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TECHNICAL NOTE

# Isolation and characterization of polymorphic microsatellite markers from the spotted eagle ray (*Aetobatus narinari*)

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**Abstract** Ten polymorphic microsatellites were isolated from the spotted eagle ray using an enrichment protocol. Primers were designed and tested on 30 individual samples collected in eastern Gulf of Mexico waters off Sarasota, FL. The number of alleles for these loci ranged from 2 to 19 and observed heterozygosities ranged from 0.345 to 0.966. All loci conformed to Hardy–Weinberg and linkage equilibrium. These markers are the first published microsatellites from *A. narinari* and will be valuable to studies investigating the population structure and conservation genetics of this near threatened species.

**Keywords** Myliobatidae · *Aetobatus* · Spotted eagle ray · Microsatellites

Spotted eagle rays are large, mainly coastal, myliobatid rays with a reported circumglobal distribution in tropical and warm temperate waters. This species is listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species as near threatened globally with a decreasing population trend (IUCN 2006 Assessment, Kyne et al. 2006). Maintaining a mainly inshore and coastal habitat range makes spotted eagle rays vulnerable to the impact of many fisheries. In the U.S., there are records to support the prevalence of this species as bycatch in Atlantic shark fisheries (Trent et al. 1997, Morgan et al. 2010) and in the northern Gulf of Mexico shrimp trawl industry (Shepherd and Myers 2005). To date, little is known about the population structure of spotted eagle rays. Here, we develop a novel set of microsatellite markers that will be used to examine population structure in this species.

Microsatellite markers were developed using an enrichment protocol (Glenn and Schable 2005). Two restriction digestions were performed on approximately 4 mg of genomic DNA (gDNA) from one individual. The first digestion employed Eco53KI and XmnI while the second used DpnI and XmnI. SuperSNX24 linkers were ligated onto the ends of gDNA fragments and served as priming sites for subsequent polymerase chain reactions. Biotinylated tetranucleotide [$(AAAG)_8$, $(AAAC)_6$, $(ACCT)_6$, $(AGAT)_8$, and $(ACAT)_8$] and trinucleotide probes [$(AAC)_6$, $(AAG)_8$, $(ACT)_{12}$, $(ATC)_8$, $(AAT)_{12}$ and $(ACG)_6$] were hybridized to gDNA from the Eco53KI digest while dinucleotide [$(TG)_{12}$ and $(TC)_{12}$] and tetranucleotide probes [$(AACC)_8$, $(AAGG)_8$, $(ACTC)_6$, $(ACAG)_6$, $(AGAT)_8$ and $(ACAT)_8$] were hybridized to the DpnI digest. The biotinylated probe-gDNA complex was added to steptavidin-coated magnetic beads (Dynabeads® M-280, Invitrogen). This mixture was washed twice with 2xSSC, 0.1% SDS and four times with 1xSSC, 0.1% SDS at 52°C. For the final two washes, the mixture was incubated for 1 min in a 52°C water bath. After the last wash, enriched fragments were removed from the biotinylated probe by denaturing at 95°C and precipitated with 95% ethanol and 3 M sodium acetate. To increase the amount of enriched fragments, a "recovery" PCR was performed in a 25 µl reaction containing 1× PCR buffer

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(10 mM Tris–HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 10× BSA, 0.16 mM of each dNTP, 0.52 μM of the Super-SNX24 forward primer, 1U *Taq* DNA polymerase, and approximately 25 ng enriched gDNA fragments. Thermal cycling was as follows: 95°C for 2 min, followed by 25 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 90 s, with a final elongation step of 72°C for 30 min. PCR fragments were cloned using the TOPO-TA Cloning® kit following the manufacturer's protocol (Invitrogen). Bacterial colonies containing a vector with gDNA were used as a template for subsequent PCR in a 25 μl reaction containing 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 10× BSA, 0.12 mM of each dNTP, 0.25 μM of the M13 primers, and 1U *Taq* DNA polymerase. Thermal cycling was as follows: an initial denaturing step of 95°C for 7 min, followed by 35 cycles of 95°C for 20 s, 50°C for 20 s, and 72°C for 90 s. PCR products were cleaned using Exonuclease I and Shrimp Alkaline Phosphatase according to the manufacturer's protocol (USB Corporation). DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were precipitated with ethanol and 125 mM EDTA and run on an ABI 3730 DNA Analyzer. Primers flanking core microsatellite repeats were developed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR reactions (10 μl) consisted of 1 x PCR Buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.6 μM each primer, 200 μM each dNTP, 0.6 U *Taq* and approximately 10 ng gDNA. Thermal cycling was as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, $T_a$ for 15 s (see Table 1) and 72°C for 15 s. For two of the loci, SER44 and SER61, a 30 min final extension was included to help reduce stutter peak heights. PCR products were mixed with the GSLIZ600 size standard (Applied Biosystems) and formamide and run on an ABI3130*xl* DNA Analyzer. Fragment analysis and genotyping were performed using Genemapper version 4.0 (Applied Biosystems).

