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SHORT COMMUNICATION

Isolation and characterization of 29 and 19 microsatellite loci from two deep-sea luminous lanternsharks, *Etmopterus spinax* and *Etmopterus molleri* (Squaliformes, Etmopteridae)

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Abstract

Etmopterus spinax (Linnaeus, 1758) and *Etmopterus molleri* (Whitley, 1939) are two bioluminescent deep-sea sharks, usually caught in large numbers as bycatch by deep-water fisheries. Yet, no study has ever involved population status of these two species using genetic tools. In order to investigate population genetic structure, diversity and connectivity of these two lanternsharks, 29 and 19 microsatellite loci were isolated from *E. spinax* DNA library for *E. spinax* and *E. molleri*, respectively. These loci were tested on 32 *E. spinax* individuals from the North Sea and seven *E. molleri* from the East China Sea. The number of alleles per locus ranged from 2 to 13. The observed heterozygosity ranged from 0.031 to 0.839 for *E. spinax* and from 0.000 to 1.000 for *E. molleri*, while the expected heterozygosity ranged from 0.031 to 0.903 and from 0.143 to 0.821, respectively. Almost all loci (24 and 16, respectively) were at Hardy–Weinberg equilibrium for both species and no linkage disequilibrium among loci was detected. These loci represent useful tools to better understand the population structure of these two species. Besides, they could also be suitable for other lanternsharks in general, as these latter remain largely understudied, specially in terms of understanding the basic science that will serve into their conservation.

Keywords *Etmopterus spinax* · *Etmopterus molleri* · Squaliformes · Etmopteridae · Microsatellites · Deep-sea lanternsharks

Introduction

Etmopteridae represents one of the two known bioluminescent deep-sea shark families [1]. These lanternsharks are globally distributed and are represented by at least 43 species [2]. Little is known about their population genetic diversity and structure, therefore their population health status. Few studies aimed at resolving the phylogeny of Etmopteridae based on mitochondrial and nuclear markers [e.g. 2–4]. However, no study has addressed population genetics of deep-sea luminous sharks using neutral nuclear markers such as microsatellites while providing useful information on evolutionary processes, population dynamics but also reproductive strategies. Here we focus on two closely related species of deep-sea luminous sharks: (1) *Etmopterus spinax* (Linnaeus, 1758), commonly found, inhabiting the Eastern Atlantic coast from the North of Norway to the Gabon coast and in the Mediterranean Sea, from 45 to 785 m depth [5, 6] and (2) *Etmopterus molleri* (Whitley, 1939), only known from Eastern Australia, New-Zealand, Taïwan and Japan, from 238 to 655 m depth [7]. Both are aplacental viviparous

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sharks. While stock assessments by longlines and trawling catches give information on the population status of *E. spinax* [8–10], nothing is known about the status of *E. molleri*. Lanternsharks are usually caught in large numbers as bycatch in deep-water fisheries, but directly discarded since they have no current commercial value.

Here, we used a DNA library extracted from *E. spinax* samples. We then isolated 29 and 19 microsatellite markers for *E. spinax* and *E. molleri*, respectively. Among them, 12 markers were common to both species. These genetic markers will help to evaluate the population status, dynamics and connectivity of these two species.

Materials and methods

Microsatellite library development and primer selection

DNA library preparation and sequencing were performed on *E. spinax* by Beijing Genomics Institute (BGI, Hong Kong), using Illumina sequencing system. Microsatellite library development was then performed by BGI using MISA software (MIcroSAtellite identification tool) [11] implemented with Primer3 [12, 13]. A total of 9216 sequences containing microsatellite motif were identified and primer pairs were in silico designed for each microsatellite motif, using the pipe 3 of QDD 3.1 [14] (see Online Resource 1 for the parameters used). Markers with a QDD design note equal to "A" were retained and checked for possible primer-dimers formation with Multiple Primer Analyzer on the web (ThermoFisher Scientific). Then 96 markers were selected for amplification test depending on the motif [(AT) and (CG) were removed as they can form hairpins], the number of repeats (≥ 5), the product size (≥ 110 bp), the primer alignment score to the sequence (≤ 5), the primer distance from the target region (≥ 10 bp) and the absence of primer-dimer.

