannigan Lake, King Island, TAS	n/a	2	—	Bass	King
74	Harcas R, West Montague, TAS	FISH82:HT-41+	1	10	Bass	Welcome
75	Allen Ck trib, TAS	FISH98:MT-20	1	—	Bass	Duck
76	Macquarie R, TAS	FISH82:HT-27+	1	10	Bass	Macquarie
77	Lagoon, Boobyalla R, Gladstone, TAS	FISH98:MT-51+	1	7	Bass	Ringarooma
78	Boobyalla R, Gladstone, TAS	FISH98:MT-54+	1	8	Bass	Ringarooma
79	Gladstone Lagoon, Gladstone, TAS	FISH98:MT-38+	5	9	Bass	Ringarooma
80	Icena Ck trib, Gladstone, TAS	FISH98:MT-36	6	8	Bass	Gt. Museel
81	Unnamed trib to Ansons R, TAS	FISH82:HT-2+	5	10	Ansons	Ansons
82	Patriarch River, Flinders Island, TAS	FISH84:Fl-1+	4	5	Eastern	Flinders
	Gingin Brook, WA	PU09-30	1		Outgroup	
	Margaret R, WA	MA79	1		Outgroup	
	Doggerup Ck, WA	PU09-49	1		Outgroup	
	Mitchell R, WA	n/a	1		Outgroup	

Population number (pop) refers to the localities shown in Fig. 1. Locality provides the name of the creek or river, then the local place name (unless none exists), followed by the state abbreviation (NSW, New South Wales; SA, South Australia; TAS, Tasmania; VIC, Victoria). Field codes can be used to track references to genetic material deposited in the South Australian Museum and morphological samples deposited in the Australian, South Australian and Victorian Museum collections. The next two columns provide the sample sizes examined in each component in this study as follows; cytochrome *b* (cyt*b*), pop is the final number of individuals examined for allozyme variation at 23 polymorphic loci (of 53 originally screened; Unmack *et al.* 2011). Clade represents the genetic lineage for each population; if multiple lineages were present, the number of individuals from each is given in parentheses. Basin is the name of each discrete river basin.

an average of 8.8 individuals per population (Table 1). In the mtDNA study, we included seven haplotypes representing the breadth of diversity within the closely related congener *Nannoperca obscura* (Hammer *et al.* 2010), plus two new *N. obscura* samples from population 29 (Fig. 2, Table 1). This was to provide more accurate phylogenetic coverage within *N. australis*, because our previous investigation found a distinct mitochondrial lineage of this species nested within *N. australis* due to an old introgression event (Unmack *et al.* 2011). To provide a more robust phylogenetic hypothesis of clade relationships, a subsample of 37 individuals representing all major cyt*b* lineages was also sequenced for 5699 base pairs (bp) of mtDNA. For most populations, whole specimens originally frozen in liquid nitrogen were deposited in the Evolutionary Biology Unit of the South Australian Museum, while formalin preserved representatives were deposited in the Australian, Victorian and South Australian museums. These samples can be identified based on their field code (Table 1).

GIS bathymetry manipulation

Data sets used to generate maps (e.g. Figs 1–3) were obtained from the Digital Chart of the World (ESRI 1993) and manipulated in ArcInfo version 10 and ArcView version 3.3 (Environmental Systems Research Institute, Redlands, CA). Bathymetric data were obtained from a 30 arc-second (*c.* 1 km) data set GEBCO 08 (September 2010 release, www.gebco.net) and manipulated to produce the −135 m bathymetric contour and low sea-level drainage patterns (Fig. 3) using the hydrological tools in ArcInfo (GIS data available at peter.unmack.net/gis/sea_level).

DNA isolation, amplification and sequencing

Total DNA was obtained from approximately 0.25 cm^3 of caudal fin or muscle via phenol/chloroform extraction or DNeasy Tissue Extraction Kits (QIAGEN Inc., Chatsworth, CA, USA). We amplified the cyt*b* gene using two primers that flanked the gene. Most samples were amplified using the primers Glu18 TAACCA GGACTAATGRCTTGAA and PP.Thr.41 AGGATTTT AACCTCTGGCGTCCG. When amplification was insufficient, the gene was amplified in two halves using Glu18 and HD.Alt GGRTTGTTGGAGCCTGTTTCAT and PP.505 TCAGTAGACAACGCCACCCT and PP.Thr.41. On the basis of our cyt*b* results, we selected 37 individuals broadly representing the diversity found within each clade for cyt*b* to sequence for a total of seven of the 13 mtDNA protein-coding genes (ND1, ND2, ATPase6/8, ND4L, ND4, cyt*b* and partial sequence from COIII), representing over a third of the mitochondrial genome (5699 bp). Most primers were designed from two whole mtDNA genomes (Prosdocimi *et al.* 2012). Details of those primers are in Supplementary Document 1. PCR amplification and sequencing conditions follow Hammer *et al.* (2010). All sequences obtained in this study were deposited in GenBank, accession numbers KC285906–KC286129, and the sequence alignment was deposited in Dryad, doi: 10.5061/dryad.9v852. (All GenBank numbers used in this study are listed by individual in Table S1, Supporting information.)

