CSIRO PUBLISHING

Marine and Freshwater Research, 2021, **72**, 263–274
https://doi.org/10.1071/MF20024

Population genetic structure of estuary perch (*Percalates colonorum* Gunther) in south-eastern Australia

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Abstract. Estuary perch (*Percalates colonorum* Gunther) is an estuary dependent fish native to south-eastern Australia that is in decline. There is an increasing emphasis on stocking the species. Understanding the genetic structure across its range is important for guiding optimal stocking strategies. A prior study found some evidence of population genetic structure; however, few genetic markers were used in that assessment. Here, we develop 21 novel polymorphic microsatellite markers to reassess population genetics. Analyses indicate three broad genetic clusters, with populations on mainland Australia exhibiting an isolation by distance pattern. The only known population from Tasmania is genetically and geographically isolated from mainland populations and has very low levels of genetic diversity. We provide recommendations for sourcing broodstock from mainland populations, including describing three broad areas for procuring and releasing broodstock and offspring. The markers and results reported here will prove invaluable for guiding and monitoring the outcomes of stocking and conservation activities.

Additional keywords: conservation, fishery, genetic diversity, stocking, supplementation.

Received 23 January 2020, accepted 17 June 2020, published online 20 July 2020

Introduction

Worldwide, many species of freshwater fish have declined in range and abundance due to anthropologically driven processes including habitat degradation, river regulation, introduction of exotic fish species and climate change (Arthington 1991; Mallen-Cooper 1993; Ogston *et al.* 2016). To redress the decline, supplemental stocking of hatchery-reared fish is often undertaken. Although stocking has been used as an effective management strategy (Morissette *et al.* 2018), negative consequences can arise when little regard is shown for the genetic structure of native populations (Utter 1998; Laikre *et al.* 2010; Valiquette *et al.* 2014). Uninformed stocking has, at times, led to reductions in genetic diversity, differentiation and population fitness, declines in local adaptation and population size, displacement of local gene pools and possibly even localised extinctions (Utter 1998; Araki *et al.* 2009; Laikre *et al.* 2010). To limit potential negative consequences of supplementary stocking of wild populations, it is imperative that the genetics of the species in question are considered. Although such a need is acknowledged, it is often not considered an essential step in stocking programs (Laikre *et al.* 2010; Attard *et al.* 2016).

Estuary perch *Percalates colonorum* Gunther is an estuary-dependent catadromous fish that inhabits tidal reaches of rivers, lakes and coastal lagoons from the Richmond River (28°52′S, 153°35′E) in northern New South Wales (NSW) to the mouth of the Murray River (35°31′S, 138°47′E) in South Australia (Cadwallader and Backhouse 1983; McDowall 1996). Historically, the species was also present in estuaries in the north of Tasmania, but only one remnant population, within the Arthur River, is now known to persist in that state (J. Haddy, pers. comm., 2019). Individuals are long lived (>40 years) and highly valued by recreational fisherman for their

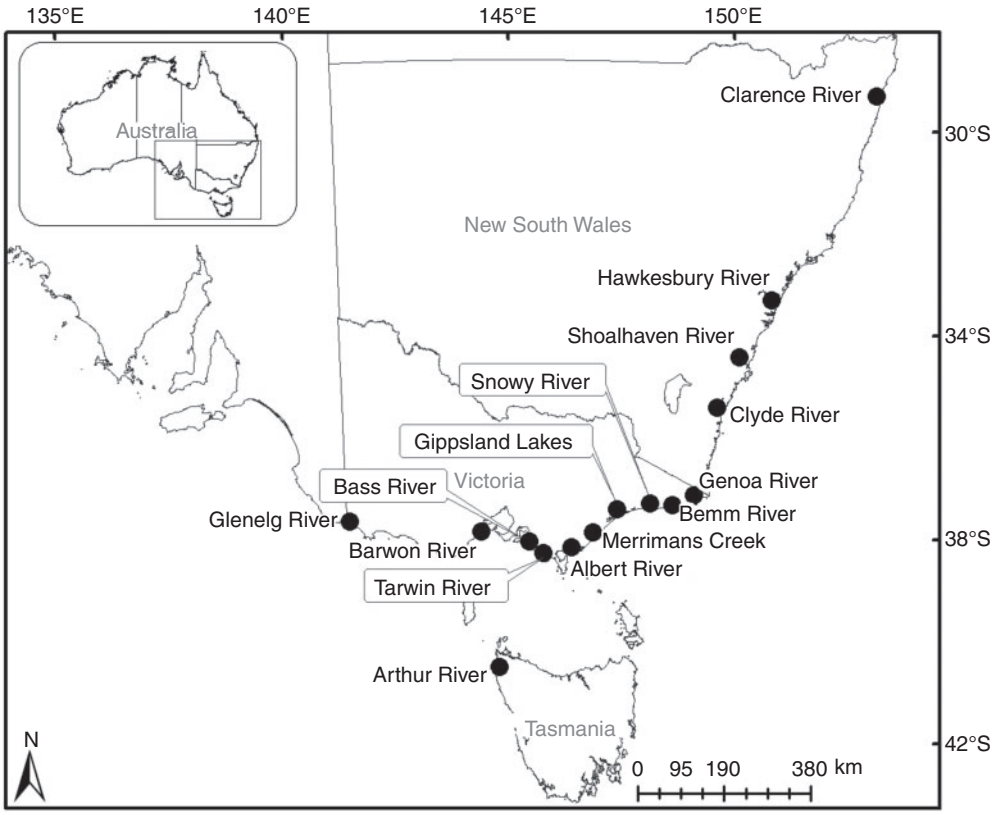

Fig. 1. Locations of the estuaries in eastern Australia where estuary perch were sampled.

fighting and eating qualities (Walsh *et al.* 2010). Estuary perch are thought to complete their entire life cycle within a specific estuary (Williams 1970; McCarraher and McKenzie 1986; Walsh *et al.* 2012, 2013); nevertheless, a small number of larvae have been captured entering the mouth of a river in central NSW from the ocean on a flood tide, and anecdotal offshore movements of adults have been reported in conjunction with large freshwater flow events (Trnski *et al.* 2005). The species was once abundant, but in recent years they have undergone a decline in distribution and abundance, likely as a response to fishing pressure, flow regulation and climate change (Walsh *et al.* 2010; Stoessel *et al.* 2018). There is now increasing emphasis on supplementing wild populations of estuary perch by using hatchery-reared fish.

