Population structure undetectable using genetic markers in Mangrove Jack *Lutjanus argentimaculatus* from its cool-water range limit in eastern Australia

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ABSTRACT

Failure to consider genetic structure in fish populations when collecting aquaculture broodstock can negatively affect fitness, and hence conservation and management goals. Here we used mitochondrial DNA from the 5' end of the control region (D-loop) and four microsatellite markers to evaluate population genetic structure in the Mangrove Jack *Lutjanus argentimaculatus* with a view to guiding broodstock collection at the southern extremity of its east Australian range.

There was no evidence of genetic structure within the entire tropical, subtropical and temperate east Australian distribution of *L. argentimaculatus*. Although this species may exhibit clinal morphological and life-history variation at its southern range limit, we did not detect wide- or fine-scale spatial genetic structure to indicate the presence of non-random evolutionary processes. Broodstock collection of *L. argentimaculatus* need not be geographically restricted in eastern Australia, but fisheries management should consider variation in life history and recruitment success at the species' edge.

Key words: *Lutjanidae*, population genetics, fish dispersal, mangrove red snapper

DOI: https://doi.org/10.7882/AZ.2019.021

Introduction

The genetic structure of animal species and populations can be used to define biologically relevant units for conservation and harvesting (Carvalho and Hauser 1994; Ovenden *et al.* 2015). In aquaculture, broodstock collection plans that consider cultured species' underlying genetic structure can contribute to protecting genetic diversity in wild populations, particularly where hatchery-bred fish could either escape or be deliberately released into the wild (Araki and Schmid 2010; Laikre *et al.* 2010). Anthropogenically facilitated hybridisation between genetically distinct populations (irrespective of population taxonomic level) has been particularly problematic in fish conservation (Allendorf *et al.* 2001), and is not uncommon in hatcheries, even at the species level (e.g. Mia *et al.* 2005). Interbreeding is of particular concern in the culture of species where gene flow is restricted and genetically distinct populations have evolved naturally. Consequently, understanding the genetic structure of wild populations plays an important role in captive breeding for stock enhancement, guiding broodstock collection from discrete populations and mitigating the harmful consequences of releasing hatchery-bred fish (Ward 2006; Ovenden *et al.* 2015).

The Mangrove Jack *Lutjanus argentimaculatus* is a medium to large (up to ca. 16 kilograms, 1.2 metres caudal fork length) member of the snapper family (Lutjanidae). It is the only Australian member of its mostly tropical marine genus to enter fresh water, where juveniles and subadults are common. The species is gonochronistic (individuals belong to only one of the two sexes) and exhibits the following life history traits: longevity exceeding fifty years, relatively late attainment of sexual maturity, and low natural mortality rates (Russell *et al.* 2003; Piddocke *et al.* 2015).

L. argentimaculatus is a popular angling and table fish that has been selected for stocking freshwater impoundments in eastern Australia. It is widespread in the Indo-Pacific and in northern and eastern Australian waters from Ningaloo Reef in Western Australia to Sydney in NSW (Allen *et al.* 2002), with occasional records from as far south as the Victoria-NSW border (Bureau of Rural Sciences 2002-2009). *L. argentimaculatus* spend juvenile years in estuarine and freshwater habitats, followed by a shift to offshore reefs, where spawning occurs, just prior to the attainment of sexual maturity at ca. eight years of age (Russell and McDougall 2008; Piddocke *et al.* 2015).

Successful hatching of *L. argentimaculatus* may be temperature dependent at ca. 27°C (Doi and Singhagraiwan 1993), generally higher than ocean temperatures in NSW (Malcolm *et al.* 2011; Manly Hydraulics Laboratory 2019). This suggests that larvae that develop in NSW estuaries have been recruited from eggs spawned on offshore reefs in Queensland, where *L. argentimaculatus* constitute a single genetic population (Ovenden and Street 2003). Eggs and larvae are likely to be carried south by the East Australian Current (EAC). Here we study genetic subdivision among *L. argentimaculatus* from NSW to far-north Queensland to determine whether broodstock for NSW freshwater impoundments must be sourced from NSW populations. Given the NSW population is likely to result from EAC-mediated dispersal from Queensland, we expected that *L. argentimaculatus* in NSW constitute a single genetic population, and that this population is an extension of the Queensland population.

