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Assessment of the frequency and consequences of hybridisation between an obligately estuarine and a migratory marine fish

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Assessment of the Frequency and Consequences of Hybridisation between an Obligately Estuarine and a Migratory Marine Fish

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Certification

I, David G. Roberts, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

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Abstract

Populations of obligately estuarine species (i.e., species that complete their entire life-cycle in estuaries) are potentially small and isolated and may lack genetic variation and display regional differentiation as a result of genetic drift and inbreeding. Low levels of hybridisation with a migratory marine congener should introduce genetic variation and reduce the effects of inbreeding depression and drift. However, high levels of hybridisation can have negative impacts including breakdown of localised adaptation, and demographic and genetic swamping and loss of species identity. Although hybridisation between marine and estuarine species has rarely been considered, estuaries may be hot spots of hybridisation and introgression may be exacerbated by anthropogenic disturbances, including fishing and mechanical modification of the frequency of estuary flushing. In south east Australia, the estuarine Black bream *Acanthopagrus butcheri*, and its migratory marine congener Yellowfin bream, *A. australis*, have overlapping distributions and spawning times and therefore potential to hybridise within estuaries. Here I use a combination of molecular population genetics and breeding experiments to determine population structure of *A. butcheri* and *A. australis*, and the frequency of occurrence and consequences of hybridisation for the two species but particularly the estuarine *A. butcheri*.

I used a broad scale survey of microsatellite and mitochondrial DNA (mtDNA) variation in 565 adult fish from 25 estuaries spanning the distributional range of the two species, to initially characterise the species and their putative hybrids, then to test the prediction that hybrids are restricted to the area of sympatry. Hybrids were widespread (68% of estuaries studied) and hybrid frequencies varied greatly among estuaries (0 to 58%). Most hybrids (88%) were classed as advanced generation backcrosses with *A.*

butcheri and displayed *A. butcheri* mtDNA haplotypes. I found most hybrids (57%) in the three estuaries within the area of sympatry, though hybrids were detected throughout the ranges of the two species.

Acanthopagrus australis is considered highly dispersive and this may have major implications for *A. butcheri*, although *A. australis* is also known to spawn in close association with estuaries which suggests populations could be genetically subdivided. I investigated the genetic structure of *A. australis* using both nuclear (microsatellite data) and mtDNA. My data from surveys of allele frequencies at six microsatellite loci for 350 fish, revealed high levels of genetic diversity within all sites but no genetic differentiation of groups collected from sites separated by up to 50 km ($F_{ST} = 0.002$, $P > 0.05$) and no differentiation of adults and juveniles spread across the distributional range of the species (several 100's of kms, $F_{ST} = 0$). I obtained similar results from analysis of sequence of the mtDNA control region for a subset of 47 fish (pairwsie $F_{ST} = 0.000 – 0.036$, $P > 0.05$). *Acanthopagrus australis* form a single panmictic population on the east coast with genetic homogeneity reflecting the predicted active northwards dispersal of adults to spawn and the southwards dispersal of larvae affected by the Eastern Australian Current.

The surprisingly high number of hybrid fish in estuaries in the area of sympatry could reflect ancient hybridisation events with little continuing introgression of the *A. australis* genome. Alternatively, *Acanthopagrus* hybrid zones may be dynamic and reflect contemporary processes. I used mtDNA sequence variation in a range wide phylogeographic study of *A. butcheri*, to test the prediction that populations within different estuaries and geographical regions are genetically differentiated and that eastern and

western *A. butcheri* represent divergent genetic lineages. Unlike many species historically fragmented by the Bassian Isthmus land bridge that form deeply divergent eastern and western lineages or incipient species, *A. butcheri* form closely related eastern and western lineages that diverged no later and perhaps considerably earlier (owing to uncertainty surrounding the rate at which mitochondrial control region DNA mutate) than the middle Pleistocene, leaving open the possibility that *Acanthopagrus* hybrid zones are maintained by contemporary processes.

As for many pairs of obligately estuarine and migratory marine fish, the degree or extent of reproductive (gamete) compatibility is unknown for *A. butcheri* and *A. australis*, though this will influence the likelihood of initial and later hybridisation. I compared the fertilisation success achieved when ova of estuary caught *A. butcheri* females were crossed with the semen of either ocean-caught *A. australis* males or estuary-caught *A. butcheri* males, to test for early acting barriers to initial and later hybridisation. The experimental crosses, which by chance included the two pure species and hybrid fish, revealed no evidence that gametic incompatibility provides a barrier to fertilisation among both pure species and their hybrids. Sperm of both *A. australis* and *A. butcheri*, and hybrid males, were apparently equally compatible with ova of *A. butcheri* and hybrid females (~ 40% viable larvae), for the single sperm concentration that was employed in the experiment.

The coastal lakes and lagoons of southern Australian are dynamic in space and time and this will influence opportunity for hybridisation. I surveyed genetic variation at microsatellite loci and the mtDNA control region of juvenile fish (collected after a single recruitment event after the known spawning time of *A. butcheri*) from five coastal lagoons

that vary in terms of their morphology and frequency of opening to the ocean (including temporal replication in two lagoons) (total n = 970) to determine the frequency and persistence of hybridisation in the area of sympatry and to test the prediction that hybrid fish occur in greater numbers in lagoons generally closed to the ocean, where the two species may be trapped together by lagoon entrance channel closure. Of 688 juvenile fish genotyped 95% were either *A. australis* (347) or hybrids (309); only 5% (32) were *A. butcheri*. Most hybrids were later generation hybrids or *A. butcheri* backcrosses that are likely multi-generational residents within lagoons. Far greater proportions of hybrid juveniles were found within two lagoons that are typically closed to the ocean (> 90% hybrid fish within closed lagoons vs. 12 – 27% in permanently or intermittently open lagoons). In both lagoons, this was consistent across multiple cohorts of fish (79 – 97% hybrid fish [n = 282]).

Although hybrid adults and juveniles are abundant, and pure *A. butcheri* extremely rare, within coastal lagoons at the approximate southern range limit of *A. australis*, my initial broad scale survey of estuarine populations uncovered hybrids within a small sample of fish from the Gippsland Lakes, a large estuary some 250 km further south of my intensive sampling of juveniles. However, the dynamic nature of both lakes and lagoons and ocean currents imply that hybridisation between migratory marine and estuarine species may vary greatly in space and time. I took advantage of the opportunity provided by the relative great stability of DNA in preserved fish within museums to describe the genotypic composition of the Gippsland Lakes population of *Acanthopagrus* spp. and test the stability of the hybrid zone over a 60 year period. I used a microsatellite DNA survey of contemporary (n=114) and historical (n=133) samples. The genetic composition of the

samples varied little over time and comprised complex hybrid swarms within which most fish were more similar to *A. butcheri*. Genetic diversity as measured by mean number of alleles/locus and H_e ranged from 8.2 to 9.2 and 0.66 to 0.70 respectively, and I detected little temporal allelic differentiation ($F_{ST} = 0.003$).

My study highlights the underemphasized importance of estuaries as sites of hybridisation and my data imply that *Acanthopagrus* hybrid zones are more widespread and persistent than previously understood. The great mobility of *A. australis* coupled with likely range expansion predicted under global warming and the complexity of introgression within lakes and lagoons on the south east coast of Australia implies that hybridisation may pose a threat to the genetic integrity of remaining *A. butcheri* populations and has yet to be investigated for large numbers of obligately estuarine species.

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Chapter 1 General introduction

1.1 Natural hybridisation

The role of hybridisation (*mating between individuals from sets of differentiated populations, regardless of taxonomic status*) in evolutionary diversification has been debated for decades stemming from uncertainty surrounding the consequences of interspecific gene flow. Whereas some authors (typically botanists) have focused on hybridisation's potential to generate novel heritable variation, functional novelty and new species, others (typically zoologists) have argued against any importance because reduced fitness or infertility of hybrids presumably compromises evolutionary potential (Harrison 1993; Arnold 1997 for review).

Many genetic studies of terrestrial plants and animals now confirm that introgression (*hybridisation resulting in the permanent incorporation of genes from sets of differentiated populations, into others*) (Anderson 1949) can generate opportunities for adaptation and evolution of new species (Rieseberg *et al*. 1995; Rieseberg *et al*. 2003; Gompert *et al*. 2006; Mavárez & Linares 2008). Indeed, genetic novelty provides the raw material for selection to act (Lewontin & Birch 1966; Grant & Grant, 1992, 1994, 1996), and many studies have demonstrated that hybrids can be as fit as, or fitter, than one or both parental species under the same or different environmental conditions (Arnold & Hodges 1995; Burke & Arnold 2001). Moreover, hybrids often express intermediate and/or unique and extreme traits (Schwarzbach *et al*. 2001; Stelkens & Seehausen 2009), which may not be particularly advantageous or useful in the parental niche, but can provide significant adaptive potential under a different set/s of environmental conditions. A hybrid that expresses a combination of parental traits might be selected for in an area that has aspects

of both parental environments. Moreover, unique or extreme traits can enable hybrids to occupy niche space that neither parent could utilise (Lexer *et al*. 2003; Rieseberg *et al*. 2003; Gross et al. 2004; Lexer *et al*. 2005).

Support for a creative role of hybridisation in aquatic environments comes from the adaptive radiation of African cichlids (Bell & Travis 2004; Seehausen 2004), including *Neolamprologus* (Salzburger *et al*. 2002) and *Metriaclima* (Smith *et al.* 2003) species complexes, Cyprinids such as *Gila* (Demarais *et al*. 1992; Dowling & Demarais, 1993), and whitefish (*Coregonas* spp.) (Rogers & Bernatchez 2007). Moreover, a number of species that hybridise are now recognised in the ocean (Van Oppen *et al*. 2000; Vollmer & Palumbi 2002; Wallace *et al*. 2004) and reticulate evolution is suspected to be important in maintaining and generating species diversity in reef building corals (Willis *et al.* 2006 for review), urchins (Addison & Hart 2005), and coral reef fish (McMillan *et al*. 1999; McCartney *et al*. 2003; Ramon *et al*., 2003; Marie *et al*. 2007; Hobbs *et al*. 2009).

While it is now clear that hybridisation can enhance the potential for persistence and adaptive evolution of species, the consequences of hybridisation can impact negatively on hybridising species and indeed discussions of hybridisation in particularly the ocean have rarely considered the possibility that hybridisation with or without introgression can result in undesirable outcomes for biodiversity conservation. These consequences include reduction to the size of populations, outbreeding depression (*a consequence of the process of segregation and recombination of distinct gene pools i.e., interbreeding between distantly related or genetically distinct individuals. Outbreeding depression impacts detrimentally on individual fitness, and may be due to disruption to favourable co-adapted*

gene complexes or loss of adaptation), contamination of gene pools (*the loss of formerly distinct gene pools through introgression*), or the merging of taxa through genetic assimilation (*the process by which all individuals within a population are hybrid by descent, because of varying numbers of generations of backcrossing and mating among hybrids i.e., a hybrid swarm*) (Ellstrand & Elam 1993; Levin *et al*. 1996; Rhymer & Simberloff 1996). Many factors will influence whether any of these potentially negative outcomes result, including the strength of reproductive isolation or gamete compatibility of parental taxa, fitness of hybrids relative to their parents, and relative or absolute population sizes of hybridising taxa (Epifanio & Philipp 2000; Wolf *et al*. 2000; Buerkle *et al*. 2003).

Even without introgression, hybridisation can pose a major threat to biodiversity as there can be decreased demographic output due to wasted reproductive effort on failed interspecific mating (e.g., Leary *et al*. 1993). This could place populations at risk of local or widespread extinction by increasing susceptibility of populations to stochastic events (e.g., fluctuating environmental conditions) or genetic effects that compromise short term survival or evolutionary potential (Frankel & Soulé 1981; Lacy 1987; Lande 1988; Caughley & Gunn 1996; Frankham 2005). Demographic swamping will be especially problematic for a rare (or less abundant) taxon that hybridises with a more common (or abundant) taxon because, as the population size of the rare (or less abundant) taxon is reduced, the probability of finding a conspecific mate will also be reduced thus impacting population size in later generations (Levin *et al*. 1996; Rhymer & Simberloff 1996).

When hybrids are both viable and interfertile, and neither ecological or geographic isolation act as barriers to repeated mating, backcrossing may lead to genetic swamping

involving one or both parental taxa, depending on whether hybridisation is symmetrical or not (e.g., Rosenfield *et al.* 2000). As later generations become increasingly more complex through continued backcrossing and mating amongst hybrids (i.e., the production of a hybrid swarm), parental taxa may be difficult to identify and eventually species boundaries may become so blurred that total loss of species identity occurs (Hails & Morley 2005; Mallet 2005).

1.2 The frequency of occurrence of marine hybridisation may be greater than currently appreciated

Studies of mating systems of marine species (reviewed by Palumbi 1994) have found prezygotic barriers to hybridisation, including mate preference, habitat specialisation, and spatial and temporal separation of spawning. However, since fertilisation occurs externally for many marine taxa, mating systems are primarily controlled by interactions of free spawned gametes, implying that whenever there is overlap of reproductive periods or spawning synchrony, resulting in the mixing of different species gametes (see Harrison *et al*. 1984), there is great potential for hybridisation. Indeed, controlled fertilisation experiments have revealed at least some level of reproductive compatibility between the gametes of numerous species-pairs (e.g., marine invertebrates, see Table 1 Geyer & Palumbi 2005). Nevertheless, the frequency of occurrence of natural hybridisation and its consequences are relatively unstudied, for many marine organisms.

Most examples of hybridisation in the ocean involve commercially important species (e.g., 51 of 108 cases of hybridisation identified by Gardner 1997 and recent studies by Seeb [1998], Roques *et al*., [2001], Nielsen *et al*., [2003], Nielsen *et al*., [2004], Nielsen

et al., [2005], and Uthicke *et al*., [2005]), and have largely relied upon morphological criteria alone to distinguish between hybrids and parentals (e.g., 81 of 108 cases of hybridisation documented by Gardner 1997). This is highly ambiguous as it assumes that hybrids will be intermediate to their parents, which is not always the case since controlled breeding experiments have shown hybrids to express many different (and often extreme) phenotypes (Gross *et al*. 2004). Moreover, distinction between different classes of hybrid (i.e. F1, BC1, and BC2 etc.) is an impossibility, as advanced generation backcrosses are often virtually indistinguishable from their parents. Also relevant is that morphological differences may not always have an underlying genetic basis, but rather reflect phenotypic plasticity and therefore is an unreliable predictor of genetic exchange between different species.

Probably the most thoroughly studied marine hybrid zone involves hybridisation between the mussels *Mytilus edulis* and *M. galloprovincialis*. The two species co-occur within an area that includes the Atlantic coast of France, southwest England and Ireland where they hybridise extensively (Gosling & Wilkins 1981; Gardner & Skibinski 1988; Coustau *et al.* 1991). Within the hybrid zone a complex 'mosaic' (*sensu* Moore 1977; Harrison 1986) pattern exists in which hybrid and pure species populations alternate (Daguin *et al.* 2001; Bierne *et al.* 2003b). The maintenance of this structure reflects site-specific selection and local adaptation (*superior fitness in a habitat, often the natal habitat, because of site-specific selection*), as hybrid genotypes genetically similar to *M. edulis* occur most often in sheltered locations, whereas *M. galloprovincialis*-like hybrids are favoured in more exposed areas (Gardner 1997 & references therein). Furthermore, sets of

maladapted genotypes are eliminated early on during larval development, presumably as a consequence of early acting outbreeding depression (Bierne *et al.* 2002, 2003a, 2006).

High resolution studies of hybridisation have been made possible by advances in molecular ecology (in terms of the availability of cost effective and powerful sets of molecular markers), coupled with new statistical methods which allow estimation of the degree of admixture or ancestry of a given individual (Pritchard *et al*. 2000; Falush *et al*. 2003a, 2003b). Nevertheless, unequivocally detecting introgression is exceptionally difficult for a number of reasons: (i) introgressed alleles may be hard to discriminate from ancestral polymorphism (see Van Oppen *et al*. 2000, Van Oppen *et al*. 2002; Miller & Van Oppen, 2003, then see Vollmer & Palumbi 2004); (ii) if there has been an extensive period of time proceeding the last episode of hybridisation, introgressed alleles could be diluted by genetic drift and selection, and mutations may have accumulated, or both, making such alleles difficult to identify; (iii) populations of one taxon may go extinct or diverge, thus their role or genetic contribution may not be recognised or extremely difficult to resolve with any degree of certainty (Woodruff & Gould 1987; Dowling & Secor 1997); and (iv) in cases where a difference in population size exists, the role of the less abundant (or rare) taxon may be difficult to detect because it contributes proportionally fewer alleles to hybrids.

Since many cases of hybridisation are probably cryptic and overlooked (Harrison 1993), there is little doubt that the frequency of occurrence of hybridisation and introgression in the world's oceans (and adjoining habitats such as coastal lakes and

lagoons) is probably greater than is currently appreciated. Many new examples await discovery and are sure to be uncovered.

1.3 Model system: the marine – estuarine interface

Coastal lakes and lagoons (or 'estuaries' – here I use these terms interchangeably) are generally recognised as important nursery habitat for larval and juvenile life-stages of many fish (e.g., Potter *et al*. 1990; Gray *et al*. 1996; Griffiths 2001), however, estuaries and coastal areas near the entrances of estuaries also provide spawning grounds for many mobile coastal or migratory marine species (Potter *et al*. 1990; Reyier *et al*. 2008). Estuaries themselves often support unique species assemblages, including estuary restricted or obligately estuarine taxa that complete their entire life within estuaries (Potter *et al*. 1990; Whitfield 1994). This raises the possibility that estuaries provide areas or zones of contact, for interbreeding between such ecologically divergent species-pairs. Indeed, estuaries may be hot spots of hybridisation between interfertile, mobile marine and estuary restricted congeners. Importantly, estuaries form the interface of marine and freshwater environments so represent areas in which there is considerable environmental variability (e.g., gradients in salinity and temperature). Thus, estuaries may form ecotonal habitat that allows the persistence of both first and later generation hybrids or stabilised hybrid derivatives that are favoured within individual estuaries.

1.4 The *Acanthopagrus* spp. complex of southern Australia

Southern Australia provides an excellent opportunity to examine connectivity among estuarine fish populations. Several genera include estuary restricted species, and species that use estuaries for nursery habitat, feeding and/or spawning but are not necessarily restricted to this type of habitat because they move between estuarine and coastal marine waters (Edgar 2000). There are also large numbers of estuaries with varying morphology and frequency of opening to the adjoining ocean (Roy *et al*. 2001). At the

extreme this varies between permanently open rivers and embayments and intermittently closed and open lakes and lagoons (i.e., ICOLLs), with the duration of entrance mouth opening to the ocean anticipated to have important implications for connectivity among populations of estuary restricted taxa as well as creating the conditions that might facilitate hybridisation. Indeed, estuary-mouth closure will not only increase the spatial isolation of populations of obligately estuarine species, but in addition provides the potential to trap visiting migrants or transient coastal marine species within the estuary. For free spawning marine and estuarine congeners with overlapping spawning times, this could conceivably result in the formation of mixed species spawning aggregations, thus creating the conditions under which hybridisation may occur.

The *Acanthopagrus* spp. complex provides an opportunity to test the prediction that estuaries provide areas of contact for hybridisation between estuary restricted and migratory marine species. *Acanthopagrus butcheri* Munro (Black bream) is considered estuary restricted, while the morphologically similar, *A. australis* Günther (Yellowfin bream) often moves between coastal and estuarine waters. *Acanthopagrus butcheri* is the mostly widely distributed of the two species. It occurs within estuaries over 3000 km of coastline in southern Australia and is considered to span at least two major biogeographic barriers corresponding to the Bassian Isthmus (BI) and the Great Australian Bight (GAB), which represent respectively the land bridge that connected Tasmania (TAS) to the Australian mainland during ancient (Plio-Pleistocene) sea level low stands (Lambeck & Chappel 2001) and an approximately 1000 km stretch of rocky coastline between South Australia and Western Australia (refer to Fig. 2.2). In partial contrast, *A. australis* inhabits both estuarine and coastal waters on the east coast and is potentially highly dispersive.

Adults are thought to migrate northwards to spawn, and larvae may spend a considerable amount of time in the coastal ocean plankton under the influence of the southward flowing East Australian Current (EAC) (Neira *et al.* 1998). Where *A. australis* and *A. butcheri* co-occur on the south east corner of the Australian mainland (SE corner), corresponding respectively to the known southern and northeastern range limit of the two species, they are thought to hybridise occasionally, with the presence of at least some natural hybrids confirmed by allozyme data (Rowland 1984).

Hybridisation is thought to occur within a few intermittently closed and open lagoons in southern New South Wales (NSW) although nothing is known of the conditions under which hybridisation occurs. It may be favoured either by flood events that temporarily displace *A. butcheri* females or when *A. australis* males enter estuaries to forage during the *A. butcheri* spawning period and become trapped for extended periods by estuary-mouth closure. Nevertheless, both the frequency of occurrence and geographical distribution of hybrids is relatively poorly documented and our understanding is completely dependent upon the correct identification of pure species and hybrids based entirely on visible morphologically differences. Importantly, the predicted great mobility of *A. australis* provides the potential for contact and interspecific gene flow over a significant portion of the distributional range of *A. butcheri*, implying that *Acanthopagrus* hybrids zones are more common and widespread than has previously been considered. Furthermore, SE Australian oceanographic conditions and coastal features imply complex dynamics, for populations of both *A. australis* and *A. butcheri*, and for hybrid zones between the two species. The SE corner corresponds to a convergence zone of two major oceanic currents, southward flowing warm-core eddies of the EAC and cold waters of the

eastward flowing Bass Straight Cascade (BSC), creating extreme spatial variation in temperature and salinity (images can be found at www.bom.gov.au: accessed June 2009). Moreover, the ocean floor underlying the Bass Straight between the Australian mainland and TAS has been subject to periodic topographical alteration resulting from ancient (Plio-Pleistocene) climatic conditions affect on sea-level (i.e., BI) (Lambeck & Chappel 2001).

Early allozyme studies of *A. butcheri* in Western Australia suggest that populations in different estuaries are effectively isolated and self seeding (F_{ST} = 0.17) (Chaplin *et al*. 1998), implying a high likelihood of local adaptation and prolonged evolution of lineages in separate drainages. However, the inferred spatial extent of dispersal varies among locations as the virtual absence of genetic structure in southeastern Australia suggests much stronger connections (F_{ST} = 0.004) (Farrington *et al*. 2000), though later restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA), and microsatellite studies, by Burridge *et al*. (2004) and Burridge & Versace, (2007) have revealed isolation by distance for those estuaries.

The population dynamics of both species may have been altered substantially in recent years by anthropogenic impacts. *Acanthopagrus butcheri* and *A. australis* form the basis of valuable and heavily exploited commercial and recreational fisheries in eastern Australia (Kailola 1993; Henry & Lyle 2003). In NSW, where the two species ranges overlap, the fishery is managed as a 'single species fishery' so the proportion of each species in the annual number of landings has effectively remained unknown since fishing commenced in the early 1900's (NSW Department of Primary Industries, unpublished data). Catches in some estuaries are in decline and under direct threat from a variety of

sources including fishing, habitat destruction and mechanical modification of the frequency of flushing of estuaries – problems that are common to estuarine fish populations both within Australia and elsewhere throughout the world (see Nielsen *et al*. 1997). In Victoria (VIC), fishery collapses have occurred repeatedly over the past century (Coutin & Morison 1997), while in WA, overfishing and other human impacts have resulted in some estuaries being restocked from hatcheries (Jenkins *et al*. 2006).

As *A. butcheri* populations in NSW are potentially self-seeding they may face major threats of localised or widespread extinction. Importantly, anthropogenic impacts namely habitat modification and overfishing, may be exacerbating the situation for *A. butcheri*. Indeed, the hydrodynamics of NSW estuaries are frequently modified by artificial opening of estuary mouths (Department of Environment, Climate Change and Water NSW, unpublished data), providing increased contact between *A. australis* and *A. butcheri* (an obvious pre-condition for hybridisation). Moreover, landings of bream within some estuaries have declined sharply over the last decade. Although reduction to the size of populations of *A. butcheri* is undesirable in its own right, reduced population size is just one of several possible detrimental consequences that may potentially challenge the persistence of heavily exploited or overfished estuarine populations. Indeed, population genetic theory predicts that accompanying reductions to the size of populations will be corresponding losses of both neutral and adaptive genetic variation (Frankham *et al*. 2002). If there were connections among estuaries, this loss of variation may be restored from surrounding *A. butcheri* populations and no further genetic effects may be apparent because gene flow will act to oppose the potential detrimental consequences associated with genetic drift and inbreeding (see Slatkin 1987). However, in the absence of dispersal of larvae or

juveniles, or migration of adults, demographic stochasticity may exacerbate the situation further. Variation in reproduction can have direct effects on reproduction and recruitment in subsequent generations (hence total population size), as well as clear genetic effects. For instance, if in a particular year total reproductive output is a consequence of mating between few individuals, the genetically effective population size (N_e) (*the subset of individuals in a population that successfully contribute offspring to the next generation*) will be small (indeed N_e may already be small anyway because of skewed reproductive success of larger more experienced fish relative to individuals that have just reached reproductive maturity), increasing the susceptibility of a continued loss of heterozygosity and allelic diversity due to genetic drift (Barrett & Kohn 1991; Caughley & Gunn 1996). Moreover, because of a small N_e inbreeding (*mating of individuals related by descent*) might reduce individual fitness through the greater expression of deleterious recessive characters resulting in a decreased probability of survival and reproduction i.e., inbreeding depression (Frankel & Soulé 1981; Charlesworth & Charlesworth 1987; Frankham 2005). Coupled with considerable uncertainty surrounding the frequency of occurrence and consequences of hybridisation, inbreeding, genetic drift, and fishing pressure may erode genetic variation and/or impact on fitness and population size, such that extinction may result for local populations of the obligately estuarine *A. butcheri.*

1.5 This study

The overall aim of my research is to use a combination of molecular population genetics and controlled breeding experiments to determine: (i) population structure of *A. butcheri* and *A. australis*; and (ii) the frequency of occurrence, and consequences of hybridisation for *A. butcheri*.

This research will contribute fundamentally to our understanding of connectivity among estuarine fish populations – an issue poorly studied worldwide. Furthermore, investigations of hybridisation between marine and estuarine fish are extremely rare. This study will contribute significantly to our understanding of the mechanisms and consequences of hybridisation, for a pair of estuary restricted (*A. butcheri*) and migratory marine species (*A. australis*). In terms of fisheries management and conservation, at a minimum, this research will determine the taxonomic composition of the *Acanthopagrus* spp. fishery, and contribute to the preservation of *A. butcheri* and *A. australis* populations.

The remainder of this thesis contains a series of chapters that seek to address the broad aims detailed above. Each chapter was written as an independent, publishable unit. I have avoided repetition by editing out sections of text common to all chapters. Many of the chapters are presented primarily as data chapters, with much of the discussion of the results presented in Chapter 8.

In Chapter 2, I test the capacity of microsatellite and mtDNA markers and morphology to initially characterise *A. australis*, *A. butcheri*, and their hybrids, and then use a broad scale genetic survey to examine the spatial extent of hybridisation to test the

prediction that the occurrence of hybrid fish is restricted to the area of sympatry of the species.

The dispersal potential of *A. australis* may have potentially serious implications for *A. butcheri*, however, *A. australis*'s close association with estuarine spawning sites suggest populations could be genetically subdivided. In Chapter 3, I use population genetic data to test the prediction that spawning site fidelity promotes fine-scale population subdivision in *A. australis*.

The surprisingly high number of hybrids found in my initial broad-scale genetic survey (Chapter 2) may reflect ancient hybridisation events with little continuing introgression. Alternatively, *Acanthopagrus* hybrid zones may be dynamic and reflect contemporary processes. In Chapter 4, I use mtDNA sequence variation in a phylogeographic study of *Acanthopagrus* spp., within a set of estuaries that are considered to span major biogeographic barriers, to investigate the evolutionary history of *A. butcheri* including the timing of divergence of genetic lineages. Specifically, I test the prediction that different estuaries and geographical regions are genetically differentiated, and that eastern and western populations represent distinct, divergent genetic lineages.

Unlike many species fragmented by the BI that form deeply divergent eastern and western lineages, *A. butcheri* form closely related eastern and western lineages, which together with a range wide lack of isolation by distance, imply a recent colonisation of estuaries throughout southern Australia (Chapter 4), leaving open the possibility that *Acanthopagrus* hybrid zones are maintained by contemporary processes. In Chapters 5 – 7

I investigate some of the processes that I anticipate will be important in the maintenance of *Acanthopagrus* hybrid zones— gamete compatibility, recruitment, and hybrid zone stability.

In Chapter 5, I use a fertilisation experiment to access gamete compatibility of pure species and their hybrids, to test for early acting barriers to initial and later hybridisation.

The dynamic nature of intermittently closed and open lagoons will mean that opportunity for hybridisation will vary considerably across lagoons. In Chapter 6, I use a fine scale genetic survey of recruits within five lagoons to further characterise the frequency of occurrence and persistence of hybridisation, and to test the prediction that frequencies of hybrids will be greatest in lagoons generally closed to the ocean, where *A. australis* and *A. butcheri* can be trapped together by lagoon entrance closure.

In Chapter 7, I use a set of museum specimens to look back through time more than 60 years to describe the genotypic composition of an estuarine population of *Acanthopagrus* spp. considered beyond the normal southern range limit of *A. australis.* Interestingly, in my initial genetic survey (Chapter 2) I uncovered rare hybrids in a small sample of fish from an estuary some 250 km south of the recognised area of sympatry (Chapter 6). In Chapter 7, I test the prediction that frequencies of hybrids are stable through time, implying that hybrids are favoured within the estuarine environment.

In Chapter 8, I discuss some of the major findings uncovered in Chapters 2 – 7.

Chapter 2 The evolutionary impacts of hybridisation and interspecific gene flow on an obligately estuarine fish

This chapter is published in *Journal of Evolutionary Biology* (including the journal cover photo, see Fig. 2.1 below). *

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*This thesis has been written as a series of manuscripts, five of which are published, in press, or in review. Each chapter has been edited to reduce the inevitable repetition that this can create.

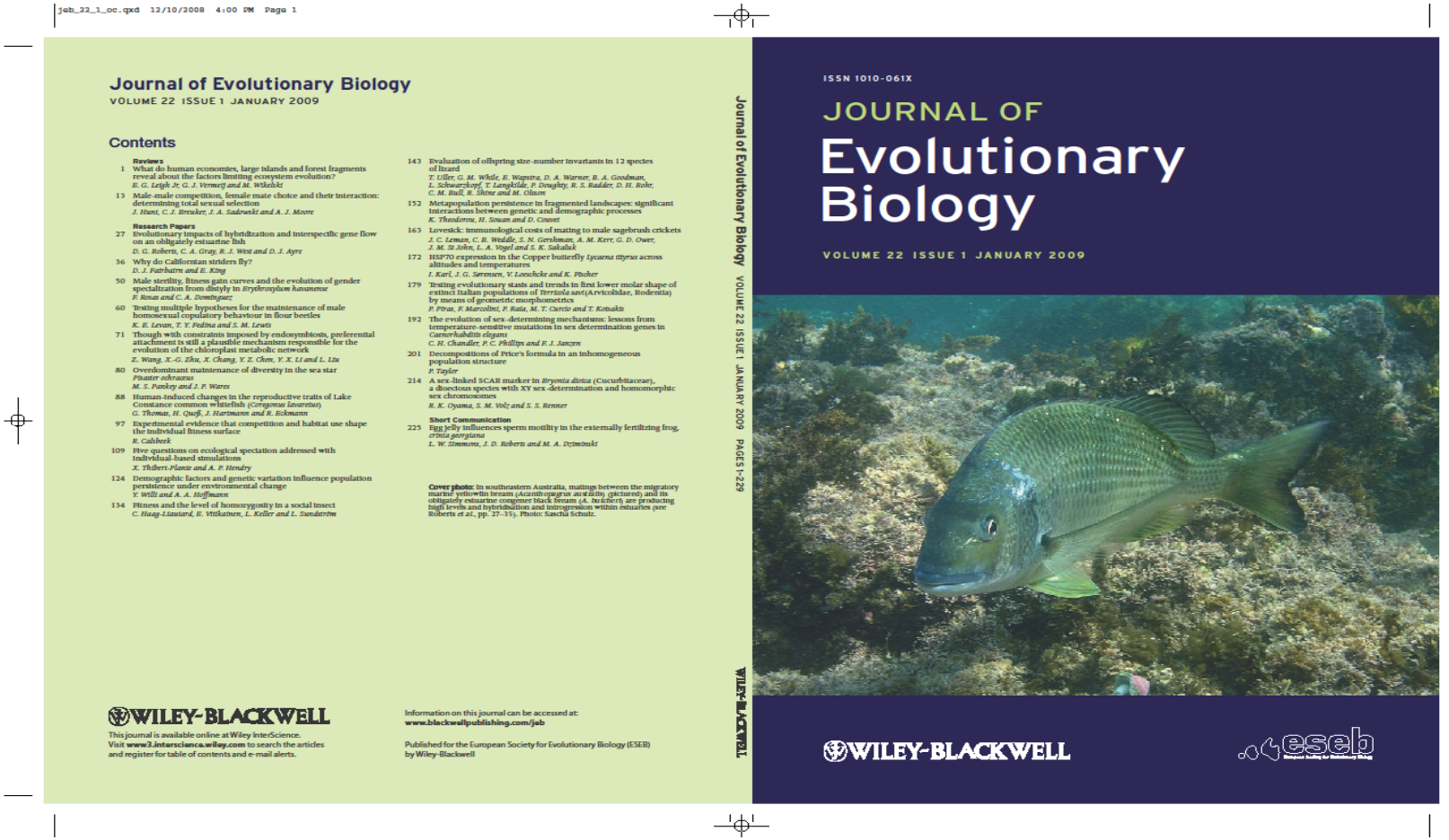

Figure 2.1 Cover photo *Journal of Evolutionary Biology*. In southeastern Australia, matings between the migratory marine Yellowfin bream (*Acanthopagrus australis*) (pictured) and its obligately estuarine congener Black bream *A. butcheri* are producing high levels of hybridisation and introgression. See Roberts *et al.*, pp 27-35. Photo by Sascha Schultz

2.1 Introduction

Populations of species that complete their entire life-cycle within estuaries (obligately estuarine) are potentially small and confined to isolated patches of suitable habitat. This creates conditions that promote rapid evolutionary divergence through the combined effects of genetic drift, founder events and site-specific selection (Frankham 2005). Given that such taxa may possess highly mobile adults or larvae, this situation may parallel that of populations restricted to mountain peaks or islands (Assefa *et al*. 2007; Lukoschek *et al*. 2007). For genetically compatible free spawning taxa however, the effects of isolation may be reduced by hybridisation with wide ranging congeners, resulting in gene flow both among estuaries and between species (Addison *et al*. 2005; Harper *et al*. 2007). These phenomena have rarely been investigated and both the likelihood of hybridisation and its ecological and evolutionary consequences are potentially both estuary-specific and susceptible to anthropogenic disturbance through processes such as harvesting and mechanical modification of estuarine hydrology (e.g., Scribner *et al.* 2000).

The consequences of hybridisation can either enhance or reduce the potential for persistence and adaptive evolution of hybridising species (Harrison 1993; Levin *et al*. 1996; Rhymer & Simberloff 1996; Arnold 1997). In particular, low levels of hybridisation may be beneficial for estuarine species since introgression may introduce genetic novelty and oppose the anticipated effects of genetic drift and/or inbreeding within otherwise isolated populations (Chaplin *et al*. 1998). However, when there is a high probability of interspecific matings then demographic and genetic swamping may occur with potentially serious consequences if hybrids are either inviable or infertile and gametes are wasted, or if outbreeding depression results because hybrids are relatively less fit than their parents

(Burke & Arnold 2001). At the extreme, frequent backcrossing could contaminate the genome of one or other parental species causing loss of species identity as the genomes of the two species recombine (e.g., Woodruff & Gould 1987). However, for estuarine dependant species the net consequences of hybridisation may reflect a complex function of the above factors and will be influenced by the site fidelity of the hybrid and the degree to which estuarine species and their hybrids compete for resources such as spawning sites and mating partners.

It seems likely that the consequences of hybridisation between marine and estuarine taxa will be greatest for locally rare, obligately estuarine species. In this circumstance, I would expect that the rare taxon is more likely to be involved in cross species matings. Moreover, these effects may be exacerbated if hybrids are both inter-fertile and remain within estuaries leading to the production of later generation hybrids and backcrosses and the replacement of the estuarine taxa by a hybrid swarm (i.e., genetic swamping).

Many of the estuaries of eastern Australia that are reported to support populations of *A. butcheri* are small, isolated and have intermittent patterns of opening to the adjoining ocean (indeed some estuaries may be landlocked for several years [Roy *et al*. 2001; DECCW NSW, ICOLL entrance condition, unpublished data]). These characteristics may potentially increase the effects of hybridisation especially if hybrids remain in residence for extended periods. We currently do not know the frequency of occurrence of hybridisation or the distribution of hybrids. Here I use a combination of microsatellite and mitochondrial DNA markers and morphology to initially characterise pure species and various classes of hybrids, and then to utilise a broad scale genetic survey to investigate the geographic extent

of hybridisation and to test whether hybrids are indeed more common in the area of sympatry.

2.2 Methods

2.2.1 Species description

Acanthopagrus butcheri has a disjunct distribution within estuaries from central NSW to WA, including TAS. *Acanthopagrus australis* is distributed along the east coast of Australia from northern QLD to the border of NSW and VIC, overlapping part of its range with *A. butcheri* (Edgar 2000) (Fig. 2.2).

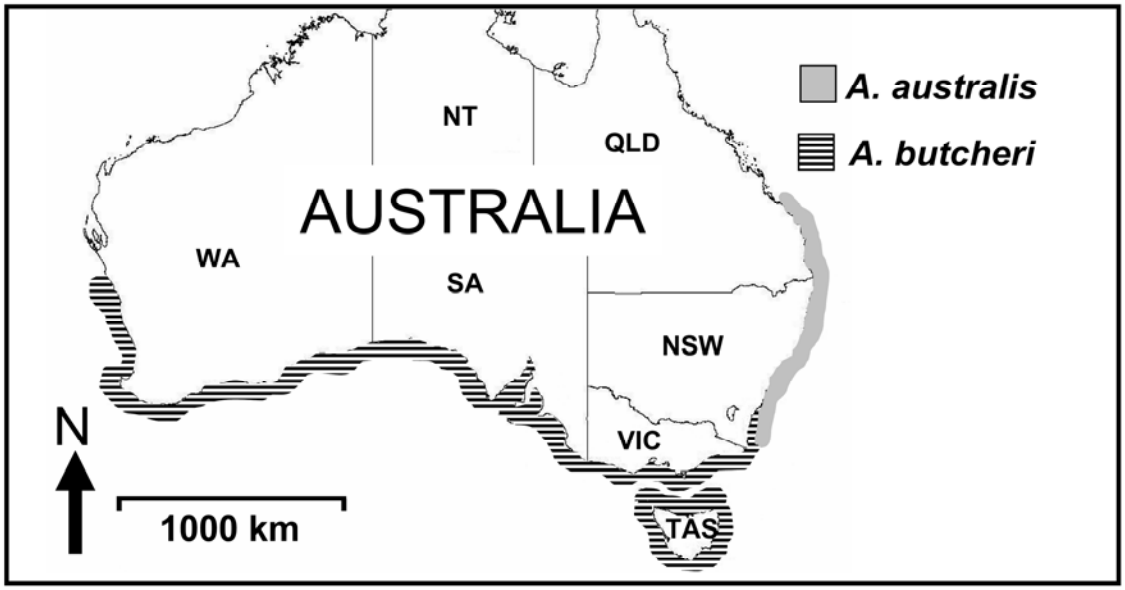

Figure 2.2 The distribution of *Acanthopagrus butcheri* (Black bream) and *A. australis* (Yellowfin bream).

