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 approxi-mately 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 mm² fin clip) was placed in a sterile 1.5 ml tube containing 5% Chelex resin in 500 µl of sterile distilled H₂O 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 × 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 STRUCTURE; 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 (FST = 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 $q_i \pm 95\%$ 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)
porting hybrid individuals (Barton & Hewitt 1985). In instant environmental conditions within the habitat sup-
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More recently, L. W. Farrington (unpubl. data) geno-
tralis and that hybrids were a rare phenomenon con-
sumed that Gippsland was beyond the range of 
Lakes. However, those authors unsurprisingly as-
to be populations of Acanthopagrus butcheri and this 
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 occur-
rting 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 as-
sumed that Gippsland was beyond the range of A. au-
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contemporary samples could reflect effects of hybridis-
atation.

Most models that seek to predict the dynamics of 
hybrid zones assume relatively stable distributions of 
parental taxa in areas of contact, and relatively con-
stant environmental conditions within the habitat sup-
porting 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. aus-
tralis is unlikely, which, together with the broad range 
of apparently later-generation hybrid or backcrossed 
genotypes detected, suggests the presence of a persis-
tent 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 Acan-
thopagrus 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 
ooliths, 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 poten-
tially 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).

Acknowledgements. This work was supported by an 
Australian Research Council Linkage grant to D.J.A, R.J.W. and 
C.A.G., which provided a PhD scholarship to D.G.R. Additional 
project support was provided by the Australian Research Council, New South Wales Department of Primary 
Industries, NSW Recreational Fishing Trust and the Univer-
sity of Wollongong’s Institute for Conservation Biology and 
Environmental Management. L. Farrington and C. Burridge 
respectively generously provided historical samples (1941 
and 1943) and more contemporary samples (collected in 
1996–97 and 2000). We thank 2 anonymous reviewers for 
comments. This is contribution number 298 of the Ecological 
Genetics Group at the University of Wollongong.
LITERATURE CITED


Roberts DG, Gray CA, West RJ, Ayre DJ (2010b) Gamete compatibility between marine and estuarine *Acanthopagrus* spp. (Sparidae) and their hybrids. J Fish Biol 77:425–431


Editorial responsibility: John Choat,
Townsville, Queensland, Australia

Submitted: February 15, 2010; Accepted: October 25, 2010
Proofs received from author(s): December 13, 2010