Materials and methods

Sampling

Lutjanus argentimaculatus samples were obtained from three sources: (1) fin clips or muscle samples from filleted skeletons (as described in Piddocke *et al.* 2015) caught on the eastern Australian coastline between Maroubra, near Sydney (~33.94°S, 151.27°E) and Fraser Island, Qld (~24.70°S, 153.28°E) from November 2011-April 2013; (2) fin clips from National Marine Science Centre[1] broodstock caught on the Tweed River in northern NSW (ca. -28.26°S, 153.51°E) in 2015; and (3) fin clips preserved in NaCl-saturated 20% DMSO solution from the study by Ovenden and Street (2003) that were collected from far north Queensland (between Cape Melville and Hinchinbrook Island; ~14-18°S, 145-146°E) between 1999-2001. Samples were assigned to five regional groups by capture location as follows: far north Queensland (FNQ; Ovenden and Street 2003), south-east Queensland (SEQ), NSW north coast from the Tweed to Clarence Rivers inclusively (NC), NSW mid-north coast from south of the Clarence River to Laurieton (MNC), and Sydney (STH; Fig. 1). The maximum distance between the most southerly and northerly sample was 1,898 kilometres. Genetic change over time was not assessed as the collection time span (ca. 16 years) was less than one generation in this long-lived species, and the east coast population was likely to be numerous and panmictic (Ovenden and Street, 2003). We assumed that temporal change due to genetic drift was unlikely.

DNA isolation

The genomic DNA of 110 *L. argentimaculatus* was isolated from 5-25mg of preserved fin or muscle tissue using a DNeasy® Blood and Tissue Kit (Qiagen) and quantified with a Qubit® 2.0 fluorometer (Invitrogen).

Genetic analyses

Mitochondrial DNA

A subset of 54 *L. argentimaculatus* were selected for mitochondrial DNA (mtDNA) analyses: five from the FNQ population, eight from SEQ, 21 from NC, 17 from MNC and three from STH (Fig. 1). Around 372 base pairs (bp) of the 5' end of the control region (D-loop) was amplified with primers Pro889U20 (CCW CTA ACT CCC AAA GCT AG) and TDKD1291L21 (CCT GAA ATA GGA ACC AAA TGC; Ovenden and Street 2003). Each 20μl amplification reaction contained 10ng genomic DNA, 2μl 1x PCR buffer, 0.2mM of each dATP, dCTP, dGTP and dTTP, 10pmol each primer, 2.5mM $MgCl_2$ and 0.25U Platinum Taq DNA Polymerase (Invitrogen). Thermal cycling was performed on an Eppendorf Mastercycler® Nexus as follows: 1.5min at 94°C, followed by 35 cycles of 5s at 94°C, 30s at 50°C and 30s at 72°C, with a final extension of 5min at 72°C (Ovenden *et al.* 2002; Ovenden and Street 2003). PCR products were purified with CleanSweep™ PCR Purification Reagent (Applied Biosystems). Sequencing using the Big Dye terminator method was performed by the AGRF (Australian Genome Research Facility, Brisbane).

Microsatellite markers

Four microsatellite loci developed for coral trout (*Plectropomus laevis*) and red-throat emperor (*Lethrinus miniatus;* van Herwerden *et al.* 2000) have been used to determine population genetic structure in a previous study of Queensland *L. argentimaculatus* (van Herwerden *et al.* 2000; Ovenden and Street 2003). These loci were amplified in 110 *L. argentimaculatus* from five geographic groups (Fig. 1) as follows: FNQ = 5, SEQ = 9, NC = 48, MNC = 45 and STH = 3. Amplification, gel separation and scoring were performed by the AGRF. Separate polymerase chain reactions (20μl) for each marker contained 15ng template DNA and primer pairs were direct labelled with Applied Biosystems fluorescent dyes as follows: BST2.33 (VIC), BST6.39TG (NED), BST6.56 (PET) and 90RTE (6-FAM). A pigtail sequence was added to the 5' end of the reverse primer. Amplicons were pooled on a single panel and fragments were separated with a five dye system on an Applied Biosystems 3730 DNA analyser and scored using GeneMapper Software (Applied Biosystems).

