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TECHNICAL NOTE

Isolation and characterisation of microsatellite loci in the Australian freshwater catfish (*Tandanus tandanus*)

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Abstract The Australian freshwater catfish (*Tandanus tandanus*) has suffered a decline in abundance and distribution, and stocking of wild populations with hatchery-bred fish has been suggested to assist with population recovery. Here we describe the isolation and characterisation of eight microsatellite markers that may be used to assess population structure of *T. tandanus* in the wild to inform future stocking programs of any major genetic boundaries between populations. We tested the variability of the loci in 28–29 individuals from three populations of *T. tandanus*, as well in 24 individuals from a population representing an undescribed species. Expected heterozygosity for these loci ranged from 0.034 to 0.920 across the four populations. All loci successfully amplified in the three *T. tandanus* populations, while in the undescribed species one locus failed to amplify and three loci were monomorphic.

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The Australian freshwater catfish *Tandanus tandanus* is a popular recreational fish species in the Murray-Darling Basin (MDB) and coastal catchments of eastern Australia (Clunie and Koehn 2001a). The species has undergone a dramatic decline in abundance and distribution, particularly in the MDB, where it has recently been declared endangered under the *Fisheries Management Act 1994*. Some populations have become established outside their native range through translocations of MDB *T. tandanus* into southern New South Wales (NSW) coastal catchments and into dams and lakes in Victoria (Harris and Battaglene 1990; Clunie and Koehn 2001a). Previous genetic studies using allozyme and mtDNA data suggested that *T. tandanus* consists of at least three species, namely *T. tandanus* in the MDB and in coastal catchments of southern Queensland (QLD), northern NSW and southern NSW, an undescribed species in coastal catchments of mid-northern NSW, and an additional undescribed species in coastal catchments of north-eastern QLD (Musyl and Keenan 1996; Jerry and Woodland 1997; Jerry 2005, 2008). The current distribution spans many catchments, some with limited or no connectivity. It is therefore expected that genetic structure exists among populations. Current management plans call for a stocking program to supplement wild populations (Clunie and Koehn 2001b), and it is critical that the underlying genetic structure within and among populations is understood. This will ensure that stocked fish will be genetically compatible with their receiving population, thus minimising any potential negative genetic impacts on these wild populations. Here we describe the development of a set of microsatellite loci that will be utilised for this purpose.

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Table 1 Primer sequences of eight *Tandanus tandanus* microsatellite loci, including number of individuals (N), number of alleles (N_a), observed heterozygosity (H_O), and expected heterozygosity (H_E) and P values for the Hardy–Weinberg equilibrium test (P)

Locus	GenBank accession no.	Repeat motif	Primer sequences (5′–3′)	Dye label	*T. tandanus* (Macintyre River MDB, $n = 28$)						*T. tandanus* (Lower Border Rivers MDB, $n = 28$)					
					Size range (bp)	N	N_a	H_O	H_E	P	Size range (bp)	N	N_a	H_O	H_E	P
Tan1_2	GQ496016	$(GT)_{20}$	F: CCGACTGTCAGTGAAAAGGAG[a]	D4	216–244	28	6	0.571	0.592	0.360	216–240	28	7	0.643	0.648	0.609
			R: AGGGTCTGGGAGTGAATGAG													
Tan1_7	GQ496017	$(GT)_{34}$	F: TGTATGGAGCTACTAACAAAACAGG[a]	D3	189–223	28	9	1.000	0.773	0.076	193–219	28	12	0.964	0.892	0.807
			R: TACTCCAGCCCTGAAGGTG													
Tan1_10	GQ496018	$(TC)_{14}$	F: TCCTGATTTCTCTCCCAAGG[a]	D2	308–310	28	2	0.357	0.408	0.643	308–314	28	4	0.607	0.665	0.300
			R: AGAAAGGTGGTGCATGTGTG													
Tan2_15	GQ496019	$(GA)_{15}$[b]	F: CGTAGTTGTTTTTGTTTCGGAAGTAG[a]	D4	176–196	28	8	0.720	0.838	0.081	160–196	27	13	0.704	0.832	0.028
			R: GTTTGCACAGGAATTAACAACAG													
Tan2_16	GQ496020	$(CTAT)_{14}$	F: TGCCTGTTGTTTCTTTCTTTC[a]	D3	227–277	28	9	0.704	0.792	0.089	219–301	28	17	0.893	0.920	0.145
			R: ATGTTCTGCCGAGCTTGAG													
Tan2_20	GQ496021	$(GT)_{17}$	F: TCCTCTGCTCCTGCTGTTTC[a]	D3	265	28	1	–	–	–	263–267	28	3	0.286	0.304	0.620
			R: ATGGGATGCCAATTCATCAC													
Tan3_27	GQ496022	$(CT)_{17}$	F: TGTGGAAGGTTGGGGTTATG[a]	D2	227–269	28	6	0.571	0.638	0.021	217–269	28	14	0.714	0.810	0.006*
			R: CGTGATCATGCAAACAGATG													
Tan3_28	GQ496023	$(CT)_{18}$	F: CCCCATTTGCTTTTTCTCTG[a]	D2	289–299	27	4	0.704	0.686	0.802	289–299	27	6	0.593	0.694	0.324
			R: TGTTGAAAGCGGCATGTTAG													
			Average ± SD			27.87 ± 0.35	5.6 ± 3.1	0.661 ± 0.196	0.675 ± 0.147			27.7 ± 0.463	9.5 ± 5.1	0.676 ± 0.206	0.721 ± 0.197	

