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TECHNICAL NOTE

Development of 18 microsatellite markers for the southern purple-spotted gudgeon (*Mogurnda adspersa*) from the lower Murray-Darling Basin through 454 pyrosequencing

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Abstract A new set of 18 microsatellite loci was developed for the threatened Australian freshwater fish southern purple-spotted gudgeon *Mogurnda adspersa* (Eleotridae) using a next generation sequencing approach. A total of 84 fish from two populations (including one rescued into captivity) were successfully genotyped at all markers using a multiplex approach. As expected for threatened species, we observed relatively low genetic variation across most loci (average allelic diversity = 5.4; average heterozygosity = 0.380). No evidence for linkage disequilibrium was detected and all loci were in Hardy–Weinberg equilibrium. This new set of microsatellite markers will benefit substantially the ongoing conservation program of a critically endangered population of *M. adspersa* that involves captive breeding, relatedness and paternity analyses, reintroduction, and landscape genetics.

Keywords Critically endangered population · Relatedness · Captive breeding · Reintroduction · Conservation program · Evolutionary significant units

The southern purple-spotted gudgeon (SPSG) *Mogurnda adspersa* (Eleotridae) is a small freshwater fish endemic to mainland southeastern Australia. Phylogeographic studies have shown the presence of an Evolutionarily Significant Unit (ESU) for this species within a distinct section of its range, in the Murray-Darling Basin (MDB) (Faulks et al. 2008). The MDB populations are classified as threatened under legislation in New South Wales, Victoria and South Australia. The species was once widespread in the MDB, but has declined dramatically in the last 30 years, being fragmented in the northern MDB, and presumed extinct from the south (Lintermans 2007). A population was recently rediscovered near the terminus of the system (southern MDB) from a wetland near Murray Bridge on the lower River Murray, however over abstraction of water exacerbated the effects of prolonged drought and saw the only known habitat dry in 2007. A fish rescue prior to drying ensures that recovery options remain (Hammer et al. 2009) including within a specific "Drought Action Plan" for fishes at very high risk of extinction in the lower MDB (Hall et al. 2009). In association with this plan, we have implemented a conservation program that involves captive breeding, relatedness and paternity analyses, reintroduction, and landscape genetics. Although microsatellite markers have been described for SPSG (Real et al. 2009), these were developed from fish collected in Queensland's central coast—a relatively divergent lineage of *Mogurnda* from an isolated catchment that likely represents a different species than the lower MDB taxon (Faulks et al. 2008). Additional highly resolving molecular markers capable of establishing kin relationships and guiding the lower MDB captive breeding program are needed.

In order to rapidly characterize a new set of microsatellite markers for *M. adspersa*, we adopted a next generation sequencing (NGS) approach. Genomic DNA ($\cong$10 μg) was extracted from muscle tissue of one specimen of *M. adspersa* from the lower MDB (35.05°S, 139.32°E). The DNA was

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Table 1 Characterization of 18 microsatellite loci isolated from *Morgunda adspersa*