Population genetic analyses

Mitochondrial DNA sequences were aligned with MEGA6 (Tamura *et al.* 2013). Estimates of genetic diversity (number of haplotypes, h; haplotypic diversity, *Hd*, number of polymorphic sites, *S*; nucleotide diversity, π), and Tajima's *D* (Tajima 1989) and Fu's *F* statistic tests for neutrality (Fu 1996) were calculated in DnaSP version 5.10.01 (Librado and Rozas 2009).

Microsatellite allele frequencies, heterozygosity and fixation indices were calculated with GenAlEx 6.5 (Peakall

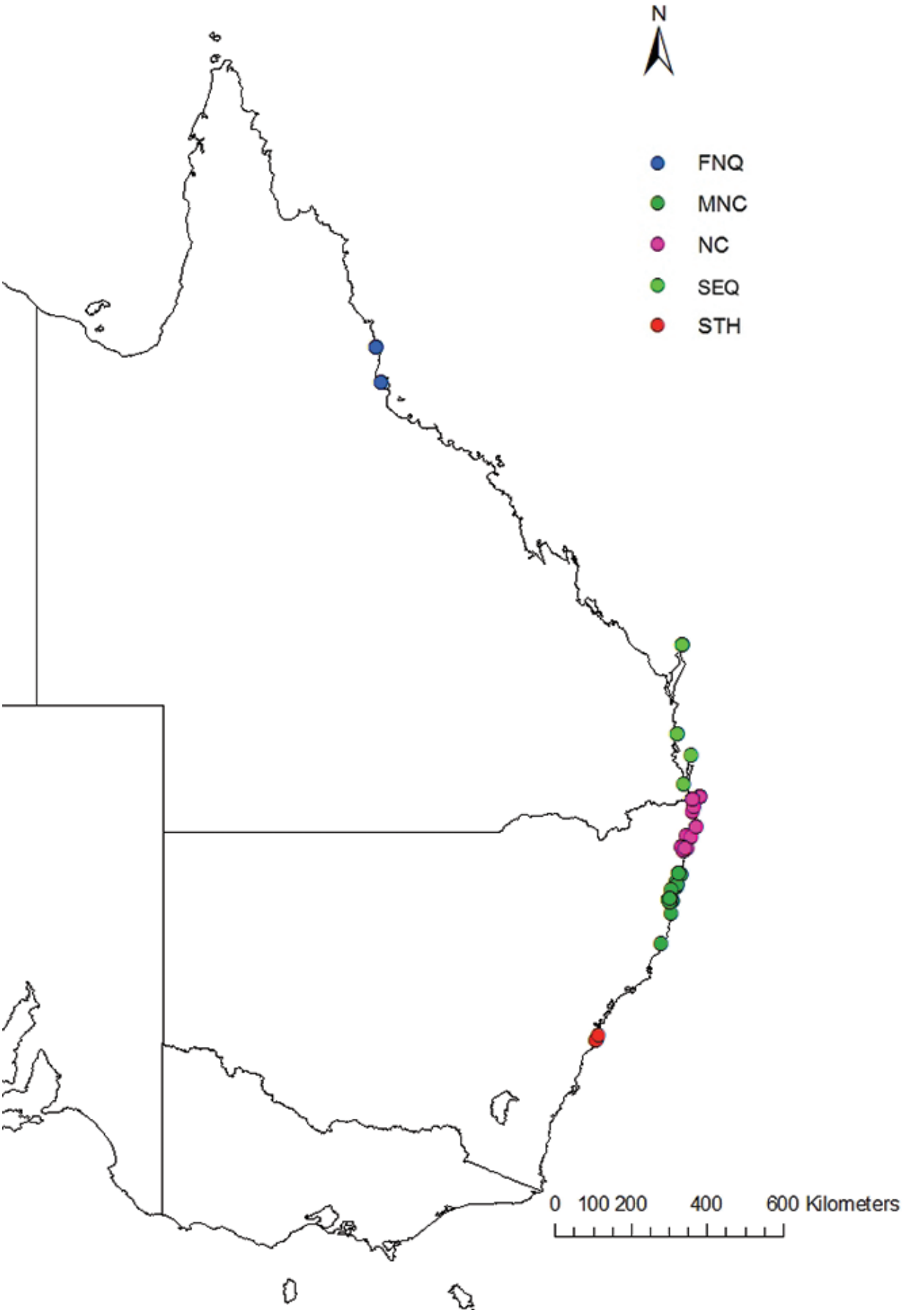

Figure 1. Collection locations of 54 Mangrove Jack Lutjanus argentimaculatus samples from Queensland and New South Wales, Australia, showing the five geographically defined populations used in genetic analyses.

