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Isolation and Characterization of Microsatellite Loci from an Ice Fish, *Neosalanx tangkahkeii* (Osmeriformes, Salangidae)

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Authors' Contribution
JZ conceived the project, performed the experiments and draft main text. BW took part in sampling and PCR, and co-drafted the manuscript. QHZ participated in study design and coordination.

Key words
Microsatellite loci, *Neosalanx tangkahkeii*, Polymorphic, Salangidae, Genetic diversity.

ABSTRACT

The icefish *Neosalanx tangkahkeii* is an endemic species in East Asia. It has a wide range of distribution in Chinese waters including coastal waters, inland lakes, and outflowing rivers south of the Yangtze River and its delta. In order to maintain stable high production of salangids, fertilized eggs from native or introduced populations of icefish were frequently introduced to various types of water throughout China mainland except for Tibet Plantae. The genetic diversity and molecular invasive mechanism are urgently needed for both native and invasive populations. Present investigation details 12 polymorphic microsatellite loci and 12 to 26 alleles per locus denoted. Whereas, d expected and observed heterozygosity values ranged from 0.902 to 0.972 and 0.750 to 0.958, respectively. Moreover, the polymorphic information content (PIC) values ranged from 0.869 to 0.949. The cross-species amplification and applicability were also evaluated on *Protosalanx chinensis*, *Neosalanx anderssoni*, *Neosalanx argentea* and *Neosalanx oligodontis*, and 6-7 loci were amplified in these species. The outcome of the present investigation would be useful in understanding genetic diversity, gene flow and the population structure of this species in the future.

INTRODUCTION

The icefish *Neosalanx tangkahkeii* belongs to the family Salangidae and order Osmeriformes. This species is endemic to the East Asia and has a wide range of distribution in Chinese waters including coastal waters, inland lakes, and outflowing rivers in the south of the Yangtze River and its delta (Xie and Xie, 1997; Zhang *et al*., 2007a; Zhao *et al*., 2008; Kim and Park, 2002; Nakabo, 2002; Nelson, 2006).

From nineteen fifties to nineteen seventies *N. tangkahkeii* remained a potential target species in the area. However, its catch appeared to be in drastic situation by mean of the common threats of habitat degradation from agricultural irrigation, reclamation, rapid industrialization as well as overfishing. On the other hand, the nationwide introduction of *N. tangkahkeii* was initiated in 1979 in Yunnan Province, which has highest biodiversity and endemism of freshwater fish fauna in China (Chen *et al*.,1998). Thirteen thousand fertilized eggs from nine pairs of parent fish of *N. tangkahkeii* were laid in Dianchi Lake in April 1979. The introduced individuals changed their reproductive strategy and establish the population successfully (Gao *et al*., 1984). By 1994, *N. tangkahkeii* has been introduced to 15 provinces, with a total inland water area of 270, 000 ha in the nation, including more than 90% of plateau lakes and reservoirs in Yunnan Province (Gu, 1998). The salangids further invaded 1,000,000 ha water area in all provinces and Regional National Autonomies in mainland China by the end of 2000, with the exception of Tibet (Li *et al*., 2002). The introduction of icefish brought in economic benefits but severely threatened biodiversity, especially in plateau lakes in Yunnan Province (Kang *et al*., 2015). The population genetics study must be conducted on both natural and introduced populations by using more sensitive gene makers since there was no clear introduction history recorded and the population genetic information before or after introduction. Recently, microsatellite markers appeared to be very popular in the studying population structure and provide valuable

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information for theoretical population to the biologists and conservationists (Newman and Squire, 2001; Liu *et al*., 2017). Besides, it is a powerful tool helping in assessing resource status. Here we describe isolation and characterization of 12 microsatellite loci extracted from *N. tangkahkeii* collected from Chinese waters.

MATERIALS AND METHODS

Collection of sample and DNA isolation

A total of 30 specimens were collected from the Dianchi Lake, Yunnan Province. The samples were preserved in 95% ethanol and brought to the laboratory for further DNA extraction. Genomic DNA was isolated from muscle tissue using standard proteinase-K/phenol-chloroform extraction (Sambrook *et al*., 1989). UV spectrophotometer was used to estimate the concentration and purity of DNA.

