RESEARCH ARTICLE

Phylogeographic structure in the threatened Yarra pygmy perch *Nannoperca obscura* (Teleostei: Percichthyidae) has major implications for declining populations

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Abstract Molecular genetic information should be a prerequisite when evaluating conservation priorities in highly structured species such as freshwater fishes. Nuclear (allozyme) and mitochondrial (cytochrome *b*) markers were used to investigate phylogeographic structure in the Yarra pygmy perch *Nannoperca obscura* (Klunzinger), a threatened freshwater fish endemic to mainland south-eastern Australia. Complementary patterns of strong, geographically defined sub-structure were observed including a major east–west divergence (at the Glenelg River), four diagnosable lineages, and statistically-significant differences between most populations. Accordingly, four Evolutionarily Significant Units (ESUs) are defined and multiple, drainage-scale Management Units (MUs) suggested. Since *Nannoperca obscura* is a relatively poor disperser with no apparent gene flow between most populations, any regional extirpation would see the irreversible loss of genetic diversity. This is problematic, as several populations, most notably a recently discovered ESU in the Murray-Darling Basin, are feared extirpated through a combination of anthropogenic threats and severe drought. The potential loss of unique evolutionarily components within *N. obscura* soon after their discovery highlights with some urgency, the need to define and protect conservation units in highly modified freshwater habitats.

Introduction

Population and evolutionary genetics can be instrumental in assessing conservation units and priorities for threatened, little-studied or morphologically-conservative groups (e.g. Firestone et al. 1999; Buhay et al. 2007; Hammer et al. 2007). There are several views on how to define conservation units (see Crandall et al. 2000), however the relevant geo-political landscape, and hence practical application of research findings, often drives the choice between the biological and/or genetic criteria employed (Waples 1995; Wood and Gross 2008). Most researchers employ two basic concepts, Evolutionarily Significant Units (ESUs) and Management Units (MUs), with the most widely adopted genetic criteria being those of Moritz (1994, 2002).

Freshwater environments have a special need for the identification of distinct evolutionary lineages. Opportunities for biological isolation and diversification are common (e.g. Fraser et al. 1995; Fagan 2002) such that fishes and other freshwater biota typically show extremely high species richness and genetic diversity (Nelson 1994; Ward et al. 1994). This biodiversity is threatened, however, by human industry associated with water resources (Cambray

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and Bianco 1998; Ricciardi and Rasmussen 1999). South-eastern Australia is a typical example, being affected by intensive development and industry (Boulton and Brock 1999), and having freshwater fishes with high levels of genetic structure (e.g. Page et al. 2004; Wong et al. 2004; Faulks et al. 2008).

The Yarra pygmy perch, *Nannoperca obscura* (Klunzinger), is a diminutive (<80 mm total length) freshwater percichthyid endemic to mainland southeastern Australia. It occurs in slow-flowing and sheltered areas of lowland streams, lakes and rivers, in association with submerged and emergent macrophytes (Kuiter et al. 1996). Biological data are few, but the species appears to be sedentary and has large, demersal larvae (Briggs 1999). There is little scope for dispersal between occupied river systems, as they are each separated by marine barriers, suggesting the likelihood of strong, within-species genetic structure (e.g. Hughes et al. 1999). *Nannoperca obscura* co-occurs across its range with the southern pygmy perch, *N. australis* Günther, and the two species are segregated by habitat and behavior, although occasional hybrids are suspected (Kuiter et al. 1996; Woodward and Malone 2002).

Nannoperca obscura has declined since European settlement, and is 'vulnerable' under IUCN criteria (IUCN 2006). Threats include water abstraction, wetland drainage, loss of stream-edge habitat and alien fishes (Wager and Jackson 1993). Its present distribution is patchy and confined to a narrow band inland from the coast (Saddlier 1993). *Nannoperca obscura* is presumed extinct in its type locality, the Yarra River, and other eastern sites (Yarra and Bunyip river basins). Decline (likely extirpation) is also evident for recently discovered populations in the western range subject to critical anthropogenic threats and now exacerbated by severe drought (i.e. Lake Alexandrina, Henry Creek and Lake Bonney: Hammer 2008, 2009).

This study uses nuclear genetic markers (allozymes) and mtDNA sequence data to generate a population genetic framework to inform current and future conservation management of *N. obscura*. Genetic data were used to (1) define genetic conservation units (ESUs, MUs) within *N. obscura* and (2) document within-population diversity. The spatial data also allow further evaluation of biogeographic hypotheses (Unmack 2001) and factors affecting gene flow and fragmentation.