Both species have annual reproductive cycles with asynchronous gonad development and group spawning occurring over a period of 2 – 5 months. Spawning time

in *A. butcheri* varies between estuaries, but generally peaks in September or October, and takes place in the extreme upper estuary, near the interface of fresh and salt water, well away from any influence of coastal currents. Fertilised eggs are pelagic, hatching ~36 – 48hrs (depending on water temperature) after fertilisation (Haddy & Pankhurst 1998). Larvae recruit to sea grass meadows and other submerged structures for growth and development. In partial contrast, the peak spawning of *A. australis* occurs in July and August in the vicinity of estuary entrances and the adjacent surf zones of ocean beaches. Some *A. australis* males may remain inside estuaries during the spawning season, and perhaps more significantly, large numbers may be trapped within estuaries for several years by closure of estuary mouths. However, little is known about how *A. australis* males behave in the presence of *A. butcheri* spawning females. As for *A. butcheri*, larvae recruit to shallow sea grass meadows and submerged structures in estuaries (Griffiths 2001) however, larvae are thought to spend time in the coastal ocean plankton under the influence of the East Australian Current (EAC) and are therefore thought to disperse great distances before recruiting to sea grass meadows in the lower reaches of estuaries (Neira *et al*. 1998).

2.2.2 Molecular markers and population sampling

I genotyped an average of 22.6 ± 4.0 (SE) adults from 25 estuaries between QLD and WA (Fig. 2.3) (Table 2.1) using eight microsatellite loci from a selection that were developed for *Acanthopagrus schlegelii* (Jeong *et al*. 2003) and *A. butcheri* (Yap *et al*. 2000). The microsatellite loci (accession number) I used were: pAb2B7 (AF284352), pAb2A5 (AF284354), pAb2D11 (AF284355) (Yap *et al*. 2000), Acs1*(AB102864), Acs3* (AB095008), Acs6* (AB095010) (Jeong *et al*. 2003), Acs-16* (AB095012) and Acs-21* (AB095014) (Jeong *et al*. unpublished).

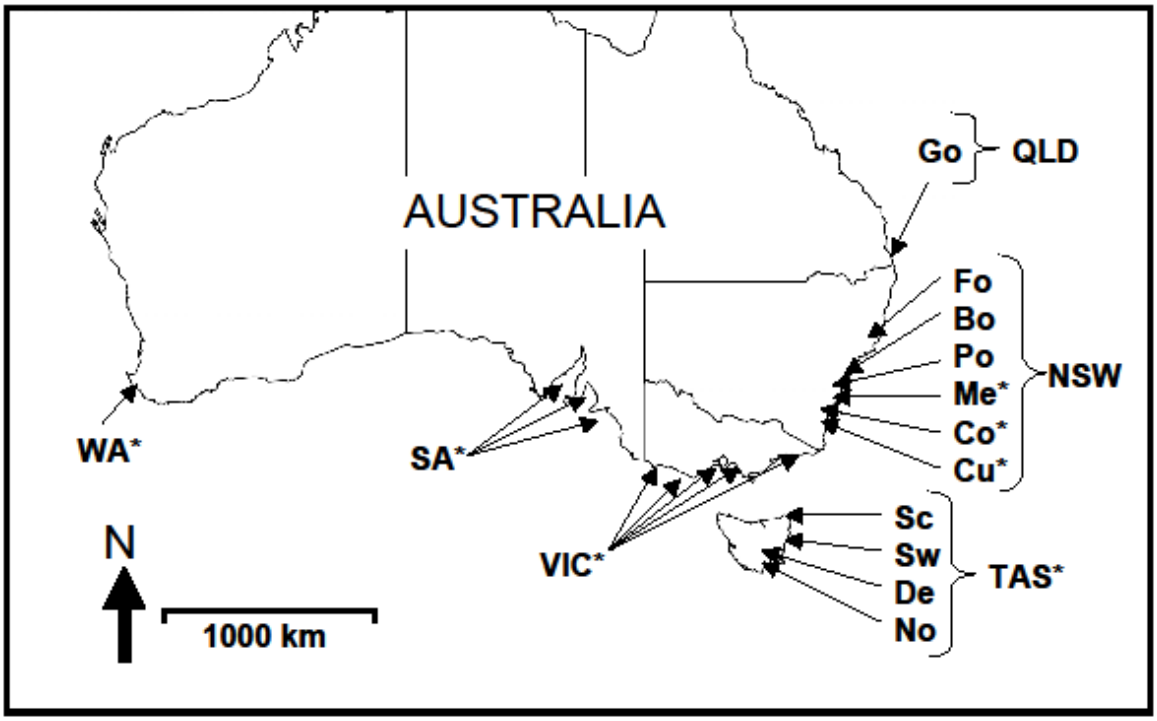

Figure 2.3 The location of study estuaries. Asterisks indicate *Acanthopagrus butcheri* sampling sites.

The eight microsatellite loci were amplified using a combination of standard and touch-down PCR. After an initial denaturing cycle of 10 minutes at 95 ºC, 25 'touchdown' cycles (the annealing temperature dropped 2 ºC every 5 cycles) of 30 seconds at 94 ºC, 30 seconds at the annealing temperature, and 45 seconds at 72 ºC was used. The touchdown cycles were followed by an additional 10 cycles of 30 seconds at 94 ºC, 30 seconds at the final annealing temperature and 60 seconds at 72 ºC, which was followed by a 10 minute final extension period at 72 ºC. The temperature of the first touchdown cycle was 8 ºC above the final annealing temperature, which was either 59 ºC (pAb2B7, pAb2A5, pAb2D11 & Acs-21*) or 52 ºC (Acs1*). For all other loci, PCR conditions were: 10 minutes at 95 ºC then 35 cycles of 30 seconds at 94 ºC, 30 seconds at either 51 (Acs6*) or

58 ºC (Acs3* & Acs-16*) and 60 seconds at 72 ºC followed by 10 minutes at 72 ºC. One unit of *Taq* polymerase was used in each 20µl reaction with the buffer supplied by the manufacturer. Reactions were 1.5 – 2.5 mM for $MgCl_2$, 2.0 mM of each dNTP, between 0.0625 mM and 0.25 mM of each primer 5′ labelled with one of 4 fluorescent dyes (6FAM, HEX, NED or PET) and approximately 5 – 20 ng of template DNA. Conditions were not altered for multiplex reactions. Negative controls were included in each PCR reaction to verify the absence of contaminating DNA. Up to four PCR products were combined in a single reaction mix and visualised with an ABI 3130 automated capillary sequencer. Assignment of allele size (bp) was achieved with reference to LIZ size standard present in each lane and scoring was performed with the aid of GeneMapper software (Applied Biosystems) (Fig. 2.4). I conducted multiple runs of randomly selected templates to ensure repeatability of allele scoring. Overall, the proportion of missing genotypes across loci ranged between 0.004 and 0.016.

I also amplified ~400bps of the 3' domain of the mtDNA control region using primers LPW and HPX of Jean *et al*. (1995). Cycling conditions for the PCR were: 10 minutes at 95 ºC then 35 cycles of 45 seconds at 94 ºC, 45 seconds at 55 ºC seconds, and 1 minute 30 seconds at 72 ºC, followed by an additional 10 minutes at 72 ºC. I digested the fragment at 37 ºC for 2 hrs using *Alu I* and *Dde I* restriction enzymes (separate reactions) and scored the subsequent profiles under ultraviolet light, after electrophoresis on 1.5 % ethidium bromide stained gels. Representative haplotypes were sequenced to confirm restriction sites (Roberts *et al*. unpublished data).

Table 2.1 Collections of adult *Acanthopagrus* spp. (*A. australis*, *A. butcheri* or their hybrids) from Australian estuaries. The number of samples genotyped using microsatellites (Msat.), mitochondrial DNA (mtDNA), and examined for morphology (Morp.) is indicated.

Estuary	Geographic location	Species	Number		
			Msat.	mtDNA	Morp.
Gold Coast (Go)	Queensland	*A. australis*	41	33	20
Forster (Fo)	New South Wales		74	59	
Botany Bay (Bo)			30	28	
Port Hacking (Po)			39	37	
Meroo (Me)		*A. butcheri*	25	11	
Coila (Co)			50	21	20
Cuttege (Cu)			24	8	
Scamander (Sc)	Tasmania		20	20	
Swan (Sw)			49	48	
Derwent (De)			21	21	
Northwest (No)			49	44	
Victoria (VIC) *	Victoria		79	47	
South Australia (SA) E	South Australia		15	12	
Western Australia (WA)	Western Australia		49	32	
Total			565	421	40

*E Pooled samples of between 5 and 18 bream collected from 3 and 10 estuaries respectively.

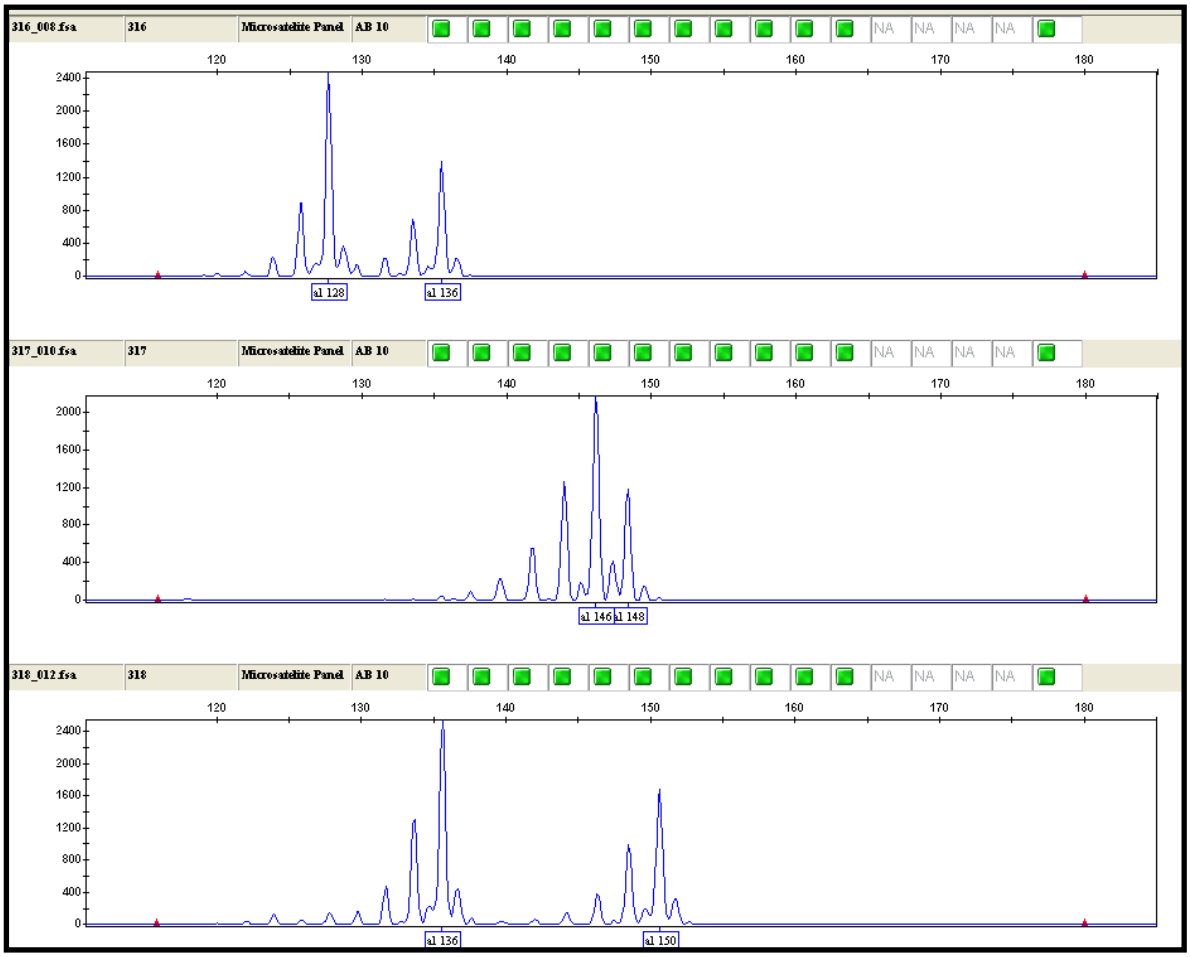

Figure 2.4 A chromatogram for microsatellite locus Acs-16*.

2.2.3 Genetic analyses

2.2.3.1 Factorial correspondence analysis

Factorial Correspondence Analysis (FCA) (performed in GENETIX 4.03 [Belkhir *et al*. 2002]) based on the eight locus nuclear genotypes of all 565 samples was used to visualise the genetic relationship among individuals to see if there was an obvious separation between species. This type of analysis is particularly suited to describing the genetic structure of hybrid zones because the genetic differentiation, including the contribution of each allele to any difference, is decomposed in a nested fashion across the axes: the between-species differentiation is apparent on the first axis of the FCA, whereas

differentiation between populations of the same species emerges on the secondary axes (see [Daguin *et al*. 2001; Bierne *et al*. 2003b] for application of FCA to hybrid zones).

2.2.3.2 Formulation of a hybrid index

By determining the extent to which alleles are characteristic of parental species within a hybrid zone it may be possible to identify simple diagnostic markers or at least formulate a hybrid index. The plotted position of alleles (i.e., position on axis one of the FCA described above) indicates which alleles are responsible for the genetic differences between the species. I found that alleles were generally distributed as two distinct clusters along the plane of axis one, from alleles characteristic of *A. butcheri* to alleles characteristic of *A. australis*. Thus, at each locus I considered that alleles with positive or negative co-ordinates along axis one were characteristic of *A. australis* and *A. butcheri*, respectively (data not shown) (e.g., Daguin *et al*. 2001; Bierne *et al*. 2003b) and pooled these alleles to form a compound allele representative of each species. For each individual, I counted the *A. butcheri* compound allele, and summed over the eight loci to form a simple hybrid index. The index varied between 0 and 16 i.e., a score of 16 meant that both alleles at all eight loci were characteristic of *A. butcheri*. Assignment of hybrid index scores to all fish allowed a simple qualitative assessment of the relative frequency of pure species and hybrids within individual lagoons.

2.2.3.3 Admixture analysis

Since both the FCA and hybrid index analyses suggested hybrids were present within my samples, I tested all bream for evidence of mixed ancestry using an assignment test. Rather than using a pooled compound allele, the assignment approach used population

allele frequencies under the assumptions of both linkage and Hardy-Weinberg equilibrium, while simultaneously assigning individuals to populations based on their eight-locus microsatellite genotype. Assignment testing was conducted using the admixture model implemented in STRUCTURE (version 2.0) (Pritchard *et al*. 2000; Falush *et al*. 2003) to calculate q_i (the mean posterior proportion of ancestry ± 95% CI's). I used a two-population model, after an initial burn-in period of 100 000 iterations, and collected data for 1 000 000 iterations. Default values were used for all other parameters. I present q_i ± 95% CI's as the inferred proportion of *A. butcheri* ancestry. An arbitrarily chosen q_i-value threshold of 0 – 0.05, and 0.95 – 1.0 was used to identify pure *A. australis* and *A. butcheri* respectively. Any individual with a q-value from greater 0.05 to less than 0.95 was classified as hybrid. I performed additional analyses varying both the q threshold (q = 0.1 – 0.2) and the number of loci (4 most diagnostic), with little difference to my findings. Three independent runs confirmed the consistency of the inference.

2.2.3.4 Inferring the pattern of mating and direction of hybridisation

Mitochondrial DNA is maternally inherited and so by determining the mtDNA haplotypes of hybrid fish I could determine whether hybridisation and backcrossing involves particular species × gender combinations i.e., if backcrossing or hybridisation always involve pure species females or both males and females (e.g., Rosenfield *et al*. 2000). The direction of hybridisation can potentially be inferred from the presence and frequency of hybrid individuals with genotypes more similar to one or other parental species. In this instance, I used mtDNA (in conjunction with information from the microsatellites) to infer both the pattern of mating and the likely direction of hybridisation. This approach could potentially be confounded if *A. butcheri* undergoes frequent sex

change as is seen in some fish. There is little evidence that sex change occurs in either *A. butcheri* or *A. australis*, though Rowland and Snape (1994) reported finding rare specimens of *A. butcheri* (8%) with ovotestis, however the ovarian component was non functional.

2.2.3.5 Standard population genetic parameters

I used POPGENE (Yeh *et al.* 1999) and GenAlEx (Peakall & Smouse 2006) to calculate standard population genetic parameters for the total sample of each lagoon and the separate groupings identified by the admixture analysis.

2.2.3.6 Classifying hybrids into distinct classes

Although my initial analyses demonstrated the presence of pure species and hybrids within my samples, the potentially complex breeding biology of these fish raise the possibility that estuaries contain not just simple F1 hybrids but also second or later generation hybrids or backcrosses between hybrids and either parental species. I have therefore used a series of simulated matings using genotype data from the set of relatively pure parentals (detailed above) and the software package HYBRIDLAB (Nielsen *et al*. 2006 see also Nielsen *et al*. 2003) to test the capacity of my microsatellite markers to distinguish the different classes of hybrids potentially contained within estuaries. I generated random genotypes of each of the six possible 'genotypic classes' of parentals and hybrids that are possible for two generations of matings between two species and their hybrids: parental *A. butcheri* ($P_{A.\ butcheri}$); parental *A. australis* ($P_{A.\ australis}$); F1; F2; BC1 in the direction of *A. australis* ($BC1_{A.\ australis}$) and BC1 in the direction of *A. butcheri* ($BC1_{A.\ butcheri}$). I produced four population samples (total n = 300). Each sample consisted of different proportions of genotypes (Table 2.4) designed to represent different hybridisation

dynamics in the *Acanthopagrus* spp. complex. Samples 1 and 2 could be viewed as potentially representative of a population within a estuary in which *A. butcheri* and *A. australis* mate infrequently and F1's tend to mate assortivately, mating with other F1's or *A. butcheri* more than with *A. australis* (Sample 1), or mating with *A. australis* more than with *A. butcheri* (Sample 2). The latter situation could occur if both species were trapped together in the one place (i.e., in a landlocked lagoon), while the former is perhaps representative of a permanently open lake or lagoon where estuarine generated hybrids have dispersed from the estuary, backcrossed to *A. australis*, and larvae including *A. australis*-like backcrosses, recruit to the lagoon from the plankton. In Sample 3, the two species are almost completely reproductively isolated, maintained by spatially discrete spawning sites, however, this breaks down occasionally when *A. australis* stray to the spawning sites of *A. butcheri* (or vice versa). Sample 4 is a combination of all possible classes of hybrid with very few *A. australis* and *A. butcheri* — the beginning of a hybrid swarm.

Here I again used STRUCTURE to estimate the ancestry, and thus the specific status of all 1200 simulated fish; however, the analysis was refined by incorporating the allele frequency distributions of genotypic classes estimated from the set of relatively pure parentals or 'reference collection'. The threshold q_i-values for classifying individuals into pure parentals vs. hybrids did not change. The *accuracy* of the inference was scored as: the ratio of the number of correctly assigned individuals per genotype class to the total number per class, while *efficiency* was scored as: the ratio of the total number of correctly assigned individuals per group (i.e. parental vs. hybrids) to the total per group (Vaha & Primmer 2006).

I acknowledge that this approach is relatively simplistic but I argue it is the most conservative test of my ability to distinguish genotypic classes. Given a more detailed sampling, it would arguably be possible to repeat this process within specific estuaries or regions. However, this is unlikely to be useful given the relatively great mobility of *A. australis* (Chapter 3) and the high frequency of hybridisation in some areas (Chapter 6).

2.2.4 Morphological analysis

Rowland (1984) detected relatively few hybrid bream in earlier allozyme surveys but argued that hybrids could be distinguished from pure parentals using meristic and morphological measurements. I used Principal Coordinates Analysis (PCA) (using the program PCO [Anderson 2003]) to determine if a sample of 40 adult bream again formed discrete clusters of putatively pure species and hybrid fish and then compared morphological assignments with assignments based on my more sensitive mtDNA and microsatellite genetic data. The analysis was based on the range-standardized Euclidean distance matrix of four meristic and 26 body ratio measurements. The measurements I recorded were: anterior margin of the mandible – branchiostegal rays – base of the anterior margin of the pelvic fin – base of the anterior margin of the first anal spine – base of the ventral margin of the pectoral fin; base of the anal fin; length and width of first anal fin; ventral caudal peduncle; ventral posterior margin of the caudal peduncle – lateral line; ventral posterior margin of the caudal peduncle – posterior margin of the lower lobe of the caudal fin; posterior margin of the upper lobe of the caudal fin – dorsal posterior margin of the caudal peduncle; junction of the lateral line and the anterior margin of the caudal fin – posterior margin of the caudal fin; dorsal caudal peduncle; dorsal fin base; base of the anterior margin of the dorsal fin – lateral line; base of the anterior margin of the dorsal fin –

the position on the nape, perpendicular with the subopercle – the position on the nape, perpendicular with the opercle – the position on the nape, perpendicular with the preopercle; body depth, subopercle, opercle, preopercle; base of the dorsal margin of the pectoral fin – base of the ventral margin of the pectoral fin; base of the dorsal margin of the pectoral fin – posterior margin of the pectoral fin; pre orbital; total length, snout to maximum tail length. I also counted the number of scales both above and below the lateral line, and recorded the colour of the pelvic and anal fins.

2.3 Results

2.3.1 Microsatellite diversity

There was variation in every lagoon for seven of the eight microsatellite loci. Overall, numbers of alleles per locus ranged between seven (pAb2D1) and 30 (Acs-16*), with six out of the eight loci displaying greater than 15 alleles per locus, the average (± SE) was 18.4 (± 2.5). Across all loci, I detected a total of 147 alleles (Appendix 1). Often null alleles are encountered in cross-species amplification of microsatellite loci. However, controlled breeding experiments revealed simple Mendelian inheritance (4 pairs; 30 larvae per pair) (Appendix 2).

2.3.2 Separation of pure species and the identification of hybrids using microsatellite genotypes

FCA revealed the presence of two genotypic clusters that generally correspond to the sets of fish collected as *A. australis* and *A. butcheri*. Although there were relatively few fish within the entire sample that were truly genetically intermediate between the species (i.e., F1 hybrid), there was a large set of fish with genotypes that were outside but most

closely aligned with the cluster of pure species *A. butcheri* (black unbroken circle) while very few fish outside the two species groups were similar to the *A. australis* cluster (grey unbroken circle) (Fig. 2.5). Importantly, the fish that clustered inside the unbroken black circle (pure *A. butcheri*) were the Tasmanian, western Victorian, and South and Western Australian fish that were expected to be beyond the range of hybridisation(i.e., outside the described range of *A. australis*). The set of fish outside the two species groups i.e., putative hybrids were, as expected largely fish caught in the area of known sympatry in southern New South Wales and eastern Victoria.

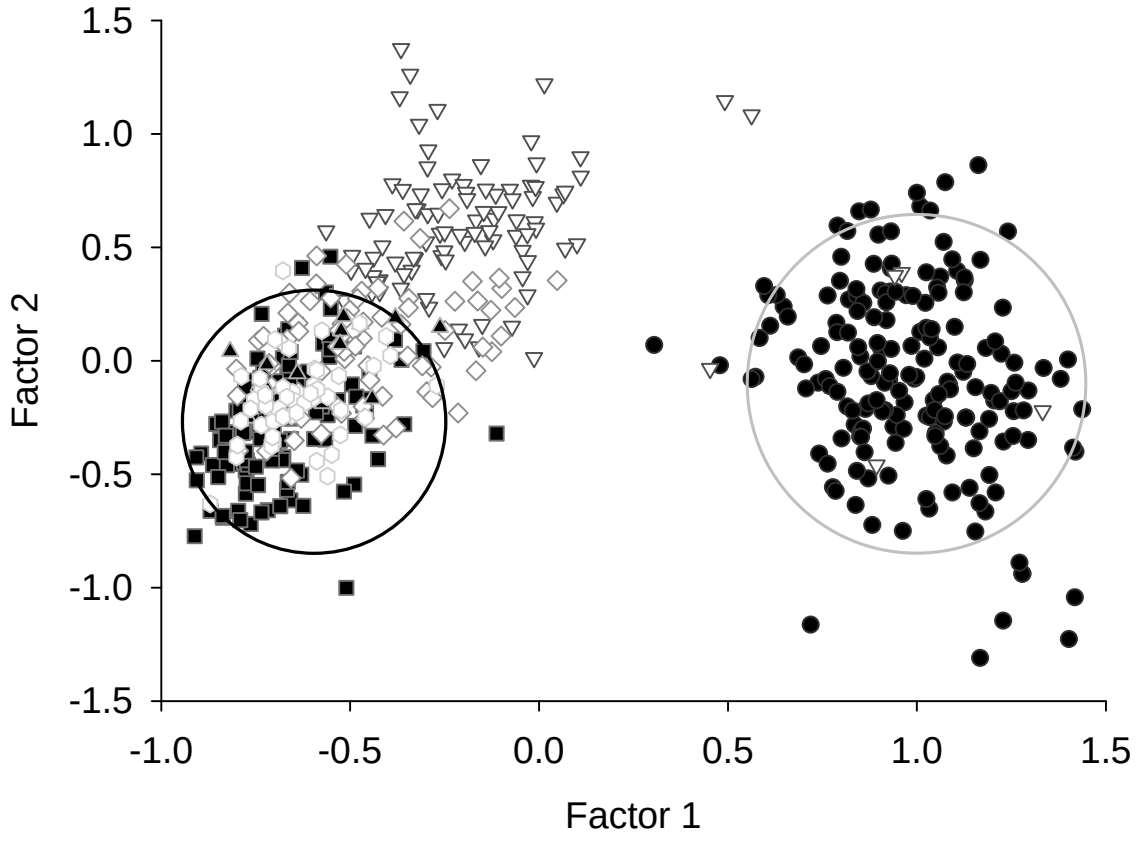

Figure 2.5 Factorial correspondence analysis based on the eight locus genotype of all 565 *Acanthopagrus* spp. Fish were sampled 'blindly' with little indication of their likely species status (the two species have extremely similar morphology). Putative *A. australis*, closed black circles inside the grey unbroken circle (Queensland & New South Wales); putative *A. butcheri* from beyond the recognised range of *A. australis*, closed black squares (Tasmania), closed triangles (South Australia), open hexagons (Western Australia), all inside the black unbroken circle; fish within estuaries in the area of known sympatry, open triangles (New South Wales), and open diamonds (Victoria).

Although distinct clusters were obvious in the FCA, frequency histograms of hybrid index scores provided a simple means of visualising the genotypic composition of fish within each lagoon and in each case these were surprisingly variable. Although hybrid index scores also reveal a clear transition from *A. australis* dominated populations in the north (QLD and northern NSW) through to *A. butcheri* dominated populations in the south (TAS) and far west (WA), these two groupings of populations typically display values ranging from 0 – 4 and 12 – 16 respectively. Within southern NSW, specifically Meroo, Coila and Cuttege Lakes (the area of greatest range overlap), hybrid index scores were highly variable, but suggested that most fish were more genetically similar to *A. butcheri* than to *A. australis* (range = 9 – 12) (Fig. 2.6).

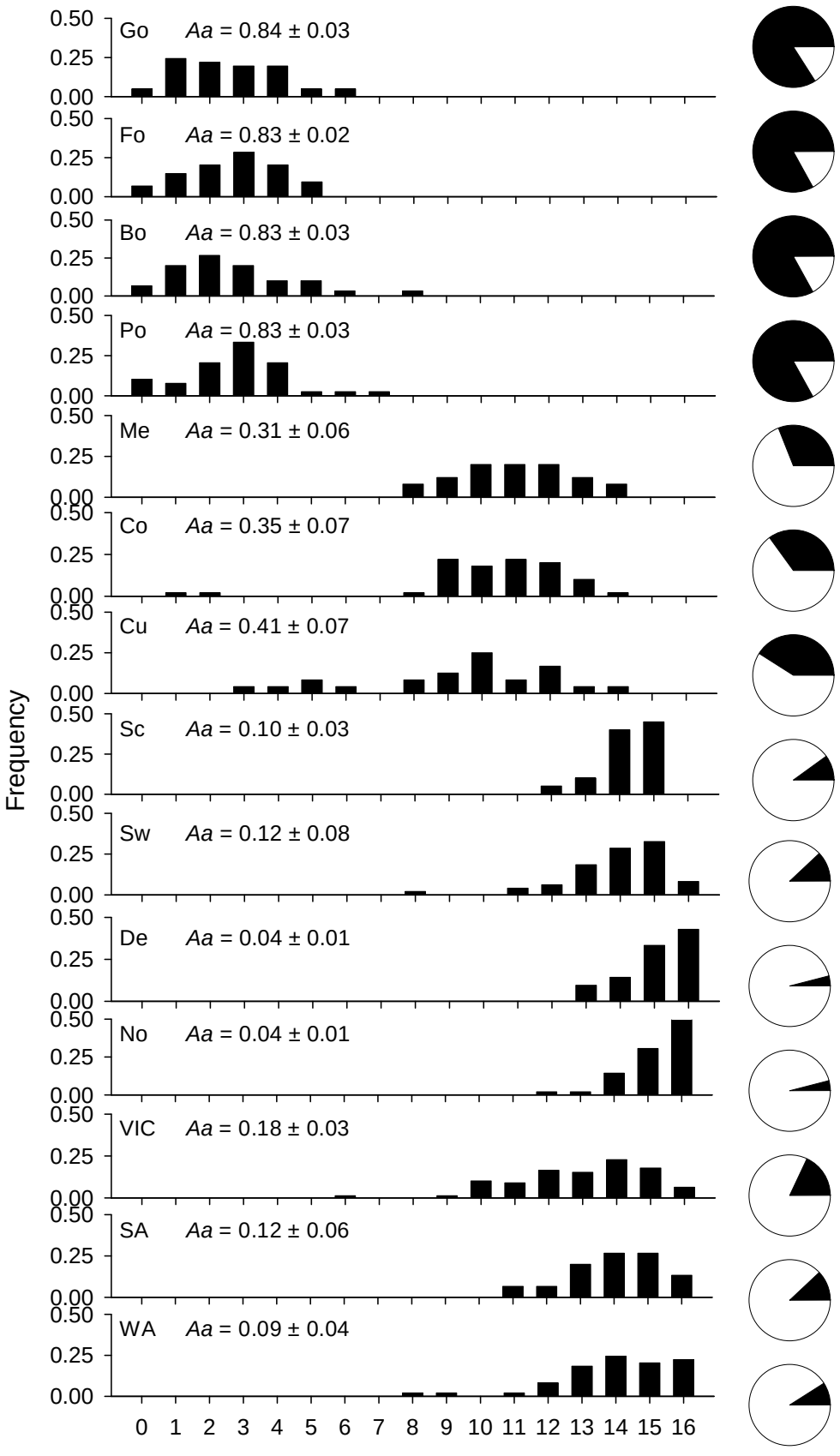
Go Aa = 0.84 ± 0.03
Fo Aa = 0.83 ± 0.02
Bo Aa = 0.83 ± 0.03
Po Aa = 0.83 ± 0.03
Me Aa = 0.31 ± 0.06
Co Aa = 0.35 ± 0.07
Cu Aa = 0.41 ± 0.07
Sc Aa = 0.10 ± 0.03
Sw Aa = 0.12 ± 0.08
De Aa = 0.04 ± 0.01
No Aa = 0.04 ± 0.01
VIC Aa = 0.18 ± 0.03
SA Aa = 0.12 ± 0.06
WA Aa = 0.09 ± 0.04
Frequency
0.00
0.25
0.50
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Hybrid Index

Figure 2.6 The distribution of hybrid index scores and the frequency of the *A. australis* and *A. butcheri* compound allele for each lagoon. The compound alleles were formed by pooling alleles at each locus based on their contribution to differences between the species in FCA analysis. To generate the hybrid index I counted the *A. butcheri* compound allele and summed over the eight loci i.e., a score of 16 meant that both alleles at all loci were characteristic of *A. butcheri*. The shaded portion of each pie chart corresponds to the average (± SE) frequency of the compound *A. australis* allele (*Aa* =). Orientation, abbreviations and geographic location of estuaries are as per Table 2.1.

Assignment tests allowed me to estimate the proportion of *A. butcheri* ancestry (q_i) for each fish. This meant I could determine both the specific status of each individual and the overall genetic composition of each lagoon (i.e., hybrids vs. pure species). I classified 178 and 304 fish as pure *A. australis* and *A. butcheri* respectively, while there were 83 hybrids (15% of the total). Hybrids were found in 68% of estuaries (range of % hybrids per estuary = 0 – 58). However, ~70% of hybrids (n = 58) were in NSW, with the majority of hybrids in Meroo, Coila and Cuttege Lakes (57%). Varying q and the number of loci made no substantive difference to my conclusions. Analyses revealed that for 4 and 8 loci and application of q of 0.1 between 52 and 64% of estuaries supported hybrids and that the range of the percentage of hybrid fish per estuary was 0 – 42 or 0 – 56%. Even applying an extremely relaxed q of 0.2, I still detected hybrids in between 44 and 56% of estuaries, while the percentage of hybrids per estuary was between 0 – 21 and 0 – 32% (Table 2.2).

Table 2.2 The effect of using different numbers of loci to estimate ancestry (q_i = the average proportion of *Acanthopagrus butcheri* genome), and q-threshold values (q = 0.05 – 0.20) to distinguish pure species and hybrids, on classification (the overall proportion of hybrids) of 565 *Acanthopagrus* spp. from 25 estuaries. The proportion of estuaries with hybrids, the range of the proportion of hybrids per estuary, and the geographic areas with the greatest proportions of hybrids are included. *

Number of loci used in the analysis Ê	q-threshold for distinguishing pure species and hybrids	Overall proportion of hybrids detected	Proportion of estuaries with hybrids	Range of the proportion of hybrids per estuary	Hybrids within New South Wales as a proportion of the total number of hybrids ^	Hybrids within estuaries in the area of sympatry as a proportion of the total number of hybrids ¤
4	0.05	0.27	0.68	0 – 0.80	0.68	0.42
	0.10	0.17	0.64	0 – 0.56	0.67	0.53
	0.15	0.13	0.64	0 – 0.48	0.64	0.55
	0.20	0.11	0.56	0 – 0.32	0.61	0.54
8	0.05	0.15	0.68	0 – 0.58	0.69	0.57
	0.10	0.09	0.52	0 – 0.42	0.73	0.65
	0.15	0.07	0.44	0 – 0.33	0.79	0.69
	0.20	0.05	0.44	0 – 0.21	0.73	0.60

*Estimates of ancestry ranging between 0 and 0.05, and 0.95 and 1.0 were used to classify each fish as either a pure *A. australis* or *A. butcheri* respectively, while fish with their estimate in between these values were classed as hybrid.
ÊFor the analysis with 4 loci, the most diagnostic loci were used [pAb2A5, pAb2D1, Acs1* & Acs3*].
^All NSW estuaries [Fo, Bo, Po, Me, Co & Cu].
¤Southern NSW estuaries [Me, Co & Cu].

2.3.3 Inferring the direction of hybridisation

Strikingly few of the 83 hybrids were seemingly simple F1's that displayed both *A. australis* and *A. butcheri* alleles at each locus. Instead most were either later generation hybrids or backcrosses. Most strikingly however, the genotypes of most of these fish (70 of 83) were more genetically similar to *A. butcheri* than *A. australis* ($q_i > 0.5$) implying that backcrossing most often involves the obligately estuarine *A. butcheri*, although I did identify some 13 hybrids whose genotypes were more similar to *A. australis* ($0.05 < q_i < 0.50$) (Fig. 2.7).

2.3.4 Inferring the pattern of mating

Examination of the mtDNA haplotypes of the 83 hybrid fish revealed that 95% of fish displayed the mtDNA haplotype of the species with which they shared the greater nuclear DNA similarity i.e., fish that received a q_i-value between greater than 0.5 and less than 0.95 possessed the mtDNA of *A. butcheri*. The three exceptions were two hybrids that on the basis of their microsatellite genotypes were similar to *A. australis* ($q_i < 0.5$) but displayed an *A. butcheri* mtDNA haplotype, and one hybrid that was similar to *A. butcheri* (based on microsatellites i.e., $q_i > 0.5$) that displayed the mtDNA of *A. australis* (all fish were caught in NSW – Cuttege Lake). For the majority of hybrids this implies that backcrossing usually involves the estuarine resident *A. butcheri* females and either hybrid or *A. australis* males. Restriction digests of mitochondrial DNA (mtDNA) revealed five composite haplotypes, of which two were specific to fish previously assigned to *A. butcheri* and three were specific to fish assigned to *A. australis*. For each species, however, more than 90% of fish displayed only one of these haplotypes (Appendix 1).

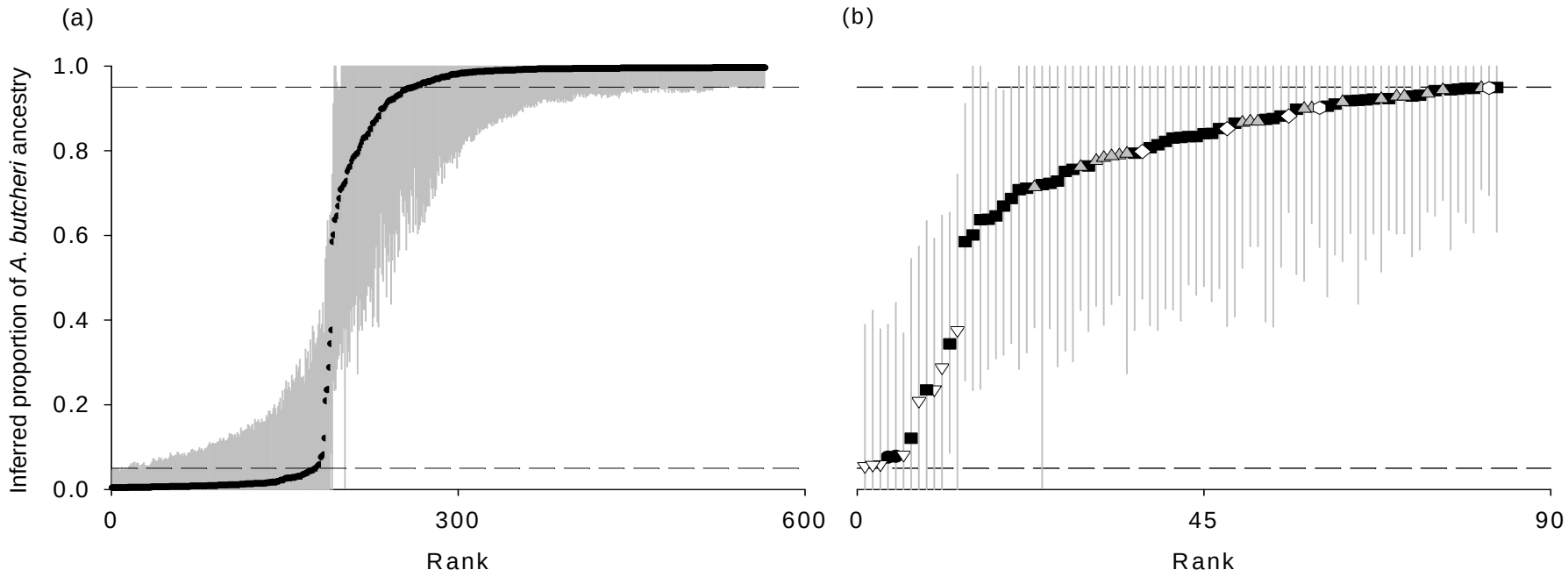

Figure 2.7 Ancestry (q_i) of all 565 *Acanthopagrus* spp., measured as the average (± 95% CI's) proportion of *A. butcheri* genome (based on eight microsatellite loci) (a). Estimates of ancestry between 0 and 0.05, and 0.95 and 1.0 (the lower and upper broken black line), I classified as pure *A. australis* and *A. butcheri* respectively, while all others were hybrids. The geographic distribution of hybrids (b); the majority (58/83) were in New South Wales estuaries (closed black squares [Meroo, Coila & Cuttege], open triangles [Forster, Port Hacking & Botany Bay]). Hybrids were also present in Queensland (closed circles), Tasmania (open diamonds [Swan & North West]), Victoria (closed grey triangles), and South Australia (open hexagons).

