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Short Communication

Microsatellite Loci Identified by Cross-Species Amplifications in the Globally Vulnerable Relict Gull, *Larus relictus*

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LW , XL and KC degined the study and LW wrote the article. LW and YG collected the samples and performed the molecular experiments. YG and HW helped in data analysis and manuscript writing.

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ABSTRACT

Relict gull, *Larus relictus* is listed as vulnerable species by IUCN. For improving the research on conservation of this species, we tested the cross-species amplification of 90 microsatellite loci developed for eight other species. Eleven out of them were successfully amplified and polymorphic with 2-10 alleles. The observed heterozygosities ranged from 0.481 to 0.827 and the polymorphic information content ranged from 0.373 to 0.775. Significant linkage disequilibrium was found only between two markers. These microsatellites could be used to enhance our understanding of genetic information and breeding biology of Relict Gull.

The Relict gull *Larus relictus* is a rare species breeding at a small number of grounds in salt marshes of Asia which could be strongly influenced by climate change, thus it was classified as a vulnerable species by the International Union for Conservation of Nature (2016). Since 1971 when it was recognized as a separate species, several aspects of the status (*i.e.* distribution, breeding ecology and population structure) have been investigated (He *et al*., 2002; Yang *et al*., 2015). However, this species remains one of the least known birds. Currently, changes in water level and loss of ephemeral wetland habitats in semi-arid region of China have already threatened the breeding population stability (*e.g.* He *et al*., 2002).

Delineating population genetic structure, resulting from limited gene flow and genetic drift, is critical for identifying evolutionary processes and for effective conservation (Frankham *et al*., 2004; Abbas *et al*., 2017). Previous genetic studies in Relict gull used mitochondrial DNA (mtDNA) and a nuclear gene to reveal structure and it was suggested that more effective molecular markers were needed to fully address the relationship among different subpopulations (Yang *et al*., 2015). Microsatellites provide a powerful tool for analyzing recent and contemporary events, and maybe more effective in fine scale studies and parental analysis (Wan *et al*., 2004; Zhu *et al*., 2017). However, microsatellite loci have not been developed in Relict gull to date. Isolating microsatellites from the genome of this species seems necessary to explore how genetic diversity and connectivity varies among the remaining population. Cross-species amplification is a convenient and fast method to identify microsatellite loci in birds (Loyau and Schmeller, 2009). Our aim was therefore to screen polymorphic microsatellites by cross-amplification in the Relict gull.

Methods

Fifty-two Relict gull muscle samples were taken from the natural dead chicks from 52 different nest sites in Hongjian Nur Nature Reserve in China (38°13' N - 39°27'N, 109°42' N - 110°54'E) in 2016 and were stored in Absolute Ethyl Alcohol. Genomic DNA was isolated from the samples using a standard phenol: chloroform based extraction technique.

We tested the cross-amplification of 90 microsatellite

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primer pairs developed for other closely species within the Charadriiformes: nine on Saunders's Gull *Larus saundersi* (Jiang *et al*., 2011), 22 on Ivory Gull *Pagophila eburnea* (Yannic *et al*., 2011), seven on Red-billed Gull *Larus novaehollandiae scopulinus* (Given *et al*., 2002), six on Herring Gull *Larus argentatus* (Gregory and Quinn, 2005), five on American Herring Gull *Larus smithsonianus* (Crochet *et al*., 2003), seven on Black-legged Kittiwake *Rissa tridactyla* (Verkuil *et al*., 2009), 31 on Little Terns *Sternula albifrons* (Noreikiene *et al*., 2012) and three on Roseate Tern *Sterna dougallii* (Szczys *et al*., 2005) for variability in Relict Gull.

PCR amplification was run in 25 µL volume containing 2.5 µL 10×PCR Buffer, 0.5 µL dNTP mix (10 mmol/µL), 2 µL $MgCl_2$ (25 mmol/µL), 0.2 µL Taq polymerase (5U/µL), 0.5 µL of each primer, 17.8 µL H_2O and about 50 ng DNA. PCR reactions were carried out in one cycling profile (an initial denaturation of 3 min at 95 °C, followed by 10 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, and 20 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and 6 min of final elongation at 72 °C. PCR products were analyzed by Songon Biotech, Shanghai, China, on ABI PRISM 3730XL Genetic Analyzer for polymorphism testing with GeneMapper v3.7 for the size range reading.

