Improved genetic markers for monitoring recruitment dynamics in the endangered Mary River cod (*Maccullochella mariensis*).

Running title: *Novel microsatellites for Mary River cod*

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SUMMARY

The Mary River cod (*Maccullochella mariensis*) is a large predatory freshwater fish identified as a potential flagship for freshwater ecosystem conservation in Australia. The species is endemic to the Mary River catchment in eastern Australia, and is listed as Endangered. Previous conservation genetic assessment of Mary River cod was based on a small set of microsatellite loci developed for congeneric *Maccullochella* species. Here we develop a novel set of 15 microsatellite loci specific to *M. mariensis*, and demonstrate that these markers exhibit higher variability than those used previously. Mean number of alleles per locus was 4 and mean expected heterozygosity was 0.57. We genotyped 35 Mary River cod larvae belonging to a single cohort using the 15 novel loci and eight previously used loci, and found 10 full-sib family groups along with clear genetic differentiation between individuals collected from the two sub-catchments – Tinana Creek and Mary River. Microsatellites presented here will be useful for cost-effective monitoring of genetic diversity and recruitment dynamics in this endangered fish species.

INTRODUCTION

The Mary River cod (*Maccullochella mariensis*), is a large-bodied (max. length 1.2m) predatory freshwater species of the family Percichthyidae, endemic to a small coastal catchment in eastern Australia, the Mary River (Pusey, Kennard, & Arthington, 2004). The Mary River cod is listed as endangered under the Australian Government *Environment Protection and Biodiversity Conservation Act 1999* due to its limited natural range and small fragmented remnant populations. Specific threats include impoundment of streams, loss of riparian vegetation and invasive fish. The species has been identified as a potential flagship for freshwater ecosystem conservation in Australia due to its size, trophic position and conservation status (Ebner et al., 2016). Monitoring genetic diversity and assessing recruitment responses to management actions are priorities identified for Mary River cod (Threatened Species Scientific Committee, 2016) . Monitoring demographic properties of a threatened population by estimating the number of breeders or estimating variation among individuals in breeding success can be achieved using polymorphic genetic markers (e.g. Ackerman et al., 2017). Microsatellites are a class of genetic marker that has long played a major role in this area of conservation genetics due to their high polymorphism, and they remain a cost-effective method for assessing relationships among closely-related individuals (Hodel et al., 2016). Nowadays microsatellite loci may be isolated efficiently by processing of genomic DNA data produced by next-generation DNA sequencing methods (e.g. Islam, Schmidt, & Hughes, 2017; Tsukagoshi, Terui, Sato, & Abe, 2017; Xiong et al., 2017).

A previous microsatellite analysis of Mary River cod populations demonstrated low levels of genetic variability overall and suggested two distinct gene pools exist within the Mary River catchment (Huey, Espinoza, & Hughes, 2013). The two genetically distinct populations occur in two sub-catchments separated by an estuary – Mary River and Tinana Creek. The microsatellite analysis of Huey et al. (2013) was based on genotyping eight loci that were originally developed for use in congeneric *Maccullochella* species. Here we contribute a new panel of microsatellite loci developed specifically for *M. mariensis*, and provide a direct comparison of variation in these markers with the panel of loci previously used by Huey et al. (2013). We also evaluate the ability of these microsatellite markers to distinguish family-level relationships among a cohort of wild-caught larvae, demonstrating their utility for monitoring recruitment dynamics of the endangered Mary River cod.

1. MATERIALS AND METHODS

Field sampling of Mary River cod larvae used quatrefoil light traps baited with 150mm yellow glow sticks. Sampling occurred between the 14th and 16th October 2014. Larval samples were obtained from two tributaries of the Mary River main channel, ‘Six Mile Creek’ and ‘Obi Obi Creek’, and also from a downstream tributary, ‘Tinana Creek’, which is isolated from the Mary River by an estuary and a saltwater barrage. Sampling was

performed in accordance with Griffith University animal ethics permit number ENV/08/13/AEC. Larvae were identified to species and preserved in 100% ethanol for genotyping. All Mary River cod larvae were at postflexion stage of development, and otolith daily ring counts indicated they were between 9 and 16.5 days old (T. Espinoza, A. Dunlop, unpublished data).

