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A hybrid zone and bidirectional introgression between two catadromous species: Australian bass *Macquaria novemaculeata* and estuary perch *Macquaria colonorum*

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The presence and distribution of hybrid individuals and the existence of a hybrid zone between the catadromous Australian bass *Macquaria novemaculeata* and estuary perch *Macquaria colonorum* were investigated throughout the range of both species in Australia. Bayesian analyses and genotypic simulations identified 140 putative hybrids (11·5% of the total sample) with varying levels of introgression. Most hybrids were observed in an area extending from the Snowy River to the Albert River suggesting a hybrid zone in the eastern Bass Strait region. Sixteen hybrids, however, were found outside this zone, possibly reflecting the movement of hybrid offspring between estuaries or their inadvertent release during fish stocking programmes. Biparental backcrossing was found to occur suggesting that hybrids were fertile. These results have implications for the management of the extensive stocking programme in *M. novemaculeata* and for understanding the potential role of habitat degradation and reduced water flow in facilitating hybridization in species with migratory life histories.

Key words: backcrossing; catadromy; conservation genetics; fish stocking; hybridization; hybrid zone.

INTRODUCTION

Natural interspecific hybridization among taxonomically discrete fish species is relatively common worldwide (Schwartz, 1972, 2001). The hybridizing potential of two species is often correlated with their phylogenetic relatedness. Recently diverged species are more likely to hybridize than those that diverged deeper in evolutionary time (Barton & Hewitt, 1989; Arnold, 1997; Goodman *et al*., 1999). Therefore,

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hybrid viability may be a good indicator of the evolutionary proximity of parental genotypes (Hubbs & Drewry, 1959). Where hybrid progeny are fertile and capable of backcrossing with the parental species (Verspoor & Hammar, 1991; Bartley *et al*., 2001; Schwartz & Beheregaray, 2008), the transfer of genetic material between species can result in a process known as introgression (Campton, 1987; Billington & Hebert, 1991; Burke & Arnold, 2001).

Low levels of hybrid introgression into the parental gene pool can significantly contribute to adaptive genetic variation *via* the transfer of adaptations between taxa and evolutionary diversification in some fish species (Lewontin & Birch, 1966; Arnold, 1997; Dowling & Secor, 1997; Burke & Arnold, 2001). Hybridization, however, is generally viewed as a detrimental process, at odds with the central tenet of dichotomous divergence and therefore a matter of conservation concern (Smith, 1992; Epifanio & Nielsen, 2001). Hybridization and introgression can lead to genetic swamping (Rhymer & Simberloff, 1996) and accelerate the loss of local adaptations threatening the persistence of the two parental species (Gharett & Smoker, 1991; Allendorf & Waples, 1996; Rhymer & Simberloff, 1996). Some hybrids may exhibit hybrid vigour in the F1 generation, whereby the interaction between the parental genotypes produces superior offspring (Falconer & MacKay, 1996). In the absence of selection against hybrids, hybrid superiority can result in the formation of hybrid swarms which can cause the extinction of one or both of the parental species (Allendorf & Leary, 1988; Arnold, 1997). Hybridization and introgression are particularly important when considering moribund or endangered fish populations (Allendorf *et al*., 2001).

In recent decades, there has been an increase in worldwide rates of hybridization and introgression through anthropogenic interference (Rhymer & Simberloff, 1996). Human-mediated habitat modification and destruction, introduction of non-native species and the translocation of fishes through stocking activities can all increase the incidences of hybridization in fishes (Hubbs, 1955; Hume *et al*., 1983; Karl *et al*., 1995; Rhymer & Simberloff, 1996; Jerry *et al*., 1999). Further, human activities have often foreshadowed an increase in the amalgamation of historically isolated species, possibly facilitating hybridization (Olden *et al*., 2004).

Australian bass *Macquaria novemaculeata* (Steindachner 1866) and estuary perch *Macquaria colonorum* (Günther 1863) are closely related, catadromous fishes that reside in coastal rivers and estuaries throughout south-eastern Australia (Harris & Rowland, 1996; Jerry *et al*., 2001). The distributions of *M. novemaculeata* and *M. colonorum* overlap between the Richmond River and Wilson's Promontory (Fig. 1 and Table I), and earlier work has shown that they produce natural, viable interspecific hybrids in Victorian populations (Williams, 1970; Jerry *et al*., 1999; Schwartz & Beheregaray, 2008). Both species are reliant on environmental cues to initiate migration and spawning behaviour including temperature, salinity levels, flooding and river flow (McCarraher & McKenzie, 1986; Kirwin, 2000). The south-east coast of Australia is one of the most densely populated regions in the country. Barriers to migration in the form of dams, weirs and road crossings can severely impede or block the movement of *M. novemaculeata* both downstream and upstream. Harris (1984) concluded that access to around one-third to one-half of the available habitat of Australian bass was hindered by barriers to migration isolating fishes from large areas of otherwise viable habitat. The overexploitation of the species by recreational fishermen, habitat degradation and thermal pollution have further reduced

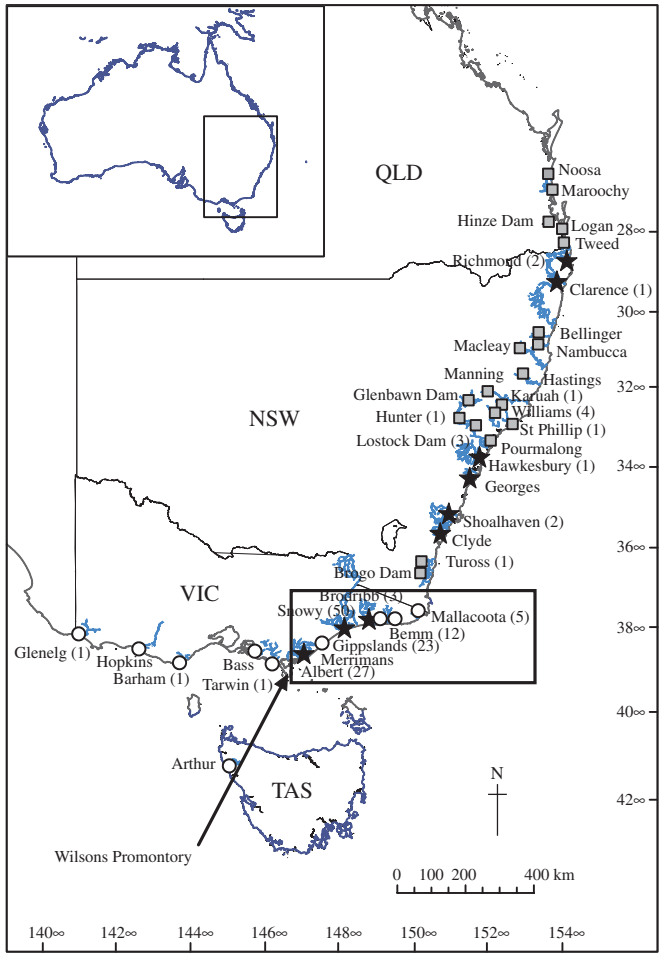

FIG. 1. Map of rivers and dams sampled for *Macquaria novemaculeata*, *Macquaria colonorum* and their hybrid offspring along the south-eastern coast of Australia. Rivers where putative *M. novemaculeata* (▪), putative *M. colonorum* (○) and both putative *M. novemaculeata* and *M. colonorum* (★) were collected are indicated. The boxed area delineates the sites that represent the potential hybrid zone. The presence and number of hybrids within each river is indicated in parentheses. NSW, New South Wales; QLD, Queensland; TAS, Tasmania; VIC, Victoria.