A prior population genetic study of estuary perch based on mitochondrial (mt) DNA and microsatellite DNA markers provided evidence for two historically distinct groups associated with the Pleistocene emergence of the Bassian land bridge in southern Australia connecting Tasmania and the mainland (Shaddick *et al.* 2011*a*). Generally, that study identified large-scale patterns of contemporary connectivity in estuary perch influenced by both large- and fine-scale oceanographic currents and processes. However, that study used a small number of microsatellite markers isolated from the closely related Australian bass (*Percalates novemaculeata*; Shaddick *et al.* 2011*b*). The small number of markers, combined with their low allelic diversity (mean number of alleles per locus), prevents comprehensive analyses of population genetic structure in species expected to exhibit large population sizes and moderate to high connectivity (see Grummer *et al.* 2019).

In this study we used next-generation sequencing (NGS) to develop 21 novel polymorphic microsatellite markers and used these to genotype 372 estuary perch samples from 15 estuaries across their range in south-eastern Australia. The overall aim of the study was to determine fine-scale population genetic structure and patterns of connectivity to guide future management of wild estuary perch populations.

Materials and methods

Samples

Samples were genotyped across the range of estuary perch in eastern Australia to provide a comprehensive assessment of genetic structure. We sampled caudal fin clips non-lethally from 154 putative estuary perch and 60 putative Australian bass in five estuaries from Victoria, Australia (Fig. 1; Table 1). Samples were collected in spring 2015 by the authors or supplied by the Victorian Fisheries Authority (State Government of Victoria). Australian bass were sampled from the Snowy and Genoa estuaries to determine the cross-amplification of the new microsatellite markers, and as a baseline for determining hybrid individuals between estuary perch and Australian bass (see below). Fin clips from 34 individuals were also obtained by the authors from a population in the Arthur River, Tasmania. All fin clip samples were stored in 95% ethanol at –20°C until DNA extraction. In addition, 238 estuary perch samples (as DNA

Table 1. Location of the 15 estuaries sampled and the total number of samples obtained in each estuary (total $n = 486$)

The number of estuary perch (EP), Australian bass (AB), their hybrids (identified through microsatellite analyses) and the number of individuals for which genotyping was unsuccessful is indicated. Subsequent genetic analyses only include samples of EP. Genotyping was deemed unsuccessful when no alleles were amplified for >50% of the 21 microsatellite markers. ARI, Arthur Rylah Institute for Environmental Research, Department of Environment, Land, Water and Planning; IMAS, Institute for Marine and Antarctic Studies, University of Tasmania; NSW, New South Wales; Tas., Tasmania, Vic., Victoria

Population	Region	Latitude	Longitude	Source	Total (*n*)	Number of EP	Number of AB	Number of hybrids	Number unsuccessfully genotyped
Arthur River	Tas.	−41.0519	144.6654	IMAS	34	34	–	–	–
Glenelg River	Vic.	−38.0581	140.9911	Shaddick *et al.* (2011*a*)	12	10	–	–	2
Barwon River	Vic.	−38.2718	144.5048	ARI	30	30	–	–	–
Bass River	Vic.	−38.4958	145.4326	Shaddick *et al.* (2011*a*)	43	36	–	–	7
Tarwin River	Vic.	−38.6951	145.8434	Shaddick *et al.* (2011*a*)	25	22	–	–	3
Albert River	Vic.	−38.6372	146.6407	Shaddick *et al.* (2011*a*)	14	9	–	1	4
Merriman Creek	Vic.	−38.3811	147.1835	Shaddick *et al.* (2011*a*)	39	30	–	–	9
Gippsland Lakes	Vic.	−37.943	147.7293	Shaddick *et al.* (2011*a*)	25	15	–	8	2
Snowy River	Vic.	−37.7651	148.5158	ARI and Shaddick *et al.* (2011*a*)	117	82	28	7	–
Bemm River	Vic.	−37.7622	148.9836	ARI	27	27	–	–	–
Genoa River	Vic.	−37.4751	149.6403	ARI	40	10	22	8	–
Clyde River	NSW	−35.6979	150.1589	Shaddick *et al.* (2011*a*)	28	25	–	–	3
Shoalhaven River	NSW	−34.8604	150.7406	Shaddick *et al.* (2011*a*)	19	17	–	–	2
Hawkesbury River	NSW	−33.5566	151.2401	Shaddick *et al.* (2011*a*)	26	18	2	–	6
Clarence River	NSW	−29.4274	153.3623	Shaddick *et al.* (2011*a*)	7	7	–	–	–
Total					486	372	52	24	38

extractions) from 11 estuaries were obtained from Shaddick *et al.* (2011*a*) for reanalysis with the new microsatellite loci (Table 1).

Genetic samples collected by the authors were sampled under Victorian Fisheries Authority Permit numbers RP827 and RP1196, Tasmania Inland Fisheries Service Permit number 2014-51, Arthur Rylah Institute, Department of Environment Land Water and Planning Animal Ethics Permit number AEC 15/11 and University of Tasmania Animal Ethics Permit number A0014546.

DNA extraction

Caudal fin clip tissue from each of the 248 specimens was used for DNA extraction. DNA was extracted using a modified Chelex extraction protocol (Walsh *et al.* 1991). Tissue samples were placed into separate 1.5-mL microcentrifuge tubes containing 3 µL of proteinase K and 200 µL of 5% Chelex solution. Samples were incubated at 55°C for 60 min and then at 95°C for 15 min with periodic vortexing. Extractions were stored at –20°C until required. Prior to polymerase chain reaction (PCR), all extractions were centrifuged at 15 493*g* for 2 min at 21°C and the supernatant from just above the Chelex resin was used for PCR amplification. Samples from Shaddick *et al.* (2011*a*) were also extracted from caudal fin clips but using the salt extraction method with a proteinase K digestion step.

Microsatellite marker development

NGS library preparation

To develop the microsatellite markers, DNA was extracted from ~10 µg of tissue from a single estuary perch using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified genomic DNA was randomly fragmented to an average size of 500 bp using an M220 Focused Ultra-sonicator (Covaris, Woburn, MA, USA). Library preparation (end-repair, A-tailing, Illumina (San Diego, CA, USA) adaptor ligation and PCR enrichment) was performed using a NEBNext Ultra DNA library prep kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. The library was quantified using a Tapestation 2200 (Agilent, Santa Clara, CA, USA) and sequenced on the MiSeq Desktop Sequencer (Illumina) located at the Monash University Malaysia Genomics Facility (Subang Jaya, Malaysia) using a configuration of two 250-bp runs.