A new Mary river cod-specific microsatellite library was developed from randomly-sheared genomic DNA, prepared using the DNeasy Blood and Tissue kit (Qiagen) and the TruSeq (Illumina) library preparation kit with targeted insert size of 500bp. Paired-end sequencing was performed on the Illumina MiSeq Sequencer at Australian Genomics Research Facility (AGRF), using a 600 cycle MiSeq reagent kit v3. The library generated 2.4 x 10^6 paired-end reads. Overlapping paired reads were merged with Geneious v9.1.5 software (Kearse et al., 2012), using the Flash v1.2.9 plugin (min overlap 20bp, max overlap 200bp). Merged reads containing microsatellites were selected using the QDD pipeline version 3.1 (Meglécz et al., 2014). Selected loci contained >= 20 uninterrupted repeats and had minimal target region complexity indicated by the QDD design category 'A'.

A total of 48 primer pairs were screened using a sample of eight specimens to check for successful amplification and polymorphism. Four unique 20-mer oligonucleotide tails were added to the 5' end of the forward primers as described by Real et al. (2009). Microsatellite genotyping and polymerase chain reaction (PCR) protocols followed Real et al. (2009), except an annealing temperature of 55°C was used for all loci. Characteristics of the new loci and their primer sequences are provided in Table 1.

Screening of 48 potential primer pairs produced a final set of 15 loci that were selected on the basis of amplification success, and polymorphism. The 15 new loci were genotyped on a sample of 35 *M. mariensis* larvae collected from two upstream tributaries of the Mary River (n = 18), and from Tinana Creek (n = 17). Direct comparison was made between the 15 new loci developed here and eight loci previously used for characterizing genetic structure of *M. mariensis* by Huey et al. (2013). The 35 larvae were genotyped using both sets of loci. The eight "old" loci were originally developed for congeneric species of freshwater cod: *M. peelii* and *M. ikei.* (Loughnan, Baranski, Robinson, Jones, & Burridge, 2004; Nock & Baverstock, 2008; Rourke et al., 2007). Names of the eight loci used were: Mik1F, Mik2B, MpeD5, MpeG7, Mpe3.B11, Mpe2.E01, Mpe3.G04 and Mpe1.H04.

The number of alleles (NA), observed heterozygosity (H_O) and expected heterozygosity (H_E), Hardy-Weinberg equilibrium (HWE) tests, linkage disequilibrium (LD) tests and fixation indices (F_{ST}) were calculated using Arlequin version 3.5.2.2 (Excoffier & Lischer, 2010). The significance threshold for multiple pairwise LD tests was adjusted using the false discovery rate approach via the R function "p.adjust" with the "BH" adjustment method. Allelic richness was calculated using the R package hierfstat (Goudet, 2005) and statistical comparison of diversity metrics (allelic richness and expected heterozygosity) between the new 15 loci and previously developed 8 loci were performed using the "t.test" function in R.

We used the Sibship Assignment (SA) method implemented in Colony version 2.0.6.2 for estimating the effective number of breeders (Nb) of the parental generation that produced the cohort of sampled larvae – assuming a random sample of individuals from a single cohort in a population with overlapping generations (Jones & Wang, 2010; Wang, 2009).

