Temporal stability of a hybrid swarm between the migratory marine and estuarine fishes *Acanthopagrus australis* and *A. butcheri*

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ABSTRACT: We predict estuaries to be hotspots of hybridisation between migratory marine and estuary-restricted species, although hybridisation rates may vary in space and time, reflecting the dynamic nature of estuaries and potentially widespread but erratic dispersal of marine taxa. Within estuaries, genotype frequencies may reflect past hybridisation events, with genetically intermediate and backcrossed individuals contributing to persistent hybrid swarms. In southeastern Australia, hybridisation has occurred between estuarine black bream *Acanthopagrus butcheri* and marine yellowfin bream *A. australis*, but it is unclear whether this reflects a contemporary process. We recently found that, within lakes and lagoons at the southern range limit of *A. australis*, hybrids were abundant and *A. butcheri* extremely rare, and surprisingly, we detected hybrids within a small sample of fish from the Gippsland Lakes, an estuary 250 km further south. In the present study, we compare the genotypic composition of the contemporary Gippsland Lakes population of *Acanthopagrus* spp. with the historical composition revealed by analysis of museum specimens. The genetic makeup of samples varied little over time, with ancestral *A. butcheri* virtually absent, and most introgressed individuals matching expectation for later-generation hybrids or *A. butcheri* backcrosses, suggesting that the lakes have supported persistent hybrid swarms. At each sampling time, the samples were genetically diverse, as measured by mean number of alleles per locus, which ranged from 8.2 to 9.2, and expected heterozygosity (H_e), which ranged from 0.66 to 0.70; however, we detected little allelic differentiation (F_{ST} = 0.003) across sampling times. Our data imply that introgressed populations of *Acanthopagrus* spp. are more widespread and persistent than previously predicted.

KEY WORDS: Coastal lakes · Lagoons · Estuaries · East Australian Current · Inter-specific gene flow · Introgression · Museum specimens

INTRODUCTION

For migratory marine and estuary-restricted taxa (i.e. taxa that complete their entire life cycle within estuaries) with overlapping spawning times, the opportunity for primary hybridisation to occur, and for hybrids to persist, may depend upon the reproductive (gametic) compatibility of the parental species, together with a range of factors including ocean-current movements, estuary-entrance channel opening, and hybrid fitness and behaviour. Given the often

erratic nature of ocean currents (Ridgway 2007) and variability in the accessibility of estuarine habitat (Jones & West 2005, Rustomji 2007), primary hybridisation within estuaries may therefore be rare, although once hybrid individuals are produced, introgressed or hybrid swarms may persist beyond the normal contemporary distribution of parental taxa. Indeed, persistent introgressed swarms may form if viable, and interfertile hybrids and backcrosses remain and inter-breed within estuaries (Roberts et al. 2010a,b). Such systems have rarely been investigated, but the intermittently

closed and open lakes and lagoons (ICOLLs) of the southeast Australian coast support populations of estuary-restricted black bream *Acanthopagrus butcheri* Munro, in which hybridisation may be mediated by variable ocean-current flow and a range of anthropogenic impacts.

On the southeast coast of Australia, hybridisation has occurred between the migratory marine yellowfin bream *Acanthopagrus australis* Günther and the estuary-restricted *A. butcheri* within the area representing the southern range limit of *A. australis* and the northeastern range limit of *A. butcheri* (Rowland 1984, Roberts et al. 2009). In this area of sympatry in southern New South Wales (NSW), *A. australis* introgression has made a considerable contribution to the genotypes of *Acanthopagrus* spp., occupying 5 ICOLLs (Roberts et al. 2010a). However, very little is known about the longer-term, multi-generational persistence of such introgressed swarms of *Acanthopagrus* spp. Fortunately, the high incidence of fish specimens archived within museum collections and the stability of DNA within preserved biological material such as scales and otoliths provide the opportunity to test for temporal variability in the genotypic composition of fish populations (Wandeler et al. 2007, Nielsen & Hansen 2008, Hansen et al. 2009).