Allozyme electrophoresis

Allozyme electrophoresis of caudal muscle homogenates was carried out on cellulose acetate gels (Cellogel®) according to standard procedures (Richardson *et al.* 1986). Details of sample preparation, locus abbreviations and allozyme nomenclature are presented in Unmack *et al.* (2011). Twenty-three of 53 loci examined in Unmack *et al.* (2011) satisfied the two criteria necessary for inclusion in any analysis of geographic structure, in that they displayed (i) allelic variation within *N. australis*, *N.* 'flindersi' or both species and (ii) unambiguous electrophoretic phenotypes for all putative genotypes. The 23 loci examined were as follows: *Acon1, Ada*, *Ca*, *Ck, Fum*, *G6pd*, *Got1*, *Got2*, *Gp1*, *Gpi1*, *Gpi2*, *Gsr*, *Idh1*, *Idh2*, *Mdh1*, *Me1*, *Me2*, *Mpi*, *PepA2*, *PepB*, *PepD2*, *Pgk* and *Pgm1*. Six of these loci were among the 10 found by Unmack *et al.* (2011) to display fixed differences between *N. australis* and *N.* 'flindersi', while 14 have previously been shown to unequivocally diagnose *N. australis* from *N. obscura* (Hammer *et al.* 2010).

Analysis of DNA sequence data

Sequences were edited using Chromas Lite 2.0 (Technelysium, Tewantin, Queensland, Australia) and imported into BIOEDIT 7.0.5.2 (Hall 1999). Sequences coding for amino acids were aligned by eye and checked via amino acid coding in MEGA 5.05 (Tamura *et al.* 2011) to test for unexpected frame shift errors or stop codons. Phylogenetic analyses were performed with maximum likelihood (ML) using GARLI 2.0 (Zwickl 2006). We identified the best-fitting model of molecular evolution using the Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada & Crandall 1998) using PAUP* 4.0b10 (Swofford 2003). For both cyt*b* and the multigene mtDNA data sets, Modeltest identified GTR+I+G as the best model. To get the ML topology, we ran GARLI with 10 search replicates with the following default setting values changed: streefname = random; attachmentspertaxon = 196; genthreshfortopoterm = 100 000; scorethreshforterm = 0.05; significanttopochange = 0.00001. For bootstrapping, we ran 1000 replicates with the previous settings with the following changes: attachmentspertaxon = 80; genthreshfortopoterm = 10 000; significanttopochange = 0.01; treerejectionthreshold = 20 as suggested in the GARLI manual to speed up bootstrapping. Trees were rooted with four *Nannoperca vittata* samples (Table 1) as these were the nearest sister species based on previous analysis of mtDNA variation in the genus (Unmack *et al.* 2011). In the multigene mtDNA analysis, we also included data from two individuals with whole mtDNA genomes from GenBank (*N. australis*, JF519732.1 and *N. obscura*, JF519733.1, Prosdocimi *et al.* 2012). The ML trees were deposited in TreeBASE, accession number 13674 (http://purl.org/phylo/treebase/phylows/study/TB2:S13674). We calculated mean within- and among-lineage and population variation based on cyt*b* using p-distances in MEGA, after excluding populations that proved geographically or genetically anomalous (see Results).

To estimate molecular divergence times, we used our multigene data set in *BEAST (Heled & Drummond 2010) as incorporated into BEAST 1.7.1 (Drummond *et al.* 2012).

Input files were generated using BEAUTI 1.7.1. The analysis used an uncorrelated lognormal relaxed molecular clock with rate variation following a tree prior using the Yule model. We used a single partition with the GTR + I + G model (identified using the AIC in Modeltest) and a random starting tree. Divergences based on pairwise comparisons in this study were assumed to occur at approximately 1.0% per million years. Other studies on teleost fishes have calibrated molecular clocks for protein-coding mtDNA genes and obtained values between 0.68% and 1.66% pairwise divergence per million years (summarized by Burridge *et al.* 2008). Other studies on Australian freshwater fishes have used a rate of 1.0% (Unmack & Dowling 2010) or have obtained a similar rate (0.84%) based on biogeographic calibrations (Unmack *et al.* 2011). Although molecular clock estimates vary, and in many cases provide crude estimates of divergence times (Magallon 2004; Donoghue & Benton 2007; Pulquerio & Nichols 2007), they can provide important insights into relative patterns of divergence. Hence, we interpret our molecular clock findings herein with an appropriate level of caution. Multiple shorter runs were conducted to check for stationarity and that independent runs were converging on a similar result. Final results from the *BEAST analyses were based on the combination of two separate runs for 100 million generations each, with parameters logged every 5000 generations. Tree and logfile outputs were combined in LOGCOMBINER 1.7.1 with a burn-in of 10%. The combined logfile was examined in TRACER 1.5, while the age estimates were summarized using TREEANNOTATOR 1.7.1 with the mean age values placed on the maximum clade credibility tree found in the sample of trees generated from BEAST.

For our estimates of divergence within regions relative to testing the influence of continental shelf width, we quantified within-region levels of cyt*b* divergence for each of the four regions by calculating average p-distances, the percentage of haplotypes that were identical between river basins and the number of river basins that shared haplotypes (river basins are defined in Table 1), with the latter two measures, being more influenced by sampling intensity, given less weight for ranking purposes. For these estimates, we excluded several populations that would otherwise bias relationships relative to continental shelf characteristics (see Results).

Analysis of allozyme data

The multivariate ordination technique of principal co-ordinates analysis (PCO) was initially employed to assess the extent to which allozyme data would (i) confirm existence of two species as found by Unmack *et al.* (2011) within *N.* 'australis', and (ii) identify any hybrids or instances of introgression between them. PCO was also used to explore the genetic affinities of regions and sites within and among mtDNA clades within each species. All methodological details follow Hammer *et al.* (2007).

Thereafter, the allozyme data for all nonhybrid sites were analysed for evidence of any (i) departure from Hardy–Weinberg expectations, (ii) linkage disequilibrium within populations and (iii) heterogeneity of allele frequencies between populations (restricted to pairwise comparisons with Δp values >40%, reflecting the limited statistical power of our small sample sizes; Table 1). These statistical tests were carried out by estimating exact probabilities using Genepop 4.0.10 (Rousset 2008), and all probability values were adjusted for multiple tests using the sequential Bonferroni correction factor (Rice 1989). F-statistics were also used to assess the degree of population genetic subdivision within and among regional populations and clades. F_{ST} and F_{IS} values and their 99% confidence intervals were obtained using FSTAT 2.9.3 (Goudet 2001). Attempts to use the programs Geneland and Structure to identify populations from first principles using Bayesian inferencing produced inconsistent outcomes, perhaps reflecting our small sample sizes, large number of populations, and high levels of inferred admixture.

