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Low inter-basin connectivity in a facultatively diadromous fish: evidence from genetics and otolith chemistry

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Running title: Low connectivity in a facultatively diadromous fish

Abstract

Southern smelts (*Retropinna* spp.) in coastal rivers of Australia are facultatively diadromous, with populations potentially containing individuals with diadromous or wholly freshwater life-histories. The presence of diadromous individuals is expected to reduce genetic structuring between river basins due to larval dispersal via the sea. We use otolith chemistry to distinguish between diadromous and non-diadromous life-histories and population genetics to examine inter-basin connectivity resulting from diadromy. Otolith strontium isotope (^{87}Sr:^{86}Sr) transects identified three main life history patterns: amphidromy, freshwater residency and estuarine/marine residency. Despite the potential for inter-basin connectivity via larval mixing in the marine environment, we found unprecedented levels of genetic structure for an amphidromous species. Strong hierarchical structure along putative taxonomic boundaries was detected, along with highly structured populations within groups using microsatellites (F_{ST} = 0.046 – 0.181), and mtDNA (Φ_{ST} = 0.498 – 0.816). The presence of strong genetic subdivision, despite the fact that many individuals reside in saline water during their early life-history, appears incongruous. However, analysis of multi-elemental signatures in the otolith cores of diadromous fish revealed strong discrimination between river basins, suggesting that diadromous fish spend their early lives within chemically distinct estuaries rather than the more homogenous marine environment, thus avoiding dispersal and maintaining genetic structure.

Introduction

Connectivity is a key determinant of population structure in nature, and strongly influences the genetic diversity, adaptive potential and resilience of species (Allendorf & Luikart 2007). There may be strong relationships between the habitats occupied by

a species and the level of connectivity among local populations. For example, freshwater fish generally have much higher levels of genetic structuring between spatially distinct populations than marine species because of the presence of natural (e.g. mountain ranges) and artificial (e.g. dams) barriers to dispersal in freshwater ecosystems (Ward *et al.* 1994). Diadromous species (i.e. species that migrate between marine and freshwater) tend to have highly variable levels of population structure, reflecting the wide range of life history strategies exhibited by these species.

There are three widely recognised modes of diadromy in fishes (McDowall 1992; Myers 1949): "catadromous" species spend most of their adult life in freshwater and migrate to the sea to spawn; "anadromous" species spend most of their adult life at sea and migrate to freshwater to spawn; and "amphidromous" species migrate between fresh and marine habitats but not for the purpose of spawning (McDowall 1992). Most amphidromous fishes exhibit "freshwater amphidromy", with spawning occurring in freshwater and the eggs and/or larvae drifting downstream until they reach the estuary and/or the open ocean (McDowall 2007; Thuesen *et al.* 2011). The juveniles generally return to freshwater after 4-6 months in the estuarine/marine environment and sexual maturation occurs in freshwater. A small number of species are also thought to exhibit "marine amphidromy" in which spawning occurs in the sea and freshwater is utilized by juveniles before migration back to sea (see Gross 1987; McDowall 1988).

Catadromous species tend to exhibit low levels of population structure or panmixia over large geographic scales (e.g. angullid eels, Als *et al.* 2011), whereas anadromous species are often highly structured due to natal homing (e.g. many salmonids, Vaha *et*

al. 2007). Amphidromous species generally exhibit low genetic structuring between populations over moderate spatial scales (i.e. 100s of km), presumably due to extensive mixing of eggs and/or larvae in the marine environment (Cook *et al.* 2009; Crandall *et al.* 2010; Page *et al.* 2013). For example, the Australian Grayling *Prototroctes maraena* exhibits obligate amphidromy and is panmictic across southern Victoria, Australia (Schmidt *et al.* 2011).

Most studies of the population structure of diadromous fishes have categorised the life-history of the study species *a priori* and then used genetic markers to test the level of among-population connectivity. However, broad categorisations of species may fail to take into consideration important intra-population variation in migratory behaviors. Such variation has been documented for a wide range of diadromous fishes and has been referred to as "facultative diadromy" (Hicks *et al.* 2010; McDowall 1988). For example, anguillid eels have long been viewed as the "classic" example of catadromy, yet otolith chemistry analyses have shown that many individuals spend their entire lives in the marine environment (Tsukamoto *et al.* 1998). Similarly, many populations of "anadromous" salmonids contain individuals that live their entire lives within freshwater (Chapman *et al.* 2012). Facultative migration amongst amphidromous species has received much less attention, although its existence has been confirmed for several species including giant kokopu *Galaxias argenteus* (Hicks *et al.* 2010) and common bully *Gobiomorphus cotidianus* (Closs *et al.* 2003). The effects of variable life-history on genetic connectivity among populations of fishes that exhibit amphidromy have not been examined.

Members of the family Retropinnidae (the Southern Hemisphere smelts) exhibit wide variation in life history modes (McDowall 1988). The New Zealand smelt *Retropinna retropinna* appears to exhibit facultative diadromy, with some individuals within populations undertaking either anadromous or amphidromous migrations, and others residing in freshwater throughout their lives (Northcote & Ward 1985). However, it is possible that these two forms are different cryptic species, as there is some evidence of reproductive isolation between anadromous and non-diadromous individuals (Northcote & Ward 1985).