Intriguingly, our initial broad-scale survey of estuarine and coastal *Acanthopagrus* spp. populations (Roberts et al. 2009) revealed the presence of a small number of introgressed bream within the Gippsland Lakes; a large, complex set of interconnected coastal lakes and lagoons 250 km south of the area of our earlier intensive sampling of adults and recruits (Roberts et al. 2010a) and an area considered beyond the normal southern range limit of *Acanthopagrus australis* (see Fig. S1 in the supplement at www. int-res.com/articles/suppl/m421p199_supp.pdf). Nevertheless, within this region, the East Australian Current (EAC) provides predominantly southward but erratic water movements (Nilsson & Cresswell 1980, Bowen et al. 2005) that may cause infrequent migration of *A. australis* beyond its accepted range limit. Indeed, *A. australis* effectively forms a panmictic population over its east coast distribution, with genetic homogeneity reflecting the active dispersal of adults to spawn and the southwards dispersal of larvae by the EAC (Roberts & Ayre 2010). Thus, within the Gippsland Lakes, opportunities for primary hybridisation involving *A. australis* and *A. butcheri* seem likely to be rare and we would expect introgressed bream to be ephemeral, unless inter-fertile introgressed bream persist and inter-breed within the lakes. In the present study, we use a set of museum specimens to describe the genotypic composition of the Gippsland Lakes population of *Acanthopagrus* spp. over a 60 yr period.

These data will allow us to determine whether levels of introgression are comparable to those seen further north in the area of sympatry (Roberts et al. 2009, 2010a) and to test the prediction that the population represents a stable introgressed swarm of *Acanthopagrus* spp.

MATERIALS AND METHODS

Species distribution. *Acanthopagrus butcheri* occurs within coastal lakes and lagoons from central NSW to Western Australia, including Tasmania. Within NSW, *A. butcheri* is known to hybridise with *A. australis*, which inhabits a range of habitats encompassing offshore reefs and the surf zone of coastal beaches, as well as estuaries, from northern Queensland to approximately the border of NSW and Victoria (see Roberts et al. 2009, 2010a and references therein for relevant background).

Specimens and genetic analyses. Contemporary specimens consisted of 2 samples of fin clips (from 1996–97 and 2000, total n = 114) stored in DMSO-saturated salt solution (DMSO 20% v/v, 0.25 M EDTA, saturated with NaCl; pH 8.0). The museum specimens (or 'historical samples') consisted of dried scales from fish caught in 1941 and 1943 (n = 133), and subsequently archived at room temperature in envelopes within the Arthur Rylah Research Institute for Environmental Research (Victorian Department of Primary Industries), Heidelberg, Victoria, Australia.

To extract DNA, a scale (or a <5 mm^2 fin clip) was placed in a sterile 1.5 µl tube containing 5% chelex resin in 500 µl of sterile distilled H$_2$O and 15 µl of Proteinase K (10 mg ml^{-1}), and heated at 65°C for 12 h. Before the supernatant was used directly in PCR, the solution was vortexed for 10 s, heated at 100°C for 5 min, and centrifuged at 12 000 $\times$ *g* for 7 min. We genotyped all 247 samples using 6 microsatellite markers described in Roberts et al. (2009). We conducted multiple independent DNA extractions and performed PCR and genotyping for a randomly selected subset of both contemporary and historical samples to ensure repeatability of our results. Overall, the average (±SE) proportion of missing genotypes per locus was consistently low for all samples (1941: 0.02 ± 0.01; 1943: 0.01 ± 0.01; 1996–97: 0.03 ± 0.01; 2000: 0.03 ± 0.02).

Temporal changes in allele frequencies could reflect not only the effects of hybridisation but also other processes, including genetic drift and genetic exchange with other genetically distinct *Acanthopagrus butcheri* populations (Chaplin et al. 1998). Distinguishing among these possibilities requires detailed descriptions of allelic diversity and comparison to alleles known to be present within the parental populations of

A. butcheri and *A. australis*. We therefore tested for homogeneity of allele frequencies among collections (1941, 1943, 1996–97 and 2000), and calculated standard measures of genetic diversity for each year, i.e. number of alleles per locus, and observed and expected heterozygosity (using POPGENE, Yeh et al. 1999). We estimated allelic richness (the standardised number of alleles per locus [n = 40 sample^{-1}]) for each year. Weir & Cockerham's (1984) formulation of Wright's (1969) *F*-statistics were used to estimate genetic differentiation among years (FSTAT, Goudet et al. 1996). The estimates were based on allele frequencies for individual loci and as an average across loci. Bootstrapping and jackknifing procedures across loci were used to estimate standard errors. We tested the statistical significance of heterozygous deficits and heterozygote excesses for each locus and overall using Exact tests implemented in GENEPOP (Raymond & Rousset 1995).