Microsatellite Identification

Raw paired-end reads were adaptor trimmed using Trimmomatic (ver. 0.39, see http://www.usadellab.org/cms/?page=-trimmomatic, accessed 26 June 2020) (Bolger *et al.* 2014) and subsequently assembled *de novo* using IDBA-UD (ver. 1.1.3, see https://kbase.us/applist/apps/kb_IDBA/run_idba_ud/release, accessed 26 June 2020) with the modified setting of –mink 31 and –maxk 251 (Peng *et al.* 2012). Bacterial- and viral-derived contigs were identified using Kraken (ver. 2, see https://ccb.jhu.edu/software/kraken/, accessed 26 June 2020; Wood and Salzberg 2014) and removed from the assembly. Microsatellite identification and optimal primer set selection was performed using the QDD3 pipeline program (see http://net.imbe.fr/~emeglecz/qdd.html, accessed 26 June 2020; Meglécz *et al.* 2014).

Microsatellite screening

Primers for 48 microsatellite loci were screened for polymorphism in PCR trials. Loci were screened using eight template DNA samples of estuary perch. Primers were pooled into groups of four where they were coamplified by multiplex PCR using a Qiagen multiplex kit. To distinguish PCR products upon capillary separation, each primer was tagged with a unique fluorescent label during the PCR using the method outlined by Blacket *et al.* (2012). Reaction matrices for PCR amplification consisted of 5 μL of Qiagen multiplex mix, 4 μL of primer mix (0.2 μM each primer) and 2 μL of template DNA. The PCR conditions consisted of an initial 15 min denaturing step at 94°C, followed by 40 cycles of 94°C for 30 s, 59°C for 1.5 min, and 72°C for 1 min, with a final extension step of 60°C for 30 min. Genotyping was performed using a 3730 capillary analyser (Applied Biosystems, Foster City, CA, USA), and product lengths were determined relative to a GS500LIZ_3730 size standard (Applied Biosystems). Microsatellite profiles were examined and scored manually and assessed for polymorphisms using GeneMapper (ver. 4.0, see https://www.thermofisher.com/order/catalog/product/4440915#/4440915, accessed 26 June 2020; Applied Biosystems).

Hybrid identification

Hybridisation of estuary perch and Australian bass occurs in the wild, particularly in east Gippsland in Victoria, which is a hot spot for such occurrences (Schwartz and Beheregaray 2008 Shaddick *et al.* 2011*b*). As a result, all individuals were screened using the 21 new microsatellite markers developed herein, as well as microsatellite markers AB001, AB006 and AB107 from Schwartz and Beheregaray (2008), which have unique alleles associated with each species. By screening the Australian bass samples collected in this study, we also identified unique alleles associated with the new microsatellite markers for each species, which enabled hybrid individuals (and back-cross individuals) to be identified. We also undertook a discriminant analysis of principal components (DAPC; see below) to help identify hybrid and back-cross individuals. All hybrids and back-crossed individuals identified were then removed from further analyses.

Microsatellite analysis

The software MICROCHECKER (ver. 2.2.3, http://www.nrp.ac.uk/nrp-strategic-alliances/elsa/software/microchecker/, accessed 26 June 2020; Van Oosterhout *et al.* 2004) was used to assess microsatellite loci for null alleles and scoring errors using the formulas outlined by Brookfield (1996). The following statistics were calculated for the microsatellite data using FSTAT (ver. 2.9.4, see https://www2.unil.ch/popgen/softwares/fstat.htm, accessed 26 June 2020; Goudet 1995): observed and expected heterozygosity, allelic richness per population averaged over loci, Weir and Cockerham's measure of inbreeding coefficients (F_{IS}), a global estimate of fixation index (F_{ST}; with 95% confidence limits (CL); Weir and Cockerham 1984), population pair-wise measures of F_{ST} and their significance determined using permutations (1000) and pairs of loci tested for linkage disequilibrium using a log-likelihood ratio test. To overcome potential limitations of F_{ST} calculations using multiallelic loci (Jost 2008), additional estimates of population differentiation (D_{est}), global D_{est} and population pair-wise measures of D_{est} (significance determined using 10 000 permutations) were generated using GenAlEx (ver. 6.5, see https://biology-assets.anu.edu.au/GenAlEx/Download.html, accessed 26 June 2020; Peakall and Smouse 2006). Deviations from Hardy–Weinberg equilibrium (HWE) were assessed for significance using GDA (ver. 1.1, see https://phylogeny.uconn.edu/software#, accessed 26 June 2020). Bottleneck tests were undertaken on population samples using BOTTLENECK (ver. 1.2.02, see http://www1.montpellier.inra.fr/CBGP/software/Bottleneck/bottleneck.html, accessed 26 June 2020; Cornuet and Luikart 1997), using the infinite allele (IAM), stepwise mutation (SMM) and two-phase (TPM) models and testing for significance using the Wilcoxon signed-rank test. The Dunn–Sidak method (Sokal and Rohlf 1995) was used to adjust significance to the $\alpha' = 0.05$ level when undertaking multiple comparisons.

A Bayesian analysis was then conducted to estimate the number of populations within the sample data using STRUCTURE (ver. 2.3.4, see https://web.stanford.edu/group/pritchardlab/structure_software/release_versions/v2.3.4/html/structure.html, accessed 26 June 2020; Pritchard *et al.* 2000). Based solely on genetic data, STRUCTURE identifies the number of distinct clusters or populations, assigns individuals to clusters and identifies migrants and admixed individuals. To determine the number of populations (K), 10 independent simulations for $K = 1$–5 with 100 000 burn-in and 1 000 000 data iterations were run for each analysis. The STRUCTURE analysis was performed using the admixture model of population structure (i.e. each individual draws some fraction of their genome from each of the K populations) and allele frequencies were set as independent among populations. STRUCTURE HARVESTER (ver. 0.6.94, see http://taylor0.biology.ucla.edu/structureHarvester/, accessed 26 June 2020; Earl and vonHoldt 2012) was then used to implement the method of Evanno *et al.* (2005) to determine the true number of populations (K).