2.3.5 Morphological analysis

Although the overall appearance of *A. australis* and *A. butcheri* was similar, a PCA plot based on my detailed analysis of 26 morphological variables clearly distinguished two clusters of points corresponding to the species groupings identified using microsatellite data. There was considerable variation amongst individuals of *A. australis* reflected in a wide scatter of points along axis two. This is not entirely unexpected considering the diversity of environments *A. australis* inhabit; indeed, my collections encompassed various areas of the lower reaches of estuaries, at the extremes these included the highly marine influenced and exposed estuary bar and training wall, as well as protected seagrass meadows within estuaries themselves. As might be predicted from the genetic similarity of *A. butcheri* and the majority of the hybrids, there was little difference in morphology between hybrids and *A. butcheri*. Based on the PCA plot it would be difficult, perhaps impossible, to separate *A. butcheri* from hybrids based solely on morphology (Fig. 2.8). Unfortunately there were no *A. australis*-like hybrids in my collections preventing comparison.

2.3.6 Genetic diversity estimates for groups indentified by admixture analysis: *A. australis, A. butcheri* and *A. butcheri*-like hybrids

I calculated standard measures of genetic diversity for the separate groupings identified with the assignment test. Although analysis of the microsatellite data set revealed clear allele frequency differences between species, 79 of 147 alleles across the eight loci were common to both species. *Acanthopagrus australis* was the most diverse with an average (± SE) expected heterozygosity of 0.815 (0.037) compared to 0.610 (0.084) for *A. butcheri*, and with nearly twice as many alleles per locus. Of 68 private alleles i.e.,

alleles unique to a particular taxon, 57 were detected within *A. australis*. In general, *A. butcheri* backcrosses (hybrids) had both fewer alleles per locus and less heterozygosity than parental *A. australis*, but greater numbers of alleles and more heterozygosity than *A. butcheri* (Table 2.3).

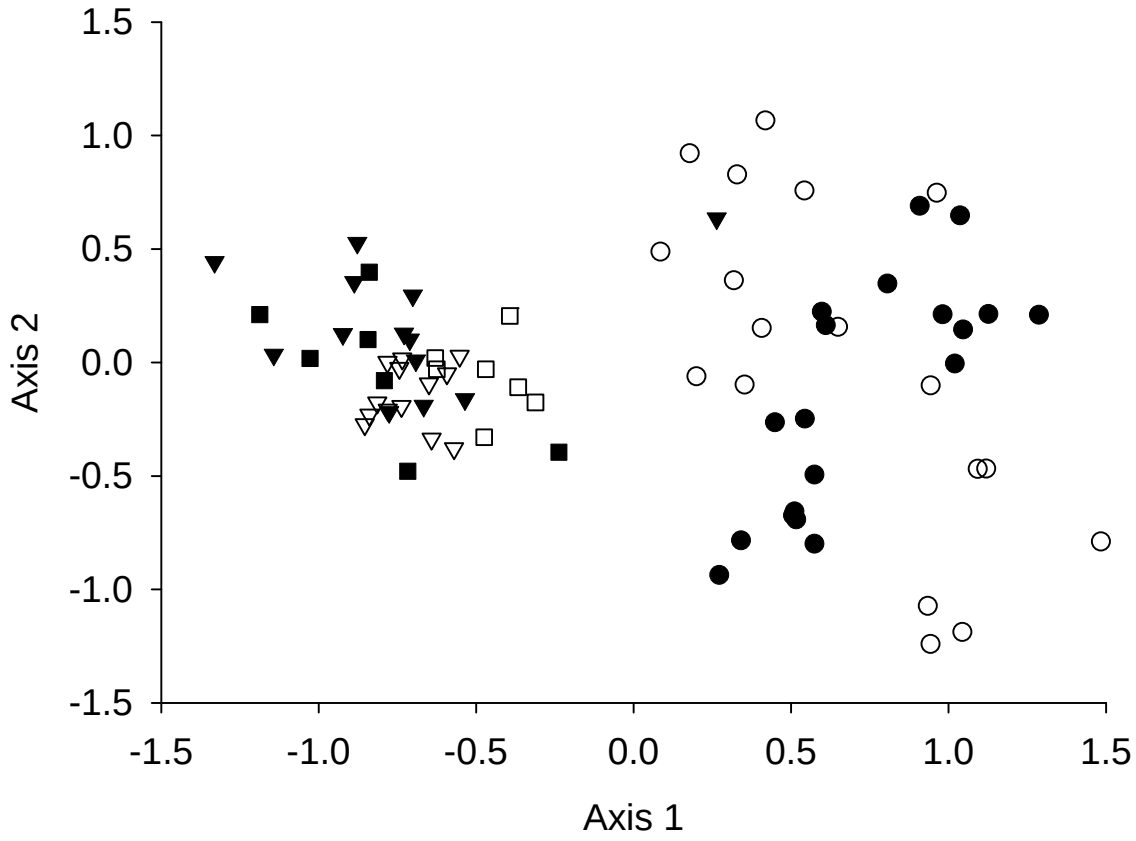

Figure 2.8 Principal coordinates analysis based on 26 meristic and body ratio measurements used to compare the morphology (closed symbols) of *Acanthopagrus* spp. (n = 40). The ancestry was estimated and each fish was classified as either *A. australis* (circles), *A. butcheri* (triangles), or their hybrid (squares [*A. butcheri* backcrosses or later generation hybrids]). The genetic relationship, based on FCA analysis of the eight locus genotype of all individuals is also displayed (open symbols).

Table 2.3 Number of alleles (A), Allelic richness (A_R), number of private alleles (alleles unique to a particular group) (P_A), observed heterozygosity (H_o) and Nei's 1973 expected heterozygosity (H_e) for *Acanthopagrus australis* (N = 178), *A. butcheri* (N = 304) and their hybrids (N = 73).

	Locus								
	pAb2B7	pAb2A5	pAb2D1	Acs1*	Acs3*	Acs6*	Acs-16*	Acs-21*	Mean (± SE)
A. australis									
A	18	19	6	11	21	14	29	21	17.4 (2.5)
A_R	15.9	16.8	5.4	9.6	17.4	12.4	24.4	17.0	14.8 (2.0)
P_A	2	15	1	7	14	3	13	2	7.1 (2.1)
H_o	0.84	0.64	0.61	0.71	0.70	0.71	0.88	0.87	0.75 (0.04)
H_e	0.89	0.89	0.62	0.74	0.81	0.76	0.93	0.88	0.83 (0.04)
A. butcheri									
A	17	4	5	6	9	12	17	10	10 (1.8)
A_R	14	3.5	3.9	4.8	5.5	9.9	14.3	8.6	8.1 (1.5)
P_A	1	0	0	2	2	1	1	1	1.0 (0.3)
H_o	0.75	0.48	0.09	0.64	0.52	0.70	0.85	0.50	0.56 (0.08)
H_e	0.82	0.49	0.12	0.72	0.55	0.76	0.85	0.58	0.61 (0.08)
Hybrid									
A	16	8	5	10	15	10	18	13	11.9 (1.6)
A_R	15.9	7.9	5.0	9.9	14.9	10.0	18	13	11.8 (1.5)
P_A	0	0	1	0	1	1	0	0	0.4 (0.2)
H_o	0.82	0.45	0.52	0.70	0.75	0.78	0.79	0.56	0.67 (0.05)
H_e	0.89	0.50	0.62	0.77	0.74	0.83	0.83	0.67	0.73 (0.05)

2.3.7 Testing the capacity of the microsatellite markers to distinguish different classes of hybrids

I used simulated crosses to test the capacity of the microsatellite markers to distinguish the different classes of hybrids potentially contained within lakes and lagoons. Although this analysis does confirm that I am able to distinguish hybrids from parentals, based on the estimates of ancestry (q_i) of simulated data it is clear that using microsatellite data alone, my data has relatively low power to accurately classify individual fish into distinct classes of hybrids (i.e., F1's, F2's, BC1) (Fig. 2.9 a – d). Across all four samples, *accuracy* of identification of specific hybrid or pure species classes often fell below 100%, so accordingly *efficiency* was also never 100%. The cause of this was that in a few rare cases *A. australis* and *A. butcheri* BC1's had their estimate of ancestry within the range I arbitrarily chose to represent pure parentals, and the converse was true as well. There was also great variation in my estimates of q_i for F1, F2 and BC1's. Because there was a high proportion of shared polymorphism (79/147 alleles) and few fixed differences between the species, I would not expect, for example, all F1's to display a q-value of ~0.5 (as I would predict for a cross between parental genotypes fixed for alternate alleles). Indeed, the estimates of F1's generally ranged between ~0.2 and 0.7, and this range overlaps values obtained for both F2 and BC1 hybrids (Table 2.4). Taken together this implies that I may in fact have misidentified some pure species fish as hybrids (and vice versa), though this should account for only a small proportion of the total sample because average overall *efficiency* (± S.E) of identification was 0.95 (0.04, 0.02) for both parentals and hybrids.

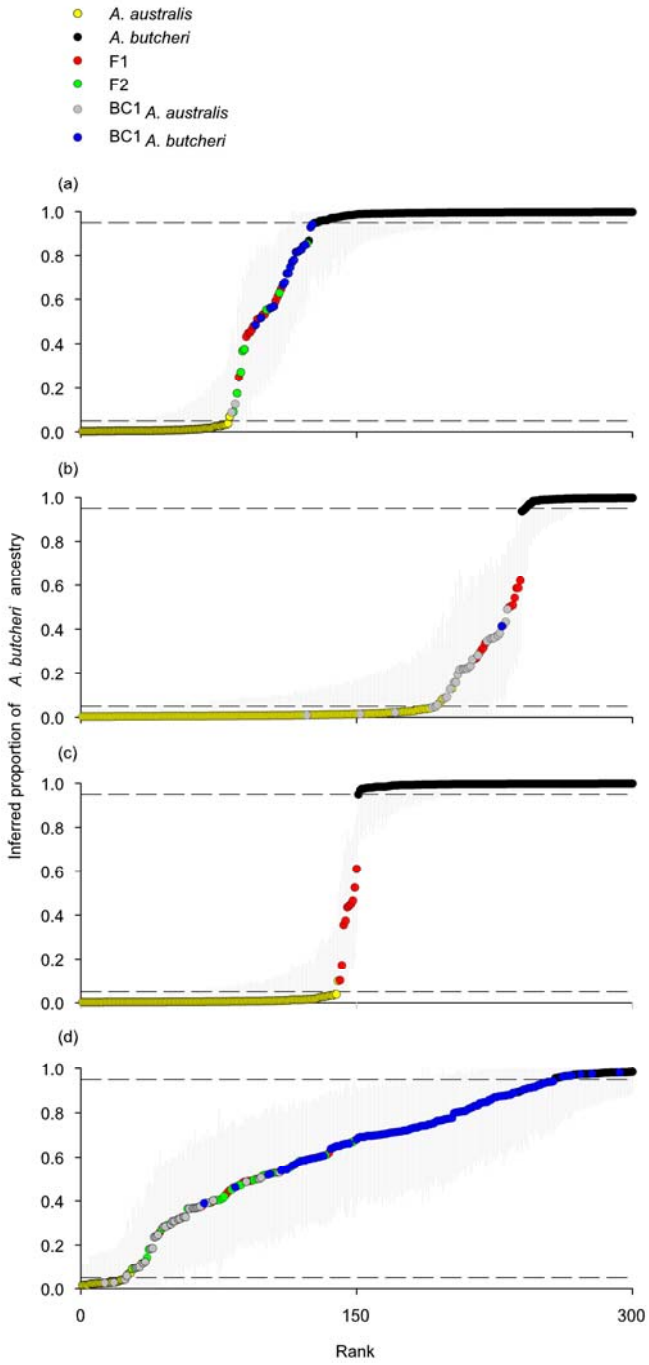

Figure 2.9 Estimates of ancestry for simulated genotypes of each of the six possible 'genotypic classes' of parentals and hybrids that are possible for two generations of matings between two species.

Table 2.4 The inferred ancestry (q_i) of simulated *Acanthopagrus* spp., measured as the average (± 95% CI's) proportion of *A. butcheri* genome (based on eight microsatellite loci). *

Genotype Class	N [E]	Range of q_i	Average q_i	*Accuracy* [^]	*Efficiency* [¤]
Sample 1					
$P_{A.\ australis}$	81	0.003 – 0.053	0.009	0.988	
$P_{A.\ butcheri}$	175	0.905 – 0.997	0.993	0.994	0.992
F1	15	0.234 – 0.690	0.507	1.000	
F2	8	0.070 – 0.863	0.410	1.000	
$BC1_{A.\ australis}$	2	0.060 – 0.124	0.092	1.000	
$BC1_{A.\ butcheri}$	19	0.497 – 0.986	0.755	0.895	0.955
Sample 2					
$P_{A.\ australis}$	195	0.003 – 0.303	0.014	0.970	
$P_{A.\ butcheri}$	61	0.954 – 0.997	0.992	1.000	0.977
F1	12	0.270 – 0.621	0.451	1.000	
F2	2	0.122 – 0.354	0.238	1.000	
$BC1_{A.\ australis}$	29	0.008 – 0.486	0.213	0.793	
$BC1_{A.\ butcheri}$	1	0.408	0.408	1.000	0.864
Sample 3					
$P_{A.\ australis}$	140	0.002 – 0.094	0.008	0.993	
$P_{A.\ butcheri}$	150	0.966 – 0.998	0.995	1.000	0.997
F1	10	0.069 – 0.607	0.403	1.000	1.000
Sample 4					
$P_{A.\ australis}$	25	0.015 – 0.115	0.035	0.840	
$P_{A.\ butcheri}$	45	0.869 – 0.984	0.961	0.800	0.814
F1	28	0.259 – 0.675	0.477	1.000	
F2	32	0.090 – 0.701	0.452	1.000	
$BC1_{A.\ australis}$	35	0.025 – 0.530	0.273	0.914	
$BC1_{A.\ butcheri}$	135	0.39 – 0.981	0.757	0.948	0.957

*Each sample of 300 individuals contained up to six different genotype classes in different proportions.

[E]Number of each genotype class per simulation.

[^], [¤]Accuracy was scored as the ratio of the number of correctly assigned individuals per genotype class to the total number per class. *Efficiency* scored as the ratio of the number of correctly assigned individuals per group (i.e., parental vs. hybrids) to the total number per group.

2.4 Discussion

My study demonstrates the importance of lakes and lagoons (estuaries) as sites of hybridisation for a pair of migratory coastal and obligately estuarine species. Estuaries provide similar zones of contact for a range of species worldwide (Beheregaray & Sunnucks 2001; Pampoulie *et al*. 2004; Watts & Johnson 2004), leading me to predict that this phenomena is more widespread and is likely to be exacerbated by increased effects of anthropogenic disturbance (Waples *et al*. 2008). Indeed, this situation may bear remarkable similarity to hybridisation and introgression that has recently been described for highly mobile invasive plant species, or introduced freshwater and anadromous fish species, that are impacting on native species through hybridisation, with consequent demographic and genetic swamping (Ayres *et al.* 1999, 2004; Scribner *et al*. 2000; Allendorf *et al*. 2004, 2005). Here, I was able to demonstrate that it is possible to characterise pure species and hybrid bream using nuclear and cyctoplasmic DNA markers, although separation of their groupings based on morphology is extremely difficult, and as for most hybrid zones, separation of different classes of hybrids is problematic.

2.4.1 Characterisation of the hybrid zone

The combination of microsatellite and mitochondrial DNA markers allowed me to distinguish hybrids from each of their parental species. I was able to demonstrate that the great majority of hybrids are both more closely related to *A. butcheri* and display *A. butcheri* mtDNA haplotypes. Although much remains to be determined about the factors that facilitate hybridisation in this system, these findings clearly imply that most hybridisation and backcrossing involve the obligately estuarine *A. butcheri* females. This pattern matches what is known of the reproductive synchronicity of these species since

'ripe' *A. australis* males are thought to have the potential to overlap the spawning period of *A. butcheri* whereas *A. australis* females are typically 'spent' when *A. butcheri* spawning occurs. The presence of a small number of *A. australis* like hybrids with *A. australis* mitochondrial haplotypes however, also demonstrates that hybridisation can proceed through *A. australis* females. The limitation of my data set, as is typical of most attempts to formally distinguish different classes of hybrids is that I cannot with confidence distinguish the majority of F1, backcross and later generation hybrids (see Boecklen & Howard 1997 for discussion).

2.4.2 Distribution of hybrids

As might be expected the hybrids were not evenly distributed over the geographical range of the species but rather were concentrated within the area of greatest sympatry. Interestingly, within southern NSW three estuaries that were most similar in terms of their physical, hydrodynamic, and habitat characteristics (information can be found at: http://www.naturalresources.nsw.gov.au/estuaries/inventory/index_ns.shtml) accounted for 57% of the overall number of hybrids detected. Future work will be needed to determine whether the high incidence of hybrids in this zone of greatest species overlap reflects a greater opportunity for hybridisation within this area or alternatively whether characteristics of these waterways favour hybridisation and hybrid survival. As in other hybrid zones, selection favouring certain genotypes or local adaptation to some environmental or ecological factor may play a role in the differential survival of genotypic classes (e.g. Harrison 1986; Lexer *et al.* 2003; Gross *et al*. 2004). In this instance, the apparent *A. butcheri* backcrosses and/or later generation hybrids appear to be favoured as other classes such as the putative *A. australis* backcrosses (i.e., fish that both had their q_i based on

microsatellites < 0.5 and possessed mtDNA characteristic of *A. australis*) were detected relatively rarely (n = 13) and only at northern locations in strongly marine influenced lower reaches of permanently open estuaries.

Chapter 3 Panmictic population structure in the migratory marine Sparid *Acanthopagrus australis* despite its close association to estuaries

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3.1 Introduction

Mobile marine organisms such as fish may be expected to form large populations strongly interconnected by dispersal and hence display little genetic subdivision. However recent studies have highlighted both the surprisingly restricted dispersal of many marine taxa (Jones *et al*. 1999; Taylor & Hellberg 2003; Jones *et al*. 2005; Almany *et al*. 2007) and consequent high level of population subdivision even within taxa with long-lived larva or mobile adults (Ayre & Duffy 1994). Many factors including ocean currents (Cowen *et al*. 2000; James *et al*. 2002), coastal features such as estuaries or embayments (Watts & Johnson 2004), and complex larval and/or adult behaviour (Gerlach *et al*. 2007; Dixson *et al*. 2008) have been implicated. Hence, predictions that the life-history of fish should result in widespread dispersal must always be treated with caution.

On the east coast of Australia *A. australis* is considered highly mobile and treated as a single (fisheries) stock ranging over more than 2000 km from southern QLD to the NSW and VIC state border (NSW DPI, unpublished). Indeed, two studies have shown bream,

which the authors assume were *A. australis* rather than the estuary restricted *A. butcheri*, tagged within both central and northern NSW estuaries were recaptured in locations in southern QLD (Henry 1983; West 1993). However, aspects of the life-history of *A. australis*, and eastern Australian oceanographic conditions, suggest that populations could be genetically subdivided. Although adults are thought to migrate northwards to spawn and pelagic larvae are thought to be dispersed over a wide area by the southward flowing EAC, *A. australis* may display spawning site fidelity. Indeed the adults of many species of fish return to their place of birth to spawn (i.e., natal site spawning) (e.g., Thorrold *et al*. 2001; Svedäng *et al*. 2007; Rooker *et al*. 2008). Spawning behaviour is relatively unstudied in *A. australis*, though spawning is thought to occur in entrance channels or lower reaches of coastal lakes and lagoons or in the surf zone of beaches adjacent to the entrances of estuaries (Pollock 1982, 1984). This close association with estuarine spawning sites has the potential to promote fine-scale genetic differentiation of both adults and juveniles if *A. australis* lineages maintain prolonged associations with individual estuaries. Alternatively if spawning is simply opportunistically associated with estuaries in general, and larvae are mixed and transported over a wide area by the EAC, then I would expect little or no population subdivision.

The dispersal potential of *A. australis* could have serious implications for its estuary restricted congener, as on the SE corner of Australia where the two species distribution is overlapping *A. australis* have made a major contribution to the genotypes of *A. butcheri* through hybridisation and introgression (Roberts *et al*. 2009). Here I use population genetic data to test the prediction that spawning site fidelity promotes fine-scale population subdivision in *A. australis*. Specifically, I compare the degree of genetic differentiation of

sets of *A. australis* recruits within different estuaries with that of *A. australis* adults caught near estuaries but on the open coast. Since little is known of the reproductive biology of *A. australis*, including whether both sexes undertake spawning migrations, I used a combination of nuclear (microsatellite) and mtDNA markers to examine population structure. Both my microsatellite and mtDNA markers provide sensitive tests of population differentiation and because mtDNA is typically maternally inherited I would expect greater mtDNA differentiation if only males migrate to spawn and females remain close to their natal spawning sites.

3.2 Methods

I examined microsatellite genotypes for 30 juvenile *A. australis* from 2 or 3 sites, for each of three lagoons on the NSW south coast, Tuross, Corunna and Wallaga Lakes separated by up to 50 km (total n = 240 fish). I also genotyped sets of ocean caught adults from each of three locations: the Gold Coast (QLD) (n = 40), Forster (n = 40) and Botany Bay (n = 30) (NSW) (total n = 110) (Fig. 3.1). These fish were included to compare the genetic similarity of juvenile bream on the south coast of NSW, to adult bream from throughout the described range of the species.

Because it is possible that there is gender dependent variation in patterns of dispersal or the degree of philopatry I also compared patterns of sequence variation for the control region of maternally inherited mtDNA. I gathered mtDNA data for between 6 and 11 juveniles (average ± SE = 9.0 ± 1.5) per lagoon on the south coast, as well as between 6 and 8 adult bream (6.7 ± 0.7) per ocean site on the north coast (total n = 47). The microsatellite and mtDNA markers and the PCR cycling conditions are described in

Chapter 2. The sequencing reactions were performed at Macrogen Inc. (Korea) (information can be found at http:\\ www. macrogen.com) using a Big Dye™ terminator cycle sequencing kit (Applied Biosystems).

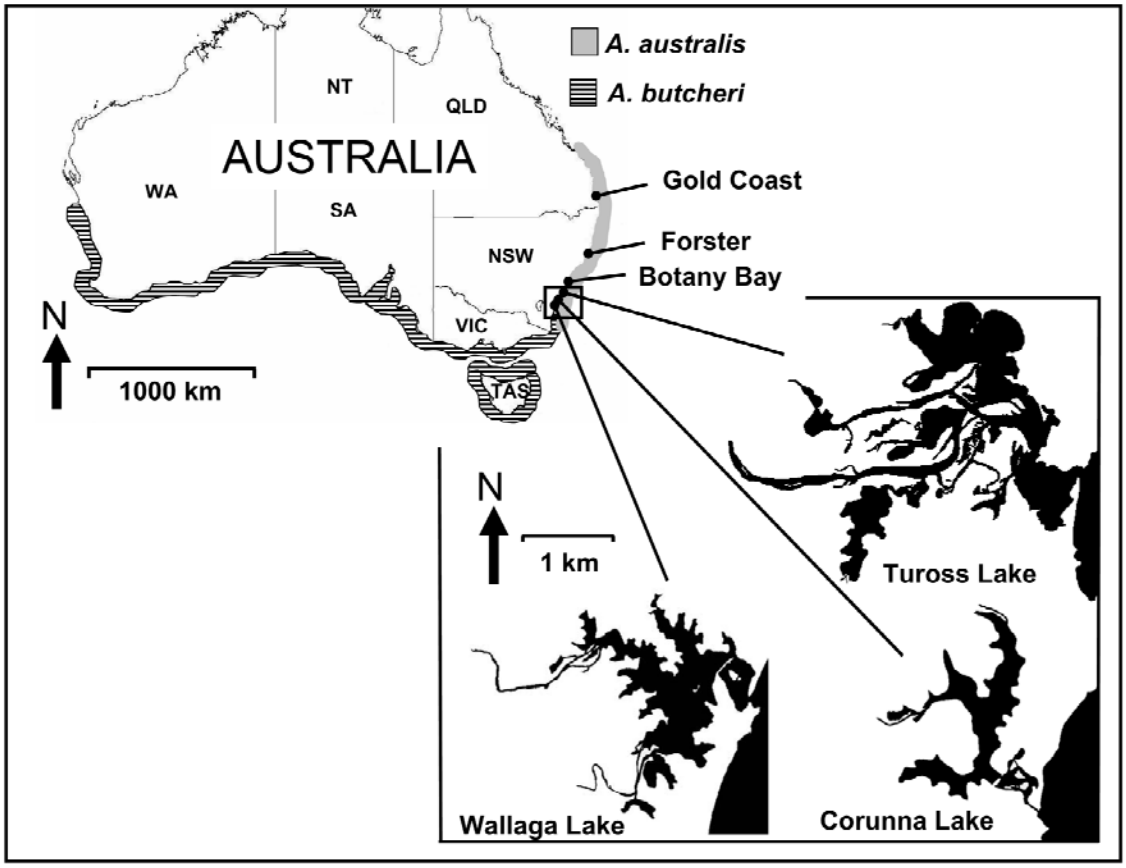

Figure 3.1 The distribution of *Acanthopagrus australis* and its estuary restricted congener *A. butcheri*, and the location of sampling sites for juvenile (Tuross, Corunna & Wallaga Lakes) and adult (Gold Coast, Forster & Botany Bay) *A. australis*.

3.2.1 Genetic analyses

3.2.1.1 Microsatellites

For each site within a lagoon I calculated the average number of alleles per locus, and the average observed and expected heterozygosity (using POPGENE Yeh *et al*. 1999). I estimated Weir & Cockerham's (1984) formulations of Wright's (1969) *F*-statistics, using the program TFPGA (Miller 1997).

Because incorrect interpretation of microsatellite data can occur when there are genotyping errors associated with null alleles (e.g., Pemberton *et al*. 1995), stutter bands on account of replication slippage during PCR (Shinde *et al*. 2003), and/or large allele dropout (Wattier *et al*. 1998) I determined if the data were affected by these potential sources of error using the program Micro Checker (van Oosterhout *et al*. 2004). Separate analyses on the overall collection of juveniles and adults for each lagoon and ocean site revealed that two loci (pAb2A5 & Acs3*) had apparent large excesses of homozygotes consistent with null alleles. Although a small number of controlled crosses involving *A. butcheri* × *A. butcheri*, and *A. australis* × *A. butcheri* previously revealed simple Mendelian inheritance and no evidence of null alleles for the eight microsatellite loci (4 pairs; 30 larvae per pair) (Chapter 2), here I have opted to present the results based on just six loci acknowledging the possibility of null alleles at pAb2A5 and Acs3* in *A. australis*. I did additional analyses that included pAb2A5 and Acs3* and these confirmed that including these loci made no substantive difference to the results and thus my interpretations and conclusions (data not presented).

My hierarchical sampling of juveniles within south coast lagoons allowed me to partition genetic variation (based on microsatellites) into separate variance components. In the present study the hierarchical levels in the data were denoted by F_{SL} and F_{LT}, and represent respectively genetic differentiation among sites within a specified lagoon, and among lagoons relative to the total. F_{IS} and F_{IT} are presented in the conventional way and are a measure of the deviation from Hardy-Weinberg expectations within subpopulations (i.e., lagoons) and in the total population (sample) respectively. The estimates were based on microsatellite allele frequencies for individual loci, and as an average across loci. Bootstrapping and jacknifing over loci were used to estimate standard deviations and 95% CI's. Comparable *F*-statistics for ocean caught adults were also included, though my sampling did not permit a hierarchical assessment of population differentiation. I tested for differentiation of adult and juvenile *A. australis* by calculating F_{ST} among the pooled sets of adult and juvenile fish. Finally, to visually display any geographic structuring within my microsatellite data set I performed Factorial Correspondence Analysis (FCA) (in GENETIX 4.03 [Belkhir *et al*. 2002]) on the pooled sample of juveniles from each lagoon together with the sample of ocean caught adults.

3.2.1.2 Mitochondrial DNA

I obtained 400 bps of sequence data for the mtDNA control region of 20 adult and 27 juvenile *A. australis*. I discarded the first 50 and last 30 bps of sequence due to the presence of some ambiguous or unresolved sites. The remaining 320 bps of sequence I aligned using Clustal W (Thomson *et al*. 1994) implemented in the software Bioedit (Hall *et al*. 1999). For each lagoon, I estimated haplotype (h) and nucleotide diversity (π). I estimated F_{ST} for all pairwise comparisons of juveniles and adults, and determined the level

of statistical significance of each comparison with 10 000 permutations. The analysis was performed in Arlequin (Excoffier *et al*. 2005) and was based on the haplotype frequencies.

3.3 Results

3.3.1 Microsatellites

The overall collection of juvenile *A. australis* comprised a genetically diverse group with little evidence of genetic subdivision. Across all samples, numbers of alleles per locus ranged between 9 and 30, and the average (± SE) was 17.7 (± 3.0). I detected similar levels of genetic variation across all sites within each lagoon. The mean number of alleles per locus ranged between 11.3 and 13.0, while mean observed heterozygosity was greater than 0.75 for each site (range = 0.76 – 0.83). Between 64.2 and 73.6% of all alleles were present at each site. Private alleles (alleles unique to a single site) were extremely rare (16/106 alleles detected), and when they did occur they were found at low frequency (< 0.05). Private alleles were distributed evenly among sites within lagoons (Table 3.1). Allele frequencies are presented in Appendix 3.

My estimates of genetic variation among sites within lagoons F_{SL} (0.001 ± 0.002) and among lagoons F_{LT} (0.002 ± 0.002) were extremely low and not significantly different from zero (as judged by 95% CI's) indicating that there was no genetic subdivision at either spatial scale. My estimates of F_{IS} and F_{IT} were also consistently close to zero (0.021 ± 0.008 and 0.022 ± 0.009 respectively) which together with the lack of spatial variation in allele frequencies implies a single outcrossed and panmictic population (Table 3.2).

Table 3.1 Mean (± SE) number of alleles (A), number of private alleles (alleles unique to a particular site) (P_A), observed heterozygosity (H_o) and Nei's 1973 expected heterozygosity (H_e) based on six microsatellite loci, for juvenile (representing the same year of birth) *Acanthopagrus australis* in three coastal lagoons in southeastern Australia. *

	Tuross			Corunna			Wallaga	
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2
A	11.3	11.8	11.5	11.8	11.7	11.3	11.7	13.0
	(1.9)	(2.1)	(2.6)	(2.3)	(2.0)	(1.7)	(2.2)	(1.8)
A_P	0.33	0.50	0.00	0.17	0.50	0.50	0.00	0.67
	(0.21)	(0.22)	(0.00)	(0.17)	(0.22)	(0.22)	(0.00)	(0.21)
H_o	0.833	0.788	0.806	0.778	0.759	0.817	0.811	0.783
	(0.092)	(0.107)	(0.184)	(0.136)	(0.090)	(0.089)	(0.103)	(0.161)
H_e	0.813	0.790	0.783	0.790	0.801	0.812	0.796	0.807
	(0.083)	(0.104)	(0.133)	(0.123)	(0.106)	(0.099)	(0.112)	(0.120)

*The sample size per site was 30 fish.

Table 3.2 Hierarchical F-statistics estimated for six microsatellite loci, and overall, for juvenile *Acanthopagrus australis* within three coastal lagoons. Genetic differentiation among sites within lagoons, and among lagoons, is denoted by F_{SL} and F_{LT}, respectively. F_{IS} and F_{IT} are presented in the conventional way and represent a measure of the degree of deviation from Hardy-Weinberg expectations within lagoons and within the total sample.

Locus	F_{IS}	F_{SL}	F_{LT}	F_{IT}
pAb2B7	0.045	0.004	0.003	0.049
pAb2D1	-0.020	0.002	-0.002	-0.018
Acs1*	0.031	0.006	0.011	0.037
Acs6*	0.033	-0.005	0.004	0.028
Acs-16*	0.014	0.001	-0.001	0.015
Acs-21*	0.013	-0.001	-0.004	0.012
Overall ± SD	0.021 ± 0.008	0.001 ± 0.002	0.002 ± 0.002	0.022 ± 0.009
95% CI	0.004 – 0.008	-0.001 – 0.004	-0.002 – 0.006	0.004 – 0.037

There was no population differentiation among samples of ocean caught *A. australis* from locations spread across the described range of the species (F_{ST} = 0.002 ± 0.001; 95% CI = 0.000 – 0.004) (Table 3.3).

Table 3.3 *F*-statistics estimated for six microsatellite loci, and overall, for ocean caught adult *Acanthopagrus australis*.

Locus	F_{IS}	F_{ST}	F_{IT}
pAb2B7	0.085	-0.005	0.085
pAb2D1	0.034	0.005	0.039
Acs1*	0.059	0.000	0.059
Acs6*	0.034	0.005	0.039
Acs-16*	0.035	0.001	0.036
Acs-21*	0.024	0.001	0.026
Overall ± SD	0.046 ±0.010	0.002 ± 0.001	0.047 ± 0.010
95% CI	0.031 – 0.066	0.000 – 0.004	0.033 – 0.066

Perhaps not surprisingly the FCA plot used to compare the genetic similarity of individual juveniles within lagoons on the south coast to north coast ocean-caught adults revealed a wide scatter of points along both axes but with the plotted positions of points for the juveniles and adults overlapping (Fig. 3.2). None of the sets of juveniles or adults formed clusters of individuals from the same sampling location. Moreover, F_{ST} based on comparison of the overall data sets for adult and juvenile fish revealed no differentiation (F_{ST} = 0.000) (Table 3.4).

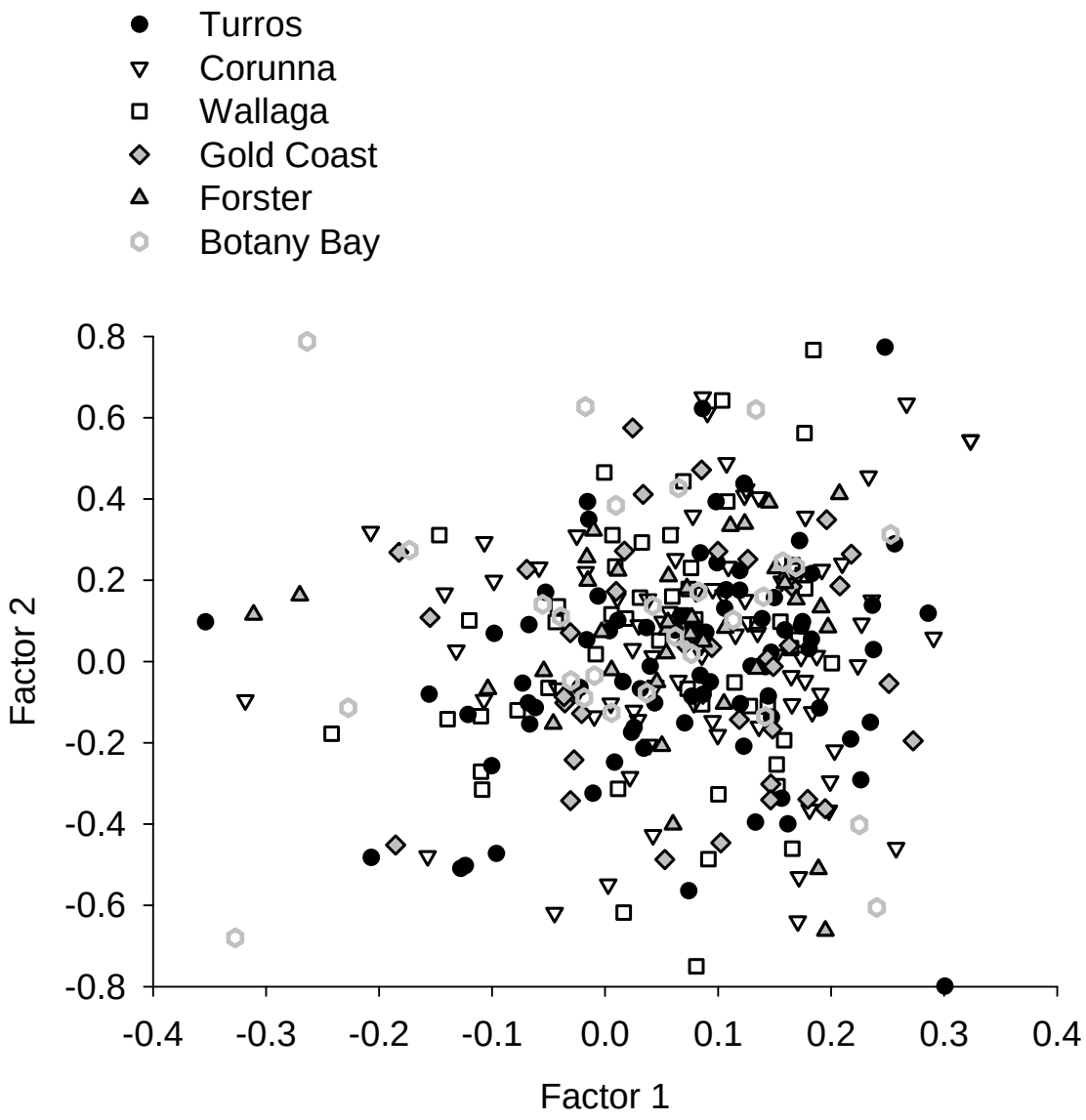

Figure 3.2 Factorial correspondence analysis based on the pairwise genetic distance matrix of all 350 *Acanthopagrus australis* (n = 240 juveniles [Tuross, closed black circles; Corunna, open triangles & Wallaga, open squares] n = 110 adults [Gold Coast, closed grey diamonds; Forster, closed grey triangles & Botany Bay, open grey circles]). The juveniles were caught within coastal lagoons, while the adults represent ocean caught fish.

Table 3.4 *F*-statistics estimated for six microsatellite loci, and overall, for the pooled sets of juvenile and adult *Acanthopagrus australis*.

Locus	F_{IS}	F_{ST}	F_{IT}
pAb2B7	0.059	-0.001	0.059
pAb2D1	-0.001	0.001	-0.001
Acs1*	0.041	-0.003	0.039
Acs6*	0.031	-0.001	0.030
Acs-16*	0.021	0.001	0.022
Acs-21*	0.017	-0.001	0.016
Overall (± SD)	0.029 ± 0.008	-0.001 ± 0.001	0.029 ± 0.008
95% CI	0.015 – 0.045	-0.002 – 0.001	0.015 – 0.044

3.2.2 Mitochondrial DNA

As for my survey of nuclear microsatellite loci, the variation in mtDNA control region sequence revealed that *A. australis*, as a species, is genetically diverse but with little genetic variation among sites. Overall, I found 39 polymorphic sites which defined 31 haplotypes. Gene diversity (h ± SD) was 0.949 ± 0.021, while corrected average nucleotide diversity (π ± SD) was 0.013 ± 0.007. There was virtually no variation in haplotype and nucleotide diversity both among lagoons and ocean sites, for both juveniles and adults. Gene diversity (h) ranged between 0.929 and 1.00, while π ranged between 0.009 and 0.019 (Table 3.5). Estimates of F_{ST} for the mtDNA data revealed virtually no genetic differentiation among any pair of sites (range = 0.000 – 0.036). Moreover, none of the F_{ST} values were statistically significantly different from zero, as revealed by a permutation test ($P > 0.05$) (Table 3.6).

Table 3.5 Genetic diversity for *Acanthopagrus australis* juveniles (Tuross – Wallaga) and adults (Gold Coast – Botany Bay), based on 320 bps of the mtDNA control region. The sample size (N), number of haplotypes detected (N_H), haplotype diversity (h), and nucleotide diversity (π) is shown.