Locus	GenBank accession no.	Repeat motif	Primer sequences (5′–3′)	Dye label	*T. tandanus* (Richmond catchment north-coast NSW, $n = 29$)						*Tandanus* sp. (Bellinger catchment, mid-north coast NSW, $n = 24$)					
					Size range (bp)	N	N_a	H_O	H_E	P	Size range (bp)	N	N_a	H_O	H_E	P
Tan1_2	GQ496016	$(GT)_{20}$	F: CCGACTGTCAGTGAAAAGGAG[a]	D4	224–240	29	5	0.793	0.675	0.086	236–242	24	4	0.500	0.489	0.931
			R: AGGGTCTGGGAGTGAATGAG													
Tan1_7	GQ496017	$(GT)_{34}$	F: TGTATGGAGCTACTAACAAAACAGG[a]	D3	181–227	29	16	0.862	0.915	0.006*	181–183	22	2	0.091	0.087	1.000
			R: TACTCCAGCCCTGAAGGTG													
Tan1_10	GQ496018	$(TC)_{14}$	F: TCCTGATTTCTCTCCCAAGG[a]	D2	298–300	29	2	0.034	0.034	1.000	302	23	1	–	–	–
			R: AGAAAGGTGGTGCATGTGTG													
Tan2_15	GQ496019	$(GA)_{15}$[b]	F: CGTAGTTGTTTTTGTTTCGGAAGTAG[a]	D4	160–186	29	11	0.759	0.786	0.397	184–190	16	3	0.563	0.498	0.793
			R: GTTTGCACAGGAATTAACAACAG													
Tan2_16	GQ496020	$(CTAT)_{14}$	F: TGCCTGTTGTTTCTTTCTTTC[a]	D3	251–301	24	9	0.292	0.833	0.000*	229–261	22	9	0.682	0.756	0.090
			R: ATGTTCTGCCGAGCTTGAG													
Tan2_20	GQ496021	$(GT)_{17}$	F: TCCTCTGCTCCTGCTGTTTC[a]	D3	261–263	29	2	0.034	0.034	1.000	269	24	1	–	–	–
			R: ATGGGATGCCAATTCATCAC													

Table 1 continued

Locus	GenBank accession no.	Repeat motif	Primer sequences (5′–3′)	Dye label	*T. tandanus* (Richmond catchment north-coast NSW, $n = 29$)					*Tandanus* sp. (Bellinger catchment, mid-north coast NSW, $n = 24$)					
					Size range (bp)	N	N_a	H_O	H_E	P	Size range (bp)	N	N_a	H_O	H_E	P
Tan3_27	GQ496022	$(CT)_{17}$	F: TGTGGAAGGTTGGGGTTATG[a]	D2	215–223	29	4	0.690	0.613	0.506	No amplification	–	–	–	–	–
			R: CGTGATCATGCAAACAGATG													
Tan3_28	GQ496023	$(CT)_{18}$	F: CCCCATTTGCTTTTTCTCTG[a]	D2	291–301	29	6	0.655	0.620	0.954	281	23	1	–	–	–
			R: TGTTGAAAGCGGCATGTTAG													
			Average ± SD			28.4 ± 1.77	6.9 ± 4.8	0.515 ± 0.342	0.564 ± 0.343			22 ± 2.8	3 ± 2.9	0.459 ± 0.257	0.458 ± 0.276	

* Significant at 5% level after sequential Bonferroni correction (Rice 1989)

[a] 5′ End of each forward primer was appended with an 19-bp 'M13' sequence (CACGACGTTGTAAAACGAC, to facilitate the incorporation of a dye label during PCR)