and Smouse 2012). Exact tests for Hardy-Weinberg equilibrium and linkage disequilibrium (demorization = 1000, batches = 100, iterations per batch = 1000) were performed with GenePop on the Web (Raymond and Rousset 1995; Rousset 2008).

GenAlEx 6.5 was used to analyse population genetic structure within and between the five populations. As the FNQ samples were collected ca. 15 years before other samples, analyses were run with and without FNQ samples; reported results include the FNQ samples after it was determined that they did not change the magnitude or direction of results. Genetic variation between populations was calculated with Hedrick's further standardised G_{ST} for codominant data with a small number of populations (Meirmans and Hedrick 2011). Values for F_{ST} (Wright 1931, 1965; Weir and Cockerham 1984) were also calculated to facilitate comparison with other studies, including Ovenden and Street (2003). The Analysis of Molecular Variance (AMOVA) framework was then used to test whether there was significant genetic variation between populations relative to within population variation (Excoffier *et al.* 1992; Peakall *et al.* 1995). Random permutation (1000 permutations) was used to test for significant departure from the null hypothesis of no genetic variation between populations.

Mantel tests were conducted with GenAlEx 6.5 to test for isolation-by-distance (Peakall and Smouse 2012). Matrices containing pairwise genetic distances between individuals were correlated with corresponding matrices of geographic distance between capture locations (Rxy; Smouse *et al.* 1986; Smouse and Long 1992). Geographic distances were calculated from GPS coordinates taken at individual points-of-capture. Statistical significance of Rxy was tested by 1000 random permutations against a null hypothesis of no relationship between genetic and geographic distance. Evidence for fine scale genetic structure was tested with spatial autocorrelation (Smouse and Peakall 1999; Peakall *et al.* 2003). This method correlates two matrices: one using multi-allele, multi-locus data to calculate genetic distance and the other containing pairwise Euclidean distance. An autocorrelation coefficient, *r*, ranging from -1 to 1, is generated. Correlograms were produced with *r* varying as a function of distance, with 1000 random permutations used to generate 95% confidence intervals around a null hypothesis of no spatial genetic structure. Values of *r* above the upper 95% confidence interval indicate positive genetic structure, and the first distance class where *r* is no longer significant estimates the extent of detectable structure (Peakall *et al.* 2003).

Results

Mitochondrial DNA

The edited alignment contained 372 bp at the 5' end of the control region, of which 98 (26.3%) were variable. Estimates of genetic diversity and tests for neutrality are presented in Table 1. There was no evidence of genetic structure associated with shared haplotypes in the sampled population (46 haplotypes among 54 individuals), or within geographic subpopulations (Table 1). A subset of ten NC *L. argentimaculatus* caught on the same stretch of the Tweed River, which when considered alone could be potentially constitute a non-random sample, had ten unique haplotypes.

Control region sequence diversity was 3-5% within populations and along the east coast, with 4-5% divergence when sample sizes were greater than five. There was no evidence from neutrality tests (Table 1) that *L. argentimaculatus* populations had been subject to non-random evolutionary processes such as selection or demographic change. Neither was there evidence from the Mantel test that *L. argentimaculatus* had been subject to historic limits on dispersal (isolation-by-distance; Rxy = -0.046, p = 0.317).

Microsatellite markers

Two samples from MNC and one from NC failed to amplify at BST6.39 and BST2.33 respectively. Microsatellite variability ranged from 6 to 32 alleles. Significant F_{IS} estimates (the correlation of alleles within individuals) indicated locus BST6.56 was heterozygote deficient (excess observed homozygosity) in two populations (NC: p = 0.038, MNC: p = 0.042). Locus BST2.33 was also heterozygote deficient in MNC. However, excess homozygosity was not observed in other

Table 1. Estimates of genetic diversity and tests for neutrality in mtDNA control region sequences of Mangrove Jack *Lutjanus argentimaculatus* from the east coast of Australia.