Location	N	N_H	$h \pm$ SD	$\pi \pm$ SD
Gold Coast	6	5	0.933 (0.122)	0.010 (0.007)
Forster	8	6	0.929 (0.084)	0.015 (0.009)
Botany Bay	6	5	0.933 (0.122)	0.009 (0.006)
Tuross	10	8	0.956 (0.059)	0.016 (0.009)
Corunna	6	6	1.000 (0.010)	0.019 (0.012)
Wallaga	11	10	0.982 (0.046)	0.014 (0.009)

Table 3.6 Estimates of pairwise F_{ST} for juvenile and adult *Acanthopagrus australis* based on mtDNA haplotype frequencies. *

	Tuross	Corunna	Wallaga	Gold Coast	Forster	Botany Bay
Tuross	-	NS	NS	NS	NS	NS
Corunna	0.022	-	NS	NS	NS	NS
Wallaga	-0.032	-0.023	-	NS	NS	NS
Gold Coast	0.022	0.006	-0.033	-	NS	NS
Forster	-0.042	0.036	-0.023	0.027	-	NS
Botany Bay	-0.011	0.033	-0.033	-0.017	-0.014	-

*NS not significant

3.4 Discussion

My findings demonstrate that despite potential for population subdivision suggested by the close association of *A. australis* with estuaries and unidirectional flow of the EAC this species forms a single genetically diverse and genetically homogeneous population. These results are consistent with the predicted great mobility of this species that has been inferred from tagging studies (Henry 1983; West 1993) and implies that fish have no

persistent associations with their natal spawning sites or nursery estuaries. Indeed, all populations were genotypically diverse and genetically homogenous over spatial scales ranging from several hundreds of meters to several hundreds of kilometres. Estuaries can provide unique opportunities for divergence (see Watts & Johnson 2004 for review), and indeed there are many examples where species that are otherwise 'good dispersers' (i.e., mobile species, and species with dispersive pelagic larvae) exist as isolated, self-seeding populations within estuaries (Johnson 1986; Gold *et al*. 1999; Meggs *et al*. 2003). My data show however, that despite the acknowledged association of adult *A. australis* with estuaries as potential feeding and spawning sites, dispersal is clearly sufficient to prevent any geographic differentiation of *A. australis* populations.

The complete lack of spatial variation in allele frequencies for collections of ocean-caught adult, and juvenile, *A. australis* from several estuaries suggests that this species effectively forms a single large panmictic population on Australia's east coast. My data for ocean-caught adults alone could reflect the post-spawning migration and possible mixing of adults from genetically distinct estuary associated subpopulations (i.e., marine admixture). Species that display strong philopatry and consequent genetic subdivision of spawning aggregations often forage over, or migrate, vast distances before returning to natal-sites to spawn. The limitation of my data set based on ocean-caught adults alone is therefore that I am unable to distinguish between range-wide genetic homogeneity and marine admixture of a set of genetically distinct estuary associated subpopulations. However, the genetic homogeneity of juveniles provides compelling evidence for a lack of any persistent genetic subdivision of populations attributable to an association with estuaries.

My data based upon analysis of both nuclear (microsatellite data) and mitochondrial DNA are consistent with what is known of the breeding biology of *A. australis*, and suggest that both the northwards migration of adults and southward dispersal of larvae via the EAC provide sufficient mixing to prevent population differentiation. The EAC is the major ocean current on the east coast. It produces a reliable north – south flow of warm water originating in the tropics, but becomes progressively weaker and less reliable from about latitude 33° S (central NSW) as in this area the majority of the flow is deflected seaward (Godfrey *et al*. 1980; Marchesiello & Middleton 2000; Ridgeway & Dunn 2003). Several authors have speculated on the EAC's effectiveness in transporting larvae to the far south coast of eastern Australia (Ayre 1990; Ayre *et al*. 1991; Billingham & Ayre 1996; Murray-Jones & Ayre 1997), however, genetic analysis of population differentiation often reveal genetic homogeneity, for widely separated collections of species with long lived pelagic larvae, including several species that are relatively immobile as adults because they exhibit site fidelity or exist attached to, or within, benthic substrata (Banks *et al*. 2007; Curley & Gillings 2009; though see Sherman *et al*. 2008a; Miller & Ayre 2008). This is most simply explained by a high degree of population connectivity maintained by dispersal of larvae within warm-core eddies of the EAC. Although the flow of the EAC is deflected seaward, eddies break-off and continue to flow southwards, sometimes reaching the Bass Straight between Tasmania and the Australian mainland (40 ° S) (Nilsson & Cresswell 1981; Bowen *et al*. 2005). Although the physical properties and southward penetration of these eddies vary greatly in space and time (Roughan & Middleton 2004; Bowen *et al*. 2005; Ridgeway 2007), with important implications for the growth, survival and transport of larvae, a recent survey of the frequency of occurrence and abundance of tropical (coral) reef fish in temperate subtidal rocky reef habitat in southern NSW highlights their

effectiveness in affecting larval transport (Booth *et al*. 2007). The authors reported recurring recruitment of a diverse set of reef fish along the NSW coast, remarkably this included at locations near the NSW and VIC state border (38°S), some 1700 km from the typical southern range limit of these fish on the Great Barrier Reef.

Chapter 4 Unexpectedly shallow divergence for a widespread estuarine fish, *Acanthopagrus butcheri*

4.1 Introduction

The coastline of southern Australia has undergone a series of dramatic changes over the last 25 000 years (Lambeck & Chappel 2001; Roy *et al*. 2001) with large numbers of estuaries located on ancient coastal plains having recent origins and biota that must typically have arisen from recent colonisation. Intriguingly however, many apparently estuary restricted species are also geographically widespread suggesting either that such species have altered their behaviour since colonisation or that they are more mobile than has been predicted.

A range of contemporary coastal, oceanographic, and ecological features contribute to the maintenance of three recognised marine biogeographical provinces in southern Australia; the Peronian (east), Flindersian (west) and Maugean (southeastern) (Bennett & Pope 1953; Knox 1963; O'Hara & Poore 2000; Hidas *et al*. 2007; Waters 2008). Phylogenetic studies on the SE corner indicate that phylogeographic structure is generally concordant with biogeographic structure, and that a point of division for eastern and western lineages occurs at Wilson's Promontory (WP) (Burridge 2000; Waters & Roy 2003; Waters *et al*. 2004; Dawson 2005; Waters *et al*. 2005; York *et al*. 2008), which also represents the eastern sill of the BI land bridge (Lambeck & Chappel 2001). Authors have disproportionately studied intertidal rocky shore invertebrates with dispersive pelagic larvae (though see Burridge 2000; Dawson 2005), and above all other possible explanations (i.e., contemporary oceanography) the deep east – west split of genetic lineages has been

interpreted as a consequence of ancient vicariance. More recently however, a comparative study by Ayre *at al*. (2009) revealed that for some exposed rocky shore specialists the strong phylogenetic structure is maintained by contemporary conditions namely a major biogeographic barrier directly northeast of WP– the Ninety Mile Beach which represents a vast area that generally lacks rocky intertidal habitat (though see Hidas *et al*. 2007). As for many species whose ranges apparently span the SE corner (Edgar 2000), the affect of this complex coastal region on the genetic structure of populations of estuary restricted species has yet to be investigated.

Across southern Australia *A. butcheri* has an uncertain origin or history of colonisation. Its distribution spans the SE biogeographic barrier, but in addition a notable lack of estuarine habitat in the area representing the GAB (a ~1000 km stretch of exposed cliffs and rocky shore) between SA and WA suggests the species is rare or absent in this area and that this area may conceivably represent a major contemporary biogeographic barrier for the species.

Earlier genetic studies of population differentiation in *A. butcheri* have revealed conflicting results, as the implied spatial extent over which *A. butcheri* disperse varies between locations (see Chapter 1). Although this difference in population structure may be explained simply by poorly documented life-history variation or more frequent estuary flooding promoting dispersal in southeastern Australia, both earlier allozyme and microsatellite studies of southeastern *A. butcheri* are potentially confounded by the presence of large numbers of hybrids between *A. butcheri* and its migratory marine

congener, *A. australis* (Chapter 2) Such hybridisation provides the potential for increased gene flow among SE Australian estuaries.

The dynamic and transient nature of estuaries, coupled with ancient topographical alteration in the area representing the SE corner, the possibility that the GAB represents a major contemporary biogeographic barrier, and hybridisation involving *A. australis* means that predicting range wide population structure in *A. butcheri* is extremely difficult. Indeed, it might be predicted that different estuaries and drainage basins would support genetically differentiated populations of *A. butcheri*, but in addition that there would be an incremental affect of the physical barrier provided by the BI on divergence, such that distinct genetic lineages would exist both to the east and west of WP, and south in TAS. Moreover, the potential barrier provided by the GAB may mean that western *A. butcheri* are further subdivided. Alternatively, it might be predicted that the geographical distribution of lineages would overlap, reflecting secondary intergradation due to contemporary dispersal or retention of a signature of a recent colonisation or radiation of *A. butcheri* throughout its distributional range. Indeed, a shoreline reconstruction of the SE corner through the last glacial cycle suggests that when the relative sea-level had risen a sufficient amount it entered a large depression in the centre of the BI (i.e., the Bass Basin [BB]) from the west, adjacent to the Australian mainland, to form a vast coastal lagoon that reached to the northwest coast of TAS well before the land connection in the east was broken i.e., the eastern sill representing WP (see fig 4.1) (Lambeck & Chappell 2001). It seems probable that such a large expanse of potentially habitable coastal lagoon would have both created the conditions that promote rapid population growth and facilitated range expansion, for an estuary restricted species such as *A. butcheri*.

Since little is known of the evolutionary history of *A. butcheri*, including whether distinct genetic lineages correspond to different geographical regions or the nature of colonisation of estuaries, here I have used a survey of mitochondrial DNA sequence variation of adult *Acanthopagrus* spp. from coastal lagoons, in a range wide phylogeographic study to examine phylogenetic structure. Mitochondrial DNA is typically maternally inherited so should not be subject to recombination by interspecific hybridisation, potentially providing a sensitive test of population differentiation across the 3000 km range of *A. butcheri*. Moreover, patterns of sequence differentiation potentially allow exploration of the timing and nature of population expansion across a set of estuaries that are considered to span at least two major biogeographic barriers. Here, I test the prediction that estuaries and geographic regions are genetically differentiated, but in addition that *A. butcheri* displays distinct eastern (i.e., east of WP) and western (west of WP) mtDNA lineages. Moreover, I test the prediction that a signature of spatial expansion persists within the mtDNA sequence of *A. butcheri*.

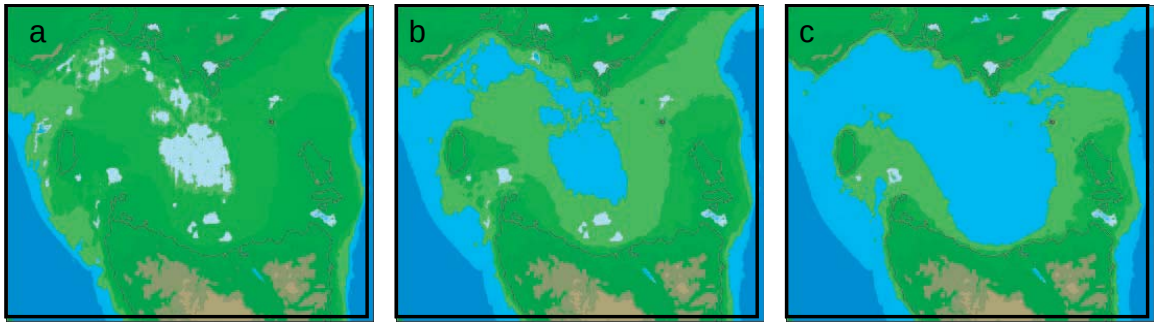

Figure 4.1 A shoreline reconstruction of southeastern Australia through the last glacial cycle (a) 25 000 years ago; (b) 17 500 years ago (c) 14 000 years ago (from Lambeck & Chappell 2001).

4.2 Methods

4.2.1 Study sites and sampling

I sequenced 400 bps of the 3' domain of the mtDNA control region of an average (± SE) of 10.8 (1.1) adult *Acanthopagrus* spp. (total n = 358) within each of 33 estuaries across the described range of *A. australis* and *A. butcheri* (Table 4.1, Fig. 4.2). Fish were essentially sampled 'blindly' with little indication of their likely species status. However, I expected *A. australis* would be found within fish caught in marine dominated northern rivers in QLD and NSW, and *A. butcheri* in lakes and lagoons in the far south and west (TAS, western VIC, SA & WA) beyond the described range of *A. australis*, while my previous genetic survey (Chapter 2) has revealed that *Acanthopagrus* spp. within estuaries in the area of sympatry are mostly *A. butcheri*-like hybrids or *A. butcheri* backcrosses with *A. butcheri* mtDNA haplotypes. The assignment of specific names to the specimens was conducted *a priori*, based on the mtDNA haplotype of each fish.

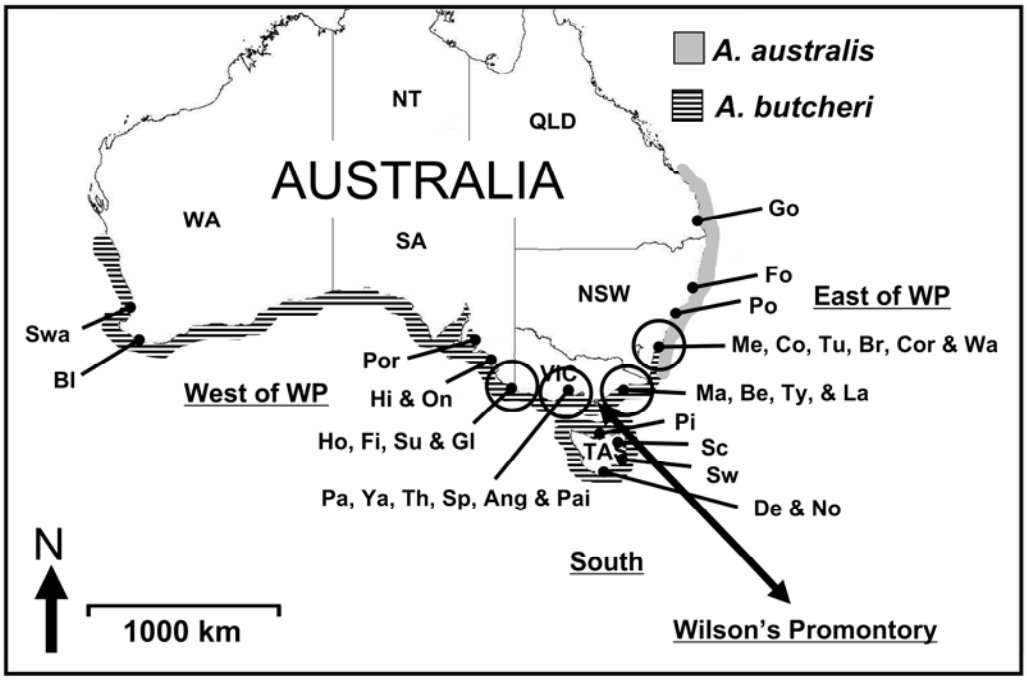

Figure 4.2 The location of study estuaries (see Table 4.1 for further explanation).

Table 4.1 Sampling location, geographical area, and numbers of *Acanthopagrus* spp. sequenced.

Location	Geographical area	Sample size
	East of Wilson's Promontory	
Gold Coast (Go)	Queensland	6
Forster (Fo)	New South Wales	8
Port Hacking (Po)		6
Meroo (Me)		5
Coila (Co)		20
Tuross (Tu)		17
Brou (Br)		18
Corruna (Co)		20
Wallaga (Wa)		12
Mallacoota (Ma)	East-Victoria	7
Bemm (Be)		9
Tyers (Ty)		5
Lakes Entrance (La)		10
	West of Wilson's Promontory	
Patterson (Pa)	Central-Victoria	9
Yarra (Ya)		5
Thompson (Th)		9
Spring Creek (Sp)		6
Anglesea (Ang)		8
Painkalac (Pai)		9
Hopkins (Ho)	West-Victoria	10
Fitzroy (Fi)		7
Surrey (Su)		8
Glenelg (Gl)		10
Hindmarsh (Hi)	South Australia	5
Onkaparinga (On)		4
Port Adelaide (Por)		3
Blackwood (Bl)	Western Australia	13
Swan (Swa)		20
	South	
Pipers (Pi)	North West-Tasmania	25
Scamander (Sc)	North East-Tasmania	20
Meredith (Me)	Central East-Tasmania	25
Derwent (De)	South East Tasmania	10
Northwest (No)		9
Mean ± SE		10.8 ± 1.1

4.2.2 Genetic markers and PCR

Detailed information concerning the PCR primers and the PCR cycling conditions used to amplify mtDNA control region sequences can be found in Chapter 2. The sequencing reactions were performed at Macrogen Inc. (Korea) (information can be found at http:\\ www. macrogen.com/eng/sequencing) using a Big Dye™ terminator cycle sequencing kit (Applied Biosystems).

4.2.3 Genetic analyses

I discarded the first 50 and last 30 bps of mtDNA sequence due to ambiguous or unresolved sites. I aligned the remaining 320 bps of sequence using Clustal W (Thomson *et al*. 1994) implemented in the software Bioedit (Hall *et al*. 1999).

4.2.3.1 Mitochondrial DNA phylogeny

I examined the mtDNA phylogeny of the sampled *Acanthopagrus* spp. using a Bayesian analysis ([using Mr Bayes] Huelsenbeck & Ronquist 2001) based on the best-fit model of sequence evolution (HKY + G). The HKY+G model was chosen from 24 different models of sequence evolution, based on the results of hierarchical likelihood ratio tests used to evaluate the most likely mode of sequence evolution, given the observed pattern of variation in the mtDNA sequence (MrModeltest, Nylander 2004). All indels were coded as binary characters to include their information in the phylogenetic reconstruction (an additional analysis that excluded the indels as a fifth character state converged on an almost identical phylogeny, data not presented). I used the following priors in the analysis: set partition=indel_mtdna; Lset applyto=(1); coding=variable; Lset applyto=(1) rates=gamma; Prset applyto=(2); statefreqpr=dirichlet(1,1,1,1); Lset

applyto=(2); nst=2 rates=gamma; prset applyto=(all) ratepr=variable; unlink shape=(all); pinvar=(all); statefreq=(all); revmat=(all). Four chains of the MCMC were used, each set for 10^5 generations, and trees were sampled 100 generations. The first 25% of trees were discarded as 'burn in' before calculating the consensus tree. To ensure that independent runs converged on a similar topology, I conducted three separate analyses. Runs converged on the same topology. Sequences of the congeners (GenBank accession number) *A. schlegelii* (AF381099), *A. berda* (AF381066), *A. macrophthalmus* (AF381081), *A. latus* (AF381094) were included in the analysis. The closely related *Rhabdosargus sarba* (AF381118) was included in the analysis to root the trees.

4.2.3.2 Levels of genetic diversity

For the *A. australis* and *A. butcheri* clades resolved by the phylogenetic analysis, and for each estuary containing fish with *A. butcheri* haplotypes, I estimated haplotype (h) and nucleotide diversity (π), and the mean number of corrected pairwise sequence differences among haplotypes (d) (Arlequin [Excoffier *et al*. 2005]).

4.2.3.3 The genealogical relationship among *A.butcheri* haplotypes

I constructed a minimum spanning network (MSN) of the *A. butcheri* mtDNA haplotypes allowing me to display the pattern of molecular evolution of haplotypes, and their spatial distribution and frequencies. In this analysis, haplotypes are joined to one another using a 95% confidence criterion to form the most parsimonious network, with the putative ancestral haplotype distinguished by assigning an 'outgroup weight' to each haplotype, based on its frequency and the number of connections emanating from it. The analyses were performed in TCS1.13 (Clement *et al*. 2000) and drawn using Network

4.2.0.1 (Bandelt *et al*. 1999). The network was compared and checked for consistency with the topology of the tree obtained using the Bayesian phylogenetic analysis.

4.2.3.4 Population structure

I performed a series of hierarchical AMOVA analyses to determine how genetic variation was partitioned within and among geographical groupings of estuaries (see Table 4.1, refer back to Fig. 4.1 & 4.2). The analyses were based on the pairwise distance matrix between haplotypes (with gamma correction), with the level of significance of the resulting Phi-statistics (Φ), formulations of Wright's (1969) *F*-statistics for sequence data, determined with 10000 permutations. These analyses were repeated, but instead of considering the sequence variation between haplotypes, the analyses were based on haplotype frequencies (i.e., F_{ST}). In the present study Φ_{ST} (respectively F_{ST}) represents fixation in individual estuaries relative to the total, while Φ_{RT} measures fixation in the specified group of estuaries relative to the total. These analyses were carried out in Arlequin (Excoffier *et al*. 2005). I also calculated pairwise Φ_{ST} and F_{ST} between all estuaries, with the level of statistical significance determined with 10000 permutations. I generated a plot of genetic distance measured as: (i) $\Phi_{ST}/(1-\Phi_{ST})$ and (ii) $F_{ST}/(1-F_{ST})$, against geographic distance (Great Circle Distance, information can be found at http://www.ga.gov.au: accessed September 2009) for all pairwise comparisons among estuaries.

4.2.3.5 Estimates of the timing of divergence between genetic lineages

The ability to estimate the timing of divergence between genetic lineages or groupings of haplotypes depends on an accurate molecular clock estimate. For the

Acanthopagrus spp. complex, a calibrated molecular clock is unavailable so here I have used mtDNA mutation rates reported in the literature for estuarine and marine fish to estimate the time to most recent common ancestor for the major *A. butcheri* lineages or 'haplogroups' identified in the MSN (see above).

Mutation rates reported in the literature for mtDNA control region of marine and estuarine fish include 3.6%/MY between lineages for snook (1.8×10^{-8} within lineages) (Donaldson & Wilson 1999), 10% /MY for pygmy angel fishes (5×10^{-8}) (Bowen *et al*. 2006), 15.4 – 33.6%/MY for Indo-Pacific tasselfishes ($7.7 \times 10^{-8} – 1.68 \times 10^{-7}$) (Chenoweth & Hughes 2003), 15 – 20%/MY for Indo-Pacific sardines ($7.5 \times 10^{-8} – 1.07 \times 10^{-7}$) (Bowen & Grant 1997), 33 – 100%/MY for butterfly fishes ($1.68 \times 10^{-7} – 5 \times 10^{-7}$) (McMillan & Palumbi 1995), and 5.8 – 48.5% for damsel fishes ($2.9 \times 10^{-8} – 2.4 \times 10^{-7}$) (Domingues *et al*. 2006). As the rate of mutation of mtDNA control region varies considerably across taxa (above), and possibly as a function of the timescale over which mutation rates are estimated (see Burridge *et al*. 2008 for example of time dependency), I regard my coalescence estimates based on mutation rate estimates within lineages of 1.8×10^{-8} (slow clock), 5.0×10^{-8}, and 1.5×10^{-7} (fast clock) as best approximations on the scale of geological epochs, as reflected in my conclusion that groupings of *A. butcheri* haplotypes or 'haplogroups' diverged no later than the middle Pleistocene. It might be argued that a more sophisticated analysis, such as the 'isolation with migration' model implemented in the program IM (Hey & Nielsen 2004), would provide a more precise estimate of the timing of divergence between lineages. However, a signature of high gene flow together with very shallow divergence among lineages has the effect of lowering the resolution of estimates of parameters in the IM model (i.e., ancestral and derived population

sizes, migration rates between populations, and a time point for splitting of the ancestral population). I conducted several analyses in IM however examination of the posterior density plots for the parameters in the model revealed that, values for the parameters but particularly *t* (the parameter for the time of divergence), peaked then formed a plateau that extended to the right at an even higher value, implying an infinitely wide range of parameter values. Hence, the limitation of my data set is that using just one locus and a relatively small read length of sequence (i.e., 320 bps) there is not enough information, and it seems highly likely that the results obtained with IM were unreliable or indeed wrong. I have therefore estimated time since divergence by dividing percent divergence between haplogroups (based on corrected Kimura 2P distance [Kimura 1980]) by two times the within lineage mutation rate (estimated from the literature, see above) (Hedrick 2005).

4.2.3.6 Population history

Population growth and decline affect the distribution of nucleotide site differences between pairs of individuals in a population, providing a signature of the demographic history of a species. I compared the pattern of mutation to the expectation of neutral evolution using Fu's F_s (Fu 1997) and Taijmas's *D* (Taijma 1989). An excess of young mutations (low frequency haplotypes) results in negative values of both F_s and *D* and can be interpreted as evidence of a population or spatial expansion, whereas a lack of low frequency haplotypes results in a shift towards positive values of F_s and is an indication of a more stable population history, while positive values of *D* indicate low levels of both low and high frequency haplotypes and may indicate a decrease in population size (a bottleneck) or a population that consists of a mixture of genetically distinct lineages. Second, the mismatch distribution (the frequency distribution of pairs of individuals who

differ by *i* sites [*i* = 0, 1…]) in a population that has experienced a rapid expansion event in the recent past shows a smooth wave-like distribution (Rogers & Harpending 1992; Rogers 1995) as opposed to the erratic and ragged distribution of a more stable population or bimodal distribution of an admixed population. In partial contrast, an L-shape distribution can be interpreted as evidence of a recent reduction to population size (a bottleneck) or recent founder event, but with subsequent population growth (Slatkin & Hudson 1991; Harpending 1994). I compared the observed mismatch distribution with the mismatch expected under a model of spatial expansion. I tested the fit of the data to the model using the sum of the squared deviations (SSD) between the observed and expected mismatch distributions. I also estimated Harpending's raggedness index (*r*), which is an index of the variation around the observed and expected mismatch distribution (Harpending 1994).

4.3 Results

4.3.1 Phylogenetic analysis of *Acanthopagrus* spp.

The phylogenetic reconstruction revealed a striking disjunction with two strongly supported clades (posterior probability = 100) separated by a corrected average of 56.1 pairwise sequence differences (17.1%). These clades correspond to *A. australis* and *A. butcheri* respectively, and revealed these taxa as sister taxa. Within each clade there was shallow divergence among haplotypes, reflected in the relatively short branch lengths of the phylogenetic tree (Fig. 4.3).

4.3.2 Overall mtDNA diversity for *A. butcheri* and *A. australis* clades

For the *A. butcheri* clade of haplotypes (308 fish), control region sequence variation and haplotype diversity were relatively low. Overall, there were 29 polymorphic sites

which defined 32 haplotypes. Average gene diversity ($h \pm$ SD) was 0.74 ± 0.02, while the corrected average nucleotide diversity ($\pi \pm$ SD) and the average number of pairwise sequence differences among haplotypes ($d \pm$ SD) was 0.007 ± 0.005 and 2.5 ± 1.4 respectively. For *A. australis*, there were 31 haplotypes in 50 samples, and h, π and d were 0.95 ± 0.02, 0.013 ± 0.007 and 4.3 ± 2.2, respectively.

4.3.3 Inter-estuary variation in mtDNA diversity for *A. butcheri*

Perhaps not surprisingly, fish with *A. butcheri* mtDNA haplotypes were common within lakes and lagoons in the far south (TAS) and west (VIC – WA) beyond the described range of *A. australis*, but also occurred within lagoons in the area of sympatry in NSW (Table 4.2). Both h and π were highly variable among estuaries, but nevertheless values for these indices of diversity were highest for the set of estuaries immediately to the east (NSW & eastern VIC; Me – La, n = 10) and west (central & western VIC; Pa – Gl, n = 10) of WP on the southeast corner of the Australian mainland. For these estuaries, h and π ranged between 0.20 – 0.93 and 0.001 – 0.013, and averaged respectively (± SE) 0.79 (0.03) and 0.70 (0.07), and 0.010 (0.001) and 0.007 (0.001). In contrast, corresponding values for estuaries in the extreme west in SA and WA (Hi – Bl, n = 5), and south in TAS (Pi – No, n = 5), were, on average, just 0.35 (0.11 & 0.14) and 0.002 (0.001) (range = 0 – 0.67 & 0 – 0.006) (Table 4.2).

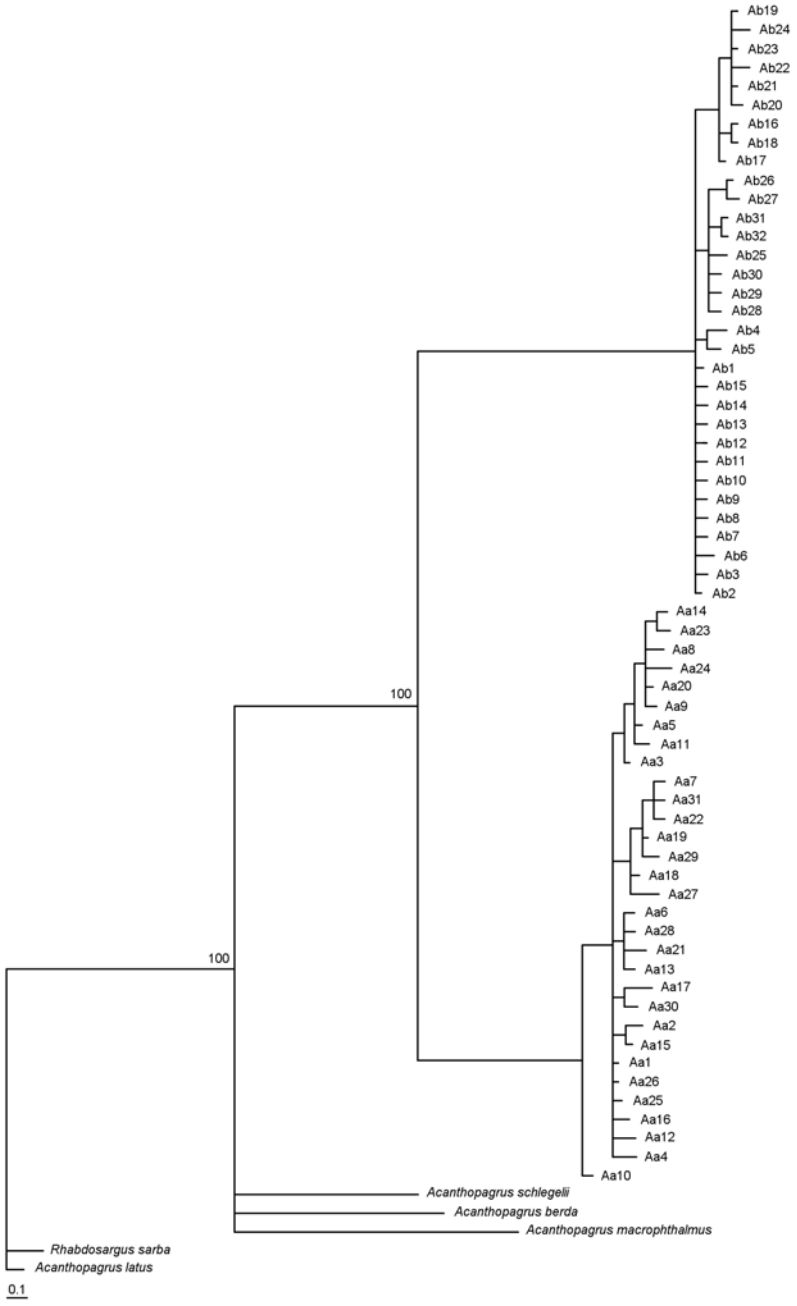

Figure 4.3 Bayesian phylogenetic analysis of *Acanthopagrus* spp. mtDNA control region haplotypes (based on 320 bps). Numbers at nodes are posterior probability values. Aa, *Acanthopagrus australis*; Ab, *Acanthopagrus butcheri*.

4.3.4 The genealogical relationship among *A. butcheri* haplotypes, and their spatial distribution

The genealogy (resolved by the MSN) of *A. butcheri* mtDNA haplotypes consisted of the putative ancestral haplotype (haplotype 1), and three, shallow, star-like lineages or haplogroups. Each lineage consisted of a relatively common, geographically widespread central haplotype (hap. 2, 25 or 19), and several private singleton, or rare, geographically restricted haplotypes (Fig 4.4). The MSN was in agreement with the Bayesian phylogenetic tree (Fig 4.3).

Table 4.2 Genetic diversity indices based on 320 bps of the mtDNA control region for *A. butcheri* within each of 30 southern Australian estuaries. The number of *A. butcheri* (N_{Ab}), number of detected haplotypes (N_H), number of private haplotypes (N_{PH}), haplotype identity (H_{ID}), haplotype (h) and nucleotide diversity (π), and the average number of pairwise sequence differences (d) is shown. Overall and values are the result of pooling estuaries (where possible) within geographical areas. *

Geographical area Location	N_{Ab}	N_H	N_{PH}	H_{ID} †	h ± SD	π ± SD	d ± SD
East of WP							
NSW							
Me	5	4	0	2, 18, 19, 26	0.900 (0.161)	0.013 (0.009)	4.0 (2.4)
Co	18	4	0	2, 18, 19, 26	0.752 (0.056)	0.011 (0.007)	3.6 (1.9)
Tu	7	5	0	2, 18, 19, 26, 27	0.857 (0.137)	0.013 (0.008)	4.0 (2.3)
Br	17	4	0	2, 18, 19, 26	0.750 (0.058)	0.011 (0.007)	3.5 (1.9)
Cor	14	6	1	2, 7, [8], 18, 19, 26	0.736 (0.109)	0.007 (0.005)	2.2 (1.3)
Wa	1	1	0	26	-	-	-
Overall	62	7	1	2, 7, [8], 18, 19, 26, 27	0.764 (0.028)	0.011 (0.006)	3.5 (1.8)
E VIC							
Ma	7	5	3	2, [5], [12], 18, [20]	0.857 (0.137)	0.013 (0.008)	4.0 (2.3)
Be	9	5	1	2, [17], 18, 19, 25	0.861 (0.087)	0.009 (0.006)	2.7 (1.6)
Ty	5	3	0	2, 19, 26	0.700 (0.218)	0.011 (0.008)	3.4 (2.1)
La	10	5	2	2, [16], 18, 19, [21]	0.667 (0.163)	0.005 (0.004)	1.4 (1.0)
Overall	31	11	6	2, [5], [12], [16], [17], 18, 19, [20], [21], 25, 26	0.832 (0.049)	0.009 (0.005)	2.8 (1.5)
West of WP							
C VIC							
Pa	9	5	2	2, [13], 25, 29, [30]	0.806 (0.120)	0.006 (0.004)	1.8 (1.1)
Ya	5	3	0	2, 19, 25	0.700 (0.218)	0.007 (0.005)	2.2 (1.4)
Th	9	5	2	2, 6, 19, [24], [32]	0.806 (0.120)	0.012 (0.007)	3.7 (2.1)
Sp	6	5	2	2, 19, [23], 25, [31]	0.933 (0.121)	0.013 (0.009)	4.1 (2.4)
Ang	8	3	0	2, 19, 25	0.678 (0.122)	0.008 (0.005)	2.5 (1.5)

Pai	9	4	0	2, 6, 19, 25	0.583 (0.183)	0.005 (0.004)	1.5 (1.0)
Overall	46	11	6	2, 6, [13], 11, [23], [24], 25, 29, [30], [31], [32]	0.765 (0.047)	0.009 (0.005)	2.8 (1.5)
W VIC							
Ho	10	6	1	2, 18, 19, 25, [28], 29	0.889 (0.075)	0.009 (0.006)	3.0 (1.7)
Fi	7	4	1	2, 19, [22], 25	0.857 (0.102)	0.009 (0.006)	3.0 (1.7)
Su	8	2	0	2, 25	0.571 (0.095)	0.004 (0.003)	1.1 (0.8)
Gl	10	2	0	2, 11	0.200 (0.154)	0.001 (0.001)	0.2 (0.3)
Overall	35	8	2	2, 11, 18, 19, [22], 25, [28], 29	0.686 (0.068)	0.007 (0.004)	2.1 (1.2)
SA							
Hi	5	2	1	2, [14]	0.400 (0.237)	0.001 (0.001)	0.4 (0.4)
Op	4	1	0	2	0.000 (0.000)	0.000 (0.000)	0
Po	3	2	1	2,[4]	0.667 (0.314)	0.006 (0.006)	2 (1.5)
Overall	12	3	2	2, [4], [14]	0.318 (0.164)	0.002 (0.002)	0.7 (0.5)
WA							
Bl	13	4	1	2, 6, 10, [15]	0.423 (0.165)	0.001 (0.002)	0.5 (0.4)
Swa	20	3	1	2, [3], 10	0.279 (0.124)	0.001 (0.001)	0.3 (0.3)
Overall	33	5	2	2, [3], 6, 10, [15]	0.330 (0.102)	0.001 (0.001)	0.3 (0.3)
South							
NW TAS							
Pi	25	3	0	2, 7, 19	0.580 (0.081)	0.005 (0.003)	1.5 (0.9)
NE TAS							
Sc	20	4	0	2, 6, 9, 10	0.500 (0.122)	0.002 (0.002)	0.6 (0.5)
CE TAS							
Mer	25	5	1	[1], 2, 9, 11, 25	0.650 (0.087)	0.003 (0.002)	0.9 (0.1)
SE TAS							
De	10	1	0	25	0.000 (0.000)	-	-
No	9	1	0	25	0.000 (0.000)	-	-
Overall	19	1	0	25	0.000 (0.000)	-	-

[†]Private haplotypes are indicated in parenthesis

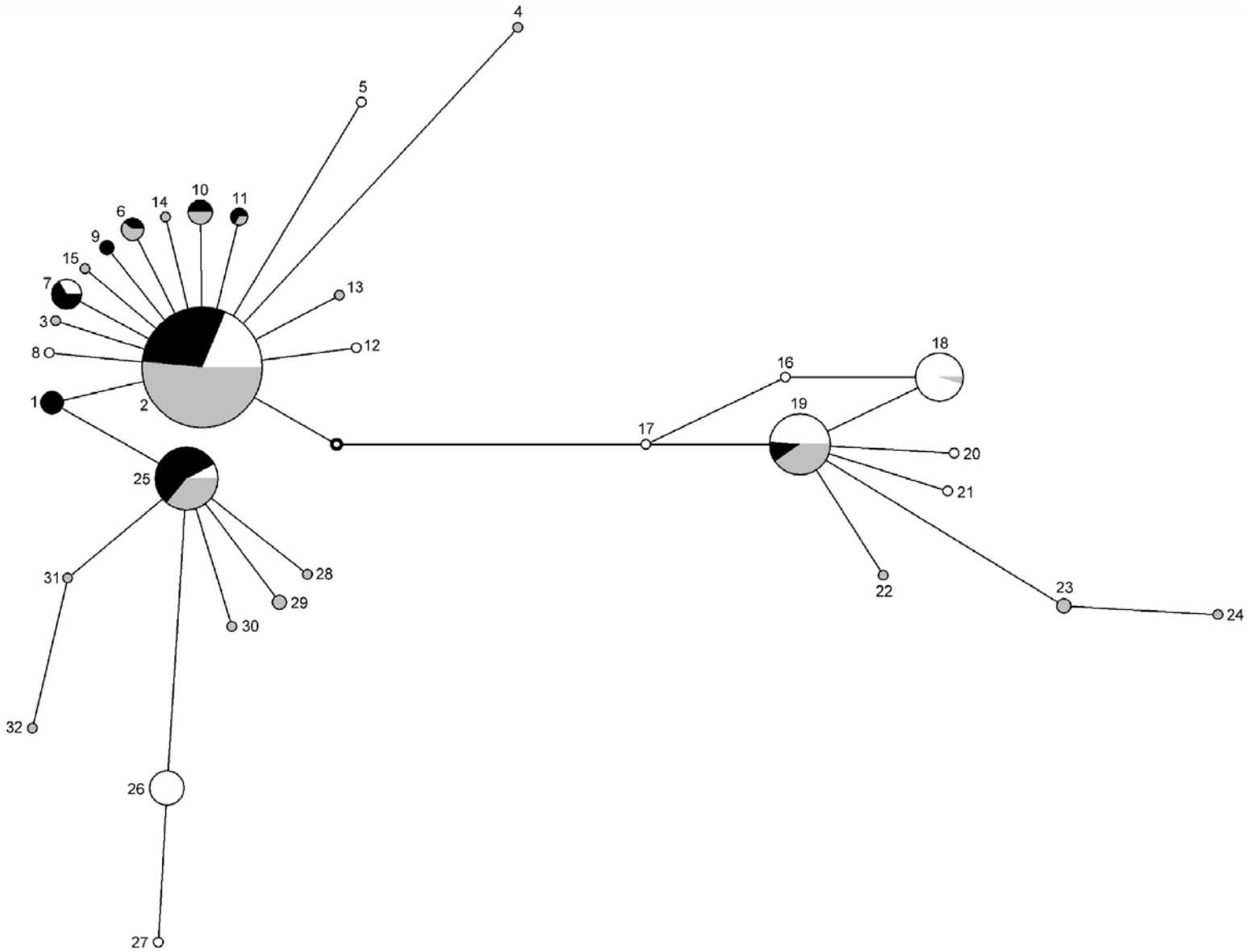
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Figure 4.4 Medium spanning or statistical parsimony network among haplotypes of *Acanthopagrus butcheri*. The area of the circle representing the different haplotypes is proportional to the frequency of occurrence of the haplotype in the total sample. The shading (white, grey and black) corresponds to the relative frequency and spatial distribution of each haplotype divided broadly into eastern (NSW & eastern VIC), western (central and western Victoria, SA, and WA) and southern (TAS) groupings, respectively. The pattern of molecular evolution of haplotypes is represented by the connections among haplotypes formed with the solid black lines, with the length of the smallest lines each proportional to one substitution. The different haplotypes are labelled 1 – 32, with haplotype 1 identified as the putative ancestral haplotype at the 95% connection limit. An inferred mutational step (i.e., a missing haplotype, either extant, but unsampled, or extinct) connected the major lineages consisting of the most frequently occurring haplotypes 1-2-25 and 18-19, and is represented in the figure by a partially filled black circle.