Phylogeographic analysis of the allozyme data was undertaken by constructing unrooted neighbor-joining (NJ) trees from matrices of pairwise Nei Distances among selected populations, as described in Unmack *et al.* (2011).

Results

Sequence analyses

For the cyt*b* only data, 988 of the 1140 bp were constant (excluding the outgroup taxon, *N. vittata*), 54 variable characters were parsimony uninformative and 98 characters were parsimony informative. ML recovered one tree with a −ln score of −4108.663303 (Fig. 4). For the multigene mtDNA data set 5699 bp (excluding the outgroup taxa, *N. vittata*), 5078 were constant, 146 variable characters were parsimony uninformative and 475 characters were parsimony informative. ML recovered one tree with a −ln score of −17725.424184 (Fig. 5A). Both data sets found eight larger clades. In the multigene data set, most of the deeper nodes had strong bootstrap support (>99% of bootstrap replicates), with two having lower support values (88% and 61%). These clades fall out into separate geographic regions referred to as Eastern, Ansons (both within *Nannoperca* 'flindersi'), Bass, Corangamite, Western, Murray (all within *Nannoperca australis*), and two clades containing *Nannoperca obscura*

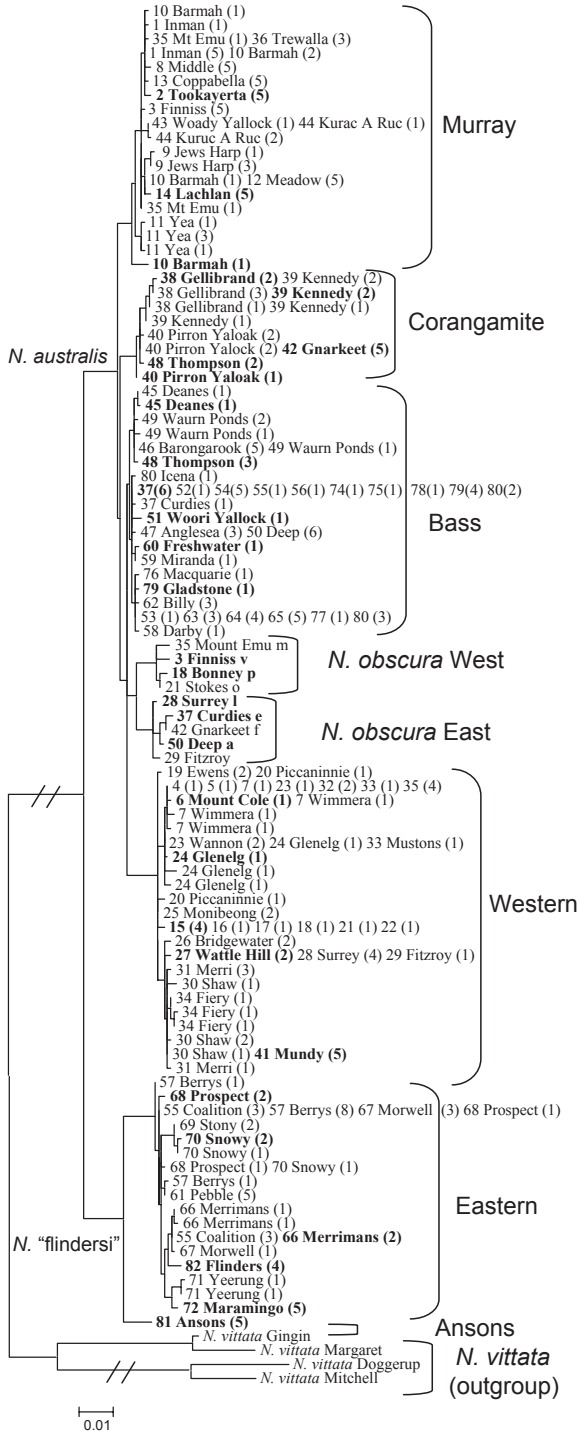

Fig. 4 Maximum likelihood tree for *Nannoperca australis*, *Nannoperca* 'flindersi' and *Nannoperca obscura* based on analysis of cytochrome *b* haplotypes (1140 bp). Each OTU code is based on the population number and locality described in Table 1 and Fig. 2, and the distribution of these clades is shown in Fig. 3. The value at the end of the OTU code indicates the number of individuals with the same haplotype from each population. OTU codes in bold represent individuals used to create Fig. 5. For *N. obscura*, the letter at the end of the OTU code is the haplotype designation from Hammer *et al.* (2010).