The 96 primer pairs were tested for amplification on eight individuals of *E. spinax* and seven of *E. molleri*. Total genomic DNA was extracted from a small piece of muscle (10–20 mg) using DNeasy Blood & Tissue kit (Qiagen™) following the manufacturer's protocol. Forward primers were indirectly fluorochrome labelled (6-FAM) by adding a universal 19-bp M13 tail at their 5′-end (5′-CACGAC GTTGTAAAACGAC-3′) [15]. PCRs were then performed with Veriti™ Thermal Cyclers (Applied Biosystems), in a total volume of 10 µL with 1X of MasterMix Applied (Applied Biosystems), 0.025 µM of forward primer tagged with the M13 tail, 0.25 µM of reverse primer, 0.25 µM of fluorescent dyed M13 tail and 2 ng μL^{-1} of genomic DNA. The thermocycling program was the following: 94 °C for 5 min + 7 × (94 °C for 30 s, 62 °C [− 1 °C at each cycle] for 30 s, 72 °C for 30 s) + 30 × (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) + 8 × (94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) + 72 °C for 5 min. PCR products were genotyped using an ABI3730XL sequencer (Applied Biosystems) at the Plateforme Gentyane (INRA, Clermont-Ferrand, France). Allelic sizes were determined with GeneMapper 4.0 (Applied Biosystems), using an internal size standard (Genescan LIZ-500, Applied Biosystems). Primer pairs were selected for further development when (1) they did amplify at least five and six individuals for *E. molleri* and *E. spinax*, respectively, (2) they did not amplify multiple fragments, and (3) genotype scoring was deemed reliable.

Polymorphism testing

The characterization of the loci selected for *E. spinax* was performed by genotyping 32 *E. spinax* individuals from the North Sea, near the Norwegian coast (60°43′03″N; 03°18′58″E) while those for *E. molleri* were amplified on the same seven *E. molleri* individuals previously used for amplification tests, sampled in the East China Sea, near the Sesoko Island coast, Okinawa, Japan (26°28′94″N; 127°41′20″E) and their juveniles ($N = 35$; data not used for further analyses as the individuals were related). Genotyping was performed using the same protocol as described previously. PCR products were multiplexed post-PCR in six panels for each species according to their amplified fragment sizes and dyes (6-FAM, VIC, NED) were assigned accordingly to each locus (Online Resource 2). Panels were made independently for each species, even for the cross-amplifying loci, to maximize efficiency and minimize costs. Allelic sizes were determined with GeneMapper 4.0 (Applied Biosystems), as previously.

Data analysis

For each species and for each locus, the number of alleles (*Na*), the observed and expected heterozygosities (*Ho* and *He*, respectively) and the inbreeding coefficient (F_{IS}) [16] were calculated with FSTAT 2.9.3.2 [17]. Departures from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested with GenePop 4.7.0 [18, 19], using False Discovery Rate (FDR) correction for multiple testing [20]. Null alleles and other potential technical biases were tested using Micro-Checker 2.2.3 [21].

Results and discussion

Among the 96 initially tested primer pairs, following our criteria, 12 loci were selected for both species (from Et01 to Et12 in Table 1). Additionally, 17 loci were selected specifically for *E. spinax* (Et13-Et29; Table 1) and seven for *E. molleri* (Et30–Et36; Table 1). Thus, 29 and 19 microsatellite

Table 1 Microsatellite loci developed for *Etmopterus spinax* and *Etmopterus molleri*