Contrary to the expectation that populations in different estuaries would be highly genetically distinct, examination of the spatial distribution of haplotypes revealed that the most commonly occurring mtDNA haplotypes were often extremely geographically widespread (Fig. 4.4). The most common haplotype, haplotype 2 (overall frequency = 0.47), occurred within nearly every estuary sampled (28/30) (Table 4.2); the exceptions were two estuaries in the extreme south in TAS (De & No), with fish in these estuaries fixed for haplotype 25. Although haplotype 25 (freq. = 0.13) was generally absent from estuaries east of WP (Me – La) and in the extreme west (i.e., SA & WA; Hi – Bl), it was nevertheless relatively frequently encountered within estuaries between WP and the VIC/SA border (Pa – Gl, 8/10 estuaries). Haplotype 19 (freq. = 0.12) was also widespread, occurring within 8 of 10 estuaries east of WP, and 7 of 10 estuaries between WP and the VIC/SA border. In partial contrast, haplotypes 18 and 26 were widespread east of WP (7 & 8/10 estuaries respectively) but were absent west of WP and in Tasmania. Perhaps most striking was that haplotype 10 was detected within estuaries located at the extreme eastern and western range limit of *A. butcheri*, (i.e., south in Tasmania [Sc] and west in WA [Swa & Bl]), but not in any other estuary between these areas (a distance of > 3000kms) (Table 4.2). In spite of these apparently widespread haplotypes, just under half (47 %) of the surveyed estuaries contained one private haplotype (i.e., a haplotype unique to a particular estuary), though up to three haplotypes per estuary were detected (e.g., Ma) (total n = 19/32 haplotypes). Private haplotypes occurred at about equal frequency east and west of WP (8 & 10 respectively), but they were extremely rare in Tasmania (Table 4.2, Fig 4.4). In all cases, private haplotypes were closely related to one or other of the widespread central haplotypes of the lineages recognised above.

4.3.5 Population genetic subdivision

Surprisingly, population differentiation, as judged by estimates of pairwise Φ_{ST} and F_{ST}, revealed that 52% and 63% respectively of 435 possible comparisons among estuaries were not statistically significantly different ($P > 0.05$). Although non significant tests of population differentiation often involved neighbouring or geographically proximate estuaries, there were many cases where *A. butcheri* within estuaries separated by 100's to 1000's of kilometres were not genetically differentiated ($P > 0.05$), although this could simply reflect the effects of small sample size. Nevertheless, the great genetic similarity of *A. butcheri* over its distributional range is most easily displayed using a plot of geographic distance versus genetic distance (Fig. 4.5).

AMOVA analyses revealed moderate statistically significant genetic heterogeneity for the overall set of estuaries arrayed east (Φ_{ST} & $F_{ST} = 0.09$, $P < 0.05$) and west of WP ($\Phi_{ST} = 0.24$, $P < 0.001$; $F_{ST} = 0.14$, $P < 0.001$). For both of these regions, further analyses that treated sets of neighbouring estuaries as 'geographical groupings' or 'regions' (Table 4.3, refer also to Table 4.2) revealed little evidence of regional genetic differentiation (range Φ_{RT} & $F_{RT} = -0.02 – 0.11$, $P > 0.05$) (Table 4.3). *Acanthopagrus butcheri* within estuaries in northern TAS were similarly genetically differentiated (0.14 & 0.07, $P < 0.05$), but in this area divergence clearly increases with the degree of isolation, as Φ_{ST} and F_{ST} increased markedly to 0.49 and 0.40 ($P < 0.001$) respectively, when estuaries to the extreme south (i.e., SE-TAS) were included in the analysis. Interestingly, when estuaries to the east and west of WP, and south in TAS, were respectively pooled to form broad geographical groupings, analyses revealed statistically significant east – west, and east – south genetic division ($\Phi_{RT} = 0.14$ & 0.21 $P < 0.01$; $F_{RT} = 0.05$ & 0.07 $P < 0.01$ & $P =$

0.07). In contrast however, there was no west – south division, (Φ_{RT} & F_{RT} = 0.0, P > 0.05) (Table 4.3).

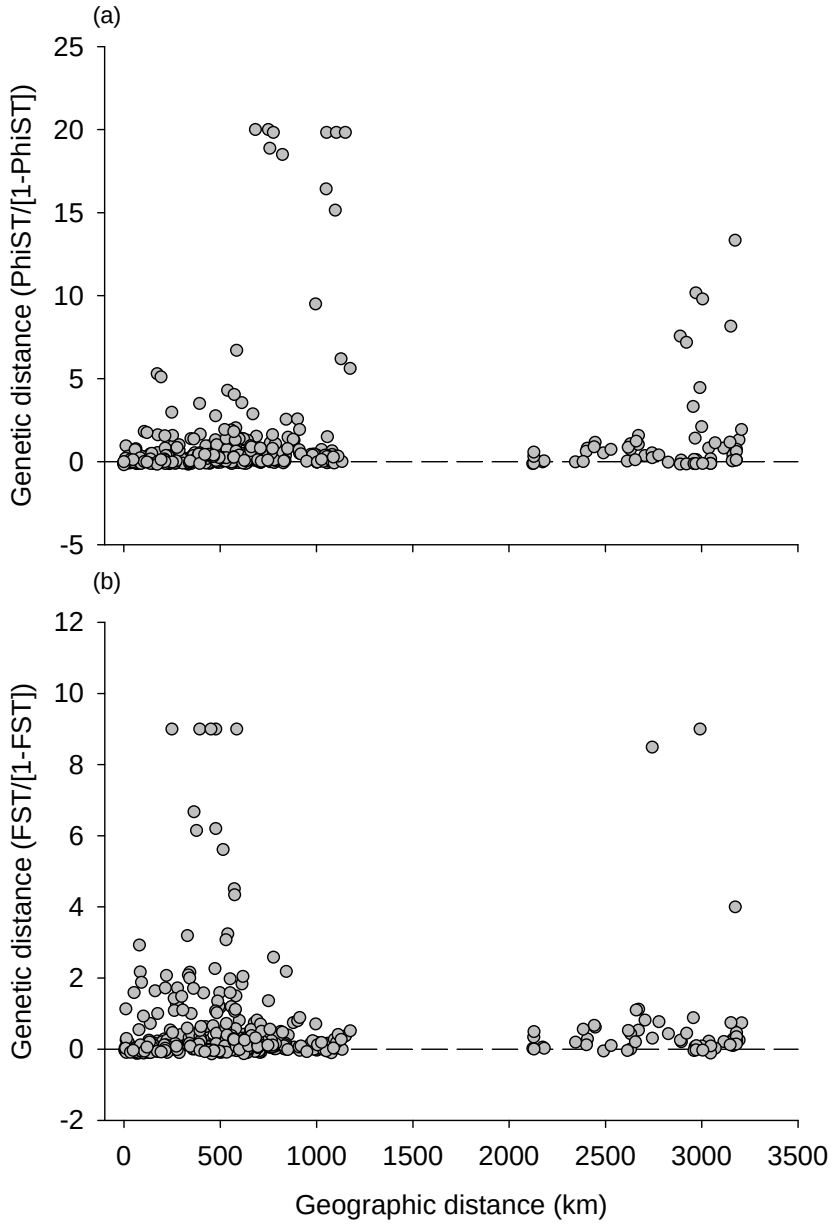

Figure 4.5 Plot of genetic distance (a) $\Phi_{ST}/(1-\Phi_{ST})$ and (b) $F_{ST}/(1-F_{ST})$ against geographic distance (Great Circle Distance) for all pairwise comparisons among estuaries, for *Acanthopagrus butcheri*. The black broken line represents zero genetic differentiation (Φ_{ST} & F_{ST} = 0).

4.3.6 The geographical distribution of *A. butcheri* mtDNA lineages

The spatial distribution of mtDNA lineages revealed by the MSN were in agreement with the broad east – west and east – south genetic divisions revealed by AMOVA. The lineage of haplotypes which included haplotype 2 and its immediate allies (Lineage 1) (Fig. 4.3) spanned the distributional range of the species though these haplotypes were most frequently encountered west of WP and south in TAS (freq. = 0.68 & 0.71 *c.f.* 0.36). Lineage 2 haplotypes (hap. 25 & allies) occurred at about the equal frequency east and west of WP, and south in TAS (0.17, 0.16 & 0.25), while lineage 3 haplotypes were (hap. 19 & allies) most common east of WP (0.47 *c.f.* 0.16 & 0.05) (Fig 4.6).

Table 4.3 Results of AMOVA analyses used to estimate genetic differentiation within and among geographical groupings of estuaries (e.g., 'NSW' – Me, Co, Tu, Br, Cor &Wa) located east and west of Wilson's Promontory (WP) on the Australian mainland, and south in Tasmania. Φ_{ST} (respectively F_{ST}) (P-value) represents differentiation in individual estuaries relative to the total, while Φ_{RT} measures differentiation in the specified group of estuaries relative to the total. *

	Φ_{ST}	Φ_{RT}	F_{ST}	F_{RT}
All estuaries	**0.28 (0.00)**	-	**0.21 (0.00)**	-
East of WP				
NSW	0.05 (0.13)	-	-0.01 (0.55)	-
E VIC	0.05 (0.21)	-	**0.09 (0.03)**	-
NSW/E VIC (East of WP) [a]	**0.09 (0.03)**	0.05 (0.09)	**0.09 (0.01)**	**0.06 (0.04)**
West of WP				
C VIC	0.10 (0.06)	-	0.03 (0.20)	-
W VIC	**0.20 (0.01)**	-	**0.14 (0.02)**	-
SA	0.09 (0.25)	-	-0.04 (0.70)	-
WA	-0.02 (0.71)	-	-0.03 (0.73)	-
C VIC/W VIC	**0.12 (0.00)**	-0.02 (0.55)	**0.07 (0.03)**	-0.01 (0.55)
C VIC/W VIC/SA	**0.17 (0.00)**	0.05 (0.19)	**0.10 (0.01)**	0.03 (0.15)
C VIC/W VIC/SA/WA (West of WP)	**0.24 (0.00)**	0.11 (0.07)	**0.14 (0.00)**	0.07 (0.06)
South				
NW TAS/NE TAS/CE TAS	**0.14 (0.00)**	-	**0.07 (0.05)**	-
NW TAS/NE TAS/CE TAS/SE TAS (South)	**0.49 (0.00)**	-	**0.40 (0.00)**	-
Inter-regional				
East of WP/West of WP	**0.26 (0.00)**	**0.14 (0.01)**	**0.11 (0.00)**	**0.06 (0.00)**
East of WP/South	**0.37 (0.00)**	**0.21 (0.01)**	**0.27 (0.00)**	0.07 (0.06)
West of WP/South	**0.31 (0.00)**	-0.02 (0.45)	**0.23 (0.00)**	-0.01 (0.40)

*Significant values are indicated in bold. Refer to Table 1 for detail concerning the number of estuaries per grouping

[a] Grouping used for inter-regional analysis

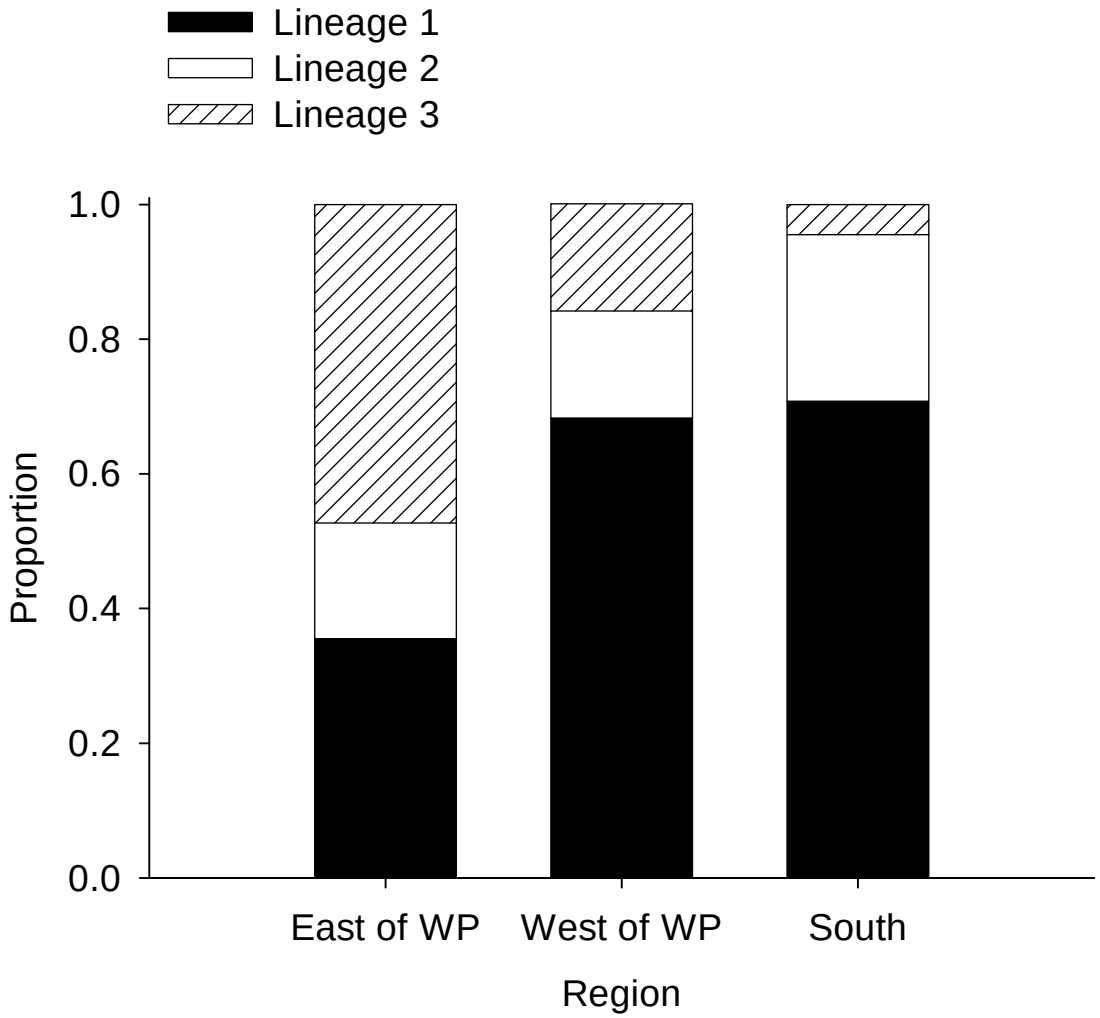

Figure 4.6 The proportion of fish representing three *A. butcheri* mtDNA lineages, east and west of Wilsons Promontory (WP), and south in Tasmania.

4.3.7 Estimates of the timing of divergence among lineages

Net sequence divergence among lineages based on corrected Kimura 2P distance were shallow implying a relatively recent coalescence (percent divergence Lin. 1/Lin. 2 = 0.65%, Lin. 1/Lin. 3 = 1.03% & Lin. 2/Lin. 3 = 1.04%). Indeed, based on a slow molecular clock (i.e., a within lineage mutation rate of 1.8×10^{-8}) timing of divergence among lineages dates to the middle Pleistocene (0.126 – 0.781 MY). However, considerable

uncertainty surrounds these estimates of divergence as just a modest increase in the mtDNA control region mutation rate to 5.0×10^{-8} within lineages places coalescence in the late Pleistocene (0.117 – 0.126 MY), while using a fast molecular clock (1.5×10^{-7} within lineages) the most recent common ancestor to these lineages may have existed as recently as ~ 0.021 to 0.034 MY years ago, just outside the Holocene (present – 0.010 MY).

4.3.8 Analyses used to infer the demographic history of *A. butcheri*

A series of analyses based on the distribution of nucleotide site differences between pairs of individuals were used to infer the demographic history of *A. butcheri*. When separate analyses were performed on the broad geographical groupings, as for AMOVA, these revealed an excess of low frequency haplotypes (young mutations), reflected in negative values of Fu's F_s and Tajima's D. However, only F_s for the sample west of WP was statistically significant ($P < 0.01$) (Table 4.4). Examination of the mismatch distribution for each broad grouping revealed a bimodal distribution with peaks at 0 and 4 pairwise sequence differences for the sample east of WP, whereas the mismatch for samples west of WP and south were clearly L-shaped (Fig. 4.7). For each grouping of estuaries east and west of WP and south in TAS, the observed mismatch distribution was not different to the mismatch distribution of a simulated population modelled to have experienced a sudden spatial expansion ($P > 0.05$).

Table 4.4 Mismatch analysis parameters and results of tests of neutrality for *A. butcheri* east and west of Wilsons Promontory (WP), and south in Tasmania. [a]

	D	*P*(D)	*Fs*	*P*(*Fs*)	SSD	*P*(SSD)	*r*	*P*(*r*)
East of WP	0.496	0.733	-1.070	0.396	0.040	0.093	0.072	0.142
West of WP	-1.314	0.074	-9.308	0.002	0.026	0.516	0.098	0.461
South	-0.618	0.311	-1.603	0.249	0.008	0.507	0.046	0.746

[a] *F*s and *D* and the corresponding significance level [*P*(*F*s) and *P*(*D*)] are from Fu and Tajima's tests of neutrality. The mismatch analysis compared the distribution of observed pairwise sequence differences with the distribution expected under a model of sudden population expansion. To test whether the observed data was consistent with the model, the sum of the squared deviations (SSD) between observed and expected mismatches was used as the test statistic, with the level of significance [(*P*SSD)] determined with a bootstrapping procedure. Harpending's raggedness index (*r*), which is an index of the variation around the observed and expected mismatches, was also calculated. The level of significance [*P*(*r*)] was similarly found using a bootstrapping procedure.

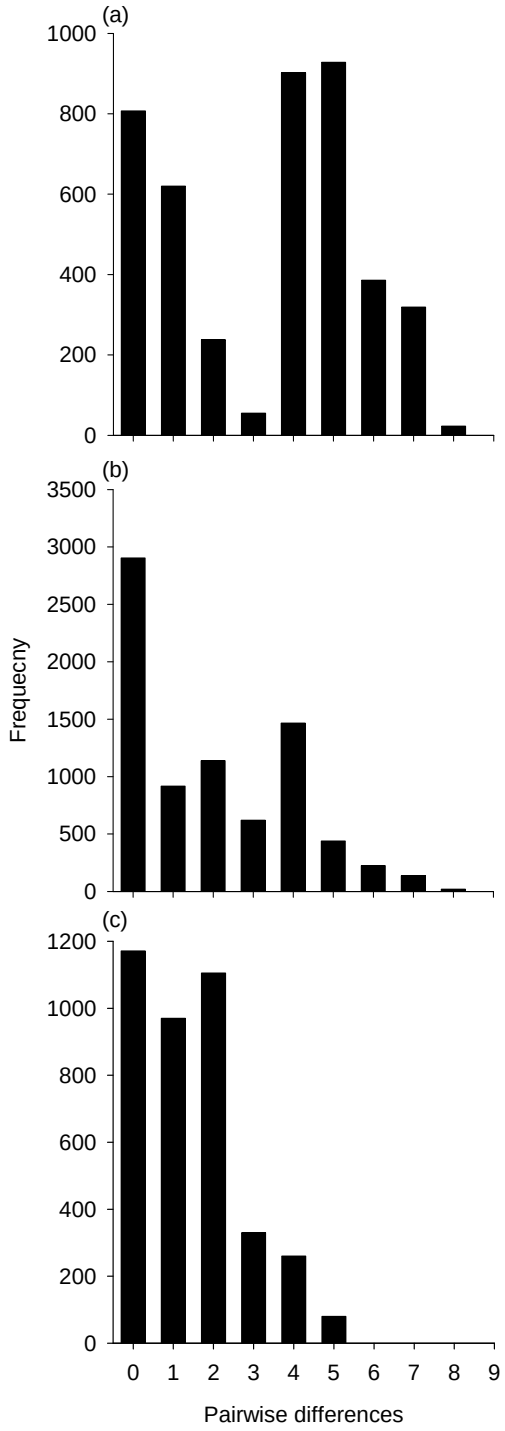

Figure 4.7 Mismatch distribution for *A. butcheri* within estuaries (a) East of WP (b) West of WP (c) and South in Tasmania. The plot for overall data set was similar to the subset of data representing fish within estuaries East of WP.

4.4 Discussion

Against expectation my phylogenetic survey revealed shallow divergence dating to no later than the middle Pleistocene, for mtDNA lineages of an extremely widespread estuary restricted species, *A. butcheri*. The dynamic and transient nature of estuaries implies that interpretation of the genetic structures of estuary restricted species almost certainly must consider non-equilibrium population dynamics, and indeed range wide population structure in *A. butcheri* is most simply explained by a recent range expansion or colonisation of estuaries throughout southern Australia. Indeed, the situation described bears remarkable similarity to the situation described for the estuarine species, *Polynemus sheridani* (Indo-Pacific tasselfish) (Chenoweth & Hughes 2003), that similarly exhibits shallow divergence between lineages and a signature of a recent colonisation of estuaries across its described northern Australian range, which includes a major paleo-barrier and recognised phylogenetic break (e.g., Chenoweth *et al*. 1998 a & b). Using a survey of mitochondrial control region sequence variation, here I was able to show that: (i) mtDNA genetic diversity is highest for *A. butcheri* populations within lakes and lagoons on the SE corner of the Australian mainland, and diversity decreases with distance respectively south and west to the range edge of *A. butcheri*'s distribution; (ii) the genealogical relationship among *A. butcheri* mtDNA haplotypes consists of three, shallow 'star-like' lineages that are geographically widespread, (iii) *A. butcheri* display a clear lack of isolation by distance, across its distributional range, and (iv.) a signature of a recent spatial or range expansion persists within the mtDNA sequence of *A. butcheri*, leading to the implication that *A. butcheri* colonised its recognised range relatively recently.

My findings based upon analysis of mitochondrial DNA sequence variation demonstrate that, unlike many species that have been historically fragmented by the BI and form deeply divergent eastern and western lineages or incipient species (see introduction), *A. butcheri* form three shallow lineages or groupings of closely related haplotypes ('halogroups'). My data showed very little phylogeographic structure, as many of the haplotypes that comprised each lineage were widely distributed both east and west of WP, and south in Tasmania. Indeed, the most frequently encountered haplotype of lineage 1 (haplotype Ab2) occurred in almost every estuary sampled across the 3000 km range of *A. butcheri*. Nevertheless, the genotypes of a large proportion of fish sampled within estuaries east of WP comprised lineage 3 haplotypes, whereas the genotypes of fish sampled west of WP and south in TAS comprised typically lineage 1 haplotypes. Fish that possessed lineage 3 haplotypes were equally distributed east and west of WP, and south in TAS. All three lineages were closely related, with net sequence divergence between 'eastern' and 'western' lineages just 0.65 %, and ~ 1% between both the eastern and western lineage and lineage 3. The implication of these data is that the three recognised *A. butcheri* lineages have a very recent coalescence.

My broad scale survey of mitochondrial DNA sequence variation provided a sensitive test of population differentiation in *A. butcheri*, across a set of estuaries considered to span two major biogeographic barriers. Not surprisingly given the occurrence of several relatively common (high frequency), widespread haplotypes, there was a lack of isolation by distance across the range of *A. butcheri* suggesting strong historical or contemporary connections among estuaries. Interestingly, estimates of genetic diversity as measured by haplotype and nucleotide diversity were highest for the set of

estuaries on the SE corner of the Australian mainland, with diversity decreasing with distance both west and south to the range edge of *A. butcheri* in southern TAS & WA respectively. Reduced genetic variability in these southern and western populations may reflect recent colonisation or founding from a southeastern Australian refuge. Indeed, a lack of isolation by distance suggests that populations within southern and western estuaries may have been formed by chance migration events from a refuge or source population rather than from the edge of a constantly expanding population.

The SE corner undoubtedly has a complex geological history and we lack much of the information necessary for a thorough understanding of its role in shaping southern Australian marine biodiversity. However, a recent modelling study of topographical alteration in the Bass Straight region through the last glacial cycle has revealed that, when the relative sea-level had risen a sufficient amount, it entered a large depression in the centre of the BI from the west to form the Bass Basin, a vast coastal lagoon or estuary (see fig 4.1 herein, refer to Lambeck & Chappell 2001 for specific detail). For an estuary restricted species such as *A. butcheri*, a vast ancient lagoon such as the BB may have been habitable and so potentially provided a major south east Australian refuge or source population. Indeed, the BB may have provided conditions that promoted rapid population growth (i.e., a productive environment capable of supporting a large population size). Moreover, the BB may have facilitated range expansion and colonisation of estuaries across the SE corner, including estuaries east of WP and south in TAS. Lambeck and Chappell's (2001) modelling study suggests that the BB stretched from the Australian mainland to as far south as the northern coast of TAS, but in addition breached the land connection in the east at WP. My analyses used to infer the demographic history of *A. butcheri* revealed a

significant excess of low frequency haplotypes or young mutations, for *A. butcheri* west of WP and south in TAS, which together with an L-shaped mismatch distribution imply recent and rapid population expansion (Slatkin and Hudson 1991; Harpending 1994). For *A. butcheri* east of WP, the mismatch was clearly bimodal, reflecting large differences in the distribution of nucleotide site differences between pairs of individuals i.e., mixing of divergent lineages.

My range wide phylogeographic study in *A. butcheri* has revealed little phylogenetic structure other than a shallow split between eastern and western populations. This most likely reflects a recent origin or colonisation of estuaries throughout southern Australia. The limitation of my data set is that I have relatively little power to distinguish the direction and magnitude of gene flow, precluding assessment of likely migration pathways. Nevertheless, my results leave open the possibility that *Acanthopagrus* hybrids zones are maintained by contemporary processes.

Chapter 5 Gamete compatibility between marine and estuarine *Acanthopagrus* spp. (Sparidae) and their hybrids

This chapter is published in *Journal of Fish Biology*.

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5.1 Introduction

Many free spawning coastal marine fish use coastal lakes and lagoons (or estuaries) as spawning grounds (e.g., Reyier *et al*. 2008), providing the potential for interbreeding with estuary restricted congeners. However, for most pairs of obligately estuarine and coastal marine fishes, the degree or extent of reproductive (gamete) compatibility is unknown although this will influence the likelihood of initial and later hybridisation.

At face value the large numbers of hybrids detected in my earlier survey (Chapter 2) suggest that ongoing hybridisation between *A. australis* and *A. butcheri* is common but that levels of hybridisation vary among lagoons, seemingly reflecting lagoon opening to the adjoining ocean. However, in a singular survey the frequency of hybrids may have been skewed by the disproportionate success of particular hybridisation events. Alternatively, hybrid formation may be a rare event with hybrid fish representing the outcome of past hybridisation events and current F1 hybrid formation may be rare or improbable. Here, I performed a fertilisation experiment to assess the compatibility of pure species *A. australis*,

A. butcheri and hybrid sperm, and pure species *A. butcheri* and hybrid ova, to test for evidence of early acting barriers to initial and later hybridisation.

5.2 Methods

I used naturally spawning ocean caught *A. australis* from beyond the range of *A. butcheri* in northern NSW (Forster), and hybrid and *A. butcheri* within Coila Lake in the area of sympatry in southern NSW. Fish were caught with rod-and-reel and transferred directly from the water to a 70 L aerated live well where they were held in total darkness in the field for up to two hours. Prior to collecting gametes, all fish were anaesthetised in aerated sea or lake water containing 0.35 ml^{-1} of 2-phenoxy ethanol (Jenkins *et al*. 1999). My intention was to cross *A. butcheri* and *A. australis* although my earlier work had shown that distinguishing between these species and their hybrids based on morphology was problematic (Rowland 1984; Roberts *et al*. 2009). Consequently, I genotyped all fish used in my experiments using both microsatellite and mtDNA markers and performed admixture analysis to determine the specific status of every individual (see Chapter 2 for detail of PCR conditions). This analysis revealed that I had crossed three *A. butcheri*, three hybrid (presumably some advanced generation hybrid or *A. butcheri* backcross in that each was genetically similar to pure parental *A. butcheri* in terms of their nuclear genome and had mtDNA characteristic of *A. butcheri*), and six *A. australis* males with each of two different *A. butcheri*, and five hybrid females (Fig 5.1).

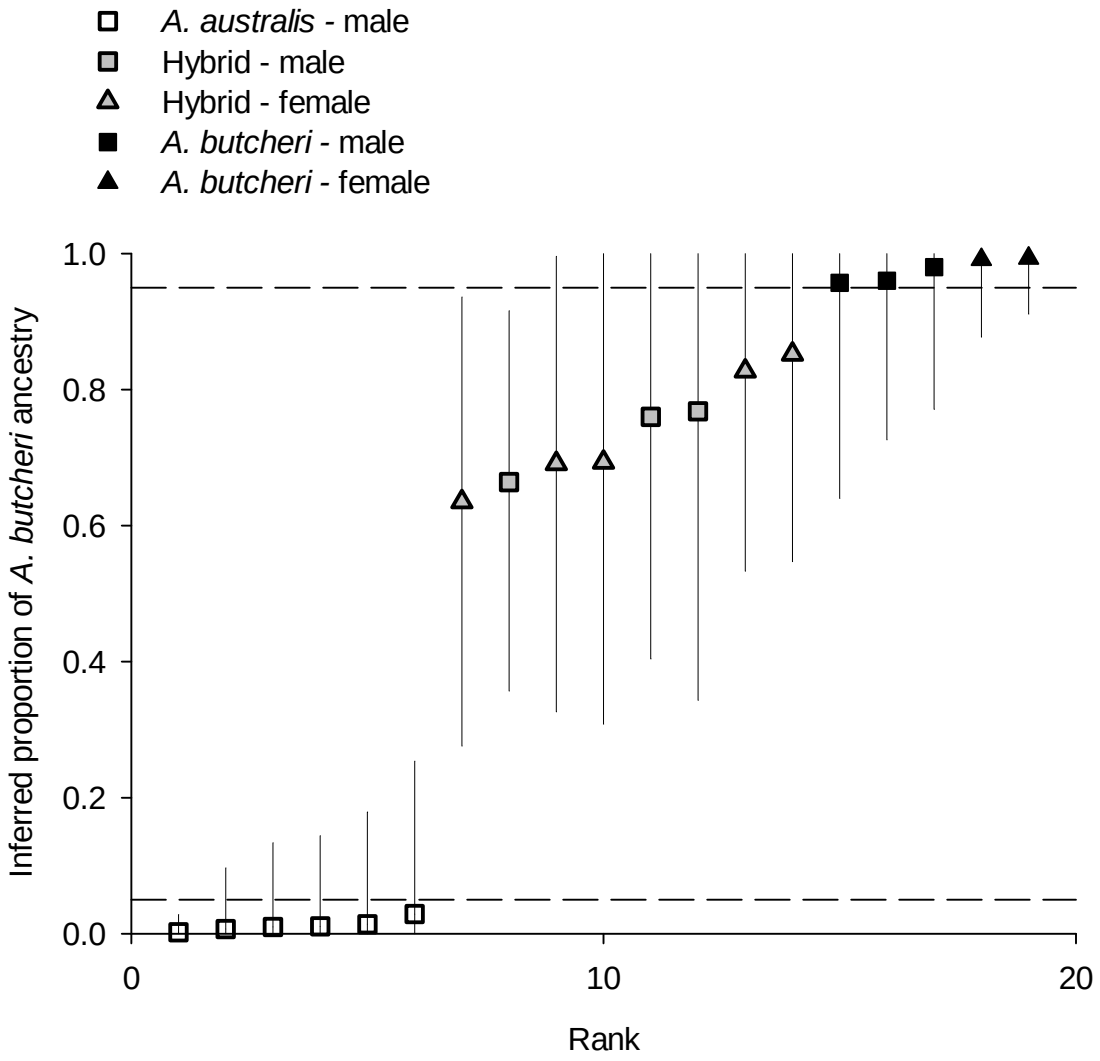

Figure 5.1 Ancestry of 19 *Acanthopagrus* spp. used in fertilisation experiments. The data are presented as the average (± 95% CI's) proportion of *A. butcheri* genome (based on eight microsatellite loci). Fish with estimates of ancestry ranging between 0 and 0.05, and 0.95 and 1.0 (the lower and upper broken black line) were classed as pure *A. australis* and *A. butcheri* respectively. All others were classed as hybrid. Pure *A. australis* and *A. butcheri* possessed species-specific mtDNA haplotypes. Hybrids possessed the mtDNA of the species they were most similar to i.e., $0.05 < q_i < 0.5$ *A. australis*, $0.5 < q_i < 0.95$ *A. butcheri*.

The combined spawning seasons of the two species extend for 2 – 5 months and overlap in at least some years because *A. australis* males can remain 'ripe' up to the peak spawning of *A. butcheri* in August or September. In most years however there is greater certainty in catching male *A. australis* in spawning condition some 4 – 6 weeks prior to peak spawning in *A. butcheri*. To ensure that *A. australis* sperm were available for use in the fertilisation experiment and to ensure all sperm were treated similarly I therefore cryopreserved the gametes of all males prior to performing the crossing experiments. Sperm was stripped and collected with a 5ml syringe, with 100 µl aliquots of fresh sperm subsequently diluted 10-fold with Hanks Balanced Salt Solution (HBSS). To facilitate freezing, I added a cryoprotectant (2M glycerol in HBSS) to the diluted sperm solution at a ratio of 1: 1 allowed the mixture to equilibrate for three minutes at ~4 °C. Each aliquot was subsequently frozen and stored in the vapour of liquid nitrogen.

I collected ova from running ripe (ovulated) females into a 1000 ml graduated flask. I only used positively buoyant, clear and spherical ova in the experiment, as ova with these characteristics generally result in high fertilisation success (Jenkins *et al*. 1999). For each experimental cross an average (± SE) of 90 ± 2.3 ova were transferred into a 7 ml scintillation tube containing 5 ml of filtered lake water. I used six replicates per male-female combination. Just prior to performing the crosses an aliquot (1000 µl) of the cryopreserved semen of each male was transferred from storage in liquid nitrogen to a water bath at room temperature (20 – 22 °C) where it was thawed for three minutes. The thawed solution was stored on ice at ~4 °C before 100 µl of the semen was added to each of the six replicate tubes per male. After 10 minutes (the time it took to perform all 72 fertilizations for a given female [12 males × 6 replicates], I transferred the contents of each

tube to separate 70 ml vessels containing 50 ml of filtered lake water. All experimental procedures were conducted in the field.

Fertilised ova were stored at room temperature in total darkness for 36 hrs, after which time fertilisation success was scored as the ratio of the number of larvae alive to the total number of ova. This was the simplest measure as at 36 hrs larvae were well developed (i.e., in the process of hatching) and positively buoyant if viable, whereas unfertilised ova or ova fertilised but which had ceased to develop were opaque and had descended to the bottom of the vessel.

Because cryopreserving semen could reduce fertilisation success relative to fresh semen (e.g., Lahnsteiner *et al*. 2004, though see Chereguini *et al*. 2003) I estimated the viability of sperm from each male using a LIVE/DEAD sperm viability assay as per the manufacturers instructions (Molecular Probes) (e.g., Sherman *et al*. 2008). Briefly, semen was stained and visualised using fluorescence microscopy. The membrane permeant nucleic acid stain used in the assay, SYBR 14, fluoresces with bright green in live sperm with intact cell membranes, while propidium iodide (the dead cell stain) fluoresces with bright red when cells are damaged or dead. To facilitate counting, each 1000 µl aliquot (of cryopreserved semen) was serially diluted to a concentration of 10^2 in HBSS. Two microlitres of a 30-fold dilution of SYBR 14 in HEPES-buffered saline (10mM HEPES, 150mM NaCl, 10% BSA, pH 7.4) and 2 µl of propidium iodide were added to 1 µl of the diluted semen. The mixture was incubated at room temperature for 10 minutes, after which time a drop of the solution was placed on a microscope slide and the viability of the first 100 sperm cells encountered were scored at 40-times magnification. I estimated the density

of sperm per ml for three replicates of each aliquot by counting the numbers of sperm in five of the 1/25 sq. mm squares on a haemocytometer. Sperm concentration and viability were quantified *a priori* using aliquots of semen from each male that were different to the aliquots used in the experiment. Across 31 aliquots of cryopreserved semen, both the average (± SE) concentration of sperm per ml, and the average proportion of viable sperm, were highly variable ranging between 6.1×10^8 (5.0×10^6) and 1.4×10^9 (2.0×10^6) and between 0.04 (0.01) and 0.61 (0.05). Such variation in the concentration of viable sperm may explain some of the variation in the fertilisation success achieved for the six different kinds of pairing (Fig. 5.2); however, because I was primarily interested in whether or not fertilisation could occur, it did not effect my interpretations.

5.3 Results

My experimental crosses revealed no evidence that gametic incompatibility provides a barrier to fertilisation among both pure species and their hybrids. Although there was considerable variation in fertilisation success among replicates of specific crosses, on average the proportions of ova fertilised were remarkably similar for all six different kinds of pairing (Fig. 5.2). Fertilisation trials involving hybrid and *A. australis* males crossed with *A. butcheri* females revealed almost identical levels of gamete compatibility to trials involving pure species *A. butcheri* (average proportion of viable larvae [± SE] = 0.43 [0.22] & 0.35 [0.21] vs. 0.40 [0.16]). Moreover, gametes of *A. butcheri*, hybrid and *A. australis* males were highly compatible with ova of hybrid females, with the average level of compatibility comparable to the set of crosses detailed above (range = 0.32 – 0.43).

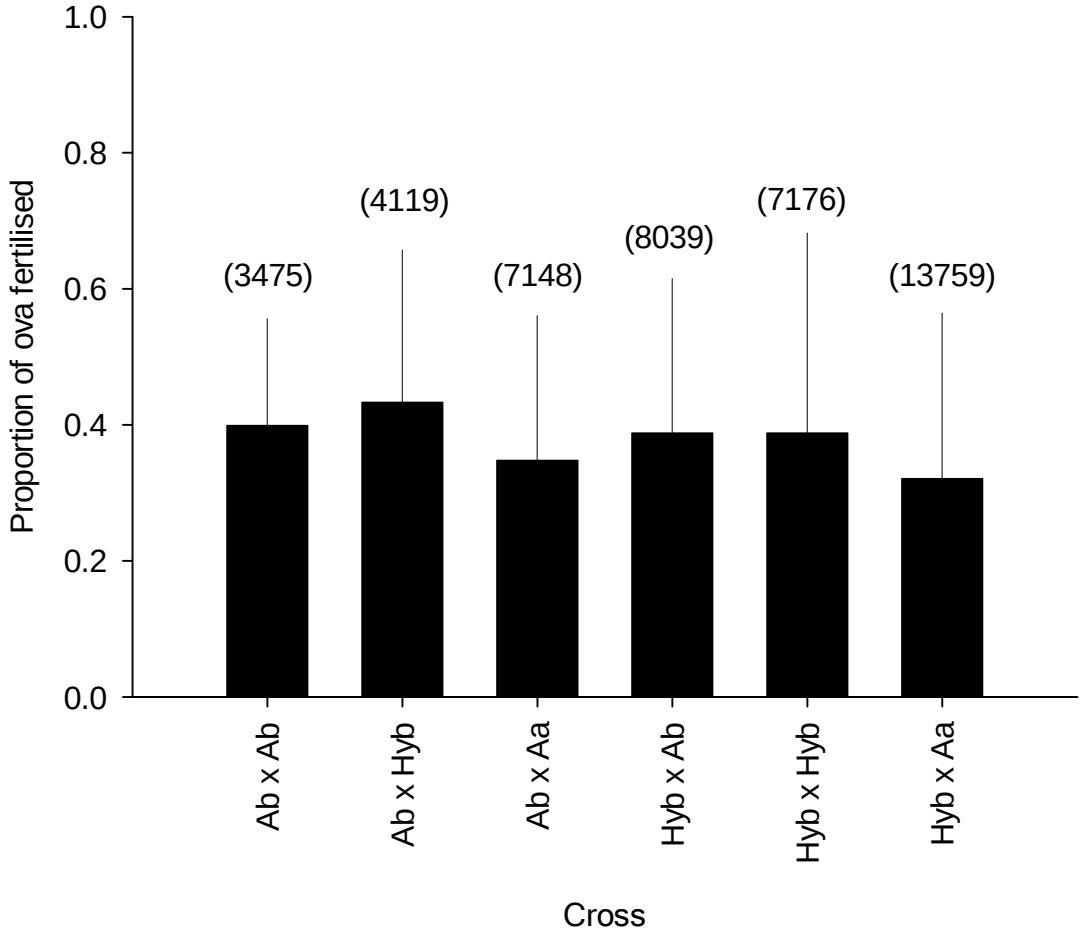

Figure 5.2 Fertilisation success scored as the proportion of ova fertilised for six different classes of cross involving *Acanthopagrus australis* (Aa), *A. butcheri* (Ab), and their hybrids (Hyb). The crosses were between *A. butcheri* and hybrid females (represented first [Ab x … & Hyb x …] on the x-axis labels), and *A. australis*, *A. butcheri*, and hybrid males. The error bars represent SE and the numbers in parentheses are the total number of ova per class of cross.