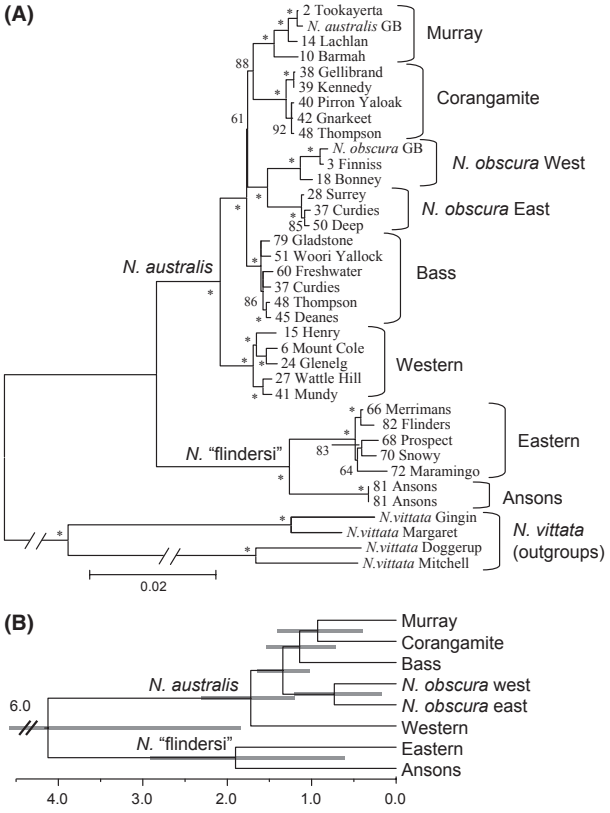

Fig. 5 Maximum likelihood (ML) tree (A) for *Nannoperca australis*, *Nannoperca* 'flindersi' and *Nannoperca obscura* based on analysis of 5699 bp of mitochondrial DNA. Bootstrap values shown are based on 1000 replicates, the * indicates nodes with bootstrap support >95. Each OTU code is based on the population number and locality described in Table 1 and Fig. 2, and the distribution of these clades is shown in Fig. 3. Sequences representing the whole mtDNA genomes deposited in Genbank are labelled as 'species GB'. *BEAST Bayesian tree (B) based on the same data set. Molecular clock estimates (in millions of years) are provided along the scale bar, grey bars represent the 95% highest posterior density. As discussed in the text, the nodes for *N. obscura* and Bass clade were flipped between the ML and Bayesian trees.

(Figs 4 and 5). Mean cyt*b* p-distances between clades within each species varied between 1.1% and 2.0% (Table S2, Supporting information). Mean p-distances within clades for *N. australis* and *N.* 'flindersi' varied from 0.16% to 0.43% (Table S2, Supporting information), while Ansons clade had no variation (it contains only a single population). Molecular clock estimates (in millions of years) obtained from the *BEAST analysis are presented based on their mean values and 95% highest posterior density (HPD) in Fig. 5B; most estimates had effective sample sizes >2600. The Bayesian topology obtained by *BEAST differed slightly from ML in that the positions of Bass and *N. obscura* were flipped, a node that had low support in both analyses though.

Table 2 Results of continental shelf width comparisons with measures of within-clade variability for *Nannoperca* from the four regions defined in Fig. 1 based on the cytochrome *b* and allozyme data

	p-distance	Shared haps. (%)	Shared rivers (%)	mtDNA Rank	Nei Ds (range)	F_{ST} (99% CI)	Allozyme rank
Eastern	0.00527	4/28 (14)	2/8 (25)	4	0.050 (0.00–0.10)	0.598** (0.019–0.805)	3
Bass	0.00149	49/67 (73)	14/20 (70)	1	0.040 (0.00–0.14)	0.383** (0.161–0.476)	2
Western	0.00355	16/32 (50)	4/7 (57)	2	0.023 (0.00–0.05)	0.221** (0.069–0.353)	1
Murray	0.00373	6/44 (14)	2/9 (22)	3	0.144 (0.05–0.34)	0.606** (0.470–0.717)	4

Shared haps refer to number of shared haplotypes between populations, shared rivers refers to the number of shared haplotypes between independent coastal rivers. For the F-statistics, **indicates statistical significance at $P < 0.01$, none of the F_{IS} values were statistically different from zero. Regions are ranked overall 1–4 in order of increasing levels of between-site genetic divergence.

Our estimates of divergence within cyt*b* for each of the four regions relative to testing the influence of continental shelf width included average p-distances, the percentage of haplotypes that were identical between river basins and the number of river basins that shared haplotypes. For these estimates, we excluded the following populations (Fig. 2) that would otherwise bias relationships relative to continental shelf characteristics: 23–24, 32–33 (influenced by headwater dispersal); 34–36 (hybridisation between lineages); 55–57 (hybridisation between species); 38–46 (mostly noncoastal drainages, that is, Corangamite clade and Lake Colac); pop. 1 (not part of the contiguous Murray Basin); pop. 81 (beyond shelf regions and lineage found in only a single population); and pop. 61 (geographic outlier for Eastern lineage). Together, these three measures of within-region cytb divergence (Table 2) indicated that regions were ranked overall Bass < Western < Murray < Eastern, in order of increasing levels of within-region genetic divergence.

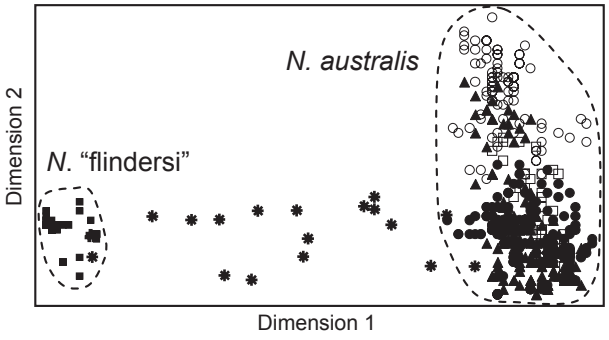

Fig. 6 Principal co-ordinates analysis (PCO) of the pairwise genetic distance matrix among all 634 *Nannoperca australis* and *Nannoperca* 'flindersi' genotyped. Relative PCO scores have been plotted for the first and second dimensions, which individually explained 55% and 16%, respectively, of the total multivariate variation present. Individuals from the two hybrid sites (pops. 55 and 57, Fig. 2) are represented by asterisks, all other individuals are identified by symbols reflecting their mtDNA clade, following Figs 3 and 4.

