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Using genotype simulations and Bayesian analyses to identify individuals of hybrid origin in Australian bass: lessons for fisheries management

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Simulated genotypes, Bayesian analyses and molecular genetic data were used to detect individuals of hybrid origin and hybrid introgression between the Australian bass (*Macquaria novemaculeata*), a species extensively stocked in Australia, and estuary perch (*Macquaria colonorum*). Based on this analytical framework, 93% of the hybrids up to three generations later could be distinguished from the Australian bass. Individuals of hybrid origin were identified in all three rivers sampled. In addition, this study verified the fertility of hybrids between Australian bass and estuary perch as determined through genomic introgression. This study exemplifies an analytical procedure that has implications for identifying suitable individuals for use in breeding and restocking programmes for other species. © 2008 The Authors
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Key words: assignment tests; conservation genetics; genetic structure; hybridization; introgression; microsatellites.

INTRODUCTION

A low level of introgression among closely related species is a natural occurrence and an important source of genetic variation for a species (Arnold, 1997; Dowling & Secor, 1997). However, hybridization becomes a conservation concern when its prevalence is increased as a result of human disturbances. Introductions of exotic species, habitat modification causing increased mixing of native species and overexploitation leading to species declines are all factors known to increase interspecific hybridization (Hubbs, 1955; Karl *et al.*, 1995; Rhymer & Simberloff, 1996). Declining species can be negatively affected by hybridization in several ways. If hybrids are not viable or are sterile, the negative affect is loss of reproductive output (Leary *et al.*, 1993). If the hybrids are viable and fertile, they can be afflicted by outbreeding depression caused by

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mitotic mismatches or disruption of coadapated gene complexes that would also result in loss of reproductive output (Burke & Arnold, 2001). Further, introgression of hybrid genes into the parental gene pool can result in the loss of local adaptations and further decline of the parental population (Gharrett & Smoker, 1991; Allendorf & Waples, 1996; Rhymer & Simberloff, 1996). Even if the outbred hybrids are under strong negative selection, introgression is still possible and parental population decline is likely (McMillan & Wilcove, 1994; Arnold, 1997; Epifanio & Philipp, 2001). Alternatively, if hybrids are under positive selection, *e.g.* heterosis or hybrid superiority, introgression can result in hybrid swarms that may out compete, and thereby drive to extinction, one or both parental species (Allendorf & Leary, 1988; Levin *et al.*, 1996; Arnold, 1997).

Hybridization is well documented in fish and is generally considered a major concern in conservation and management practices (Allendorf & Leary, 1988; Epifanio & Nielsen, 2001: and references therein; Allendorf *et al.*, 2004). Conservation concerns over hybridization and introgression typically focus on species of important economical value, which have been overexploited or threatened by an introduced species. Breeding programmes, as a management technique, have become a common practice for declining species of economic importance. Thus, to maintain the genetic integrity of the species, breeding programmes need to ensure that breeders are of hybrid-free origin. The aims of this study were to use simulations and Bayesian analyses with molecular genetic data to detect individuals of hybrid origin for exclusion from breeding programmes, to determine if introgression is occurring and to convey how this information can be used for fisheries management. For this purpose, this study focuses on the Australian bass (*Macquaria novemaculeata* Steindachner 1866).

Australian bass is a catadromous fish found in coastal river drainages of eastern Australia from Queensland to Victoria (Harris & Rowland, 1996). This species feeds in freshwater reaches of streams and migrates to estuaries to spawn. The distributional range of the Australian bass largely overlaps with that of the estuary perch (*Macquaria colonorum* Günther 1863), a species morphologically and evolutionarily related to Australian bass (Jerry *et al.*, 2001). These species spawn in different salinities and possibly at different times (Williams, 1970; Harris & Rowland, 1996), and hence may be spatially and temporally reproductively isolated from each other. Nevertheless, previous work based on morphology, allozymes and mtDNA markers has verified that the Australian bass hybridizes with sympatric populations of the estuary perch (Jerry *et al.*, 1999). The hybrid zone was likely restricted to Victorian rivers, particularly rivers afflicted by habitat degradation or those with declining bass populations (Jerry *et al.*, 1999).

Australian bass is one of the most popular freshwater sport fish in Australia and sustains large recreational fisheries in coastal catchments of south-eastern Australia (Harris & Rowland, 1996). Wild stocks of this species have declined dramatically as a result of the construction of dams and weirs on coastal streams, separating freshwater habitats from estuarine breeding areas (Harris & Rowland, 1996). As a consequence, the stocking of waterways upstream of dams and weirs with hatchery-produced juveniles has been an increasingly common management response since captive breeding techniques were

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developed in the 1980s (NSW Fisheries, 2003). In New South Wales alone, a total of 2 856 146 Australian bass had been stocked up until 2003 (NSW Fisheries, 2003). Given the scale of bass stocking as a management activity and given that Australian bass and estuary perch can be notoriously difficult to distinguish morphologically, accidental hybridization of these two species in captive breeding programmes is a conservation concern. This is particularly true if hybrids are fertile and can backcross to the parental bass species. To assist fisheries management, a rapid and efficient method of identifying 'pure' bass strains is needed to avoid using individuals of hybrid origin as breeders in hatchery programmes.

