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NOTE

Development and characterization of novel microsatellite loci for an endangered hammerhead shark *Sphyrna lewini* by using shotgun sequencing

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ABSTRACT: The scalloped hammerhead shark *Sphyrna lewini* is a migratory shark species, highly valued commercially for its meat and fins. Although this species has been listed on the CITES Appendix II, its genetic structure in the West Pacific is still little known. In this study, shotgun sequencing technique was used to sequence millions of small fragmented DNA sequences simultaneously. Nine novel polymorphic microsatellite markers were isolated from sequencing reads and then tested on 54 individuals collected from the western North Pacific. The number of alleles detected in each locus ranged from seven to 21. Observed and expected heterozygosity of these loci were from 0.370 to 0.870 and from 0.505 to 0.724, respectively. These nine novel polymorphic loci can further be used to reveal genetic connectivity patterns among *S. lewini* in the West Pacific.

KEY WORDS: Allele frequency, Heterozygosity, Genetic stock, Elasmobranch, Genetic connectivity.

INTRODUCTION

Stock assessment and fishery management of sharks is based on a unit stock assumption. Therefore, it is essential to understand the stock structure or genetic connections before conducting any stock assessment (King, 2007). The scalloped hammerhead, *Sphyrna lewini*, a coastal-pelagic, semi-oceanic shark species, belongs to Carcharhiniformes, characterized by its hammer-like head, called a cephalofoil, and its schooling behavior (Klimley, 1983). Due to the high commercial value of its meat and fins, *S. lewini* has become a targeted species in global shark fisheries. Cardeñosa et al. (2018) reported that *S. lewini* comprises 40.8 % of the fins of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed shark species imported to Hong Kong in 2015. Baum *et al.* (2009) documented that the abundance of *S. lewini* declined 50 – 90 % based on both species-specific estimates and grouped estimates for *Sphyrna* spp. Due to significant decline of this species in several regions, it was included in CITES Appendix II in 2013.

The scalloped hammerhead shark is one of the major shark by-catch in Taiwanese offshore longline fisheries. However, despite the reproductive biology (Chen et al. 1988), age and growth (Chen *et al.*, 1990), and demographic analysis (Liu and Chen, 1999), the stock identification and stock assessment of this species are lacking in the western North Pacific, which may hinder the implementation of conservation and management. In addition, the migratory behavior and ability to travel long distances of *S. lewini* make it difficult to be managed properly (Duncan *et al.*, 2006; Bessudo *et al.*, 2011; Hoyos-Padilla *et al.*, 2014). Therefore, genetic connectivity and stock structure of *S. lewini* in this region are urgently needed. Molecular marker analysis is a cost-effective way to monitor populations of elusive species (Freeland, 2005). Microsatellite or simple sequence repeats (SSR) is a relatively neutral, co-dominant, and highly variable molecular marker. It has been the most widely used molecular marker in recent years when it comes to population level research for marine fishes (Daly-Engel *et al*., 2012; Glover *et al.*, 2011; Takagi, 1999).

Population structure of *Sphyrna lewini* has been widely investigated on a global and regional scale (Quattro et al. 2006, Nance et al. 2011, Daly-Engel *et al.* 2012, Quintanilla *et al.*, 2015). In addition, previous regional studies were only focused on populations of the East Pacific (Quattro *et al.*, 2006; Nance *et al.*, 2011; Quintanilla *et al.*, 2015). For the global study conducted by Daly-Engel *et al.* (2012), they applied 13 loci to reveal the global genetic structure, with only 10 among them were species-specific loci. And 3 out of the 10 loci showed significant heterozygosity deficiency which were excluded for further analyses. Thus, the aim of this study was to isolate novel microsatellite loci from *S. lewini* by using next-generation DNA sequencing (NGS) on the Illumina Miseq platform. Combining novel loci we

Table 2. Primer sequence of *Sphyrna lewini* microsatellite loci. T_a: annealing temperature, N_a: number of alleles per locus, H_O and H_E are observed and averaged expected heterozygosities.

Locus	Motif	Product Size	Primer Sequence	Flurochrome	T_a	N_a	H_O	H_E	P_{HWE}	Access. No.
SL13	$(GT)_{15}$	266	F-CTTGGCGCATCTACAACAGC R-CCAGCATGTACGCACACAAG	TAMRA	60	12	0.481	0.532	0.281	MN137242
SL15	$(TG)_{16}$	186	F-CCTGACAATCAAAATGGCCCA R-CAAAGACGTGCAGGAAACGG	FAM	60	18	0.870	0.724	0.367	MN137244
SL16	$(GT)_8(GA)_{11}$	235	F-AGCAAGAGCCCAGCACATTA R-ACCCACTTAAGGAGCCATCA	FAM	60	7	0.815	0.551	0.208	MN137245
SL17	$(CA)_{15}$	264	F-AGTCAGGGTGGAATGCATGG R-GTGTGTCGGATTGGGCTACA	FAM	60	9	0.759	0.625	0.164	MN137246
SL18	$(CA)_{30}$	152	F-CAGGCCTTCTAACGGTGTGT R-ATGGCATTGTGTACCGCAGA	FAM	60	21	0.778	0.667	0.452	MN137247
SL25	$(CT)_{14}$	226	F-TTCCGCCTGTGTGTGTGAAT R-TTCCAAACAGAGGGCAGTGG	TAMRA	60	10	0.685	0.656	0.158	MN137248
SL26	$(AC)_{14}$	266	F-CCACAGCAGTCAGTTGGAGT R-ATGACAGTGGGTGTGTGCAT	TAMRA	64	10	0.722	0.600	0.416	MN137249
SL27	$(AC)_{14}$	279	F-GCACTTCAAGGGGATTTCACAG R-GAATTGTGCCCAGGTTCAGC	FAM	60	10	0.370	0.505	0.135	MN137250
SL29	$(GT)_{14}$	277	F-TAGTCATGTCAGTGGCGCTG R-TCACGCGAAACAAAGCAGTG	FAM	60	19	0.704	0.662	0.433	MN137251

