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LETTER TO THE EDITOR

Isolation and characterization of polymorphic microsatellite loci from pale-edged stingray, *Telatrygon zugei* (Elasmobranchii, Dasyatidae)

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INTRODUCTION

Sharks, skates and rays originated 400 Ma, with a further radiation throughout every ocean, and play a vital role in maintaining the balance of marine ecosystems as top predators (Weigmann 2016). There are more than 1000 species of sharks, skates, rays and chimaeras in the world, 244 of which are distributed in Chinese seas. That is, Chinese chondrichthyan fauna make up at least one-fifth of the world's extant species. Recent habitat degradation and overexploitation have caused sharp declines in many populations of elasmobranchs (White 2007; Dulvy *et al.* 2017). Environmental changes and loss of oceanic apex predators due to overfishing could affect the migration routes and distribution of batoids (rays), resulting in community restructuring in the coastal ecosystem (Yamaguchi *et al.* 2005; Myers *et al.* 2007). Despite the combination of known high biodiversity and heavy exploitation of elasmobranchs in China, there is little reliable information on the population genetics of many shark and ray species. Consequently, clarification of the population structure is crucial for conservation and management, especially for endangered elasmobranchs.

Dasyatidae is one of the largest families of elasmobranchs, comprising 19 genera and at least 86 living species. They are distributed circumglobally in tropical, subtropical and temperate waters (Nelson 2006; Last *et al.* 2016). The pale-edged stingray, *Telatrygon zugei* (Müller & Henle, 1841), is an inner continental stingray of the Indo-West Pacific, typically found along the continental shelf of the Gulf of Thailand, the Java Sea, and the coastal waters off southern China and western Japan at <100m (Kyne *et al.* 2012). *T. zugei* is consumed locally but also forms a significant component of the elasmobranch bycatch within the demersal fishery throughout its range (White 2007). The fecundity of *T. zugei* is very low, with females usually possessing only a single embryo (White 2003). Because of high levels of exploitation and low resource resilience, *T. zugei* is listed as near threatened (NT) by the IUCN (2017).

Microsatellite markers are widely used co-dominant genetic markers and have proven to be optimal in assessing population structure for conservation genetics (Harper *et al.* 2003; Guyomard *et al.* 2006). In this study, we developed 9 highly polymorphic microsatel-

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lite primers in the pale-edged stingray, which will allow for investigating the genetic diversity and population genetic structure of *T. zugei*, as well as proposing scientific evidence for management and conservation.

MATERIALS AND METHODS

The screening process for microsatellites mainly adhered to the FIASCO protocol (Zane *et al.* 2002). Genomic DNA was extracted from the fin tissue of one individual using the DNeasy Blood & Tissue kit (Qiagen). Approximately 250 ng DNA was digested by *Mse*I, and DNA fragments were ligated to the double-strand *Mse*I AFLP adaptor (MseI R:5′-GAC GAT GAG TCC TGA G-3′ and MseI F: 5′-TAC TCA GGA CTC AT-3′). Then, these DNA fragments were amplified with AFLP adaptor-specific primers (MseI-N: 5′-GAT GAG TCC TGA GTA A-3′) for enrichment. The enriched DNA fragments were hybridized with 5′-biotinylated probe $(AC)_{12}$ and $(AG)_{12}$ for selection. The hybrid was restored to a double-strand pattern by PCR. The DNA strand was ligated into the pEASY-T1 Simple Cloning Vector (TransGen) and transformed into Trans5α chemically competent cells (TransGen). The cells were plated on LB-ampicillin plates supplemented with IPTG and X-Gal. Recombinants were screened by PCR with vector primers and AC/AG primers. Clones with PCR products showing multiple bands in 2% agarose electrophoresis were considered as positive clones and picked out for sequencing. Sequences were checked for microsatellites by Tandem Repeat Finder (Benson 1999). We eliminated sequences with any of the following conditions: (i) repeat units number less than 10; (ii) either side of flanking sequence was not long enough for designing primer; and (iii) the core sequence had compound repeat units, such as diuncleotide and triuncleotide compound repeat motifs.