There are currently two recognised species of Southern Hemisphere smelts in Australia. The Australian smelt *Retropinna semoni* is a primarily freshwater species found in inland and coastal drainages throughout south-eastern Australia. Although most populations of the species are non-diadromous, a recent otolith chemistry study showed that the majority of individuals sampled from a coastal river in southern Victoria had spent their early life history in saline habitats, suggesting these fish were most likely amphidromous or catadromous (Crook *et al.* 2008). The Tasmanian smelt *R. tasmanica* is found only in the lower reaches of coastal rivers and estuaries in Tasmania. Little is known of the species' life history, although it has been suggested that they exhibit anadromy (Fulton 1990; McDowall 1988). Allozyme analyses of *Retropinna* from throughout southern Australia recently found that *R. tasmanica* from Tasmania form a clade with *R. semoni* from the Murray Darling Basin and western Victoria, suggesting that *R. tasmanica* may not be a distinct species (Hammer *et al.* 2007). The analyses of Hammer *et al*. (2007) also suggest the presence of two or more cryptic *Retropinna* species, so it is possible that life-history variation corresponds with putatative taxonomic status. Here we examine population genetic structure and

life-history variation within and between populations of the two currently recognized Australian *Retropinna* species. However, as taxonomic boundaries are yet to be clarified within Australian *Retropinna*, we hereafter refer to fish by their geographic location, or more generally as *Retropinna* spp.

In this study, we use otolith chemistry analysis to examine life-history variation within and among populations of Australian smelt from coastal rivers in southern Victoria and Tasmania, Australia. We also use a combination of mitochondrial sequence data and microsatellite analyses to examine genetic variation among populations in different river basins. The results of the otolith chemistry and genetic analyses are then used to test the hypothesis that populations will be structured genetically when most individuals are non-amphidromous, whereas populations consisting largely of amphidromous individuals will be genetically homogeneous due to mixing via marine dispersal pathways.

Material and methods

Fish and water collection

Retropinna spp. were collected using seine or dip nets from the freshwater reaches of 10 coastal rivers in Victoria, and were sampled from the lower freshwater/upper estuarine reaches of four coastal rivers in Tasmania (Table 1; Fig. 1a). Where possible, 30 individuals were sampled from each site for genetic analysis, with a subset of 10 fish per site used for otolith chemistry analysis. Fish were preserved in 100% ethanol in plastic containers prior to dissection. Water samples were taken for analysis of Sr isotope ratios (^{87}Sr:^{86}Sr) from the 11 rivers where otoliths were sourced (Table 1). Water was sampled in 125 ml acid-washed polyethylene bottles, filtered in

the field through 0.2 µm Acrodisc syringe-mounted filters, acidified with nitric acid and refrigerated at 4ºC. Samples were taken from each catchment at the lowest accessible point upstream of estuarine influence. The ^{87}Sr:^{86}Sr ratio from this sample was therefore an integration of the water chemistry of the upstream catchment, and represents the first freshwater ^{87}Sr:^{86}Sr signature encountered by juveniles immigrating upstream from the estuary or sea.

Water and otolith chemistry analysis

We analysed ^{87}Sr:^{86}Sr in water and otoliths to discriminate between wholly freshwater and diadromous smelt. ^{87}Sr:^{86}Sr is essentially invariant in the marine environment (global value: 0.70916, McArthur & Howarth 2004), but varies widely in fresh water due to heterogeneity in the underlying geology and local hydrology (Barnett-Johnson *et al.* 2008; Crook *et al.* 2013). As ^{87}Sr:^{86}Sr incorporation into otoliths directly reflects ambient water (Amakawa *et al.* 2012), it is possible to directly match otolith and water ^{87}Sr:^{86}Sr to determine residence in saline versus freshwater. Fish with ^{87}Sr:^{86}Sr values close to 0.70916 near the otolith core (i.e. early life history), that subsequently transitioned to values closely reflecting the catchment's freshwater ^{87}Sr:^{86}Sr value, were identified as diadromous (Schmidt *et al.* in press).

Water chemistry – ^{87}Sr:^{86}Sr

For water ^{87}Sr:^{86}Sr analysis, 20 ml aliquots were dried overnight in a HEPA-filtered fume cupboard. Matrix elements were washed off the resin with 2M and 7M nitric acid, followed by elution of clean Sr in 0.05M nitric acid. Sr isotope analyses were carried out on a "Nu Plasma" multicollector ICP-MS interfaced with an ARIDUS desolvating system (see Maas *et al.* 2005). Mass bias was corrected by normalizing to

^{88}Sr:^{86}Sr = 8.37521 and results are reported relative to a value of 0.710230 for the SRM987 standard. Internal precisions (2SE) based on at least 30 ten-second integrations averaged <0.00002. Reproducibility (2SD) was <0.00004.

Otolith chemistry – ^{87}Sr:^{86}Sr

Sagittal otoliths were removed, cleaned and stored dry in 0.5 mL polypropylene microtubes. One otolith from each fish was then mounted individually, proximal surface downwards, on an acid-washed glass slide in thermoplastic glue and prepared for ^{87}Sr:^{86}Sr analysis following the procedures of Schmidt *et al.* (in press). We measured otolith ^{87}Sr:^{86}Sr using a "Nu Plasma" multicollector ICP-MS coupled to a HelEx 193 nm ArF excimer laser ablation system (see Woodhead *et al.* 2005; Schmidt *et al.* in press for further details). Otoliths were ablated from the core to the dorsal margin at the widest radius using a 6 ×100 µm rectangular laser slit. Pre-ablation was conducted along each transect to remove potential surface contaminants, and data were acquired from a second analysis along the same transect, with the laser operated at 90 mJ, pulsed at 10 Hz and scanned at 3 µm sec^{-1}. A 20-30 sec background was measured prior to data acquisition for each sample. Iolite Version 2.13 (Paton *et al.* 2011) was used to process data offline, with corrections made for isobaric interferences from Kr, Rb and Ca argide/dimer species following the methods of Woodhead *et al.* (2005) and Schmidt *et al.* (in press). Instrumental mass bias was corrected by internal normalisation to ^{88}Sr:^{86}Sr = 8.37521. We integrated ^{87}Sr:^{86}Sr values measured across 2-sec intervals, resulting in a spatial resolution along the transect of 6 µm horizontal distance per integration. A modern marine carbonate standard was analysed after every 10 otolith samples to calculate external precision. Mean (±1 SD) ^{87}Sr:^{86}Sr in the standard (n = 24) was 0.70917 ± 0.00013.