Allozyme analyses

The final allozyme data set comprised 634 individual fish genotyped at 23 putative allozyme loci. No individual displayed any allozyme evidence of introgression with *N. obscura*, based on the 14 diagnostic loci surveyed herein. An initial PCO of all specimens supported confirmed the presence of two readily diagnosable species, that is, *N. australis* and *N.* 'flindersi' (Fig. 6), and demonstrated that fish from two sites in the upper Tarwon (sites 55 and 57, Fig. 2B, Table 1) reflect hybridisation between these species. Both conclusions are supported by an examination of allele frequency data for all other sites (Table S3, Supporting information), which indicates that (i) the two species are fully (i.e. no shared alleles) or effectively (i.e. no shared allele >5%) diagnosable at each of six diagnostic loci (*Ada*, *Ck*, *Fum*, *Mdh1*, *Mpi* and *Pgk*) found previously by Unmack *et al.* (2011), and (ii) these two upper Tarwon sites possess both sets of alleles at all these loci as well as a mix of cyt*b* haplotypes from both species. Given their hybrid nature, both sites were excluded from all subsequent allozyme analyses.

Statistical analyses of the allozyme data revealed no evidence to reject the standard null hypotheses operating at the outset of a population genetic study, that is, individual populations are panmictic, and no two loci are in linkage disequilibrium. In contrast, the NJ trees (Fig. 7 and Fig. S1, Supporting information), pairwise statistical comparisons (Table S4, Supporting information) and supplementary PCOs (analyses not shown) together revealed considerable genetic heterogeneity in each species, both at regional and local scales.

Using mtDNA clades to provide the nomenclatural framework, three primary allozyme groups (Murray, Corangamite, and Western/Bass) and six allozymically admixed sites (sites 35, 36, 41, 44, 45 and 46, all from the zone of regional proximity among clades; Fig. 3) were evident within *N. australis* (Fig. 7). Pairwise comparisons of allele frequency among sites within each

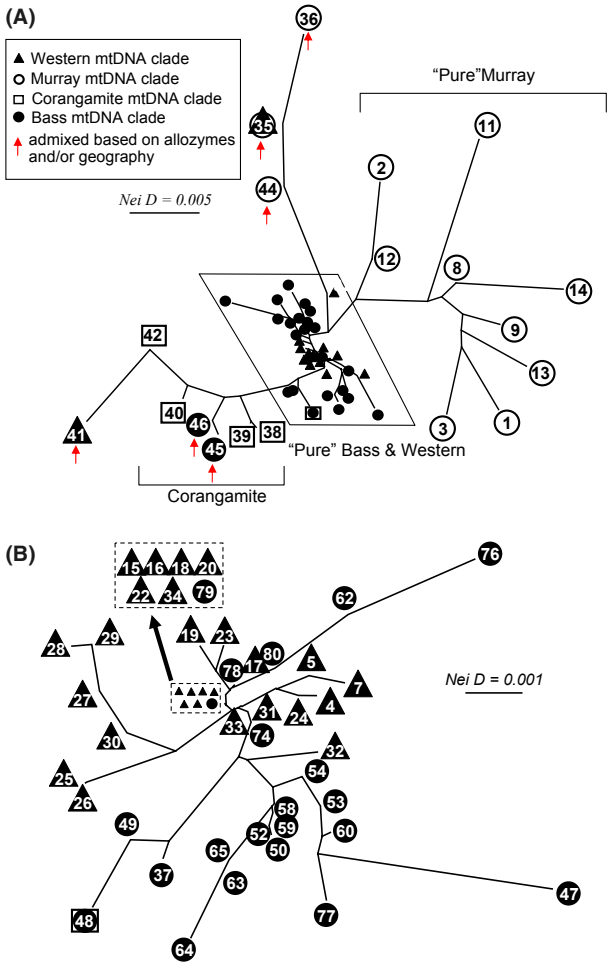

Fig. 7 Unrooted neighbor-joining (NJ) trees of allozyme data for *Nannoperca australis*, depicting the phylogenetic affinities among 62 sites, based on unbiased Nei Ds. Sites are coded as per Table 1 and carry symbols reflecting their mtDNA profile (s). (A) Unrooted NJ tree for all sites, showing the three primary allozyme subgroups (Murray, Western/Bass, Corangamite) and the six sites that were either mitochondrially anomalous or obvious geographic-outliers (arrowed). (B) Follow-up unrooted NJ tree for only 'pure' Bass/Western sites, as defined in (A).

allozymically pure group revealed numerous statistically significant differences in allele frequency, often for multiple loci (Table S4, Supporting information). As predicted from the NJ networks, all allozyme measures of between-site heterogeneity were greatest in the Murray group, intermediate in the Corangamite group, and lowest in the Western/Bass group. Indeed, regarding the latter, it is noteworthy that a number of Bass and Western sites displayed little or no evidence of allozyme divergence between one another (Fig. 7B; Table S4, Supporting information), despite being geographically well separated and readily diagnosable by having very distinctive mitochondrial clades (Figs 4 and 5).

For *N.* 'flindersi', the primary allozyme lineages were not concordant with the two mtDNA clades (Fig. S1, Supporting information), largely due to the genetic distinctiveness of the most easterly population (site 72) from all others. However, as in *N. australis*, pairwise comparisons of allele frequencies revealed statistically significant evidence of genetic divergence among most sites, usually at multiple loci (Table S4, Supporting information).

Although the allozyme data provided a somewhat different perspective on phylogeographic structure in *N. australis*, all assessments of allozyme diversity in relation to continental shelf width were conducted on groups as defined by the cyt*b* clades, in order to provide strictly comparable data sets. These analyses excluded all introgressed (sites 55, 57) and allozymically admixed (sites 35, 36, 41, 44, 45 and 45) sites. Two measures of within-clade allozyme divergence, F_{ST} and average unbiased Nei D, were calculated for each clade. All F_{ST} values were large (0.221–0.606) and significant, and the concordantly ranked Nei Ds ranged from 0.023 for Western clade to 0.114 for Murray clade (Table 2). Together, these measures ranked regions as Western < Bass < Eastern < Murray, in order of increasing levels of within-region genetic divergence.