A total of 92 *T. zugei* individuals were collected along the Indo-West Pacific coastline from 6 locations: Ariake Bay in Japan (JP), the Gulf of Thailand (TLD) and the Andaman Sea (ADM), and 3 localities along the coast of the East China and South China Seas, including Shandong (SD), Guangdong (GD) and Hainan (HN) (Table 1). Primers were tested on 92 *T. zugei* individuals to check the polymorphism of loci. PCR reactions were performed in 20-μL volumes: 100 to 200 ng of genomic DNA, 10 μL 2 × EasyTaq PCR Supermix (TransGen Biotech) and 2.5 pmol of each primer pair (forward primer fluorescently labeled with FAM, HEX or TAMRA). The amplification was performed on an Applied Biosystems (ABI) 2720 thermal cycler under the following conditions: an initial denaturing step at 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, with the annealing temperature at 50 °C or 55 °C for 30 s, and 40 s at 72 °C, and a 10-min extension at 72 °C. The PCR products were separated on an ABI PRISM 3730 genetic analyzer (Applied Biosystems) with a GS500 size standard.

To test the transferability of the microsatellite loci, we also tested these loci for cross-amplification using species from a different genus: *Hemitrygon akajei*, *Hemitrygon bennetti*, *Neotrygon kuhlii* and *Himantura microphthalma*. Extractions and genotyping were conducted following the same protocol as above.

Genotypes were analyzed using GENMARKER (version 1.3, SoftGenetics LLC). Micro-Checker (van Oosterhout *et al.* 2004) was used to detect genotyping errors due to null alleles, stuttering or allele dropout. The number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and polymorphic information content (PIC) were calculated with software Cervus 3.03 (Marshall *et al.* 1998). Departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested with GENEPOP v3.4 (Raymond & Rousset 1995).

Table 1 Sampling site information

Abbreviation	Locality (sea area)	Longitude	Latitude	Sample size
JP	Japan (Arike Sound)	130.14–130.60°E	32.62–33.18°N	20
SD	Shandong, China (Yellow Sea)	118.92–122.55°E	36.95–37.86°N	6
GD	Fujian and Guangdong, China (East China Sea)	113.62–1189.20°E	22.45–25.37°N	14
HN	Hainan, China (South China Sea)	108.63–110.15°E	20.05–18.25°N	20
TLD	Thailand (Gulf of Thailand)	100.60–101.56°E	12.59–13.51°N	12
ADM	Andaman Sea (Indian Ocean)	100.60–101.56°E	12.59–13.51°N	20

RESULTS

A total of 108 positive clones were picked up for sequencing and 90 of them were sequenced successfully; 50 unique sequences were chosen for primer design and 20 primers were designed using Premier 5. These 20 primers were tested on 92 *T. zugei* individuals by amplification. Of these 20 primers, 9 produced stable amplification results and showed high polymorphism. The numbers of alleles per locus ranged from 20 to 56, whereas the observed and expected heterozygosity ranged from 0.604 to 0.935 and from 0.874 to 0.981, respectively (Table 2). There is no evidence for scoring error due to stuttering or large allele dropout and no existence of null alleles was found at any loci. No significant departure from the genotype frequencies expected under the null hypothesis of Hardy–Weinberg equilibrium was detected after Bonferroni correction. No significant linkage association was found among all these loci. We also detected the genetic diversity of the 9 loci in 6 populations. The number of alleles (N_A) per locus ranged from 6 (locus *Dzu*I45) to 56 (locus *Dzu*I25). Observed heterozygosities (H_O) and expected heterozygosities (H_E) ranged from 0.429 (locus *Dzu*I57) to 1.0 (multiple loci) and 0.621 (locus *Dzu*I45) to 0.989 (locus *Dzu*II15), respectively. The PIC value ranged from 0.56 (locus *Dzu*I45) to 0.96 (locus *Dzu*II15). Overall, all loci were highly polymorphic in all 6 populations (Table 3).

Nine microsatellite loci were tested for cross-species amplification. We used all primer pairs to amplified sequences successfully in *Hemitrygon akajei*, *Hemitrygon bennetti*, *Neotrygon kuhlii* and *Himantura microphthalma*. These highly polymorphic and non-species-specific microsatellites will be useful for further research to investigate the genetic diversity, population structure and conservation genetics of *T. zugei* and other Dasyatid species.

Table 2 Characteristics of the 9 microsatellite loci isolated from *Telatrygon zugei* locus name, primer sequences, repeat motif, allele sizes, annealing temperature (*T*a), number of alleles (N_A), polymorphic information content (PIC), observed (H_O) and expected (H_E) heterozygosities, and GenBank accession number