Locus	Primer sequences (5′–3′)*,†	Repeat structure	N_A	Size range (bp)	MDB		NSW		GenBank accession no.
					H_O/H_E	H–W	H_O/H_E	H–W	
PlexA									
Mog5	F AGGGAGGACTCGTGCTTTTC[VIC] R GGACCCACTGGTGAGCTG	(CAG)16	7	126–252	0.444/ 0.598	0.0156*	0.567/ 0.515	0.1051	JN674515
Mog14	F CTTCCCCTGCCACTAAAAAC[FAM] R ACGTTGCAGCCACACTACAC	(ATA)12	4	239–293	0.296/ 0.252	0.5831	0.600/ 0.616	0.7961	JN674520
Mog18	F CAGGGTTGAGGTAGCTGGAG[NED] R GATAATCGCAATGCCTCAGTC	(ATT)11	3	280–376	–	–	0.621/ 0.499	0.2816	JN674522
Mog26	F GGCGGGATATTCTCATAAGG[NED] R GCATATTAGACTGTGACTGGATTTG	(ATCT)12	8	190–230	0.833/ 0.805	0.5513	0.500/ 0.789	0.033	JN674526
Mog28	F GGGTGTGGGAGAGTGAATG[PET] R CCTGGGTGGATAAGACATCG	(ATT)11	5	223–238	0.389/ 0.375	0.6719	0.069/ 0.067	1.000	JN674527
PlexB									
Mog10	F TAACTACTCTGCCGCTGTGC[VIC] R TTGGTTTAGGTTAGTCCAATTTAAGTC	(TTA)16	9	202–256	0.426/ 0.531	0.3346	0.633/ 0.596	0.4476	JN674518
Mog11	F GTCCTTGTAGGCGCTCATTG[NED] R ATGGTCTTGTGCTGGCTATC	(ATA)8	2	212–224	0.444/ 0.494	0.5763	0.433/ 0.495	0.4825	JN674519
Mog22	F TTGACCCAAGGCAGAGGTAG[PET] R TTTGCAGTAGAAACTCCAGTCC	(CAG)13	4	181–208	0.056/ 0.088	0.0924	0.567/ 0.433	0.1981	JN674523
Mog30	F GGCATCAGCAACCTGAGAAG[FAM] R TAGACTGTTGGCGTGGTACG	(ATA)13	7	245–263	0.648/ 0.634	0.5098	0.167/ 0.206	0.3254	JN674528
Mog32	F AAGCCCAGACTAAACCAGGTC[PET] R TCTGCACTCTGACAAATAGCAC	(CA)14	3	268–272	0.472/ 0.493	0.5526	–	–	JN674529
PlexC									
Mog7	F TGTGGAGGTCTCATGTGGAG[NED] R TGGGCCTTTGCCAGTAGTAG	(ATTT)10	7	230–286	–	–	0.704/ 0.632	0.1142	JN674516
Mog16	F GGCATTGTTGAGAGAACACG[VIC] R TGCACCGCCTTATAAACATC	(CTT)9	5	226–244	0.444/ 0.353	0.1592	0.600/ 0.532	0.8493	JN674521
Mog25	F CCTTGAATTAACCAGGACTAAACAG[PET] R GGTGTGGGTCGAATTGGTAG	(GA)14	4	180–190	0.537/ 0.574	0.5439	–	–	JN674525
Mog34	F AATGGTGTGAATCCGTCAGC[FAM] R ATGGCAGTGAGTGAGACTCC	(CA)12	3	248–256	–	–	0.467/ 0.498	0.7264	JN674531
PlexD									
Mog4	F CCGCTTCTGGACTTTACAGAG[PET] R TTGACATGAAACAGCAGACG	(GACA)11	5	106–134	0.370/ 0.395	0.6488	–	–	JN674514
Mog8	F CTGGGGCAAACACACTGAC[FAM] R CTTAGTTGCGGCTTGGAGAG	(CTTAT)13	7	204–279	0.778/ 0.748	0.8967	0.267/ 0.278	1.000	JN674517
Mog24	F TCCAGAGGTGAAGACCAAGG[VIC] F GCAGCATCCACTTTATTGACAG	(AC)22	8	156–228	0.259/ 0.710	0.000*	0.033/ 0.033	–	JN674524
Mog33	F GGAACACCACGGAAACCTC[NED] R CCTCCTCGAGTAAGACCAATATTTAC	(AC)13	6	214–264	0.444/ 0.598	0.0395	0.310/ 0.348	0.5942	JN674530

N_A number of alleles, range of allele size, H_O observed heterozygosity, H_E expected heterozygosity and Hardy–Weinberg P values (H–W) are based on 54 specimens from the lower Murray-Darling Basin (MDB) and 30 specimens from a New South Wales (NSW) population (details in the text)

* Significant deviation from Hardy–Weinberg equilibrium after Bonferroni adjustment

† Forward primers were tagged with a 5′M13 universal sequence (5′TGTAAAACGACGGCC) and label using four different fluorescence (i.e. 6-FAM, NED, PET, VIC)

sent to the Australian Genome Research Facility (http://www.agrf.com.au) and sequences were nebulised and ligated with 454 sequencing primers. Each fragment was tagged with a common sequence, which was used to separate the pooled sequences from the whole plate's fragment using post run bioinformatics tools. The sample was then subjected to high throughput DNA sequencing on 1/8 of a 70 9 75 PicoTiterPlate using the Roche GS FLX (454) system as described elsewhere (Margulies et al. 2005). The software MSATCOMMANDER 0.8.2 (Faircloth 2008), MICROFAMILY (Meglécz 2007) and PRIMER 3 (Rozen and Skaletsky 2000) were used to select contigs and design primer sets as previously described (Carvalho and Beheregaray 2010). From a total of 303,160 reads obtained through the NGS, 3,168 had putative microsatellite motifs. MICROFAMILY recovered 378 unique and UnBlastTable loci, from which the best 30 loci (17 tri, 7 di, 3 penta e 3 tetra-nucleotide) were chosen for polymerase chain reaction (PCR). Amplification followed the method described by Schuelke (2000) in which PCR products are fluorescently labeled through the inclusion of a third (fluorescent M13) primer in each reaction. Reactions and PCR conditions followed (Beheregaray and Sunnucks 2000) and used their 61–52°C touch down. After initial PCR optimization, 18 microsatellite primer sets amplified consistently and were selected for further work. MULTIPLEX MANAGER (Holleley and Geerts 2009) was used to design the four distinct multiplex PCRs: PlexA (5 loci), PlexB (5 loci), PlexC (4 loci) and PlexD (4 loci) (Table 1). A total of 54 wild fish from the lower MDB population now held in captivity and 30 fish from a NSW population (upper Hunter River) were used for microsatellite genotyping.