Different techniques have been used in the past to identify hybrids between Australian bass and estuary perch (Jerry *et al.*, 1999). First generation hybrids (F1) can be identified by allozyme and mtDNA markers, but these techniques cannot detect different levels of introgression or backcrossing (Jerry *et al.*, 1999). Biparentally inherited nuclear DNA markers, such as microsatellite DNA, offer several advantages over protein and mtDNA markers for the analysis of hybridization. Microsatellites are hypervariable markers, represented by genotypic arrays, which provide considerable power for individual identification (Sunnucks, 2000). These sensitive and highly discriminatory DNA markers, when used in conjunction with modern analytical approaches for individual assignment, overcome many limitations of previous methods of hybrid identification (Randi *et al.*, 2001; Vazquez-Dominguez *et al.*, 2001; Vähä & Primmer, 2006). A major advantage of these modern approaches, as recently shown in simulation (Vähä & Primmer, 2006) and empirical studies (Russello *et al.*, 2007), is that large samples from hybrid-free areas are not necessarily needed as references. In addition, given that microsatellites can be assayed *via* the polymerase chain reaction (PCR), they are appropriate for non-lethal sampling of breeders used in hatcheries.

This study uses microsatellite markers developed for Australian bass to identify hybrids between the Australian bass and the estuary perch, and more specifically to identify purebred individuals that should be used in an Australian bass breeding programme. The data are analysed with a statistical framework that combines a Bayesian clustering method to identify individuals that are pure species from those of hybrid origin, and a genotypic simulation approach to evaluate the effectiveness of this method in identifying various classes of hybrid individuals. The fertility of these hybrids was assessed by testing for introgression of perch microsatellite alleles into the bass genome. Additionally, this study explores the utility and limitations of this genotyping technique and analysis method in the conservation management and development of breeding programmes for threatened fish species.

MATERIALS AND METHODS

Ethanol-preserved fin clips from the Snowy ($n = 81$), Bemm ($n = 18$) and Brodribb ($n = 10$) rivers in south-east Australia were provided by the Victorian Department of Primary Industries and Fisheries (DPI, Victoria). These 109 fish included: 89 'unknown' individuals of questionable species status, 10 putative bass and 10 putative perch. In addition to these fish, this study included 10 purebred individuals from hybrid-free

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areas, the Logan River in Queensland ($n = 6$ bass) and the Hopkins River in western Victoria ($n = 4$ perch), for a total number of 119 individuals. The species are thought not to overlap in distribution in these two hybrid-free areas (Harris & Rowland, 1996). DNA was isolated from the fin clips using a modified salt extraction with an additional phenol–chloroform purification step (Sambrook *et al.*, 1989). Samples were genotyped at six Australian bass microsatellite markers (Table I) using primers, PCR conditions and protocols described in Schwartz *et al.* (2005). These microsatellite primers also amplify homologous loci in the estuary perch (Schwartz *et al.*, 2005).

To determine the ancestry of individual fish, a model-based clustering method was implemented in STRUCTURE (Pritchard *et al.*, 2000), which estimates the proportion of an individual's genotype originating from each of a set of potentially hybridizing taxa. Genotyped fish were assigned to two species groups ($k = 2$; *i.e.* bass and perch) using 500 000 burn-ins and 1 000 000 repetitions. The six pure bass and four pure perch individuals from the hybrid-free zones were not used as 'reference samples', as Vähä & Primmer (2006) determined that Bayesian-based assignment tests are minimally affected by the inclusion of reference data for correct assignments. Rather, these samples were used as controls to evaluate the accuracy of the purebred assignments. Thus, for this STRUCTURE run, all 119 fish were classified as unknown. Additional parameters used in STRUCTURE included the admixed model with default parameters and independent allele frequencies between the two species. This analysis resulted in a q-value for each individual that ranged from 1 to 0 and represented the mean posterior proportion of ancestry (*i.e.* the proportion of the fish's genome that had bass or perch ancestry; Table II). This analysis was run over five iterations to verify the robustness of the data-set. Additionally, the data were tested for sensitivity to sample size by analysing random sub-samples consisting of 95, 90, 85, 80 and 75% of the original 119 individuals.

A recently published simulation study by Vähä & Primmer (2006) and other recent hybridization studies (Beaumont *et al.*, 2001; Lancaster *et al.*, 2006) have used a q-value threshold of $\leq 0{\cdot}1$ as an efficient cut-off for defining parental populations having an F_{ST} of 0·2. This study used a more stringent threshold q-value of $\leq 0{\cdot}05$ on either side of the distribution (*i.e.* $q \geq 0{\cdot}95$ for bass and $q \leq 0{\cdot}05$ for perch) for two reasons. First, this level is considered conservative in that, although some purebred individuals may be classified as hybrids, there is a reduced chance that hybrids will be misclassified as pure species (Vähä & Primmer, 2006). Second, q-values for the 10 (six bass and four perch) purebred individuals from the hybrid-free zones fell within these thresholds (Table II). F_{ST} values were calculated with ARLEQUIN 3.0 (Excoffier *et al.*, 2005), initially using the six bass and four perch from the hybrid-free zones, then including the additional individuals that fell within the 0·05 threshold. These individuals were also used to calculate observed and expected heterozygosities with ARLEQUIN 3.0.

