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Species delineation, phylogeography and conservation of temperate perches (Actinopterygii: Percichthyidae) from an endemism and climate change hotspot

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ABSTRACT

Context. The south-western corner of Australia is a biodiversity hotspot that includes a freshwater fauna with a high proportion of endemic species. The temperate perches comprise nearly half of the obligate freshwater fishes of the region, representing important components of local ecosystems and are of significant conservation concern. **Aims.** Provide a spatially comprehensive molecular genetic assessment of species boundaries and major substructure for all local members of the family to better understand the interplay of ecology and environment across a common landscape. **Methods.** Nuclear markers (allozymes) and matrilineal (*cytb*) datasets were generated to infer genetic groupings and any instances of hybridisation or introgression in relation to the current taxonomy, regional geography and ecological understanding. **Key results.** There were contrasting patterns of diversification across genera, with *Nannoperca* housing four likely species-level splits, *Nannatherina* having three distinct geographically and ecologically separated subpopulations, and *Bostockia* comprising several refugial subpopulations that appear partially introgressed. Repeated genetic patterns were identified across particular biogeographic features, most notably the Margaret River and Shannon River. **Conclusions.** This study highlighted the value of comparative range-wide molecular studies to inform taxonomy, ecology and conservation planning. **Implications.** These analyses pave the way for taxonomic revision, management of key habitat refuges, and other conservation actions.

Keywords: allozymes, aquatic biodiversity, biogeography, conservation genetics, cryptic species, freshwater fishes, mtDNA, species delineation.

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Introduction

Freshwater habitats support high biodiversity, but also face significant and increasing threatening processes, with, for example, one quarter of all freshwater fishes now being considered at risk of extinction (Arthington *et al.* 2016; Liu *et al.* 2017; Reid *et al.* 2019; Sayer *et al.* 2025). The temperate perches (Percichthyidae) are a group of obligate freshwater fishes of Australia and South America, being diverse in terms of distribution patterns, physical attributes and ecology (McDowall 1996; GR Allen *et al.* 2002; Arratia and Quezada-Romegialli 2019), and are of special conservation concern, with an alarming number of species (55%) being considered threatened with extinction (Lintermans *et al.* 2020; International Union for Conservation of Nature 2024). The family contains 22 valid species in 8 genera (Jerry *et al.* 2001; Near *et al.* 2012; Fricke *et al.* 2024), ranging from small (<10 cm) habitat specialists of streams and wetlands to large (>1 m) iconic top-order predators in riverine and floodplain habitat, and including species of significant cultural and recreational value (Lintermans 2007; Saddlier *et al.* 2013; Humphries 2023). Detailed molecular studies have found many taxa within this group to harbour cryptic species, some now having been formally described and others yet to receive taxonomic attention (Musyl and Keenan 1992; Ruzzante *et al.* 2006; Nock *et al.* 2010; Morgan *et al.* 2013; Unmack *et al.* 2013; Hammer *et al.* 2014; Buckley *et al.* 2018). Moreover, molecular data have also

revived ongoing contentions around higher-level classification, such as the status of some genera, and resolution of two diadromous species now considered to belong to their own, yet to be defined, family (Jerry *et al.* 2001; Betancur-R *et al.* 2017; Arratia and Quezada-Romegialli 2019).

The bulk of the Percichthyidae in Australia includes 13 valid species in the temperate south-east of the continent, predominantly in close association with the Great Dividing Range, but with singular outliers in the Wet Tropics (small habitat pocket) and drier inland Lake Eyre Basin (Pusey and Kennard 2001; GR Allen *et al.* 2002; Beheregaray *et al.* 2017). A smaller contingent also occurs in the Mediterranean climatic region of south-western Australia, represented by three genera and four locally endemic species (Morgan *et al.* 2011). The monotypic nightfish (*Bostockia porosa* Castelnau, 1873), is the largest of these (up to 17 *v*. <10 cm for other perches in the region), being widespread across varied habitats and considered Near Threatened (Morgan 2020). The western pygmy perch (*Nannoperca vittata* Castelnau, 1873), is also a widespread generalist species that is considered stable but Vulnerable, having undergone major historical declines following land clearance and secondary salinisation (Morgan *et al.* 2003; Beatty *et al.* 2011; Morgan 2019). This contrasts markedly with two more restricted and narrow-range habitat specialists, the little pygmy perch (*Nannoperca pygmaea* Morgan, Beatty & Adams, 2013), and Balston's pygmy perch (*Nannatherina balstoni* Regan, 1906), both considered Endangered because of either a range reduction or extent of occupancy as well as ongoing localised threats in stream and seasonal wetland habitats (Morgan *et al.* 1995, 2014*a*; Beatty *et al.* 2011; Beatty and Morgan 2019; Morgan and Beatty 2019; MG Allen *et al.* 2020).

The south-western corner of Australia is a unique, yet precarious, environment for freshwater biota. It has been historically well isolated from other aquatic systems as essentially a small fringe of stable coastal habitat in a higher-rainfall zone enveloped by hot arid conditions and the Southern and Indian Oceans. The region, consequently, boasts very high species diversity and endemism, being globally recognised as a biodiversity hotspot (Myers *et al.* 2000). This is largely based on terrestrial attributes, with the species richness of aquatic fauna low in comparison, but still displaying high endemism, especially for groups more tied to a reliance on aquatic habitat; namely obligate freshwater fishes (9 native species, 100% endemism), decapod crayfishes (n = 14, 100%), frogs (n = 27, 97%), turtles (n = 2, 100%), caddisflies, stoneflies and mayflies (n = 91, 75%), and isopods and amphipods (n = 28, 82%) (see full review in Davies and Stewart 2013). Although the regional context appears to have promoted endemism, the same geographic fixation and isolation leaves the biota susceptible to anthropogenic perturbations. In particular, the interplay of rapid climate change combined with the increasing industry of human land-use is seen as a significant threat to south-western freshwater fishes, with major declines in stream flow concomitant to reduced rainfall already observed (Morrongiello *et al.* 2011; Beatty *et al.* 2014; Ogston *et al.* 2016; MG Allen *et al.* 2020) and even more likely in the future as a region under elevated risk (Tims and Saupe 2023; Buckley *et al.* 2024).

Limited previous systematic treatment has been undertaken on the Percichthyidae within south-western Australia, with the alpha taxonomy being largely stable since the early 1900s (McCulloch 1929; Kuiter and Allen 1986; GR Allen *et al.* 2002). Broad work on a molecular phylogeny to understand continental east–west relationships affirmed the local genera and species endemism and flagged initial insights on cryptic diversity present as two major lineages within *Nannoperca* (Unmack *et al.* 2011). *Nannoperca pygmaea* was only recently discovered during environmental monitoring surveys as an obviously distinct phenotypic form hidden in a discrete area, and which was subsequently investigated and described (Morgan *et al.* 2013) using a combined-lines-of-evidence approach considering molecular, morphological and ecological data (Page *et al.* 2005; Hammer *et al.* 2013, 2018). This species was also included in subsequent phylogenomic assessments of pygmy perches (Buckley *et al.* 2018, 2024), as one of four proposed candidate species across the two major lineages previously observed (Unmack *et al.* 2011), with the selection of populations included by Buckley *et al.* (2018, 2024) being guided by the unpublished results of the current study. Herein, we present the first comprehensive spatial genetic assessment of all south-western percichthyids to map species boundaries and contemporary genetic connectivity, within a comparative focus to assess how levels and patterns of genetic divergence might be explained by ecological traits across genera and groups (Tibbets and Dowling 1996; Hughes *et al.* 2009; Hammer *et al.* 2019*a*). The baseline of ecological data to draw comparative observations from is significant owing to a sustained 30-year research program in the region (e.g. Morgan *et al.* 1998, 2004, 2011; Morgan and Gill 2000; Beatty *et al.* 2011, 2014; MG Allen *et al.* 2020).

The objectives of this study are to undertake a specific assessment of species boundaries across the south-western Australian percichthyids, and document patterns of divergence in relation to geography, including any signs of hybridisation or introgression (common in freshwater fishes and recorded in percichthyids: Unmack *et al.* 2011, 2013; Valenzuela-Aguayo *et al.* 2022). This study employs a combined molecular approach using multiple, moderately conservative nuclear genetic markers (allozyme loci) and a matrilineal gene (mitochondrial DNA, mtDNA; gene cytochrome *b*, *cytb*) to assess genetic and taxonomic diversity with a geographically comprehensive dataset. The generation of these data aims to pave the foundations for somewhat urgent conservation programs and follow-up studies in a biodiverse region experiencing increasing anthropogenic change.

Materials and methods

Sampling

The hydrological organisation for surface waters followed here is the Australian Hydrological Geospatial Fabric, which groups smaller single catchments or sub-basins into larger river basin reporting units (Atkinson *et al.* 2008). The temperate south-west of Australia is an expansive region occupying a coastal fringe of temperate river, stream and wetland habitats comprising three main sections relevant to obligate freshwater fish habitat. First, there is a series of 'northern' river basins running ~500 km, draining east–west to the coast, largely disembarking from the Darling Range, from the Arrowsmith River (Greenough River Basin) in the north, through the large Swan Coast–Avon River Basin covering the major city of Perth, and south to the Collie–Preston River Basin; there is a second series of ~200 km 'southern' flowing river basins between the Donnelly River Basin and the Angove River (Albany Coast River Basin); and wedged in between these regions is the small Busselton Coast River Basin (including the prominent Margaret River catchment), which occurs on a small peninsula (western flowing), plus the Blackwood River, which extends well inland and divides both northern and southern regions (Fig. 1). The region experiences a Mediterranean climate with strong seasonality in rainfall and stream flow (winter–spring dominated) that is regionally variable (mean annual rainfall ranges between 500 and 1200 mm) and highest along the southern coast (Morgan *et al.* 2002, 2014*b*; Buckley *et al.* 2024).

Percichthyids were sampled on multiple trips over 15 years by using a combination of methods from passive (traps and fyke nets) and active (dip net, seine net, backpack electrofishing) gear types. Sampling covered the extant range of all south-western percichthyids on the basis of review of historic records, baseline sampling and detailed regional investigations (e.g. Morgan *et al.* 2010, 2013, 2014*b*; Murphy 2010). Sampling effort thus represented a combination of dedicated surveys and value-adding to other projects to build up a bank of tissues for genetic assessment. Sampling was spatially comprehensive across the region, with material collected from some 81 sites between 1999 and 2014, representing different combinations of the three genera at each (Fig. 1, Table 1).

