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PRIMER NOTE

Twelve microsatellite loci for marine and riverine tucuxi dolphins (*Sotalia guianensis* and *Sotalia fluviatilis*)

HAYDÉE A. CUNHA* and PHILLIP C. WATTS†
*Laboratório de Biodiversidade Molecular, Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil/MAQUA — Departamento de Oceanografia, Universidade do Estado do Rio de Janeiro, 20550-013, Brazil, †School of Biological Sciences, The Biosciences Building, Crown Street, University of Liverpool, Liverpool L69 7ZB, UK

Abstract

Twelve dinucleotide polymorphic microsatellite loci were isolated from the marine tucuxi dolphin (*Sotalia guianensis*). Levels of genetic diversity were assessed using 34 individuals from the coasts of Rio de Janeiro and Pará, Brazil. Numbers of alleles varied between two and 14, and observed and expected heterozygosities ranged from 0.040 to 0.704, and from 0.093 to 0.818, respectively. Moreover, eight of these loci were variable in the riverine tucuxi *Sotalia fluviatilis*. This is the first description of microsatellite primers from a dolphin that does not belong to the Delphininae. These loci are currently being used in the analysis of population structure of both tucuxi species.

Keywords: cetacean, Delphinidae, genetics, kinship, population structure, social structure

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Marine and riverine tucuxi dolphins (*Sotalia guianensis* and *Sotalia fluviatilis*) have been recently assigned to different species (Cunha *et al*. 2005). *Sotalia guianensis* has a coastal Atlantic distribution from Honduras to southern Brazil, while *S. fluviatilis* is restricted to the Amazon River basin. Although many tucuxi populations are under threat, largely because of human activities, neither species has been assigned a conservation status as knowledge of their basic biological parameters is lacking (IUCN 2006). Microsatellites are well suited for the study of population structure and kinship and accordingly many loci have been isolated for some dolphin species. While a large proportion of these loci cross-amplify in other cetaceans (e.g. Valsecchi & Amos 1996; Coughlan *et al*. 2006), potential problems arise when using heterologous primers, including an ascertainment bias and null alleles because of mutation in the flanking sequence. This is particularly relevant for tucuxi dolphins because they are not members of the Delphininae, the subfamily from which all presently known dolphin microsatellites have been isolated. Therefore, we developed a panel of polymorphic dinucleotide microsatellite loci for the marine tucuxi and tested these loci in the congeneric riverine tucuxi.

Correspondence: Haydée A. Cunha, Laboratório de Biodiversidade Molecular, Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro. Av. Pau Brazil 211, Bl. A, Sl. A2-098, Ilha do Fundão, Rio de Janeiro, Brazil, 21941-590. Fax: 55 21 25626333; E-mail: haydeecunha@ufrj.br

Total genomic DNA for both library construction and genotyping was isolated from *S. guianensis* frozen skin or muscle samples using a phenol–chloroform protocol (Sambrook *et al*. 1989). To construct the partial genomic library, 4 μg of pooled DNA (from six individuals) was digested with *Sau*3AI (Boehringer-Mannheim) and ligated to 25 pmol of phosphorylated linkers (S61 5′ -GGCCAGAGACCCCA-AGCTTCG-3′ annealed to S62 5′-PO4-GATCCGAAGCTT-GGGGTCTCTGGCC-3′, Refseth *et al*. 1997). DNA fragments between 500 bp and 1000 bp were excised from a 1.8% NuSieve gel (FMC Bioproducts) and purified using a gel extraction kit (QIAGEN). Full details of the enrichment procedure (based on Gardner *et al*. 1999) are provided elsewhere (Bloor *et al*. 2001); briefly, we hybridized the DNA fragments with M2-80 streptavidin-coated magnetic beads (Dynal) incubated with 3′-biotin-labelled $(CA)_{12}$ oligonucleotides (MWG Biotech). After a series of differential stringency washes in 2× SSC and 1× SSC, the enriched DNA was made double stranded and amplified in a 25-μL polymerase chain reaction (PCR) primed with 30 pmol S61 and 1.25 U of *Taq* polymerase (ABgene) at: 95 °C 5 min, 30× (95 °C 50 s, 56 °C 1 min, 72 °C 2 min), 72 °C 10 min. The DNA was purified using a PCR purification kit (QIAGEN), ligated into pGEM-T vector (Promega) and transformed into JM109 competent cells (Promega). Recombinant clones

were identified using black/white screening on S-gal (Sigma) agar/ampicillin plates. Plasmids containing a microsatellite insert were identified by two or more amplified products after PCR primed with 40 pmol S61 and 20 pmol of (non-biotinylated) microsatellite oligonucleotide. Fifty-four positive clones were isolated and cycle sequenced using M13 universal primers and BigDye chemistry on an ABI PRISM 3100 (Applied Biosystems). Primers flanking microsatellite regions were designed using PRIMER2 software (S.J. Kemp, unpublished data). We were able to design primers around 18 loci only, because of insufficient or unsuitable sequence flanking the microsatellite region.

Microsatellite alleles were amplified in a 10-μL PCR using ReddyMix PCR mix (ABgene) on a Dyad DNA Engine (MJ Research Inc.). A tailed primer method, whereby forward primers are synthesized with a 5′ (or tail) sequence of a primer that is labelled with either 6-FAM, NED, PET or VIC fluorophores (Applied Biosystems), was used to label PCR products (see Schuelke 2000) and therefore each PCR contained three primers — tailed, labelled and reverse. The PCR consisted of 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, 5–50 ng template DNA, 4 pmol of each primer, 10 μg BSA and 0.25 U *Taq* polymerase (ABgene). PCR conditions were 95 °C for 1 min, 5× (95 °C 30 s, 57 °C 45 s, 72 °C 45 s), 25× (92 °C 30 s, 57 °C 45 s, 72 °C 55 s), 72 °C 10 min (all loci amplified at the same annealing temperature). PCR products were pooled with a 500-bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI PRISM 3100 and sized using GENEMAPPER software (Applied Biosystems).