Otolith chemistry – trace elements

Trace element analyses were also conducted for a subset of the Victorian sites to examine connectivity between diadromous populations. The second otolith of each fish was mounted on a clean glass slide as described above; however, the polishing step was omitted. Vertical ablation transects were run from the distal surface of the otolith to the proximal surface, through the primordium, using the depth profiling method of Macdonald *et al*. (2008). A suite of elements (^{24}Mg, ^{43}Ca, ^{55}Mn, ^{88}Sr, ^{138}Ba) were measured using a Varian 810 quadrupole ICP-MS coupled to the HelEx laser ablation system described above (see also Crook *et al.* 2013). Otolith core material accreted between 2-10 days post-hatch was selected to represent the environment experienced by the diadromus individuals during early larval life (Macdonald *et al.* 2008). Data was processed using Iolite Version 2.13 (Paton *et al.* 2011) and followed standard protocols for background subtraction, normalisation to Ca and calibration to an external glass standard (National Institute of Standards Technology, NIST 612) (see Crook *et al.* 2013). Data were expressed as element:Ca molar ratios (i.e. Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca) and we created a multi-elemental signature for each fish by combining all four molar ratios. Measurement precision (% relative standard deviation, R.S.D.) was determined based on (n = 9) analyses of NIST 610 and MACS-3 (n = 8) reference standards. Mean % R.S.D. was Mg:Ca 2.59/1.58, Mn:Ca 3.19/5.02, Sr:Ca 3.06/2.37, Ba:Ca 3.47/4.52 for NIST 610 and MACS-3 respectively. A single-factor multivariate analysis of variance (MANOVA) and quadratic discriminant function analysis (DFA) were conducted on $\log_{10}$ transformed data to examine variation in multi-elemental signatures among diadromous populations.

In rivers where the difference between freshwater $^{87}Sr:^{86}Sr$ and the global marine value was too small to resolve diadromous movements using this marker alone (i.e. Glenelg River, Darlots Creek, Hopkins River), we also examined otolith Sr:Ca and Ba:Ca transects across the full life history of individuals using the depth profiling method outlined above. Mn peaks observed during data processing were used as a proxy for the otolith core (Brophy *et al.* 2004; Macdonald *et al.* 2008; Ruttenberg *et al.* 2005), and Sr:Ca and Ba:Ca were integrated across 2-sec intervals..

Genetic methods

Genomic DNA was extracted from ethanol-preserved tissue samples using a standard salting-out protocol modified for 96-well plate throughput as per Aljanabi and Martinez (1997). A 950bp fragment of mitochondrial DNA spanning ATP synthase subunit 8 and ATP synthase subunit 6 genes was amplified and sequenced using primers and protocols described by Woods *et al*. (2010). Sequence data was deposited in GenBank under accession numbers KC150089 – KC150135 (Supporting information, Table S1). Additional sequences representing a range of *Retropinna* geographic localities and outgroups were obtained from GenBank (Hughes & Hillyer 2006; Ishiguro *et al.* 2003; Page & Hughes 2010; Schmidt *et al.* 2011; Woods *et al.* 2010). Seven microsatellite loci were amplified and genotyped using the multi-tailed primer tagging method of Real *et al.* (2009). Microsatellite primer sets included *sm26*, *sm49*, *sm77*, *sm80* (Hillyer *et al.* 2006); *smE8*, *smG7* (Schmidt *et al.* 2011); and a previously unpublished locus *smC7* (*smC7*_F: GCACCGTATGCCTGTCTACCAC; *smC7*_R: CATCTGTTGCTGTTGTTGATGGTT).

Genetic analyses

For mtDNA data, haplotype diversity (*h*), nucleotide diversity (π) and pairwise estimates of fixation indices (F_{ST}, Φ_{ST}) among 14 sample sites were calculated using ARLEQUIN v3.5.1.2 (Excoffier & Lischer 2010). Genealogical relationships among the sampled mtDNA haplotypes were estimated by statistical parsimony using TCS v1.21 (Clement *et al.* 2000). A gene tree incorporating exemplar haplotypes from each sampled mtDNA clade, outgroups, and published sequences representing variation within *Retropinna* was reconstructed using BEAST v1.7.4 (Drummond & Rambaut 2007). A lognormal relaxed clock model was first used to estimate divergence times of ingroup clades. However the data could not reject a strict clock (ucld.stdev included zero), therefore a strict clock model was used along with a coalescent constant size tree prior with a fixed substitution rate of 0.65×10^{-8} substitutions/site/year (Bermingham *et al.* 1997). An appropriate substitution model (GTR + I) was selected using MrMODELTEST v2.3 (Nylander 2004). Convergence, mixing and effective sample size of model parameters (>200) was assessed using the program Tracer v1.5 (Drummond & Rambaut 2007) after running the analysis for 10^8 generations. A maximum clade credibility tree was produced using the programs TreeAnnotator v1.7.4 and Fig Tree v1.3.1 (Drummond & Rambaut 2007).