Table 1. Details of the specimens of *Sphyrna lewini* used in the present study.

Year	Month	Male	Female	Unknown	Coordinates
2016	April	6	6	0	24°34'57.1"N 121°52'09.8"E
2017	April	0	0	6	"
2017	November	5	1	0	"
2018	March	7	11	0	"
2018	April	3	3	0	"
2018	May	0	0	6	"
Total		21	21	12	

developed with published loci, we hope to increase the resolution with more loci use in the future to examine the population connectivity of *S. lewini* in the West Pacific.

MATERIALS AND METHODS

Sample Collection

Muscle samples were collected from 54 *S. lewini* individuals landed at the Nanfangao fish market (24°34'57.1"N, 121°52'09.8"E), northeastern Taiwan (Table 1). These sharks were caught by offshore longline fishing vessels in the waters between Taiwan and Diaoyutai Island, western North Pacific. Muscle samples were preserved in 95% ethanol (ETOH) before being processed. Genomic DNA was extracted using a Tissue & Cell Genomic DNA Purification kit (Biokit Biotechnology Inc., Miaoli County, Taiwan). DNA extracts were eluted in TE buffer and stored in freezers at −20°C.

Microsatellite Development

Extracted DNA was sonicated, and DNA fragments in the range of 300-550 base pairs were selected by DNA gel extraction. These DNA fragments were used to construct a shotgun library (Illumina TruSeq Nano DNA Sample Preparation kits). A Miseq 300 sequencer was used for paired end sequencing, and the raw sequence data went through a series of quality control steps using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Sequences which passed the quality test were assembled with SPAdes v3.10.1 (Bankevich *et al.*, 2012), and the detection of microsatellites was carried out using MISA-Microsatellite identification tool (Thiel, 2003).

Genotyping and analyses

Forward primers were 5' labeled with either FAM or TAMRA fluorescent dye. For each microsatellite marker, a total of 12 μL reaction mixture, containing 30-50 ng template DNA and Master Mix RED (Ampliqon A/S, Denmark) with specific primer pair, was pre-mixed for PCR reaction in a PCR thermocycler. The thermocycling profile starts with an initial denature step at 94°C for 3 min; then 94°C for 30 s, followed by annealing at the appropriate temperature for 30 s for each locus, and an extension step at 72°C for 30 s for 35 cycles, and a final step at 72°C for 2 min. Genotyping was carried out using an ABI 3730XL sequencer (Applied Biosystems) with purified PCR products (Genomics BioSci and Tech).

Alleles were identified and scored with Peak Scanner 2.0 software (Applied Biosystems). GenAlEx 6.5 (Peakall and Smouse, 2012) was used to estimate the number of alleles (N_a), observed heterozygosity (H_O) and expected heterozygosity (H_E). Testing for Hardy - Weinberg equilibrium (HWE) and linkage disequilibrium was carried out using Genepop (Rousset, 2008), and the frequencies of null alleles were calculated using Microchecker (Van Oosterhout *et al.*, 2004) to search for possible evidence of null alleles, and to dropout large alleles.

RESLUTS AND DISCUSSION

In total, 41,812 contigs were obtained after quality control and assembly. One hundred and forty-one loci with perfect SSR were isolated, comprising 125 di-nucleotide loci, one tri-nucleotide locus, six tetra-nucleotide loci, one penta-nucleotide locus, one hexa-nucleotide locus, and seven complex nucleotide loci. The

primers were designed by Primer 3 (see Supplementary). Among the 141 loci, 30 of them were selected and synthesized for further PCR testing. Nine out of 30 loci were amplified successfully and were polymorphic (Table 2). The number of alleles detected ranged from seven (SL16) to 21 (SL18). Observed and expected heterozygosity of these loci were 0.370 to 0.870 and 0.505 to 0.724 respectively among 54 specimens. No significant disequilibrium significant deviation from HWE ($P < 0.01$) and no evidence for the presence of null alleles were found.

The present study isolated 141 loci by using shotgun sequencing method, and nine highly polymorphic microsatellite loci were characterized. These microsatellite loci have perfect motifs, and the polymorphism is comparable with those used in Daly-Engel *et al.* (2012). The nine novel loci developed in the present study can be applied to examine the connectivity and genetic stock structure of *S. lewini* in the West Pacific and benefit the regional conservation and management strategies planned for this species in the future.

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Supplementary materials are available from Journal Website.