Methods

Specimen collection

Samples of *N. obscura* were collected from across its extant range, including river basins (AWRC 1976) and major rivers within basins (Fig. 1; Table 1). Its threatened status, nationally and locally, meant that collections were necessarily small (where possible, up to $n = 10$ per site). Field sampling was undertaken in 2000–2004 and utilized seine and dip nets. Samples of *N. australis* as outgroups were collected concurrently. Fish were euthanased in an aqueous solution of clove oil, and muscle or whole individuals were snap-frozen in liquid nitrogen and returned to the Australian Biological Tissues Collection, Adelaide (ABTC) and stored at −70°C. Two collections could be stored only in 100% ethanol and so were limited to DNA analysis, although adjacent sites were available for allozyme analysis (Fig. 1; Table 1). Given a small sample size ($n = 2$), one site was pooled with its near neighbor to provide increased statistical power (i.e. sites 4–5). Voucher specimens were preserved in formalin and lodged with the South Australian or Victorian museums (identified by field codes, Table 1).

Allozyme electrophoresis

Muscle homogenates were screened for allozyme variation on cellulose acetate gels (Cellogel™, Milan, Italy), following Richardson et al. (1986). Thirty-six proteins (both enzymes or non-enzymatic markers) displayed sufficient activity and resolution after staining to permit their use in this study: ACON, ADA, ADH, AK, ALD, AP, CA, CK, ENOL, EST, FDP, FUM, G6PD, GAPD, GDA, GDH, GLO, GOT, GPI, GSR, IDH, LDH, MDH, ME, MPI, NDPK, PEPA, PEPB, PEPD, PGAM, 6PGD, PGK, PGM, PK, SORDH and TPI. Enzyme and locus abbreviations, enzyme commission numbers, electrophoretic conditions and stain recipes are given in Richardson et al. (1986). Allozyme alleles were designated alphabetically and multiple loci, where present, were designated numerically, in order of increasing electrophoretic mobility (e.g. $Ada^a < Ada^b$; $Ca1 < Ca2$).

An overview study was conducted on four populations across the geographic range of *N. obscura*. Having identified a suite of genetic markers, all remaining samples were genotyped. Details of localities and sample sizes for both stages are shown in Table 1, and the geographic arrangement of localities is displayed in Fig. 1.

Data were subjected to Principal Co-Ordinates analysis (PCO) to reveal the genetic affinities among individuals from first principles (see Hammer et al. 2007). The raw genotypic data for individual sample sets were then examined for statistical evidence of departures from Hardy–Weinberg expectations and of linkage disequilibrium. Where no evidence was found to refute the null hypothesis of panmictic sample sets and unlinked loci, pairwise comparisons for statistically-significant differences in allele frequency were undertaken. All statistical

Fig. 1 Geographic relationships and Evolutionarily Significant Units (ESUs) in *Nannoperca obscura* samples subjected to molecular analyses. Sites codes as per Table 1 and ESU codes as per the text. River Basins (RB) and place names mentioned in the text are also shown
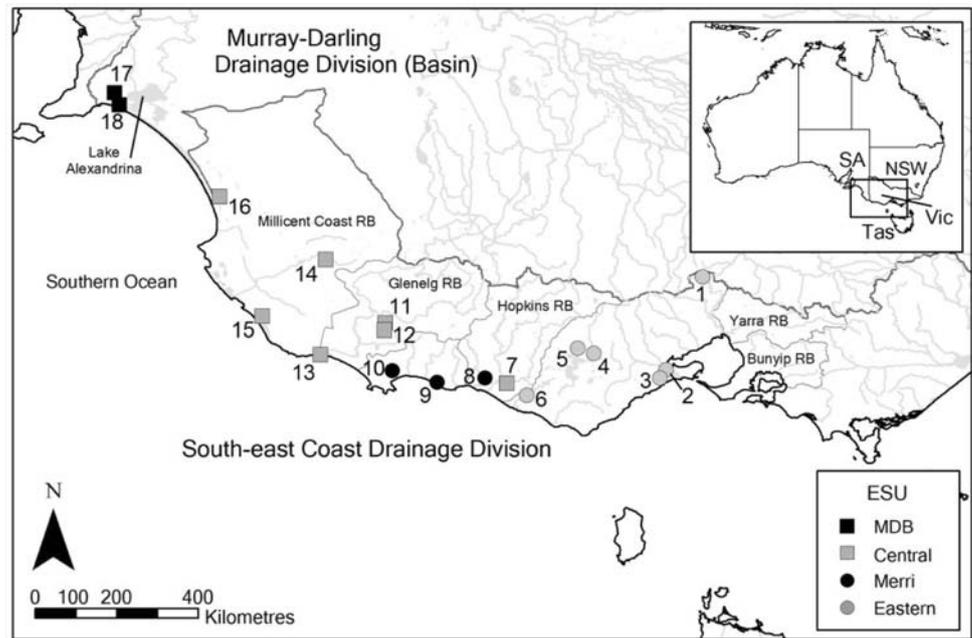
Table 1 Locality and sample size information for the allozyme overview (ov) and population (pop) studies and mtDNA analyses