For microsatellite data, tests for deviation from Hardy-Weinberg Equilibrium (HWE) for each locus-population combination were carried out using exact tests implemented in ARLEQUIN. Genetic structure was quantified by estimating pair-wise and global F_{ST} values in ARLEQUIN. These were tested for significant deviation from panmictic expectations by 10,000 permutations of individuals among populations. For significance testing, the critical value (α) was corrected for multiple tests using the

False Discovery Rate method (FDR, Narum 2006). To examine the contribution of individual population samples to overall F_{ST} we computed population-specific F_{ST} values for microsatellite data using GESTE v2.0, (Foll & Gaggiotti 2006); and for mtDNA by calculating the average pairwise Φ_{ST} of each focal population. Population-specific F_{ST} analyses were calculated separately for three regional groups of populations that were genetically distinct (West, East, Tasmania; see Results).

Structuring of individual multi-locus genotypes was investigated independently of sample groupings using a model-based Bayesian clustering method. The probability of an admixture model was tested for clusters (K) ranging from one to 15 using STRUCTURE 2.3.1 (Pritchard *et al.* 2000). Models were tested using eight independent MCMC simulations, each consisting of 2×10^6 iterations after a burn-in of 10^5 iterations. The most likely number of homogeneous clusters was assessed by standardising the second order rate of change of the mean likelihood of K (i.e. ΔK, Evanno *et al.* 2005), using the online application STRUCTURE HARVESTER (Earl & Vonholdt 2012). A hierarchical approach to STRUCTURE was used (see Vaha *et al.* 2007).

Contemporary migration rates (*m*, the proportion of immigrants in a focal population that arrive from a source population) over the past few generations were estimated using a Bayesian assignment method implemented in the software BAYESASS v1.3 (Wilson & Rannala 2003). This method is based on the principle that immigrants and their progeny show temporary disequilibrium in their microsatellite genotypes relative to the focal population under the assumption that background migration rate is relatively low ($F_{ST} > 0.05$) and that loci are in linkage equilibrium. To achieve

convergence and consistency of posterior probability estimates among replicate runs, analyses were run for 60 million iterations, sampling every 2000 with a burn-in of 10 million iterations. Delta values were adjusted to 0.2 to ensure chain swapping occurred in approximately 50% of the total iterations as recommended by Wilson and Rannala (2003). Unidirectional estimates of *m* were made for all pairs of sites and rates were reported that fell outside the 95% confidence interval simulated for uninformative data (Wilson & Rannala 2003).

Results

Otolith Chemistry

Analysis of $^{87}Sr:^{86}Sr$ in the otoliths of *Retropinna* spp. indicated three broad life-history patterns: diadromy, freshwater residence and estuarine/marine residence. Samples of mainland *Retropinna* from the Tarwin, Snowy and Bemm Rivers in Victoria showed evidence of diadromy, with otolith $^{87}Sr:^{86}Sr$ close to the global marine value of 0.70916 in the core region and $^{87}Sr:^{86}Sr$ closely reflecting the freshwater $^{87}Sr:^{86}Sr$ signature near the margin (Fig. 2, traces i, m and o). Diadromous individuals dominated in the three rivers where diadromy was detected, with only two non-diadromous fish observed out of the 30 analyzed. One fish from the Snowy River had $^{87}Sr:^{86}Sr$ values indicative of freshwater residence throughout its life (Fig. 2, trace n), while one from the Bemm River had $^{87}Sr:^{86}Sr$ values indicative of residence in saline water throughout life (Fig. 2, trace p). This latter instance likely indicates that the fish had only recently arrived from the estuarine habitat, as the freshwater collection site on the Bemm River was located immediately (~200 m) upstream of the estuary.

Examination of the multi-elemental signatures of the otolith cores (i.e. saline residence phase) of diadromous *Retropinna* from the Bemm, Snowy and Tarwin rivers demonstrated highly significant differences between rivers (MANOVA: df = 8, 42, Pillai's trace = 0.723, P = 0.01). Jack-knifed classification accuracy from the DFA was 100% for the Snowy and Bemm, and 89% for the Tarwin (one fish from the Tarwin was misclassified to the Bemm). The first discriminant function (DF) accounted for 98% of the variance and Ba was by far the most influential element (standardised coefficients for DF1: Ba = 1.056; Sr = 0.308; Mn = 0.131; Mg = -0.006).

Mainland *Retropinna* from the Yarra and Thomson rivers were clearly non-diadromous, with no marine ^{87}Sr:^{86}Sr in the core region and the ^{87}Sr:^{86}Sr at the otolith margins closely reflecting the water from which they were sampled (Fig. 2, e.g. traces g and k). The existence of diadromy in the Glenelg River, Hopkins River and Darlots Creek samples could not be resolved using ^{87}Sr: ^{86}Sr because the freshwater end-member values were too close to the seawater value of 0.7016 to clearly identify shifts in ^{87}Sr:^{86}Sr between residence in estuarine or marine water and fresh water (Fig. 2, traces a, c and e). Subsequent examination of otolith Sr:Ca and Ba:Ca profiles provided no evidence of diadromous movements for any individuals sampled from the three rivers, with stable and relatively low Sr:Ca values observed (< 2.7 mmol mol^{-1}) and the absence of negative relationships between Ba:Ca and Sr:Ca that can indicate a shift between estuarine or marine and freshwater residence in this species (Fig. 3) (Crook *et al.* 2008). In contrast, Crook et al. (2008) found clear peaks in Sr:Ca of ~3.5 mmol mol^{-1} and strong negative relationships between Sr:Ca and Ba:Ca in otolith chemistry profiles of diadromous smelt from the Tarwin River.