Locus	Primer sequence (5′-3′)	Motif	Size (bp)	Pop	*N*	*Na*	*Ho*	*He*	F_{IS}	*r*	GenBank accession no.
Etmopterus spinax/molleri common loci											
Et01	F: M13-CAGATATAAGTTTCCATCGCCTG	$(AC)_6$	143–161	S	32	2	0.031	0.031	-0.008^{NS}	−0.016	MH990863
	R: CAAACACAACTGATCCTGAGACA			M	7	4	0.429	0.738	0.419^{NS}	0.182	
Et02	F: M13-TAAATCCCAACGCTGACTAACAT	$(AC)_6$	135–143	S	31	2	0.097	0.094	-0.030^{NS}	−0.050	MH990864
	R: AGACAAGGAATTTGTTGGACTCA			M	6	4	1.000	0.667	-0.499^{NS}	−0.358	
Et03	F: M13-ACTCTAGCCGTGCTGCTTTCT	$(AC)_6$	154–162	S	32	3	0.375	0.389	0.036^{NS}	0.005	MH990865
	R: GAGCTTCCTGGCTTAGTACATCA			M	7	3	0.286	0.500	0.429*	0.225	
Et04	F: M13-TGAAAGGCGTAGCATTGAATAAT	$(AC)_7$	163–173	S	31	4	0.129	0.292	0.558**	**0.195**	MH990866
	R: GCCTAACTCCATATACCAGCCTT			M	7	3	0.286	0.274	-0.043^{NS}	−0.148	
Et05	F: M13-TTTAGACTTTGTACTCCAGCACG	$(AC)_7$	172–184	S	27	5	0.519	0.746	0.305**	**0.144**	MH990867
	R: GGCAGAGACTTTGTGATAGGAAA			M	7	3	0.143	0.405	0.647^{NS}	0.230	
Et06	F: M13-TCACGTCATTGCTATTTGCTAGA	$(AG)_7$	172–180	S	32	4	0.406	0.379	-0.072^{NS}	−0.051	MH990868
	R: ATGAGATTACCAAATGTTGCCTG			M	7	2	0.000	0.571	1.000*	**0.411**	
Et07	F: M13-AAGTGGCCTTTGTGTTAGTTTGA	$(AC)_8$	172–214	S	31	13	0.839	0.903	0.071^{NS}	0.028	MH990869
	R: AAGTTGCACTCATCCTCAGCTAC			M	7	5	0.714	0.821	0.130^{NS}	0.039	
Et08	F: M13-TATTCACCAATGCAATAACCACA	$(AC)_9$	161–171	S	32	5	0.625	0.661	0.054^{NS}	0.023	MH990870
	R: CAGCAATGATTTCTTCACTCACA			M	7	2	0.143	0.143	0.001^{NS}	−0.074	
Et09	F: M13-CTCCATCCCATTATTCAGCATT	$(AG)_{11}$	153–165	S	31	4	0.484	0.496	0.024^{NS}	0.005	MH990871
	R: AAAGCTGGTTTGAACAGTTTCAC			M	7	4	0.571	0.488	-0.171^{NS}	−0.303	
Et10	F: M13-GAACTCTTTCCAGTCGAACTCAG	$(AAG)_5$	147–156	S	32	3	0.250	0.279	0.104^{NS}	0.086	MH990872
	R: CACACCTGGGCTAATAACTCTCA			M	7	2	0.143	0.143	0.001^{NS}	−0.074	
Et11	F: M13-AGCGTTTCAGTAGCTTCTGCTC	$(AGG)_5$	150–162	S	31	2	0.065	0.063	-0.024^{NS}	−0.033	MH990873
	R: TCTCCCACCACATAGACTTAACC			M	7	3	0.571	0.583	0.020^{NS}	−0.042	
Et12	F: M13-GTGAACAGGACAACGGCAC	$(AGG)_6$	144–153	S	31	4	0.645	0.520	-0.241^{NS}	−0.189	MH990874
	R: GTCTCAAGAGGCTCTGTCTTGC			M	7	2	0.143	0.143	0.001^{NS}	−0.074	
Etmopterus spinax specific loci											
Et13	F: M13-CCGTCAACATCCAATTTCTAAAG	$(AC)_6$	130–140	S	32	5	0.438	0.658	0.335*	**0.165**	MH990875
	R: CATTCCACTGTTGACCCAAGTAT										
Et14	F: M13-ATCCTACCTCACCATAAAGGCTC	$(AC)_6$	140–154	S	31	5	0.548	0.573	0.043^{NS}	0.024	MH990876
	R: AATGTAGAGCCCTCCACAGAAG										
Et15	F: M13-CCACCATTCTTCCCTTTATTACC	$(AC)_6$	160–172	S	32	6	0.688	0.635	-0.083^{NS}	−0.052	MH990877
	R: CAAATAGCCCACTGAAAGTCAAT										
Et16	F: M13-TGCAGCTTGTATTATGATGTTGG	$(AC)_6$	174–176	S	32	2	0.281	0.451	0.376*	0.162	MH990878
	R: CGGAACAGTGAACCACTTAGTTT										
Et17	F: M13-CAGCTCTGTACATCGAGGAAAGT	$(AG)_6$	170–180	S	31	5	0.548	0.498	-0.101^{NS}	−0.068	MH990879
	R: AAACAGCACTGTGCCAGAGATAG										