We tested for the presence of hybrids by performing an admixture analysis (using the program STRUC-TURE; Falush et al. 2003) incorporating our previously identified 'reference collection' of pure species in the analysis (see also Roberts et al. 2010a). For this analysis, we used only the data from the 4 microsatellite loci that we had shown previously had the greatest power to distinguish hybrids and indeed display almost fixed differences between the 2 parental species (Roberts et al. 2009). To statistically test for differences in the overall genotypic composition of *Acanthopagrus* spp. among years, we compared the distribution of q-values (i.e. the inferred proportion of *A. butcheri* ancestry) for all pairwise comparisons using a Kolmogorov-Smirnov test (implemented in the program PAST; Hammer et al. 2007). We performed factorial correspondence analysis (FCA) (in GENETIX 4.03, Belkhir et al. 2002) on the overall pooled sample of historical and contemporary fish with the previously categorised fish of our reference collection (Roberts et al. 2009, see also Roberts et al. 2010a) to simply visualise the genetic similarity of hybrids to pure *A. butcheri*.

RESULTS

Contrary to the expectation that levels of genetic diversity would vary between the samples of historical and contemporary *Acanthopagrus* spp., the average number of alleles per locus (±SE) was remarkably similar across all 4 collections (range: 8.2 ± 1.2 to 9.2 ± 0.8), as was allelic richness (range: 7.4 ± 1.0 to 8.1 ± 1.2 alleles per locus) and expected heterozygosity (~0.70). We detected 3 and 4 rare private or 'ghost' alleles (i.e. alleles that were present in the historical but not the contemporary sample) within the 1941 and 1943 samples

respectively, and similarly, we recorded 3 rare alleles in each contemporary sample that were not in either historical sample (data presented in Table S1 in the supplement at www.int-res.com/articles/suppl/m421p199_supp.pdf). However, each of these private or 'ghost' alleles has previously been detected in contemporary estuarine *Acanthopagrus* spp. populations in southeastern Australia, and such minor variation in the occurrence of rare alleles would be expected as a consequence of sampling variation (Roberts et al. 2009, 2010a, D. G. Roberts unpubl.). Tests for homogeneity of allele frequencies revealed statistically significant differences for just 2 loci, *pAb2B7* and *Acs1** (Fig. 1). Not surprisingly, genetic subdivision across the 4 sampling times was not statistically significantly different from zero (F_{ST} = 0.003 ± 0.002; 95% CI: 0.000 to 0.007).

Admixture analysis, using a q-threshold of 0.05 to distinguish pure species and introgressed or hybrid bream, revealed that a high percentage of both the contemporary and historical samples were introgressed rather than pure ancestral *Acanthopagrus butcheri* (95 to 99%) (Fig. 2). This same pattern was evident even when we used an extremely relaxed q-threshold of q = 0.2; the percentage of hybrids in each year ranged from 69 to 80% of all fish genotyped (Table S1 in the supplement). Analysis of the distribution of q-values for all pairwise comparisons among samples did not reveal statistically significant differences (Kolmogorov-Smirnov tests: p > 0.05), suggesting that the proportion of introgressed bream has not changed over 60 yr. Moreover, all samples included fish with genotypes characteristic of complex, later-generation hybrids and backcrosses. In all cases, however, q-values were skewed by the greater similarity of hybrids to *A. butcheri* rather than to *A. australis* (Fig. 2). The greater similarity of introgressed bream and *A. butcheri* is most easily displayed using an FCA plot of the genetic similarity of our Gippsland Lakes sample and our reference collections of pure species (Roberts et al. 2009) (Fig. 3).