All sampling was approved by the participants' Animal Ethics committees under approval codes W2237/09, RW2521/12 and RW2793/15 (Murdoch University) plus IACUC 07-0403 (Brigham Young University), and conducted under wildlife collecting permits SF006431 and SF007216. Ethical approval was not required by the SA Museum because all samples were sourced from existing tissue collections and involved fish that were ethically collected under valid collecting permits. Most tissues (either whole fish or a portion of muscle from the anterior flank on one side) were snap frozen in liquid nitrogen for subsequent ultra-cold storage as part of the South Australian Museum's Australian Biological Tissues Collection (ABTC). A few tissues were available only by preservation in 100% ethanol (and therefore not useable for allozymes). In most cases, formalin-fixed fish, either the same individual sampled for muscle or representatives from the same site, were retained as matching voucher specimens and lodged with the Western Australian and South Australian Museums or held in the Murdoch University reference collection. All vouchers can be identified on the basis of their field code (Table 1).

Choice of nuclear markers

Seeing as this study was conceived in the early 2000s and commenced in 2011 after suitable tissues became available (with an early pilot study: Hammer 2001), we chose allozymes as our primary nuclear genetic markers. Although largely supplanted by genomic datasets since then, allozymes have a long and successful history as reliable and proven genetic markers for delineating species, detecting between-species admixture and identifying major phylogeographic breaks within species (Richardson *et al.* 1986; Horner and Adams 2007; Adams *et al.* 2014). Attesting to this, allozyme datasets have provided comparable insights to a companion genomic dataset for these three above-mentioned systematic endeavours in every study we have undertaken or tracked thus far (Blom *et al.* 2016; Unmack *et al.* 2017, 2019, 2022, 2023; Thacker *et al.* 2022; Mossop *et al.* 2023; M. Adams and P. J. Unmack, unpubl. data), and can occasionally even match genomic data for showing fine-scale population structure (e.g. Jusaitis and Adams 2005; Brooks *et al.* 2022). Most importantly, allozymes have been shown to be pivotal in the identification of more than 50 candidate species thus far among the Australian freshwater fish fauna alone (Adams *et al.* 2023) and the systematic and phylogeographic framework they provide allows any follow-up genomic studies to optimally target key individuals and populations during sampling design.

Allozyme analyses

Three separate allozyme studies were undertaken, one for each percichthyid genus. Because it can be problematic to detect their hybrids *a priori* and distinguish juvenile *Nannoperca* from *Nannatherina*, each of these two studies also 'accidentally' included several examples of the other genus or a single F_1 hybrid. Excluding these individuals, the allozyme sample sizes for each study were as follows: *Nannoperca*, 359 individuals from 67 sites; *Nannatherina*, 89 individuals from 21 sites; and *Bostockia*, 97 individuals from 38 sites (Fig. 1, Table 1).

Allozyme electrophoresis of muscle homogenates was conducted on cellulose acetate gels (Cellogel), according to standard principles and procedures (Richardson *et al.* 1986). All individuals were screened for the full suite of enzymes or

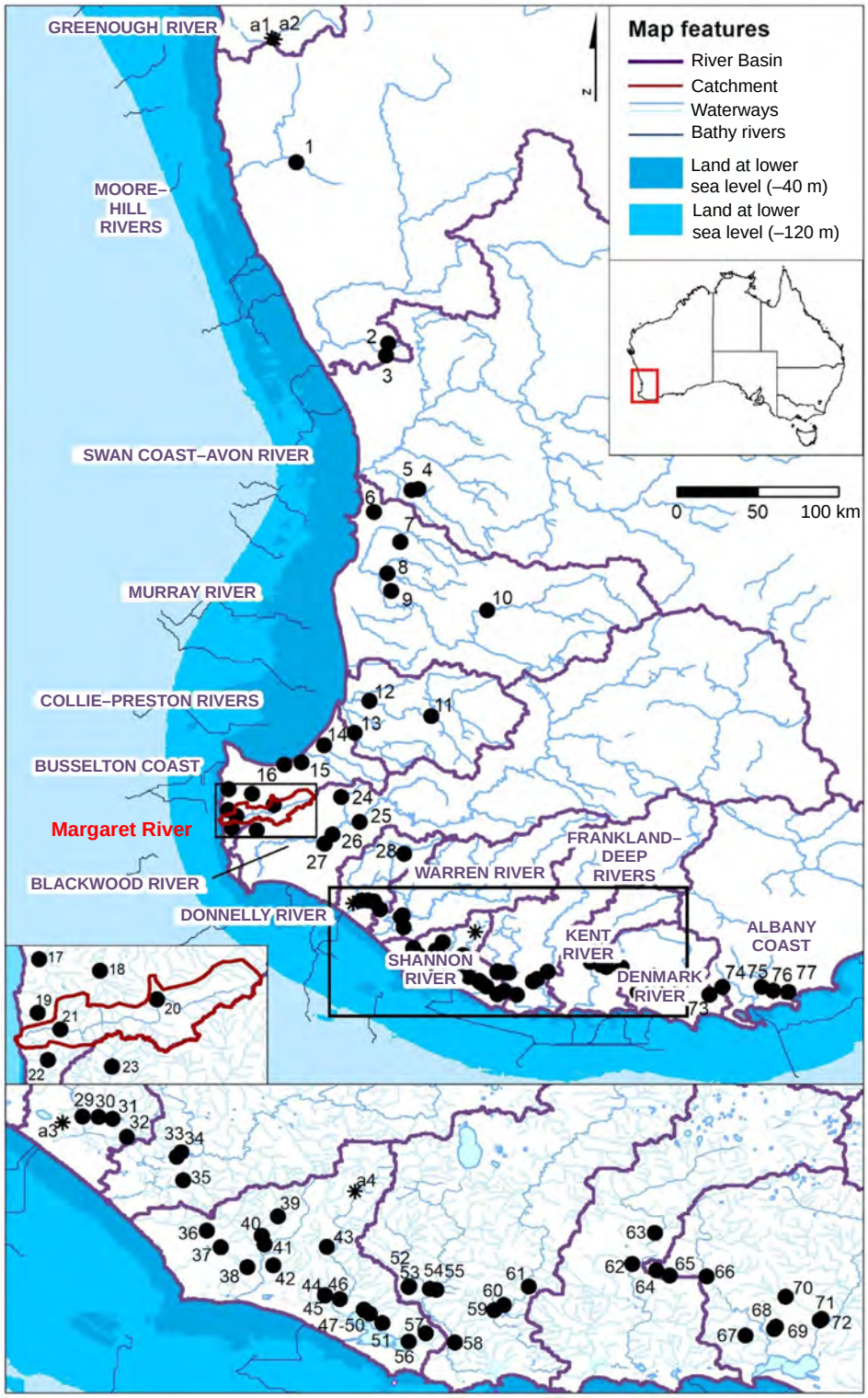

Fig. 1. Map of sampling sites for south-western Australia's percichthyids that were subjected to combined allozyme and mitochondrial DNA (mtDNA) genetic analysis (asterisks show that Sites a1–4 represent alcohol-preserved tissues only, i.e. mtDNA exclusive assessment). The hydrological framework of catchments grouped into river basins follows the Australian Hydrological Geospatial Fabric (Atkinson *et al.* 2008). Past sea levels are approximated from bathymetric data (General Bathymetric Chart of the Ocean 2024) and bathy rivers are reconstructed drainage patterns at low sea level after Unmack (2001).

Table 1. Site, taxon and sampling details for all percichthyids examined in this study.

Site	ABTC site code	Year	River Basin	Locality	Latitude	Longitude	NP	VWI	VMA	VSH	NTHB	BP
1	DM10-HI	2010	Moore–Hill	Hill River	−30.3050	115.4110						5/2
2	PU09-30	2009	Moore–Hill	Gingin Brook	−31.3120	115.9230		5/5				5/1
3	DM09-m04	2009	Moore–Hill	Lennard Brook	−31.3800	115.9100		3/3				
4	MA08-64	2008	Swan Coast–Avon	Canning River	−32.1229	116.0895		5/5				
5	DM02-m01	2002	Swan Coast–Avon	Canning River #2	−32.1300	116.0500		3/1				3/2
6	MA08-69	2008	Murray	Brunswick River	−32.2504	115.8414		5/5				
7	MA08-68	2008	Murray	Dirk Brook	−32.4173	115.9882		3/3				1/1
8	MA08-67	2008	Murray	South Dandalup River	−32.5923	115.9166		5/5				
9	MA08-66	2008	Murray	Murray River tributary	−32.6900	115.9375		3/3				
10	PU09-36	2009	Murray	Hotham River	−32.7990	116.4720		1/1				
11	PU09-60	2009	Collie–Preston	South Collie River	−33.3870	116.1630		5/5				
12	MA08-70	2008	Collie–Preston	Collie River	−33.3027	115.8176		5/5				
13	MA08-72	2008	Collie–Preston	Preston River	−33.4802	115.7335		5/5				3/1
14	MA08-73	2008	Busselton Coast	Capel River	−33.5505	115.5643						1/1
15	MA08-75	2008	Busselton Coast	Abba River	−33.6462	115.4382		5/5				2/1
16	MA08-78	2008	Busselton Coast	Vasse River	−33.6571	115.3422		5/4				4/1
17	MA08-80	2008	Busselton Coast	Wilyabrup Brook	−33.7941	115.0313		5/4				
18	JM12-CACA	2012	Busselton Coast	Carbunup Brook	−33.8191	115.1623		4				
19	JM12-ELCA	2012	Busselton Coast	Ellen Brook	−33.9093	115.0286		1				3
20(A)	PU09-58	2009	Busselton Coast	Canebrake Pool 2009	−33.8810	115.2830			4[A]/4		1/1	3/1
20(B)	JM12-CBMA	2012	Busselton Coast	Canebrake Pool 2012	−33.8810	115.2830			8		3/1	5
21	MA08-79	2008	Busselton Coast	Margaret River	−33.9446	115.0768			7/7			1/1
22	JM12-BOST	2012	Busselton Coast	Boojidup Brook	−34.0106	115.0500						3
23	JM12-UCBDA	2012	Blackwood	Upper Chapman Brook	−34.0241	115.1879		4				3
24	JM12-MILBAK	2012	Blackwood	Mill Brook	−33.8376	115.6597		4				3
25	MA08-81	2008	Blackwood	Blackwood River	−33.9760	115.7620						4/2
26	JM12-MIBL	2012	Blackwood	Milyeannup Brk	−34.0454	115.6104		4			6	3
27	PU09-53	2009	Blackwood	Milyeannup Brook	−34.0980	115.5680		4/4			1/1	4/1
28	MA08-82	2008	Donnelly	Donnelly River	−34.1546	116.0088		3/3				
29	PU09-52	2009	Donnelly	Donnelly River #2	−34.4150	115.7720		4/4				
30	JM12-CARDO	2012	Donnelly	Carey Brook	−34.4158	115.8100		1				1
31	JM12-BEEDO	2012	Donnelly	Beedilup Brook	−34.4199	115.8422						1
32	JM12-FLYD	2012	Donnelly	Fly Brook	−34.4623	115.8751		5				1
33	PU09-51	2009	Warren	Treen Brook	−34.4970	116.0020		5/5				
34	JM12-WARV	2012	Warren	Warren River	−34.5078	115.9912		1				3
35	JM12-DOWA	2012	Warren	Dombakup Brook	−34.5635	116.0064		4				2
36	PU09-50	2009	Shannon	Meerup River	−34.6800	116.0610		10/5				1
37	PU09-49	2009	Shannon	Doggerup Creek	−34.7190	116.0930				5/5		3/1
38	MH00-m14	2000	Shannon	near Chesapeake Brook	−34.7650	116.1560				7/2	2/2	3/2
39	PU09-48	2009	Shannon	Boorara Brook	−34.6470	116.2270		4/3				3/1
40	JM12-BOOGA	2012	Shannon	Boorara Brook #2	−34.6922	116.1887		5				3
41	JM12-CAGAR	2012	Shannon	Canterbury River	−34.7120	116.1950		5				1
42	JM12-BUGA	2012	Shannon	Buldania Creek	−34.7604	116.2162		5				3

(*Continued on next page*)

Table 1. (*Continued*).