Table 1 (continued)

Locus	Primer sequence (5′-3′)	Motif	Size (bp)	Pop	*N*	*Na*	*Ho*	*He*	F_{IS}	*r*	GenBank accession no.
Et18	F: M13-ACGGATTAAACGACAATCACACT R: GATCGAGAAACTCCTGAACTACG	$(AG)_6$	177–183	S	32	4	0.406	0.546	0.256^{NS}	0.103	MH990880
Et19	F: M13-TGCTGATGTTTCAATATCTCGTG R: GTCAGAAGCCAATATAATGCGAC	$(AG)_7$	148–150	S	32	2	0.188	0.350	0.464*	**0.183**	MH990881
Et20	F: M13-CAAACCACGGGTTTAAGTAACAG R: CAGTGGATAATTGCTGCCTAGTT	$(AG)_7$	174–180	S	32	4	0.438	0.510	0.142^{NS}	0.070	MH990882
Et21	F: M13-TCGAAGAGTGCAAGTGTAGTGAA R: TAATTTGCATCTTGTGCAGACAC	$(AC)_8$	129–131	S	32	2	0.250	0.222	-0.126^{NS}	−0.134	MH990883
Et22	F: M13-GTTCCCTTCTCCGTCAATTAGTG R: ACTCGAAAGCAGCAAATTGTAAA	$(AG)_8$	150–158	S	32	3	0.188	0.175	-0.071^{NS}	−0.097	MH990884
Et23	F: M13-GGAGAGCTGAACAGAAGTTGAAA R: GTACTGAACCCGATCAGACAGAC	$(AG)_9$	178–180	S	32	2	0.156	0.146	-0.070^{NS}	−0.081	MH990885
Et24	F: M13-CGAGTACAAACATGAGTGATCGT R: CAAATCGCTTGGACATTACAACT	$(AAC)_5$	176–182	S	31	3	0.452	0.480	0.059^{NS}	0.044	MH990886
Et25	F: M13-CAGATGATGAATAGCCCGAGAT R: AAGTCCGGCATAACTACCAGAG	$(AGG)_5$	153–156	S	32	2	0.031	0.031	-0.008^{NS}	−0.016	MH990887
Et26	F: M13-AGAAGGAGTCATCCGATAAGGTG R: GAGAGGTCAGTGCGAAGAGTG	$(AGG)_5$	171–186	S	32	6	0.656	0.685	0.042^{NS}	0.017	MH990888
Et27	F: M13-ACTCCTGGATCAGGTCTTCGT R: AGCTGACGAACCTGAAGAAGTT	$(AGG)_5$	175–178	S	32	2	0.063	0.061	-0.025^{NS}	−0.032	MH990889
Et28	F: M13-CACAACCAGCAACAGGTCAG R: AGTTCTCCAAACTCTGCCTCCT	$(CCG)_5$	126–147	S	32	7	0.688	0.678	-0.014^{NS}	−0.012	MH990890
Et29	F: M13-AGACTCTTGGTCGGAGTATGATG R: TGATGGTCACCTCACTGTTACTG	$(AGG)_6$	173–179	S	32	3	0.156	0.148	-0.056^{NS}	−0.080	MH990891
Etmopterus molleri specific loci											
Et30	F: M13-TTTACAGTCAGCTCCTCTCCATC R: ATTATTTGGATGGTTGTGGTGTC	$(AC)_6$	174–176	M	7	2	0.286	0.262	-0.091^{NS}	−0.155	MH990892
Et31	F: M13-TCTTGGACCTTAGATGGCAAATA R: AACTTTCCCAACAAGACAGTCAC	$(AG)_6$	131–133	M	7	2	0.143	0.143	0.001^{NS}	−0.074	MH990893
Et32	F: M13-TGTTGGGATTTCTAACTGCTTTC R: AACATTTGAAGAAACTGGTGCTG	$(AG)_6$	155–159	M	7	3	0.429	0.381	-0.125^{NS}	−0.229	MH990894
Et33	F: M13-AGTAAGCACACTTGTGCACCTTT R: ATAGAACTGTTCCGCGGTGAT	$(AG)_6$	149–153	M	7	3	1.000	0.571	−0.751*	−0.696	MH990895
Et34	F: M13-CCATAAGCCGTTTACAACTCAAC R: AGTGGCAACAGTGAGCTGAAG	$(AG)_6$	167–171	M	7	2	0.429	0.548	0.218^{NS}	0.069	MH990896