Population	n	*h*	*Hd*	*S*	ϖ	*D*	*Fs*
FNQ	5	5	1.000	30	0.037	-0.415	0.056
SEQ	8	7	0.964	35	0.044	1.157	0.441
NC	21	20	0.995	74	0.048	-0.587	-6.366
MNC	17	15	0.985	77	0.052	-0.712	-2.125
STH	3	3	1.000	21	0.379	n/a	n/a
All samples	54	46	0.992	98	0.048	-0.637	-21.777

n = sample size, ***h*** = number of haplotypes, ***Hd*** = haplotypic diversity, ***S*** = number of segregating (polymorphic) sites, ϖ = nucleotide diversity, ***D*** = Tajima's ***D*** statistic, ***Fs*** = Fu's ***F*** statistic. Asterisks denote significant ***D*** and ***Fs*** values (nil).

loci within these populations, or in any locus when all individuals were considered. Otherwise, observed and expected proportions of genotypic heterozygosity met the Hardy-Weinberg principle of equilibrium. Significant linkage disequilibrium in the loci pair BST6.39 and BST6.56 (Fisher's $\chi^2 = 9.637$, df = 4, $p < 0.05$) could be attributed to linkage disequilibrium in the NC population only ($p = 0.019$).

There was no significant genetic differentiation between populations (Table 2).

AMOVA confirmed that genetic variation in the dataset could not be used to partition individuals into populations ($F_{ST} = 0.000$, df = 4, $p = 0.525$). There was no positive relationship between genetic and geographic distance that would indicate restricted gene flow between populations (Rxy = -0.071, p = 0.080). Neither was there any detectable spatial genetic structure over a fine geographic scale.

Discussion

We found no evidence that the NSW population of *L. argentimaculatus* was genetically divergent from populations further north. Mitochondrial control region sequence divergence (4-5%) was within the range reported from across northern Australia (Ovenden and Street 2003). Control region haplotype diversity was high and similarity was not linked to geographic proximity. From microsatellite data, there was no significant genetic subdivision between our geographically defined subpopulations. There was no evidence of isolation-by-distance that would indicate restricted gene flow, and no evidence of contemporary fine-scale spatial genetic structure. We conclude that *L. argentimaculatus* from temperate NSW to tropical far north Queensland waters constitute a single population with contemporary interconnectedness encompassing its east coast range.

The absence of significant genetic structure over long distances has been found in other marine species with ontogenetic migration. Ovenden and Street (2003) cite tailor *Pomatomus salatrix*, which also spawn offshore but are often found as subadults in estuaries and show no genetic structure in North American (Graves *et al.* 1992) or Australian populations (Nurthen *et al.* 1992). Vermilion snapper *Rhomboplites aurorubens*, another lutjanid that spawns pelagic eggs, also constitute a single genetic population in the South Atlantic Bight and Gulf of Mexico, where larval dispersal is affected by strong Gulf Stream currents (Bagley *et al.* 1999). However, yellowtail snapper *Ocyurus chrysurus* in Florida and the US Caribbean show small but significant genetic differentiation over similar distances to our study (1600km) and significant isolation-by-distance resulting in genetically distinct populations (Saillant *et al.* 2012). Gene flow in this species may be restricted by the conflicting directions of ocean currents that affect different spawning sites in this region (Saillant *et al.* 2012). In eastern Australia, the EAC dominates the coastline from Queensland to Tasmania and is the key environmental factor in offshore fisheries (Hobday and Hartmann 2006), with seasonal changes in its reach determining the distribution of many marine species (Ridgway 2007). Provided spawning sites are not overfished, extant genetic variability is likely to be maintained in *L. argentimaculatus* through the full extent of its east Australian range. Other similar species with larval stages transported by the EAC should also show little or no genetic structure except across very large geographic scales (Bagley *et al.* 1999).