Two loci that amplified spurious bands were dropped, leaving 16 loci to be tested for polymorphisms in 34 *S. guianensis* from the coast of Rio de Janeiro (23°01′S) and Pará (0°16′N), Brazil, and 11 specimens of *S. fluviatilis* from the Brazilian Amazon (2°57′S, 64°48′W). The online version (3.4, http://wbiomed.curtin.edu.au/genepop/) of GENEPOP (Raymond & Rousset 1995) was used to calculate basic measures of genetic diversity, the significance of any deviations from expected Hardy–Weinberg equilibrium (HWE) conditions and also for linkage disequilibrium between all pairs of loci.

Four loci were monomorphic, while the remaining 12 loci resolved between two and 14 distinct alleles and had observed and expected heterozygosities varying between 0.040 and 0.704, and between 0.093 and 0.818, respectively (Table 1). No pairs of loci showed significant linkage

Table 1 Levels of variability at 12 polymorphic microsatellite loci in the marine tucuxi (*Sotalia guianensis*) from Brazil (n = 34). N_a indicates number of alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity. The columns to the right show the N_a, H_O and H_E found in the riverine tucuxi (*Sotalia fluviatilis*) from the Brazilian Amazon (n = 11). NP, lack of amplification product

Locus accession no.	Primer sequence (5′–3′)	Repeat motif	Size range* (bp)	N_a	H_O H_E	*S. fluviatilis* N_a	*S. fluviatilis* H_O	*S. fluviatilis* H_E
Sgui-002	F: GGATGTCACTGAACACAGAGC	$(CA)_{18}$	207–211	3	0.182	2	0.500	0.521
BV693806	R: ACCTATCTACATTTCCCAGAGG				0.444			
Sgui-003	F: TCCAATCTCCAACCAAATCCC	$(GT)_{28}$	148–162	6	0.273	3	0.636	0.610
BV693807	R: GTCGCTAAGTTCATCATCTGC				0.508			
Sgui-004	F: TGAATGGGTCAGAGTTTTGCC	$(GT)_{37}(GA)_{19}$	251–259	5	0.200	NP	—	—
BV693808	R: TCAATGGGAGGCACTGTAGG				0.573			
Sgui-005	F: AGCACAATCACAACGAAGACC	$(GT)_{19}$	131–139	5	0.533	NP	—	—
BV693809	R: TTGCCTCAGTTCAGGAAGCC				0.660			
Sgui-006	F: CTATGATGGACGGTTGAAGG	$(GT)_{21}$	201–215	6	0.444	4	0.556	0.660
BV693810	R: TCTCTTGGTCATTGCCTTCC				0.745			
Sgui-007	F: CCATTTAGAGGTTGGGGTGC	$(GT)_{22}$	227–229	2	0.040	2	0.500	0.400
BV693811	R: GGGATTCCATAGTGACAAGC				0.115			
Sgui-010	F: ATTAGCCACAGACAAGATCG	$(CA)_{31}$	283–289	4	0.345	1	—	—
BV693812	R: CATGGGATTTCTGGAAGCC				0.471		0.800	0.800
Sgui-011	F: ACAGAGAAGCAAGTGGGAAACC	$(GT)_{26}$	398–446	14	0.567	5		
BV693813	R: TTCCCCGCCACTAAGATTCC				0.795			
Sgui-014	F: TCCAGTAGGGTTTTCTGTTGC	$(GT)_{28}$	244–256	4	0.161	1	—	—
BV693814	R: GAATGTGGGCACTTCTTCCC				0.439			
Sgui-016	F: TTCTCTGGGCAAACACTGC	$(GT)_{28}$	158–162	2	0.095	3	0.556	0.464
BV693815	R: CATTATTGCCGAACTGATGC				0.093			
Sgui-017	F: GTGGTGGAGTAGAGGATAGG	$(CA)_{22}$	150–166	6	0.704	2	0.200	0.190
BV693816	R: ACATTGGGCTTCAACGCACG				0.818			
Sgui-018	F: CTGGAAAAAGAGTAGTTGGC	$(GT)_{29}$	234–252	10	0.704	6	0.500	0.717
BV693817	R: GTGCAAGACCTCAAAATCC				0.782			

*Allele sizes discounting the tailed extension of the primers.

disequilibrium and deviations from expected HWE conditions were not detected ($P > 0.05$, after sequential Bonferroni corrections, Rice 1989). However, six loci (Sgui-002, 003, 004, 006, 011 and 014) had almost significant heterozygote deficits, probably due to Wahlund effect since samples were collected from two distinct areas. Four loci (Sgui-004, 007, 014 and 016) showed occasional amplification failure and might suffer from null alleles. In *S. fluviatilis*, two loci failed to amplify, and Sgui-010 and 014 were monomorphic. Despite this and the lower numbers of alleles found at the remaining eight loci, possibly due to the limited sample size, levels of heterozygosity were comparable to those observed in *S. guianensis* (Table 1).

These markers are currently being used to analyse the population structure of both tucuxi species and clearly have applicability to study the social structure of tucuxi dolphins. Moreover, these loci probably are better candidates for testing in other related dolphin species such as *Sousa* dolphins and *Steno bredanensis* than primers for loci that were isolated from more distantly related delphinids.

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