the ability of *M. novemaculeata* to maintain sustainable populations in some rivers (Harris, 1984, 1988; Jerry, 1997). This questionable ability to sustain viable populations together with a perceived decline in range and abundance (Lake, 1967; Harris, 1986; Battaglene *et al*., 1989) has seen the species listed as potentially threatened in Victoria (Koehn & Morison, 1990), and as requiring continuous monitoring in New South Wales (NSW) (Klippel, 1992). In *M. colonorum*, commercial and recreational fishing pressures may have contributed to a purported decline in population numbers (Harris, 1986; Allen, 1989; McDowall, 1996) and in NSW the species is now protected from commercial fishing under the Fisheries Management Act (1994) (www.austlii.edu.au/au/legis/nsw/consol_act/fmal1994193). In addition to a ban on commercial fishing, conservative bag and size limits apply to recreational anglers.

TABLE I. Number and percentage (in parentheses) of samples classified as pure *Macquaria novemaculeata* (AB), pure *Macquaria colonorum* (EP) and putative hybrids based on genotypes across six nuclear loci. The geographical distribution of AB and EP is shown together with the area of overlap (shaded in grey). The suggested hybrid zone between Mallacoota Inlet and the Richmond River is outlined. The last seven sites on the bottom of the table are dams or impounded populations

River	AB	Hybrids	EP	State	Range	
					AB	EP
Noosa	29	—	—	Qld	X	
Logan	5	—	—	Qld	X	
Maroochy	27	—	—	Qld	X	
Tweed	29	—	—	NSW	X	
Richmond	33 (82·9)	2 (5·7)	4 (11·4)	NSW	X	X
Clarence	38 (75·6)	1 (2·2)	10 (22·2)	NSW	X	X
Bellinger	9	—	—	NSW	X	X
Nambucca	22	—	—	NSW	X	X
Macleay	35	—	—	NSW	X	X
Hastings	13	—	—	NSW	X	X
Manning	17	—	—	NSW	X	X
Karuah	29 (96·7)	1 (3·3)	—	NSW	X	X
Williams	55 (93·2)	4 (6·8)	—	NSW	X	X
Hunter	22 (95·7)	1 (4·3)	—	NSW	X	X
Hawkesbury	34 (53·1)	1 (1·6)	29 (45·3)	NSW	X	X
Georges	5	—	5	NSW	X	X
Shoalhaven	66 (71·7)	2 (2·0)	27 (26·3)	NSW	X	X
Clyde	21 (48·8)	—	22 (51·2)	NSW	X	X
Tuross	24 (96·0)	1 (4·0)	—	NSW	X	X
Mallacoota	—	5 (35·7)	9 (64·3)	Vic	X	X
Snowy	50 (41·3)	50 (41·3)	21 (17·4)	Vic	X	X
Brodribb	—	3 (37·5)	5 (62·5)	Vic	X	X
Bemm	—	12 (25·5)	35 (74·5)	Vic	X	X
Gipps Lakes	24 (37·5)	23 (35·9)	17 (26·6)	Vic	X	X
Merrimans	—	—	27	Vic	X	X
Albert	3 (5·2)	27 (47·4)	27 (47·4)	Vic	X	X
Tarwin	—	1 (3·7)	26 (96·3)	Vic		X
Bass	—	—	30	Vic		X
Barham	—	1 (16·7)	5 (83·3)	Vic		X
Hopkins	—	—	35	Vic		X
Glenelg	—	1 (11·1)	8 (88·9)	Vic		X
Arthur	—	—	9	Tas		X
Hinze	19	—	—	Qld		
St Philips	29 (96·7)	1 (3·3)	—	NSW		
Lostock	20 (87·0)	3 (13)	—	NSW		
Pourmalong	24	—	—	NSW		
Lake Yarrunga	5	—	—	NSW		
Glenbawn	20	—	—	NSW		
Brogo	20	—	—	NSW		

NSW, New South Wales; Qld, Queensland; Tas, Tasmania; Vic, Victoria.

Finally, the harvesting of high flows in large dams, whereby floodwater runoff is collected for productive use, can interfere with spawning cues and subsequently successful reproduction in both the species (Harris, 1984, 1988; Kirwin, 2000; Growns & James, 2005).

Despite an overlapping distribution, *M. novemaculeata* and *M. colonorum*, generally occupy distinct growth and spawning habitats (Harris, 1986; McCarraher & McKenzie, 1986). *Macquaria novemaculeata* primarily reside in the freshwater reaches of rivers and during winter months (May to August) they migrate downstream to the brackish waters of upper estuaries (Harris, 1986; Jerry & Baverstock, 1998) to breed in salinities of *c*. 8–10 and in temperatures ranging from 11 to 18° C (Harris, 1986; McCarraher, 1986*a*). *Macquaria colonorum*, on the other hand, remain in the upper estuaries for most of their life cycle but migrate between July and December to spawn in lower estuaries, in waters open to the ocean. Spawning transpires at salinities close to sea water (*c*. 10–24) and temperatures ranging from 14 to 19° C (McCarraher & McKenzie, 1986). Consequently, even in areas where they are considered sympatric, *M. novemaculeata* and *M. colonorum* exhibit fine-scale temporal and spatial isolation, breeding at different times and in different areas of the estuary (Williams, 1970; Jerry *et al*., 1999).

Both *M. novemaculeata* and *M. colonorum* provide important recreational fisheries and a decline in the abundance of wild stocks has led to an increased emphasis on conservation and management issues (Koehn & Morison, 1990; Klippel, 1992; Hodgkin, 1994). To support the significant recreational fishery, and to provide fisheries in dams and rivers upstream of fish passage barriers, *M. novemaculeata* have been stocked as a management activity since the introduction of captive breeding techniques in the 1980s (NSW Fisheries, 2003). Hatchery-produced *M. novemaculeata* fingerlings have been stocked into several rivers and impoundments throughout the distribution of the species. Stocking has been particularly prevalent in the NSW waters where *c*. 5·1 million fish were released between 1980 and 2010 (Doolan, 2009).