A DAPC was also used to identify and describe clusters of genetically related individuals using the R package adegenet (ver. 1.3–9.2, see https://adegenet.r-forge.r-project.org/, accessed 26 June 2020; Jombart *et al.* 2010). This multivariate method does not assume a particular population genetics model and is free of assumptions on HWE and linkage equilibrium (Jombart *et al.* 2010). To avoid overfitting of the discriminate functions, we retained 15 principal components (>80% of the variation).

Finally, to explore the relationship between geographic and genetic distance, we undertook regressions and Mantel tests of Slatkin's linearised F_{ST} transformation (i.e. $F_{ST} \div (1 - F_{ST})$; Rousset 1997) with the natural log of geographic distance calculated using GenAlEx (ver, 6.5.03, see https://biology-assets.anu.edu.au/GenAlEx/Download.html, accessed 26 June 2020; Peakall and Smouse 2006). The significance of Mantel tests was determined by permutation (10 000 randomisations).

Results

Microsatellite marker development, characterisation and hybrid determination

In all, 1 125 201 reads and 271 961 082 bp of data were obtained from the MiSeq platform. Of the 7780 microsatellite loci identified with QDD3, primers were developed for 48 loci, which

Table 2. Characteristics of the 21 microsatellite loci isolated from estuary perch, with primer combinations for each of the multiplex reactions
AB, Australian bass; EP, estuary perch; N_a, number of alleles; NA, no amplifiable product

Locus	Forward Primer	Reverse Primer	Repeat motif	N_a for EP (AB)	EP size range (bp)	AB Size range (bp)
Multiplex 1						
EP09	ACAATACCCAGAGTGCCGAG	ATACAACGTCCCACCGAGAC	AC	3 (2)	103–125	103–117
EP26	GATGGAGCAGAGGTGGTAGG	TCACTCAACCAGACAGTGCC	AG	4 (9)	134–140	136–154
EP41	AAAGGGATAGTCCGTGTAGTGC	GGTGTAACTGGATCTGTTGGG	AAG	4 (2)	249–257	246–261
EP18	CACCATCAATAACACGGCAG	AAACAGTTGAACCAGGACCG	AC	6 (3)	206–228	202–216
EP36	AGTCCTCCTGGACACCAGC	TATTGAGAAGGGCCAACAGG	AGC	4 (5)	234–249	234–249
EP04	TCCTGCTGTCCCTTTATGAAAC	ACAGGTGAGGAGGTCAGAGG	ATC	7 (3)	260–281	270–280
EP38	GCTGACATCAAGGCCAATTC	ATTTGAGGCCAACCATCAAC	AC	7 (3)	330–342	403–429
Multiplex 2						
EP30	CTGTCCAGGATTCATCTCCC	AAGATTGGCCATGAACAACAG	AC	5 (2)	138–150	88–138
EP39	TTGTGCACCTGTGTCAACTG	TGTTTGAACTTGTCTCCCTGG	AC	13 (4)	146–169	131–145
EP03	GCCACTGATCACTCAGCAAC	TCAGTGGTTCTGCAGACAGG	AC	6 (8)	203–217	195–219
EP22	CATTCCAGGCAATAGAAGTGC	TCCTTTATCATCGGTTTGGC	AC	6 (NA)	214–226	NA
EP32	AGCCGTTACCGTCCACATAC	TTTGTGATCCAGGCTGTTTG	ACT	29 (8)	162–298	223–277
EP19	ATGGGAAAGCAGGCAGTG	AGTCACCCAGGATTACGCAC	AC	3 (20)	319–323	347–403
EP42	TCATTATATGGGAATGCAGACG	CCAATCTGCCAAGGGATAAG	AC	11 (7)	331–365	331–353
Multiplex 3						
EP16	TTGCATACAGGAACGTCCG	TAGGCCAGTGTGAGTGGTTG	AC	2 (1)	123–125	123
EP47	ACTGAGAGGCCCTGAGAGTG	AATACCCTGTGACACTGCCC	AC	3 (1)	135–139	128
EP08	TGTTAGTCCACAGTGGTGGC	AAAGGAGATGCGTCAAGGAG	AGAT	7 (2)	248–276	276–280
EP44	TAATGCATGGCATGAAGTCC	TCATGTTTGGCTCTGAAACG	AC	4 (2)	198–208	204–206
EP14	TCCACTGGTTGCATGTCTTC	AATGTTGGTGCCTCAATGTG	AC	3 (1)	214–222	220
EP23	GGTCTGCAGGGTTTGGTATG	GCACTAAGAGCTTTCCTCCG	AG	3 (4)	323–329	327–339
EP33	AGAGGGAGCAACTGGGACTC	CCAGAGGCTTACAGTCCGTC	AC	5 (8)	329–343	327–347

were then screened for polymorphism. Of the 48 loci, 25 were found to be polymorphic and 21 were selected for the genetic assessment of estuary perch samples. The coamplification of these markers was optimised (Blacket *et al.* 2012) and consisted of three multiplex reactions with seven markers per reaction. These microsatellite loci were characterised across all samples for both estuary perch and Australian bass (Table 2). In all, 135 alleles were detected in estuary perch across the 21 microsatellite markers from 372 successfully genotyped individuals, whereas 95 alleles were detected in Australian bass from 52 individuals. Several loci (EP14, EP16, EP47) were monomorphic in Australian bass, and one locus (EP22) did not amplify. The highest number of alleles in estuary perch was detected at locus EP32 (29 alleles), whereas the highest number of alleles was detected at locus EP19 (20 alleles) for Australian bass.

We identified 24 hybrid individuals using the different allelic profiles of the 21 microsatellite loci and screening the three additional loci (AB001, AB006, AB107) from Schwartz and Beheregaray (2008). The DAPC analysis confirmed the hybrid status of these individuals (Table 1). Hybrid individuals were found in Victorian estuaries only, from the Snowy River ($n = 7$), Genoa River ($n = 8$), Gippsland Lakes ($n = 8$) and Albert River ($n = 1$). Forty samples did not produce genotypes at >50% of the 21 loci and were therefore not included in further analyses.

Population genetic analyses in estuary perch

In all, 372 estuary perch representing 15 sampling locales were genotyped at most of the 21 microsatellite loci. One population (Albert River) had three individuals where genotypes were not obtained for seven loci (missing data). Similarly, one locus (EP32) also had a higher frequency of missing data (~30%) across all populations (this marker was removed from population structure analyses). Otherwise, genotyping success was ~96% across all loci and individuals. Marker independence was confirmed across all sample locations, with linkage disequilibrium analysis indicating no significant linkage between loci after corrections for multiple comparisons. MICROCHECKER found no significant evidence for null alleles, scoring errors or allele dropout in samples from any population, and therefore all markers were used in subsequent analyses.