To identify the possible range of q-values for potential purebreds and different hybrid classes, the programme HYBRIDLAB (Nielsen *et al.*, 2001, 2006) was used to simulate parental and hybrid genotypes. In brief, HYBRIDLAB estimates allele frequencies from user-specified parental populations (either actual genotyped populations or simulated populations). These allele frequencies are then used to create, or simulate, multilocus genotypes for the offspring of the two specified parental populations. The genotyped fish that grouped with the known bass and perch individuals (q-value $\geq 0{\cdot}95$ for bass and $\leq 0{\cdot}05$ for perch) in the first STRUCTURE run were used as the 'source' parental populations for genotype simulations. Genotypes were then simulated for 500 individuals in each parental population (*i.e.* bass and perch). These simulated parental populations were then used to simulate F1 hybrids, F2 hybrids (*i.e.* F1 $\times$ F1), B2 backcrosses to each species (*e.g.* F1 $\times$ bass) and B3 backcrosses to each species (*e.g.* B2-bass $\times$ bass). These simulated samples were then analysed in STRUCTURE, using the same parameters as the first STRUCTURE run described above, to determine the q-value ranges for each hybrid class [Fig. 1(b–d)].

To further estimate an individual's probability of having a hybrid origin in the past two generations, STRUCTURE was run a second time on the genotyped individuals. In this run, each fish was defined as belonging to one of two species groups based on their q-value from the first STRUCTURE run ($q > 0{\cdot}5$ grouped with bass and

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$q < 0{\cdot}5$ grouped with perch). STRUCTURE was run using this prior information (population information, $k = 2$), the generation option set to 2, and other settings as previously described. This specifically tested each individual for having an ancestor of the other species in the past two generations (Pritchard *et al.*, 2000).

For comparison, NEWHYBRIDS (Anderson & Thompson, 2002) was also used. NEWHYBRIDS has a Bayesian model-based clustering framework and uses Markov Chain Monte Carlo to determine posterior probabilities to place the individuals in a hybrid category. Eight genotype frequency classes were defined that correspond to the simulated populations described above for HYBRIDLAB. The programme was first run on the 119 genotyped fish, then on the simulated genotypes using no prior species information.

RESULTS

The microsatellite loci revealed moderate genetic variability in the samples (up to seven alleles per locus). Five of the loci had at least one species-specific allele, and three of the loci were diagnostic of the species (Table I). In evaluating the genotypes based on diagnostic alleles and Mendelian inheritance, some individuals have loci with two diagnostic bass alleles at one locus and a hybrid genotype at other loci (*e.g.* individual 98 in Table II), thereby providing clear evidence of hybrid fertility due to either hybrids backcrossing with bass parental species or breeding between F1 hybrids. A comparison of the individual genotype data to the simulated data (Fig. 1) indicated that backcrossed perch may have been present in the sample. However, the actual genotypes of these individuals based on Mendelian inheritance provide no conclusive evidence (*i.e.* a locus with only diagnostic perch alleles and a locus with hybrid genotype) for backcrossing of hybrids to the perch parental species.

In the first STRUCTURE run, the six bass from the hybrid-free area had q-values ranging from 0·971 to 0·978, and the four perch from the hybrid-free area had q-values ranging from 0·022 to 0·034. Based on the cut-off criteria of 0·05, the 55 bass had a q-values ranging from 0·967 to 0·980, and 19 perch had q-values ranging from 0·022 to 0·042 (Table II). F_{ST} between the six bass and four perch from the hybrid-free area was 0·681 ($P < 0{\cdot}01$). When all individuals below the 0·05 threshold were included, F_{ST} between species was 0·675 ($P < 0{\cdot}001$).

The test for robustness in the data over five iterations in STRUCTURE resulted in a maximum S.D. of 0·0005 over the 119 individuals; thus, the data results were highly consistent. The test of sensitivity of the dataset to sample size showed that with a 15% decrease in sample size (*i.e.* with 85% of the data), the results remained highly consistent (maximum S.D. of 0·016) and none of the individuals was assigned to a different class. With a decrease of 20%, however, the maximum S.D. (0·152) increased sharply, and eight hybrid individuals changed between the hybrid classes (*i.e.* from F2 to F1).

The q-value distributions of the simulated genotypes appear in Fig. 1(b–d). The q-values of the simulated F1 hybrids ranged from 0·46 to 0·69 with a mean of 0·50 and were clearly distinct from the simulated parental population ranges: bass (0·95–0·96) and perch (0·04–0·16). F2 hybrid (*i.e.* F1 $\times$ F1) q-values ranged from 0·06 to 0·96 with a mean of 0·51. The range of q-values for B2 and B3 backcrosses of the hybrids to the parental species were as follows:

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TABLE I. Microsatellite alleles (in base pairs), sample sizes (*n*), allele frequencies and observed heterozygosity (Ho) and expected heterozygosity (He) for each locus in the Australian bass (*Macquaria novemaculeata*), estuary perch (*Macquaria colonorum*) and hybrids as defined by STRUCTURE. Alleles from hybrid-free zones are in bold (bass) or italics (perch)

		Australian bass			Estuary perch			Hybrids	
Locus	Alleles	*n*	Allele frequencies	Ho/He	*n*	Allele frequencies	Ho/He	*n*	Allele frequency
AB01		55		0·51/0·54	18			42	
	224					1·00			0·40
	228								0·01
	232		0·08						0·01
	234		0·61						0·40
	236		0·03						
	238		0·27						0·17
AB06		47		0·87/0·62*	19			36	
	180					1·00			0·43
	188		0·30						0·11
	190		0·51						0·36
	192		0·19						0·10
AB09		48		0·46/0·43	19		0·21/0·25	39	
	281					0·03			
	283					0·89			0·34
	285		0·72			0·08			0·51
	295		0·28						0·15
AB97		54		0·26/0·28	19			43	
	104		0·83			1·00			0·84
	112		0·17						0·16
AB107		52			19			43	
	290		1·00						0·51
	300					1·00			0·49
AB114		53		0·51/0·44	19		0·32/0·32	42	
	115		0·01						
	117		0·25						0·15
	119								0·01
	131		0·72			0·16			0·45
	133		0·01						0·03
	141					0·84			0·35