Otolith chemistry analysis of *Retropinna* sp. from the three Tasmanian rivers revealed no evidence of freshwater residence at any stage during life. Otolith ^{87}Sr:^{86}Sr values were generally close to the global marine value and did not reflect the freshwater ^{87}Sr:^{86}Sr values of the river basin from which the fish were collected (Fig. 2, traces s and u). This pattern suggests that *Retropinna* sp. from Tasmania have a very different life history strategy to the diadromous and non-diadromous *Retropinna* collected from mainland Australia (see Discussion).

Genetics

A total of 255 mtDNA sequences were obtained from fish across 14 coastal rivers (Supporting information, Table S1). The edited alignment was 413 bp in length, including 97 variable positions and 47 haplotypes resolved into four disconnected networks (Clades I-IV, Fig. 1b). Genetic variation was evident within all of the 14 river samples (Fig. 1a; Table 2), and strong phylogeographic structure was evident between the samples (Fig. 1a,b). This phylogeographic pattern consisted of a west-east mainland division centered on Wilson's Promontory (WP, Fig. 1a), dividing the distribution of clades I and II (Fig. 1a,b). Clade III was restricted to the Yarra R. site (Fig. 1a,b); and clade IV was confined to sites from Tasmania (Fig. 1a,b). A single individual sampled from the Tarwin R. on the west side of WP carried Clade II mtDNA and was the only exception to this phylogeographic pattern.

Phylogenetic analysis showed that *Retropinna* from Tasmania (Clade IV, Fig. 1b) were nested within the paraphyletic mainland *Retropinna*. The west-east phylogeographic division of clades I and II corresponded with two highly supported

clades in the gene tree, with mean uncorrected pairwise divergence of 15.3% corresponding to a median divergence time of 21 million years (Fig. 1b; Supporting information, Table S2, Fig. S1). Clade I haplotypes have a close affinity with *R. semoni* sequences collected from inland rivers of the Murray-Darling Basin. Clade II haplotypes correspond to the southeast coastal (SEC) genetic grouping of *R. semoni* designated by Hammer *et al.* (2007). Clades III (Yarra R.) and IV (Tasmania) form a strongly supported grouping (posterior probability 0.99, Fig. 1b) with a mean pairwise divergence of 3.5%.

There was extremely strong mtDNA structure among mainland populations west of WP. Indeed, network and phylogenetic analyses showed the Yarra River population contains a unique mtDNA clade that shares a most recent common ancestor (MRCA) with Tasmanian populations (Fig. 1b; Supporting information Table S2). The four other western rivers also exhibit heterogeneity in haplotype frequencies (Fig. 1a). After correction for multiple tests, all pairwise Φ_{ST} values were highly significant, except between the Hopkins and Tarwin Rivers (Supporting information, Table S3). The overall Φ_{ST} value among western rivers was 0.816 and all population level Φ_{ST} values were very high, especially the Yarra ($\Phi_{ST} = 0.93$, Table 2). The eastern mainland populations showed less structure overall $\Phi_{ST} = 0.498$, $P<0.001$. The five rivers all shared a common haplotype, but the Thompson River also contained a unique clade (population level $\Phi_{ST} = 0.61$, Table 2) and was significantly different from the other four, which were not significantly different from each other. *R. tasmanica* were also less structured than the western mainland group, although there were some differences in frequencies of haplotypes (Table 2; Table S3).

The STRUCTURE analysis incorporating all individuals suggested the most likely number of clusters was two, one containing all Tasmanian and western mainland populations and the other containing all eastern mainland populations (Fig. 4). When these two groups were analyzed separately, the Tasmania+western cluster was divided into separate Tasmanian and western groups, each of which was further subdivided into a third level of hierarchical structure featuring each of the five western population samples clustering into separate groups (Fig. 4). The eastern group produced two clusters when analyzed in isolation, with the Thomson River population distinct from all others. When the Thompson was removed and the remaining four populations analyzed alone, no further clustering was detected (Fig. 4), although pairwise F_{ST} values for all eastern populations were significant, except the Snowy versus the Bemm and the Avon versus Macalister (Supporting information, Table S4).

There was remarkable concordance between the microsatellite and mtDNA results in terms of pairwise comparisons, with the exception of the Hopkins and Tarwin, which were not differentiated on mDNA, but were differentiated with microsatellites, possibly due to smaller sample sizes for the mtDNA (Supporting information, Tables S3, S4).

Contemporary migration among the coastal river populations sampled in this study appears very limited. The BAYESASS analysis of microsatellite data revealed only five sampled populations containing individuals that could be distinguished as potential immigrants (Table 1). In cases where migration was detected, the putative source population was a nearby river, for example from the Leven to the Rubicon and Great Forester in Tasmania and from the Macalister to the Avon and the Snowy to the

Bemm in eastern Victoria (Table 1). In eastern Victoria, some migration was detected from Darlots Creek to the Hopkins. Assignment testing of microsatellite data therefore indicates long distance movement among rivers via the sea is absent or rare in smelt.