Site	ABTC site code	Year	River Basin	Locality	Latitude	Longitude	NP	VWI	VMA	VSH	NTHB	BP
43	JM12-SHNE	2012	Shannon	Shannon River #2	−34.7180	116.3401						1
44	JM12-CHCH	2012	Shannon	Chesapeake Brook #1	−34.8305	116.3359		6		7	2	2
45	JM13-CHBI	2013	Shannon	Chesapeake Brook #2	−34.8305	116.3362		4		7	10	
46	JM13-SHBI	2013	Shannon	Shannon River #3	−34.8388	116.3706		5		2	4	
47	JM13-4THBI	2013	Shannon	Fourth River	−34.8636	116.4259		18		4	4	
48	JM12-4THR	2012	Shannon	4th River	−34.8636	116.4259		10		4	1	1
49	JM13-KIBI	2013	Shannon	Kingsman River	−34.8656	116.4288				7		
50	MH00-m15	2000	Shannon	Broke Inlet	−34.8730	116.4400					2	
51	JM13-BIBI	2013	Shannon	Big Creek	−34.8946	116.4691		2		1	3	
52	MH00-m16	2000	Shannon	Deep River	−34.8110	116.5300				3/3	4/1	
53	JM13-BPDE	2013	Shannon	Beardmore Pool	−34.8085	116.5319		2		12[B]	10	
54	JM12-WEBE	2012	Shannon	Weld River	−34.8150	116.5793		5			5	1
55	JM12-DEFE	2012	Shannon	Fernhook Falls	−34.8170	116.5951		1				1
56	JM13-INBI	2013	Shannon	Inlet River	−34.9380	116.5307				1	2	
57	JM12-IRSWH	2012	Shannon	Inlet River #2	−34.9184	116.5697				3	6	3
58	JM12-DERXRA	2012	Shannon	Deep River #2	−34.9396	116.6380		5				
59	JM13-ELFA	2013	Frankland–Deep	Elsie Brook	−34.8643	116.7292		13			5	
60	JM13-WEFA	2013	Frankland–Deep	Wedding Brook	−34.8523	116.7519		1				
61	JM12-FACA	2012	Frankland–Deep	Frankland River	−34.8102	116.8097		7				
62	JM12-KEMI	2012	Kent	Kent River	−34.7572	117.0499		5				
63	DM14-m03	2014	Kent	Kent River #2	−34.6857	117.1027	7					
64	DM14-m01	2014	Denmark	Dennmark River	−34.7718	117.1053	1					
65	DM14-m02	2014	Denmark	Dennmark River #2	−34.7841	117.1365	6					
66	JM13-DENM	2013	Denmark	Denmark River #3	−34.7866	117.2231					6	
67	JM12-SCLI	2012	Denmark	Scotsdale Brook	−34.9232	117.3124		2				
68	DM09-m03	2009	Denmark	Quickup River	−34.9090	117.3790		3/3				
69	DM09-m02	2009	Denmark	Quickup Dam	−34.9030	117.3840		3/3				
70	PU09-37	2009	Denmark	Mitchell River	−34.8340	117.4060		3/3				
71	DM09-m01	2009	Denmark	Mitchell River #2	−34.8880	117.4860	8[C]/7	5/5			4/2	5/3
72	JM13-MTHR	2013	Denmark	Hay River	−34.8847	117.4894					6	
73	PU09-38	2009	Denmark	Marbelup Brook	−34.9400	117.7120		5/5				2
74	PU09-41	2009	Albany Coast	King River	−34.8950	117.7830		4/4				
75	MA08-85	2008	Albany Coast	Kalgan River	−34.8958	118.0023		4/4				
76	MH04-m02	2004	Albany Coast	Goodga River	−34.9170	118.0670		3/3				
77	MH04-m01	2004	Albany Coast	Angove River	−34.9230	118.1520		3/3			1	
a1	DM10-m02	2010	Greenough	Arrowsmith #1	−29.6184	115.2904		0/2				
a2	DM10-m03	2010	Greenough	Arrowsmith #2	−29.5170	115.4410		0/1				
a3	DM14-SMI	2014	Donnelly	Lake Smith	−34.4291	115.7260	0/5					
a4	PU99-SHA	1999	Shannon	Shannon River	−34.5887	116.4063						0/1
							22/12	255/119	19/11	62/10	89/9[D]	97/23

Taxon abbreviations as follows: NP, *Nannoperca pygmaea*; VWI, VMA and VSH, codes designated in the text for the three candidate species of *Nannoperca*; NTHB, *Nannatherina balstoni*; BP, *Bostockia porosa*. Sample sizes per site are shown as allozymes/*cytb* (a single number means that no *cytb* sequences were generated).
[A]A single *Nannatherina balstoni* was also included in the *Nannoperca* allozyme study.
[B]Includes a single (VSH × VWI) × VSH backcross.
[C]One *N. balstoni* and one *N. balstoni* × *N. pygmaea* F_1 fish were also included in the *Nannoperca* allozyme study.
[D]Includes a Genbank sequence with no identifiable locality.

non-enzymatic proteins employed in our previous studies on percichthyids (Hammer *et al.* 2010; Unmack *et al.* 2011, 2013; Morgan *et al.* 2013). Details of enzyme and locus abbreviations, enzyme commission numbers, electrophoretic conditions, stain recipes and allozyme nomenclature are presented in these papers or in Richardson *et al.* (1986).

For each generic dataset, the initial analysis involved the use of stepwise principal co-ordinates analysis (PCA) on individuals to identify discrete genetic lineages and any instances of likely admixture, independent of other taxonomic or geographic expectations. This procedure is always coupled with a comparison of the raw allozyme profiles of each major PCA cluster to assess cluster diagnosability (multiple fixed differences in sympatry being an unequivocal indication of the presence of two species at that site), detect obvious genetic heterogeneity at individual sites, and identify likely hybrids and individuals with significant admixture. The methods and protocols for conducting these stepwise PCAs are detailed elsewhere (Horner and Adams 2007).

Informed by our PCA characterisations of individuals, unique taxon–site combinations and primary genetic lineages, subsequent analyses for each genus involved calculating pairwise matrices of genetic distance and divergence among the chosen operational taxonomic units (whether individuals, sites, lineages or taxa). For assessments of species boundaries and lineage distinctiveness, we used the number of fixed differences (FDs) as the appropriate measure of genetic divergence and diagnosability. Consistent with the standard practice in morphological taxonomy of employing diagnostic characters that share character states at low frequency (Hammer *et al.* 2007; Horner and Adams 2007), our FD calculations allowed up to a 10% tolerance for the combined frequency of any rare shared alleles (as advocated by Adams *et al.* 2014).

We used two approaches to explore within-species genetic substructure for our ecological comparisons. Following standard methods (see Hammer *et al.* 2007; Horner and Adams 2007; Unmack *et al.* 2011), we constructed unrooted neighbour joining (NJ) networks by using Nei's unbiased distance among sites (for species with adequate sample sizes at most sites, herein *Nannoperca* taxon 'VWI') or Rogers' genetic distance among individuals (for *Nannatherina* and *Bostockia*). We also used *STRUCTURE* (ver. 2.3.4, see https://web.stanford.edu/group/pritchardlab/structure.html; Pritchard *et al.* 2000) to provide an independent assessment of population structure in all widespread candidate species plus one pair of parapatric taxa, with each input dataset limited to only those allozyme loci that were polymorphic in that species–taxon pair, after excluding loci with only rare alleles (i.e. sum of all rare alleles <0.05 in the metapopulation and <0.10 for each PCA and NJ cluster). Each *STRUCTURE* run employed the default settings for an admixture model with correlated allele frequencies and consisted of five replicates each for $K = 1$–8, with burn-in of 200,000 and 1,000,000 iterations. The results were then uploaded to the *CLUMPAK* website (see http://clumpak.tau.ac.il/; Kopelman *et al.* 2015) to provide estimates of the optimum K by using the ΔK method (Evanno *et al.* 2005) plus the raw input files required by *CLUMPP* (ver. 1.1.2, see https://rosenberglab.stanford.edu/clumpp.html; Jakobsson and Rosenberg 2007) to produce an optimum bar plot for each value of K. It should be noted here that, compared with genomic datasets, the numbers of informative allozyme loci are too low to provide any rigorous or detailed assessment of population structure in any taxon. Nevertheless, these initial explorations will help guide any future genomic studies aiming to provide such insights.

Mitochondrial DNA

For our matrilineal marker, we chose the *cytb* gene to build on an existing and informative dataset already available for pygmy perches (Unmack *et al.* 2011, 2013). In addition to sampling the geographic range of each genus, the choice of individuals to sequence was initially informed by the lineages identified in the allozyme datasets plus some preliminary mtDNA sequences (control region; Morgan *et al.* 2010; Murphy 2010), and, ultimately, by the phylogenetic and phylogeographic insights evident after the initial round of sequencing was completed for each group. Thus, there were considerably more *cytb* sequences generated for *Nannoperca* (four candidate species, structure evident within two of these) compared with the other two genera (little matrilineal structure evident and minimal or no support for the allozyme subpopulations). The final sample sizes for each genus were as follows (including sequences already on GenBank): *Nannoperca*, 152 individuals from 38 sites; *Nannatherina*, 9 individuals from 6 sites; and *Bostockia*, 23 individuals from 18 sites. All sequences were checked by amino acid coding to test for unexpected frame shift errors or stop codons.

All details regarding DNA isolation, amplification, sequencing and sequence editing are presented in Unmack *et al.* (2013). Phylogenetic analyses of the final sequence dataset employed maximum likelihood (ML), as implemented using *IQ-TREE* (ver. 1.6.12, see http://www.iqtree.org/; Nguyen *et al.* 2015) run on the *W-IQ-TREE* server (see http://iqtree.cibiv.univie.ac.at/; Trifinopoulos *et al.* 2016). These analyses employed the model selection procedure of *IQ-TREE* (-m TEST; Kalyaanamoorthy *et al.* 2017), resulting in the selection of the TIM2 + F + I + G4 model, followed by 10,000 replicates of ultrafast bootstrapping (Hoang *et al.* 2018).