5.4 Discussion

Although my earlier genetic study suggests that hybrid and backcrossed bream are common in the estuaries of the NSW south coast it is unclear whether they are the product

of past or continuing hybridisation. The likelihood that interspecific matings can currently produce F1 hybrids or backcrosses may reflect both the relative and absolute frequencies of each class of genotype within spawning aggregations (i.e., *A. butcheri, A. australis* and their hybrids) and the strength and nature of isolating mechanisms. For instance, F1 hybrid formation may be a rare event if egg-sperm recognition or gamete incompatibility systems prevent most interspecific fertilisations (Palumbi & Metz 1991; Palumbi 1999; Lessios 2007), however, backcrossing might occur more frequently because of assortative mating facilitated by gametic selection i.e., strong compatibility between hybrids and the parental group hybrids most resemble genetically (e.g. Rieseberg *et al*. 1998). Using genetic markers to distinguish pure *A. australis, A. butcheri*, and hybrids, I was able to utilise a genotypically diverse array of naturally spawning fish in fertilisation experiments to examine the likelihood of initial and later hybrid formation. My results confirmed that first generation (*A. australis* × *A. butcheri*) and later generation hybridisation (hybrid × hybrid), as well as backcrossing (hybrid × *A. butcheri*) are indeed possible and seemingly equally likely. These data imply that hybridisation and backcrossing could occur readily within mixed spawning aggregations of hybrid, *A. australis* and *A. butcheri* when spawning seasons overlap and both species males have access to *A. butcheri* females.

Additional work will be needed to determine the composition of breeding aggregations within lagoons and to establish whether mate choice acts as a barrier to hybridisation. Moreover, recent reproductive compatibility assays in free spawning taxa have revealed marked preferences for conspecific sperm in situations where ova are exposed to heterospecific sperm mixtures (a result which would not have been predicted from only conducting no-choice experiments) (Geyer & Palumbi 2005; Harper & Hart

2005; Willis *et al*. 2006). It now seems crucial to establish whether fitness differences exist between pure species and hybrids. At a minimum, we need to know if the high level of hybridity detected in some NSW lagoons reflects a selective advantage of hybrids relative to *A. butcheri*, for example, greater survival, or increased growth rates of hybrids and therefore ability to exploit resources more effectively than *A. butcheri* (see Einum & Fleming 1997; Rosenfield & Kodric-Brown 2003; Facon *et al*. 2005).

My intention was to conduct a more powerful examination of early acting barriers to initial and later hybridisation, including determining the likelihood that hybridisation will occur within mixed species aggregations. Ideally, crosses would be conducted to test for gamete compatibility, the potential for sperm competition in mixed sperm situations, and then larvae reared to test for fitness differences between pure species and hybrids. This could be achieved by conducting the following intra- and inter-specific matings: $eggs_{species\ 1} \times sperm_{species\ 1}$; $eggs_{species\ 2} \times sperm_{species\ 2}$; $eggs_{species\ 1} \times sperm_{species\ 2}$ and the reciprocal mating; plus to test for interspecific competition: $eggs_{species\ 1} \times sperm_{species\ 1}$ and 2 and the reciprocal mating. Moreover, each cross would ideally be conducted across a range of different sperm concentrations, and varying the concentrations of intra-and interspecific sperm in situations where ova are exposed to homo- and heterospecific sperm. Then, by determining the proportion of pure species and hybrids produced in mixed sperm situations by genotyping and determining paternity, then comparing between the fertilisation success resulting from intra-specific mating in 'sperm choice' and 'no choice' situations, it would be possible to determine the relative importance of genetic incompatibility versus sperm competition in limiting or facilitating hybridisation.

The high frequency of hybrids and lack of, or uncertain origin of, *A. butcheri* in NSW estuaries (Chapter 2), together with my dependence on wild caught fish in spawning condition for gametes, precluded my being able to conduct such crosses. I did however attempt the experimental protocol described above at Challenger TAFE Aquaculture Development Unit, Fremantle Maritime Centre, WA. Although techniques for captive breeding and rearing of *A. butcheri* are well established at this facility (Jenkins *et al*. 1999), my experiments were largely unsuccessful. Reproductive and stress hormones are known to have a detrimental impact on post-ovulatory egg viability in teleost fish (e.g., Hobby & Pankhurst 1997), and in this instance, low egg viability was a major impediment to the success of my experiments. I was unable to obtain sufficient numbers of viable or 'good quality' ova from broodstock held at the facility (nor were WA *A. butcheri* females in spawning condition able to be caught in the wild during the six week period I worked at the hatchery). Indeed, ova from broodstock held at the facility were either underdeveloped, overdeveloped or of poor quality, preventing my being able to conduct crosses in the first instance, or when crosses were performed with ova that appeared of good quality under a microscope (in hatchery rearing of *A. butcheri*, the use of clear and spherical ova between 700 – 800 μm in diameter, with a single spherical oil globule approximately 250 μm in diameter usually results in high fertilisation success), the resulting fertilisation success was extremely low (including within a set of control crosses in which fresh sperm were added to the ova – as in my preliminary experiment, hybrid, *A. australis*, and *A. butcheri* male gametes were cryopreserved). Hence, the data were insufficient to report.

Chapter 6 Marine genetic swamping: hybrids replace an obligately estuarine species

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6.1 Introduction

Obligately estuarine taxa exist within a series of naturally isolated populations often with small effective population sizes and, as a consequence of drift and inbreeding, are expected to display low levels of genetic variation and possible inbreeding depression (Chaplin *et al*. 1998; Yap *et al*. 2000). Low levels of hybridisation with a wide-ranging marine congener could therefore be beneficial as a source of genetic novelty and could increase the fitness of local populations. When offspring are viable and interfertile, however, hybridisation may have detrimental effects if outbreeding depression results from the breakdown of locally adapted genotypes or co-adapted gene complexes (Templeton 1986; Edmands 2007). High levels of hybridisation can threaten the persistence of hybridising species (Rhymer & Simberloff 1996; Levin *et al*. 1996) through genetic swamping leading to the loss of species identity (Woodruff & Gould 1987; Allendorf *et al*. 2001, 2004, 2005). This problem is likely to be exacerbated when hybrids are interfertile and remain resident in estuaries as further interbreeding may lead to the production of advanced generation hybrids and backcrosses. Even when hybrids are not interfertile,

obligately estuarine taxa may be threatened by demographic swamping with the gametes of the estuarine taxon being completely wasted in failed interspecific matings (e.g. Leary *et al*. 1993). For most taxa the degree or extent of gamete compatibility is unknown and this will influence the likelihood of initial and later hybridisation.

Reduction to the size of populations of obligately estuarine taxa by fishing may exacerbate demographic swamping, with consequent elevation of rates of hybridisation due to the estuarine taxon becoming relatively or absolutely rare. The impacts of this process are however likely to be estuary specific with hybrid fitness and the likelihood of hybridisation and subsequent introgression influenced by local conditions including the frequency of an estuary opening to the adjoining ocean.

In my initial broad scale genetic survey (Chapter 2) I showed that hybrids between *A. australis* and *A. butcheri* are common, with most hybrids occurring in the area of sympatry, though I detected hybrids beyond the recognised range of *A. australis*. There are essentially two competing explanations for the situation uncovered in the area of sympatry. First, the large numbers of introgressed bream may reflect ancient hybridisation events that have led to the formation of an essentially stabilised hybrid taxon, with little continuing introgression. Alternatively, the area may represent an underappreciated dynamic hybrid zone in which the frequency of hybrids may vary across both estuaries and generations. My phylogeographic study revealed that, unlike many taxa that have historically been fragmented by the Bassian Isthmus landbridge and form deeply divergent eastern and western lineages (see Introduction Chapter 4), *A. butcheri* form very shallow lineages that show between 0.65 and 1.04% sequence divergence. This, together with a clear lack of

isolation by distance and several haplotypes found throughout the recognised range of the species, imply a recent history of colonisation of estuaries throughout southern Australia, leaving open the possibility that contemporary processes are important in the maintenance of *Acanthopagrus* hybrid zones. Indeed, preliminary indications are that hybridisation is so frequent that genetic and demographic swamping threatens the persistence of *A. butcheri* (Chapter 2). Moreover, it seems likely that anthropogenic disturbances such as modification of estuarine hydrodynamics through alteration of the frequency of estuary flushing, and fishing, are in some years changing the interactions of the species by increasing the potential for contact between *A. australis* and potentially isolated and thus self-seeding populations of *A. butcheri* or between *A. butcheri* and hybrids.

Here I conducted a fine scale survey of fish (in the *Acanthopagrus* spp. complex) in estuaries in the area of sympatry to further characterise the frequency of occurrence and persistence of hybridisation. I hypothesise that the dynamic nature of estuaries and their varying morphologies will mean that opportunity for hybridisation will vary among years and the proportion of hybrids should vary both across estuaries and age classes. Indeed, I predict that frequencies of hybrids will be greater in lagoons that are generally permanently closed to the ocean, where *A. australis* and *A. butcheri* can become trapped together by lagoon-entrance channel closure. I examined large numbers of juvenile fish from the same year of birth (i.e., recruits) at multiple sites in five lagoons that varied in terms of their frequency of closure to the ocean, and included temporal replication of a broad but known age range of adult fish in two lagoons to determine if hybridisation is a general phenomenon, and if frequencies of hybrids are stable over generations.

6.2 Methods

6.2.1 Study lagoons and history

I surveyed five estuaries distributed over approximately 100 kms of coast in the area of sympatry of *A. butcheri* and *A. australis* in southern NSW (Fig. 6.1). The entrances of four of these lagoons are intermittently open to the ocean, but, Coila, Corunna and Brou Lakes rarely open naturally and remain open for much less time than Wallaga Lake (several weeks per year *c.f.* several months). The entrance channels to these lagoons are periodically mechanically opened so that water can discharge, though the frequency with which this occurs is highly variable (DECCW NSW, ICOLL entrance condition, unpublished data). The entrance of Tuross Lake is permanently open to the ocean, except in times of extreme drought. Since the early 1900's commercial fishers have harvested *Acanthopagrus* spp. in these estuaries, though the proportion of each species in landings is not known.

6.2.2 Collection of juvenile fish

I surveyed large numbers of juvenile fish (i.e. fish < 3 cm in length) following a single recruitment event resulting from the 2001winter/spring spawning of *A. australis* and *A. butcheri*, respectively. Across all five estuaries I collected 688 juvenile *Acanthopagrus* spp (81 – 170 per estuary). I sampled over a two-month period beginning in February 2002, approximately three months after *A. butcheri* spawn (i.e., 2001 juveniles). Examination of ICOLL entrance condition data (DECCW NSW, unpublished data) for the 12 month period prior to my sampling (thus encompassing both *A. australis* and *A. butcheri* spawning and recruitment) revealed that Coila Lake was closed to the ocean, as was Lake Brou except for an approximately three month period between the end of October 2001 and

the end of January 2002 (i.e., after *A. australis* recruitment but during *A. butcheri* spawning and recruitment). In contrast, the entrance channel of Tuross Lake was open for both *A. australis* and *A. butcheri* recruitment and spawning, whereas the entrance channels of Corunna and Wallaga Lakes were open from July and August 2001 respectively, corresponding with the end of *A. australis* spawning and peak *A. australis* recruitment, and all of the *A. butcheri* spawning and recruitment (the lagoons closed again in respectively August and January 2002). I collected between 20 and 66 fish from seagrass meadows at 3 – 4 haphazardly chosen sites per estuary. The locations of sites were determined in part by the ease of access to the shore, but sites were always separated by more than 500 m. Fish were captured with a 10 × 2 m haul-seine net (6 mm mesh) over an approximately 25 m^2 area. I genotyped all 688 juveniles using eight microsatellite loci and characterised their mitochondrial DNA (mtDNA) using restriction digests of 400bps of the 3' domain of the control region.

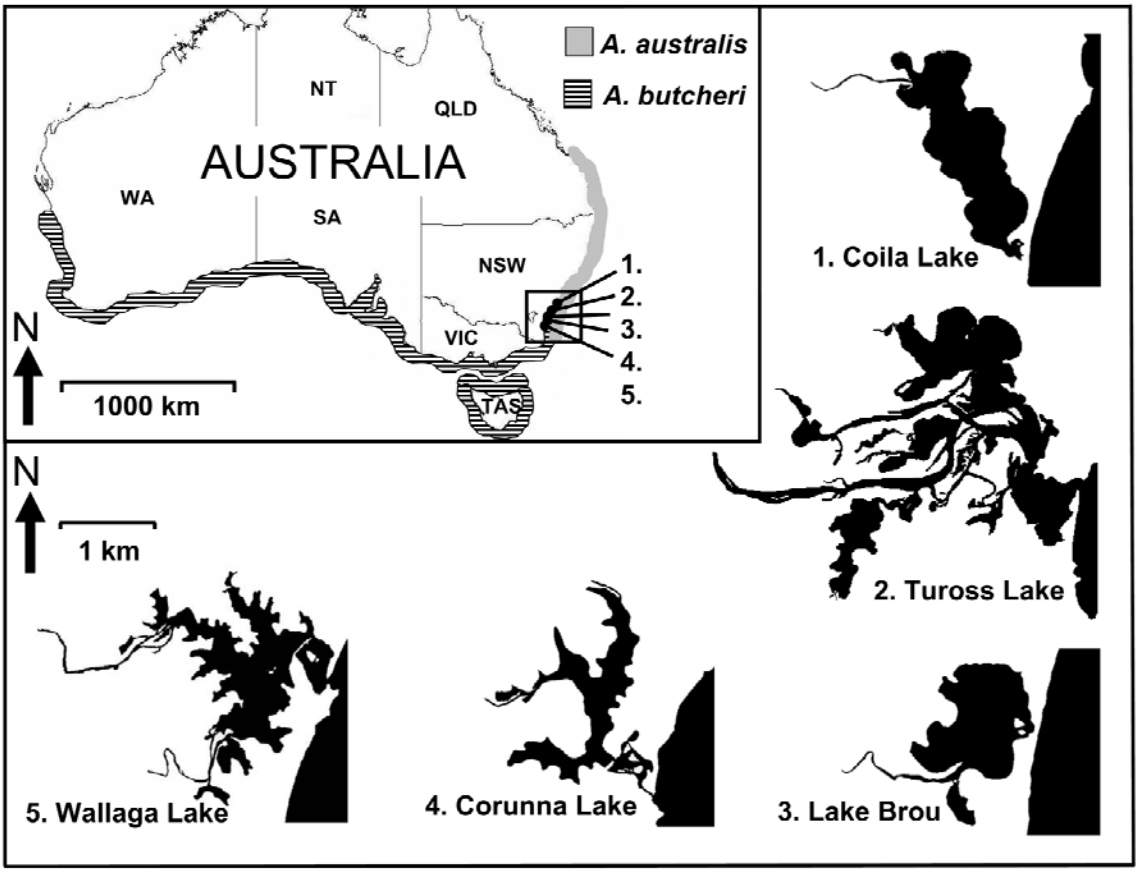

Figure 6.1 The location of study sites, and the distribution of *Acanthopagrus butcheri* and *A. australis*.

6.2.3 Temporal replication: adults of known ages

In order to determine if hybridisation had occurred in multiple cohorts of fish and whether the frequency of hybrids was stable over time I genotyped a total of 168 and 114 adult fish from Coila and Brou Lakes respectively. This could only be done using microsatellites, as the DNA of these stored specimens was too degraded for PCR amplification of the larger mtDNA fragment (400 bps). These fish were captured between 2004 and 2005 and their ages estimated from otoliths (NSW DPI, unpublished data). Based on their ages, fish were separated into specific cohorts i.e., year of birth (YOB) (Coila

Lake: 1999, n = 89 and 2003, n = 79; Brou Lake: 1995, n = 19; 1999, n = 38 and 2003; n = 57).

6.2.4 Genetic analyses

I used the microsatellite 'genetic reference' collection (see Chapter 2) to assign my current samples to genotype classes (Roberts *et al*. 2009). At the simplest level, I performed Factorial Correspondence Analysis (FCA) (in GENETIX 4.03 [Belkhir *et al*. 2002]) on the overall sample of 2001 juveniles for each lagoon together with the previously categorised fish of my reference collection. I again used STRUCTURE to estimate the ancestry, and thus the specific status of all 942 fish (688 juveniles & 254 adults of known ages); however, as for the simulated data in Chapter 2, the analysis was refined by incorporating the allele frequency distributions of genotypic classes estimated from the reference collection. The threshold q_i-values for classifying individuals as either pure species or hybrid were as detailed in Chapter 2. Although the estimation of the frequency of hybrids using STRUCTURE strongly depends on the arbitrarily q_i value threshold chosen to classify an individual as parental, I found that relaxing the threshold ($q_i = 0.1$ & 0.2) made no substantive difference to my conclusions (see Roberts *et al*. 2009).

6.3. Results

6.3.1 Genetic composition of juvenile fish within lagoons

The FCA used to visualise the genetic relationship among the samples revealed that the composition of the 2001 juveniles in each lagoon was highly variable with many fish displaying genotypes that overlapped strongly the genotypes of pure *A. australis* (within my adult reference collection), smaller numbers of apparently pure *A. butcheri* and variable

numbers of genetically intermediate fish (Fig. 6.2a) (allele frequencies for the juveniles are contained in Appendix 4). The admixture analysis revealed that an extremely low proportion of the 688 juvenile fish surveyed were in fact pure *A. butcheri* (0.05), and approximately half of the sample was classified as hybrid (Fig. 6.2b). When I relaxed the estimate of the q-threshold used to classify pure *A. butcheri* this unsurprisingly increased the number of apparently pure *A. butcheri* detected, however, application of q values of 0.1 and 0.2 still implied that the 2001 juveniles included a very low proportion of *A. butcheri* (0.09. & 0.22 respectively) (Table 6.1).

Although juvenile hybrids were present in every estuary studied, the proportion of hybrids was much greater in Coila and Brou Lakes (> 0.90 vs. 0.12 – 0.27 hybrids). In Coila, Brou and Corunna Lakes the hybrids were evenly distributed among the sites (data not shown) indicating that the sample was almost certainly representative of the *Acanthopagrus* population of each lagoon. In partial contrast relatively few hybrids were detected within Tuross and Wallaga Lakes (0.14 & 0.12 hybrids) and these samples were dominated by *A. australis* (Table 6.1).

The juvenile hybrids within Coila and Brou Lakes were genetically similar to pure *A. butcheri*, based on microsatellites. In addition, these fish possessed mtDNA characteristic of *A. butcheri*, thus these are most likely the offspring of later generation hybrids or *A. butcheri* backcrosses. In contrast, in Corunna, Tuross, and Wallaga Lakes, there was a disproportionate abundance of juvenile hybrids (range = 0.62 – 0.93) that were more genetically similar to *A. australis* than *A. butcheri* i.e., most of their microsatellite

alleles and their mtDNA were characteristic of *A. australis* and thus they were presumably *A. australis* backcrosses (Fig. 6.2).

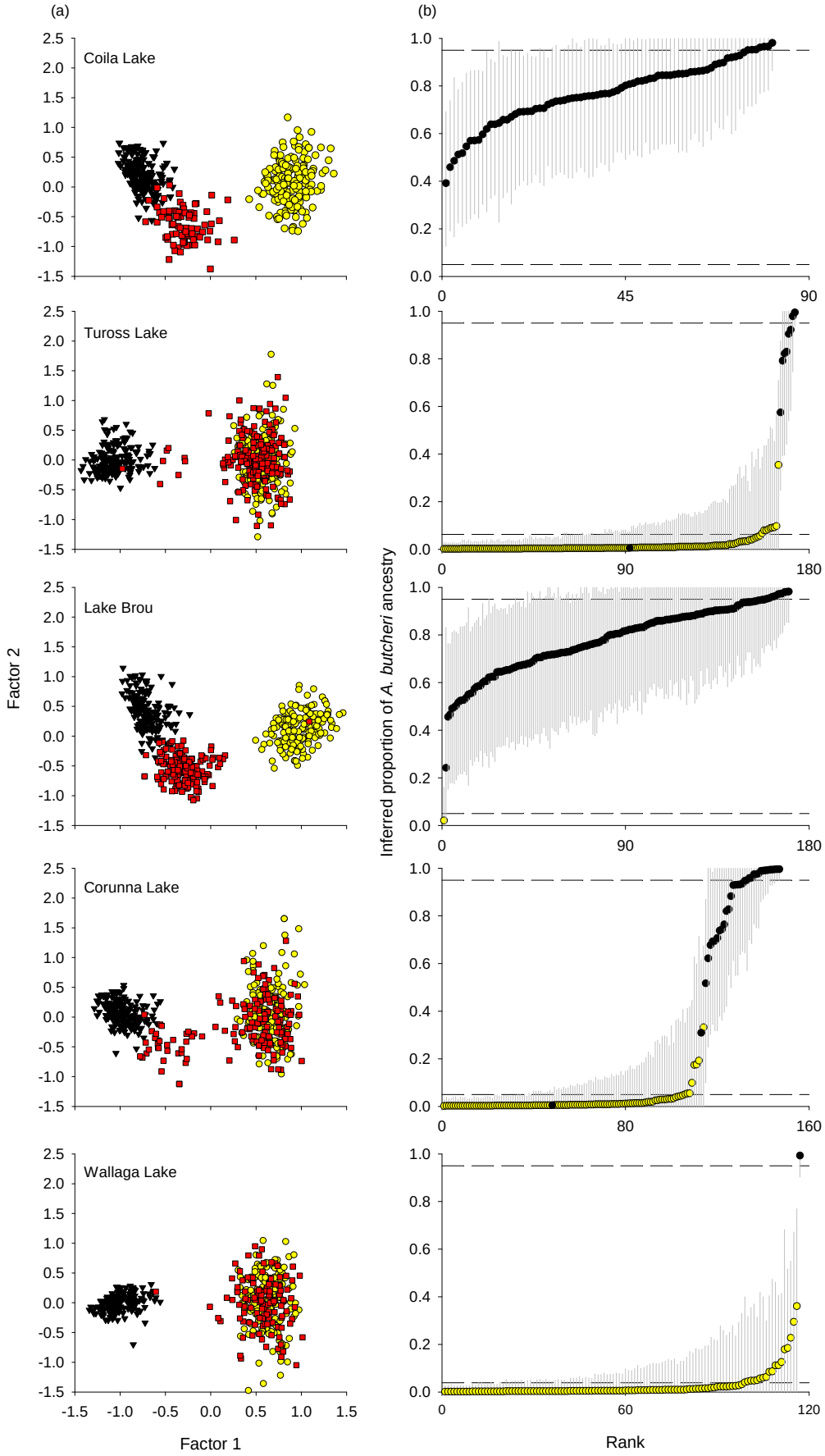
(a)
(b)
Coila Lake
Tuross Lake
Lake Brou
Corunna Lake
Wallaga Lake
Factor 2
Factor 1
Inferred proportion of A. butcheri ancestry
Rank

Figure 6.2 Factorial correspondence analysis (a) based on the eight-locus microsatellite genotype of juvenile *Acanthopagrus* spp. (year of birth = 2001) within five coastal lagoons (n = 81 – 170 per lagoon [red solid squares]) in southeastern Australia. A genetic 'reference collection' (Roberts *et al*. 2009) of allopatric *A. australis* (yellow solid circles) and allopatric *A. butcheri* (black solid triangles) were included and plotted for each analysis. To assign the sample of juveniles to genotype classes, the ancestry of each individual was estimated using the allele frequencies of the reference collection (b). Ancestry is presented as the inferred proportion of *A. butcheri* genome (average $q_i \pm$ 95% CIs). Based on the estimate of ancestry we classified each individual as either pure *A. australis* ($q_i \leq 0.05$) or *A. butcheri* ($q_i \geq 0.95$) or hybrid ($0.05 < q_i < 0.95$). Mitochondrial haplotypes characteristic of *A. australis* and *A. butcheri* are indicated by yellow and black shading respectively.

6.3.2 Comparison of hybrid proportions across year classes

Examination of the genotypes of adult fish of known ages in Coila and Brou Lakes revealed high proportions of putative later generation hybrids and/or *A. butcheri* backcrosses that matched my findings for juvenile fish from the 2001 spawning. I detected high proportions of hybrid fish in two year classes for Coila Lake (YOB: 1999 = 0.88; 2003 = 0.97) and three year classes for Brou Lake (1995 = 0.79; 1999 = 0.79; 2003 = 0.81) (Table 6.1).

Table 6.1 Proportion of juvenile *Acanthopagrus australis* (Aa), *A. butcheri* (Ab), and hybrids (Hyb) in five southeastern Australian lagoons (including temporal replication in two lagoons, Coila and Brou Lakes). *

Lagoon, year	q-threshold 0.05			0.10			0.20		
	Aa	Ab	Hyb	Aa	Ab	Hyb	Aa	Ab	Hyb
Coila, 1999 (n = 89) Ê	0	0.12	0.88	0	0.20	0.80	0	0.47	0.53
Coila, 2001 (n = 81)	0	0.09	0.91	0	0.15	0.85	0	0.46	0.54
Coila, 2003 (n = 79)	0	0.03	0.97	0	0.13	0.87	0	0.34	0.65
Tuross, 2001 (n = 179)	0.85	0.01	0.14	0.91	0.01	0.08	0.95	0.02	0.03
Brou, 1995 (n = 19)	0	0.21	0.79	0	0.26	0.74	0	0.42	0.58
Brou, 1999 (n = 38)	0	0.21	0.79	0	0.34	0.63	0	0.5	0.5
Brou, 2001 (n = 170)	0.01	0.07	0.92	0.01	0.22	0.78	0.01	0.52	0.48
Brou, 2003 (n = 57)	0	0.19	0.81	0	0.28	0.72	0	0.39	0.61
Corunna, 2001 (n = 147)	0.66	0.07	0.27	0.73	0.08	0.19	0.74	0.14	0.12
Wallaga, 2001 (n = 117)	0.87	0.01	0.12	0.92	0.01	0.07	0.96	0.01	0.03
Overall, 2001 (n = 688)	0.50	0.05	0.45	0.55	0.09	0.36	0.56	0.22	0.22

*The specific-species status of each individual was determined using admixture analysis based on eight microsatellite loci. The effect of varying the q-threshold value (q_i = the average proportion of *A. butcheri* genome) used to distinguish parentals and hybrids is also displayed.

Ê All 2001 fish were caught as juveniles just after spawning in *A. butcheri*, whereas all other fish were caught as adults, with the age of each fish estimated from its otoliths. Based on the age and date of capture, individual fish were separated into specific cohorts i.e., year of birth – 1995, 1999 & 2003.

6.4 Discussion

My findings demonstrate that hybridisation and introgression involving the obligately estuarine *A. butcheri*, and its migratory coastal marine congener *A. australis*, make a massive contribution to the genotypes of *Acanthopagrus* spp. within coastal lagoons

in the zone of greatest species overlap. Although discussions of hybridisation have rarely considered hybridisation between a marine and obligately estuarine taxon, the situation uncovered may be similar to hybridisation and introgression described for freshwater and anadromous species worldwide that are being impacted by introduced species, with consequent genetic swamping and genomic extinction of locally rare or geographically restricted taxa, or loss or breakdown of local adaptation (Perry *et al*. 2001a, 2001b; Allendorf *et al*. 2004, 2005). Using microsatellite and mitochondrial DNA marker surveys, here I was able to show (i): that hybrids can make up a considerable proportion of the population of a given lagoon, (ii.) hybridisation has occurred in multiple cohorts of fish with the frequency of hybrids stable across years, and (iii.) hybrids persist through to adulthood and are long-term residents (multi-generational) within lagoons.

The genetic composition of the set of fish present within Coila and Brou Lakes highlights the difficulty of characterising the outcome of introgression and perhaps the elasticity of species concepts. It could be argued that the virtual absence of pure *A. butcheri* within these lagoons implies that genetic swamping has indeed eliminated the resident *A. butcheri* population. On the other hand it seems likely that these populations which contain few *A. australis*-like or even F1 hybrids are receiving less contemporary genetic input from *A. australis*, and thus the extent of genetic swamping is being tempered by infrequent entry of *A. australis* into these lagoons.

My genetic data revealed that in contrast to Coila and Brou Lakes, Tuross, Corunna, and Wallaga Lakes supported large numbers of *A. australis* juveniles. This is perhaps not surprising given that an open-entrance channel is a necessary prerequisite for *A. australis*

larvae to recruit from the plankton and settle within seagrasses in ICOLLs (Griffiths 2001), and indeed the entrance channel of each lake was open to the ocean for at least part of *A. australis* spawning and recruitment in 2001. I would not have expected large numbers of juvenile *A. australis* within Coila and Brou Lakes, since both of these lakes were closed to the ocean for *A. australis* recruitment, and my initial genetic survey of adult *Acanthopagrus* spp. within Coila Lake implies that just a small proportion of the resident adult population trapped within the lake (at the time of my sampling of juvenile fish) was *A. australis* (I detected 2/50 = 0.04 adult *A. australis*, Chapter 2).

Importantly, the contrasting genotypic composition of the set of bream within frequently open and typically closed lakes provides powerful evidence that primary hybridisation between *A. butcheri* and *A. australis* is an important contemporary process. My data imply that lakes which are open to the ocean for both *A. australis* and *A. butcheri* spawning and recruitment, such as Tuross, Corunna, and Wallaga Lakes, provide sites for primary hybridisation between *A. australis* and *A. butcheri*, but in addition provide nursery habitat for apparent *A. australis* backcrosses and/or early generation hybrids that have presumably recruited to such lakes from the plankton and which are therefore likely produced within *A. australis* spawning aggregations (Chapter 2, for detail of the distribution of adult *A. australis*-like hybrid fish). Indeed, examination of the genotypes of hybrid juveniles within those lakes revealed that, the hybrid fish intermediate between the two gene pools based on microsatellites, possessed mtDNA characteristic of *A. butcheri* and were presumably first generation hybrids produced by mating between *A. australis* males and *A. butcheri* females within the lakes themselves. In contrast, most hybrid juveniles with microsatellite genotypes closely aligned with *A. australis* possessed mtDNA

characteristic of *A. australis* and were presumably *A. australis* backcrosses, with hybridisation proceeding through *A. australis* females. Although much remains to be learnt about the factors facilitating hybridisation in this system, my data clearly demonstrate that the timing of lake entrance channel opening to the ocean can influence both opportunity for initial and later hybridisation, and therefore it seems likely that the frequency and duration with which lakes are open to the ocean would influence the net outcome of hybridisation for the estuary restricted *A. butcheri.*

Chapter 7 Temporal stability of a hybrid zone between migratory marine and estuarine fish

This chapter has been submitted to *Marine-Ecology Progress Series* and is currently with referees.

Roberts, D. G., Gray, C. A., West, R. J. & Ayre, D. J. 2010 Temporal stability of a hybrid zone between migratory marine and estuarine fish *Marine-Ecology Progress Series* in review.

7.1 Introduction

Hybridisation between migratory marine and estuary restricted taxa can lead to the formation of hybrids when spawning overlaps within estuaries. However, the opportunity for hybridisation to occur and for hybrids to persist may depend upon the reproductive compatibility of parental species together with a range of factors including water temperatures and movement, estuary-mouth opening, and hybrid fitness and behavior. Given the often erratic nature of ocean currents (e.g., Ridgeway 2007) and variability in the accessibility of estuarine habitat (Jones & West 2005; Dye 2006; Rustomji 2007), primary hybridisation within estuaries may therefore be rare and hybrid zones ephemeral. However, persistent hybrid swarms may form if viable and inter-fertile hybrids remain within estuaries.

In Chapter 6 I used microsatellite and mitochondrial DNA data to show that hybridisation and introgression have made a considerable contribution to the genotypes of

Acanthopagrus spp. occupying five ICOLLs in southern NSW. Within these five lagoons the extremely high frequency of hybrids and virtual absence of *A. butcheri* (as identified elsewhere in the species range) suggests that these hybrid zones are dynamic. However, nothing is known about the longer-term stability of hybrid populations and the multi-generational persistence of hybrids within these lakes and lagoons. Fortunately the high incidence of fish specimens archived within museum collections and stability of DNA within preserved 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 my initial broad-scale survey of estuarine and coastal *Acanthopagrus* spp. populations revealed the presence of hybrid fish within the Gippsland Lakes, a large coastal lagoon 250 km south of the area of my earlier intensive sampling (Chapter 6) and an area considered beyond the normal southern range of *A. australis* (Fig. 7.1). Nevertheless, within this region of southern Australia the EAC provides predominantly southward although erratic water movements that may cause infrequent migration of *A. australis* (larvae or adults - see Chapter 3) beyond its accepted range limit. Moreover, in recent times several authors have documented unusually high levels of southward transport of tropical taxa by the EAC (Booth *et al*. 2007), unusual establishment or 'overwintering' of tropical taxa beyond their accepted ranges (Johnson *et al*. 2005; Figueira & Booth 2009), and oceanographic modelling predicts a warmer and strengthened southward flow of the EAC as a consequence of global climate change (Poloczanska 2008). Here opportunities for hybridisation seem likely to be rare and I would expect that populations containing hybrids would be ephemeral unless hybrids persist and backcross within the lakes. In this

chapter I use a set of museum specimens to describe the genotypic composition of the Gippsland Lakes population of *Acanthopagrus* spp. over a 60 year period. These data allow me to determine whether levels of hybridity are comparable to those seen further north in the area of sympatry (Chapters 2 & 6) and to test the prediction that the population represents a stable hybrid swarm implying that hybrids are favoured within estuarine habitats.

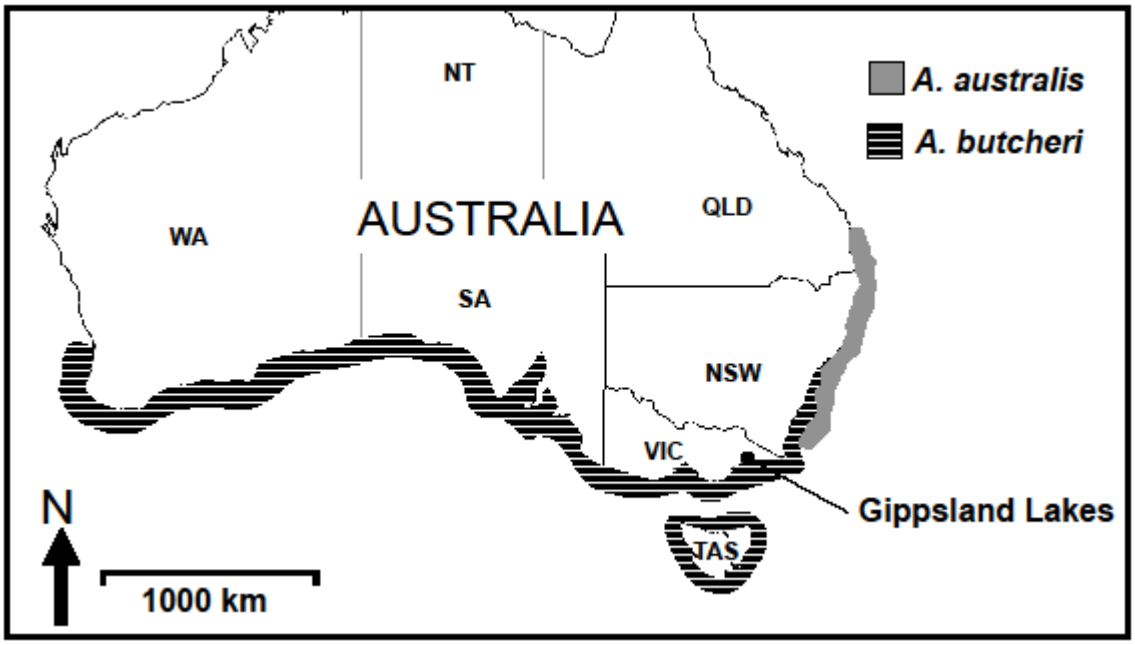

Figure 7.1 The location of the Gippsland Lakes, Victoria. The distribution of the estuary restricted *Acanthopagrus butcheri* and migratory marine *A. australis* is also displayed.

7.2 Methods

7.2.1 Material for genotyping

Contemporary specimens consisted of two samples of fin clips (from 1996/97 and 2000, total n = 114) stored in Dimethyl sulfoxide saturated salt solution (DMSO 20%,

0.25M Ethylenediaminetetraacetic acid, NaCl PH 8.0). The museum specimens (or historical sample) 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).

7.2.2 DNA extraction, molecular markers and genotyping

To extract DNA, a scale (or a < 5 mm^2 fin clip) was placed in a sterile 1.5µl tube containing 5% chelex resin in 500 µl of sterile distilled H_20 and 15µl of Proteinase K (10mg/ml), and heated at 65°C for 12hrs. Before the supernatant was used directly in PCR, the solution was vortexed for 10 seconds, heated at 100°C for 5 minutes, and centrifuged at 12 000k for 7 minutes. I genotyped all 247 samples using six microsatellite markers described in Roberts *et al*. (2009) (Chapter 2). I 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 my results. Overall, the average (± SE) proportion of missing genotypes (i.e., genotypes that could not be resolved, even after repeated DNA extraction and PCR) per locus was consistently low for all samples (1941, 0.02 ± 0.01; 1943, 0.01 ± 0.01; 1996, 0.03 ± 0.01; 2000, 0.03 ± 0.02).

7.2.3 Genetic analyses

7.2.3.1 Temporal stability of allele frequencies

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 *A. 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*. I therefore tested for homogeneity of alleles frequencies among collections (1941, 1943, 1996, & 2000) (allele frequencies are contained in Appendix 5), and calculated standard measures of genetic diversity for each year i.e., number of alleles per locus, observed and expected heterozygosity (using POPGENE, Yeh *et al*. 1999). I estimated allelic richness (the standardised number of alleles per locus [n = 40 per sample]) for each year. Weir & Cockerham's (1984) formulation of Wright's (1969) *F*-statistics were used to estimate genetic differentiation among years (FSTAT [Goudet 1996]). The estimates were based on allele frequencies for individual loci and as an average across loci. Bootstrapping and jacknifing procedures across loci were used to estimate standard errors. I tested the statistical significance of heterozygous deficits and heterozygote excesses for each locus and overall using Exact tests implemented in GENEPOP (Raymond & Rousset 1995).

7.2.3.2 Levels of hybridity

I tested for the presence of hybrids by performing an admixture analysis (using the program STRUCTURE; Falush *et al*. 2003) incorporating my previously identified 'reference collection' of pure species in the analysis. For this analysis I used only the data from the four microsatellite loci that I had shown previously had the greatest power to distinguish hybrids and indeed display almost fixed differences between the two parental species (Roberts *et al*. 2009; Chapter 2). Because estimates of the proportion of hybrids in a sample largely depend on the arbitrarily q-value threshold (q = the inferred proportion of ancestry, in this instance we present data as the inferred proportion of *A. butcheri* ancestry)

chosen to classify an individual as pure parental, I present the results for a range of q (q = 0.05 – 0.2), though as in my previous work relaxing q made no substantive difference to my conclusions. To statistically test for differences in the overall genotypic composition of *Acanthopagrus* spp. among years, I compared the distribution of q-values for all pairwise comparisons using a Kolmogorov-Smirnov test (implemented in the program PAST; Hammer *et al*. 2007.). I 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 my reference collection to simply visualise the genetic similarity of hybrids to pure *A. butcheri*.