Discussion

Our otolith chemistry analyses demonstrated substantial life-history variation among populations of *Retropinna*, as hypothesized by Crook *et al*. (2008). In the Victorian coastal rivers, five of the eight sampled populations consisted entirely of non-diadromous individuals, whilst the remaining populations were dominated by individuals that had spent their early life history in saline waters and had subsequently migrated back upstream into freshwater. As spawning has only ever been recorded in freshwater by this species (Koster & Dawson 2010), the life history mode of these fish appears to most closely resemble "freshwater amphidromy" (McDowall 1988; Myers 1949). The otolith chemistry analysis of *Retropinna* collected from Tasmania provided little evidence of residence in freshwater at any time during the life history, with fish appearing to have resided in estuarine and possibly marine waters throughout life. Unlike the Victorian samples that were sampled in freshwater, these fish were collected from the uppermost estuarine reaches of the study systems, and one possibility is that they were sampled before having an opportunity to migrate upstream into freshwater. We consider this unlikely, however, as all of the Tasmanian fish analyzed were greater than 60 mm TL, which is much larger than the size at which upstream migration in mainland *Retropinna* has been shown to occur (~15-40 mm TL, Crook *et al.* 2008; Crook and Macdonald unpubl. data). These findings are also consistent with previous descriptions of the distribution of *Retropinna* in

Tasmaina, suggesting that they are restricted to estuaries and the lower reaches of rivers (Allen *et al.* 2002; Fulton 1990). Based on these observations, it would appear that Tasmanian *Retropinna* have a life-history that occurs primarily within estuarine/marine habitats. A similar habitat association and pattern of genetic structure was found for the co-distributed species Tasmanian whitebait (*Lovettia sealii*), which also undertakes an annual migration into upper estuarine reaches around the Tasmanian coast (Schmidt *et al.* in press).

We had predicted that if populations contained wholly freshwater fish only, they would be genetically divergent, whereas if some populations contained amphidromous individuals, populations would be homogeneous or panmictic. This was because all previous studies of species thought to be amphidromous had shown minimal genetic structure over scales much larger than examined here (e.g. Cook *et al.* 2009; Crandall *et al.* 2010; Page *et al.* 2013), so with a mixture of population life-histories, we predicted that any amphidromous individuals would cause homogeneity among rivers. This prediction was not supported. Rather, we found extreme variation in life-history, ranging from wholly freshwater, to amphidromous to estuarine/marine, as well as extremely high levels of genetic structure. Many of the wholly freshwater populations were highly genetically divergent from all others, suggesting that they had remained isolated for considerable evolutionary time (possibly more than 60,000 years in the case of the Yarra population; supplementary Table S2, lower 95% HPD). Yet, surprisingly all these rivers have open access to the sea, so amphidromous individuals from other estuaries have had the potential to invade over time but appear not to have done so. Only very limited migration seems to occur between rivers, and then only between nearby rivers.

These findings present a conundrum. If some populations contain amphidromous individuals, why have these populations not provided a source of migrants into other rivers and therefore prevented genetic divergence? One possible scenario is that the species is capable of natal homing. This has never before been reported in an amphidromous species, although it is common in anadromous species that leave their natal habitat as juveniles (not eggs or larvae as in amphidromy) and return to freshwater as mature adults (Dionne *et al.* 2008; Fontaine *et al.* 1997). We have no other evidence to suggest that *R. semoni* is capable of such a feat, but studies on another amphidromous species (*Galaxias maculatus*) have shown that larvae and juveniles are sensitive to differences in water quality (Hale *et al.* 2008), so it is possible, though unlikely, that juvenile smelt can identify chemical cues from their natal river. Furthermore, larvae of a number of coral reef fish species, which also disperse as eggs and larvae, have been shown to be capable of natal homing (Thorrold *et al.* 2001).

Alternatively, it has been suggested by a number of authors that selection against immigrants can promote genetic structure among populations (Hendry 2004; Nosil *et al.* 2005). This hypothesis predicts that local adaptation results in residents having higher fitness in a given habitat than immigrants that will not be adapted to the new environment. Bradbury *et al*. (2008) showed that in the anadromous rainbow smelt *Osmerus mordax*, population structure among rivers was much more pronounced in spawning adults than in juveniles and suggested that selection was resulting in immigrant juveniles not surviving to spawning age. It seems unlikely that such a mechanism could explain the results observed here though. In their study, F_{ST} values

were between 0.001 and 0.05, much smaller than observed for some populations in our study.

A final possibility is that individuals that resided in saline water during the early life history are retained within the estuary of their natal river basin: that is, they are only "marginally amphidromous" (*sensu* McDowall 1988). This suggestion is strongly supported by the multi-elemental otolith analyses - the highly significant inter-basin variation in trace element signatures in the otolith cores (representing the saline residence phase) of amphidromous fish suggests that they most likely resided in saline water within the estuary of their natal catchment, rather than in the more chemically homogenous open ocean. This contrasts with findings for the closely related Australian grayling *Prototroctes maraena* from coastal Victorian streams where there were no significant differences in otolith core chemistry between spatially distinct samples (Crook *et al.* 2006). Subsequent genetic analyses of Australian grayling demonstrated panmixia among samples, further supporting the contention that they spend their early life history in the ocean and are, thus, amphidromous (Koster *et al.* 2013; Schmidt *et al.* 2011).

Recent studies of some northern hemisphere estuarine species have suggested that larval retention within estuaries can occur through vertical migration of individuals correlated with tides (Bilton *et al.* 2002). However, even where such mechanisms have been proposed, such as in the sand goby (*Pomatoschistus minutus*), the levels of genetic structure are considerably lower than those observed here for the smelt (F_{ST} = 0.026 for sand goby versus mean population F_{ST} = 0.19 for western mainland and F_{ST} = 0.07 for eastern mainland smelt). Presumably this is because, even though most

sand goby larvae are contained within the estuary, occasionally a small number of individuals are washed to the sea and result in limited mixing. This appears to happen very rarely for southern smelts.