*Deviation from Hardy–Weinberg expectations, $P \leq 0{\cdot}05$

B2-bass 0·46–0·96, mean 0·67; B3-bass 0·50–0·96, mean 0·83; B2-perch 0·04–0·56, mean 0·26 and B3-perch 0·04–0·51, mean 0·15. From the simulations, 0·2% of the F2 hybrids, 2·4% of the B2-bass and 24·2% of the B3-bass overlapped with the simulated bass parental population. Similarly, 0·02% of the F2 hybrids, 17% of the B2-perch and 56% of the B3-perch overlapped with the simulated perch parental population. Overall, 86% of the simulated genotypes with hybrid origin in the last three generations were distinguishable from both

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TABLE II. Genotyped individuals sorted by q-values from the first run of STRUCTURE. Individuals from the hybrid-free zones appear in bold. Columns labelled second and third indicate the probability of being a second (F1) or third (backcross) generation hybrid based on the second run of STRUCTURE, with asterisk indicating the significance level of being a hybrid. ST and NH list the species and hybrid class designation based on the two STRUCTURE runs and in NEWHYBRIDS

ID	River	*AB01*		*AB06*		*AB09*		*AB97*		*AB107*		*AB114*		q	Second	Third	P	ST	NH
81	Snowy	N	N	B	B	N	N	B	B	B	B	N	N	0·98	0·00	0·00		B	B
88	Snowy	B	B	B	B	N	N	B	B	B	B	N	N	0·98	0·00	0·00		B	B
55	Snowy	B	B	B	B	N	N	N	B	B	B	B	N	0·98	0·00	0·00		B	B
65	Snowy	B	B	B	B	N	N	N	B	B	B	N	N	0·98	0·00	0·00		B	B
86	Snowy	N	B	B	B	N	N	N	B	B	B	B	N	0·98	0·00	0·00		B	B
96	Snowy	B	B	B	B	N	N	N	B	B	B	B	N	0·98	0·00	0·00		B	B
113	**Logan**	**B**	**B**	**B**	**B**	**N**	**N**	**N**	**B**	**B**	**B**	**B**	**N**	**0·98**	**0·00**	**0·00**		**B**	**B**
32	Bemm	B	B	B	B	N	N	N	B	B	B	N	N	0·98	0·00	0·00		B	B
84	Snowy	B	B	B	B	N	N	N	B	B	B	B	N	0·98	0·00	0·00		B	B
101	Snowy	N	B	B	B	N	N	N	B	B	B	N	N	0·98	0·00	0·00		B	B
8	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
44	Brodribb	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
49	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
69	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
104	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
1	Snowy	B	B	—	B	N	N	N	B	B	B	N	N	0·98	0·00	0·00		B	B
2	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
7	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
9	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
29	Bemm	N	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
48	Brodribb	B	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
60	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
66	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
68	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
73	Snowy	N	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
79	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
82	Snowy	N	B	B	B	N	N	N	N	B	B	N	N	0·98	0·00	0·00		B	B
99	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
100	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
102	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
108	Snowy	B	B	B	B	N	N	N	N	B	B	B	N	0·98	0·00	0·00		B	B
112	**Logan**	**B**	**B**	**B**	**B**	**N**	**N**	**N**	**N**	**B**	**B**	**B**	**N**	**0·98**	**0·00**	**0·00**		**B**	**B**
115	**Logan**	**B**	**B**	**—**	**B**	**N**	**N**	**N**	**B**	**B**	**B**	**B**	**N**	**0·98**	**0·00**	**0·00**		**B**	**B**
4	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·97	0·00	0·00		B	B
56	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·97	0·00	0·00		B	B
64	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·97	0·00	0·00		B	B
93	Snowy	N	B	B	B	N	N	N	N	B	B	N	N	0·97	0·00	0·00		B	B
103	Snowy	B	B	B	B	N	N	N	N	B	B	N	N	0·97	0·00	0·00		B	B
106	Snowy	B	B	B	B	—	—	N	B	B	B	B	B	0·97	0·00	0·00		B	B
6	Snowy	B	B	B	B	N	—	N	N	B	B	B	N	0·97	0·00	0·00		B	B
54	Snowy	B	B	—	B	N	N	N	N	B	B	B	N	0·97	0·00	0·00		B	B
61	Snowy	B	B	B	B	N	N	N	B	B	B	N	N	0·97	0·00	0·00		B	B