Discussion

Continental shelf width predictions

Based on the degree of isolation, we predicted populations within regions that experienced the highest potential degree of river coalescence (i.e. during times of lowered sea level) or actual river coalescence (i.e. the Murray) would show lower average levels of genetic divergence when compared to regions where catchments remained more isolated. Under this prediction, the four regions investigated in this study should be ranked, from lowest to highest, Murray < Bass < Eastern/Western (both regions have a similar continental shelf width). Neither of our two molecular data sets produced rankings that were consistent with this prediction. For cyt*b*, Bass region displayed the lowest average levels of genetic divergence, followed by Western < Murray (similar average p-distances but the latter displaying fewer shared haplotypes), followed by Eastern (Table 2). Our multilocus allozyme data revealed a different pattern, with by far the lowest average levels of genetic divergence present in Western region, followed by the ranking Bass < Eastern < Murray. Thus, a consensus of these two molecular data sets would rank regions in the order Bass/Western < Eastern/Murray, again not compatible with our prediction. It is clear from these data that factors other than continental shelf width have affected between-

population levels of genetic divergence in the Eastern and Western regions. We now explore in greater depth the patterns within each region, in an attempt to identify these factors.

The comparatively high degree of genetic similarity within Bass clade (Table 2, Fig. 1) is most easily explained by predicted connections during lowered sea levels, when virtually all of the drainages integrated into a single river system once sea levels dropped below −67 m (Fig. 3; Blom 1988; Blom & Alsop 1988). Although the river basins within Bass clade have been completely isolated over the last 18 000 years as sea levels gradually rose to their present state (Cupper *et al.* 2003), a pattern of repeated connection and isolation has occurred during each glacial cycle, at roughly 100–150 000 year intervals throughout the Pleistocene (Bennett 1990). Most cyt*b* haplotypes within Bass clade only differed by 1–3 bp, and many river basins shared haplotypes (Table 2, Fig. 3). There were also low levels of genetic divergence among populations at our allozyme markers (Fig. 7; Table S4, Supporting information). The fish *Gadopsis marmoratus* Richardson, *Galaxiella pusilla* (Mack) and the crayfish *Engaeus cunicularius* (Erichson) are the only other aquatic species that have been examined genetically from both southern Victoria and northern Tasmania. Ovenden *et al.* (1988) and Miller *et al.* (2004) found no or only minimal divergence between *G. marmoratus* populations, suggesting they were also similarly affected by repeated connectivity during low sea levels. Horwitz *et al.* (1990) found one fixed allozyme difference and a Nei's genetic distance of 0.048 in *E. cunicularius*, which is also consistent with relatively low divergence across Bass Strait. In contrast, *G. pusilla* showed higher levels of divergence, consistent with increased isolation between populations which Unmack *et al.* (2012) suggested may have been due to lower tolerance of drier conditions that occur during glacial periods (White & Mitchell 2003).

Despite the high current hydrological connections within Murray region, there are only moderate levels of genetic similarity among populations (Table 2 and Fig. 3 for mtDNA; Table 2 and Fig. 7 for allozymes). This is surprising, as *Nannoperca australis* is thought to have been distributed continuously throughout most of its range in the Murray region based on historical records (e.g. Llewellyn 1974; Cadwallader 1977). In contrast to contemporary hydrological connectivity and a supposed contiguous distribution, molecular data suggest restricted gene flow within the region. Factors may include current day aridity and higher temperatures in lowland areas of the Murray region (*N. australis* is essentially a temperate species, with the Murray representing its northern range limit), distance between core populations and perhaps competition (the region has higher species richness than coastal systems), with the added historical influence of increased aridity during glacial periods (White & Mitchell 2003). Natural factors have no doubt been compounded by significant fragmentation and hydrological variation caused by recent human modifications.

We expected populations within the Eastern and Western regions to display similar levels of within-region genetic diversity as they drain onto a continental shelf of similar width. However, populations in Eastern region had substantially higher levels of genetic divergences across both data sets (Table 2). Four factors may have confounded predictions. First, our sampling was far more contiguous in Western region, with almost every separate drainage basin sampled, whereas Eastern region was far less thoroughly sampled. Second, the Western region has lower topographic differentiation, party due to the extensive lava deposited across the region from the Pliocene through until Holocene (Hills 1975; Joyce *et al.* 2003), which could make 'overland' movement of fish between river basins easier (e.g. lateral connections during wetter periods). In contrast, much of Eastern region is topographically more diverse close to the coastline, making overland movements more difficult. Third, a large proportion of the Eastern region between Victoria and Flinders Island/Tasmania is only available as habitat during low sea levels (Figs 1 and 3). Fourth, as a distinct species, *Nannoperca* 'flindersi' may differ ecologically from its western congener, leading to increased divergence.

Concordance between mitochondrial and nuclear data sets

With two exceptions, our independent molecular assessments of phylogeographic structure were generally concordant in recovering the same broad phylogeographic structure in both species, namely separate Anson and Eastern lineages in *N.* 'flindersi' and distinct Murray, Bass, and Corangamite lineages in *N. australis*. Minor discordance was evident in the phylogeographic affinities of the most easterly site (pop. 72) in *N.* 'flindersi', which was a distinctive third lineage based on nuclear data but just another Eastern population in the mtDNA gene tree. Unexpectedly, however, there was a stark discrepancy between the two data sets in their ability to define a distinctive Western lineage in *N. australis*. In particular, the Western mtDNA clade was the most divergent of the four mitochondrial clades evident in this species (Figs 4 and 5), whereas the allozyme analyses found no evidence to support the existence of a simple phylogeographic dichotomy between Western and Bass lineages (Fig. 7A), despite finding moderate levels of genetic heterogeneity in the composite Western/Bass

metapopulation (Table S4). Given how comprehensive both data sets are, this discrepancy requires some examination.