While there are clearly differences among populations in terms of both life-history and genetic distinctness, there also appear to be more general differences, at least between the two mainland groups east and west of Wilson's Promontory, a major biogeographic break point in the Australian marine fauna (see Supporting information, discussion S1). This geographic division coincides with a putative species-level boundary identified by Hammer *et al*. (2007) within the taxon currently referred to as *R. semoni*. The western group (clade I, Fig. 1a,b) contained a greater proportion of non-diadromous populations than the eastern group (clade II, Fig. 1a,b). Whether this reflects a real difference in life-history characteristics between the two groups would needs to be confirmed with additional otolith analysis. The patterns in Tasmania (clade IV, Fig. 1a,b) are different again, with no fish showing evidence of having spent time in fresh water, yet there is still significant genetic structure and 95% confidence intervals of all population level F_{ST} values do not overlap with zero. This suggests that even this group of populations, currently recognized as *R. tasmanica* and suggested by McDowall (1996) to be anadromous, show evidence of limited dispersal among estuaries.

In conclusion, our findings of strong genetic structure between populations of smelt, despite the potential for marine dispersal, contrast with the findings of previous studies of amphidromous organisms (e.g. Cook *et al.* 2012; Dennenmoser *et al.* 2010). The drivers of this highly unusual genetic structuring are unclear at present. Previous

research has suggested that the expression of amphidromous life histories is strongly influenced by the physical attributes of the river basin, with steep, hydrologically unstable systems more likely to encourage amphidromy than gradually sloping, hydrologically stable systems (Thuesen *et al.* 2011). Our findings of life history variation at large spatial scales and in accordance with putative taxonomic boundaries (e.g. wholly freshwater and amphidromous in Victoria versus estuarine/marine in Tasmania) suggest that local environmental factors are not the sole drivers of life history variation in smelt. A future research need in this area is to determine the extent to which behavioural and environmental factors interact to influence life history expression and dispersal, and thus, regulate population structure.

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Data Accessibility

DNA sequences: Genbank accessions KC150089 – KC150135

Otolith ^{87}Sr:^{86}Sr profiles, microsatellite genotype data and mitochondrial DNA sequence alignment: Dryad entry doi:10.5061/dryad.3dh2c

Figure Captions

Figure 1. Study area and mtDNA variation in *Retropinna* spp. (a) Map of study area showing position of 14 sampled coastal rivers. Inset map shows position of study area on the Australian continent with Murray-Darling Basin (MDB) in grey. Frequency pies show distribution of mtDNA haplotypes coloured according to haplotype network in Fig. 1b. Uncoloured pie segments correspond to singleton haplotypes. Arrow marks the position of Wilson's Promontory (WP); (b) Maximum clade credibility tree showing relationships among mtDNA clades and matching haplotype networks showing relationships among the full set of sampled haplotypes within each clade. Exemplar sequences from the literature are designated by their GenBank accession number. Letter codes applied to clades correspond to putative taxa proposed by Hammer *et al*. (2007): MTV, Murray-Darling Basin+Tasmania+western coastal Victoria; COO, Cooper Creek River Basin; SEQ, south-east Queensland; SEC, south-east coast. Uncoloured haplotypes represent singletons. Smelt illustration used under licence © R.Swainston-anima.net.

Figure 2. Strontium isotope ratios ($^{87}Sr:^{86}Sr$) for fish collected from 11 rivers in Victoria and Tasmania, Australia (see Fig. 1a). Dashed lines show the water $^{87}Sr:^{86}Sr$ value for sea-water. Solid lines show the $^{87}Sr:^{86}Sr$ value for the freshwater from the sampled catchment. Marine $^{87}Sr:^{86}Sr$ is invariant, whereas freshwater $^{87}Sr:^{86}Sr$ reflects local geology and may be higher or lower than the marine value. Otolith transects were run from the core to the edge of the otolith. Each vertical bar represents an individual fish and shows the range of values across the transect. Open circles represent the value at the otolith core and closed triangles represent the value at the otolith edge. Representative transects for each population are shown below the bar graphs.

Figure 3. Core to edge transects of otolith Sr:Ca (black line, left hand y-axis) and Ba:Ca (grey line, right hand y-axis) for *R. semoni* captured from the Glenelg River (a,b), Darlots Creek (c,d) and the Hopkins River (e,f).

Figure 4. STRUCTURE plot showing admixture proportion of 392 individuals genotyped with seven microsatellite loci. Three hierarchical levels of analysis are shown. Population abbreviations: Tho, Thomson; Mac, Macalister; Avo, Avon; Sno, Snowy; Bem, Bemm; Dar. Darlots; Hop, Hopkins; Yar, Yarra; Tar, Tarwin; Lev, Leven; Rub, Rubicon; Gre, Great Forester; Der, Derwent.

Author contributions

JMH and DAC conceived and designed the study. DJS, JIM, DAC, JAH collected data and performed data analyses. JMH, DAC, DJS wrote manuscript with contributions from all authors.