The correct identification of broodstock used in supplementation programmes is an important consideration given the extensive supplementation of *M. novemaculeata*. To prevent the liberation of hybrid individuals and to preserve the genetic integrity of natural populations, it is imperative that broodstock are indigenous and non-hybrid in origin (Allendorf *et al*., 2004; Vaha & Primmer, 2006; Schwartz & Beheregaray, 2008). Hybridization has traditionally been detected in fishes using morphology, allozyme electrophoresis and mitochondrial DNA (Campton, 1987). The limitations of these methods have been largely overcome by the development of biparentally inherited, polymorphic, molecular markers such as microsatellites. The rise of innovative statistical methods that utilize information derived from these hypervariable markers has heralded a major advance in the field of population genetics (Vaha & Primmer, 2006). For instance, model-based Bayesian statistical methods and genotype simulations can use microsatellite data to accurately identify hybrid individuals (Nielsen *et al*., 2003; Vaha & Primmer, 2006), together with varying levels of introgression (Schwartz & Beheregaray, 2008). While earlier studies have reported the presence of hybrid individuals in Victorian rivers and the introgression of *M. novemaculeata* alleles into the *M. colonorum* genome (Jerry *et al*., 1999; Schwartz & Beheregaray, 2008), the geographical extent of hybridization between the species and the presence of bidirectional introgression remains undetermined.

For the purpose of ongoing stocking programmes, it is important to accurately detect hybrids and introgressed individuals in order to ascertain whether *M. novemaculeata* broodstock contain *M. colonorum* alleles. Additionally, a comprehensive assessment of hybridization between *M. novemaculeata* and *M. colonorum* could provide insight into the potential role of habitat degradation and reduced flow in facilitating hybridization in species with migratory life histories, that is, those that rely on coastal marine environments for dispersal and recruitment.

In this study, hybridization between the two species was investigated using a sample size comprising 1218 individuals collected from 39 populations spanning the geographic distribution of the two species. The aim was to combine genetic data with genotype simulations and Bayesian analyses (Schwartz & Beheregaray, 2008) to: (1) identify hybrid individuals in populations of *M. novemaculeata* and *M. colonorum*, (2) thoroughly define the extent and prevalence of hybridization, (3) determine the existence of a hybrid zone, (4) test for the presence of bidirectional introgression and (5) provide management advice for the two species.

MATERIALS AND METHODS

SAMPLES

Ethanol-preserved fin clips were obtained from 1218 individuals from 32 catchments and seven dams throughout south-eastern Australia (Fig. 1 and Table I). Samples included 357 putative *M. colonorum* and 734 putative *M. novemaculeata*. Further, 18 hybrid individuals identified in Jerry *et al*. (1999) and an additional 109 individuals of mixed origin (45 hybrids, 19 pure *M. colonorum* and 45 pure *M. novemaculeata* identified with genetic testing) from Schwartz & Beheregaray (2008) were included. Included in this sampling design were purebred *M. novemaculeata* and *M. colonorum* collected from areas that are presumably hybrid free: Noosa River ($n = 29$ *M. novemaculeata*), Maroochy River ($n = 27$ *M. novemaculeata*), Tweed River ($n = 29$ *M. novemaculeata*), Hopkins River ($n = 35$ *M. colonorum*), Bass River ($n = 30$ *M. colonorum*) and Arthur River ($n = 9$ *M. colonorum*). The purebred *M. novemaculeata* ($n = 45$) and *M. colonorum* ($n = 19$) individuals identified from Schwartz & Beheregaray (2008) were used as controls to determine the precision of assignment tests.

GENETIC METHODS

Total genomic DNA was extracted from caudal fin clips using a standard proteinase K digestion and salt extraction method (Sambrook *et al*., 1989). Samples were genotyped at six *M. novemaculeata* microsatellite loci (AB001, AB006, AB009, AB097, AB107 and AB114) using polymerase chain reaction (PCR) conditions outlined in Schwartz *et al*. (2005). Previous studies (Schwartz *et al*., 2005; Schwartz & Beheregaray, 2008) have shown these loci to be unlinked and not to display technical problems associated with null alleles, scoring errors or large allele drop out in either *M. novemaculeata* or *M. colonorum*. Critically, these six loci provide sufficient, putatively neutral, allelic and genotypic information not only to conclusively identify individuals of hybrid origin between *M. novemaculeata* and *M. colonorum* but also to identify up to three generations of introgression (Schwartz & Beheregaray, 2008). To determine species-specific maternal lineages, a 396 bp fragment of the hypervariable section of the mitochondrial DNA (mtDNA) control region was also amplified and sequenced for a subset of the samples ($n = 819$). This was achieved using oligonucleotide primers A and E (Lee *et al*., 1995) and PCR conditions and protocols outlined in Beheregaray & Sunnucks (2001). Mitochondrial DNA control region sequences were aligned by eye with existing *M. novemaculeata* and *M. colonorum* mtDNA control-region data sets (Jerry & Baverstock, 1998; Shaddick *et al*., 2011).

DATA ANALYSIS

Statistical analyses of microsatellite data were conducted using a procedure that combined genotypic simulations and Bayesian analyses of molecular genetic data (Schwartz & Beheregaray, 2008). The assignment programme Structure version 2.2.2 (Pritchard *et al*., 2000) was used to infer the ancestry of individual fishes. In the context of the study, Structure's model-based clustering method can be used to determine the proportion of a hybrid individual's genome that arises from each of the parental species. In the initial Structure run, all 1218 genotyped individuals were classified as unknown and assigned to either *M. novemaculeata* or *M. colonorum* species groups ($k = 2$). The *M. novemaculeata* or *M. colonorum* identified in Schwartz & Beheregaray (2008) were used to evaluate the accuracy of the assignment of purebred individuals into their correct species groups. The posterior probabilities of the data were determined with 1 000 000 iterations after a burn-in period of 500 000. The programme was run five times using the admixture model and independent allele frequencies between *M. novemaculeata* and *M. colonorum*.