Discrepancies between matrilineal and nuclear gene trees are common in intraspecific phylogeographic studies (Toews & Brelsford 2012) and can reflect a variety of underlying causes such as selection, stochastic lineage sorting, gender specific differences in dispersal ability and parental genetic effects (Avise 2004; Knowles & Chan 2008; Toews & Brelsford 2012; Yoshizawa *et al.* 2012). Although we are unable to determine herein which of these factors might be operating, we anticipate that multiple causes are involved, given the breadth of geographic coverage and presence of several disjunct zones of overlap/hybridisation between lineages and clades. Regardless of what these causal factors are, it is indisputable that the strong phylogeographic signal revealed by the mtDNA data demonstrates unequivocally that Western clade populations ought to be regarded as a distinct phylogeographic entity.

Drainage divide crossings

We found genetic evidence, based on the presence of shared or almost identical haplotypes and/or admixtures of mtDNA haplotypes and allozyme alleles (Table 1 and Table S4, Supporting information, Figs 4 and 7), for five exchanges across drainage divides; two across the major drainage divide separating southern and northern draining rivers in western Victoria and three across drainage divides between different coastal drainages. None of these exchanges are supported by clear evidence for drainage rearrangements based on the geological literature (e.g. Joyce *et al.* 2003). Instead, most are associated with regions that have little to no topographic differences across their drainage divide. It seems most likely that fishes have been able to swim across shallow drainage divides, presumably during very rare periods of extraordinary rainfall or flooding (e.g. Unmack 2001; Thacker *et al.* 2007, 2008).

At least two major drainage divide exchanges were identified. The first, within Western clade, was between Wimmera drainage (pops. 4–7) and either or both Glenelg (pops. 23–24) and Hopkins drainages (pops. 32, 33 and 35). The second was from Murray clade (Figs 1–3) into the southern draining Mt Emu Creek (pops. 35–36). Multiple crossings between southern draining rivers were also evident. Murray and Western clade individuals presumably moved from Mt Emu Creek (pops. 35–36) into Corangamite drainage (pops. 41, 43–44), while Corangamite clade haplotypes were exchanged between Corangamite (pop. 40) and Gellibrand drainages (pops. 38–39). Movement may have occurred between the Glenelg (pops. 23–24) and Hopkins drainages (pops. 32, 33 and 35) within Western clade, although this route could have alternatively been via Wimmera drainage. The last example was from *N.* 'flindersi' in LaTrobe drainage (pop. 67) into Tarwin drainage (pops. 55–57) in *N. australis* (Figs 3 and 4).

Hybridisation and introgression

The drainage divide crossings identified above all resulted in exchange between lineages and/or species. In some cases, the result is ongoing hybridisation between the invading and the resident lineages as reflected in their admixture of mtDNA and allozyme genotypes. In other cases, the result appeared to be a homogeneous genetic pattern within the geographic region involved, with all traces of former lineages that presumably occupied the habitat prior to invasion being lost. We have assumed that admixture reflects natural range expansion; nevertheless, our data cannot exclude anthropogenic disturbance (e.g. the so-called 'bait bucket' transfer, as observed for other Australian species; Lintermans 2004) as a means of dispersal in some cases.

Upper regions of the Tarwin River (pops. 55–57) contain a mixture of genotypes from *N. australis* and *N.* 'flindersi'. Most individuals had mtDNA haplotypes from *N.* 'flindersi' (Fig. 4). Allozyme data provide better evidence for hybridisation with PCO clearly demonstrating the intermediate position of fish from sites 55 and 57 (Fig. 6). The single population examined from a tributary to the lower Tarwin River (pop. 54) contained a pure population typical of Bass lineage (Fig. 7B). This demonstrates the introgression has not expanded throughout the entire Tarwin drainage.

Both Mt Emu Creek and portions of the Corangamite drainage show patterns of admixture among Murray, Western and Corangamite lineages. Mt Emu Creek (pops. 35–36) contained a mixture of Western and Murray lineage genotypes (Figs 4 and 7A). Woady Yallock drainage (pops. 43–44) had Murray mtDNA, Mundy Gully (pop. 41) had Western mtDNA and both displayed allozyme admixture between Murray and southern populations (Fig. 7A; Table S3, Supporting information).

Thompson Creek (pop. 48) was unusual within this study in that it contained three separate mtDNA lineages and had an allozyme profile more typical of Bass clade rather than Corangamite clade (Fig. 6). Of the seven individuals examined at cyt*b*, three had Bass clade haplotypes, two had Corangamite clade haplotypes and two displayed an identical haplotype to *Nannoperca obscura* from the same locality (Fig. 4, same haplotype as *N. obscura* 42 Gnarkeet). This latter result represents a secondary introgression that was originally from *N. australis* into *N. obscura* (around 1.1 Ma, 95% HPD of

0.7–1.5 Ma, Fig. 5B) and then recently back into *N. australis*. No evidence of nuclear introgression with *N. obscura* was found within *N. australis* from Thompson Creek, either in our allozyme data (14 diagnostic loci) nor in two other nuclear introns (472 bp from the first intron of S7 and 502 bp from the second intron of RAG1; Unmack unpub. data). Failure to identify nuclear alleles from *N. obscura* indicates this introgression was historic rather than contemporary, although the presence of a mtDNA haplotype identical to one found in sympatric *N. obscura* (Fig. 4) infers it occurred in the recent past. The origins of the Corangamite haplotypes found in Thompson Creek remain obscure. Intriguingly, these haplotypes differed by only one base pair from the most common haplotype found in Corangamite basin (Fig. 4), inferring a recent shared phylogeographic history. We can only speculate that perhaps Corangamite lineage was previously more widespread and has now mostly been replaced by Bass lineage.