7.3 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 four collections (range = 8.2 ± 1.2 – 9.2 ± 0.8), as was allelic richness (7.4 ± 1.0 – 8.1 ± 1.2) and expected heterozygosity (~ 0.70). I detected three and four 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, I recorded three rare alleles in each contemporary sample that were not in either historical sample (Table 7.1). However, each of these private or 'ghost' alleles has previously been detected in contemporary estuarine *Acanthopagrus* spp. populations in southeastern Australia (Roberts *et al*. 2009; Chapter 6; Roberts, D.G., unpublished data) and such minor variation in the occurrence of rare alleles would be expected as a consequence of sampling variation. Tests for homogeneity of allele frequencies revealed statistically significantly differences for just two loci, pAb2B7 and Acs1* ($P < 0.05$) (Fig.

7.2). Not surprisingly, genetic subdivision across the four sampling times was not statistically significant different from zero (F_{ST} = 0.003 ± 0.002, 95% CI = 0.000 – 0.007).

Table 7.1. Number of alleles (A), allelic richness (A_R), number of private alleles (alleles unique to a particular collection) (P_A), observed heterozygosity (H_o), Nei's 1973 expected heterozygosity (H_e) and, the estimator f of the inbreeding coefficient, F_{IS} (Weir & Cockerham 1984), with corresponding statistical significance for departure from Hardy-Weinberg equilibria, for two historical and contemporary samples of *Acanthopagrus* spp. caught within the Gippsland Lakes in southeastern Australia, based on six microsatellite loci. The proportion of *A. australis* (Aa), *A. butcheri* (Ab) and hybrid (Hyb) fish in each collection and a comparison of the effect of varying the q-threshold (q_i = the average proportion of *A. butcheri* ancestry) used to distinguish pure species and hybrids is also shown. [1]

Year	Locus	A	A_R	P_A	H_o	H_e	f	q-threshold: proportion Aa/Ab/Hyb [*]
1941	pAb2B7	12	10.8	0	0.733	0.823	0.120^{**}	
n = 91	pAb2A5	9	6.7	1	0.473	0.590	0.200^{**}	q = 0.05: 0.00/0.01/0.99
	pAb2D1	7	6.1	1	0.685	0.626	-0.090^{NS}	q = 0.10: 0.00/0.07/0.93
	Acs1*	7	6.5	0	0.615	0.739	0.173^{***}	q = 0.20: 0.00/0.23/0.77
	Acs3*	9	7.4	2	0.635	0.698	0.096^{*}	
	Acs6*	11	9.0	0	0.753	0.748	-0.001^{NS}	
	Mean (SE)	9.2 (0.8)	7.8 (0.7)	0.7 (0.3)	0.649 (0.042)	0.704 (0.036)	$0.083\ (0.045)^{***}$	
1943	pAb2B7	14	13.7	3	0.762	0.808	0.070^{NS}	
n = 42	pAb2A5	6	6.0	0	0.357	0.601	0.415^{***}	q = 0.05: 0.00/0.05/0.95
	pAb2D1	6	6.0	0	0.619	0.691	0.115^{NS}	q = 0.10: 0.02/0.08/0.90
	Acs1*	7	7.0	0	0.561	0.716	0.229^{*}	q = 0.20: 0.02/0.19/0.79
	Acs3*	8	8.0	0	0.575	0.679	0.166^{***}	
	Acs6*	8	8.0	0	0.786	0.714	-0.089^{NS}	
	Mean (SE)	8.2 (1.2)	8.1 (1.2)	0.5 (0.5)	0.610 (0.064)	0.702 (0.027)	$0.151\ (0.069)^{***}$	
1996	pAb2B7	15	13.7	1	0.704	0.840	0.171^{***}	
n = 55	pAb2A5	5	4.7	0	0.455	0.489	0.080^{*}	q = 0.05: 0.00/0.02/0.98
	pAb2D1	6	5.6	1	0.456	0.625	0.136^{NS}	q = 0.10: 0.00/0.02/0.98
	Acs1*	8	7.7	1	0.615	0.756	0.196^{***}	q = 0.20: 0.02/0.18/0.80
	Acs3*	8	7.7	0	0.604	0.690	0.135^{*}	
	Acs6*	8	7.5	0	0.673	0.726	0.083^{*}	
	Mean (SE)	8.3 (1.4)	7.8 (1.3)	0.5 (0.2)	0.599 (0.037)	0.687 (0.049)	$0.134\ (0.019)^{***}$	
2000	pAb2B7	13	12	1	0.684	0.815	0.169^{**}	
n = 59	pAb2A5	10	8.0	1	0.407	0.500	0.195^{***}	q = 0.05: 0.00/0.03/0.97
	pAb2D1	5	4.6	0	0.542	0.582	0.077^{NS}	q = 0.10: 0.00/0.03/0.95
	Acs1*	7	6.3	0	0.655	0.685	0.052^{NS}	q = 0.20: 0.02/0.29/0.69
	Acs3*	7	6.4	1	0.690	0.620	-0.104^{NS}	
	Acs6*	8	7.4	0	0.755	0.732	-0.022^{NS}	
	Mean (SE)	8.3 (1.1)	7.4 (1.0)	0.5 (0.2)	0.622 (0.052)	0.656 (0.046)	$0.061\ (0.046)^{***}$	

[1] The specific-species status of each individual was determined using admixture analysis based on the four most diagnostic microsatellite loci. Estimates of ancestry ranging between 0 and 0.05, and 0.95 and 1.0 (i.e., in the case of q = 0.05) were used to classify each fish as either a pure *A. australis* or *A. butcheri* respectively, while fish with their estimate in between these values were classed as hybrid (see Roberts *et al*. 2009).

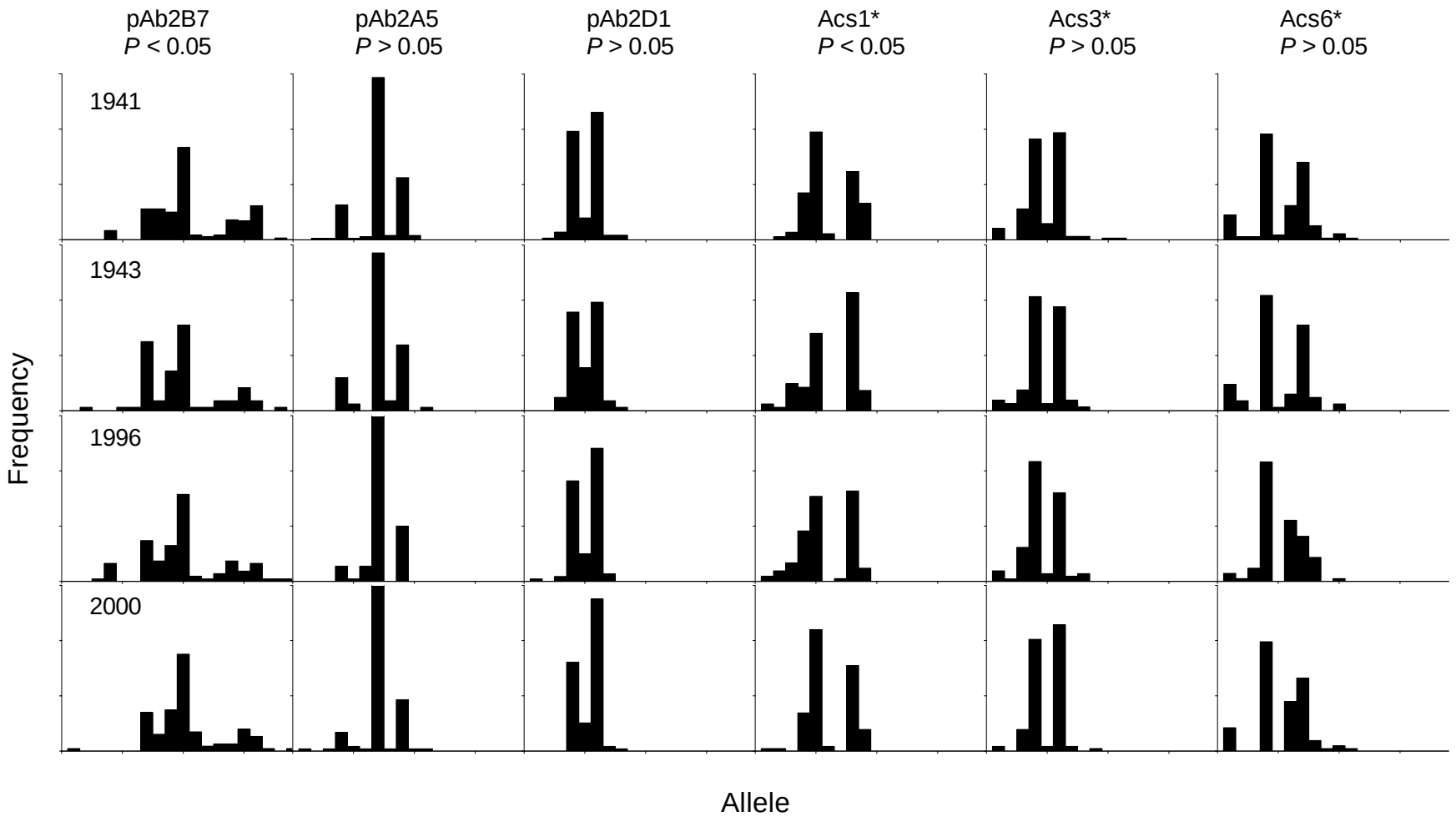

Figure 7.2 Allele frequencies at six microsatellite loci for four samples (collected at different times; 1941, 1943, 1996 and 2000) of *Acanthopagrus* spp. caught within the Gippsland Lakes. *P*-values are from tests of homogeneity of allele frequencies among collection times.

Admixture analysis using a q-threshold of 0.05 to distinguish pure species and hybrids revealed that a high percentage of both the contemporary and historical samples were hybrid fish rather than pure *A. butcheri* (95 – 99%) (Fig. 7.3). This same pattern was evident even when I 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 (refer to Table 7.1). 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 hybrids has not changed over 60 years. Moreover, all samples included fish with genotypes characteristic of genetically intermediate and more complex later generation hybrids and backcrosses. In all cases however, q-values were skewed by the greater similarity of hybrids and *A. butcheri* rather than *A. australis* (Fig. 7.3). The greater similarity of hybrids and *A. butcheri* is most easily displayed using a FCA plot of the genetic similarity of our Gippsland Lake sample and my reference collections of pure species (Fig. 7.4).

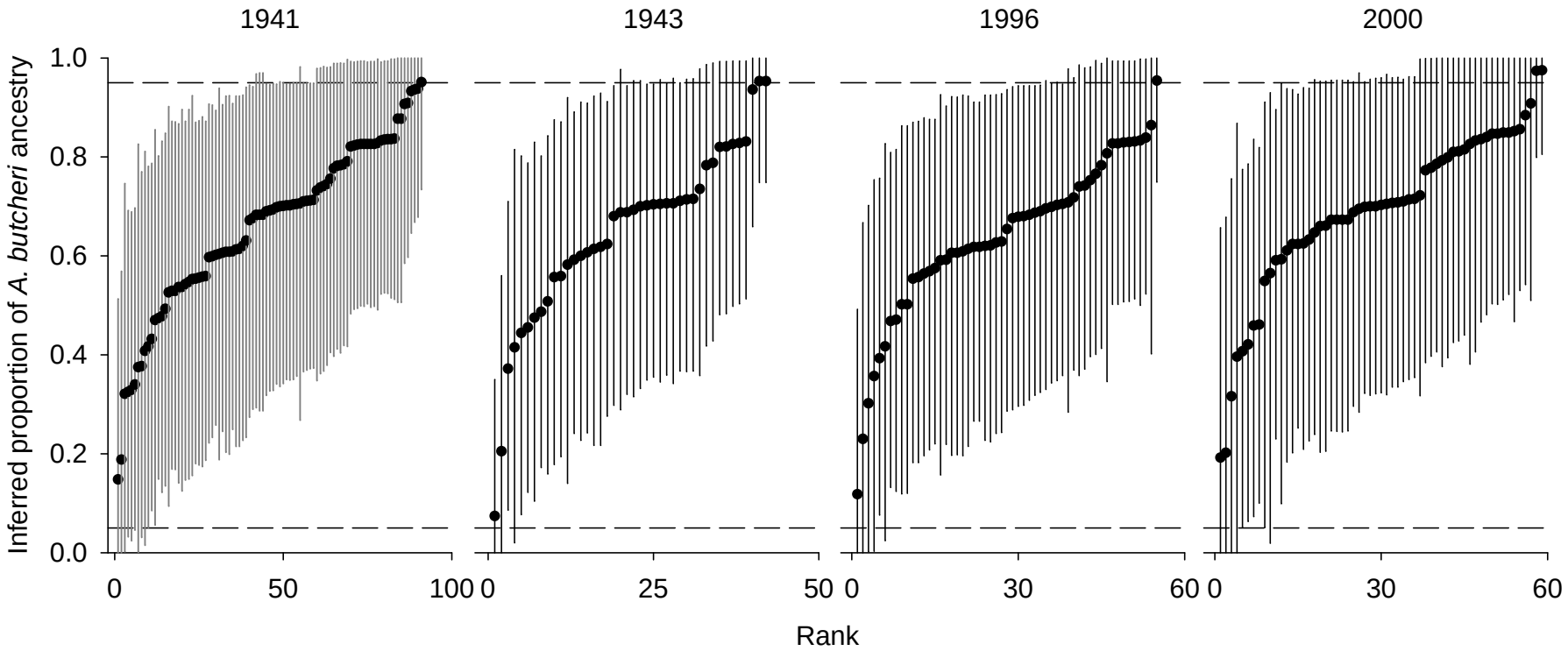

Figure 7.3 Estimates of ancestry for *Acanthopagrus* spp. within the Gippsland Lakes based on four relatively diagnostic microsatellite loci, for four collection times (1941, 1943, 1996 and 2000). The data are presented as the inferred proportion of *A. butcheri* ancestry (average $q_i \pm$ 95% CIs). 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 ($0.05 < q_i < 0.95$). I varied the q-value used to distinguish pure species and hybrids with no substantive difference to my conclusions (see results reported in Table 7.1).

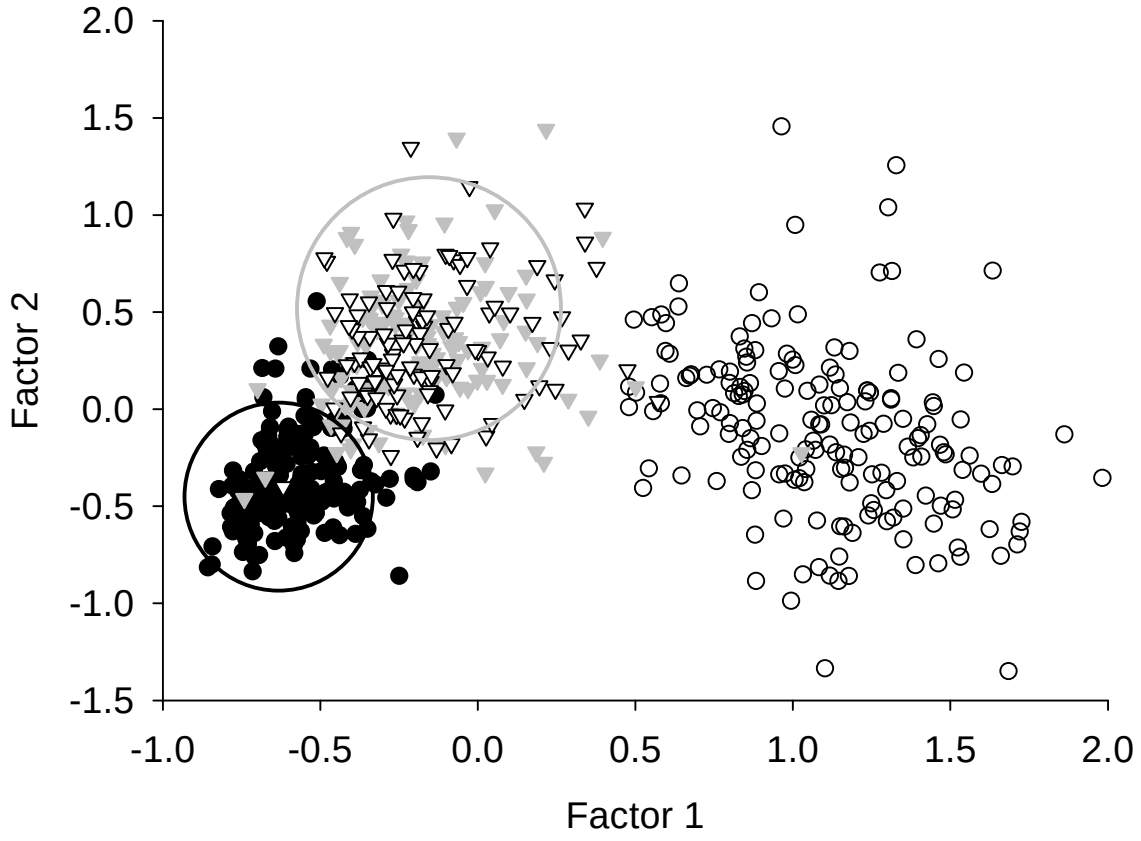

Figure 7.4. Factorial correspondence analysis based on the six-locus microsatellite genotype of contemporary and historical *Acanthopagrus* spp. within the Gippsland Lakes (closed grey triangles: historical; open black triangles: contemporary, enclosed within the grey circle). A genetic reference collection of pure *A. australis* (open circles) and *A. butcheri* (closed black circles) were included in the analysis and plotted on the figure. Fish within the reference collection that cluster inside the unbroken black circle were *A. butcheri* from outside the described range of *A. australis* from Tasmania, western Victoria and South and Western Australia respectively and were therefore expected to be beyond the range of hybridisation (Chapter 2; Roberts *et al*. 2009).

7.4. Discussion

My longitudinal survey of the frequency of *A. butcheri* x *A. australis* hybrids highlights the importance of historical museum collections in describing the genetic composition of populations through time (Wandeler *et al*. 2008) and the stability of hybrid zones (Buggs 2007). These data show that despite potentially infrequent contact between the two parental species the Gippsland Lakes *Acanthopagrus* spp. population is a complex and stable hybrid swarm. Samples from 68, 66, 13, and 9 yrs 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*. The implication of these data is that hybridisation has been occurring for a very long time, and hybrids are neither eliminated by selection or emigration.

My data clearly demonstrate that introgression has impacted *A. butcheri* populations occurring beyond the accepted range of *A. australis* and that this has been a consistent phenomenon for at least 60 years. Taken at face value, my findings clash with the earlier findings of Farrington *et al*. (2000) and Burridge *et al*. (2004), and Burridge and Versace (2007) who respectively used allozyme and microsatellite data to describe the genetic structure of what they considered to be populations of *A. butcheri* within the Gippsland Lakes. However, those authors were unsurprisingly assuming that Gippsland was beyond the range of *A. australis* and that hybrids were a rare phenomenon confined to almost intermittently closed and open lakes and lagoons in southern NSW (Rowland 1984). More recently, Farrington (unpublished data) genotyped a subsample of the historical fish surveyed here and inferred that slightly higher levels of diversity in contemporary samples could reflect effects of hybridisation.

Chapter 8 General discussion

The conventional wisdom has always suggested that *A. australis* × *A. butcheri* hybrids were extremely rare and geographically restricted to intermittently closed and open lagoons in southern NSW (Rowland 1984). However, I have now used population genetic data to show that: hybrids can comprise almost the entire population of *Acanthopagrus* spp. of a given lake or lagoon; hybridisation has occurred in multiple cohorts of fish; hybrids persist through to adulthood and are long term residents (multigenerational) within lakes and lagoons; and hybrids are more geographically widespread than previously understood.

As an exploited estuary restricted taxon *A. butcheri* should be expected to face the threat of local extinction, throughout its range, as a consequence of over harvesting and the genetic effects of resulting small population sizes (Frankel & Soulé 1981; Charlesworth & Charlesworth 1987; Slatkin 1987; Frankham 2005). Indeed, in WA, populations in different estuaries are genetically divergent and Chaplin *et al*. (1998) concluded that connections among estuaries are relatively weak and populations are potentially exposed to the detrimental effects associated with genetic drift and inbreeding. The occurrence of hybridisation may have either positive or negative consequences for *A. butcheri*. At low levels, hybridisation could provide genetic novelty to estuarine populations and may provide the raw material for adaptive change. However, there are numerous examples where hybridisation between populations of locally adapted fish results in severe outbreeding depression (Gharrett *et al*. 1999; Utter 2000; Allendorf *et al*. 2004).

My data imply that although in allopatry to the south (TAS) and west of WP (western VIC, SA and WA) *A. butcheri* may suffer some of the effects of small population

size outlined above, introgression and genetic and demographic swamping by hybrids is a major threat to the persistence of populations of *A. butcheri* in the presence of its congener *A. australi*s. I detected few pure *A. butcheri* in my genetic surveys of adults and juveniles in southern NSW, and uncovered a situation where hybrids were dominant in lagoons generally closed to the ocean (Meroo & Cuttege Lakes, Chapter 2; Coila & Brou Lakes, and to a lesser extent Corunna Lake, Chapter 6). This seems most simply explained if the inter-fertile hybrids are mutigenerational residents within estuaries. Indeed, controlled breeding experiments revealed weak barriers to initial and later hybridisation (Chapter 5), and examination of the genotypes of adult fish of known ages in Coila and Brou Lakes revealed comparably high and stable proportions of hybrids across two and three additional cohorts of fish respectively (Chapter 6). This clearly demonstrates both that hybrids are common within many lagoons, and that the extreme hybridity detected in the 2001 cohort of juvenile fish (Chapter 6) is typical rather than an unusual exception. Moreover, it is clear that the hybrid juveniles that are produced in these lagoons can remain at high frequencies i.e., they are neither eliminated by selection or emigration because hybrids persist through to adulthood. It would therefore be unsurprising that as successive generations of hybrids and backcrosses have accumulated in each population, demographic and genetic swamping of *A. butcheri* has occurred, with pure individuals becoming relatively or absolutely rare as seen in many lakes and lagoons on the south east coast.

In the absence of hybridisation the genetic structure of *A. butcheri* on the south east coast might be expected to resemble that of WA populations in that they too would be highly genetically differentiated (F_{ST} = 0.17) with populations in different estuaries effectively isolated, self-seeding, and potentially highly locally adapted (Chaplin *et al.*

1998). However, in the presence of its migratory marine congener, it appears that east coast populations have experienced an overwhelming amount of interspecific gene flow and consequent genetic homogenisation precluding any formal estimate of gene flow among *A. butcheri* populations. It seems less likely that introgression would have a major impact on the genomic integrity of *A. australis*, since their great mobility as adults and their pelagic larval phase means that they form a single vast panmictic population on the east coast and are not confined to lagoons (Chapter 3). Moreover, the majority of hybrids appear to have been formed by mating between *A. australis* males and *A. butcheri* females and subsequent backcrossing with *A. butcheri* (Chapters 2 & 3).

8.1 Is it too late for *A. butcheri* on the east coast

The presence of hybrid zones and the rarity of *A. butcheri*, within each of the coastal lagoons that I surveyed, raise two key questions about the evolution and conservation of this species on the south east coast of Australia.

First we have to ask whether 'genetic rescue' involving stocking of lakes and lagoons with pure *A. butcheri* (as identified elsewhere in the species range) should be attempted for these populations or indeed whether it is possible or indeed desirable. Distinguishing between anthropogenically induced and natural hybridisation seems problematic for this and many other hybrid systems (Allendorf *et al*. 2001) and it is possible that these hybrid zones are both ancient and should themselves be conserved as a source of raw material for natural adaptive change (Lexer *et al*. 2003; Gross *et al*. 2004).

Indeed, it is possible that estuaries on the south east coast support what is in effect a hybrid species. My examination of contemporary and historical samples from the Gippsland Lakes (Chapter 7) revealed remarkably stable proportions of hybrids across decades (> 60 yrs) and in each case appeared to represent later generation hybrids or *A. butcheri* backcrosses that were most similar to the estuary restricted *A. butcheri*. My phylogeographic study based upon analysis of mitochondrial DNA variation of *A. butcheri* was consistent with a recent range expansion or colonisation of estuaries throughout southern Australia (Chapter 4), a finding which I interpreted in terms of providing the potential for contemporary process to be important in the maintenance of *Acanthopagrus* hybrid zones. However, coupled with the remarkable genotypic stability in the Gippsland (an area considered to beyond the normal range of *A. australis*), there is the possibility that historical events have influenced *Acanthopagrus* hybrid zones. Indeed, the high incidence of hybrids within east coast estuaries may reflect secondary contact between *A. butcheri* and *A. australis* following range expansion of *A. butcheri* (or *A. australis*), and therefore a single episode of hybridisation with little continuing introgression of the *A. australis* genome. However, distinguishing between contemporary and historical process is notoriously difficult (Endler 1977; Endler 1982) and beyond my current data set, although the almost virtual absence of genetic structure in south east compared to south west *A. butcheri* populations (see Chapter 1) does suggest that contemporary process is important in the maintenance of *Acanthopagrus* hybrid zones.

If the current hybrid zones are a consequence of anthropogenic disturbance of a set of effectively closed *A. butcheri* populations (paralleling the situation in WA [Chaplin *et al*. 1998]) restoration of the historical genetic structure would be an impossibility. Even if

remnant *A. butcheri* stocks exist within the upper reaches of the streams feeding east coast coastal lakes and lagoons (providing a source of broodstook) their genetic makeup would almost certainly reflect some level of introgression and attempts at restocking would be compromised since all east coast lakes and lagoons probably have some proportion of resident hybrids and allow at least periodic entry of *A. australis*. Indeed, stocking may merely lead to a different, more complex hybrid swarm. Alternatively, there could be detrimental consequences due to interbreeding between wild and hatchery stocks (e.g., Hansen *et al*. 2009).

Second, we have to consider the possibility that anthropogenic disturbance has not only facilitated hybridisation in the surveyed lagoons but it may continue to increase the number and geographic range of hybrid dominated lagoons. This process could be a threat to *A. butcheri* elsewhere within its range. Eastern Australia is an area predicted to experience severe modification of major current flows as a result of climate change. Indeed, there are already reports of urchins (*Centrostephanus rodgersii*) being transported to the far south of their previous distributional range limit by the movement of larvae within warm-core eddies of the EAC, with adult populations now established in Tasmania and having devastating effects on subtidal kelp forests and associated fauna (Johnson *et al*. 2005). In addition, Booth *et al*. (2007) and Figueira & Booth (2009) have reported unusual transport and 'overwintering' of tropical fish beyond their accepted ranges, and oceanographic modelling predicts a warmer and strengthened EAC as a consequence of global climate change (Poloczanska *et al*. 2007). For the *Acanthopagrus* spp. complex, a range expansion by *A. australis* may have little direct impact on *A. australis* population structure, but it could have major implications for *A. butcheri.* A southward range

expansion by *A. australis*, facilitated by warmer, more frequent or further than usual southward penetration of EAC warm-core eddies could simply increase the size of this panmictic east coast population (Chapter 3). Alternatively however, the southward spread of *A. australis* may increase both the geographic range within which hybridisation occurs and number of hybrid dominated lakes and lagoons. Indeed, my broad scale genetic survey based on relatively small sample sizes has already uncovered rare hybrids in Tasmanian estuaries (Roberts *et al*. 2009; Chapter 2). Frequent westward dispersal seems less likely for *A. australis* (though see discussion below), as we now know that the SE corner of Australia is a major biogeographic barrier, for several species regarded as 'good dispersers' (Waters & Roy 2003; Dawson 2005; Waters *et al*. 2005; York *et al*. 2008; Ayre *et al*. 2009; Fraser *et al*. 2009). The SE corner corresponds to a convergence zone of southward flowing warm-core eddies of the EAC and cold waters of the eastward flowing Bass Straight Cascade and therefore represents an area of extreme spatial variation in water temperature and salinity (refer to Chapter 1) that presumably blocks along shore dispersal of adults and/or transport of larvae.

We currently lack detailed knowledge of the reproductive biology, behaviour and hybrid fitness of the *Acanthopagrus* species complex that is needed to allow either a thorough understanding of the factors facilitating and limiting hybridisation, or to allow prediction of the speed with which introgression will occur. When hybrids are viable and interfertile and remain or are retained within one species parental habitat, as is the case for *A. butcheri* within estuaries on the east coast, the potentially negative effects of introgression (Levin *et al*. 1996; Rhymer & Simberloff 1996) are expected to develop relatively rapidly. Studies modelling the amount of time it takes for introgression to cause

loss of species identity have shown that, once high levels of hybridisation are initiated (even if hybrids display reduced fitness relative to their parents), genetic swamping leading to loss of species identity can occur within 2 – 5 generations (Huxel 1999; Epifanio & Philipp 2000; Wolf *et al*. 2001). On the east coast, rates of hybridisation may have become elevated because *A. butcheri* are both relatively and absolutely rare.

Clearly additional large scale geographical surveys are needed to fully resolve the current spatial extent of the *A. australis* x *A. butcheri* hybrid zones and to monitor the anticipated south and westward spread of *A. australis* and/or their hybrids (see Hobday, A. J., in press, for predictions on the southward spread of Australian pelagic fish). Further longitudinal surveys that determine both genotypes and otolith microchemistry may provide the best opportunity to study the dynamics of these hybrid swarms. By subjecting samples of fish to both genotyping and sectioning of otoliths it should be possible to compare the fitness of hybrid and parental fish 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 *A. butcheri* and their hybrids and so indicate whether the geographic spread of hybrid zones depends entirely on contact between *A. australis* and *A. butcheri* or if it involves allopatric introgression resulting from the migration of hybrid fish. Indeed, Elsdon & Gillanders (2005) have shown bream, which the authors assume were *A. butcheri* because they studied estuarine fish from beyond the range of *A. australis* in western VIC and South Australia, within the Glenelg River can display strikingly different behaviours during the known spawning period of *A. butcheri*, including resident, diadromous, and anadromous migratory patterns. Interestingly, a genetic survey (using microsatellites) has revealed that this estuary supports a genotypically

diverse *Acanthopagrus* population, including *A. butcheri* and both hybrid fish that are genetically similar to *A. australis* and *A. butcheri* (Roberts D. G., unpublished data), thus different genotypic classes may display strikingly different migratory behaviours. Otolith microchemistry can potentially be used to determine whether *A. butcheri* and their hybrids spend periods of their life in the ocean (providing the opportunity for dispersal) and may indicate if fish have moved between (chemically) different estuaries (e.g., Elsdon & Gillanders 2006; Arai & Gotto, 2008; Bradbury *et al*. 2008; Kuroki *et al*. 2008; Vasconcelos *et al*. 2008). Alternatively, traditional fish tagging, but with precise identification of genotypic classes through genotyping would also potentially uncover whether *A. australis* introgression is through ongoing mating between *A. australis* and *A. butcheri* or by the movement and mating of hybrid fish with *A. butcheri*. Indeed, these methods represent perhaps the only way to truly understand connectivity among estuarine populations of *Acanthopagrus* spp. on the east and south east coasts (NSW & VIC).

It seems critical that managers in southern and western states discontinue the practice of artificially opening lakes and lagoons since this may increase the probability of contact between *A. butcheri* and *A. australis* or between *A. butcheri* and hybrids and decrease the availability of brackish habitat for *A. butcheri* and other resident taxa. Ideally this should be combined with monitoring of genotype frequencies within these southern and western Australian estuaries with the aim of determining baseline levels of *A. australis* and/or their hybrids and responses to natural and artificial impacts on these estuaries, to determine whether the zone of hybridisation is stable or expanding as a result of global climate change.

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Appendix 1 Microsatellite allele and mtDNA haplotype frequencies for *Acanthopagrus* spp. in estuaries of Australia.*

Locus	Putative *A. australis*								Putative *A. butcheri*					
allele	Go	Fo	Bo	Po	Me	Co	Cu	Sc	Sw	De	No	VIC	SA	WA
n	41	74	30	39	25	50	24	20	49	21	49	79	15	49
pAb2B7														
090	-	0.007	-	-	-	-	-	-	-	-	-	-	-	-
092	-	0.007	-	-	-	-	-	-	-	-	-	0.013	-	-
094	0.012	0.014	-	0.038	0.060	0.010	-	-	0.010	-	-	0.006	-	-
096	-	0.014	0.017	0.038	0.020	0.040	0.021	-	-	-	-	-	-	-
098	0.037	0.034	0.050	0.038	-	-	0.028	-	-	-	-	-	0.033	-
100	0.073	0.075	0.050	0.051	0.080	0.010	0.042	0.025	0.020	-	-	0.070	0.067	-
102	0.183	0.103	0.067	0.090	0.160	0.110	0.104	0.100	0.071	0.024	0.020	0.063	0.067	0.115
104	0.073	0.116	0.100	0.064	0.100	0.110	0.083	-	0.020	-	-	0.082	0.033	0.042
106	0.195	0.130	0.233	0.282	0.240	0.200	0.271	0.375	0.245	0.190	0.276	0.329	0.333	0.438
108	0.073	0.144	0.083	0.038	0.060	0.150	0.083	-	-	-	-	0.032	0.067	0.010
110	0.146	0.123	0.133	0.128	0.040	0.110	0.021	-	-	0.024	-	0.025	-	0.010
112	0.098	0.110	0.150	0.179	0.040	0.040	0.167	-	0.041	-	-	0.006	-	0.021
114	0.012	0.048	0.050	0.013	0.140	0.140	0.167	-	0.031	0.024	-	0.070	0.033	0.063
116	0.061	0.027	0.033	-	0.020	0.020	-	0.125	0.214	0.381	0.439	0.165	0.300	0.031
118	-	0.027	-	0.013	0.040	0.040	0.021	0.375	0.245	0.357	0.265	0.089	-	0.198
120	0.012	0.014	0.017	0.013	-	-	-	-	0.082	-	-	0.025	0.067	0.021
122	0.012	0.007	0.017	0.013	-	0.020	-	-	-	-	-	0.019	-	-
124	0.012	-	-	-	-	-	-	-	0.020	-	-	-	-	-
126	-	-	-	-	-	-	-	-	-	-	-	0.006	-	0.052
pAb2A5														
084	0.050	0.081	0.067	0.051	-	-	-	-	-	-	-	-	-	-
086	-	-	0.017	-	-	-	-	-	-	-	-	-	-	-
100	-	0.007	0.017	0.013	-	-	-	-	-	-	-	-	-	-
102	0.075	0.054	0.017	-	-	0.010	-	-	-	-	-	-	-	-
104	0.038	0.020	0.017	-	-	-	-	-	-	-	-	-	-	-
106	0.175	0.095	0.050	0.077	0.060	0.061	0.045	-	-	-	-	0.032	-	-
108	0.138	0.189	0.183	0.359	0.020	0.010	0.045	-	-	-	-	-	-	-
110	0.125	0.122	0.050	0.077	-	-	0.045	-	-	0.024	0.031	-	-	0.010
112	0.100	0.155	0.100	0.154	0.780	0.714	0.705	0.500	0.429	0.619	0.551	0.639	0.667	0.615
114	0.063	0.088	0.150	0.064	-	-	0.045	-	-	-	-	-	-	-

116	0.050	0.034	0.067	0.038	0.120	0.194	0.114	0.500	0.571	0.357	0.418	0.316	0.333	0.375
118	0.038	0.020	0.100	0.026	0.020	-	-	-	-	-	-	0.013	-	-
120	0.063	0.027	0.017	0.051	-	-	-	-	-	-	-	-	-	-
122	0.013	0.054	0.083	0.064	-	-	-	-	-	-	-	-	-	-
124	0.013	0.014	0.017	-	-	-	-	-	-	-	-	-	-	-
126	-	0.014	-	-	-	0.010	-	-	-	-	-	-	-	-
128	0.050	0.027	0.033	0.013	-	-	-	-	-	-	-	-	-	-
130	-	-	0.017	0.013	-	-	-	-	-	-	-	-	-	-
132	0.013	-	-	-	-	-	-	-	-	-	-	-	-	-
pAb2D11														
100	-	-	-	-	0.040	-	-	-	-	-	-	-	-	-
104	0.146	0.149	0.172	0.141	0.660	0.660	0.500	1.000	0.969	1.000	1.000	0.778	1.000	1.000
106	0.280	0.203	0.345	0.244	0.100	0.190	0.292	-	-	-	-	0.019	-	-
108	0.549	0.581	0.466	0.462	0.200	0.130	0.188	-	0.010	-	-	0.177	-	-
110	0.012	0.061	0.017	0.090	-	0.020	0.021	-	0.010	-	-	0.019	-	-
112	-	0.007	-	0.064	-	-	-	-	0.010	-	-	0.006	-	-
114	0.012	-	-	-	-	-	-	-	-	-	-	-	-	-
Acs1*														
105	0.012	0.007	-	-	-	-	-	-	-	-	-	-	-	-
111	-	-	-	0.013	-	-	-	-	-	-	-	-	-	-
113	0.012	0.020	-	-	-	-	-	-	-	-	-	-	-	-
117	0.024	-	-	0.038	0.020	-	-	-	-	-	-	0.006	-	-
119	-	0.014	0.017	-	-	-	-	-	-	-	-	0.006	-	-
121	0.098	0.095	0.083	0.090	0.040	0.06	-	-	-	-	-	-	-	0.020
123	0.207	0.182	0.333	0.269	0.060	-	0.021	-	-	-	-	0.025	-	-
125	0.451	0.473	0.317	0.385	0.340	0.280	0.354	0.211	0.194	0.048	0.082	0.259	0.464	0.296
127	0.098	0.095	0.167	0.090	0.240	0.290	0.104	0.447	0.561	0.452	0.439	0.335	0.286	0.418
129	0.085	0.101	0.067	0.077	-	0.050	0.021	-	-	-	-	-	-	-
131	0.012	0.014	0.017	0.038	-	-	-	-	-	-	-	-	-	0.010
133	-	-	-	-	0.260	0.280	0.458	0.184	0.051	0.071	0.102	0.266	0.036	0.184
135	-	-	-	-	0.040	0.060	0.042	0.158	0.194	0.429	0.378	0.101	0.214	0.071
Acs3*														
067	-	-	-	-	-	-	-	-	-	-	-	-	-	0.011
071	0.049	0.095	0.017	0.038	0.020	0.010	0.021	-	-	-	-	0.006	-	-