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TABLE II. Continued

ID	River	*AB01*	*AB06*	*AB09*	*AB97*	*AB107*	*AB114*	*q*	Second	Third	*P*	ST	NH
111	**Logan**	**B B**	**— B**	**N N**	**N N**	**B B**	**B N**	**0·97**	**0·00**	**0·00**		**B**	**B**
59	Snowy	N B	B B	N N	N N	B —	N N	0·97	0·00	0·00		B	B
67	Snowy	B B	— B	N N	N N	B B	B N	0·97	0·00	0·00		B	B
70	Snowy	B B	B B	N N	— —	B B	— —	0·97	0·00	0·00		B	B
83	Snowy	B B	B B	— N	N N	B B	N N	0·97	0·00	0·00		B	B
110	**Logan**	**B B**	**— B**	**N N**	**N N**	**B B**	**N N**	**0·97**	**0·00**	**0·00**		**B**	**B**
72	Snowy	B B	B B	— N	N N	B B	N N	0·97	0·00	0·00		B	B
92	Snowy	B B	B B	N N	N N	B —	N N	0·97	0·00	0·00		B	B
114	**Logan**	**B B**	**— B**	**N N**	**N N**	**B B**	**N N**	**0·97**	**0·00**	**0·00**		**B**	**B**
50	Snowy	B B	B B	— —	N N	B B	B N	0·97	0·00	0·00		B	B
53	Snowy	B B	B B	— —	N N	B B	B N	0·97	0·00	0·00		B	B
24	Bemm	B B	B B	— N	N B	— —	N N	0·97	0·00	0·00		B	B
57	Snowy	B B	— —	N N	N B	B B	— —	0·97	0·00	0·00		B	B
90	Snowy	B B	B B	N N	N N	B P	B N	0·89	0·00	0·01		B3+B	B
40	Brodribb	B B	B B	N N	N N	B P	N N	0·89	0·00	0·02		B3+B	B
98	Snowy	B B	B B	N —	N N	B P	B N	0·88	0·00	0·01		B3+B	B
11	Snowy	— —	B B	N N	N B	B P	N N	0·88	0·00	0·02		B3+B	B
78	Snowy	P B	B B	N N	N N	B B	B —	0·87	0·00	0·27		B2/B3-B	B/B2-B
89	Snowy	B B	P —	N N	N N	B B	N N	0·86	0·00	0·4		B3+B	B
105	Snowy	B B	B B	N N	N B	P P	B N	0·81	0·00	0·02		F2/B3+B	B
97	Snowy	B B	P B	N N	N B	B B	N P	0·79	0·01	0·91	***	B2-B	B2-B
52	Snowy	B B	P B	— N	N B	B B	N P	0·78	0·01	0·92	***	B2-B	B2-B
107	Snowy	B B	B B	N N	N N	P P	N N	0·77	0·00	0·03		B3+B	B
94	Snowy	P B	P B	N N	N N	B P	N —	0·63	0·11	0·87	***	B2-B	F1
63	Snowy	P B	P B	P N	N B	B P	N N	0·60	0·67	0·33	***	F1	F1
19	Snowy	P B	P B	P N	N B	B P	N N	0·59	0·67	0·33	***	F1	F1
74	Snowy	P B	P B	— —	N N	B P	B N	0·59	0·46	0·53	***	F1	F1
95	Snowy	P B	P B	N N	N N	B P	B P	0·57	0·56	0·44	***	F1	F1
20	Snowy	P B	— B	P N	N B	B P	N P	0·56	0·84	0·16	***	F1	F1
3	Snowy	P B	P B	P N	N N	B P	B N	0·56	0·63	0·37	***	F1	F1
51	Snowy	P B	P B	— N	N N	B P	B P	0·54	0·88	0·12	***	F1	F1
15	Snowy	P B	P B	P N	N N	B P	N N	0·54	0·74	0·27	***	F1	F1
12	Snowy	— —	P B	— N	N N	B P	N P	0·53	0·79	0·21	***	F1	F1
58	Snowy	P B	— —	P N	N B	B P	B P	0·53	0·84	0·16	***	F1	F1
76	Snowy	P B	P B	P N	N B	B P	N P	0·52	0·91	0·09	***	F1	F1
43	Brodribb	P B	P B	P N	N B	B P	N P	0·51	0·91	0·09	***	F1	F1
17	Snowy	P B	P B	P N	N B	B P	N P	0·50	0·91	0·09	***	F1	F1
14	Snowy	P B	— B	P N	N N	— —	N P	0·50	0·44	0·26	*	F1	F1
91	Snowy	P B	P —	N N	N N	B P	N P	0·49	0·05	0·80	**	B2-P	F1
27	Bemm	N B	P B	P N	N N	B P	N P	0·48	0·83	0·15	***	F1	F1
5	Snowy	P B	P B	— —	N N	B P	B P	0·48	0·84	0·16	***	F1	F1
75	Snowy	P N	P —	P N	N B	B P	B P	0·47	0·12	0·86	***	B2-P	F1
62	Snowy	P B	P B	P N	N N	B P	— —	0·47	0·80	0·18	***	F1	F1
16	Snowy	P B	P B	P N	N N	— —	B P	0·46	0·80	0·19	***	F1	F1
46	Brodribb	P B	P B	P N	N N	B P	N P	0·46	0·88	0·12	***	F1	F1
33	Bemm	P B	P B	P N	N N	B P	N P	0·46	0·85	0·14	***	F1	F1
10	Snowy	P B	P —	P N	N B	B P	N P	0·45	0·09	0·84	***	B2-P	F1