Structure infers a q-value ranging from 1 to 0 for each individual fish that denotes the mean posterior proportion of the genome of each individual fish with either *M. novemaculeata* or *M. colonorum* ancestry (Table II and Appendix). Schwartz & Beheregaray (2008) used a q-value threshold of $\leq$0·05 to identify parental populations of *M. novemaculeata* and *M. colonorum* ($q \geq 0{\cdot}95$ for *M. novemaculeata* and $q \leq 0{\cdot}05$ for *M. colonorum*). These q-value cut offs were considered conservative enough to ensure that hybrids would not be misclassified as purebred species. Further, the q-values for the purebred controls used in their study fell within these thresholds. While this level is considered conservative, individuals collected from putative hybrid-free zones and purebred individuals from this study fell within an even more stringent threshold q-value of $\leq$0·01, a result that may be due to the present much larger sample size. Consequently, a q-value cut off of $q \geq 0{\cdot}99$ was used to identify purebred *M. novemaculeata* and $q \leq 0{\cdot}01$ for purebred *M. colonorum*. Arlequin 3.01 (Excoffier *et al*., 2005) was used to estimate F_{ST} values, first with individuals collected from hybrid-free zones and then including individuals identified as purebred from threshold q-values. Observed and expected heterozygosities and allele frequencies were calculated in GenAlEx version 6 (Peakall & Smouse, 2006).

Schwartz & Beheregaray (2008) used HybridLaB (Nielsen *et al*., 2006) to simulate parental and hybrid genotypes in order to identify the range of potential q-values for the purebred individuals and different hybrid classes. HybridLaB uses nuclear datasets to simulate the genotypes of interspecific hybrid offspring from user-specified parental populations. In their study, Schwartz & Beheregaray (2008) used parental genotypes simulated from a source population comprising individuals that grouped with purebred *M. novemaculeata* (AB) or *M. colonorum* (EP). Simulated parental populations were then used to generate ranges of q-values that define F1 hybrids, F2 hybrids (F1 × F1), B2 backcrosses to each purebred parental (F1 × purebred EP or AB) and B3 backcrosses to each purebred parental species (*e.g*. B2-AB × AB). This study then used these simulated q-values, adjusted for $q \leq 0{\cdot}01$, to classify individuals of hybrid origin and assess the level of introgression. To determine the probability of an individual having a hybrid origin over the past two generations, Structure was run a second time dividing the genotyped individuals into one of two species groups ($k = 2$) as determined by q-values from the initial Structure run ($q > 0{\cdot}5$ grouped with *M. novemaculeata* and $q < 0{\cdot}5$ grouped with *M. colonorum*). The generation option was set at 2, while all other settings remained the same.

RESULTS

Analysis of microsatellite data revealed moderate levels of genetic variability with an average of 5·8 alleles per locus (Table III). Three loci were diagnostic for each species and five of the loci contained putatively species-specific alleles (Table III). A total of 140 of the 1218 individuals sampled (11·5%) were identified as having hybrid origins based on the presence of both species diagnostic alleles in a single

TABLE II. Summary statistics indicating number of individuals (n) that were identified as either purebred *Macquaria novemaculeata* (AB) or *Macquaria colonorum* (EP) or from different hybrid classes. The range of q-values for each of these classes along with the range of probabilities of being a second or third generation or later hybrid and the mtDNA haplotypes for each class are shown. Individual results are in Appendix

Species–hybrid class	n	q-value range	Probability of being a second generation hybrid (F1)	Probability of being a third generation or later hybrid (backcross)	mtDNA haplotypes
Pure AB	727	0·996–0·998	0	0·1%	625 sequenced – all AB
Third generation or later hybrid backcrossed to AB (B3+AB)	32	0·760–0·951	0	2·5–18·6%	19 sequenced – all AB
Second generation hybrid backcrossed to AB (B2-AB)	59	0·534–0·752	0·1–26·9%	73·3–100%	45 sequenced – 44 AB, 1 EP (H55)
B2-AB or F1 hybrid	4	0·482–0·576	41·3–57·6%	42·8–58·7%	2 sequenced – both AB
First generation hybrid (F1)	17	0·587–0·852	85·2–92·1%	7·6–14·8%	8 sequenced – all AB
Second generation hybrid backcrossed to EP (B2-EP)	14	0·337–0·494	7·0–24·2%	78–98·4%	9 sequenced – 8 AB, 1 EP (H126)
Second generation or later hybrid backcrossed to EP (B2+EP)	2	0·180–0·187	0	68·2–84·3%	1 sequenced – AB (H129)
Third generation or later hybrid backcrossed to (B3+EP)	12	0·064–0·191	0	2·2–32·9%	6 sequenced – all EP
Pure EP	351	0·002–0·005	0	0·1%	All EP

TABLE III. Microsatellite alleles, sample sizes (n) and observed (H_o) and expected (H_e) heterozygosities for *Macquaria novemaculeata*, *Macquaria colonorum* and hybrid individuals

		Macquaria novemaculeata			*Macquaria colonorum*			Hybrids	
Locus	Alleles	n	Allele frequencies	H_o/H_e	n	Allele frequencies	H_o/H_e	n	Allele frequencies
AB01		718		0·45/0·45	349		0·01/0·01	139	
	224		—			0·999			0·406
	226		—			0·001			—
	228		—			—			0·004
	230		0·001			—			—
	232		0·041			—			0·022
	234		0·718			—			0·338
	236		0·126			—			0·036
	238		0·115			—			0·176
	240		—			—			0·011
	242		—			—			0·007
AB06		720		0·60/0·57	350		0·01/0·01	133	
	174		0·003			—			
	178		0·001			—			
	180		–			0·999			0·459
	184		–			—			0·019
	186		0·003			0·001			0·023
	188		0·269			—			0·143
	190		0·586			—			0·278
	192		0·135			—			0·079
	194		0·001			—			—
	196		0·001			—			—
AB09		705		0·39/0·41	350		0·01/0·01	137	
	281		—			0·003			—
	283		—			0·997			0·252
	285		0·707			—			0·588
	295		0·293			—			0·161
AB97		725		0·30/0·31	350			140	
	104		0·809			1·000			0·823
	112		0·191			—			0·177
AB107		724			350			138	
	290		1·000			—			0·554
	300		—			1·000			0·442
	302								0·004
AB114		716		0·51/0·50	350		0·09/0·10	140	
	115		0·002			—			—
	117		0·378			—			0·168
	119		0·010			—			0·004
	131		0·602			0·049			0·418
	133		0·008			—			0·043
	141		—			0·951			0·368

individual's genotype and the results from Structure. Hybrids were detected in 17 of the 32 wild populations sampled and two of the seven impoundment populations; the percentage hybrid fishes per estuary ranged from 0 to 47% (Table I). Hybrids were mostly found in the Victorian rivers between Mallacoota Inlet and Albert River ($n = 123$, 87·9%); however, 16 individuals were found outside of this area (Table I and Fig. 1). The majority of hybrids detected were backcrossed individuals with only 17 potential first generation (F1) hybrids identified. Interestingly, nearly all of the F1 individuals were sampled from the Snowy River ($n = 13$). Ninety-five of the 140 hybrid individuals (67·8%) had genotypes with closer affinities to *M. novemaculeata* ($0{\cdot}99 > q > 0{\cdot}50$) while 28 (20%) were more similar to *M. colonorum* ($0{\cdot}01 < q < 0{\cdot}50$).