Table 1 (continued)

Locus	Primer sequence (5'-3')	Motif	Size (bp)	Pop	*N*	*Na*	*Ho*	*He*	F_{IS}	*r*	GenBank accession no.
Et35	F: M13-TAAAGTCCGTCAATCTGAGCAAT R: CTGCAATTCAGCATATCAGAACA	$(AC)_7$	169–171	M	7	2	0.286	0.262	-0.091^{NS}	−0.155	MH990897
Et36	F: M13-AATCTTACAAGGCATCCATAGCA R: TTCGGTTTAAGCTAATGGTTGAG	$(AG)_7$	138–148	M	7	2	0.143	0.143	0.001^{NS}	−0.074	MH990898

Null allele frequencies (*r*) [21] are issued from Micro-Checker 2.2.3 and presence is indicated by values in bold

Accession Numbers Primer sequences are deposited on GenBank with accession numbers from MH990863 to MH990898

For each locus, are indicated the primer sequences (F: forward; R: reverse), the repeat motif, the size range (specific size ranges for the common loci to both species are indicated in Online Resource 2) and the GenBank accession number. The number of amplified individuals (*N*; over 32 for *E. spinax* and seven for *E. molleri*), the number of alleles (*Na*), the observed and expected heterozygosities (*Ho* and *He*, respectively) and the inbreeding coefficient (F_{IS}) [16] are calculated for each locus in each population (Pop; S: *E. spinax*; M: *E. molleri*). Deviation from Hardy–Weinberg equilibrium is indicated: ^{NS}: non-significant; $^{*}p < 0.05$; $^{**}p < 0.01$

loci were selected and characterized for *E. spinax* and *E. molleri*, respectively.

All of these loci were polymorphic (i.e. at least one bi-allelic genotype different from others) in both species, with 2 to 13 alleles per locus for *E. spinax* and 2 to 5 for *E. molleri* (Table 1). Observed heterozygosity (*Ho*) ranged from 0.031 to 0.839 for *E. spinax* and from 0.000 to 1.000 for *E. molleri*, while expected heterozygosity (*He*) ranged from 0.031 to 0.903 and from 0.143 to 0.821, respectively (Table 1). For *E. spinax*, 24 over 29 loci were at Hardy–Weinberg equilibrium. All the other loci showed significant heterozygote deficiency (F_{IS} ranging from 0.305** to 0.558**). For *E. molleri*, 16 loci were at Hardy–Weinberg equilibrium. Among the three other loci, two showed significant heterozygote deficiency (Et03: $F_{IS} = 0.429$*; Et06: $F_{IS} = 1.000$*) and one showed significant heterozygote excess (Et33: $F_{IS} = -0.751$*). These heterozygotes deficiencies are potentially due to the presence of null alleles, which were detected in four *E. spinax* loci (Et04, Et05, Et13 and Et19) and only in one *E. molleri* locus (Et06), or to the small sample size for *E. molleri* (seven adult individuals). However, their frequency remained low according to Micro-Checker estimations (Table 1). Finally, for both species, no linkage disequilibrium was found among all pairs of loci.

These loci will be very useful in studying the genetic diversity, reproductive strategies and gene flow among populations of both species, but more generally of lanternsharks. Up to now, genetic information remain poorly documented for deep-sea elasmobranchs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval *Etmopterus spinax* individuals were collected in Norway under the experimental fish care permit number 12/14048 obtained at UCLouvain by J.M. *Etmopterus molleri* individuals were fished and handled according to Churaumi aquarium (Okinawa, Japan) husbandry and veterinary rules for fish experimentations. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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