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TABLE II. Continued

ID	River	*AB01*		*AB06*		*AB09*		*AB97*		*AB107*		*AB114*		*q*	Second	Third	*P*	ST	NH
13	Snowy	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·83	0·15	***	F1	F1
77	Snowy	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·83	0·15	***	F1	F1
80	Snowy	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·83	0·15	***	F1	F1
23	Bemm	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·84	0·15	***	F1	F1
28	Bemm	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·81	0·16	***	F1	F1
71	Snowy	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·83	0·15	***	F1	F1
87	Snowy	P	B	P	B	P	N	N	N	B	P	N	P	0·45	0·83	0·15	***	F1	F1
109	Snowy	P	B	P	B	P	N	N	B	P	P	B	P	0·43	0·52	0·47	***	F1	F1
37	Bemm	P	B	P	B	P	N	N	N	B	P	N	P	0·39	0·34	0·60	***	F1/B2-P	F1
18	Snowy	P	B	P	—	P	N	N	N	B	P	N	P	0·38	0·04	0·77	**	B2-P	F1
85	Snowy	P	—	P	B	P	N	N	N	B	P	N	P	0·38	0·28	0·08		F1/B2+P	F1
30	Bemm	P	P	P	P	P	N	N	—	P	P	P	P	0·04	0·00	0·00		P	P
31	Bemm	P	P	P	P	P	N	N	N	P	P	P	P	0·04	0·00	0·00		P	P
34	Bemm	P	P	P	P	P	N	N	N	P	P	P	P	0·04	0·00	0·00		P	P
116	**Hopkins**	**P**	**P**	**P**	**P**	**P**	**P**	**N**	**N**	**P**	**P**	**N**	**P**	**0·03**	**0·00**	**0·00**		**P**	**P**
22	Bemm	P	P	P	P	P	P	N	N	P	P	N	P	0·03	0·00	0·00		P	P
47	Brodribb	P	P	P	P	P	P	N	N	P	P	N	P	0·03	0·00	0·00		P	P
117	**Hopkins**	**P**	**P**	**P**	**P**	**P**	**P**	**N**	**N**	**P**	**P**	**N**	**P**	**0·03**	**0·00**	**0·00**		**P**	**P**
118	**Hopkins**	**P**	**P**	**P**	**P**	**P**	**P**	**N**	**N**	**P**	**P**	**N**	**P**	**0·03**	**0·00**	**0·00**		**P**	**P**
119	**Hopkins**	**P**	**P**	**P**	**P**	**P**	**P**	**N**	**N**	**P**	**P**	**N**	**P**	**0·03**	**0·00**	**0·00**		**P**	**P**
21	Bemm	P	—	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
25	Bemm	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
26	Bemm	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
35	Bemm	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
36	Bemm	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
38	Bemm	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
39	Brodribb	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
41	Brodribb	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
42	Brodribb	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P
45	Brodribb	P	P	P	P	P	P	N	N	P	P	P	P	0·02	0·00	0·00		P	P

B, Australian bass specific allele or species designation; B2, second generation hybrid through a backcross to one of the species (B or P); B3+, third generation or later hybrid backcrossed to one of the species (B or P); F1, first generation hybrid; N, non-diagnostic allele; P, estuary perch specific allele or species designation; —, questionable allele or non-genotyped locus, dismissed from analyses.

$***P < 0{\cdot}001$, $**P < 0{\cdot}01$, $*P < 0{\cdot}05$.

the perch- and the bass-simulated parental populations, and more specifically, 93% of these simulated hybrids could be distinguished from the simulated parental bass. In summary, this method distinguished 93% of the simulated fish of hybrid origin (up to three generations) from the Australian bass with six microsatellite markers and a cut-off threshold of $q < 0{\cdot}05$ in STRUCTURE.

Based on the cut-off criteria, the simulated data and the STRUCTURE analyses, the 45 hybrid individuals were classified into three distinct groups: six individuals with q-values of 0·857–0·889, four with q-values of 0·771–0·806 and 35 with q-values of 0·380–0·628 [Fig. 1(a)]. Genotypic details of the individuals

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of hybrid origin appear in Table II. Hybrid individuals appeared in each of the rivers: Bemm, five hybrids (28% of fish); Brodribb, three hybrids (33%) and Snowy, 37 hybrids (46%).

Results from NEWHYBRIDS were less stringent in the definition of pure species and differed from results for STRUCTURE and HYBRIDLAB. Many individuals defined as backcrosses by STRUCTURE, based on the 0·05 q-value threshold, were identified as pure species by NEWHYBRIDS (Table II). Similarly, results from the simulated data correctly assigned all parental species and F1 hybrids, but assignments of simulated backcrossed individuals were much less accurate, with only 48% of F2 hybrids, 33% of B2-bass, 0% of B3-bass, 49% of B2-perch and 43% of B3-perch correctly assigned. Overall, NEWHYBRIDS distinguished 71% of simulated hybrids from the pure species and identified 79% as not being pure bass.

DISCUSSION

This study demonstrates that microsatellite DNA data used in conjunction with simulations and assignment tests have the power to discriminate between the Australian bass and estuary perch, to detect interspecific hybrids and to assess levels of introgression. This analytical framework enables the identification of purebred Australian bass to be used in breeding programmes and stocking activities. The outcomes of different assignment programmes show that the simulation of genotypes is useful for determining cut-off values of different hybrid classes and for predicting the probability to distinguish between backcrossed and purebreds. This procedure could also be used for other species prone to hybridization to identify suitable individuals for breeding and restocking.