DISCUSSION

Our longitudinal survey of the frequency of *Acanthopagrus butcheri* × *A. australis* hybrids highlights, through the first such study with fish, the importance of historical museum collections in describing the genetic composition of populations through time (Wandeler et al. 2007) and the stability of a hybrid swarm. Importantly, our data show that, despite potentially infrequent contact between the 2 parental species, the Gippsland Lakes *Acanthopagrus* spp. population is genotypically complex, and genotype frequencies are surprisingly stable. Samples from 69, 67, 14 and 10 yr
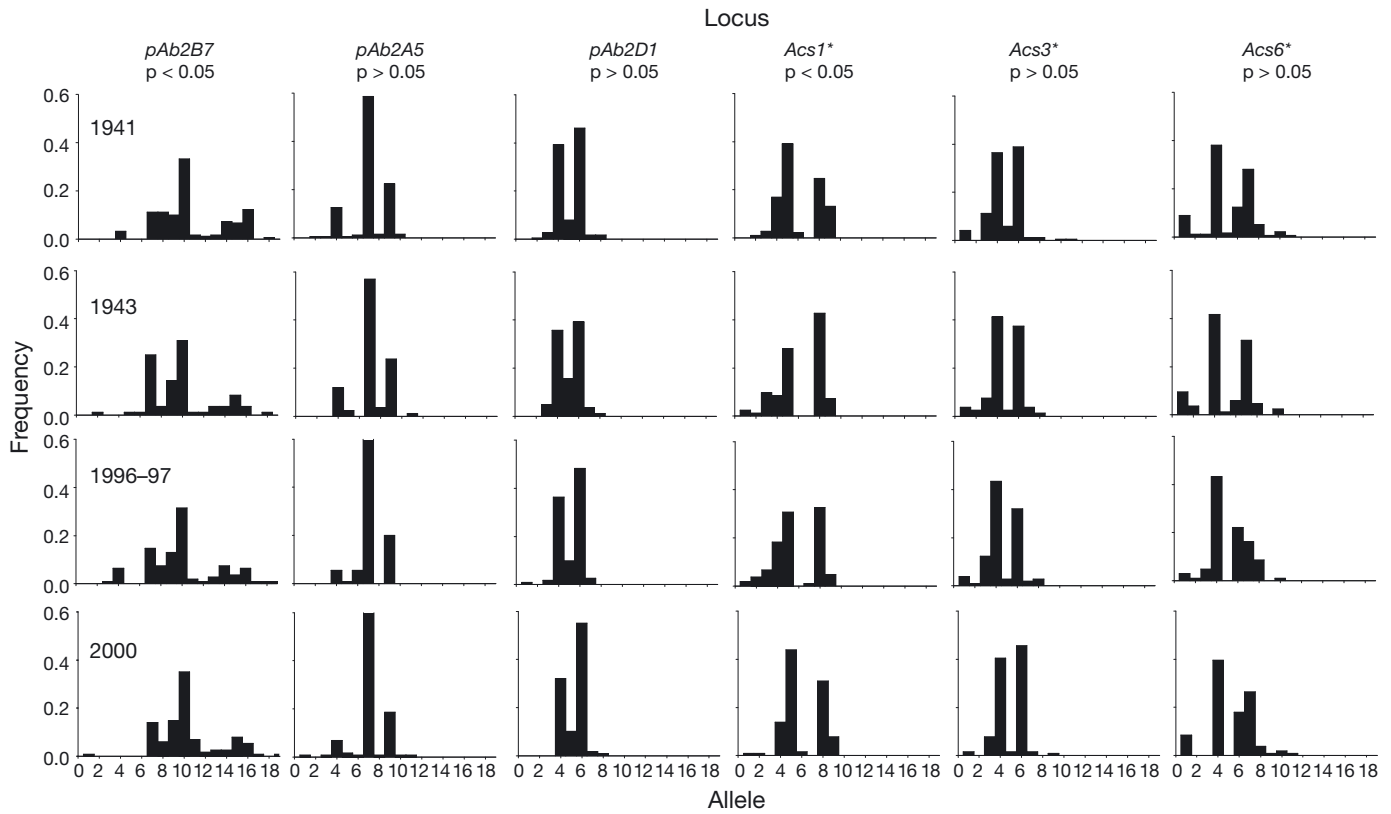
Fig. 1. *Acanthopagrus* spp. Allele frequencies at 6 microsatellite loci for 4 samples (collected at different times: 1941, 1943, 1996–97 and 2000) caught within the Gippsland Lakes. The p-values are from tests of homogeneity of allele frequencies among collection times
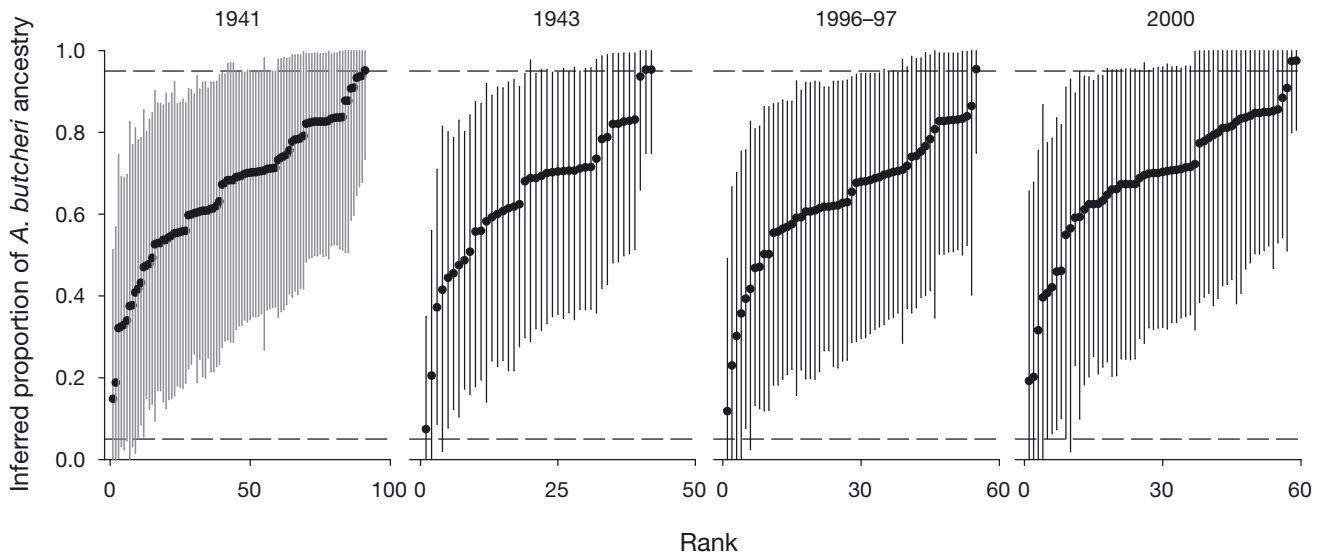