[b] Interrupted microsatellite repeat

We collected *T. tandanus* from the Macintyre River (−29 45.754, 151 07.226, Border Rivers catchment, MDB) and extracted genomic DNA from one individual using a phenol–chloroform procedure (Sambrook et al. 1989) and a QIAGEN DNeasy tissue kit (QIAGEN). The combined products of these extractions resulted in sufficient high-molecular weight DNA for the enrichment. We followed an enrichment technique to isolate microsatellites (Fischer and Bachmann 1998 as modified in; Beheregaray et al. 2004). Briefly, approximately 3 µg of genomic DNA was digested with *Rsa*I and *Hae*III, and oligo adapters were ligated onto the blunt ends. The resultant ligation mix was annealed to CA, GA, AGAT, AACT and ACAT biotinylated probes. Fragments containing the annealed probes were purified using Streptavidin magnetic beads (Promega) and amplified in a polymerase chain reaction (PCR) using one of the oligo adaptors. The process was repeated using the PCR product of the first enrichment as template, thus resulting in a double enrichment. Enriched DNA was purified using an UltraClean 15 DNA purification kit (MoBio Laboratories), ligated into a pCR 2.1-TOPO vector (Invitrogen), transformed into TOP10 cells (Invitrogen) and plated onto LB agar containing ampicillin (50 µg/ml) and X-gal (40 mg/ml).

A total of 127 positive clones were PCR amplified using M13 forward (−20) primers and sequenced using the BigDye terminator chemistry and an ABI 377 DNA automated DNA sequencer (PE Applied Biosystems). ChromasPro 1.41 (www.technelysium.com.au/ChromasPro.html) was used to assemble sequences into contigs to facilitate the identification of duplicate sequences, and to remove vector sequence. Sequences were also screened using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) to confirm that all vector sequence was removed. We used PRIMER 3 (Rozen and Skaletsky 2000) to design primers for 28 microsatellite repeat sequences.

The 28 loci were tested for variability in individuals from two populations in the Murray–Darling Basin [(the Macintyre River ($n = 28$) and lower Border Rivers catchment ($n = 28$)] and in two coastal catchments [the Richmond River catchment ($n = 29$) and the Bellinger River catchment ($n = 24$)]. The latter catchment represents individuals from the undescribed species from the mid-north NSW coast as proposed elsewhere (Musyl and Keenan 1996; Jerry and Woodland 1997; Jerry 2008). PCRs were carried out in 12 µl volumes containing 5–20 ng DNA, 0.125 µM of forward primer, 0.25 µM of reverse primer and 0.375 µM of M13 fluorescent-labelled primer (D4, D3 or D2; CACGACGTTGTAAAACGAC, Sigma), 0.5 mM of dNTP (Astral Scientific), 2.5 mM of $MgCl_2$, 1.25 µl of 5× reaction buffer and 0.5 U of GoTaq Flexi DNA polymerase and (Promega). Thermal cycling conditions consisted of 95°C for 2 min, 30 cycles of 95°C

for 30 s, 55°C for 45 s, and 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were sized using CEQ 8000 Genetic Analysis System and assessed for scoring ease and polymorphism using the CEQ 8000 software (Beckman Coulter).

Of the 28 loci screened across 109 samples of *T. tandanus*, eight were polymorphic. These loci were successfully amplified in samples from the Macintyre River, lower Border Rivers catchment and Richmond River catchment, while Tan3_27 failed to amplify in any of the Bellinger River catchment samples (undescribed species) and three loci were monomorphic (Tan1_10, Tan2_20 and Tan3_28). The mean observed heterozygosities for these four catchments respectively were 0.661, 0.676, 0.515 and 0.459; mean expected heterozygosites were 0.675, 0.721, 0.564 and 0.458; mean number of alleles per locus were 5.6, 9.5, 6.9 and 3 (Table 1). We used GENEPOP (Raymond and Rousset 1995) to conduct tests for Hardy–Weinberg equilibrium (HWE) at each locus, and for linkage disequilibrium between pairs of loci from each population. Three loci (Tan1_7, in the Richmond catchment, Tan2_16 in the Richmond catchment and Tan3_27 in the lower Border Rivers catchment) deviated from HWE ($P < 0.05$) after sequential Bonferroni correction (Rice 1989) due to heterozygosity deficits. However, given that these loci only departed from HWE in a single population each, null alleles are unlikely to be responsible. None of the loci-pair combinations showed evidence of linkage disequilibrium in our data set. These loci are currently being used to assess genetic diversity and population structure of *T. tandanus* across its extensive distribution to assist with the future management of the species.

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