Initial analyses based on the presence of diagnostic alleles and Mendelian inheritance confirmed hybrid fertility through the existence of genomic introgression through backcrossing to either *M. novemaculeata* or *M. colonorum*. This is evident from the presence of individuals homozygous for diagnostic *M. novemaculeata* and *M. colonorum* alleles at one locus, but heterozygous for diagnostic alleles at another. While hybrid individuals predominantly had *M. novemaculeata* mtDNA, a number exhibited *M. colonorum* mtDNA haplotypes (Table II and Appendix). This provides further evidence that hybrids can backcross to both *M. novemaculeata* and *M. colonorum* parental species. It is also possible, however, that this observation simply indicates the maternal species of the initial hybridization event, rather than the one involved with the backcross.

In the preliminary structure analysis, the 85 *M. novemaculeata* and 74 *M. colonorum* from the hybrid-free zones had q-values ranging from 0·992 to 0·996 and 0·010 to 0·004, respectively. A further 642 *M. novemaculeata* and 276 *M. colonorum* individuals were identified as purebred based on the q-value threshold of $q \leq 0{\cdot}01$ [these numbers include the control purebred parentals identified in Schwartz & Beheregaray (2008)]. High F_{ST} values were obtained between *M. novemaculeata* and *M. colonorum* from the hybrid-free zones ($F_{ST} = 0{\cdot}712$, $P < 0{\cdot}001$). When all individuals designated as purebred species from the q-value cut-off criterion were included, F_{ST} between species was also high 0·726 ($P < 0{\cdot}001$).

Using a q-value cutoff of $q \leq 0{\cdot}01$ and simulated data, it was possible to use the Structure analyses to identify varying levels of introgression within the 140 hybrid individuals (Table I). Introgressed individuals were classified into a number of groups based on simulated (Schwartz & Beheregaray, 2008) and actual q-values. These included 32 individuals with q-values of 0·760–0·951 and a low probability of having an *M. novemaculeata* grandparent (third generation or later backcrossed to AB), 63 individuals with q-values of 0·534–0·893 (second generation hybrid backcrossed to *M. novemaculeata*), 14 individuals with q-values of 0·337–0·494 (second generation hybrid backcrossed to EP) and 14 individuals with q-values of 0·064–0·191 (third generation or later hybrid backcrossed to perch) (Table II and Appendix).

DISCUSSION

This study used a large sample size, both in number of individuals and number of localities, together with an effective analytical framework to demonstrate

the existence of biparental backcrossing in *M. novemaculeata* and *M. colonorum*. Additionally, a geographical zone, coinciding with an area of increased contact between *M. colonorum* and *M. novemaculeata*, where hybridization is amplified was described. These findings have implications for conservation management and stocking practices and contribute to the general understanding of hybridization between species that rely on the coastal marine environment for dispersal.

Hybrids were concentrated within the Snowy River and Gippslands region in the eastern Bass Strait, Victoria. The high number of statistically robust hybrids in this area may be a consequence of both historical and contemporary processes. Natural hybridization often results from secondary contact between formerly isolated populations (Barton & Hewitt, 1989). It is conceivable that historic sea level change, that led to the emergence of a land bridge in the Bass Strait, the Bassian Isthmus, between Tasmania and Victoria, isolated a single ancestral coastal species into two populations, from which *M. novemaculeata* and *M. colonorum* evolved. The presence of a phylogeographic break in this region is evident in several other coastal marine organisms (Waters *et al*., 2005; Waters, 2008; Ayre *et al*., 2009). A subsequent rise in sea levels that inundated the isthmus could have allowed the two daughter species, *M. novemaculeata* and *M. colonorum*, to extend their ranges, causing a zone of overlap around Wilson's Promontory, where a high number of hybrids have been detected. This historical scenario is consistent with observations that hybridization often takes place due to heightened responses to selection when populations move into new environments (Arnold, 1997; Seehausen, 2004). For example, an historical divergence followed by a recent colonization event has been proposed for the existence of a hybrid zone between the marine yellowfin bream *Acanthopagrus australis* (Günther 1859) and the estuarine black bream *Acanthopagrus butcheri* (Munro 1949) in south-eastern Australia (Roberts *et al*., 2010).

Alternatively, contemporary processes may have contributed to the increased number of hybrids detected in Victorian rivers. Lower population densities of *M. novemaculeata* at the southern extremity of their geographical range coupled with short coastal rivers may lead to restriction and convergence of the viable spawning sites and aggregations (McCarraher, 1986*a*, *b*). Competition for overcrowded and limited spawning sites increases the chances of non-conspecific fertilization and the production of hybrid offspring (Avise & Saunders, 1984; Jerry *et al*., 1999). In addition, discrepancies in species maturation time may contribute to the distribution and occurrence of hybridization in Victorian rivers where male *M. colonorum* and female *M. novemaculeata* reach sexual maturity earlier than their conspecific counterparts (McCarraher, 1986*a*). Subsequently, there are increased opportunities to breed with non-conspecifics resulting in hybrid offspring (Jerry *et al*., 1999).

Hybridization in Victorian populations may also reflect anthropogenic factors such as stocking practices, habitat degradation and habitat modification. The widespread construction of dams on Australian coastal rivers has caused dramatic changes in downstream flow and sediment regimes, modifying estuarine hydrodynamics and affecting riparian ecology (Poff *et al*., 1997; Erskine *et al*., 1999). Alterations to natural riverine flow regimes can cause a reduction in the duration and volume of flow, changes in the seasonal pattern and variability of flows and a decrease in the scale and frequency of flooding events (Poff *et al*., 1997; Rose & Bevitt, 2003). Further, physical changes to sediment regimes can affect the quality and accessibility of habitat for riverine fauna (Erskine, 1985; Benn & Erskine, 1994; Rose & Bevitt,

2003). Spawning as well as recruitment and cohort abundance in *M. novemaculeata* and *M. colonorum* are intricately related to the influx of flood waters and availability of spawning habitat (Harris, 1984, 1988). Subsequently, any changes to the downstream hydrologic regime will affect spawning habitat and interfere with the spawning cues of both the species (McCarraher & McKenzie, 1986; Kirwin, 2000; Growns & James, 2005). This may explain the presence of large numbers of hybrids in the Snowy River, where the Snowy Mountains hydroelectric scheme has dramatically altered the natural flow regime of the river (Erskine *et al*., 1999; Rose & Bevitt, 2003).