Results

Identification of primary taxa and lineages in *Nannoperca*

The final nuclear dataset for *Nannoperca* comprised the allozyme genotypes of 359 fish at 57 putative loci. For simplicity, we have employed the following informal nomenclature throughout for

the three diagnosable taxa currently assigned to *N. vittata sensu lato*: VWI, *vittata* 'widespread'; VMA, *vittata* 'Margaret River catchment'; and VSH, *vittata* 'Shannon River Basin'. The allozyme profiles and heterozygosity measures for all pure taxa and two 'hybrid' individuals, along with a reference profile for *N. balstoni*, are presented in Supplementary Table S1.

An initial PCA on all individuals (Fig. 2*a*) displayed a primary split in the first dimension between taxa VWI + VMA (Lineage I) *v.* taxon VSH + *N. pygmaea* (Lineage II). Also

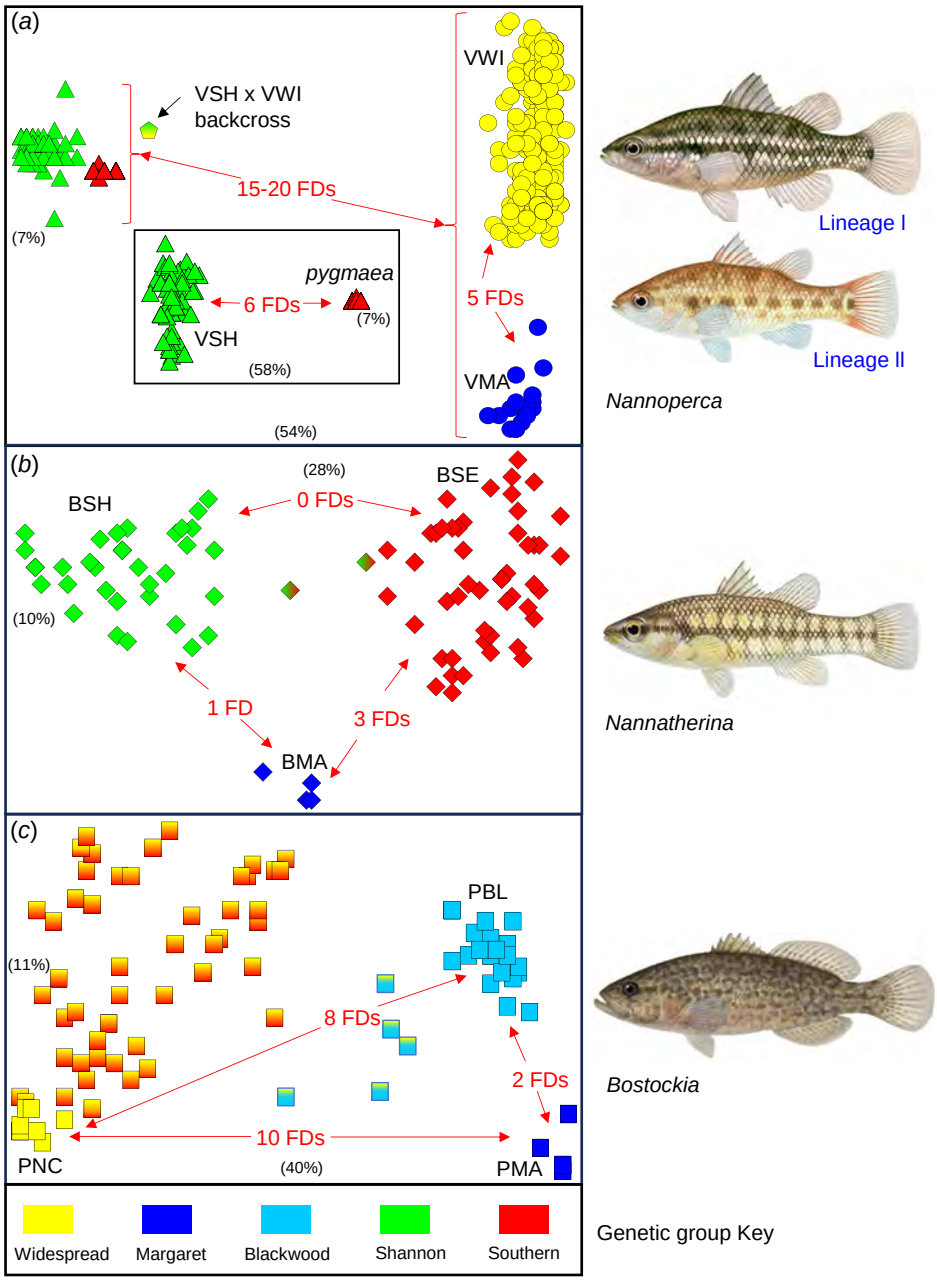

Fig. 2. Scatterplots of principal co-ordinates analysis (PCA) scores in the first two dimensions for the three allozyme datasets. The relative contribution of each dimension is shown in parentheses (axes not scaled accordingly). Primary genetic groups are identified according to the colour key provided and likely admixed individuals are indicated by using a colour gradient representing their putative parental groups. Also shown are the number of fixed differences (FDs, as defined in the text) that diagnose each 'pure' primary group. (*a*) Initial PCA of all 359 *Nannoperca*; inset shows a second PCA focusing only *N. pygmaea* plus taxon VSH ($n = 83$). (*b*) Initial PCA for *Nannatherina* ($n = 89$). (*c*) Initial PCA for *Bostockia* ($n = 97$) with hypothesised 'pure' groups (PNC, PBL and PMA) labelled. Fish images © R. Swainston (see animafish.com), reproduced with permission.

evident was a single outlier individual whose allozyme profile and observed heterozygosity (H_O) count were consistent with being a (VSH × VWI) × VSH backcross (Table S1) and was collected from a site harbouring both parental taxa (Site 53; Table 1). All four primary taxa were readily distinguishable by PCA once additional dimensions were considered (dimensions 2 and beyond for VWI *v*. VMA, inset follow-up PCA for VSH *v*. *N. pygmaea*; Fig. 2*a*) and all were unequivocally diagnosable from one another by multiple fixed allozyme differences (minimum five FDs, range 5–20; Fig. 2*a*, Table S1). These levels of diagnosability become even more substantial given there are two instances of sympatry among the four taxa (VWI and VSH at multiple sites; VWI and *N. pygmaea*; Table 1) plus taxon VMA is fully enveloped by VWI sites (Table 1; Fig. 1), although technically these two taxa are parapatric. For reference purposes, *N. balstoni* was diagnosable from the four *Nannoperca* taxa by 25–31 FDs (Table S1).

In addition to detecting the backcrossed individual at Site 53, allozyme profiling also showed the presence of a single F_1 hybrid between *N. pygmaea* and *N. balstoni* at Site 71 (Table S1). This individual was highly heterozygous ($H_O > 0.5$, compared with $H_O < 0.01$ for both parental species), notably at all 27 diagnostic loci.

Population structure in taxon VWI

Of the four taxa evident in *Nannoperca*, only the broadly distributed VWI displayed sufficient within-taxon genetic heterogeneity to warrant an exploration of population structure. Fig. 3 presents the *STRUCTURE* plot for $K = 3$ (the optimum ΔK value), matched with a NJ network among those sites with $n > 1$. Both analyses are broadly concordant in identifying three subpopulations corresponding to 'northern', 'Blackwood' (all Blackwood sites plus Site 11 in the upper Collie), and 'southern', albeit with considerable admixture among all three subpopulations at a number of sites that mostly occupy geographically intermediate positions.

Admixture between taxa VWI and VMA

Despite the presence of five FDs between the two parapatric taxa VWI and VMA, there was some indication of potential admixture in the initial PCA (Fig. 2*a*) and a follow-up PCA (not shown). A *STRUCTURE* plot ($K = 4$, the optimum ΔK value) involving all individuals of both taxa (Fig. 4) indicates that all but one VMA fish were pure exemplars of a distinctive VMA lineage. As expected, it is likely that the single anomalous individual had a hybrid ancestry involving pure VMA introgressed with VWI (presumably from a regional VWI population, with Site 19 being particularly close). Although this admixed individual (from Site 21 in the township of Margaret River itself) presented as the most prominent PCA outlier (Fig. 2*a*), its allozyme profile was not consistent with being an F_1 hybrid, indicating that VWI × VMA F_1 hybrids must be at least partially fertile.

Nannatherina allozyme analyses

The final allozyme dataset for *Nannatherina* contained genotypes for 89 fish at 61 putative loci. An overview PCA of all individuals (Fig. 2*b*) inferred the existence of three primary genetic groups, namely (a) BMA (Site 20 in the Margaret River catchment, (b) BSH (Sites 38,44–48, 50, 51 and 57 in the Shannon River Basin), and (c) BSE (='southern'; Sites 26 and 27 in the Blackwood, all sites to the east of the Shannon, plus Sites 52–54 in the eastern portion of the Shannon). There was also some evidence of modest admixture at Sites 47 (1 of 4 fish) and 57 (1 of 6 fish) between BSH and BSE, an observation consistent with an absence of fixed differences. By contrast, BMA was diagnosable by the FD counts, most notably from the widespread BSE subpopulations (three FDs), despite occupying adjacent systems. However, such modest levels of diagnosability, coupled with small sample sizes and a lack of sympatry or shallow parapatry, are clearly insufficient to infer that these three groups warrant recognition as anything other than distinct subpopulations. The allozyme profiles and heterozygosity measures for the three genetic groups and the two outlier fish are presented in Table S2.

Further exploration of population structure in *N. balstoni* is summarised by the *STRUCTURE* analyses and NJ network (Fig. 5). The optimum ΔK estimate predicted $K = 2$ for *STRUCTURE*, corresponding to all BSH sites *v*. BMA plus BSE sites plus a single admixed at Site 57 (Fig. 5). Also presented is the detailed plot for $K = 5$, this being both the next most optimum value and the lowest value that consistently delineated the Margaret and Blackwood River sites from one another (as found by PCA and FD counts, Fig. 2*b*). Both this second analysis and the NJ network broadly supported the presence of the three primary PCA subpopulations plus two admixed individuals (Sites 47 and 57; individuals marked with an asterisk), although *STRUCTURE* further split the 'southern' BSE subpopulation into separate 'Blackwood + Angove', 'Shannon' and 'east of Shannon' subunits, with sporadic admixture between the latter two.