Locus	Primer sequences (5′–3′)	Repeat motif	*T*a (°C)	Size range (bp)	N_A	H_O	H_E	PIC	GenBank accession number
DzuI25	F: TAMRA-AGCCAACCATGAGTCTTCTTT R: AATGCCTCTTTCTTTCTATGCTA	(AC)58	50	142–230	56	0.611	0.981	0.975	KF555248
DzuI31	F: TAMRA-GCATCTGCATTCCGTCATA R: TTGCTCCTGCCAGATAAAGTC	(AC)16	50	206–282	39	0.880	0.954	0.946	KF555250
DzuI45	F: FAM-GCCCAGCAGAATACAAGGTG R: CCATATCCAGGTTTACAGACAA	(GT)23	50	187–261	25	0.728	0.918	0.907	KF555249
DzuI46	F: HEX-CCGACGGAGGGATTACTTACT R: TCTCCATAGCAGCAAACAATCA	(AC)40	50	365–485	43	0.859	0.955	0.948	KF555251
DzuI57	F:TAMRA-TGCCAAACAAAGTCACCCTCAC R: GGACCAACAGTTCTAACCATCATC	(AC)18	55	228–256	20	0.604	0.874	0.857	KF555253
DzuI71	F: HEX-AGTCATCCGCCATCATCTACAAAT R: GGTCAAGTGAAAGGGGAGGAAGT	(AC)59	55	236–258	43	0.880	0.952	0.944	KF555254
DzuI82	F: HEX-CACCTATCCCACCCCCTCTA R: CGTCCCATTGTGTCTGTGATAAA	(AC)24	55	130–204	43	0.912	0.965	0.958	KF555255
DzuII15	F: FAM-GATGCAAACTGTCTCCCTGTA R: GTCTGATGCCTTGCCCACTA	(AG)41	55	178–306	56	0.935	0.976	0.970	KF555256
DzuII64	F: HEX-TTTCTCCGGTGTTGCATTATTTGT R:AACTTCTTGCCTCCCTGTCATTTT	(CT) 30	50	234–356	52	0.633	0.969	0.962	KF555257

Table 3 Polymorphism information of the 9 loci in 6 populations

Locus	JP				SD				GD			
	N_A	H_E	H_O	PIC	N_A	H_E	H_O	PIC	N_A	H_E	H_O	PIC
*Dzu*I25	27	0.976	0.8	0.949	7	0.933	0.8	0.82	14	0.929	0.429	0.887
*Dzu*I31	12	0.889	0.9	0.852	8	0.894	1	0.8	14	0.915	0.714	0.873
*Dzu*I45	6	0.621	0.6	0.56	10	0.955	1	0.864	14	0.923	0.857	0.881
*Dzu*I46	11	0.88	0.9	0.84	9	0.939	1	0.847	11	0.923	0.857	0.88
*Dzu*I57	10	0.876	0.85	0.837	7	0.933	0.6	0.82	12	0.921	0.429	0.877
*Dzu*I71	15	0.872	0.95	0.835	8	0.924	1	0.83	15	0.926	0.786	0.884
*Dzu*I82	14	0.862	0.85	0.826	9	0.978	1	0.868	13	0.929	1	0.886
*Dzu*II15	18	0.915	1	0.884	10	0.97	0.833	0.879	24	0.989	1	0.952
*Dzu*II64	11	0.842	0.75	0.8	5	0.893	0.25	0.746	18	0.958	0.714	0.919
Locus	HN				TLD				ADM			
	N_A	H_E	H_O	PIC	N_A	H_E	H_O	PIC	N_A	H_E	H_O	PIC
*Dzu*I25	21	0.966	0.526	0.937	15	0.946	0.583	0.899	18	0.951	0.6	0.92
*Dzu*I31	20	0.95	0.85	0.922	21	0.92	1	0.945	19	0.954	0.85	0.926
*Dzu*I45	15	0.912	0.9	0.88	11	0.989	0.167	0.871	15	0.905	0.9	0.873
*Dzu*I46	17	0.945	0.8	0.916	17	0.97	0.833	0.926	23	0.965	0.85	0.939
*Dzu*I57	8	0.821	0.45	0.773	6	0.754	0.583	0.691	9	0.845	0.65	0.803
*Dzu*I71	19	0.954	0.85	0.926	16	0.967	0.833	0.922	23	0.964	0.9	0.937
*Dzu*I82	25	0.974	1	0.948	20	0.982	0.833	0.938	20	0.955	0.85	0.927
*Dzu*II15	31	0.986	0.9	0.96	16	0.96	1	0.915	15	0.885	0.85	0.85
*Dzu*II64	24	0.969	0.7	0.942	16	0.96	0.667	0.915	19	0.96	0.45	0.933

H_E, expect expected heterozygosity; H_O, observed heterozygosity; N_A, number of alleles; PIC, polymorphic information content.

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SUPPLEMENTARY MATERIALS

Supplemental data

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