Taxonomy and conservation

Molecular data from both matrilineal (Fig. 5) and nuclear genetic markers (Fig. 6) have demonstrated that *N. australis* and *N.* 'flindersi' represent two largely parapatric species and that each species is genetically heterogeneous across its geographic range. Within each species, the combined mtDNA and allozyme analyses broadly support the recognition of a major genetic dichotomy, that is, Murray clade vs. all others for *N. australis* and the Eastern vs. Ansons clade for *N.* 'flindersi' (Figs 4 and 5, Fig. S1, Supporting information). Within *N. australis*, there is also overall support for three other phylogeographic lineages, corresponding to the Western, Corangamite and Bass mtDNA clades, albeit with some admixture in zones of regional overlap. For simplicity, the discussion that follows concentrates primarily on regional groupings as defined by the data set that reveals the simplest geographic pattern, namely mtDNA.

Substantial genetic structuring is present within each species. This heterogeneity is likely to have important conservation and biological implications (Frankham *et al.* 2010); therefore, it deserves to be recognized more formally. Here, we apply the twin concepts of 'Evolutionarily Significant Units' (ESUs) and 'Management Units' (MUs) to both species, based on Moritz (1994).

The most consistent differentiation within *N. australis* for both mtDNA and allozymes is the distinctiveness of Murray clade relative to coastal clades. This outcome argues conservatively for the recognition of Murray clade populations as one ESU and remaining *N. australis* clades as the coastal ESU. This distinction mirrors apparent differences in breeding biology (Humphries 1995) plus will focus conservation attention on Murray populations, all of which have been in rapid decline (Hammer *et al.* 2009). The primary dichotomy within *N.* 'flindersi' warrants recognition of an Ansons ESU from northeast Tasmania and an Eastern ESU encompassing remaining populations from eastern Flinders Island and Gippsland, Victoria.

The decision as to whether additional ESUs or multiple MUs can be defined in either species is problematic as the nuclear data provide only modest evidence that the major phylogeographic breaks among populations within *N.* 'flindersi' or *N. australis* ESUs (Figs 4–7, Fig. S1, Supporting information) correspond to these specific geographic patterns. Importantly, many individual populations have significantly different allele frequencies at numerous allozyme loci from those in adjacent rivers (Table S4, Supporting information), suggesting such populations would merit designation as distinct MUs under the definition employed herein. As a compromise, we suggest that an appropriate starting point is to recognize all 'pure' (i.e. geographically contiguous) mtDNA clades (i.e. Eastern, Ansons, Bass, Corangamite and Western) as MUs, while acknowledging that considerable nuclear genetic heterogeneity exists within most of these MUs.

Conclusions

Despite widespread acceptance of the importance of sea water barriers in determining freshwater biogeographic patterns (Myers 1938; Banarescu 1990; Unmack 2001), few studies have specifically addressed hypotheses relating to sea-level changes. This study critically tests whether major differences in continental shelf width during the Pleistocene predict within-region levels of genetic divergence in an obligate freshwater species. Our results recovered only limited support for our continental shelf width hypothesis (Fig. 1, Table 2), although patterns within Bass clade were largely congruent with reconstructed low sea-level drainage patterns (Fig. 3). By inference, discordant outcomes for the other three regions imply that continental shelf width is not influential on levels of genetic divergence in southern pygmy perches during episodes of lowered sea levels. Having established a clearer framework for testing these hypotheses, additional studies are now required across multiple taxa within regions as well as in contrasting geographic settings in other parts of the world, to determine the generality of our results.

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Data accessibility

Details of the primers used to amplify mtDNA genes: uploaded as online supporting information.

Table with p-distances between and within clades: uploaded as online supporting information.

DNA haplotype sequences: Genbank accessions KC285906–KC286129.

Genbank numbers for each haplotype: uploaded as online supporting information.

Cytochrome *b* sequences from all individuals: DRYAD entry doi:10.5061/dryad.9v852.

All DNA data sets used in our phylogenetic analyses: DRYAD entry doi:10.5061/dryad.9v852.

Raw allozyme data at 23 allozyme loci for 72 *Nannoperca* populations: DRYAD entry doi:10.5061/dryad.9v852.

Phylogenetic trees from our DNA analyses: TREEBASE Study accession no. 13674.

Allele frequencies at 23 allozyme loci for 72 *Nannoperca* populations: uploaded as online supporting information.

Summary of the statistically significant differences in allele frequency: uploaded as online supporting information.

Unrooted neighbor-joining tree of allozyme data for *Nannoperca* 'flindersi': uploaded as online supporting information.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 GenBank numbers for (A) all cytochrome *b* sequences used in the study, (B) the mitochondrial sequences used in the phylogenetic study.

Table S2 Between clade cytochrome *b* average p-distances for eight *Nannoperca* clades and the outgroups.

Table S3 Allele frequencies for *Nannoperca australis* and *N.* 'flindersi' at 23 allozyme loci for 72 populations.

Table S4 Summary of the number of statistically significant differences in allele frequency detected among pairwise comparisons of populations for various hierarchical levels of geographical relationship in southern pygmy perch.

Fig. S1 Unrooted neighbor-joining tree of allozyme data for *Nannoperca* 'flindersi,' depicting the phylogenetic affinities among 10 sites, based on unbiased Nei Ds.

Fig. S2 Details of the PCR primers used to amplify mtDNA genes.