The mean number of alleles across loci at each sampling locale ranged between 1.524 and 5.476, whereas allelic richness (correcting for sample size differences in sampling locales with 10 or more samples) ranged between 1.512 and 3.022 (Table 3). Expected heterozygosities (H_E) ranged between 0.227 and 0.534. Most sampling locations had moderate levels of genetic diversity, except the Arthur River sample, which had significantly lower genetic diversity (mean number of alleles, allelic richness, observed heterozygosity and H_E; $P < 0.05$ for all pairwise comparisons with a sign test) than all other locations. All sampling locations conformed to HWE expectations and there were no significant positive or negative F_{IS} (inbreeding coefficient) values in any sampling locale. BOTTLENECK detected only one population (Arthur River) that exhibited a significant excess of heterozygotes (Wilcoxon signed-rank test, $P < 0.001$) than that expected under all three mutation models

Table 3. Population genetic statistics for estuary perch collected from 15 estuaries in eastern Australia, screened with 21 microsatellite loci
Mean values over loci are presented for the number of alleles (N_a), allelic richness (r), observed (H_O) and expected (H_E) heterozygosities, Hardy–Weinberg equilibrium (HWE) P-values and inbreeding coefficients (F_{IS}; no F_{IS} values were significant after corrections for multiple comparisons). NSW, New South Wales

Population	Region	n	N_a	r	H_O	H_E	HWE	F_{IS}
Arthur River	Tas.	34	1.524	1.512	0.227	0.227	0.923	0.003
Glenelg River	Vic.	10	2.667	2.398	0.419	0.427	0.884	0.019
Barwon River	Vic.	30	3.143	2.329	0.395	0.416	0.829	0.051
Bass River	Vic.	36	4.095	2.630	0.419	0.445	0.486	0.059
Tarwin River	Vic.	22	3.667	2.625	0.445	0.448	0.930	0.009
Albert River[A]	Vic.	9	3.143	–	0.472	0.506	0.841	0.076
Merriman Creek	Vic.	30	4.571	2.961	0.480	0.495	0.822	0.032
Gippsland	Vic.	15	3.619	2.801	0.446	0.496	0.162	0.105
Snowy River	Vic.	82	5.476	2.881	0.471	0.482	0.313	0.023
Bemm River	Vic.	27	4.429	2.934	0.506	0.488	0.101	−0.039
Genoa River	Vic.	10	3.429	2.831	0.533	0.496	0.996	−0.079
Clyde River	NSW	25	3.524	2.687	0.549	0.491	0.061	−0.114
Shoalhaven River	NSW	17	3.952	3.022	0.487	0.522	0.193	0.069
Hawkesbury River	NSW	18	3.762	2.930	0.530	0.534	0.473	0.006
Clarence River[A]	NSW	7	3.048	–	0.531	0.515	0.954	−0.032

[A]Allelic richness was not estimated for populations with <10 samples.

(IAM, SMM and TPM), indicating that this population has gone through a recent bottleneck.

Estimates of $F_{ST} \div D_{est}$ across all loci and sampling locations were significantly different from zero ($F_{ST} = 0.090$ (95% CL 0.069–0.114); $D_{est} = 0.084$ (95% CL 0.057–0.117)), indicating genetic structure across sampling locations. Similarly, pairwise estimates of $F_{ST} \div D_{est}$ also indicated genetic structure, with 71 and 75 of 105 (90 for D_{est}) pairwise comparisons significant after corrections for multiple comparisons for F_{ST} and D_{est} respectively (Table 4). Most significant pairwise comparisons were generally between the Arthur River population and all other populations, populations in western Victoria (Glenelg and Barwon rivers) and all other populations, and populations in NSW (Clarence, Hawkesbury, Clyde and Shoalhaven rivers) and all other populations for both F_{ST} and D_{est}. Generally, pairwise comparisons between populations in close geographic proximity were not significant for either F_{ST} or D_{est} estimates (e.g. Snowy, Genoa and Bemm rivers).

DAPC cluster detection using genotypes from the 21 microsatellite loci identified the optimal number of population clusters as three ($K = 3$). The three clusters and the relationship between individuals from the different sampling locales are shown in Fig. 2. The first cluster only includes samples from the Arthur River in Tasmania. This cluster is clearly differentiated on the x-axis from all other population samples. The second and third clusters are separated largely on the y-axis, with the second cluster including samples from western Victoria (Glenelg and Barwon rivers). The third cluster is made up of all remaining samples. If samples from the Arthur River are removed from the analysis and the DAPC is repeated, the optimal number of clusters is again three ($K = 3$; see Fig. S1, available as Supplementary material to this paper). The first cluster contains samples from the Clarence, Hawkesbury and Shoalhaven rivers in NSW. The second cluster includes samples from the Clyde River (NSW) and samples from east (Genoa, Bemm and Snowy rivers and the Gippsland Lakes), south (Merriman Creek and Albert River) and west (Tarwin and Bass rivers) Gippsland. The third cluster included samples from western Victoria (Barwon and Glenelg rivers). The differentiation appears to follow a west–east geographic pattern of differentiation between sampling locations.

Bayesian analysis using STRUCTURE identified three population clusters ($K = 3$), using the method of Evanno *et al.* (2005) in STRUCTURE HARVESTER (Fig. 3). Generally these clusters were congruent with the DAPC results, with the Arthur River sample clearly differentiated from all other samples, and samples from estuaries on mainland Australia showing a longitudinal geographic pattern of differentiation; samples from western Victoria (Glenelg and Barwon rivers) were generally all in Cluster 2, samples from northern NSW (Clarence, Hawkesbury and Shoalhaven rivers) were generally all in Cluster 3 and samples in between were shared between Clusters 2 and 3. When population samples from mainland estuaries are arranged longitudinally (e.g. Fig. 3), there is a clear west–east pattern to the proportion of the membership coefficient belonging to each of the two clusters.