The construction of the Snowy Mountains hydroelectric scheme in the period between 1949 and 1974 resulted in the capture of 99% of the natural flow from the Snowy River diverting the water into hydroelectric power stations (Erskine *et al*., 1999; Rose & Bevitt, 2003). Flow volume has not exceeded >4% of natural levels since completion of the scheme. This reduction in downstream flow volume has severely affected the river's ecology (Rose and Bevitt, 2003; Gilligan & Williams, 2008). In this study, over 41·3% of *M. colonorum* and *M. novemaculeata* sampled in the Snowy River were hybrids. Further, the majority of F1 individuals ($n = 13$) found were within the Snowy River suggesting that it is a hot spot of hybridization. It is possible that the decrease in flow volume has interfered with spawning and migration cues and reduced the availability of spawning habitats bringing the two species into increased contact and promoting hybridization.

The existence of hybrids in both the eastern and western Victorian populations may also reflect the inadvertent use of hybrid fishes as broodstock in *M. novemaculeata* captive breeding and release programmes. The accidental liberation of hybrid fishes through stocking programmes is an issue of substantial conservation concern, particularly in populations like the Snowy River where a large number of hybrids were detected. While *M. novemaculeata* have been stocked into the Snowy River, the natural population of *M. novemaculeata* have not bred in this river since 1988 (Douglas, 2011). As such, *M. novemaculeata* in the Snowy River are endangered and the population is at risk of becoming a hybrid swarm. Nonetheless, although the release of *M. novemaculeata* fingerlings with *M. colonorum* alleles is problematic, it is unlikely that the presence of large numbers of hybrids observed in the Snowy River is a reflection of stocking practices. Large-scale stocking of the Snowy River has only occurred fairly recently (2007) and these recent stocking activities have only utilized broodfish tested as non-hybrids. Indeed, prior to 2007, only 200 *M. novemaculeata* fingerlings were stocked into the river (Ainsworth, 2000). Although it is possible that the broodstock used may have contained *M. colonorum* genes acquired through introgression, the small number of fish stocked makes it unlikely that stocking was a major factor facilitating the increased amount of hybridization in the Snowy River.

Whether due to historical or contemporary processes or a combination of both, the majority of hybrid individuals fall within an area of increased sympatry between *M. novemaculeata* and *M. colonorum*, which is proposed as the hybrid zone. This zone extends from the Albert River to Mallacoota Inlet in eastern Victoria. Given that there is a lack of F1 individuals outside Victorian populations and that only two individuals sampled outside the hybrid zone were hybrids with statistical certainty [H50: Lostock ($P < 0{\cdot}001$) and H24: Williams ($P < 0{\cdot}01$)], it is unlikely that hybridization occurs

commonly across the remainder of the species' distribution. Indeed, within NSW, the two species are thought to live in spatial and temporal isolation, thus inhibiting prolific hybridization in the rivers where they coexist. Both *M. novemaculeata* and *M. colonorum,* however, are capable of migrating considerable distances through coastal marine waters (Chenoweth & Hughes, 1997; Jerry & Baverstock, 1998; Jerry *et al*., 1999; Shaddick *et al*., 2011) facilitating the movement of hybrid individuals along the coastline. This may explain the presence of three putative hybrids outside the range of *M. novemaculeata* in the Tarwin, Glenelg and Barham Rivers (Fig. 1).

The supplementation of wild stocks with hatchery fish may also account for the existence of hybrid individuals outside the hybrid zone. *Macquaria novemaculeata* have been stocked into several impoundments and waterways since the 1980s (NSW Fisheries, 2003; Southern Rivers Catchment Management Authority, 2007). Introgressed individuals were detected in two stocked locations: Lostock Dam in the Hunter Valley ($n = 3$) and St Philips, a wetland near Port Stephens (Karuah catchment) ($n = 1$). Although only one of these fishes (H50) was deemed a hybrid with any statistical certainty ($P < 0{\cdot}001$), the detection of any hybrids in stocked dams provides unequivocal proof of the accidental use of *M. colonorum* or hybrid individuals as broodfish in stocking programmes. Hybrids were detected in a number of wild populations in NSW (Fig. 1 and Table I); however, of the catchments where hybrids were detected, only the Richmond and the Hunter catchments had been directly stocked at the time the tissue samples were collected. Nonetheless, given the possibility of hybrid migration between estuaries and gene leakage from dam escapees, fish stocking may have contributed to the presence of hybrid fishes in the NSW rivers.

Genomic introgression *via* hybridization can be a beneficial evolutionary process; however, it is more commonly viewed as detrimental, particularly when occurring as a direct result of human activities (Epifanio & Nielsen, 2001). The decreased reproductive fitness or complete sterility of hybrid offspring may result in a reduced total reproductive output of parental species (Dowling & Moore, 1985; Leary *et al*., 1985, 1993). Further, genomic introgression of hybrids into the parental gene pool may lead to a loss of unique genetic diversity and a breakdown of local adaptations resulting in a decline of parental populations (Dowling & Moore, 1985; Leary *et al*., 1985; Epifanio & Nielsen, 2001; Schwartz & Beheregaray, 2008). Hybrid offspring may also exhibit increased fitness or hybrid superiority resulting in the formation of hybrid swarms that can out-compete parental species and can lead to the loss of unique populations or entire species (Allendorf & Leary, 1988; Arnold, 1997; Burke & Arnold, 2001; Epifanio & Philipp, 2001; Seehausen, 2004). Thus, it is essential to establish the genetic identification of *M. novemaculeata* broodstock used in stocking programmes in order to prevent the release of large numbers of hybrid individuals.

The majority of hybrids were genotypically similar to *M. novemaculeata* and also displayed *M. novemaculeata* mtDNA indicating that the hybridization and backcrossing events predominantly proceed through *M. novemaculeata* females. Identification of backcrossed individuals denotes that hybrids are viable and fertile and that *M. colonorum* alleles are being spread *via* extensive backcrossing with pure *M. novemaculeata*. All of the F1 individuals displayed *M. novemaculeata* mtDNA suggesting F1 hybrids may be biased towards having *M. novemaculeata* mothers. One *M. novemaculeata*-like hybrid (H55), however, had an *M. colonorum* haplotype and a

small number of *M. colonorum*-like hybrids displayed *M. colonorum* mtDNA haplotypes suggesting that hybridization and introgression can also occur with *M. colonorum* females. This is the first time the possibility of bidirectional backcrossing has been observed in *M. novemaculeata* and *M. colonorum.* The predominance of *M. novemaculeata* backcrosses possibly reflects the rarity of *M. novemaculeata* in the hybrid zone which falls at the extremities of the species' range (McCarraher, 1986*a*, *b*, *c*). In the Victorian waters, south of the main distribution of *M. novemaculeata*, *M. colonorum* is by far the more common species outnumbering *M. novemaculeata* 37:1 in the Snowy River and 211:1 in the Tambo River (McCarraher, 1986*a*, *b*, *c*). This inference that the rarer species was the maternal parent in most of the hybrids is consistent with similar observations in sunfishes (*Lepomis* species) crosses (Avise & Saunders, 1984).