2. RESULTS

All 23 microsatellite loci amplified successfully and showed clear polymorphic chromatogram peaks in the sample of 35 larval *M. mariensis*. Primer sequences and summary statistics for 15 new microsatellite loci are presented in Table 1. Thirteen of the new loci conformed to HWE in both population subsamples (Mary R and Tinana Ck), while two loci exhibited deviation from HWE in a single population subsample (*mrc41*, *mrc43*; Table 1). Linkage disequilibrium was tested among all 23 loci including 15 new loci and 8 old loci, resulting in 521 pairwise tests for which there was no evidence of significant linkage between any pair of loci after controlling the false discovery rate. For the new loci, mean heterozygosity over the 35 larval samples pooled together was $H_O = 0.54$ and $H_E = 0.57$, while heterozygosity values for the old loci were $H_O = 0.36$ and $H_E = 0.39$. Considering subpopulations separately, mean expected heterozygosity was higher for the 15 new loci relative to eight old loci in both subpopulations (Mary R: $H_{E\ new\ loci} = 0.56$, $H_{E\ old\ loci} = 0.39$; Tinana: $H_{E\ new\ loci} = 0.43$, $H_{E\ old\ loci} = 0.27$). Similarly, allelic richness was higher in the new loci relative to the old loci (Mary R: $AR_{new\ loci} = 3.83$, $AR_{old\ loci} = 2.80$; Tinana: $AR_{new\ loci} = 2.71$, $A_{Rold\ loci} = 1.74$). T-tests confirmed allelic richness was significantly higher in the new loci compared to the old loci (Mary: $t\text{-value}_{(df = 20.9)} = 2.22$, $p = 0.038$; Tinana: $t\text{-value}_{(df = 20.2)} = 3.95$, $p = 0.0008$). An estimate of F_{ST} between Mary and Tinana subpopulations averaged across all 23 loci was 0.240 ($p < 0.001$), while average F_{ST} values for the 15 new loci and 8 old loci were 0.223 ($p < 0.001$) and 0.282 ($p < 0.001$) respectively. Locus-by-locus F_{ST} values for the 15 new loci are presented in Table 1, showing that 12 individual loci exhibit significant differentiation between Mary River and Tinana Creek. Genetic variation among all 35 larval samples was also visualised using principal component analysis (PCA), demonstrating clear separation between individuals sampled from Mary River and Tinana Creek based on both the new set of loci (Figure 1A) and old loci (Figure 1B).

For the 35 larval samples genotyped across all 23 microsatellite loci, the inferred effective number of breeders assuming monogamy and random mating was Nb = 14 (lower and upper 95% limits = 8, 30). Assuming male polygamy and random mating Nb = 12 (6, 26). Assuming the cohort of 35 larvae was produced by discrete sets of parents (i.e. monogamy), then the individuals can be grouped into 10 families of full-sibs, including six families from the Mary River (three from Six Mile Creek and three from Obi Obi Creek), and four families from Tinana Creek. One large full-sib family comprising 10 individual larvae from Six Mile Creek was inferred with high probability of inclusion as full-sibs (~0.99) and high probability of exclusion as full-sibs with any other individuals (~0.99). This result suggests that larvae

from the Six Mile Creek locality were mostly the product of a single pair of parents, but at least one (and possibly two) other pairs of parents were successful breeders at this locality as well. Exclusion probabilities were not as high for other inferred family groups, although up to four pairs of parents could have been responsible for producing the 17 larvae collected from the Tinana Creek site.