We tested for deviations from linkage disequilibrium (LD), and Hardy–Weinberg equilibrium (HWE) by Markov chain method using the following parameters: dememorization = 10000, number of batches = 20, and number of iterations/batch = 5000 in Genepop 4.3. Bonferroni correction (α=0.05) was applied to the two above tests. Null alleles were checked in Micro-Checker 2.2.3. The observed and expected heterozygosity, and polymorphic information content were calculated with Cervus 3.0.

Results and discussion

Out of a total of 90 microsatellites combination tested, 56 (62%) positive amplifications, of which 11 loci were polymorphic. None of the markers was observed departing from HWE and no null alleles were found in the 11 loci. The polymorphism loci had 2-10 alleles per locus and with PIC ranging from 0.373 to 0.775 (Table I). The observed heterozygosities ranged from 0.481 to 0.827.

The genetic variability estimated by microsatellite markers significantly outnumbered the genetic variability assessed from the two mtDNA and one nuclear gene that has been previously used (Yang *et al*., 2015). However, this particular set of microsatellite loci are still relatively invariant compared to many microsatellite loci examined in other species (*e.g.* Jiang *et al*., 2011; Yannic *et al*., 2011) and possibly at other loci in this species. One possibility is that the populations are small with relatively low level of gene flow. As revealed by mitochondrial and nuclear sequence data, the sampled population indeed appears to have low variation compared with that in closely related gull (Yang *et al*., 2015). And that only about 20% (11 out of 56) of tested primers were variable in our study is also consistent with this explanation.

Significant LD was found only between Locus RBG29 and LARZAP11. LD creates pseudo-replication for analysis where locus is assumed to be independent. In this scenario, one of the linked loci should be excluded to avoid increased Type I error (Selkoe and Toonen, 2006). However, for many ecological questions, application of linked loci could be beneficial. For example, LD can help understanding the patterns of gene exchange and history of changes in population size (*e.g.* Tishkoff *et al*., 1996). Therefore, these microsatellite markers could be useful for inferring population patterns and processes, and analyzing kinship among individuals in Relict Gull.

Table I.- Characteristics of eleven microsatellite loci in Relict gull. Initial species, number of alleles (N_a), size range, observed (Ho) and expected (He) heterozygosities, polymorphic information content (PIC) and Hardy-Weinberg exact test (*P*-val) are shown.

Locus[1]	Initial species	N_a	Size range (bp)	Ho	He	PIC	P-val
RBG29	Red-billed Gull	3	125-137	0.615	0.619	0.530	0.758
Lasa-8	Saunders's Gull	7	239-267	0.712	0.703	0.645	0.882
Lasa-3	Saunders's Gull	6	209-229	0.592	0.607	0.546	0.793
LARZAP11	Herring Gull	3	197-209	0.558	0.581	0.482	0.696
Salb2	Little Terns	10	186-231	0.827	0.811	0.775	0.299
IVGU-A2	Ivory Gull	2	194-196	0.627	0.472	0.358	0.044
IVGU-A137	Ivory Gull	2	195-199	0.481	0.500	0.373	0.787
IVGU-A138	Ivory Gull	2	174-180	0.577	0.504	0.375	0.404
IVGU-B125	Ivory Gull	4	264-276	0.731	0.736	0.677	0.725
IVGU-C7	Ivory Gull	4	167-183	0.635	0.614	0.530	0.932
IVGU-D110	Ivory Gull	3	199-207	0.577	0.600	0.507	0.232

[1]All of the 11 loci were successfully amplified in the 52 samples.

Conclusion

In conclusion, 11 polymorphic microsatellite loci in Relict gull were identified by cross-species amplifications. The characteristics of these loci provide useful information for further studies on genetic diversity, population structure, conservation status as well as kinship among individuals in this species.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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