All loci were polymorphic with the number of alleles ranging from 2 to 19. Observed and expected heterozygosities were calculated using ARLEQUIN (Schneider et al. 2000) and tests for linkage disequilibrium and Hardy–Weinberg equilibrium were carried out using GENEPOP (Raymond and Rousset 1995). Observed heterozygosities ranged from 0.345 to 0.966 (Table 1). None of the loci were found to be out of Hardy–Weinberg equilibrium and no significant linkage disequilibrium was detected for any pair.

Given the near threatened status of *Aetobatus narinari*, this suite of genetic markers will be useful to studies investigating the population structure and conservation genetics of this species.

**Table 1** Repeat information, primer sequences, annealing temperatures, and variability of microsatellite loci isolated from *A. narinari*

| Locus | Repeat | Primer sequence 5′–3′ (label) | $T_a$ (°C) | $N$ | $A$ | Size range | $H_O$ | $H_E$ | GenBank Accession no. |
|---|---|---|---|---|---|---|---|---|---|
| SER27 | $(ATAG)_{35}$ | F: CAATCGGTGAATGAATGAAGAA (HEX) | 54 | 30 | 17 | 249–333 | 0.964 | 0.927 | JF501638 |
| | | R: CCAAATGAAAAACAAATCACATTC | | | | | | | |
| SER44 | $(AC)_{15}$ | F: TAAGGCACTGGCACATAGCA (FAM) | 55 | 30 | 3 | 162–165 | 0.586 | 0.569 | JF501639 |
| | | R: CTGGATTTCCATGTGCAGAA | | | | | | | |
| SER61 | $(AC)_{28}$ | F: CCCAAAATCCCTGTTGGATA (FAM) | 54 | 30 | 19 | 164–210 | 0.963 | 0.939 | JF501640 |
| | | R: TTCCAGCTGATAAAGTGAGTTGA | | | | | | | |
| SER92 | $(AC)_{22}$ | F: TACATGCACACGCACACATT (HEX) | 54 | 30 | 9 | 121–147 | 0.923 | 0.883 | JF501641 |
| | | R: TGAATTTCAAAAACAAACAGCAA | | | | | | | |
| SER159 | $(TAAA)_7(ATAG)_{16}$ | F: CAGGGAGTGTGAAGCACAGA (HEX) | 56 | 30 | 10 | 210–254 | 0.827 | 0.837 | JF501642 |
| | | R: GAGTGCTGATTGAGCCCCTA | | | | | | | |
| SER243 | $(ACAT)_6$ | F: TTTTCTCGCAGACATTCACAG | 55 | 30 | 2 | 135–139 | 0.483 | 0.460 | JF501643 |
| | | R: CCAATAGGCACACTTCCAAA (HEX) | | | | | | | |
| SER263 | $(AC)_{16}$ | F: CCCCTGAGATTCACTTTCTTG (FAM) | 53 | 30 | 7 | 213–239 | 0.556 | 0.511 | JF501644 |
| | | R: CACAATCACTGGACTCCCTCT | | | | | | | |
| SER358 | $(TATC)_7$ | F: CCACAGTGGTTCTTTGCATC (FAM) | 52 | 30 | 2 | 192–196 | 0.345 | 0.334 | JF501645 |
| | | R: AAGGGTACTTGGTTATGCCTGT | | | | | | | |
| SER453 | $(TAGA)_{36}$ | F: CAACTTGTGCAAACACAAAACA (HEX) | 54 | 30 | 19 | 222–306 | 0.966 | 0.948 | JF501646 |
| | | R: TGAAATCGCTTGACTGCAAA | | | | | | | |
| SER476 | $(TATC)_{40}$ TATT$(TATC)_{13}$ | F: GCTTCCACAACCAATGGACT (HEX) | 53 | 30 | 17 | 201–321 | 0.931 | 0.910 | JF501647 |
| | | R: CCAACTTGAGCATTCAAGCA | | | | | | | |

Annealing temperature ($T_a$), sample size ($N$), number of alleles ($A$), observed heterozygosity ($H_O$), expected heterozygosity ($H_E$)

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