Site	Field codes	Locality	State	DD	RB	Latitude (S)	Long. (E)	n (ov)	n (pop)	n Cyt b
1[a]	PU00-03, PU02-106	Deep Ck, Lancefield	Vic.	II	30	37°16′	144°43′	–	10	8
2	PU00-29, PU02-84	Waurn Ponds Ck, Geelong	Vic.	II	33	38°11′	144°21′	–	10	9
3	PU02-107	Thompson Ck	Vic.	II	35	38°16′	144°17′	–	10	5
4	PU00-28[a], PU03-06	Woady Yaloak R., Cressy	Vic.	II	34	38°01′	143°38′	–	2	8
5	PU00-27	Gnarkeet Ck, Lismore	Vic.	II	34	37°58′	143°28′	3	10	5
6	PU00-24, PU02-91	Curdies R, Curdie	Vic.	II	35	38°27′	142°57′	–	10	9
7	PU00-23, PU02-112	Mount Emu Ck, Panmure	Vic.	II	36	38°20′	142°46′	–	10	7
8	PU00-22, PU02-111	Merri R., Grassmere	Vic.	II	36	38°16′	142°32′	–	10	5
9	PU00-21, PU02-113	Shaw R., Yambuk	Vic.	II	37	38°02′	142°04′	–	10	5
10	PU00-20	Surrey R., Heathmere	Vic.	II	37	38°00′	141°37′	3	10	5
11	PU00-18, PU02-119	Palmer Ck, Merino	Vic.	II	38	37°43′	141°33′	–	10	9
12	PU00-19[a]	Stokes R., Digby	Vic.	II	38	37°48′	141°32′	–	–	9
13	FISHY2	Crescent Pond, Picks Swamp	SA	II	38	38°00′	140°54′	–	11	9
14	PU00-16, FISH83	Mosquito Ck	Vic.	II	39	37°05′	140°57′	6	13	4
15	FISHY2	Drain 88, Lake Bonney	SA	II	39	37°39′	140°19′	–	10	10
16	FISH90	Henry Ck	SA	II	39	36°00′	139°53′	–	7	8
17	FISH84,FISH90	Finniss R., L. Alexandrina	SA	IV	26	35°02′	138°51′	5	7	5
18	FISH98, FISHY2	Hindmarsh Is., L. Alexandrina	SA	IV	26	35°32′	138°53′	–	7	8

Site numbers match those in Fig. 1. *DD* Drainage Division, *RB* River Basin (AWRC 1976)

[a] Tissues preserved in ethanol

tests of Hardy–Weinberg Equilibrium, linkage disequilibrium and heterogeneity in allele frequencies were made using GENEPOP 3.4 (Raymond and Rousset 2003). Probabilities were adjusted for multiple tests using the sequential Bonferroni correction factor (Rice 1989). *F*-statistics were used to compare divergence within and between sites at various hierarchical levels of population structure. F_{IS} and F_{ST} values and associated 99% confidence intervals were calculated using the program FSTAT version 2.9 (Goudet 2000). Within-site allozyme diversity was assessed via observed heterozygosity values (H_O) calculated from all 52 loci surveyed in the overview study (cf. Hammer et al. 2007). Genetic divergence was assessed using Nei's unbiased genetic distance (Nei D: Nei 1978).

PHYLIP (Felsenstein 1993) was used to construct UPGMA (Unweighted Pair-Group Method of arithmetic Averages) dendrograms and NJ (Neighbor Joining) networks. The program TREEVIEW (Page 1996) was then used to visualize the tree structure. Allele frequencies and genetic distance measures were generated following Hammer et al. (2007).

Mitochondrial DNA

Total DNA was obtained from *c.* 0.25 cm^3 of caudal fin or muscle via phenol/chloroform extraction modified after Hillis et al. (1996). We amplified the cytochrome *b* (cyt *b*) gene using two primers that flanked this gene: Glu31 5′-TGRCTTGAAAAACCACCGTTGT-3′ and PPThr41 5′-AGGATTTTAACCTCTGGCGTCCG-3′. When this failed to produce sufficient polymerase chain reaction (PCR) product, the gene was amplified in two halves, using Glu31—HDALT602 5′-GGRTTGTTGGAGCCTGTTTCAT-3′ and ppL505 5′-TCAGTAGACAACGCCACCCT-3′—PPThr41. Final concentrations for PCR components per 25 μL reaction were as follows: 25 ng template DNA, 0.25 μM of each primer, 0.625 units of Taq DNA polymerase, 0.1 mM of each dNTP, 2.5 μL of 10× reaction buffer and 2.5 mM MgCl$_2$. Amplification parameters were as follows: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 90 or 60 s, and 72°C for 7 min. PCR products were examined on a 1.5% agarose gel using SYBR safe DNA gel stain (Invitrogen, Eugene, OR, USA) and purified using a Montage PCR 96 plate (Millipore, Billerica, MA, USA). Sequences were obtained via cycle sequencing with Big Dye 3.0 dye terminator-ready reaction kits using 1/16th reaction size (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were run with an annealing temperature of 52°C following the ABI manufacturer's protocol. Sequenced products were purified by passing reactions through Sephadex columns. Sequences were obtained using an Applied Biosystems 3730 XL automated sequencer at the Brigham Young University DNA Sequencing Center.