Excess homozygosity was observed at locus BST2.33 on the NSW north coast, and at BST 2.33 and BST6.56 on the mid-north coast, and two loci were non-randomly associated among fish on the north coast. Both

Table 2. Microsatellite genetic differentiation (Hedrick's further standardised G_{ST}) between populations of Mangrove Jack *Lutjanus argentimaculatus* (n = 110) along the east coast of Australia are below the diagonal. The statistical significance of G_{ST} values were tested against 1000 random permutations; *p*-values are shown above the diagonal. Values and significance of F_{ST} for comparison are shown in parentheses.

	FNQ	MNC	NC	SEQ	STH
FNQ		0.824	0.714	0.672	0.556
		(0.884)	(0.739)	(0.675)	(0.583)
MNC	-0.084		0.113	0.649	0.626
	(0.019)		(0.144)	(0.701)	(0.763)
NC	-0.057	0.019		0.250	0.618
	(0.022)	(0.008)		(0.264)	(0.713)
SEQ	-0.073	-0.028	0.025		0.912
	(0.032)	(0.014)	(0.021)		(0.962)
STH	-0.047	-0.066	-0.054	-0.198	
	(0.061)	(0.037)	(0.038)	(0.030)	

phenomena can arise through a number of evolutionary processes, including selection, assortative mating, or undetected genetic substructure in small populations (Ridley 1996). In some fish studies excess homozygosity has indicated severe inbreeding (O'Leary *et al.* 2013), fishing pressure at spawning sites (Smith *et al.* 1991) or non-random mating (e.g. Smith 1987). Ovenden and Street (2003) also detected heterozygote deficiency in locus BST2.33 in two populations, and also in a different locus, BST6.39TG, in a single population. They stress that excess homozygosity is often and inexplicably detected in microsatellite studies of wild populations. A common cause of excess homozygosity is when 'null alleles' cause the locus to appear falsely homozygous. Given (1) departures from equilibrium were restricted to only some loci in some populations, (2) populations were demarcated by researchers and not biologically distinct; (3) markers were in Hardy-Weinberg and linkage equilibrium when applied to the entire data set; (4) there was no other evidence of genetic structure; and (5) ongoing gene flow is likely to be maintained by the EAC, we conclude that deviations from equilibrium arose from sampling and did not constitute evidence of non-random evolutionary processes.

Broodstock collection of *L. argentimaculatus* need not be geographically restricted in eastern Australia. However, despite its genetic homogeneity, Piddocke *et al.* (2015) found exaggerated life history traits of longevity, size and low natural mortality at its southern edge that may make NSW *L. argentimaculatus* particularly vulnerable to overfishing. It is unclear whether the species reproduces at its cooler range limits. The presence of large individuals with mature gonads in NSW waters suggests that it does (Piddocke, 2015), although it is possible that gonads are reabsorbed before spawning occurs or offspring do not survive (Russell *et al.* 2003). Certainly reproductive success is likely to be more variable at the edge of the species range (Myers 1991), where temporal variation in the reach of the EAC is likely to affect spawning success and larval survival, as do other Australian currents (e.g. Caputi *et al.* 1996). Generally, fisheries management should not be based solely on the absence of genetic structure among microsatellites as neutral markers can fail to detect local adaptation (Cano *et al.* 2008). Carvalho and Hauser (1994) and Gagnaire and Gaggiotti (2016) point out that large effective genetic population sizes and some migration may result in populations that are effectively self-recruiting but not genetically distinct. Further research is required to determine whether the *L. argentimaculatus* breeds at its southern limit, gather evidence of local adaptation, and determine the extent to which recruitment success among these longer-lived *L. argentimaculatus* (Piddocke *et al.* 2015) increases with breeder age (Beamish *et al.* 2006). As the EAC strengthens (Ridgway 2007), these factors have implications not only for *L. argentimaculatus* but for future stocks of other species potentially affected by interspecific competition or displacement.

Acknowledgements

The authors are grateful to NSW Recreational Fishing Grant Trust, NSW Department of Primary Industries, and the National Marine Science Centre for funding this project. We would like to thank Mel Bradbury (Fisheries NSW), Cathy Nock (Plant Science, Southern Cross University), Ken Cowden, Alison King, and Nadine Boulotte (NMSC) for their assistance. This research would not have been possible without the contributions of the recreational fishers who donated samples.

The authors declare no conflict of interest.

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