While low levels of hybridization may exist naturally, human-induced change to the habitat and hydrology of estuarine spawning sites within the hybrid zone and elsewhere (and the Snowy River in particular) may have forced the sympatric *M. novemaculeata* and *M. colonorum* to compete for spawning sites, thus increasing hybridization rates. As both species disperse to other estuaries, individuals of hybrid origin may spread to adjacent rivers where the parent species exist in isolation affecting the genetic integrity of those populations. The unintentional captive propagation and even more widespread dispersal of hatchery-produced hybrid individuals throughout the range of *M. novemaculeata* pose an even greater biodiversity risk. To help mitigate the increased threat associated with human-mediated hybridization and to preserve the genetic integrity of both *M. novemaculeata* and *M. colonorum*, it is essential to establish the genetic identity of all broodstock used in stocking programmes and to avoid habitat modifications that may decrease the spawning area of both the species. This is particularly applicable to populations falling within the proposed hybrid zone.

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APPENDIX. Hybrid *Macquaria novemaculeata*:*Macquaria novemaculeata* individuals ranked by *q*-values derived from the first Structure run. The probabilities of individuals being a second (F1; second) or third (backcross; third) generation hybrid (based on the second run of Structure) are presented

Sample	River	*q*	Second	Third	*P*	Hyb class	mtDNA
PB1	Logan	0·996	0·000	0·001		PAB	AB
PB2	Logan	0·996	0·000	0·001		PAB	AB
PB3	Logan	0·996	0·000	0·001		PAB	AB
PB4	Logan	0·996	0·000	0·001		PAB	AB
H1	Shoalhaven	0·951	0·000	0·025		B3+AB	AB
H2	Snowy	0·951	0·000	0·025		B3+AB	AB
H3	Shoalhaven	0·944	0·000	0·038		B3+AB	AB
H4	Tuross	0·944	0·000	0·039		B3+AB	AB
H5	St Philips	0·944	0·000	0·039		B3+AB	AB
H6	Lostock	0·944	0·000	0·039		B3+AB	AB
H7	Richmond	0·944	0·000	0·040		B3+AB	AB
H8	Williams	0·943	0·000	0·041		B3+AB	AB
H9	Lostock	0·943	0·000	0·041		B3+AB	AB
H10	Karuah	0·935	0·000	0·051		B3+AB	AB
H11	Williams	0·934	0·000	0·055		B3+AB	AB
H12	Hunter	0·934	0·000	0·054		B3+AB	AB
H13	Gippslands	0·934	0·000	0·054		B3+AB	AB
H14	Clarence	0·933	0·000	0·055		B3+AB	AB
H15	Williams	0·933	0·000	0·055		B3+AB	AB
H16	Snowy	0·908	0·000	0·104		B3+AB	ns
H17	Albert	0·905	0·000	0·069		B3+AB	AB
H18	Snowy	0·905	0·000	0·067		B3+AB	AB
H19	Albert	0·905	0·000	0·070		B3+AB	AB
H20	Albert	0·905	0·000	0·067		B3+AB	AB
H21	Snowy	0·893	0·000	0·106		B3+AB	ns
H22	Brodribb	0·893	0·000	0·093		B3+AB	ns
H23	Snowy	0·893	0·000	0·751	**	B2-AB	ns
H24	Williams	0·892	0·000	0·756	**	B2-AB	AB
H25	Snowy	0·892	0·000	0·074		B3+AB	ns
H26	Snowy	0·881	0·000	0·161		B3+AB	ns
H27	Snowy	0·814	0·000	0·994	***	B2-AB	ns
H28	Gippslands	0·812	0·000	0·764	**	B2-AB	AB
H29	Snowy	0·811	0·000	0·124		B3+AB	ns
H30	Snowy	0·795	0·000	0·997	***	B2-AB	ns
H31	Snowy	0·791	0·000	0·984	***	B2-AB	AB
H32	Snowy	0·788	0·000	0·816	**	B2-AB	AB
H33	Bemm	0·788	0·000	0·135		B3+AB	ns
H34	Snowy	0·788	0·000	0·134		B3+AB	ns
H35	Snowy	0·785	0·000	0·139		B3+AB	ns
H36	Snowy	0·785	0·000	0·139		B3+AB	ns
H37	Snowy	0·785	0·000	0·171		B3+AB	ns
H38	Albert	0·767	0·004	0·989	***	B2-AB	AB
H39	Bemm	0·766	0·000	0·186		B3+AB	ns
H40	Snowy	0·764	0·000	0·894	**	B2-AB	ns
H41	Snowy	0·760	0·000	0·994	***	B2-AB	ns
H42	Snowy	0·760	0·000	0·139		B3+AB	ns