DNA sequences were edited using Chromas Lite 2.0 (Technelysium, Tewantin, Queensland, Australia) and imported and aligned by eye in BioEdit 7.0.5.2 (Hall 1999). Sequences were checked via amino acid coding in MEGA 4.0 (Tamura et al. 2007) to test for unexpected frame shift errors or unexpected stop codons. Phylogenetic analyses were performed using both parsimony and likelihood approaches using PAUP (Swofford 2003). Maximum parsimony (MP) was conducted via a heuristic search with 1,000 random additions and TBR branch-swapping. For our maximum likelihood (ML) analysis we identified the best-fitting model of molecular evolution using the hierarchical likelihood ratio test (hLRT) in Modeltest 3.7 (Posada and Crandall 1998). Modeltest identified TrN + G

as the best model with the following parameter estimates: Lset Base = (0.2429 0.3199 0.1461) Nst = 6 Rmat = (1.0000 25.4495 1.0000 1.0000 10.4299) Rates = gamma Shape = 0.0922 Pinvar = 0. Robustness of nodes was estimated with PAUP by bootstrap with 1,000 replicates for MP using a heuristic search with 10 random additions of taxa and TBR branch-swapping, and 1,000 replicates for ML via a heuristic search with 10 random additions of taxa and TBR branch-swapping. All tree lengths reported for MP include both informative and parsimony uninformative characters. Within- and among-taxon variation was calculated using the Maximum Composite Likelihood Method in MEGA.

Pairwise tests between sample sets for differences in the frequency of cyt *b* haplotypes were undertaken using the GENEPOP subprogram "STRUC", which estimates Fishers exact probabilities for m × n contingency tables, regardless of sample size. Probabilities were Bonferroni-adjusted for multiple tests.

Results

Allozyme analyses

Seventeen *N. obscura* individuals were successfully screened for 52 putative allozyme loci during the overview study. The following 12 loci displayed variation: *Ada*, *Adh*, *Ald2*, *Enol1*, *Est2*, *Gapd2*, *Gsr*, *Me2*, *PepA1*, *PepB*, *6Pgd*, and *Pgm1*. Of these, only the weakly-staining *Ald2* was not employed in the follow-up population study. An additional 12 loci were screened as they are routinely stained on the same zymograms as variable loci. Thus, 24 loci in all were screened in the population study. One individual from site 13 (labeled as a suspected hybrid upon capture) was heterozygous for unique alleles at 11 loci, and subsequent comparison with *N. australis* samples confirmed an *N. obscura* × *N. australis* F$_1$ hybrid (diagnostic loci are provided in Supplement 1). This individual was excluded from all further analyses, and there were no other indications of F$_1$ hybrids nor any instances of localized introgression (final *n* = 156 fish from 17 sites).

PCO allocated individuals neatly to one of four primary genetic lineages or sub-groups within *N. obscura*, hereafter referred to as 'MDB', 'Central', 'Merri' and 'Eastern' (Fig. 2). There is an obvious east versus west dichotomy in the first dimension (accounting for 46% of all variation), with one anomaly being a single eastern site (site 7, Mt Emu Creek) which is unequivocally aligned genetically to western sites. The second PCO dimension further splits the eastern and western into the four geographically-based subgroups described above (Figs. 1, 2). Follow-up PCO (not shown) revealed unequivocal separation of the Merri and
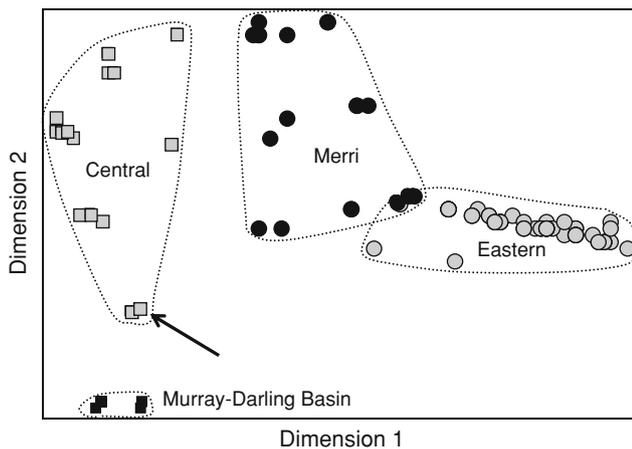
Fig. 2 Principal Coordinates Analysis of the 156 specimens in the allozyme population study. The relative PCO scores have been plotted for the first and second dimensions, which explain 46% and 13% of the total variance, respectively. Envelopes highlight major genetic groups, and regional symbols match Fig. 1. The geographically outlying Mt Emu Creek population is shown with an arrow

Fig. 3 UPGMA dendrogram depicting genetic affinities among 17 sites sampled for allozyme analysis, based on pairwise Nei Distance values. Letters represent hierarchical levels used in statistical evaluation (see Table 2)

Eastern lineages in deeper dimensions, and did not suggest admixture of sub-groups in individual sites. Thus four lineages and major sub-structure also evident in the population level analysis of the UPGMA dendrogram (Fig. 3) and NJ network (not shown) is well supported.