Amplification products were detected in an automated DNA sequencer ABI 3130 (Applied Biosystems). The resulting microsatellite profiles were examined using GENEMAPPER 4.0 (Applied Biosystems) and peaks were scored manually. GENEPOP v4 (Rousset 2008) was used to estimate expected (H_E) and observed (H_O) heterozygosity, number of alleles (N_A), linkage disequilibrium and Hardy–Weinberg proportions. Bonferroni corrections were applied when conducting multiple statistical tests (Rice 1989). The software MICROCHECKER (Van Oosterhout et al. 2004) was used to check for null alleles and scoring errors.

All loci were polymorphic for the 84 individuals genotyped. An average of 5.4 alleles per locus (range of 2–9) and low mean heterozygosity ($H_o = 0.380$) was observed. No evidence for linkage disequilibrium locus-pair/population was found between loci and no consistent deviations from Hardy–Weinberg equilibrium were detected across populations/loci. This study reports on the first set of microsatellite markers for the endangered ESU of *M. adspersa* found in the MDB. These markers are an important resource for the ongoing conservation program that involves captive breeding, relatedness and paternity analyses, reintroduction, and landscape genetics.

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References

Beheregaray LB, Sunnucks P (2000) Microsatellite loci isolated from *Odontesthes argentinensis* and the *O. perugiae* species group and their use in other south American silverside fish. Mol Ecol 9(5): 629–631. doi:10.1046/j.1365-294x.2000.00882.x

Carvalho D, Beheregaray L (2010) Rapid development of microsatellites for the endangered neotropical catfish *Conorhynchus conirostris* using a modest amount of 454 shot-gun pyrosequencing. Conserv Genet Resour 1–3. doi:10.1007/s12686-010-9365-4

Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol Ecol Resour 8(1):92–94

Faulks LK, Gilligan DM, Beheregaray LB (2008) Phylogeography of a threatened freshwater fish (*Mogurnda adspersa*) in Eastern Australia: conservation implications. Mar Freshw Res 59(1): 89–96. doi:10.1071/Mf07167

Hall A, Higham J, Hammer M, Brice C, Zampatti B (2009) Drought action plan for South Australian Murray-Darling Basin threatened freshwater fish populations 2009–2010; rescue to recovery. South Australia Department of Heritage, Adelaide

Hammer M, Wedderburn S, van Weenan J (2009) Action plan for South Australian freshwater fishes. Native Fish Australia (SA) Inc, Adelaide, p 206

Holleley CE, Geerts PG (2009) MULTIPLEX MANAGER 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. Biotechniques 46(7):511–517. doi:10.2144/000113156

Lintermans M (2007) Fishes of the Murray-Darling Basin: an introductory guide. Murray-Darling Basin Commission, Canberra

Margulies M, Egholm M, Altman WE, Attiya S et al (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437(7057):376–380. doi:10.1038/nature03959

Meglécz E (2007) MICROFAMILY (v.1): a computer program for detecting flanking-region similarities among different microsatellite loci. Mol Ecol Notes 7(1):18–20. doi:10.1111/j.1471-8286.2006.01537.x

Real K, Schmidt D, Hughes J (2009) *Mogurnda adspersa* microsatellite markers: multiplexing and multi-tailed primer tagging. Conserv Genet Resour 1(1):411–414. doi:10.1007/s12686-009-9095-7

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43(1): 223–225

Rousset F (2008) GENEPOP ' 007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour 8(1):103–106. doi:10.1111/j.1471-8286.2007.01931.x

Rozen S, Skaletsky H (2000) PRIMER3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386. doi:10.1385/1-59259-192-2:365

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18(2):233–234. doi:10.1038/72708

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4(3):535–538. doi:10.1111/J.1471-8286.2004.00684.X