A Mantel test of all population samples showed a weak significant positive correlation between genetic distance (Slatkin's linearised F_{ST}) and the natural log of geographic distance ($r = 0.433$, $P < 0.036$), indicating a weak isolation by distance (IBD) relationship. Regression analysis showed this relationship to be largely non-linear (Fig. 4*a*; $R^2 = 0.178$, $F = 20.56$, $P < 0.001$). A Mantel test with only samples from mainland estuaries showed a much stronger and highly significant positive correlation between genetic and geographic distance ($r = 0.704$, $P < 0.001$), indicating a strong IBD relationship. Regression analysis showed this relationship to be largely linear (Fig. 4*b*; $R^2 = 0.496$, $F = 74.86$, $P < 0.001$).

Table 4. Pairwise estimates of fixation index (F_{ST}; above the diagonal) and population differentiation (D_{est}; below the diagonal) among samples of estuary perch from 15 estuaries

Bold values are not significant; all other values are significant at $P < 0.001$ after 10 000 permutations and corrections for multiple comparisons. D_{est} could not be calculated for pairwise comparisons with the Albert River population

	Arthur River	Glenelg River	Barwon River	Bass River	Tarwin River	Albert River	Merriman Creek	Gippsland Lakes	Snowy River	Bemm River	Genoa River	Clyde River	Shoalhaven River	Hawkesbury River	Clarence River
Arthur River	–	0.334	0.301	0.243	0.237	**0.277**	0.222	0.277	0.222	0.264	0.298	0.266	0.324	0.302	0.400
Glenelg River	0.192	–	0.074	0.071	0.070	**0.076**	0.080	0.068	0.084	0.097	0.100	0.109	0.133	0.108	0.140
Barwon River	0.183	0.052	–	0.067	0.064	0.106	0.109	0.098	0.098	0.125	0.121	0.126	0.167	0.130	0.162
Bass River	0.156	0.056	0.042	–	**0.017**	**0.030**	0.029	0.025	0.026	0.045	**0.031**	0.061	0.116	0.082	0.118
Tarwin River	0.134	0.060	0.039	**0.012**	–	**0.024**	**0.030**	**0.017**	0.031	0.047	**0.023**	0.043	0.116	0.070	0.112
Albert River	–	–	–	–	–	–	**−0.021**	**−0.012**	**−0.006**	**0.003**	**−0.007**	0.025	**0.083**	**0.056**	**0.087**
Merriman Creek	0.152	0.078	0.092	0.025	0.029	–	–	**0.002**	**0.006**	**0.014**	**0.003**	0.032	0.080	0.058	0.084
Gippsland Lakes	0.171	0.067	0.077	0.018	**0.016**	–	**0.001**	–	**−0.002**	**0.009**	**0.006**	0.043	0.100	**0.069**	**0.097**
Snowy River	0.176	0.077	0.080	0.023	0.027	–	**0.006**	**−0.006**	–	**0.002**	**0.005**	0.041	0.100	0.076	0.106
Bemm River	0.185	0.089	0.104	0.041	0.040	–	0.012	**0.002**	**0.002**	–	**0.004**	0.043	0.089	0.071	0.101
Genoa River	0.181	0.092	0.098	0.028	**0.020**	–	**0.006**	**0.003**	**0.007**	**0.004**	–	0.038	0.107	**0.059**	**0.086**
Clyde River	0.173	0.109	0.109	0.051	0.040	–	0.031	0.038	0.038	0.038	0.038	–	0.066	0.021	0.043
Shoalhaven River	0.240	0.138	0.156	0.118	0.121	–	0.089	0.109	0.107	0.096	0.125	0.067	–	**0.024**	**0.044**
Hawkesbury River	0.230	0.111	0.114	0.082	0.068	–	0.064	0.073	0.082	0.078	0.068	**0.014**	**0.025**	–	**−0.015**
Clarence River	0.278	0.136	0.142	0.116	0.110	–	0.091	0.104	0.114	0.108	0.097	0.035	0.049	**−0.018**	–

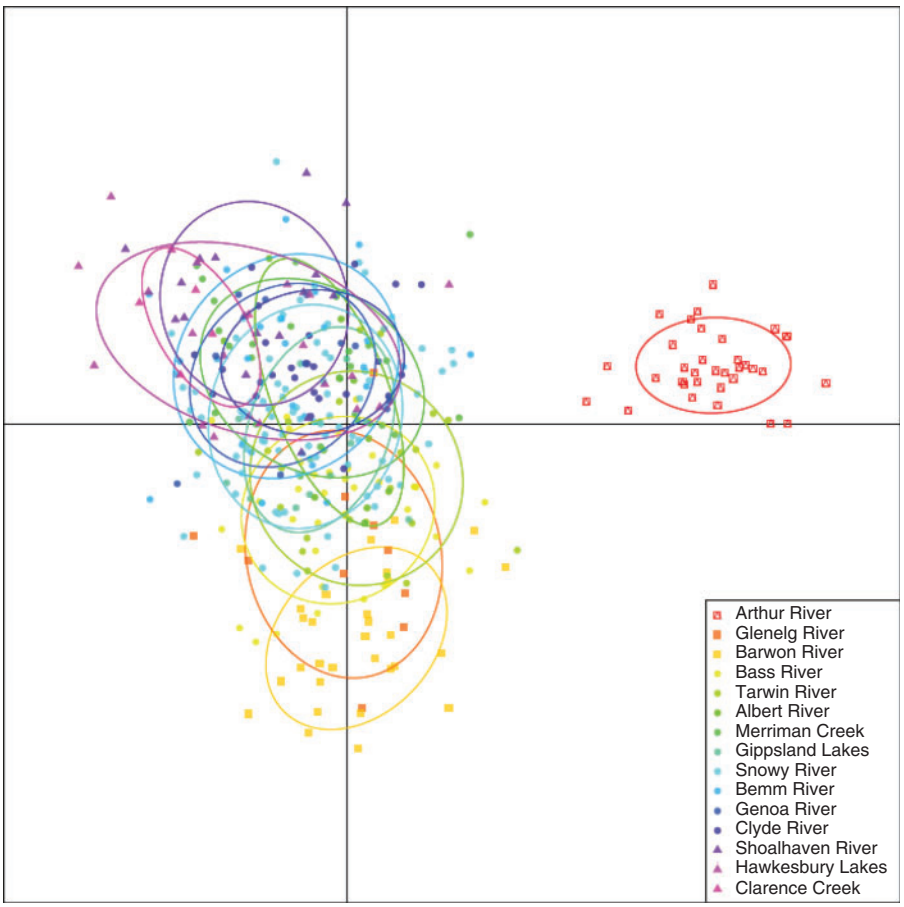

Fig. 2. Scatter plot of the discriminant analysis of principal components (DAPC) of estuary perch across 21 microsatellite loci. The first two principle components of the DAPC, which explain the majority of the variation, are shown. Clusters are indicated by different symbols ($K = 3$), whereas different shades and inertia ellipses represent the sampled estuaries and dots represent individuals.