Statistical tests provided no evidence that individual sample sets violated Hardy–Weinberg expectations or harbored loci in linkage disequilibrium, sanctioning further comparisons of allele frequency among samples sets. Pairwise comparisons of allele frequency (Supplement 2, raw data in Table 2) revealed numerous statistically significant differences. Indeed, 95 of 105 comparisons showed at least one statistically-significant difference after Bonferroni correction, and 81% of all differences were highly significant ($P < 0.001$; Supplement 2). A summary of the extent of genetic heterogeneity (mean numbers of pairwise significant differences and range of values) displayed at different levels in the population hierarchy is shown Table 3, based on the hierarchy in the UPGMA dendrogram (Fig. 3).

Another perspective on between-site divergence was obtained by calculating F-statistics for the hierarchical levels of population structure in *N. obscura* (Table 3). Large, significant F_{ST} values were obtained for all basal levels of the population hierarchy (nodes A, B and E; Fig. 3), indicating strong support for the presence of four primary genetic lineages. Further population sub-structuring was indicated within the Merri and Eastern lineages, but not the Central nor MDB lineages. Table 3 also reveals that a single F_{IS} value was significant ($p < 0.01$) for an overall excess of heterozygotes, an outcome consistent with a chance event given that 14 statistical tests were applied.
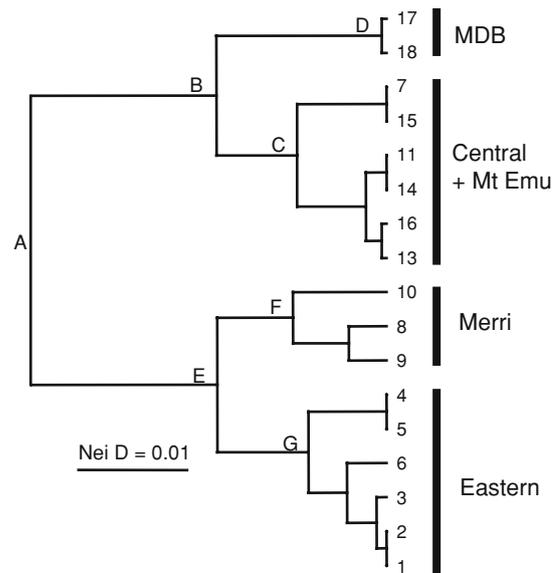
The UPGMA analysis revealed close genetic similarity of the Mt Emu and Crescent Pond sample sets. Samples from both sites were monomorphic at every locus for the most common allele present in the MDB plus Central lineages (sites 7, 11, 13–18; Table 2), ensuring that they displayed a pairwise Nei D of zero. Indeed, comparatively low levels of heterozygosity were evident at all sites, with H_O values ranging from 0.00 (the two previously-mentioned sites plus MDB site 17) to a maximum of 0.040 (overall population mean 0.017 ± 0.012), with no apparent between-lineage trends.

MtDNA analyses

Twenty-four cyt *b* haplotypes (*a–x;* GenBank Accession numbers GQ470892-GQ470915) were detected among the 128 individuals examined. Of 1,140 base pairs sequenced per individual, 1,070 were constant, 34 characters were parsimony-uninformative and 36 characters were parsimony-informative. Table 4 summarizes the distribution of haplotypes among 17 sites surveyed in the allozyme population study, plus an additional site (site 12, alcohol-preserved tissues only) referable to the Central lineage by geographic location (Fig. 1).

MP, with all characters weighted equally, recovered three most parsimonious trees of 79 steps (CI 0.911, RI 0.967). ML recovered one tree with a—ln score of—2105.58502 (Fig. 4). MP and ML analyses provided similar levels of bootstrap support, and displayed a well-supported primary dichotomy within *N. obscura*. This corresponded

Table 2 Allele frequencies at 17 variable loci for the 17 sites surveyed in *N. obscura*