1 **Table 1.** Summary of water, otolith and genetic samples analysed and genetic variation in populations of *Retropinna* spp. based on
2 mitochondrial DNA (mtDNA) and microsatellite markers. Number of specimens (*N*); Observed heterozygosity (H_O) and expected heterozygosity
3 (H_E) presented as mean across all loci with one standard deviation; haplotype diversity (*h*) and nucleotide diversity (π) presented as mean±SD.
4 Water ^{87}Sr:^{86}Sr values are point estimates or mean±SD where replicate samples were taken.
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River	Latitude, Longitude	Otoliths		Microsatellites			mtDNA		
		N	**Water ^{87}Sr:^{86}Sr**	***N***	H_O	H_E	***N***	***h***	π
Glenelg	S38°03.108', E141°00.408'	10	0.709536	8	0.625 ± 0.298	0.692 ± 0.150	8	0.679 ± 0.122	0.00476 ± 0.00342
Darlots	S38°13.186', E141°46.233'	10	0.708339 ± 0.000159	31	0.548 ± 0.167	0.626 ± 0.180	20	0.190 ± 0.108	0.00046 ± 0.00068
Hopkins	S38°23.10', E142°35.28'	10	0.709457 ± 0.000191	29	0.640 ± 0.304	0.659 ± 0.316	19	0.661 ± 0.084	0.00218 ± 0.00177
Yarra	S37°51.00', E144°54.00'	10	0.714086	18	0.417 ± 0.376	0.403 ± 0.373	17	0.324 ± 0.136	0.00082 ± 0.00096
Tarwin	S38°39.861', E145°56.651'	10	0.705729 ± 0.000017	36	0.528 ± 0.267	0.629 ± 0.286	13	0.872 ± 0.054	0.02729 ± 0.01490
Thomson	S38°08.399', E147°04.738'	10	0.71755	29	0.424 ± 0.225	0.525 ± 0.272	15	0.733 ± 0.067	0.00826 ± 0.00501
Macalister	S37°46.558', E144°40.175'	-	-	30	0.690 ± 0.163	0.794 ± 0.105	19	0.538 ± 0.133	0.00150 ± 0.00138
Avon	S37°55.141', E144°00.757'	-	-	30	0.671 ± 0.146	0.762 ± 0.149	24	0.312 ± 0.121	0.00081 ± 0.00093
Snowy	S37°42.618', E148°27.125'	10	0.714634	35	0.678 ± 0.157	0.764 ± 0.139	21	0.186 ± 0.110	0.00046 ± 0.00068
Bemm	S37°36.462', E148°54.061'	10	0.718793 ± 0.000215	31	0.733 ± 0.151	0.776 ± 0.154	17	0.515 ± 0.145	0.00142 ± 0.00134
Leven	S41°09.941', E146°04.926'	-	-	30	0.600 ± 0.408	0.597 ± 0.359	26	0.523 ± 0.116	0.00182 ± 0.00154

River	Latitude, Longitude	Otoliths		Microsatellites			mtDNA		
		N	**Water $^{87}Sr:^{86}Sr$**	***N***	H_O	H_E	***N***	***h***	π
Rubicon	S41°24.074', E146°36.349'	10	0.705645	30	0.486 ± 0.307	0.581 ± 0.303	15	0.629 ± 0.124	0.00244 ± 0.00195
Great Forester	S41°02.766', E147°37.516'	10	0.714036	27	0.497 ± 0.339	0.570 ± 0.334	21	0.771 ± 0.073	0.00270 ± 0.00205
Derwent	S42°45.544', E147°00.480'	10	0.709885	28	0.658 ± 0.304	0.707 ± 0.297	20	0.711 ± 0.089	0.00335 ± 0.00240

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9 **Table 2**. Estimates of mean population-specific genetic fixation indices for 14 populations of *Retropinna* spp. and estimates of contemporary
10 migration rates (m) for each river sample. Population-specific fixation indices were calculated separately for three regional groups (West, East,
11 Tasmania) using the microsatellite (F_{ST}) and mtDNA (Φ_{ST}) datasets. Migration rate estimates were based on unidirectional assignment of
12 microsatellite genotypes in BAYESASS v1.3. Proportion of non-migrant values represent the proportion of individuals assigned back to their
13 river of origin; mean and 95% CI values for uninformative data was 0.833 (0.675, 0.992). Putative source of migrants represents source
14 population supplying migrants into the focal population where the estimated migration rate exceeds the upper 95% CI value for uninformative
15 data (0.104). Migration rates and source of migrants were not provided for cases where the 95% CI of estimated migration rate overlapped with
16 the 95% CI value for uninformative data.
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Region	Focal River	microsatellite F_{ST}	mtDNA Φ_{ST}	Proportion of non-migrants	Putative source of migrants	Migration rate (m), mean (95% CI)
West	Glenelg	0.159	0.635	0.704	-	-
	Darlots	0.261	0.585	0.990	-	-
	Hopkins	0.089	0.507	0.678	Darlots	0.265 (0.197, 0.319)
	Yarra	0.329	0.934	0.983	-	-
	Tarwin	0.113	0.295	0.988	-	-
East	Thomson	0.196	0.609	0.977	-	-
	Macalister	0.052	0.151	0.984	-	-
	Avon	0.038	0.156	0.678	Macalister	0.261 (0.191, 0.321)
	Snowy	0.064	0.156	0.990	-	-
	Bemm	0.043	0.149	0.677	Snowy	0.273 (0.211, 0.321)
Tasmania	Leven	0.053	0.370	0.989	-	-
	Rubicon	0.132	0.361	0.687	Leven	0.240 (0.135, 0.316)
	Great Forester	0.127	0.296	0.679	Leven	0.277 (0.213, 0.323)
	Derwent	0.037	0.825	0.985	-	-

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