Fig. 2. *Acanthopagrus* spp. Estimates of ancestry for *Acanthopagrus* spp. within the Gippsland Lakes based on 4 relatively diagnostic microsatellite loci, for 4 collection times (1941, 1943, 1996–97 and 2000). Data are presented as the inferred proportion of *A. butcheri* ancestry (average q_i ± 95 % CIs). Based on the estimate of ancestry, each individual was classified as *A. australis* ($q_i \leq$ 0.05), *A. butcheri* ($q_i \geq 0.95$) or hybrid ($0.05 < q_i < 0.95$). Individuals were ranked based on their inferred proportion of *A. butcheri* ancestry (i.e. value of q_i), from lowest q_i to highest q_i. Note that the *x*-axis scale varies among sampling times, reflecting different sample sizes. We varied the q-value used to distinguish pure species and hybrids, with no substantive difference to our conclusions (see Table S1 in the supplement at www.int-res.com/articles/suppl/m421p199_supp.pdf)
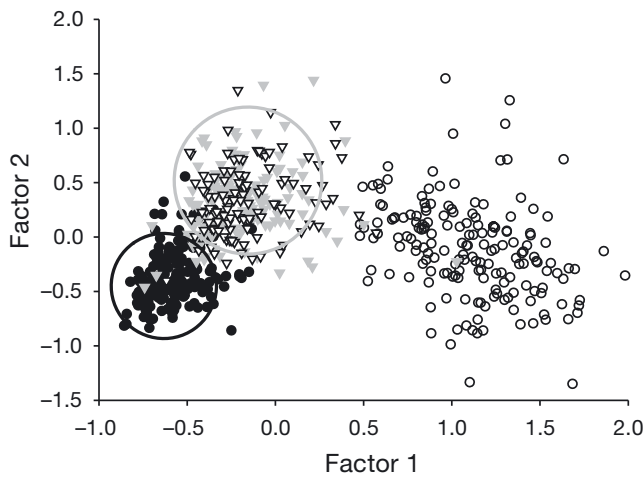
Fig. 3. *Acanthopagrus* spp. Factorial correspondence analysis based on the 4-locus microsatellite genotype of contemporary (▽) and historical (▼) *Acanthopagrus* spp. within the Gippsland Lakes (enclosed within grey circle). A genetic reference collection of *A. australis* (○) and *A. butcheri* (●) (Roberts et al. 2009) was included in the analysis. Fish within the reference collection that cluster inside the black circle were *A. butcheri* from outside the described range of *A. australis* and were therefore expected to be beyond the range of hybridisation (refer to Roberts et al. 2009)