Locus	1 (10)	2 (10)	3 (10)	4 (2)	5 (10)	6 (10)	7 (10)	8 (10)	9 (10)	10 (10)	11 (10)	13 (10)	14 (13)	15 (10)	16 (7)	17 (7)	18 (7)
Ada	c	c	c	c	c^{95},a	c	c	c	c	c	c^{80},d	c	c^{92},d	c	c	c	c
Adh	b	b	b	b	b	b	b	b	b	b	b	b	b^{96},a	b	b	b	b
Enol1	b	b	b	b	b	b	b	b	b	b	b	b	b^{96},a	b^{95},a	b	b	b^{86},a
Est2	c^{75},d	c^{55},d	c^{55},d	c^{50},d	c^{55},d	d^{60},c	d	d	d	d^{95},c	d	d	d	d	d	d	d
Gapd2	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	b	b
Gpi1	e	e	e	e	e	e	e	e	e^{95},b	e	e	e	e	e	e	e	e
Gsr	b	b	b	b	b	b	b	b^{95},a	b^{95},a	a^{85},b	a	b	a^{85},b	a^{70},b	a^{93},b	b	a^{93},b
Mdh1	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Me1	b	b	b	b	b	b	b	b	b	b^{95},a	b	b	b	b	b	b	b
Me2	c	c	c	c	c	c	a	c	c^{90},a	c^{85},a	a^{55},c	a	a^{65},c	a	a	a	a
PepA1	b	b^{95},c	b^{65},c	b^{50},c	c^{75},b	b^{70},c	b	b	b	b	b	b	b^{96},c	b	b	b	b
PepA2	b	b	c^{45},d^{40},b	b	b	b	b	b	b	b	b	b	b^{96},a	b^{95},a	b^{79},a	b	b
PepB	d^{60},c	d^{60},c	b	c	c	c	d	d	d	d^{95},b	d	d	d	d	d	d	d
PepD2	b	b	b	b	b^{80},c	b	b	b	b	b^{80},c	b	b	b	b	b	b	b
6Pgd	d	d	d	d	d	d	b	d	d^{80},e^{15},b	d^{75},b	b	b	b	b	b	b	b^{79},a
Pgm1	a	a^{85},b	a	b^{75},a	a^{50},b	a	a	a^{60},b	a	a	a	a	a	a^{90},b	a^{93},b	a	a
Pgm2	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
H_O	0.008	0.021	0.028	0.028	0.040	0.023	0.000	0.009	0.015	0.034	0.017	0.000	0.025	0.019	0.013	0.000	0.016
S.E.	0.006	0.011	0.017	0.021	0.021	0.016	0.000	0.008	0.009	0.014	0.012	0.000	0.014	0.012	0.009	0.000	0.010

For polymorphic loci, the frequencies of all but the rarest alleles are expressed as percentages and shown as superscripts (allowing the frequency of each rare allele to be calculated by subtraction from 100%). Invariant loci (not shown): *Enol2*, *Est1*, *Gapd1*, *Got1*, *Got2*, *Gpi2*, and *PepD1*. Observed heterozygosity estimates (H_O) were calculated for all 52 loci run in the overview study, as discussed in the text

Table 3 Summary of quantitative analyses of population structure in *N. obscura*, based on the allozyme data

Hierarchical level[a]	Sites	Sig. diffs[b]	F_{IS} (99% CI)[c]	F_{ST} (99% CI)[c]
Species [node A]	All	2.8 (0–7)	−0.014 (−0.156 to 0.144)	0.687** (0.429 to 0.808)
MDB/Central/Mt Emu [node B]	7, 11, 13–18	1.1 (0–2)	−0.112** (−0.205 to −0.011)	0.607** (0.049 to 0.857)
Central/Mt Emu [node C]	7, 11, 13–16	0.7 (0–2)	−0.112 (−0.213 to 0.001)	0.454 (−0.001 to 0.692)
MDB [node D]	17, 18	0	−0.125 (−0.200 to 0.000)	0.111 (0.000 to 0.167)
Merri/Eastern [node E]	1–3, 4/5, 6, 8–10	2.1 (0–6)	0.027 (−0.178 to 0.212)	0.449** (0.241 to 0.599)
Merri [node F]	8–10	1.3 (1–2)	−0.067 (−0.192 to 0.137)	0.423** (0.011 to 0.655)
Eastern [node G]	1–3, 4/5, 6	1.0 (0–3)	0.063 (−0.347 to 0.375)	0.252** (0.004 to 0.394)

[a] Nodes in the hierarchical level refer to those identified in Fig. 3

[b] Mean and range (bracketed) for the number of statistically-significant differences in allele frequency detected among pairwise comparisons of populations

[c] Confidence intervals (99% CI)

** $P < 0.01$ (all other $P > 0.05$)

Table 4 Comparative mtDNA haplotypes at each *N. obscura* site

Popn	ESU	Cyt *b* subclade	Haplotype/sample size	Sites within ESU displaying significant differences
1	Eastern	IA	a^6; b^2	all
2	Eastern	IA	c^4, d^5	all
3	Eastern	IA	f^4, g^1	all except 4 & 5
4	Eastern	IA	e^2, f^5, h^1	all except 3 & 5
5	Eastern	IA	f^5	all
6	Eastern	IA	e^9	all
8	Merri	IB	i^5	10
9	Merri	IB	i^2, j^1, k^2	10
10	Merri	IB	l^5	8 & 9
7	Central	IIA	m^7	all
11	Central	IIB	n^9	all
12	Central	IIB	o^9	all
13	Central	IIB	s^9	all
14	Central	IIB	p^4	all except 15 & 16
15	Central	IIB	o^1, p^5, q^2, r^2	all except 14 & 16
16	Central	IIB	o^3, p^2, t^3	all except 14 & 15
17	MDB	IIC	u^3, v^2	none
18	MDB	IIC	u^1, v^4, w^2, x^1	none

The number of individuals per haplotype is shown as a superscript. Also included is a summary of which pairwise comparisons of cyt *b* haplotype frequencies between sample sets were statistically significant ($P < 0.05$) within each ESU or sub-clade, after correcting for multiple tests

to that shown by allozyme data (Fig. 3): clade I was found only at Merri and Eastern sites, whereas clade II was restricted to MDB and Central sites (Table 4). Together the two major clades were further resolvable into five geographically-restricted sub-clades, all supported by bootstrap values >62%. These sub-clades have been labeled IA (Eastern sites), IB (Merri sites), IIA (Mt Emu site), IIB (Central sites), and IIC (MDB sites). Thus, the mtDNA data demonstrated the same primary genetic structure within *N. obscura* as shown by nuclear genetic data, and in addition highlighted a phylogeographic separation between Mt Emu and all other Central sites. The five sub-clades (Table 4) are statistically distinct ($P < 0.001$ in all pairwise comparisons).