3. DISCUSSION

The 15 new loci developed and characterized here, showed significantly higher within population variation than loci used previously to characterize population genetic variation of Mary River cod (Huey et al., 2013). The new markers will be useful for long-term monitoring of intraspecific diversity. In particular we've demonstrated the utility of these markers in identifying potential full-sib relatives among a cohort of wild-caught larval samples. Although the larval cohort analysed here is small, the data suggested more than one set of parents bred at each site, which is consistent with Mary river cod spawning synchronously in response to an environmental cue. This panel of microsatellites represents a cost-effective resource for monitoring recruitment success and number of breeders in Mary River cod, which is a priority for conservation management of the species (Threatened Species Scientific Committee, 2016) . Both sets of loci show clear differentiation between subpopulations in the Mary River catchment. Previous analysis of adult samples using eight loci developed for congeneric cod species gave a lower F_{ST} value between Mary and Tinana 0.086 (Huey et al., 2013). By comparison, the inflated F_{ST} estimates obtained here using either the same eight loci (F_{ST} = 0.28), or new 15 loci (F_{ST} = 0.22) are likely due to sampling a limited number of full-sib family groups within each subpopulation, which has the effect of decreasing within-population variation relative to between population variation. Nonetheless, genetic subdivision is clearly evident between larvae collected from Tinana Creek and from upper tributaries of the Mary River, which supports previous findings of Huey et al. (2013) based on analysis of adult Mary River cod samples. A range of other freshwater taxa are known to exhibit a parallel pattern of genetic divergence between Mary River and Tinana creek sub-catchments. These include crustaceans (Bentley, Schmidt, & Hughes, 2010; Sharma & Hughes, 2009), fishes (Bishop, Hughes, & Schmidt, 2018; Hughes et al., 2015; Islam, Schmidt, Crook, & Hughes, 2018) and a turtle endemic to the Mary River (Schmidt, Espinoza, Connell, & Hughes, 2018). Together these findings highlight the significance of Tinana Creek as an important reservoir of intraspecific diversity in Mary River cod and numerous other freshwater taxa.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

Table 1: Characterization of fifteen novel microsatellite loci developed for *Maccullochella mariensis*. Summary statistics based on genotyping 35 larvae including 18 individuals from Mary River and 17 individuals from Tinana Creek. † repeat motif observed in original sequence from voucher specimen; ‡ code for tail added to forward primer to facilitate fluorescent labelling of PCR product (see Real *et al*. 2009); § size range includes 20 bp tail on forward primer; N_A = total number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{ST} = fixation index calculated between Mary River and Tinana Creek subpopulations. NS = not significant; * $p < 0.05$.

Locus Name	Genbank Accession	Primer sequence (5'-3')	Repeat motif †	Fwd 5' Tail ‡	size range (bp) §	N_A	Mary River H_E/H_O	Tinana Creek H_E/H_O	F_{ST}
mrc03	MG676359	F: CTGCAAACGACCTGGTTTCT R: GGGTGCGCGTGTCTGTAG	$AC_{(22)}$	3	258-270	3	$0.417/0.475^{NS}$	NA	0.227*
mrc08	MG676360	F: GCTACATTAAGGGCAGCTCA R: TACATGCATCACTGAAACACCA	$AC_{(20)}$	4	336-343	3	$0.75/0.652^{NS}$	$0.647/0.686^{NS}$	-0.008^{NS}
mrc17	MG676361	F: GGTCCACCGACAGGAGAGTA R: TCCCTTACACAGGGATCCAG	$AC_{(22)}$	1	143-147	3	$0.529/0.487^{NS}$	$0.882/0.611^{NS}$	0.110*
mrc18	MG676362	F: GTGGCCCAACACCTTAGTTT R: TGACGAAATGTCCTGAGTGC	$AG_{(20)}$	2	178-188	5	$0.375/0.421^{NS}$	$0.692/0.563^{NS}$	0.043^{NS}
mrc19	MG676363	F: CCCTGCTCTTATCTCCTTGTCA R: GAGCCATACTAGGCAGCACA	$AC_{(24)}$	3	190-208	7	$0.857/0.844^{NS}$	$0.583/0.54^{NS}$	0.079*
mrc23	MG676364	F: CAGACCTGAGCCTTCTGACC R: ATGAACTCTTATTGTTCCAGTAACTTT	$AC_{(24)}$	3	311-328	4	$0.857/0.725^{NS}$	$0.438/0.498^{NS}$	0.204*
mrc26	MG676365	F: GCTGAGTGGAGCCACCTTAC R: TGGATCGTGACAGATGCTTT	$AC_{(24)}$	2	178-191	6	$0.313/0.389^{NS}$	$0.647/0.636^{NS}$	0.378*
mrc30	MG676366	F: GCTGCAGATGGGAACTAACC R: CAGTAATGGGTGTGGTGGAG	$AC_{(28)}$	2	263-282	3	$0.429/0.54^{NS}$	$0.267/0.239^{NS}$	0.083*
mrc31	MG676367	F: TGTGCTGGAGGTCATTGGTA R: CAGATCTGCAAGGGTGAGGT	$AC_{(23)}$	3	130-145	4	$0.667/0.608^{NS}$	$0.235/0.219^{NS}$	0.1248*
mrc32	MG676368	F: GCTGGATAACGTTACGTGTCTG R: GATTCAGTGACAGACGGCAG	$AC_{(20)}$	4	221-232	4	$0.611/0.551^{NS}$	$0.294/0.266^{NS}$	0.051*
mrc36	MG676369	F: TACAACTCAGCGTGGCCAAA R: ATCGCCACTCTAATCCCAAA	$AC_{(20)}$	4	141-151	5	$0.813/0.579^{NS}$	$0.438/0.377^{NS}$	0.455*
mrc37	MG676370	F: ATACCTCCGCTTTGGAATCA R: GTAGCATCATCTGTGAGACCG	$AC_{(20)}$	1	331-342	5	$0.8/0.616^{NS}$	$0.375/0.546^{NS}$	0.398*
mrc40	MG676371	F: TATCTGCACTTAAACGCCAA	$AC_{(21)}$	4	312-314	2	$0.556/0.457^{NS}$	$0.294/0.258^{NS}$	0.419*