APPENDIX. Continued

Sample	River	*q*	Second	Third	*P*	Hyb class	mtDNA
H43	Gippslands	0·752	0·000	1·000	***	B2-AB	AB
H44	Snowy	0·732	0·000	1·000	***	B2-AB	ns
H45	Snowy	0·722	0·000	1·000	***	B2-AB	AB
H46	Albert	0·718	0·000	1·000	***	B2-AB	AB
H47	Albert	0·718	0·000	1·000	***	B2-AB	AB
H48	Gippslands	0·691	0·000	1·000	***	B2-AB	AB
H49	Albert	0·690	0·001	0·999	***	B2-AB	AB
H50	Lostock	0·688	0·001	0·999	***	B2-AB	AB
H51	Snowy	0·685	0·000	0·917	***	B2-AB	ns
H52	Albert	0·682	0·029	0·971	***	B2-AB	AB
H53	Albert	0·654	0·116	0·884	***	B2-AB	AB
H54	Snowy	0·653	0·010	0·990	***	B2-AB	ns
H55	Bemm	0·646	0·015	0·984	***	B2-AB	EP
H56	Bemm	0·632	0·122	0·878	***	B2-AB	AB
H57	Gippslands	0·631	0·202	0·798	***	B2-AB	AB
H58	Gippslands	0·629	0·091	0·909	***	B2-AB	AB
H59	Albert	0·629	0·202	0·798	***	B2-AB	AB
H60	Albert	0·628	0·202	0·798	***	B2-AB	AB
H61	Gippslands	0·627	0·091	0·909	***	B2-AB	AB
H62	Albert	0·627	0·153	0·847	***	B2-AB	AB
H63	Gippslands	0·626	0·202	0·798	***	B2-AB	AB
H64	Albert	0·626	0·202	0·798	***	B2-AB	AB
H65	Albert	0·626	0·202	0·798	***	B2-AB	AB
H66	Gippslands	0·626	0·202	0·798	***	B2-AB	AB
H67	Albert	0·626	0·202	0·798	***	B2-AB	AB
H68	Albert	0·626	0·202	0·798	***	B2-AB	AB
H69	Snowy	0·622	0·482	0·518	***	F1/B2-AB	AB
H70	Snowy	0·621	0·482	0·518	***	F1/B2-AB	ns
H71	Snowy	0·605	0·264	0·736	***	B2-AB	ns
H72	Snowy	0·590	0·206	0·794	***	B2-AB	ns
H73	Gippslands	0·589	0·267	0·733	***	B2-AB	AB
H74	Albert	0·589	0·267	0·733	***	B2-AB	AB
H75	Albert	0·589	0·267	0·733	***	B2-AB	AB
H76	Albert	0·589	0·206	0·794	***	B2-AB	AB
H77	Albert	0·589	0·267	0·733	***	B2-AB	AB
H78	Mallacoota	0·589	0·124	0·876	***	B2-AB	AB
H79	Albert	0·589	0·267	0·733	***	B2-AB	AB
H80	Albert	0·589	0·267	0·733	***	B2-AB	AB
H81	Snowy	0·589	0·267	0·733	***	B2-AB	ns
H82	Snowy	0·587	0·857	0·143	***	F1	ns
H83	Gippslands	0·586	0·202	0·798	***	B2-AB	AB
H84	Gippslands	0·585	0·267	0·733	***	B2-AB	AB
H85	Gippslands	0·585	0·267	0·733	***	B2-AB	AB
H86	Gippslands	0·583	0·267	0·733	***	B2-AB	AB
H87	Gippslands	0·583	0·124	0·876	***	B2-AB	AB
H88	Gippslands	0·583	0·267	0·733	***	B2-AB	AB
H89	Albert	0·583	0·267	0·733	***	B2-AB	AB
H90	Albert	0·583	0·267	0·733	***	B2-AB	AB

APPENDIX. Continued

Sample	River	*q*	Second	Third	*P*	Hyb class	mtDNA
H91	Snowy	0·583	0·413	0·587	***	F1/B2-AB	AB
H92	Albert	0·582	0·267	0·733	***	B2-AB	AB
H93	Albert	0·582	0·267	0·733	***	B2-AB	AB
H94	Snowy	0·576	0·572	0·428	***	F1/B2-AB	ns
H95	Snowy	0·544	0·896	0·104	***	F1	ns
H96	Snowy	0·540	0·921	0·079	***	F1	ns
H97	Snowy	0·535	0·003	0·997	***	B2-AB	ns
H98	Snowy	0·534	0·269	0·731	***	B2-AB	ns
H99	Snowy	0·534	0·921	0·079	***	F1	AB
H100	Snowy	0·533	0·921	0·079	***	F1	ns
H101	Brodribb	0·533	0·921	0·079	***	F1	AB
H102	Snowy	0·494	0·242	0·758	***	B2-EP	ns
H103	Bemm	0·488	0·928	0·072	***	F1	ns
H104	Snowy	0·487	0·872	0·128	***	F1	ns
H105	Snowy	0·487	0·871	0·129	***	F1	AB
H106	Snowy	0·486	0·220	0·780	***	B2-EP	ns
H107	Bemm	0·485	0·924	0·076	***	F1	ns
H108	Mallacoota	0·482	0·104	0·895	***	B2-EP	AB
H109	Gippslands	0·482	0·104	0·895	***	B2-EP	AB
H110	Gippslands	0·482	0·104	0·895	***	B2-EP	ns
H111	Bemm	0·480	0·920	0·080	***	F1	AB
H112	Brodribb	0·480	0·920	0·080	***	F1	AB
H113	Snowy	0·480	0·920	0·080	***	F1	AB
H114	Snowy	0·480	0·920	0·080	***	F1	AB
H115	Snowy	0·479	0·919	0·081	***	F1	AB
H116	Snowy	0·479	0·919	0·081	***	F1	ns
H117	Snowy	0·475	0·852	0·148	***	F1	ns
H118	Snowy	0·450	0·162	0·838	***	B2-EP	ns
H119	Gippslands	0·434	0·070	0·917	***	B2-EP	AB
H120	Gippslands	0·424	0·036	0·949	***	B2-EP	AB
H121	Snowy	0·420	0·110	0·890	***	B2-EP	ns
H122	Gippslands	0·390	0·003	0·984	***	B2-EP	AB
H123	Mallacoota	0·377	0·085	0·915	***	B2-EP	AB
H124	Snowy	0·377	0·086	0·914	***	B2-EP	AB
H125	Bemm	0·376	0·085	0·915	***	B2-EP	AB
H126	Gippslands	0·337	0·000	0·919	***	B2-EP	EP
H127	Tarwin	0·191	0·000	0·146		B3+EP	EP
H128	Gippslands	0·187	0·000	0·843	**	B2+EP	ns
H129	Mallacoota	0·180	0·000	0·682	*	B2+EP	AB
H130	Snowy	0·177	0·000	0·042		B3+EP	EP
H131	Glenelg	0·177	0·000	0·042		B3+EP	ns
H132	Hawkesbury	0·096	0·000	0·329		B3+EP	EP
H133	Albert	0·096	0·000	0·329		B3+EP	ns
H134	Barham	0·092	0·000	0·129		B3+EP	EP
H135	Mallacoota	0·083	0·000	0·262		B3+EP	EP
H136	Bemm	0·066	0·000	0·022		B3+EP	ns
H137	Richmond	0·065	0·000	0·022		B3+EP	EP
H138	Bemm	0·064	0·000	0·022		B3+EP	ns

APPENDIX. Continued

Sample	River	*q*	Second	Third	*P*	Hyb class	mtDNA
H139	Bemm	0·064	0·000	0·022		B3+EP	ns
H140	Bemm	0·064	0·000	0·022		B3+EP	ns
PP1	Hopkins	0·004	0·000	0·001		PEP	EP
PP2	Hopkins	0·004	0·000	0·001		PEP	EP
PP3	Hopkins	0·004	0·000	0·001		PEP	EP
PP4	Hopkins	0·004	0·000	0·001		PEP	EP

Hyb class, the hybrid class designation; mtDNA, the mitochondrial haplotype of the fish; PAB, pure *Macquaria novemaculeata*; AB, *Macquaria novemaculeata* species designation; PEP, pure *M. colonorum*; EP, *M. colonorum* species designation; B2, second generation hybrid through a backcross to one of the species (AB or EP); B3+, third generation or later hybrid back crossed to either *M. novemaculeata* or *M. colonorum* (AB or EP), F1; first generation hybrid; ns, not sequenced.
$*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.