STRUCTURE and a threshold q-value $\leq 0{\cdot}05$ can confidently identify purebred individuals, even though the individuals from the hybrid-free areas were not used as a reference. This is consistent with Vähä & Primmer (2006), who demonstrated that the use of reference samples from known populations (or species) in STRUCTURE had little to no affect on the identification of hybrids, especially if $F_{ST} > 0{\cdot}12$ between parental populations. Furthermore, they determined that Bayesian-based analyses can identify purebred individuals with high efficiency (95%) and high accuracy (100%) with six loci (the same number used here) and a q-value $\leq 0{\cdot}05$ on admixed populations with a F_{ST} of 0·21 between parental populations and with neither diagnostic alleles nor reference samples. Thus, a q-value of 0·05 conservatively increases the chances of eliminating purebred individuals rather than including hybrids in a breeding programme. In comparison to the Vähä & Primmer (2006) simulation study, the present study found a considerably larger F_{ST} of 0·68 and diagnostic alleles at several loci. Additionally, the analyses in STRUCTURE clearly separated the majority of the samples into two distinct species groups [Fig. 1(a)]. Interestingly, inherent breaks in this dataset produced the same result for a q-value threshold between 0·03 and 0·10. Importantly, the comparative analysis based on the NEWHYBRIDS confirmed all the hybrid assignments in STRUCTURE, but it classified many backcross individuals (B2 and B3) as purebred species. Overall, STRUCTURE proved to be more stringent in classifying a purebred individual than NEWHYBRIDS.

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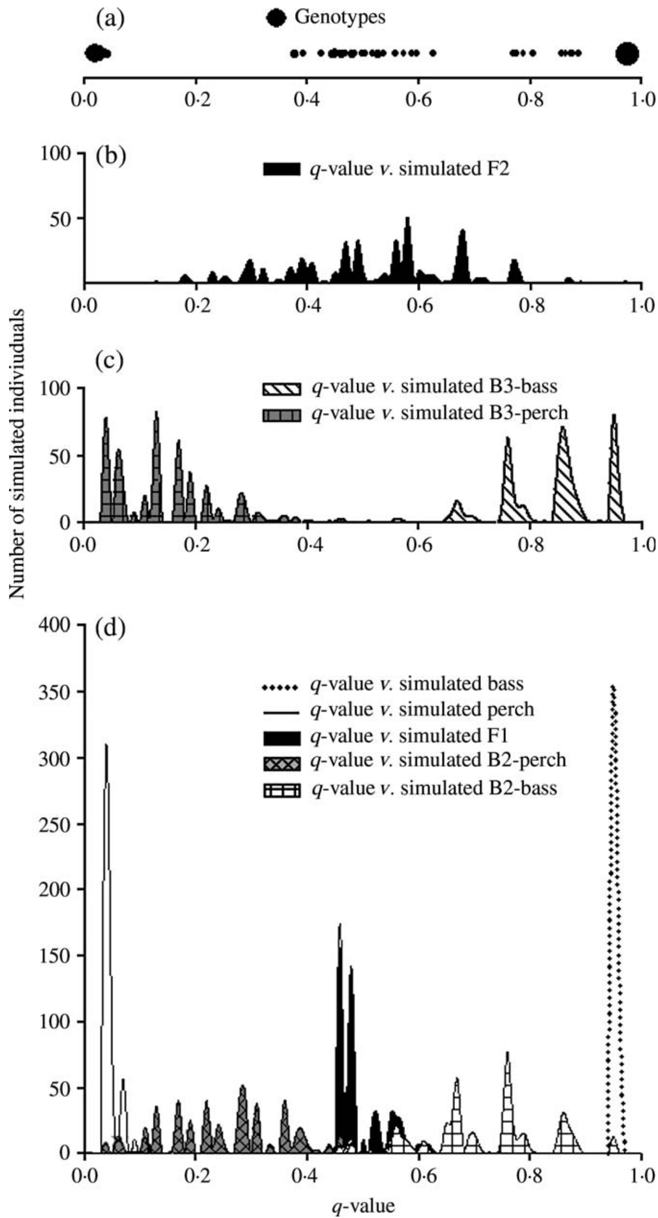

FIG. 1. Genotyped individuals sorted by *q*-value from the first run of STRUCTURE. The *q*-value represents the probability of an individual being Australian bass (*Macquaria novemaculeata*: *q*-value = 1·0) or estuary perch (*Macquaria colonorum*: *q*-value = 0·0) ancestry. (a) Bubble graph depicts *q*-values of genotypes from sampled individuals, with the size of the dot representing the relative number of individuals with that *q*-value. Graphs (b), (c) and (d) depict the ranges and frequencies of the STRUCTURE-based *q*-values of the simulated bass, perch, F1, F2 and backcrossed hybrids generated by HYBRIDLAB.

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After identification, purebred individuals were used in HYBRIDLAB to simulate genotypes of the different hybrid classes. These data were used in STRUCTURE to determine the range of *q*-values inherent to each hybrid class – this enabled the comparison of *q*-values of the genotyped hybrid individuals to determine their hybrid class. These comparisons, along with a simple Mendelian evaluation of genotypes, indicate that the F1 hybrids are in fact fertile and able to introgress into the bass genome. The identification of hybrids between Australian bass and estuary perch support a previous study that used morphology, allozymes and mtDNA to evaluate hybridization between these species (Jerry *et al.*, 1999). Interestingly, Jerry *et al.* (1999) identified 18 putative hybrids, all with bass mtDNA, indicating that in each case the maternal parent was bass. These authors suggested that early sexually mature estuary perch males are interbreeding with female bass before Australian bass males become sexually mature. This conclusion suggests that unidirectional hybridization and introgression, and assortative mating or sterility of hybrids from different crosses may be important (Dowling & Childs, 1992). It is important to note, however, that this study included wild fish collected for an Australian bass hatchery programme. Although no conclusive evidence indicated that hybrids backcross to perch parental species, these hybrids displayed a normal 'bass morphotype'. Further experiments should test whether hybridization is unidirectional, whether the apparent introgression is from hybrid backcrosses to the parental bass species or whether it results from matings between F1 hybrids.