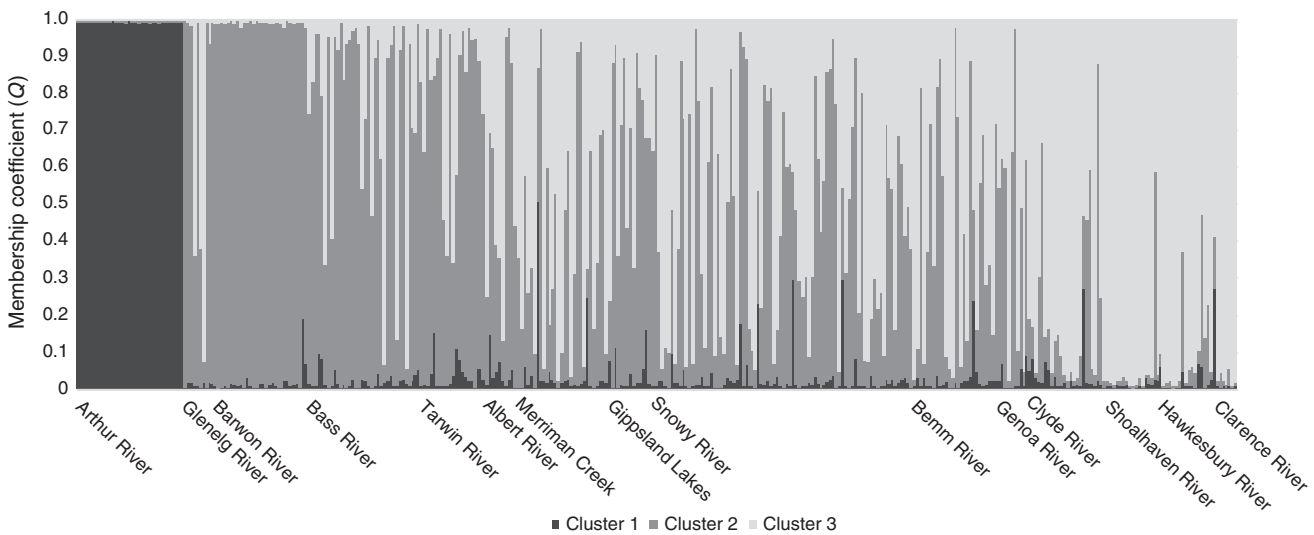

Fig. 3. Averaged estimated membership coefficient Q (y-axis) from 10 replicate runs for each estuary perch individual for $K = 3$ as determined by STRUCTURE for 21 microsatellite markers. Each individual is represented by a single vertical line broken into segments, where segments are proportional to Q for each of the population clusters. Individuals are arranged according to the populations from which they were sampled, with the population name along the x-axis below the first individual from each population.

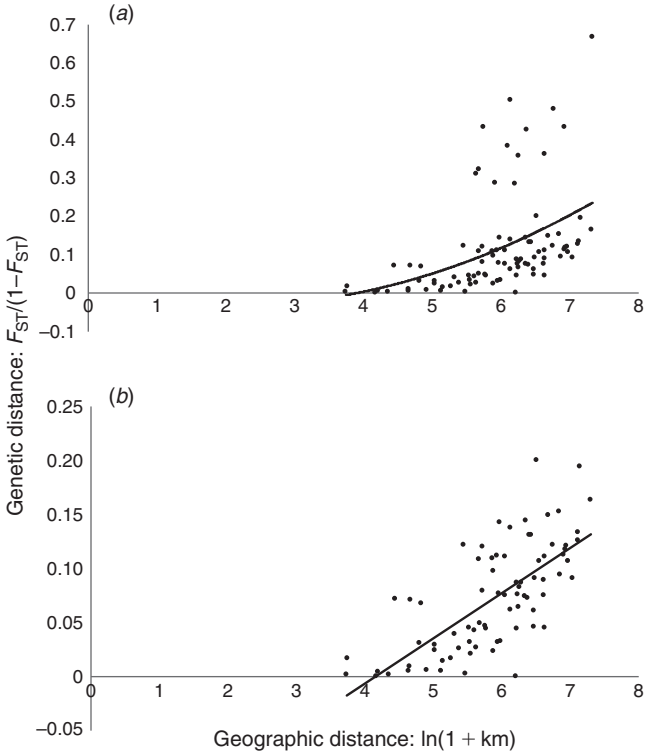

Fig. 4. Genetic distance (Slatkin's linearised fixation index, F_{ST}) plotted against the natural logarithm of geographic distance (1 + km) for (*a*) all pairs of estuary perch populations (Mantel $r = 0.433$, $P < 0.036$; $R^2 = 0.178$, $F = 20.56$, $P < 0.05$) and (*b*) all pairs of estuary perch populations excluding the Arthur River population (Mantel $r = 0.704$, $P < 0.001$; $R^2 = 0.496$, $F = 74.86$, $P < 0.001$).

Discussion

The results of this study indicate significant genetic structure of populations of estuary perch throughout its range in eastern Australia. The samples from the Arthur River in Tasmania were strongly differentiated from all other populations (F_{ST} range 0.136–0.228). However, this population also had significantly lower genetic variation than all other populations, with no unique alleles and evidence of a recent bottleneck. It appears that the population in the Arthur River was therefore either founded from very few individuals in recent times or has lost genetic variation following a population bottleneck (genetic drift and inbreeding). van Wyk (2015) suggests that, in addition, the population suffers from poor recruitment and a comparatively low number of individuals (mean ± s.d., 1594 ± 775), which may indicate some fitness effects due to inbreeding. Because all other populations of estuary perch that were known to have occurred in Tasmania are now suspected to be extinct (J. Haddy, pers. comm., 2019), genetic exchange with nearby populations is extremely unlikely. Therefore, the population may benefit from supplementation from mainland populations (i.e. genetic rescue) with the aim of increasing fitness and genetic diversity (Weeks *et al.* 2011). Such a strategy should consider appropriate mainland populations from both an ecological and genetic perspective. The strategy would also rely on a natural spawning event following supplementation to ensure the long-term viability of the population.