Bostockia allozyme analyses

For *Bostockia*, the final allozyme dataset involved 97 fish profiled at 56 putative loci. An initial PCA on all individuals (Fig. 2*c*) presented a complex geographic scenario. Reflecting the FD counts, we formulated a working hypothesis of three pure groups (diagnosable from one another by 2–10 FDs), namely (a) 'upper northern coastal' (PNC, Sites 1 and 2), (b) 'Blackwood' (PBL, Sites 22–27 plus two individuals from Site 13 in the Preston), and (c) 'Margaret' (PMA, Sites 20 and 21), plus two broad but still separable clusters of admixed individuals. One of these clusters was consistent (i.e. significantly more heterozygous and only 0–1 FDs from parental subpopulations; Table S3) with the five individuals concerned (Sites 5 and 14 plus one fish at Site 13) being contemporary 'hybrids' between PNC and PBL, whereas the other comprised

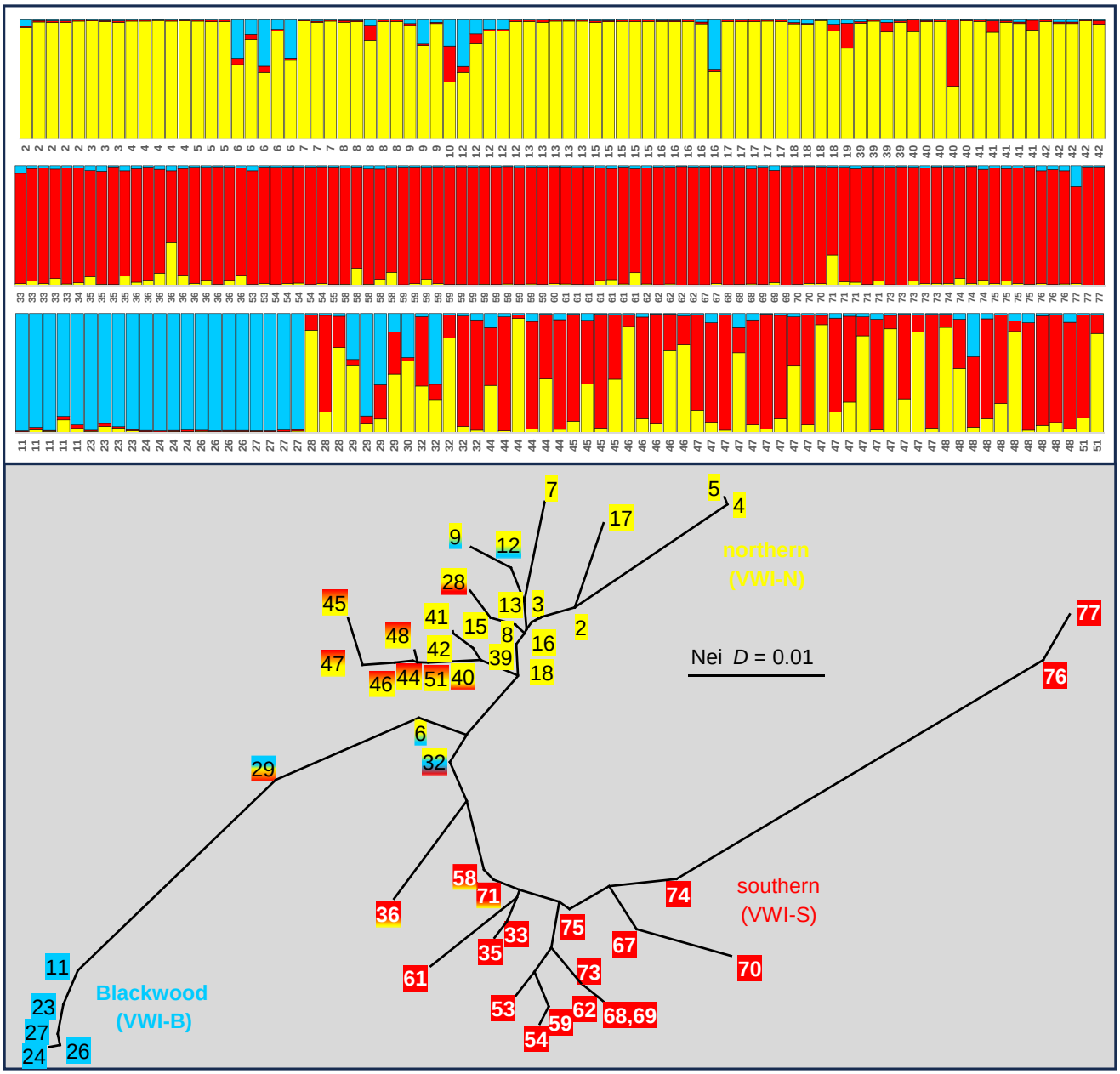

Fig. 3. Companion *STRUCTURE K* = 3 plot (all individuals) and NJ network among sites (excluding those where *n* = 1) for *Nannoperca* taxon VWI. Individuals in the *STRUCTURE* plot and termini in the NJ network are labelled by site number (following Table 1 and Fig. 1) and display matching colours that broadly reflect the geographic template designated in Fig. 2.

a geographically diverse assemblage of individuals (all other sites) that displayed varying levels of presumably ongoing or 'historic' admixture between PBL and PNC. As for *Nannatherina*, the allozyme data are too limited in sampling and genomic intensity to conclude here that *Bostockia* comprises more than a single species. The allozyme profiles and heterozygosity measures for all hypothesised pure and admixed subpopulations are presented in Table S3.

Fig. 6 presents the *STRUCTURE* results and companion NJ network among individuals for *Bostockia*. Again, we chose $K = 4$ for the *STRUCTURE* plot rather than that predicted by ΔK ($K = 2$), this being the lowest value that consistently delineated the PMA and PBL subpopulations from one another (as shown by both PCA and FD counts, Fig. 2*c*). Broadly consistent with our PCA-based expectations, the STRUCTURE analysis delineated all three hypothesised 'pure' subpopulations and supported our prediction of five 'hybrid' fish involving PBL and PNC. However, it also provided an alternative viewpoint on population structure, predicting an expanded distribution for subpopulation PNC (allocating

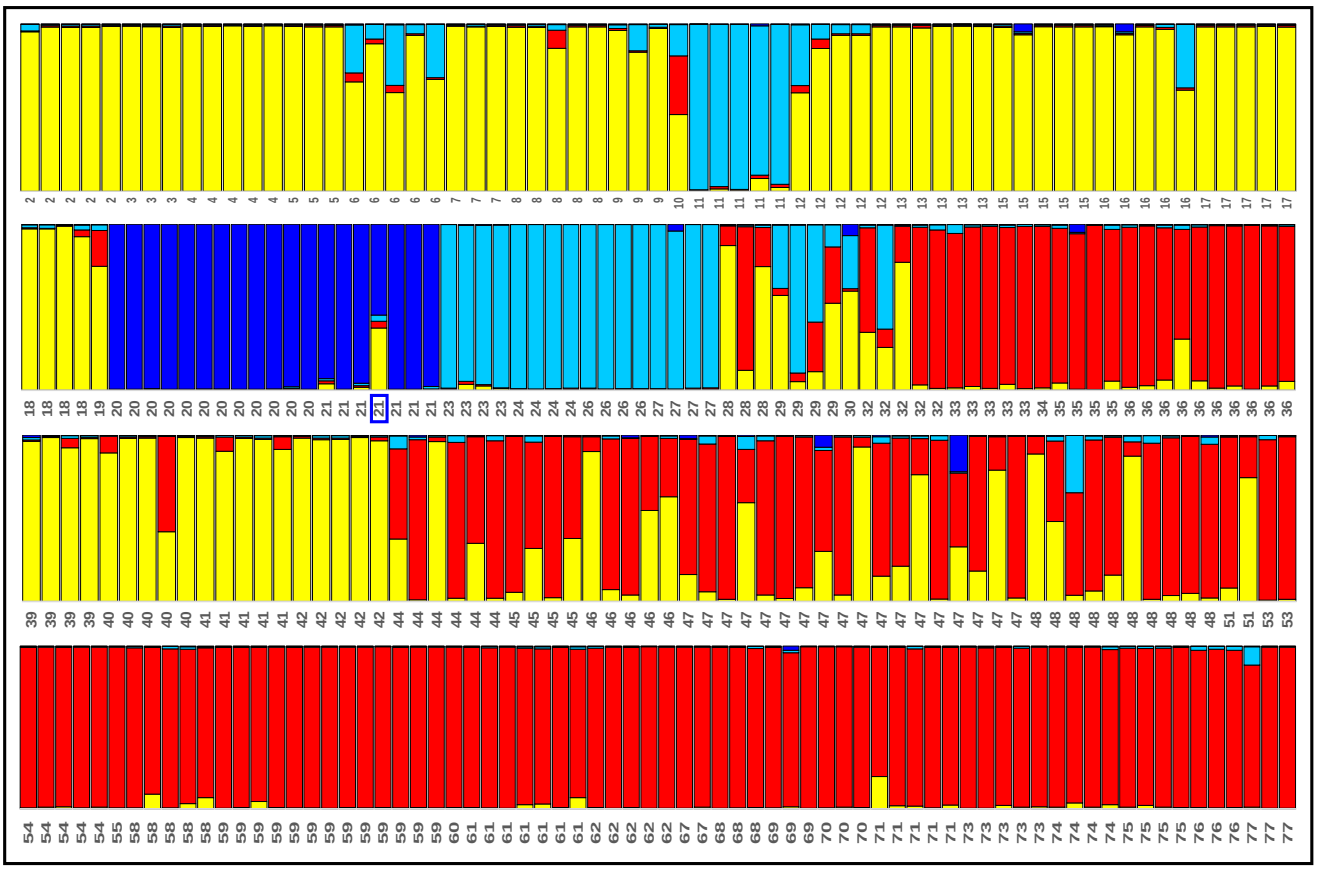

Fig. 4. *STRUCTURE K* = 4 plot for *Nannoperca* taxa VWI plus VMA (Sites 20 and 21). Individuals in the *STRUCTURE* plot are labelled by site number (following Table 1 and Fig. 1). Colours for VWI subpopulations match those used in Fig. 3. The single VMA individual displaying evidence of admixture is highlighted with a blue box.

the more southerly Sites 7, 15, 16 and 19), and inferring that a fourth 'southern' subpopulation (PSE, pure Sites 30–34, 36–40, 71–73) is the parental subpopulation contributing to our hypothesised admixture with PNC (admixed Sites 35, 41–44, 48, 54, 55 and 57; Fig. 6) rather than the PBL subpopulation. The companion NJ network (Fig. 6), although less informative, is consistent with both alternative viewpoints.

Mitochondrial gene tree across all genera

The final *cytb* dataset consisted of 1141 bp of sequence data for 184 individuals (including 10 previously deposited in Genbank) across all three genera. In addition to positioning each of the three genera as very distinctive clades, the resultant ML tree (-ln score of −4642.492695, Fig. 7) strongly supports the existence of the four *Nannoperca* species identified by our allozyme analyses. As predicted herein and found previously (Buckley *et al.* 2018, 2024), *Nannoperca* comprises two primary lineages, one for sister species *N. pygmaea* and VSH and the other containing the closely related candidate species VWI and VMA. Within-species structure is also evident for species VWI (full haplotype tree in Supplementary Fig. S1), which presents as two moderately well-supported (bootstrap value 75%) clades with broadly 'northern' *v.* 'southern' distributions, but with some overlap in the Donnelly, Warren and western Shannon River Basins plus one geographic anomaly (Site 7; Fig. 7, S1). Of note was verification in the mtDNA dataset of an additional population of *N. pygmaea* from Lake Smith (Site a3, Donnelly River Basin), well separated from other sites to the east.