A potential limitation of this study is that the diagnostic alleles observed here may not be diagnostic in all populations, due to either homoplasy (*i.e.* two same-sized alleles are not identical by descent) or lineage sorting, which allows some populations to retain the ancestral allele(s) (Allendorf *et al.*, 2004). Previous research has shown only low (allozymes) to moderate (mtDNA) levels of genetic structuring among Australian bass populations in the form of isolation by distance (Chenoweth & Hughes, 1997; Jerry, 1997; Jerry & Baverstock, 1998), making it more likely that same-sized alleles in this study are identical by descent. If these loci prove not to be diagnostic in other populations, the identification of backcrossed individuals will be difficult.

The assignment test implemented in the STRUCTURE is based on fit to Hardy–Weinberg proportions, thus the analyses of local populations should provide more robust results, as this would lessen the possibility of including genetically structured bass populations in a single group (*i.e.* Wahlund's effect). Ideally, bass, perch and hybrid populations should be examined separately. The identification of two low-frequency (0·01) private alleles in the hybrids (Table I) further indicates the need to additionally sample throughout the species' range in hybrid-free zones. This is the group's aim of the current research programme on Australian bass and estuary perch population genetics. Ongoing genotyping of samples from throughout the range of both species has identified a few new low-frequency alleles; however, the diagnostic alleles described here continued to be diagnostic for each of the two species (K. Shaddick & L. B. Beheregaray, unpubl. obs.). This result, along with tests for sensitivity to sample size, supports the conclusion that sample sizes used here were sufficient.

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UTILITY AND APPLICATION OF THIS TECHNIQUE IN FISHERIES MANAGEMENT

Estimates of divergence with microsatellites indicate that bass and perch in 'hybrid-free' areas are genetically distinct, but interestingly, the analyses also detected several hybrids among putative individuals of bass (three of 10) and of perch (two of 10) from Snowy River. Similarly, Jerry *et al.* (1999) reported that five of 18 hybrids were misclassified as bass or perch. These results highlight the difficulty in identifying hybrids and backcrosses based on morphology and thus, the importance of comprehensive genetic screenings to identify suitable individuals for the large Australian bass breeding and stocking programmes in south-eastern Australia.

Based on simulations, the six microsatellite loci (Schwartz *et al.*, 2005) used in the present study have the power to distinguish 93% of the simulated hybrids from the bass species (Fig. 1). The use of fluorescently labelled microsatellite primers for screening of potential Australian bass provides breeders with an efficient, automated, non-lethal method that can be implemented in governmental and private hatchery programmes. Based on the loci and the samples used in this study, a q-value threshold $\leq 0{\cdot}05$ was useful for identifying the best individuals for breeding programmes, even though this criterion may lead to the inclusion of backcrossed hybrids. Simulations suggest that only 74% of third generation backcrossed individuals would be detected and that further backcrosses are less likely to be detected. The inclusion of additional diagnostic microsatellite loci would improve the resolution for identifying later generation backcrosses.

Hybrid fertility and the ability of hybrids to backcross to the bass parental species is a conservation concern, as this could lead to genomic extinction through introgression of the Australian bass populations in rivers where high levels of hybridization occur (Ryman & Laikre, 1991). Jerry & Baverstock (1998) suggested that the prevalence of backcrossing would be low, as incidences of non-conspecific mtDNA were found in 300 bass and 100 perch collected over a relatively large geographic scale. In contrast, the present study identified individuals of hybrid origin in all three of the rivers sampled, with evidence for introgression in two rivers (Snowy and Brodribb). Furthermore, some individuals were incorrectly classified as bass based on morphology. If backcrossing is unidirectional in that female bass mate with male perch, and if hybrids are misclassified as bass, the detection of non-conspecific mtDNA would be unexpected.

Some populations of Australian bass may consist of hybrid swarms in which hybrids and parental types mate freely (Rhymer & Simberloff, 1996). This is especially likely in a population, such as Snowy River, where perch greatly outnumber bass (McCarraher, 1986*a*, *b*). Such populations may be extirpated, either through outbreeding depression or replacement by hybrids (Ellstrand, 1992; Levin *et al.*, 1996). To avoid accidental introductions of estuary perch genes into other bass populations, it is important that potential breeders for stocking are genetically screened. Furthermore, managers should not use broodfish from 'hybrid-free' zones to produce fingerlings for release into rivers where hybridization is of concern, as a large influx of genotypes from the

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extended range of the species may lead to outbreeding depression in the host population (Ryman & Laikre, 1991). Thus, fingerlings should be generated from populations in which they are to be released. Finally, caution should also be taken to avoid losses in heterozygosity due to the drop in effective population size that can accompany stock enhancement programmes.

The use of microsatellite markers, assignment tests and computer simulations has documented the fertility of hybrids between Australian bass and estuary perch, and the introgression of estuary perch alleles into the Australian bass genome. Further research is needed to investigate the prevalence, geographical extent and implications of this phenomenon for the conservation management of the genetic integrity of the Australian bass. Furthermore, the results of this study stress the need to genetically verify the species status of individuals to be used in breeding programmes for management. The procedures outlined here can be used to identify breeders and assess levels of introgression between morphologically similar species prone to hybridize. The use of simulated genotypes in the assignment programme to determine the *q*-value range of the hybrids and backcrossed individuals appears to be useful in determining purebred cut-off values.

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