Although strong phylogeographic structure was evident in the mtDNA phylogram, overall levels of haplotype diversity were low. Thus, mean maximum composite likelihood divergences between haplotypes within sub-clades were 0.2–0.3%, with between sub-clades values being only two- to five-fold higher (0.5–0.7% between sub-clades of the same major clade and 1.3–1.7% between sub-clades from different major clades; Supplement 3).

Additional sub-structuring was evident within several sub-clades, but did not correspond to any simple dichotomous geographic pattern (Table 4). Nevertheless, of the four geographic regions represented by multiple sites (all except Mt Emu), only the MDB region did not display statistically-significant pairwise differences in haplotype
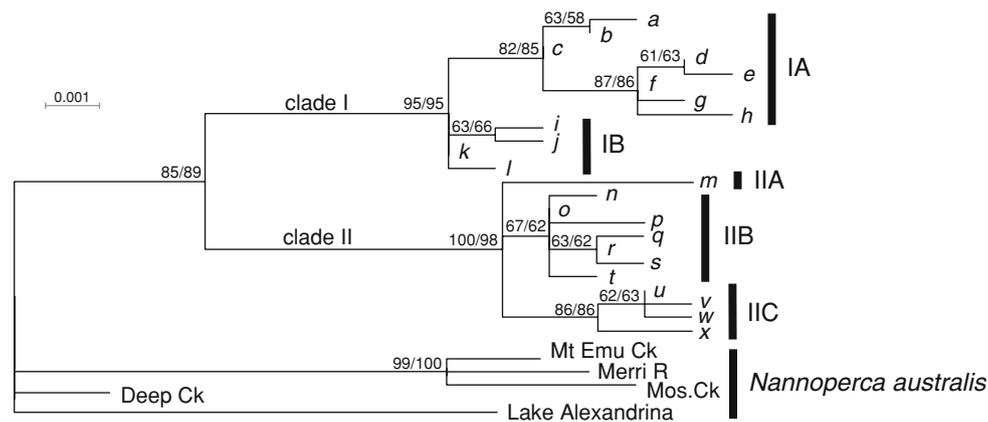
Fig. 4 Maximum likelihood phylogram for the 24 cyt *b* haplotypes detected among 128 *Nannoperca obscura*. The phylogram was rooted using five *N. australis* sequences using specimens from sites of sympatry (GenBank Accession numbers GQ470916-GQ470920; *Mos.* *Ck* Mosquito Creek). Haplotypes are labeled by letter (*a–x*) for *N. obscura*. Bootstrap values (MP/ML) above 60% are shown for all nodes. Major clades (I and II) and sub-clades (IA, IB, IIA, IIB, and IIC), are labeled as per the text

frequency (Table 4). This pattern is in agreement with that displayed by the allozyme analyses (Supplement 2).

Discussion

This study represents a comprehensive assessment of genetic sub-divisions across the extant range of *N. obscura* including recently discovered western populations. The species displays a major phylogeographic dichotomy and other sub-structuring that is largely congruent across both nuclear and matrilineal data, providing a new perspective on recent and historic gene flow in *N. obscura*. The data raise the issue of how strong genetic structure can best be incorporated into the conservation and management of threatened freshwater fishes.

Genetic sub-structure

Nannoperca obscura displays marked phylogeographic sub-structure, largely concordant with geographically definable boundaries. Populations were characterized by moderate levels of genetic differentiation between sites, implying low vagility in the species. Isolation occurs in drainages separated by marine barriers or sub-divided by features such as waterfalls (Mt Emu Creek) and land barriers (Crescent Pond, a karst sinkhole). Dispersal and gene flow within systems appear to be minimal, although more spatial replication is needed for confirmation. Some insight is provided for three sites (14–16) with similar allele frequency and mtDNA profiles in the Millicent Coast River Basin, suggesting contemporary gene flow across a naturally continuous landscape of intermittent, longitudinal wetlands. The recent and ongoing influence of drainage infrastructure which now dissects the Millicent Coast River

Basin (South Eastern Drainage Board 1980) has fragmented dispersal routes and populations, and may ultimately cause reductions in genetic diversity, or local extinction (i.e. Henry Creek and Lake Bonney).