In estuaries on the mainland of Australia, populations of estuary perch exhibit an IBD pattern of genetic differentiation, with increasing geographical distance progressively restricting gene flow between populations. Although Shaddick *et al.* (2011*a*) did not detect a strong IBD signal using three microsatellite loci, they did find genetic differentiation was positively correlated with geographical distance when using mtDNA. Therefore, the results of the present study further confirm the presence of an IBD pattern of genetic differentiation implied by mtDNA, with the genetic structure described here a likely consequence of increased resolving power provided by a larger set of polymorphic microsatellite markers. This increase in resolution is also evidenced by the much higher heterozygosity estimates found in the present study (mean population $H_E = 0.466$) compared with the very low levels found by Shaddick *et al.* (2011*a*; mean population $H_E = 0.021$).

Genetic differentiation explained by an IBD pattern is common in diadromous fish species such as estuary perch and Australian bass. For example, anadromous fishes (a form of diadromy), which spend most of their lives in the marine environment before returning to fresh water to spawn, generally exhibit an IBD pattern (Bradbury and Bentzen 2007; Schmidt *et al.* 2014), with few known exceptions. Similarly, some catadromous fish, which spend most of their adult life in fresh water before returning to spawn in a marine environment, can also show an IBD pattern, although this form of genetic structuring is generally weaker in such populations, with commonly low levels of overall genetic structuring being present (Schmidt *et al.* 2014).

Interestingly, Jerry (1997) found Australian bass had an IBD pattern of genetic structure using allozyme markers. The genetic structure and IBD pattern we found for estuary perch is likely to be similar in strength to that of Australian bass, which is unsurprising given the close sibling status of the two species, their overlapping range in distribution and the relatively high incidence of hybridisation between the two species in some populations.

The significant genetic structure found here has implications for supplementary stocking of estuary perch to prevent negative effects on the genetic integrity of populations, and to increase the likelihood for positive outcomes of stocking. First and foremost, adequate numbers of founders or parentals of captively reared broodstock should be sourced from populations in the wild to maximise genetic diversity, minimise inbreeding and reduce the deterioration in fitness that often occurs in hatchery-reared fish (see guidelines in Weeks *et al.* 2011; Attard *et al.* 2016; Frankham *et al.* 2017). Similarly, time in captivity should be reduced to a minimum to limit the negative effects of the captive environment on fitness. Furthermore, to prevent the potential breakdown of any putative local adaptation and displacement of local gene pools (Laikre *et al.* 2010; Morissette *et al.* 2018) in estuaries on mainland eastern Australia, we recommend that broodstock be sourced within three broad genetic clusters identified here and that their offspring are released into the same cluster: (1) Northern Zone, north of and including the Shoalhaven River; (2) Southern Zone, from the Clyde River in NSW to Western Port Bay in Victoria; and

(3) Western Zone, west of Port Phillip Bay in Victoria. To maintain genetic diversity more broadly, broodstock should be sourced from multiple locations within each cluster, and not simply from one or two populations (Weeks *et al.* 2011; Coleman *et al.* 2013). Such a strategy would lower the likelihood of affecting local adaptation (particularly given the IBD pattern found here) if adequate founders are sourced and contribute to released broodstock. Importantly, these broad zones are also less likely to markedly affect management and provide clear guidelines for undertaking supplemental stocking.

The microsatellite loci developed here can be used to help better identify hybrid or introgressed individuals of estuary perch and Australian bass (Shaddick *et al.* 2011*b*), with five loci having allelic profiles unique to each species (Table 2). The improved accuracy of this marker dataset should provide greater confidence to managers when selecting individuals to act as broodstock to be used in supplementary stocking programs. These new genetic markers are also an important resource for managers to monitor wild populations of estuary perch (and Australian bass), particularly if populations continue to decline. Importantly, they can also be used to monitor the success of stocking efforts, by undertaking parentage analyses to determine whether released offspring survive in locations in which they are released (e.g. Eldridge *et al.* 2002).

The continued hybridisation between estuary perch and Australian bass in natural populations is intriguing. East Gippsland in Victoria appears to be a hot spot for hybridisation, although hybridisation does occur less frequently in other regions (Shaddick *et al.* 2011*b*). Understanding the nature of hybridisation in this species should be seen as an important research question; for example, what is the relative fitness of hybrid individuals compared with parentals? Clearly, the identification of back-cross hybrids (Schwartz and Beheregaray 2008; Shaddick *et al.* 2011*b*; present study) indicates that hybrid individuals are (or can be) fertile. From an evolutionary point of view, gene flow across species may be an important component of the long-term adaptive process, particularly in times of rapid environmental change (Weeks *et al.* 2011; Hoffmann *et al.* 2015).

Conclusions

In this study we developed a suite of new microsatellite markers for estuary perch and genotyped samples from across their range, revealing moderate genetic structure consistent with an IBD pattern in estuaries of south-eastern continental Australia. We defined three regions of estuary perch for management purposes to help guide future supplementation stocking for the conservation of this important recreational fish. Samples from the Arthur River in Tasmania are isolated from mainland populations and exhibit very low levels of genetic diversity, which likely reflects a population bottleneck or recent founder event. Supplementation from mainland populations to the Arthur River could be used as a strategy to increase genetic diversity and population adaptability, but must be considered carefully from an ecological and genetic perspective. The markers developed here will enable future monitoring of both the success of supplementation stocking and the genetic effects of supplementation on wild populations. Importantly, this study provides a baseline to monitor the overall genetic health of wild populations of estuary perch throughout their range in south-eastern Australia.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Declaration of funding

The authors thank the East Gippsland Catchment Management Authority for partially funding this project.

Acknowledgements

The authors thank the Victorian Fisheries Authority for allowing the use of supplementary putative Australian bass samples, and Michael Nicol (Arthur Rylah Institute, Department of Environment, Land, Water and Planning) for creating maps used in this paper. The authors also thank Kim Shaddick (Macquarie University), Dean Gilligan (New South Wales Department of Primary Industries), Christopher Burridge (University of Tasmania), Dean Jerry (James Cook University) and Kiet Truong (Iowa State University) for allowing us to reanalyse historic estuary perch DNA extractions.

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Handling Editor: Peter Unmack