In stark contrast with *Nannoperca*, both *Nannatherina* and *Bostockia* displayed minimal *cytb* diversity across their entire geographic range that reflected all identified subpopulations (Fig. 7). This result further reinforces the hypothesis that neither harbours cryptic taxa. It also indicates that both sets of subpopulations are likely to experience genetic connectivity over time whenever environmental conditions permit.

Fig. 7 also includes the best available molecular clock estimates for the key nodes in the south-western percichthyid phylogeny, as calculated by Buckley *et al.* (2018) by using *r8s* (ver. 1.81, see https://sourceforge.net/projects/r8s/) for 13,991 concatenated single-nucleotide polymorphism (SNP) loci and calibrated using the aridification of the Nullarbor region to date the loss of connectivity between eastern and western *Nannoperca* species. These dates are also consistent

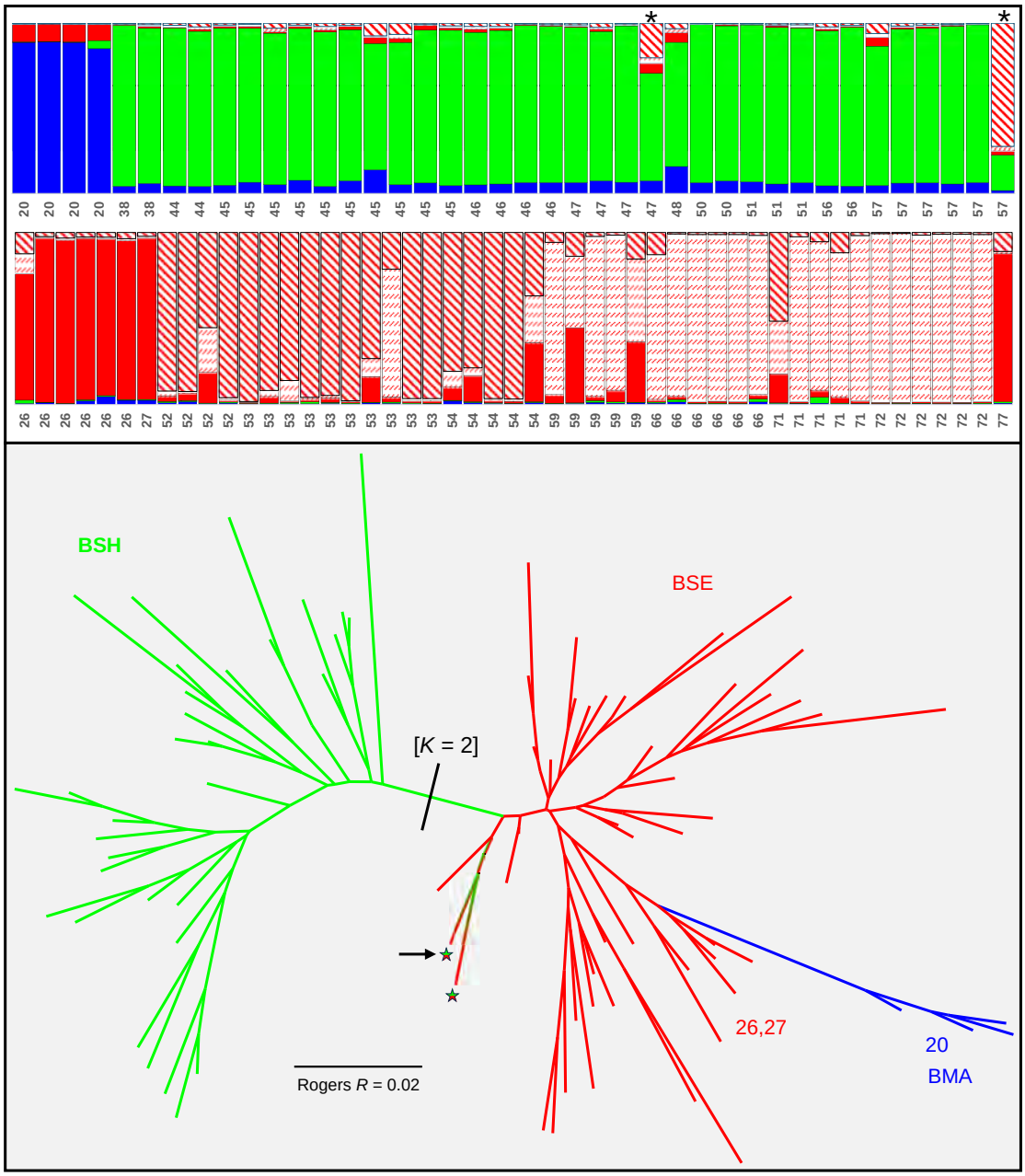

Fig. 5. Companion *STRUCTURE* $K = 5$ plot and NJ network among individuals for *Nannatherina*. Individuals in the *STRUCTURE* plot are labelled by site number (following Table 1 and Fig. 1) and putative subpopulations are coloured to match those used in the NJ network (with each of the three 'southern' subpopulations bearing unique patterning). For the NJ network, the only individuals labelled are those for the Margaret (Site 20), Blackwood (Sites 26, 27), and the two individuals (marked with an asterisk) that were intermediate in the PCA of Fig. 2*b*; instead, branches are colour coded to reflect the geographic template used in Fig. 2. Also shown is the primary *STRUCTURE* split evident using the optimum value of $K = 2$ and the single Site 57 individual (arrowed) inferred to be admixed.

with those obtained for a combined analysis of *cytb* plus *S7* plus RAG sequences (Unmack *et al.* 2011) by using *BEAST* (ver. 1.5.3, see http://beast.community/beast). For *Nannoperca*, divergence dates for both pairs of sister species fall within the Pliocene, with the primary split between Lineages I and II referable to the Miocene (Unmack 2001).

Landscape patterns

With comprehensive sampling and genetic screening across the extant distribution of the three target genera, it is possible to compare patterns observed against the broader common aquatic landscape. Table 2 summarises the comparative

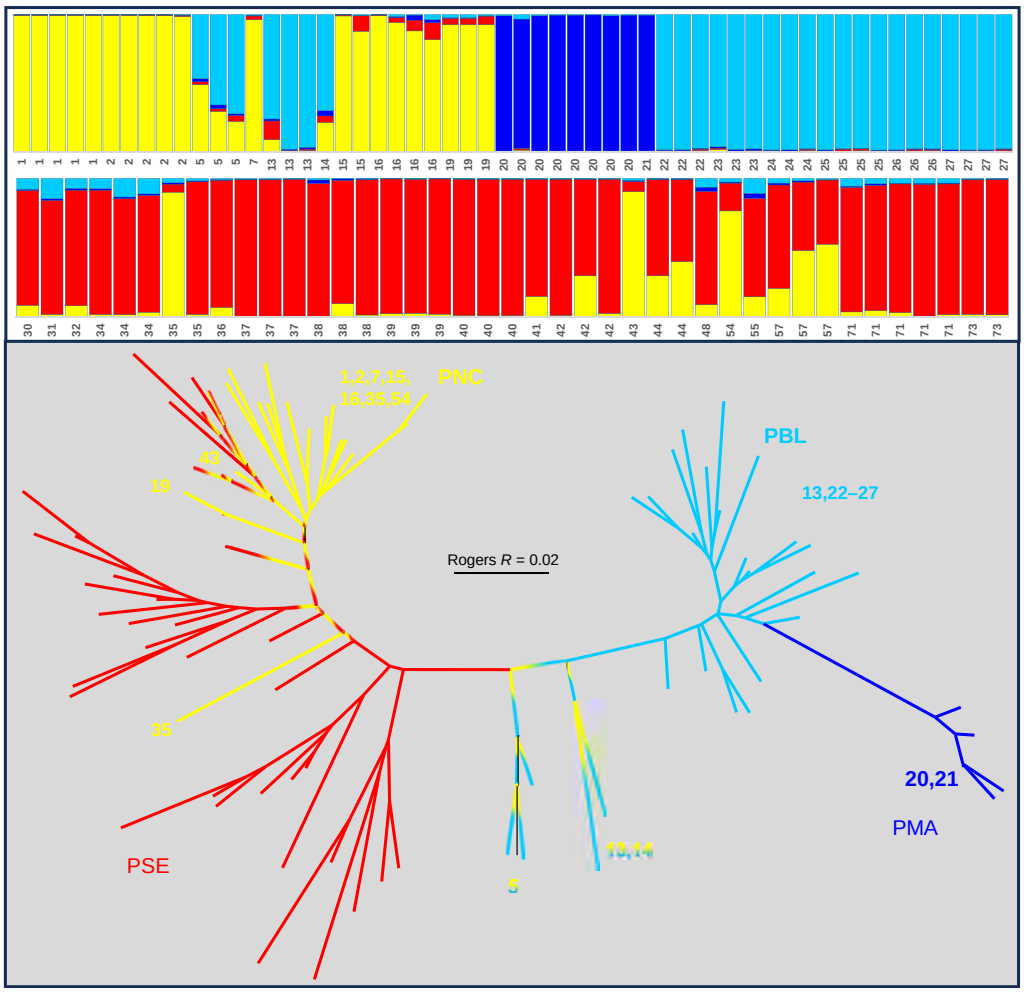

Fig. 6. Companion *STRUCTURE* $K = 4$ plot and NJ network among individuals for *Bostockia*. Individuals in the *STRUCTURE* plot are labelled by site number (following Table 1 and Fig. 1) and putative subpopulations are coloured to match those used in the NJ network. For the NJ network, only selected OTUs were labelled for cross-referencing with the PCA (Fig. 2*c*) and *STRUCTURE* plot; instead, branches are colour-coded to broadly reflect the geographic template used in Fig. 2.

levels of geographically based genetic subdivision across the four south-western percichthyid lineages (i.e. including the two for *Nannoperca*), as either unique candidate species or subpopulations. This summary is mainly based on allozyme patterns, but is also largely congruent with the mtDNA data for *Nannoperca*. The spatial distribution of these groups is also mapped and compared in Fig. 8 against major hydrological units, elevation, and historic drainage patterns and low-sea levels (Unmack 2001; Atkinson *et al.* 2008; General Bathymetric Chart of the Ocean 2024).