ago displayed strikingly similar allelic and genotypic composition, and in each case appeared to represent later-generation hybrids or *A. butcheri* backcrosses that were most similar to the estuary-restricted *A. butcheri*.

Our data clearly demonstrate that introgression has impacted *Acanthopagrus butcheri* populations occurring beyond the accepted range of *A. australis* and this has been a consistent phenomenon for at least 60 yr. Taken at face value, our conclusions clash with the earlier findings of Farrington et al. (2000) who used allozyme data and Burridge et al. (2004) and Burridge & Versace (2007) who used microsatellite data to describe the genetic structure of what they considered to be populations of *A. butcheri* within the Gippsland Lakes. However, those authors unsurprisingly assumed that Gippsland was beyond the range of *A. australis* and that hybrids were a rare phenomenon confined to ICOLLs in southern NSW (see Rowland 1984). More recently, L. W. Farrington (unpubl. data) genotyped a subsample of the historical fish surveyed here and inferred that slightly higher levels of diversity in contemporary samples could reflect effects of hybridisation.

Most models that seek to predict the dynamics of hybrid zones assume relatively stable distributions of parental taxa in areas of contact, and relatively constant environmental conditions within the habitat supporting hybrid individuals (Barton & Hewitt 1985). In the present case, however, *Acanthopagrus australis* is highly mobile, only occasionally expected to be resident within estuaries, and estuaries themselves represent a characteristically variable environment. Moreover, the Gippsland Lakes occur beyond the recognised contemporary range limit of *A. australis*, suggesting that frequent genetic input from *A. australis* is unlikely, which, together with the broad range of apparently later-generation hybrid or backcrossed genotypes detected, suggests the presence of a persistent hybrid swarm. Indeed, it is possible that bream populations within these southern Australian lakes and lagoons are the result of ancient hybridisation events, which set up introgressed swarms that have remained stable.

Clearly, additional large-scale geographical surveys are needed to fully resolve the spatial extent of *Acanthopagrus* spp. hybrid swarms. Further longitudinal surveys that determine both genotypes and otolith microchemistry may provide the best opportunity to study the dynamics of hybridisation and introgression in this *Acanthopagrus* spp. system. By subjecting samples of bream to both genotyping and sectioning of otoliths, it should be possible to compare the fitness of introgressed and parental bream in terms of growth rates, longevity and age-specific fecundity. Moreover, analysis of otolith microchemistry could potentially determine the mobility (Elsdon et al. 2008 for review) of pure *A. butcheri* and introgressed bream and so indicate whether the geographic spread of *A. australis* alleles (introgression) depends entirely on contact and inter-breeding between *A. australis* and *A. butcheri* or if it involves allopatric introgression resulting from the migration and inter-breeding of introgressed bream and *A. butcheri*. Otolith microchemistry can potentially be used to determine whether *A. butcheri* and the genotypically diverse array of introgressed bream spend periods of their life in the ocean (providing the opportunity for dispersal) and may indicate if bream have moved between (chemically) different estuaries (e.g. Elsdon & Gillanders 2006, Arai & Goto 2008, Bradbury et al. 2008, Kuroki et al. 2008, Vasconcelos et al. 2008).

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