073	0.012	-	-	0.013	-	-	-	-	-	-	-	0.006	-	-
077	0.061	0.041	0.067	0.026	-	-	0.021	-	-	-	-	0.013	-	-
079	0.061	0.034	0.033	0.064	-	0.050	0.021	-	-	-	-	-	-	-
081	0.427	0.338	0.333	0.359	0.160	0.190	0.146	0.125	0.141	0.075	0.063	0.077	0.100	-
083	0.134	0.196	0.133	0.192	0.320	0.350	0.208	0.425	0.457	0.850	0.781	0.506	0.700	0.957
085	0.073	0.074	0.067	0.038	-	-	-	-	0.098	-	0.010	0.013	-	-
087	0.024	0.054	0.033	0.090	0.400	0.330	0.521	0.450	0.304	0.050	0.125	0.372	0.200	0.033
089	0.061	0.041	0.067	0.090	-	-	0.042	-	-	-	-	0.006	-	-
091	-	0.014	-	-	-	-	-	-	-	0.025	-	-	-	-
093	-	-	-	-	-	0.030	-	-	-	-	-	-	-	-
095	0.037	0.007	0.033	-	0.080	0.010	-	-	-	-	-	-	-	-
097	-	-	-	-	0.020	0.010	-	-	-	-	-	-	-	-
099	0.024	0.034	0.050	0.038	-	0.010	-	-	-	0.010	-	-	-	-
101	0.012	0.020	0.067	0.013	-	-	-	-	-	0.010	-	-	-	-
103	-	0.007	-	0.026	-	-	-	-	-	-	-	-	-	-
105	0.024	0.007	-	-	-	0.010	0.021	-	-	-	-	-	-	-
107	-	0.007	0.050	-	-	-	-	-	-	-	-	-	-	-
109	-	0.014	0.017	-	-	-	-	-	-	-	-	-	-	-
111	-	-	0.017	-	-	-	-	-	-	-	-	-	-	-
115	-	0.014	-	0.013	-	-	-	-	-	-	-	-	-	-
117	-	0.007	-	-	-	-	-	-	-	-	-	-	-	-
121	-	-	0.017	-	-	-	-	-	-	-	-	-	-	-
Acs6*														
086	0.100	0.068	0.067	0.077	-	-	-	-	-	-	-	-	-	-
090	0.175	0.142	0.117	0.154	-	-	0.063	0.075	0.083	0.238	0.394	0.135	0.100	0.229
094	0.050	0.020	0.017	0.026	0.060	0.010	0.021	-	-	-	-	-	-	-
096	-	-	-	-	0.040	-	-	-	-	-	-	-	-	-
098	0.388	0.385	0.500	0.410	0.040	0.130	0.125	-	-	-	-	0.006	0.067	-
100	-	0.020	0.017	0.013	-	0.090	0.104	-	-	-	-	0.013	-	-
102	0.013	0.034	0.033	0.038	0.120	0.130	0.208	0.525	0.510	0.643	0.468	0.404	0.467	0.375
104	0.038	0.020	-	0.013	-	-	-	-	0.021	-	-	0.006	-	-
106	0.113	0.223	0.233	0.244	0.200	0.340	0.125	0.075	0.094	0.024	0.011	0.083	0.033	0.094
108	0.050	0.020	-	-	0.280	0.260	0.146	0.325	0.208	0.024	0.043	0.205	0.167	-
110	0.038	0.047	0.017	-	0.080	0.030	0.167	-	0.042	-	-	0.045	-	-
112	0.013	0.007	-	0.013	-	0.010	0.021	-	0.010	0.024	0.043	0.045	0.067	0.010
114	0.013	0.007	-	-	-	-	-	-	-	-	-	-	-	-

116	-	0.007	-	0.013	0.180	-	0.021	-	0.031	0.048	0.043	0.058	0.067	0.292
118	0.013	-	-	-	-	-	-	-	-	-	-	-	-	-
122	-	-	-	-	-	-	-	-	-	-	-	-	0.033	-
Acs-16*														
118	0.049	0.054	0.067	0.141	-	-	0.021	-	-	-	-	-	-	-
120	0.012	0.041	0.033	-	-	-	0.042	-	-	-	-	-	-	-
122	0.012	0.014	-	-	-	-	-	-	-	-	-	-	-	-
124	0.061	0.054	0.050	0.064	-	-	-	-	-	-	-	0.006	-	-
126	-	0.007	-	-	-	-	-	-	-	-	-	-	-	-
128	0.171	0.074	0.017	0.141	-	-	-	-	-	-	0.011	-	-	-
130	0.098	0.081	0.167	0.077	-	-	-	-	-	-	-	-	-	-
132	0.085	0.088	0.067	0.064	-	0.020	-	-	-	-	-	-	-	-
134	0.098	0.155	0.167	0.179	0.020	0.020	0.063	-	-	-	-	-	-	-
136	0.098	0.054	0.067	0.051	-	-	-	-	-	-	-	-	-	-
138	0.049	0.054	0.083	0.064	-	0.010	-	-	-	-	-	0.006	-	-
140	0.061	0.041	0.033	0.051	-	-	-	-	0.010	-	0.033	0.013	-	-
142	0.012	0.020	-	0.013	-	0.010	-	-	-	-	-	0.006	-	-
144	0.012	0.088	0.033	0.026	-	0.020	0.021	0.025	0.031	-	-	0.013	-	0.011
146	0.012	0.034	0.017	0.013	-	0.050	-	-	0.010	0.024	-	0.084	0.067	-
148	0.049	0.007	0.033	0.026	0.060	-	0.021	0.150	0.167	0.119	0.011	0.071	0.067	0.311
150	0.024	0.007	0.017	-	0.160	0.080	0.104	0.025	0.073	0.024	0.022	0.058	0.067	0.011
152	0.024	0.027	0.017	0.026	0.180	0.190	0.208	0.200	0.271	0.500	0.359	0.169	0.167	0.111
154	-	0.014	-	0.013	0.480	0.450	0.333	0.150	0.083	0.236	0.163	0.078	0.233	-
156	-	0.007	-	0.013	0.040	0.090	0.167	0.125	0.125	0.071	0.283	0.325	0.267	0.189
158	0.024	0.007	0.033	0.013	0.040	0.010	-	0.025	0.042	-	0.022	0.071	0.033	0.056
160	0.024	-	0.017	-	-	0.010	0.021	0.050	0.021	-	-	0.006	-	0.033
162	0.024	0.007	0.017	0.026	-	-	-	-	0.010	-	0.011	0.039	-	0.022
164	-	0.014	-	-	0.020	0.040	-	0.100	0.063	-	0.054	0.045	0.100	0.222
166	-	0.020	0.017	-	-	-	-	-	0.063	-	0.033	-	-	0.022
168	-	0.007	0.017	-	-	-	-	0.025	0.010	-	-	-	-	0.011
170	-	0.007	0.017	-	-	-	-	0.100	0.010	0.024	-	-	-	-
172	-	-	0.017	-	-	-	-	-	-	-	-	-	-	-
174	-	0.014	-	-	-	-	-	0.025	0.010	-	-	-	-	-
176	-	0.007	-	-	-	-	-	-	-	-	-	-	-	-
Acs-21*														

183	-	-	-	-	-	-	-	-	0.020	0.026	0.065	0.013	-	-
193	0.025	0.020	-	-	-	-	0.022	-	-	-	-	-	-	-
195	0.125	0.169	0.183	0.115	-	0.010	-	-	-	-	-	-	-	-
197	-	0.007	0.017	0.026	-	-	-	-	-	-	-	-	-	-
199	0.075	-	0.033	0.026	-	-	-	-	-	-	-	-	-	-
201	0.025	0.041	0.017	0.013	-	0.010	-	0.175	0.276	0.474	0.543	0.127	0.033	0.128
203	0.088	0.108	0.117	0.115	-	0.010	0.022	-	-	-	-	0.006	-	-
205	0.125	0.095	0.150	0.090	-	-	0.022	-	0.010	-	-	0.006	0.033	-
207	0.138	0.189	0.200	0.141	0.340	0.190	0.087	0.175	0.031	0.026	-	0.120	-	-
209	0.213	0.182	0.083	0.244	-	0.050	0.087	0.025	-	0.026	0.011	0.038	-	-
211	0.025	0.020	0.017	0.026	0.600	0.570	0.304	0.600	0.612	0.421	0.380	0.633	0.933	0.809
213	0.063	0.074	0.033	0.115	-	0.040	0.217	0.025	0.020	-	-	0.006	-	0.032
215	-	0.041	0.033	-	-	-	-	-	0.031	-	-	0.051	-	0.032
217	0.075	0.027	0.067	0.026	0.040	0.100	0.087	-	-	0.026	-	-	-	-
219	-	-	-	0.013	0.020	0.010	0.109	-	-	-	-	-	-	-
221	-	-	-	0.013	-	0.010	0.022	-	-	-	-	-	-	-
223	0.013	-	0.017	0.013	-	-	-	-	-	-	-	-	-	-
225	-	0.007	0.017	0.013	-	-	0.022	-	-	-	-	-	-	-
227	-	0.014	-	-	-	-	-	-	-	-	-	-	-	-
229	0.013	-	-	-	-	-	-	-	-	-	-	-	-	-
231	-	0.007	-	-	-	-	-	-	-	-	-	-	-	-
233	-	-	0.017	0.013	-	-	-	-	-	-	-	-	-	-
mtDNA														
n [E]	33	59	28	37	11	21	8	20	48	21	44	47	12	32
AA	1.000	0.915	0.929	0.811	-	-	-	-	-	-	-	-	-	-
AB	-	0.034	-	0.027	-	-	-	-	-	-	-	-	-	-
AC	-	-	-	-	0.273	0.333	0.250	-	0.083	-	-	0.043	-	0.031
BA	-	0.051	0.071	0.162	-	-	-	-	-	-	-	-	-	-
CC	-	-	-	-	0.727	0.667	0.750	1.000	0.917	1.000	1.000	0.957	1.000	0.969

[*] Allele frequencies are based on *n* fish per estuary, a dash indicates that the allele was absent in the sample.

[Ê] mtDNA haplotype frequencies are based on *n* pure species per estuary. We estimated the ancestry, and thus specific status of individual fish using an admixture analysis (based on eight microsatellite loci) with arbitrarily chosen q_i-value thresholds of 0 — 0.05 and 0.95 — 1.0 (q_i = the inferred proportion of *A. butcheri* ancestry) as our criteria to distinguish pure species *A. australis* and *A. butcheri* respectively. Any individual with a q-value from greater 0.05 to less than 0.95 was classified as a hybrid.

Appendix 2 Pattern of segregation of 8 microsatellite loci based on analysis of pedigrees from four experimental crosses involving *Acanthopagrus* spp. For each locus, the parental and offspring genotypes are shown, as well as the expected pattern of segregation where loci conform to Mendelian inheritance.

Locus	Cross genotype (base-pairs)	Observed segregation	Expected Segregation	Observed ratio	Expected ratio	Mendelian inheritance [a]
Cross 1						
pAb2B7	100/114 × 102/106	100/102: 100/106: 102/114: 106/114	100/102: 100/106: 102/114: 106/114	6: 6: 7: 11	1: 1: 1: 1	•
pAb2A5	112/128 × 112/112	112/112: 112/128	112/112: 112/128	16: 14	1: 1	•
pAb2D11	104/108 × 104/106	104/104: 104/106: 104/108: 106/108	104/104: 104/106: 104/108: 106/108	8: 9: 6: 7	1: 1: 1: 1	•
Acs1*	113/123 × 125/127	113/125: 113/127: 123/125: 127	113/125: 113/127: 123/125: 127	10: 5: 11: 4	1: 1: 1: 1	•
Acs3*	71/89 × 81/83	71/81: 71/83: 81/89: 83/89	71/81: 71/83: 81/89: 83/89	7: 6: 8: 9	1: 1: 1: 1:	•
Acs6*	98/112 × 106/108	98/106: 98/108: 106/112: 108/112	98/106: 98/108: 106/112: 108/112	9: 6: 7: 8	1: 1: 1: 1:	•
Acs-16*	132/140 × 146/156	132/146: 132/156: 140/146: 140/156	132/146: 132/156: 140/146: 140/156	4: 10: 7: 8	1: 1: 1: 1	•
Acs-21*	195/207 × 207/217	195/207: 195/217: 207/207: 207/217	195/207: 195/217: 207/207: 207/217	9: 6: 7: 8	1: 1: 1: 1	•
Cross 2						
pAb2B7	112/122 × 102/106	102/112: 102/122: 106/112: 106/122	102/112: 102/122: 106/112: 106/122	6: 6: 10: 6	1: 1: 1: 1	•
pAb2A5	108/112 × 112/112	108/112: 112/112	108/112: 112/112	14: 16	1:1	•
pAb2D11	104/106 × 104/106	104/104: 104/106: 106/106	104/104: 104/106: 106/106	4: 20: 6	1: 2: 1	•
Acs1*	125/131 × 125/127	125/125: 125/127: 125/131: 127/131	125/125: 125/127: 125/131: 127/131	9: 10: 6: 5	1: 1: 1: 1	•
Acs3*	81/83 × 81/83	81/81: 81/83: 83/83	81/81: 81/83: 83/83	9: 19: 2	1: 2: 1	•
Acs6*	106/106 × 106/108	106/106: 106/108	106/106: 106/108	15: 15	1: 1	•
Acs-16*	120/134 × 146/156	120/146: 120/156: 134/146: 134/156	120/146: 120/156: 134/146: 134/156	7: 11: 5: 7	1: 1: 1: 1	•
Acs-21*	195/213 × 207/217	195/207: 195/217: 207/213: 213/217	195/207: 195/217: 207/213: 213/217	9: 5: 8: 8	1: 1: 1: 1	•
Cross 3						
pAb2B7	104/110 × 102/106	102/104: 102/110: 104/106: 106/110	102/104: 102/110: 104/106: 106/110	4: 13: 6: 7	1: 1: 1: 1	•
pAb2A5	112/116 × 112/112	112/112: 112/116	112/112: 112/116	16: 14	1: 1	•
pAb2D11	104/104 × 104/106	104/104: 104/106	104/104: 104/106	16: 14	1: 1	•
Acs1*	127/133 × 125/127	125/127: 125/133: 127/127: 127/133	125/127: 125/133: 127/127: 127/133	18: 5: 1: 4	1: 1: 1: 1	×
Acs3*	83/87 × 81/83	81/83: 81/87: 83/83: 83/87	81/83: 81/87: 83/83: 83/87	7: 8: 10: 5	1: 1: 1: 1	•
Acs6*	98/102 × 106/108	98/106: 98/108: 102/106: 102/108	98/106: 98/108: 102/106: 102/108	7: 6: 6: 11	1: 1: 1: 1	•

Acs-16*	152/154 × 146/156	146/156: 146/154: 152/156: 154/156	146/156: 146/154: 152/156: 154/156	6: 7: 11: 6	1: 1: 1: 1	•
Acs-21*	211/211 × 207/217	207/211: 211/217:	207/211: 211/217:	14: 13	1: 1	•
Cross 4						
pAb2B7	108/110 × 108/114	108/108: 108/110: 108/114: 110/114	108/108: 108/110: 108/114: 110/114	8: 5: 5: 7	1: 1: 1: 1	•
pAb2A5	106/116 × 112/112	106/112: 112/116	106/112: 112/116	17: 10	1: 1	•
pAb2D11	104/110 × 104/104	104/110: 104/104	104/110: 104/104	13: 17	1: 1	•
Acs1*	125/127 × 125/127	125/125: 125/127: 127/127	125/125: 125/127: 127/127	6: 21: 1	1: 2: 1	×
Acs3*	83/83 × 83/87	83/83: 83/87	83/83: 83/87	13: 16	1: 1	•
Acs6*	106/108 × 102/106	102/106: 102/108: 106/106: 106/108	102/106: 102/108: 106/106: 106/108	7: 7: 8: 6	1: 1: 1: 1	•
Acs-16*	150/154 × 150/160	150/150: 150/154: 150/160: 154/160	150/150: 150/154: 150/160: 154/160	7: 8: 6: 7	1: 1: 1: 1	•
Acs-21*	211/217 × 207/211	207/211: 207/217: 211/211: 211/217	207/211: 207/217: 211/211: 211/217	6: 7: 10: 6	1: 1: 1: 1	•

[a]A closed black circle (•) indicates Mendelian inheritance as revealed by chi-squares tests of observed and expected genotype frequencies.

Appendix 3 Microsatellite allele frequencies for juvenile and adult *Acanthopagrus australis*.*

	Juveniles								Adults		
Locus	Tuross			Corunna			Wallaga		Gold Coast	Forster	Botany Bay
allele	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 1	Site 1	Site 1
n	30	30	30	30	30	30	30	30	40	40	30
pAb2B7											
090	-	-	-	0.017	-	0.017	0.033	-	-	0.013	-
092	-	-	-	-	0.017	0.050	-	-	-	0.013	-
094	0.017	0.033	0.017	0.033	0.017	0.017	-	-	0.013	0.013	-
096	0.017	0.017	-	0.067	-	0.050	-	-	-	-	0.017
098	0.033	0.017	0.067	-	0.100	0.033	0.050	0.050	0.038	0.038	0.050
100	0.083	0.100	0.050	0.083	0.033	0.033	0.067	0.050	0.075	0.075	0.050
102	0.050	0.133	0.050	0.050	0.167	0.100	0.100	0.067	0.188	0.113	0.067
104	0.067	0.083	0.100	0.050	0.167	0.117	0.200	0.083	0.075	0.138	0.100
106	0.267	0.283	0.183	0.183	0.150	0.100	0.083	0.133	0.188	0.163	0.233
108	0.133	0.100	0.167	0.133	0.133	0.167	0.133	0.217	0.063	0.150	0.083
110	0.133	0.017	0.133	0.100	0.050	0.100	0.117	0.150	0.150	0.088	0.133
112	0.133	0.100	0.150	0.183	0.083	0.117	0.083	0.117	0.100	0.088	0.150
114	-	0.050	-	0.017	0.033	0.017	0.050	0.017	0.013	0.063	0.050
116	0.033	0.050	0.050	0.033	-	0.050	0.017	0.017	0.063	0.013	0.033
118	0.017	-	-	-	0.017	0.017	-	0.017	-	0.038	-
120	0.017	0.017	0.033	0.033	-	-	0.033	0.050	0.013	-	0.017
122	-	-	-	-	0.017	-	-	-	0.013	-	0.017
124	-	-	-	0.017	0.017	-	0.033	0.017	0.013	-	-
132	-	-	-	-	-	0.017	-	-	-	-	-
136	-	-	-	-	-	-	-	0.017	-	-	-
pAb2A5											
078	-	-	-	-	0.017	-	-	-	-	-	-
084	0.100	0.050	0.033	0.050	0.083	0.083	0.083	0.100	0.051	0.100	0.067

086	-	0.017	-	-	-	0.017	-	0.033	-	-	0.017
098	-	-	-	-	0.017	-	-	-	-	-	-
100	-	-	0.017	-	-	0.017	0.017	0.017	-	0.013	0.017
102	0.133	0.017	0.083	0.067	0.033	0.050	-	0.067	0.077	0.013	0.017
104	0.050	0.017	0.017	-	-	0.017	0.033	0.017	0.038	0.025	0.017
106	0.083	0.150	0.150	0.100	0.067	0.133	0.167	0.033	0.179	0.063	0.050
108	0.183	0.200	0.167	0.183	0.217	0.267	0.150	0.133	0.141	0.150	0.183
110	0.067	0.117	0.117	0.183	0.133	0.067	0.150	0.217	0.115	0.125	0.050
112	0.083	0.133	0.133	0.083	0.117	0.067	0.100	0.117	0.103	0.150	0.100
114	0.133	0.133	0.100	0.083	0.183	0.217	0.083	0.117	0.064	0.125	0.150
116	0.033	0.050	0.017	0.050	0.067	0.017	0.033	0.050	0.051	0.038	0.067
118	0.017	0.017	0.050	-	-	0.017	0.033	-	0.038	0.025	0.100
120	0.017	0.017	-	0.050	-	-	0.017	0.033	0.051	0.025	0.017
122	0.083	0.017	0.100	0.100	0.017	0.017	0.100	0.017	0.013	0.075	0.083
124	0.017	0.033	-	0.017	-	-	0.033	-	0.013	0.025	0.017
126	-	0.017	-	-	-	-	-	-	-	0.013	-
128	-	0.017	0.017	0.033	0.033	0.017	-	0.033	0.051	0.038	0.033
130	-	-	-	-	0.017	-	-	-	-	-	0.017
132	-	-	-	-	-	-		0.017	0.013	-	-
pAb2D11											
098	-	0.017	-	-	-	-	-	-	-	-	-
100	0.017	-	-	-	-	-	-	0.017	-	-	-
102	-	-	-	-	-	-	-	0.017	-	-	-
104	0.150	0.150	0.167	0.083	0.250	0.117	0.083	0.117	0.150	0.088	0.172
106	0.250	0.167	0.150	0.267	0.167	0.283	0.317	0.183	0.275	0.225	0.345
108	0.433	0.500	0.567	0.567	0.517	0.500	0.483	0.600	0.550	0.600	0.466
110	0.117	0.100	0.083	0.083	0.017	0.083	0.033	0.050	0.013	0.088	0.017
112	0.033	0.017	0.033	-	0.017	-	0.083	-	-	-	-
114	-	0.050	-	-	0.033	0.017	-	0.017	0.013	-	-

Acs1*											
105	0.017	-	-	-	-	-	-	-	0.013	0.013	-
111	-	0.017	-	-	-	-	-	-	-	-	-
113	-	-	-	0.017	-	0.017	0.017	0.033	0.013	-	-
115	-	-	-	0.017	-	-	-	0.017	-	-	-
117	-	-	0.017	-	0.017	0.017	0.017	0.050	0.025	-	-
119	0.017	0.017	0.017	0.033	0.017	0.017	0.017	0.017	-	0.013	0.017
121	0.100	0.050	0.067	0.117	0.133	0.117	0.033	0.083	0.100	0.100	0.083
123	0.217	0.233	0.233	0.217	0.200	0.317	0.300	0.267	0.213	0.225	0.333
125	0.417	0.517	0.533	0.383	0.383	0.350	0.317	0.300	0.438	0.450	0.317
127	0.150	0.100	0.067	0.133	0.200	0.067	0.233	0.117	0.100	0.100	0.167
129	0.083	0.067	0.067	0.067	0.017	0.050	0.050	0.083	0.088	0.088	0.067
131	-	-	-	0.017	-	0.050	0.017	0.033	0.013	0.013	0.017
133	-	-	-	-	0.033	-	-	-	-	-	-
Acs3*											
071	0.050	0.050	0.017	0.067	0.033	0.033	0.033	0.089	0.050	0.050	0.017
073	-	0.017	-	-	-	-	-	-	0.013	-	-
077	0.033	0.033	0.067	0.017	0.033	0.017	0.050	0.089	0.050	0.038	0.067
079	-	0.083	0.033	0.050	0.050	0.067	0.033	0.054	0.050	0.050	0.033
081	0.417	0.300	0.350	0.367	0.417	0.283	0.383	0.304	0.438	0.350	0.333
083	0.233	0.150	0.150	0.200	0.100	0.117	0.233	0.179	0.138	0.213	0.133
085	0.067	0.033	0.050	0.083	0.067	0.050	0.100	0.071	0.075	0.100	0.067
087	0.033	0.050	0.067	0.100	0.083	0.133	0.017	-	0.025	0.038	0.033
089	0.050	0.050	0.133	0.033	0.067	0.017	0.067	0.036	0.063	0.025	0.067
091	-	0.033	0.017	-	0.017	0.050	-	0.018	-	0.025	-
093	-	-	-	-	-	0.017	-	-	-	-	-
095	0.017	-	0.033	0.017	0.017	0.033	-	0.036	0.038	-	0.033
097	0.017	-	-	-	-	-	-	-	-	-	-
099	0.067	0.033	-	-	0.083	0.033	-	0.071	0.025	0.038	0.050

101	-	0.050	-	0.017	-	0.067	-	0.018	0.013	0.013	0.067
103	-	0.017	0.017	0.017	-	-	0.017	-	-	0.013	-
105	-	0.033	-	0.017	-	0.017	0.017	-	0.025	-	-
107	-	0.017	-	-	-	0.017	0.033	-	-	0.013	0.050
109	-	0.017	0.017	-	0.033	0.017	-	-	-	0.025	0.017
111	-	0.017	0.017	-	-	0.017	0.017	-	-	-	0.017
113	0.017	0.017	-	-	-	-	-	0.018	-	-	-
115	-	-	0.033	0.017	-	0.017	-	0.018	-	-	-
117	-	-	-	-	-	-	-	-	-	0.013	-
121	-	-	-	-	-	-	-	-	-	-	0.017
Acs6*											
086	0.067	0.067	0.017	0.017	0.086	0.067	0.050	0.050	0.103	0.075	0.067
090	0.117	0.200	0.167	0.183	0.172	0.150	0.100	0.050	0.179	0.113	0.117
094	0.083	0.067	0.033	0.017	-	0.017	0.050	0.050	0.051	0.025	0.017
096	-	-	-	-	0.017	-	-	-	-	-	-
098	0.417	0.400	0.433	0.467	0.397	0.367	0.500	0.417	0.372	0.388	0.500
100	0.033	0.017	0.017	0.017	0.017	0.050	0.017	0.017	-	0.013	0.017
102	-	0.017	-	0.017	0.017	0.033	-	-	0.013	0.050	0.033
104	0.033	0.033	0.050	-	-	-	-	0.017	0.038	0.038	-
106	0.150	0.133	0.200	0.167	0.190	0.183	0.183	0.200	0.115	0.263	0.233
108	-	0.017	0.017	0.033	0.069	0.033	0.067	0.033	0.051	0.013	-
110	0.033	-	0.033	0.050	-	0.050	0.017	0.050	0.038	0.025	0.017
112	0.033	0.017	-	0.017	0.017	0.033	0.017	0.017	0.013	-	-
114	0.033	0.017	0.017	-	0.017	-	-	0.067	0.013	-	-
116	-	0.017	0.017	0.017	-	0.017	-	0.017	-	-	-
118	-	-	-	-	-	-	-	0.017	0.013	-	-
Acs-16*											
118	0.050	0.050	0.086	0.017	0.083	0.100	0.050	0.117	0.050	0.088	0.067
120	0.033	0.017	-	0.017	0.017	0.033	0.033	-	0.013	0.013	0.033

122	0.033	0.017	0.017	0.033	0.033	-	0.017	0.033	0.013	0.013	-
124	0.067	0.067	0.052	0.067	0.050	0.067	0.017	0.067	0.063	0.063	0.050
126	-	0.017	0.017	0.017	-	-	-	-	-	0.013	-
128	0.100	0.017	0.034	0.067	0.067	0.050	0.017	0.050	0.175	0.088	0.017
130	0.050	0.117	0.017	0.050	0.167	0.150	0.150	0.083	0.100	0.088	0.167
132	0.033	0.117	0.086	0.100	0.050	0.033	0.067	0.050	0.088	0.075	0.067
134	0.183	0.100	0.069	0.117	0.033	0.150	0.100	0.117	0.100	0.113	0.167
136	0.083	0.117	0.138	0.133	0.067	0.200	0.083	0.083	0.100	0.075	0.067
138	0.033	-	0.034	0.050	0.050	-	0.100	0.050	0.050	0.075	0.083
140	0.033	0.050	0.052	0.033	0.067	0.033	0.017	-	0.063	0.038	0.033
142	0.050	0.017	0.017	0.017	0.017	0.017	0.067	0.033	0.013	-	-
144	0.033	0.050	0.086	0.050	0.050	0.050	0.083	0.067	0.013	0.088	0.033
146	-	0.033	0.034	0.017	-	-	0.017	0.017	0.013	0.038	0.017
148	0.033	0.033	0.069	0.017	0.017	0.017	0.017	0.083	0.050	0.013	0.033
150	0.050	0.083	0.052	0.083	0.017	0.050	0.033	0.033	0.025	0.013	0.017
152	0.033	0.033	0.017	0.017	0.017	-	0.033	-	0.025	0.025	0.017
154	-	-	0.017	-	0.083	-	-	-	-	-	-
156	-	-	-	-	-	0.017	-	-	-	-	-
158	-	0.017	0.034	-	-	-	0.050	0.033	0.013	-	0.033
160	0.067	0.017	0.017	0.050	0.067	0.017	-	0.017	0.013	-	0.017
162	0.017	-	0.017	-	0.033	-	0.017	-	0.025	-	0.017
164	-	-	0.017	-	0.017	-	-	-	-	0.013	-
166	0.017	-	-	-	-	-	0.017	0.017	-	0.025	0.017
168	-	-	-	-	-	-	0.017	0.017	-	0.013	0.017
170	-	-	-	0.033	-	-	-	0.017	-	0.013	0.017
172	-	0.017	0.017	-	-	0.017	-	0.017	-	-	0.017
174	-	-	-	0.017	-	-	-	-	-	0.013	-
176	-	0.017	-	-	-	-	-	-	-	0.013	-
Acs-21*											
177	-	-	-	-	-	-	-	0.017	-	-	-

193	0.033	-	0.033	0.017	0.017	0.067	0.033	0.017	0.026	0.025	-
195	0.133	0.207	0.167	0.233	0.183	0.117	0.150	0.121	0.128	0.125	0.183
197	-	-	-	-	-	-	0.017	0.017	-	-	0.017
199	-	-	0.017	-	-	-	0.017	0.017	0.064	-	0.033
201	0.033	-	0.050	0.017	0.033	0.050	0.033	0.034	0.026	0.075	0.017
203	0.150	0.207	0.117	0.067	0.200	0.067	0.083	0.121	0.090	0.138	0.117
205	0.117	0.086	0.133	0.150	0.067	0.100	0.100	0.207	0.115	0.075	0.150
207	0.100	0.172	0.117	0.200	0.117	0.150	0.217	0.086	0.141	0.213	0.200
209	0.217	0.190	0.100	0.167	0.117	0.200	0.200	0.207	0.218	0.200	0.083
211	0.017	0.034	-	0.017	0.083	-	0.017	0.017	0.026	0.013	0.017
213	0.117	0.017	0.150	0.033	0.083	0.100	0.067	0.086	0.064	0.063	0.033
215	0.017	0.034	0.050	-	0.033	0.100	0.033	0.034	-	0.025	0.033
217	0.033	0.017	0.050	0.067	0.050	0.033	0.033	0.017	0.077	0.025	0.067
219	-	0.017	-	-	0.017	-	-	-	-	-	-
221	0.017	-	-	-	-	-	-	-	-	-	-
223	0.017	-	0.017	0.017	-	-	-	-	0.013	-	0.017
225	-	-	-	-	-	-	-	-	-	-	0.017
227	-	-	-	-	-	-	-	-	-	0.013	-
229	-	-	-	-	-	-	-	-	0.013	-	-
231	-	-	-	-	-	0.017	-	-	-	0.013	-
233	-	0.017	-	0.017	-	-	-	-	-	-	0.017

*Allele frequencies are based on *n* fish per estuary, a dash indicates that the allele was absent in the sample.

Appendix 4 Microsatellite allele frequencies for juvenile *Acanthopagrus* spp. in five coastal lagoons on the New South Wales south coast.*

Locus		Coila	Turos s	Brou	Corunna	Wallaga
allele	*n*	81	173	170	147	117
pAb2B7						
090		-	0.006	-	0.007	0.021
092		-	-	-	0.021	0.004
094		0.006	0.023	-	0.014	0.013
096		0.031	0.017	0.030	0.027	0.013
098		-	0.046	-	0.034	0.043
100		0.019	0.084	0.015	0.045	0.051
102		0.173	0.087	0.160	0.127	0.077
104		0.130	0.084	0.074	0.099	0.128
106		0.173	0.202	0.225	0.151	0.162
108		0.093	0.124	0.074	0.151	0.128
110		0.111	0.107	0.115	0.075	0.107
112		0.043	0.113	0.047	0.099	0.124
114		0.179	0.032	0.195	0.062	0.043
116		0.025	0.038	0.006	0.034	0.021
118		0.019	0.014	0.041	0.024	0.017
120		-	0.014	0.003	0.017	0.021
122		-	0.003	0.003	0.003	0.004
124		-	0.006	0.006	0.007	0.013
126		-	-	0.006	-	0.004
132		-	-	-	0.003	-
136		-	-	-	-	0.004
pAb2A5						
078		-	-	-	0.003	0.004
084		-	0.058	0.003	0.071	0.103
086		-	0.012	-	0.003	0.009
098		-	-	-	0.003	-
100		-	0.006	-	0.003	0.009
102		-	0.046	-	0.034	0.051
104		0.019	0.026	0.003	0.014	0.030
106		0.056	0.095	0.086	0.082	0.098
108		-	0.168	-	0.170	0.132
110		0.006	0.069	0.003	0.099	0.141
112		0.704	0.220	0.688	0.184	0.128
114		-	0.119	-	0.126	0.090
116		0.204	0.058	0.208	0.116	0.068
118		-	0.020	0.003	0.007	0.017
120		-	0.014	0.003	0.010	0.021
122		0.012	0.046	0.003	0.044	0.051
124		-	0.017	-	0.007	0.017

126	-	0.006	-	-	-
128	-	0.014	-	0.020	0.017
130	-	0.003	-	0.003	-
132	-	0.003	-	-	0.009
134	-	-	-	-	0.004
pAb2D11					
096	0.012	-	0.006	-	-
098	-	0.006	-	-	-
100	-	0.006	-	-	0.004
102	-	-	-	-	0.009
104	0.654	0.171	0.639	0.279	0.154
106	0.167	0.214	0.207	0.242	0.239
108	0.130	0.494	0.115	0.415	0.521
110	0.037	0.072	0.030	0.051	0.038
112	-	0.026	0.003	0.003	0.026
114	-	0.012	-	0.010	0.009
Acs1*					
105	-	0.003	-	-	-
111	-	0.006	-	-	0.004
113	-	-	-	0.010	0.021
115	-	-	-	0.003	0.004
117	0.006	0.012	-	0.010	0.034
119	-	0.014	0.003	0.014	0.021
121	0.012	0.090	0.012	0.088	0.043
123	-	0.194	-	0.184	0.286
125	0.247	0.431	0.257	0.350	0.350
127	0.333	0.165	0.281	0.180	0.141
129	0.037	0.052	0.041	0.068	0.073
131	-	0.014	-	0.017	0.013
133	0.290	0.012	0.364	0.054	0.009
135	0.074	0.006	0.041	0.020	-
Acs3*					
071	0.006	0.044	0.021	0.044	0.062
073	-	0.006	-	-	-
077	0.012	0.058	0.003	0.014	0.058
079	0.037	0.052	0.038	0.051	0.062
081	0.160	0.334	0.139	0.293	0.323
083	0.364	0.206	0.308	0.211	0.181
085	0.012	0.049	0.015	0.065	0.111
087	0.327	0.049	0.405	0.156	0.031
089	-	0.055	0.006	0.031	0.062
091	-	0.012	-	0.017	0.004
093	0.006	0.006	0.015	0.003	0.009
095	0.043	0.012	0.027	0.031	0.022
097	0.006	0.003	-	-	-

099	-	0.029	0.003	0.024	0.031
101	0.006	0.017	-	0.017	0.004
103	-	0.015	-	0.003	0.004
105	-	0.015	0.015	0.010	0.004
107	0.019	0.003	0.006	0.003	0.013
109	-	0.006	-	0.014	-
111	-	0.015	-	0.003	0.004
113	-	0.006	-	0.003	0.009
115	-	0.009	-	0.007	0.004
Acs6*					
086	-	0.049	0.003	0.041	0.043
088	-	-	-	-	0.004
090	-	0.145	0.024	0.147	0.132
094	0.012	0.049	0.003	0.017	0.034
096	-	0.003	0.003	0.003	-
098	0.074	0.402	0.112	0.339	0.406
100	0.123	0.043	0.121	0.051	0.021
102	0.191	0.014	0.174	0.034	0.017
104	-	0.029	0.003	-	0.009
106	0.235	0.179	0.288	0.212	0.201
108	0.253	0.020	0.203	0.068	0.043
110	0.105	0.026	0.059	0.051	0.038
112	0.006	0.012	0.006	0.017	0.017
114	-	0.012	-	0.003	0.026
116	-	0.012	0.003	0.014	0.004
118	-	0.006	-	-	0.004
Acs-16*					
118	-	0.052	-	0.045	0.073
120	-	0.017	-	0.017	0.021
122	-	0.017	0.009	0.024	0.013
124	-	0.055	-	0.051	0.077
126	0.006	0.009	-	0.003	-
128	-	0.067	-	0.045	0.064
130	-	0.078	0.003	0.096	0.094
132	0.006	0.064	0.006	0.045	0.068
134	-	0.108	0.006	0.079	0.103
136	-	0.105	0.003	0.089	0.085
138	-	0.041	0.003	0.034	0.047
140	0.006	0.044	-	0.031	0.009
142	-	0.026	-	0.010	0.038
144	0.025	0.061	0.021	0.041	0.073
146	0.013	0.023	0.024	0.007	0.026
148	0.006	0.023	0.030	0.027	0.026
150	0.138	0.055	0.090	0.051	0.047
152	0.213	0.038	0.219	0.072	0.017
154	0.400	0.038	0.404	0.123	0.013

156	0.081	0.006	0.060	0.041	0.017
158	0.013	0.015	0.015	0.010	0.034
160	0.006	0.017	0.030	0.031	0.009
162	0.013	0.015	0.006	0.007	0.004
164	0.075	0.003	0.072	0.007	-
166	-	0.006	-	-	0.013
168	-	0.006	-	-	0.013
170	-	-	-	0.007	0.004
172	-	0.006	-	0.003	0.009
174	-	-	-	0.003	-
176	-	0.006	-	-	-
178	-	-	-	-	0.004
Acs-21*					
177	-	-	-	-	0.004
193	-	0.020	-	0.020	0.026
195	-	0.166	-	0.122	0.129
197	-	0.003	-	0.003	0.013
199	-	0.012	-	-	0.009
201	0.019	0.041	0.009	0.037	0.026
203	0.006	0.116	0.003	0.099	0.121
205	0.031	0.105	-	0.092	0.129
207	0.219	0.137	0.170	0.150	0.177
209	0.025	0.172	0.065	0.136	0.185
211	0.556	0.061	0.604	0.177	0.039
213	0.019	0.076	0.015	0.058	0.073
215	0.013	0.038	-	0.031	0.034
217	0.100	0.032	0.125	0.058	0.017
219	0.013	0.006	0.009	0.007	0.004
221	-	0.006	-	-	-
223	-	0.009	-	0.003	-
227	-	-	-	-	0.004
231	-	-	-	0.003	0.004
233	-	0.003	-	0.003	0.004

*Allele frequencies are based on *n* fish per estuary, a dash indicates that the allele was absent in the sample.

Appendix 5 Microsatellite allele frequencies for contemporary and historical *Acanthopagrus* spp. within the Gippsland Lakes.*

Locus	Historical		Contemporary	
allele	1941	1943	1996	2000
n	91	42	55	59
pAb2B7				
082	-	-	-	-
090	-	0.012	-	-
092	-	-	0.009	-
094	0.033	-	0.065	-
096	-	0.012	-	-
098	-	0.012	-	-
100	0.111	0.250	0.148	0.140
102	0.111	0.036	0.074	0.061
104	0.100	0.143	0.130	0.149
106	0.333	0.310	0.315	0.351
108	0.017	0.012	0.019	0.070
110	0.011	0.012	0.009	0.018
112	0.017	0.036	0.028	0.026
114	0.072	0.036	0.074	0.026
116	0.067	0.083	0.037	0.079
118	0.122	0.036	0.065	0.053
122	-	-	0.009	0.009
124	0.006	0.012	0.009	-
126	-	-	0.009	0.009
pAb2A5				
100	-	-	-	0.008
102	0.005	-	-	-
104	0.005	-	-	0.008
106	0.126	0.119	0.055	0.068
108	0.005	0.024	0.009	0.017
110	0.011	-	0.055	0.008
112	0.588	0.571	0.682	0.678
114	0.016	0.036	-	0.008
116	0.225	0.238	0.200	0.186
118	0.016	-	-	0.008
120	-	0.012	-	0.008
pAb2D11				
098	-	-	0.009	-
100	0.006	-	-	-
102	0.028	0.048	0.018	-
104	0.393	0.357	0.364	0.322
106	0.079	0.155	0.100	0.102
108	0.461	0.393	0.482	0.551

110	0.017	0.036	0.027	0.017
112	0.017	0.012	-	0.008
Acs1*				
117	-	0.024	0.019	0.009
121	0.011	0.012	0.038	0.009
123	0.027	0.098	0.067	-
125	0.170	0.085	0.183	0.138
127	0.390	0.280	0.308	0.440
129	0.022	-	-	0.017
131	-	-	0.010	-
133	0.247	0.427	0.327	0.310
135	0.132	0.073	0.048	0.078
Acs3*	0.041	0.038	0.038	0.017
071	-	0.025	0.009	-
079	0.112	0.075	0.123	0.078
081	0.365	0.413	0.434	0.405
083	0.059	0.025	0.028	0.017
085	0.388	0.375	0.321	0.457
087	0.012	0.038	0.019	0.017
089	0.012	0.013	0.028	-
091	-	-	-	0.009
093	0.006	-	-	-
095	0.006	-	-	-
097				
Acs6*				
090	0.090	0.095	0.029	0.085
098	0.011	0.036	0.010	-
100	0.011	-	0.048	-
102	0.382	0.417	0.433	0.396
104	0.017	0.012	-	-
106	0.124	0.060	0.221	0.179
108	0.281	0.310	0.163	0.264
110	0.051	0.048	0.087	0.038
112	0.006	-	-	0.009
116	0.022	0.024	0.010	0.019
118	0.006	-	-	0.009

*Allele frequencies are based on *n* fish per estuary, a dash indicates that the allele was absent in the sample.