Assessing genetic structure of thornback ray, Raja clavata

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CHAPTER 2

Characterization and isolation of DNA microsatellite primers in *Raja clavata* L. (thornback ray, Rajidae)

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Published in *Molecular Ecology Notes* (2005) **5**: 427-429

Abstract

The thornback ray, *Raja clavata*, is an elasmobranch (cartilaginous fish). Since the 1950s, its stock has severely declined. In order to investigate the genetic population structure, we developed microsatellite loci. The five reported here have 8-48 alleles/locus and display an observed heterozygosity from 0.32 to 0.98 with no deviation from Hardy-Weinberg equilibrium. In the test panel of 122 individuals from three populations, there were no null alleles, stuttering, large allele drop-out or linkage disequilibrium detected.

The thornback ray, *Raja clavata*, has a widespread distribution from the Faeroe Islands to South Africa including the Mediterranean and Black Seas (Stehmann & Bürkel 1986). The species has the highest commercial value among rays and skates, and is part of the by-catch associated with demersal fisheries. Prior to 1950s, thornbacks occurred throughout the central/southern North Sea, but are now restricted in localized areas along the English east coast (Walker & Heessen 1996; Walker & Hislop 1998). Because thornbacks are characterized by slow growth rate, late maturity, low fecundity, and no pelagic larval phase, the strong and rapid decline of thornback stocks has led to a serious concern about their sustainability, and the possible replenishment of depleted areas (Dulvy & Reynolds 2002). Here, we develop and characterize five polymorphic microsatellite loci for population differentiation studies.

Muscle tissue was sampled from one North Sea individual and immediately frozen in liquid Nitrogen. DNA extraction was performed using DNeasy tissue kit (Qiagen). Two libraries enriched for CA_n and GA_n motifs were developed using a hybrid-capture method based on biotin-labelled microsatellite motifs attached to magnetic beads (Zeller & Reusch 2004). Candidate clones (n=424) were picked and sequenced with M13-primers using the Big-dye 3.1-sequencing kit (Applied Biosystems) on ABI-3100 or ABI-377 automatic sequencer (Applied Biosystems). Primer sets were designed for 37 candidate loci using Primer3 software (Rozen & Skaletski 2000) from which five polymorphic loci were subsequently identified. Primers were then tested on 122 individuals from three locations (North Sea, *N*=54; English Channel, *N*=43; and Irish Sea, *N*=25). DNA from the sample-panel was extracted using a CTAB protocol (Hoarau *et al.* 2002) or a silica-based protocol (Elphinstone *et al.* 2003). All polymerase chain reactions were performed in a 10 µL volume containing 1-3µL of extracted DNA (<1ng/µL), 1X reaction Buffer (Promega), 0.2mM of each dNTP, 0.25U *Taq* DNA polymerase (Promega) and $MgCl_2$ and primer concentrations as shown on Table 1. For each primer set, the forward primer was fluorescently end-labelled. PCR amplifications were performed either on PTC-100™ thermocycler (MJ Research, Inc.) or Mastercycler gradient cycler (Eppendorf). Cycling conditions were: initial denaturation for 1 min at 94°C; followed by four cycles of denaturation for 1 min at 94°C, annealing at 52-60°C for 1 min, and extension at 72°C for 30 s, then between 30 and 35 cycles of denaturation at 94°C for 20 s, annealing at 52-60°C for 15 s and extension at 72°C for 12 s, and a final extension step at 72°C for 10 min. PCR products were separated on a 6% polyacrylamide gel using an ABI Prism-377 automatic sequencer. Allele sizes were determined using an internal lane standard and GENESCAN™ software.

The software MICRO-CHECKER 2.2.1 (Van Oosterhout *et al.* 2004) was used to check for null alleles, stuttering and large allele drop out for all loci. Linkage disequilibrium was tested using the LinkDis procedure (Black & Krafsur 1985), and observed (H_{obs}) and non-biased expected (H_{exp}) heterozygosities (Nei 1978) estimated with the software GENETIX 4.05 (Belkhir *et al.* 2004). Significance was tested against 3000 permutations. Loci are characterized in Table 1.

The five microsatellite loci reported here showed no evidence for null alleles, stuttering, large allele drop out or linkage desequilibrium after sequential Bonferroni corrections for multiple tests (Rice 1989) (P>0.05). No deviations from Hardy Weinberg equilibrium were detected after sequential Bonferroni corrections.

Table 1 Primer sequences and characterization of five microsatellite loci for *Raja clavata* tested on 3 locations (North Sea, English Channel and Irish Sea

Locus (Accession n°)	Repeat motif	Primer sequence	T_A (°C)	C_{pr} (µM)	$MgCl_2$ (mM)	Size range (bp)	N_A	North Sea (N=54) $H_{exp}(H_{obs})$	English Channel (N=43) $H_{exp}(H_{obs})$	Irish Sea (N=25) $H_{exp}(H_{obs})$
Rc-B3 (AY822712)	$(GA)_{17}T(GA)$	F:HEX CTGGCTGGAGTTTTTGAAGG R:CACCACCGTACCATTGATGA	58	0.4	1.5	213-223	9	0.604 (0.54)	0.689 (0.71)	0.629 (0.65)
Rc-B4 (AY822713)	$(GA)_{15}(GGA)_5$ $(GA)_{21}$	F:FAM CACAGATTTACTCAGGGCAACA R:TGGTATCAGCCACTTGGACA	55	0.14	2	151-228	31	0.940 (0.87)	0.938 (0.95)	0.942 (0.83)
Rc-B6 (AY822713)	$(GA)_{17}(GGGA)_2GG$ $G(G(GA)_9GGGAGT$ $GG(GA)_6$	F:FAM CCATGATGCATTGAATCGAA R:GGCTTTCTTCACTGCCTGCT	52	0.5	2	170-291	48	0.921 (0.92)	0.911 (0.98)	0.935 (0.88)
Rc-E9 (AY822715)	$(GA)_{14}(GGGA)_2$ $(GAGGGA)_2GGG$ $(AG)_{13}$	F:HEX TGCCAAGTACTGCAGGTGTC R:TGAAGGCAGAGACATGATGC	52	0.5	2	256-301	8	0.385 (0.32)	0.363 (0.33)	0.367 (0.33)
Rc-G2 (AY822716	$(CT)_{16}GTG(CT)_3$	F:FAM GCTACAAGCATGAGCCGATT R:CAAAGCCTATGTGGTTTGATTG	60	0.66	3	170-192	9	0.598 (0.76)	0.641 (0.51)	0.660 (0.44)

N=sample size, T_A=Annealing temperature, C_{pr}=Primer concentration, N_A=Total number of alleles per locus, H_{exp}=Expected heterozygosity and H_{obs}=Observed heterozygosity

Acknowledgements

We thank Silke Carstensen for her help in the library construction, Felipe Alberto for useful advice on the protocol, Jean-Paul Delpech (IFREMER Boulogne sur mer France; CGFS survey), Jim Ellis (CEFAS, UK, CORY13/03 survey) and Henk Heessen (RIVO, Netherlands, IBTS survey) for sampling. This project was supported by NWO-*Prioriteit programma* "Sustainable Use of Marine Natural Resources" Project Nr. 885-10-311.

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