		R: TTATGCAAGACGTGGTACGC							
mrc41	MG676372	F: ACATGTTGCTGAGGCTGAGA R: CATGACAGAACTGGTGCGTT	$AC_{(21)}$	1	134-151	3	0.875/0.645*	$0.692/0.686^{NS}$	0.025^{NS}
mrc43	MG676373	F: GGTGGCAATGAATATCCTGTT R: GACATGAACTCCACCTCGTG	$AC_{(20)}$	3	161-179	7	0.692/0.751*	$0.647/0.686^{NS}$	0.367*

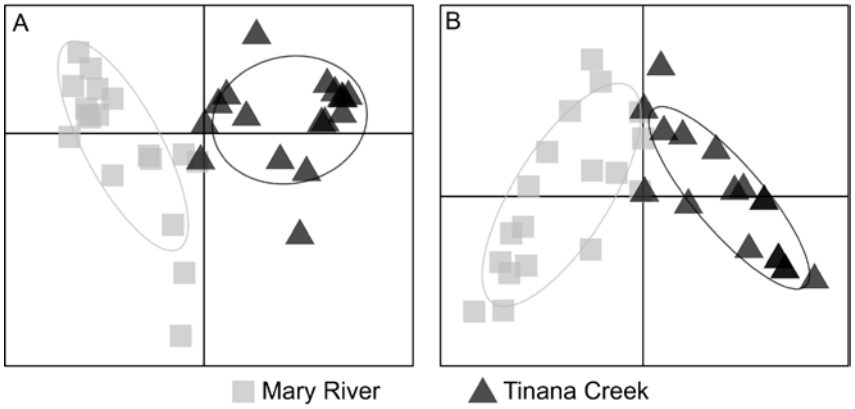

Figure 1. Genetic relationship among 35 Mary River cod larvae genotyped using two sets of microsatellite markers. Plots represent the first two axes of a principal component analysis (PCA) produced using R function "dudi.pca" from ade4 package. Individuals sampled from Mary River represented with grey squares, individuals sampled from Tinana Creek represented with black triangles. **A.** PCA based on 15 new loci; percentage of variation explained by axis 1 (x-axis) and axis 2 (y-axis) was 30.7% and 11.9% respectively. **B.** PCA based on 8 old loci; percentage of variation explained by axis 1 and axis 2 was 41.5% and 19.5% respectively.