Discussion

High endemism is often manifested through unique form and ecology, being both charismatic and irreplaceable (Allan and Flecker 1993; Ebner *et al.* 2016). The partitioned nature of freshwater environments can promote local isolation and adaptation as a forerunner to endemism, especially in dispersal-limited obligate freshwater fishes (Dudgeon *et al.* 2006; Reyjol *et al.* 2007). The south-western corner of Australia is a global biodiversity hotspot defined primarily for its terrestrial flora and fauna, but extending to a freshwater fauna that displays a very high proportion of endemic species (Morgan *et al.* 2011; Davies and Stewart 2013). The region is geographically isolated from other freshwater bioregions and features a heavily partitioned hydrological framework through a series of isolated river basins and subcatchments. Here, we explored the molecular systematics of temperate perches (family Percichthyidae) across this habitat templet, represented by the genera *Bostockia*, *Nannoperca* and *Nannatherina*, and discovered unique and contrasting patterns at and below the species level. These data have strong implications for management and conservation, thus providing a foundation for future taxonomic and biological studies.

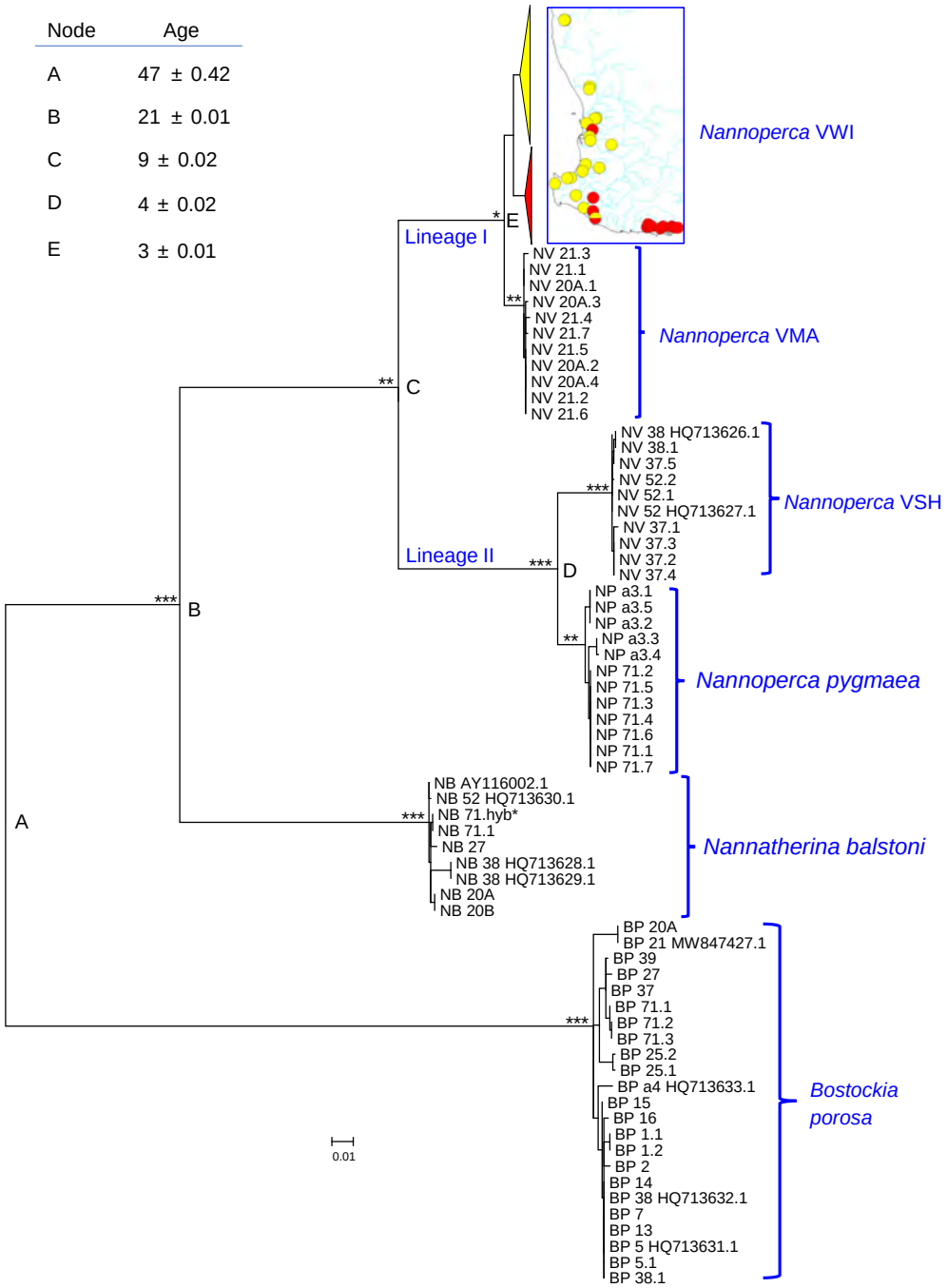

Fig. 7. Maximum likelihood tree for the 184 *cytb* sequences available across all three genera. For species VWI, only the two primary clades are shown, alongside a map of their comparative geographic distributions. The full VWI haplotype tree is presented in Fig. S1. Individuals are labelled by species code + site code (as per Table 1) + individual code (.1, .2 etc. for fish from the same site). Genbank sequences are denoted by HQ, AY or MR numbers. Bootstrap proportions are indicated for the earlier-branching nodes by asterisks (***, 00%; **, 95–99%; *, 85–94%). Also shown are molecular clock estimates (millions of years ± s.d.) for key nodes (A–E) as calculated by Buckley *et al.* (2018) by using *r8s*, being in line with those of Unmack *et al.* (2011) using *BEAST*.

Comparative ecology

Landscape-related genetic structure in freshwater fishes can be influenced by varying combinations of biological traits, such as dispersal ability, behaviour and habitat specialisation, all of which can interact with the physical aquatic environment to affect gene flow (Tibbets and Dowling 1996; Hughes *et al.* 2009; Hammer *et al.* 2019*a*). However, previous

Table 2. Summary of geographic-based genetic subdivision observed for nuclear genetic markers for the percichthyids of south-western Western Australia.

Region	*Nannoperca* I	*Nannoperca* II	*Nannatherina*	*Bostockia*
Northern	Subpopulation (VWI-N) plus admixture	Absent	Absent	Subpopulation (PNC) plus admixture
Margaret	Species level (VMA)	Absent	Subpopulation (BMA)	Subpopulation (PMA)
Blackwood	Subpopulation plus upper Collie (VWI-B)	Absent	Subpopulation plus Angove (BSE-B)	Subpopulation (PBL)
Shannon	Subpopulation with southern (VWI-S) plus admixture	Species level (VSH)	Subpopulation (BSH)	No specific substructure (admixed)
Southern	Subpopulation (VWI-S) plus admixture	Species level (*N. pygmaea*)	Two possible subpopulations? (BSE-eastern)	No specific substructure (admixed)

Taxon and subpopulation nomenclature and assignments broadly match those used in Fig. 2–6.

ecological assessments of south-western Australian percichthyids that were based on existing field data have been confounded by unrealised problems in their taxonomic foundation, i.e. the presence of cryptic species. The detection of a narrow-range candidate species nested within an otherwise widespread *Nannoperca* lineage (i.e. VMA + VWI), and a second narrow-range candidate species (VSH) forming a second lineage with *N. pygmaea*, implies that three different sets of biological attributes are likely at play within the current biological data available for *N. vittata sensu lato*.

Nevertheless, on the basis of a considered review of biological characteristics, we would expect that *B. porosa*, as a larger growing (i.e. potential better swimming ability) and more generalist species in terms of habitat occupancy (Pen and Potter 1990), might be less substructured. This was supported by mostly shallow substructure across the region, albeit with a strong hint of refuge contraction in dry evolutionary periods followed by subsequent wide dispersal (i.e. distinct subgroups, some of which showed signals of admixture). Clearly, a full elucidation of this complex biogeographic pattern is beyond the resolving power of allozymes and thus would require a comprehensive genomic dataset. Likewise, the widespread *Nannoperca* taxon VWI is likely to have fairly general habitat needs and relatively good dispersal ability (Pen and Potter 1991; data from a VWI population), as reflected in limited substructuring and signs of broad admixture across an expansive distribution from the Arrowsmith River through to the Angove River. By contrast, *N. balstoni* is a noted habitat specialist primarily in off-channel acidic pools with high structural habitat integrity (Morgan *et al.* 1995), and this is likely to restrict broad gene-flow as reflected by the presence of three distinct subpopulations within a relatively restricted distribution. *Nannoperca pygmaea* has also been shown to have highly specialised habitat requirements, including specific refuge needs, which may help explain its restricted distribution (MG Allen *et al.* 2020). However, our matrilineal data (mtDNA only for Smith Lake) indicated limited genetic substructure across two fragmented populations (Kent and Denmark *v*. Donnelly River Basins), which suggests a broader historic distribution and therefore dispersal opportunities prior to major human-mediated landscape change. Ongoing fine-scale field sampling and genomic data would help inform population genetics for this threatened species.

There was an overall pattern of strong genetic substructure within the targeted taxa, including the presence of cryptic species, a trend that is likely to apply to the remaining five endemic obligate freshwater fishes of south-western Australia, this being a general pattern for freshwater fishes in naturally occurring, heavily divided landscapes (Beheregaray and Caccone 2007; Hammer *et al.* 2013; Hending 2025). Comparative ecology studies in these habitats must be mindful to ensure that a sound systematic framework interrogated with modern taxonomic approaches is in place from the outset (Page *et al.* 2005; Feckler *et al.* 2014).

An additional ecological insight from this study was the detection of rare instances of hybridisation between taxa across both genera and lineages, which field ecologists should be mindful of when identifying samples. This issue is likely to become more problematic in the future, given that anthropogenic modifications of habitat often lead to an increase in hybridisation between species (Hasselman *et al.* 2014; McFarlane and Pemberton 2019).

Landscape patterns and conservation

The additional taxonomic complexity and spatial genetic substructure observed in south-western Australia's percichthyids, a group with already high endemism and conservation concerns (Morgan *et al.* 2014*a*; Lintermans *et al.* 2020; International Union for Conservation of Nature 2024), clearly elevates the required focus on land and natural resource management in general, plus identifies several priority focal regions.