Overall estimates of heterozygosity and haplotype diversity in *N. obscura* were relatively low. The distribution of *N. obscura* is generally reported as patchy with low abundance rather than as extensive and large populations (Saddlier 1993; Kuiter et al. 1996; Hammer 2002). Thus, low gene diversity may be the result of intermittently or permanently small effective population sizes (Nevo et al. 1984). The lack of nuclear divergence between the Mt Emu population and some others along the Millicent Coast may also reflect population genetic phenomena (e.g. founder effects, bottlenecks, selective sweeps) associated with initial and continuing small population size.

Intensive sampling throughout a species' range (or potential habitat) is essential to ensure that interesting and perhaps pivotal patterns of genetic differentiation are detected (Moritz et al. 1995). In this case, anomalies included Mount Emu Creek which was dissimilar to its neighboring populations, and the Murray-Darling Basin which was found to harbor a distinct lineage at the western edge of the species' range. Peripheral populations are often distinct genetically (Lesica and Allendorf 1995; Eckert et al. 2008), and historic oversight of this population is quite significant (cf. Kuiter et al. 1996).

Biogeographic patterns

The major phylogenetic break in *N. obscura*, along the eastern edge of the Glenelg River Basin, coincides with the extent of the Newer Volcanics in western Victoria (Johnson 1989; Joyce et al. 2003). This broad expanse of basalt (*c.* 15,000 km²) was erupted during the period 4.5–0.5 Ma

(Johnson 1989). The surprising alignment of Mt Emu Creek, a tributary of the Hopkins River, to the western genetic grouping possibly suggests a previous connection to the west. The upper Hopkins and Glenelg river basins are contiguous and dispersal may have occurred historically across low divides or swampy connections or during considerable volcanism and sedimentation in the area (Joyce et al. 2003). The genetic distinctiveness of the Murray-Darling population indicates long-term isolation due to ocean and drainage basin boundary barriers to fish movement. In addition, the occurrence of *N. obscura* within MDB only in Lake Alexandrina supports information that this water body has been a predominantly fresh habitat over thousands of years (Sim and Muller 2004; Fluin et al. 2007). These data are in contrast to the proposed future management of Lake Alexandrina which include further disconnection via new weirs, ongoing water level reductions, and the potential introduction of sea-water.

Implications for ecology

The presumed low dispersal ability of *N. obscura* is supported by observed strong genetic structure, but requires validation with field movement studies. The wide lateral spread of populations (some 1,500 km) encompass contrasting environmental conditions and fish communities, and the ecological exchangeability of major lineages and divergent populations warrant more research (Crandall et al. 2000; Palsbøll et al. 2007). Examples of critical knowledge gaps include the physiochemical tolerance of different life stages, life history, morphological variation/adaptation, and interaction with other fishes and terrestrial predators.

The reproductive and ecological isolating mechanisms of sympatric *Nannoperca* are robust (i.e. only a single F_1 hybrid was detected with no indication of introgression), but may come under threat with habitat alterations that disrupt ecological boundaries. Indeed, monitoring at the Crescent Pond site since the single *N. obscura* × *N. australis* hybrid was detected in 2004 (this study) has shown a sharp increase in the incidence of hybrids, likely due to prolonged drought and/or climate change eroding reproductive isolating barriers within a small habitat (Hammer 2009). Degraded or altered habitats have been known to foster hybridization and introgression between species in other freshwater genera (e.g. Seehausen et al. 1997; Fisher et al. 2006).

Conservation

Four diagnosable lineages were identifiable as ESUs based on genetic criteria (Moritz 1994), namely (1) the Murray-Darling Basin, (2) the Glenelg River Basin, Millicent Coast and Mt Emu Creek, (3) rivers including and immediately surrounding the Merri Catchment, and (4) eastern range populations. Mt Emu Creek represents a fifth mtDNA lineage, but did not show significant differences at nuclear markers and therefore does not strictly qualify as an ESU as defined herein. Nevertheless its phylogeographic distinctiveness and outlying geographic distribution qualifies it as an MU (Moritz et al. 1995). A logical extension of this perspective would see all independent drainage areas or catchments (i.e. those with marine barriers) regarded as distinct MUs, given that most populations screened showed significant differences at nuclear loci and/or unique mtDNA haplotypes.

The finding that ESUs in *N. obscura* occur at geographic distributional scales of singular to grouped river basins, is an outcome comparable to other small demersal fishes throughout the world (e.g. Mesquita et al. 2001; Quattro et al. 2001). Accordingly the spatial scale for the conservation of evolutionary diversity in freshwater fishes is comparatively restricted when compared to more vagile fauna such as marine vertebrates (e.g. Karl and Bowen 1998).

Several of the newly recognized conservation units have come under threat in the short time since initial sampling for genetic analysis was undertaken (i.e. Murray-Darling ESU, all South Australian populations of the Central ESU: Hammer 2008, 2009), and similar declines are likely but unheralded in eastern populations. This example illustrates that urgent research attention should be paid to declining species or regional populations to prioritize protection for important evolutionary components, and provide a baseline for restorative actions in case of local extinction.

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