The Margaret River is of high evolutionary and conservation significance for aquatic biota (MG Allen *et al.* 2017). A major level of genetic distinctness was shown for each obligate freshwater fish examined from the system, ranging between a likely species-level split in *N. vittata* and distinctive subpopulations in *Nannatherina* and *Bostockia*. This distinctiveness implies that long-term refugial habitat has existed in this catchment, presumably reflecting longer-term biogeographic isolation from adjacent catchments (e.g. narrow continental

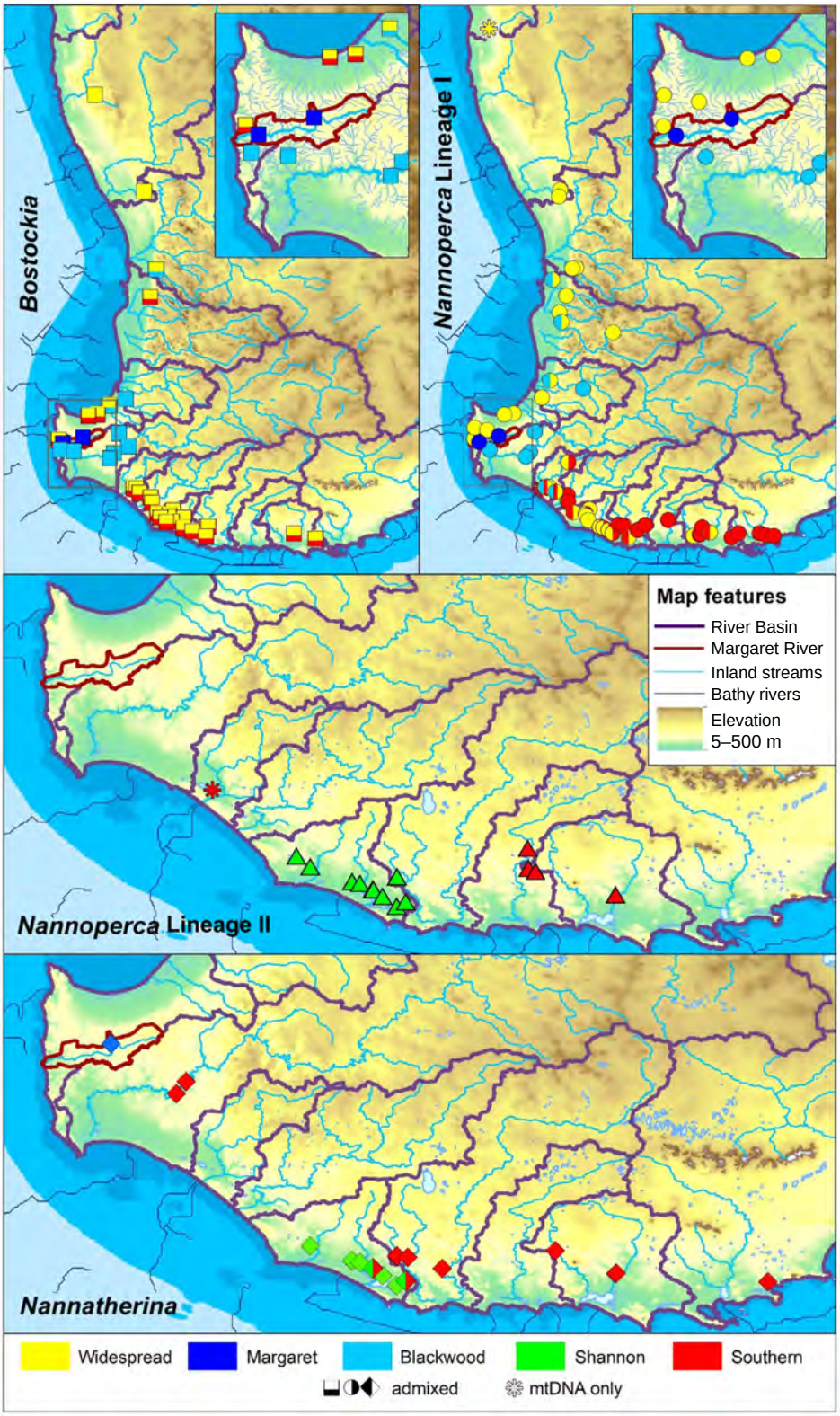

Fig. 8. Composite map of geographic genetic groups across the three percichthyid data sets (see Fig. 1 for river basin and site details). Note that historically *Nannatherina* was more widespread occurring into the region of the Moore–Hill Rivers catchment, but assessment of the genetic affinity of this region was not possible because of extirpation.

shelf width limiting coastal connections at lower sea levels with distinctive eastern flow direction; also potentially aided by the presence of small waterfalls). The conservation implications are to protect the genetic integrity and population health of the Margaret River fish community, which has already been challenged by the introduction of eastern gambusia, *Gambusia holbrooki*, to key permanent water refuges in the upper catchment (a specific threatening process for the *N. balstoni* BMA subpopulation), and faces broad land management issues (MG Allen *et al.* 2017).

The significance and extinction risk of the Margaret River aquatic fauna is re-enforced by the example of the decapod crustacean group marron (genus *Cherax*). Genetic data, followed by combined morphological and ecological characterisation, documented two species in the lineage, the hairy marron (*Cherax tenuimanus*) endemic to the Margaret River, and a widespread species occurring across the south-west, the smooth marron, *Cherax cainii* (Austin and Ryan 2002). However, the narrow-range endemic declined rapidly through aggressive competition, predation and introgressive hybridisation following human-mediated dispersal of the widespread sister species into the Margaret River (Duffy *et al.* 2014; Guildea *et al.* 2015). In pygmy perches, the observed signal of potential introgression between the narrow-range Margaret endemic candidate species and the more widespread sister species in *Nannoperca* (VMA *v*. VWI) warrants urgent investigation and careful pro-active management to limit any human-mediated dispersal into and within the system. This should include both direct fish movement plus other indirect means such as transfer of aquatic vegetation, habitat modifications altering natural barriers, and even restoring passage to artificial barriers (i.e. consideration of altered ecological condition in goal setting). Additional unique aquatic species of conservation concern in the Margaret River include the narrow-range endemic Margaret River burrowing crayfish, *Engaewa pseudoreducta* (Morgan *et al.* 2011), and a potentially species-level lineage of the freshwater mussel genus, *Westralunio* (Klunzinger *et al.* 2021).

The Shannon River Basin was also noted as an important region of diversification in the south-western percichthyid fauna. An endemic candidate species of *Nannoperca* (VSH), sister to *N. pygmaea*, was entirely restricted to the Shannon River Basin. Likewise for a distinct subpopulation of *Nannatherina,* which maintains parapatry with a separate subpopulation in surrounding areas (BSH *v*. BSE), where a low level of gene flow appears to be potentially balanced by some form of strong selection (and therefore worthy of further spatial and temporal genomic investigation). The Shannon River Basin itself (Shannon and Gardner Rivers) has low drainage divides with adjoining systems along the coast and would have converged with other systems at low sea levels. Hence, given the patterns of gene flow observed, a high level of habitat specificity is likely to be involved, possibly associated with a distinctive, low-gradient habitat of coastal swamp or similar. Other southern coast river basins are also clearly important areas for percichthyids, such as the Donnelly, Kent and Denmark River Basins being habitat of the Endangered *N. pygmaea* and of general refuge value for a diverse fauna (Morgan and Beatty 2008; MG Allen *et al.* 2020).

It is important to note that it was not possible to fully assess the genetic affinity of fish from some regions, owing to large declines from the historic distribution. The most notable example was northern populations of *Nannatherina* that ranged northward to at least the Swan Coastal Plain, a region now heavily affected by agricultural development, urbanisation and introduced fishes (WA Museum record P.27025, Gingin Brook, 1981: Morgan *et al.* 1998, 2014*a*; Hourston *et al.* 2014). Other extirpated populations of *Nannoperca* and *Bostockia* well inland would also be of interest for assessing genetic patterns, such as from the now heavily salinised upper Blackwood River (e.g. WAM P.3109, 3111 and 3112, Dumbleyung Lake area, 1947: Morgan *et al.* 2003). These and other now extirpated populations with voucher material remain a potential target for historic DNA techniques focusing on museum specimens (Appleyard *et al.* 2021; Tims *et al.* 2024).

Taxonomic considerations

The suggestion of two additional candidate species within the genus *Nannoperca* necessitates an urgent taxonomic assessment by using combined lines of evidence for species description and morphological diagnosis (i.e. undertaking a full complementary data assessment of morphological traits across different taxa and populations) (Morgan *et al.* 2013; Hammer *et al.* 2019*b*). Thereafter, a chart of action to address differing taxonomic issues needs to start by matching available names (i.e. old-type material) to morphological traits or geographic boundaries of candidate species. Both aspects can be challenging owing to the generally poor physical condition and limited information accompanying early museum vouchers, and given that historic sampling data have shown that distributions were broader in the 1800s, perhaps reflecting other patterns unrealised through lack of data.

The available names for species in the *Nannoperca* complex are *N. vittata*, with a type locality listed simply as 'fresh waters of the interior of Western Australia by Rev. Bostock' (a large type specimen in reasonable condition which should facilitate comparison; Bostock was based in Perth and Fremantle, so collection from this region is likely) and *Nannoperca viridis* Castelnau, 1873, with type locality 'interior of King George Sound by Mr Maxwell' (two small <2 cm and fairly damaged types, which may make assignment difficult). Both were assessed in the same paper (Castelnau 1873), where *N. vittata* is the senior synonym following assignment in a review of the taxonomy of the pygmy perches by Kuiter and Allen (1986), should they prove to be conspecific. Kuiter and Allen (1986) conducted the first modern data assessment for *N. vittata*; however, these data unknowingly included both *Nannoperca* lineages (VWI and VSH candidate species), with their raw summary being repeated as comparative *N. vittata* data in

the description of *N. pygmaea* (Morgan *et al.* 2013: the Lake Smith, Donnelly catchment population was not known at the time). Clearly, a complete revision will be required, including the need to re-diagnose both *N. vittata* and *N. pygmaea*.

The allozyme data are powerful in diagnosing four candidate species in *Nannoperca*. Nevertheless, additional site sampling and genomic data for the Margaret River would assist in the confidence of taxonomic assessment for the VMA taxon, especially considering the small geographic distances involved and the finding of a single admixed fish at one of the two sites surveyed (which could represent natural low-level interactions in parapatry or more disturbing signs of human-mediated gene flow). Preliminary genomic data (Buckley *et al.* 2024) comparing four populations covering the full geographic range of candidate species VWI plus one VMA site have already demonstrated a wealth of potential diagnostic genetic markers for delineating these two sister taxa (871–1223 SNPs; Fig. S2), plus shown great potential for exploring genetic substructure in VWI (over 300 SNPs differ between VWI-N and VWI-S subpopulations; Fig. S2).

Conclusions

The south-western corner of Australia is a unique region of high significance for biodiversity that faces considerable future challenges, not least of which is advancing climate change that will worsen key aquatic habitat availability and quality issues. The current study represents the first spatially comprehensive genetic assessment of a key group of aquatic fauna for the region, the obligate freshwater percichthyids. Although the data have been a while in preparation, their availability is very timely to tie into identification of natural assets and threat assessment within land and resource management, identifying urgent issues around narrow-range endemic candidate species and subpopulations in two key areas (Margaret and Shannon). Moreover, recent advances in genomic data now offer a means whereby many of the interesting questions unable to be fully addressed by allozyme datasets can be readily investigated and expanded using the broad biogeographic patterns presented here.

Supplementary material

Supplementary material is available online.

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Data availability. The raw allozyme and aligned *cytb* data are available on *Zenodo* (Adams and Hammer 2024). All novel *cytb* sequences have also been deposited in GenBank (Accession numbers PQ472737–PQ472909).

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