Isolation of microsatellite loci and polymorphism analysis

The FIASCO (Fast Isolation by AFLP of Sequences Containing) method was employed for the isolation of microsatellite loci (Zane *et al*., 2002). The purified total DNA was digested with *Mse*I to obtain suitable-sized fragments (*Mse*I F: 5'-TACTCAGGACTCAT-3' and *Mse*I R: 5'-GACGATGAGTCCTGAG-3'), then ligated to double-stranded AFLP linkers. Ligation products were hybridized to 5'-biotinylated oligonucleotides $(AG)_8$ and $(AC)_8$ probes to enrich the target fragments by the streptavidin magnetic beads. The enriched DNA fragments were amplified with the primer *Mse*I-N and cloned into the pEASY-T1 vector (TransGen Biotech) before transforming into Trans5α Chemically Competent Cells (TransGen Biotech). The transformants were grown at 37°C on Luria-Bertani (LB) agar plate containing ampicillin 11-13h for enrichment. In total, 303 positive clones were picked out screened with a standard blue-white selection. Then they were verified by Polymerase chain reaction (PCR) technique using two vector primers (M13F and M13R) and $(AG)_8/(AC)_8$ oligonucleotide and visualized by 2% agarose gel electrophoresis.

Table I.- Characterization of twelve *Neosalanx tangkahkeii* microsatellite loci: locus designation, primer sequences, repeat motif, annealing temperature (Ta), size of clone allele, number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorph information content (PIC) and GenBank accession number.

Locus	Primer sequence (5′-3′)	Repeat motif	Ta (°C)	Size rang (bp)	Na	Ho	He	PIC	GenBank accession No.
NT-I-13	F:TGGGTAACAGAACAAGCAGGGTAT R:CAGTAAAGATCCAGGGAGTTCAGG	(AC)19	58	177-215	24	0.875	0.965	0.942	MG987068
NT-I-17	F:CCCCTTTAGTGAGCCTGTGTTTGT R:TGAAGGGTGTGATAAGAGGGTGTT	(CT) 14 (AC) 25	55	44-86 268-318	23	0.917	0.966	0.944	MG987069
NT-I-19	F:GTGTGAGTGTGGGTAGGATGATGT R:GCTAGATGCACGGCTGATAAAAT	(AC)32	55	189-252	21	0.958	0.958	0.935	MG987070
NT-I-31	F:GAATCCCACCAGTCACTTATCAAT R:AGGCGTCTCCGAATCTGCT	(AC)55	61	138-247	26	0.958	0.972	0.949	MG987071
NT-I-83	F:AAGTATCGACCTGTTTCTGCTATC R:AGAACAACACAATGAGTCCGTAAC	(AC)24	55	187-235	23	0.913	0.957	0.932	MG987072
NT-I-91	F:CCGACTGCGCCACACGA R:AACCAGGATCCGAGCCACTTC	(GT)15	55	248-278	18	0.75	0.931	0.901	MG987073
NT-II-05	F:ACGGGCCAGTCACAGTC R:ATCTCCCCCATTTCTTCTCT	(AG)22	55	133-176	20	0.87	0.946	0.921	MG987074
NT-II-09	F:TGGAGAAGAACATCGACTGAACA R:TATACTATCCTCTCCGCTTTGGTG	(AG)22	55	164-208	12	0.81	0.902	0.869	MG987075
NT-II-59	F:AGAAGGCTGTATTTGTGCTGTC R:ATGATTAGGTGAGAGGAAGATGC	(CT)25	60	336-385	21	0.875	0.961	0.938	MG987076
NT-II-61	F:TAGTTTAGGAGTCTTTTGGTGATA R:AGGCCTTGCTGTTTGAT	(AC)48	55	121-217	22	0.958	0.966	0.944	MG987077
NT-II-83	F:GAACCCACTCTATCATCTGC R:ATTACACTTCCTTTCTTATCTTTG	(AG)84	55	427-594	20	0.905	0.95	0.923	MG987078
NT-II-99	F:GAACGCACCAGGCAAACTAAACA R:CCCCTCCCTGCTCTAATCGTG	(AG)23 (AC)56	55	288-334 477-587	26	0.958	0.968	0.945	MG987079

Table II.- Cross-species amplification of 12 microsatellite loci in *Protosalanx chinensis*, *Neosalanx anderssoni*, *Neosalanx argentea* and *Neosalanx oligodontis*.

	NT-I-13	NT-I-17	NT-I-19	NT-I-31	NT-I-83	NT-I-91	NT-II-05	NT-II-09	NT-II-59	NT-II-61	NT-II-83	NT-II-99
P. chinensis	+	+	+	+	-	-	+	-	-	+	-	+
N. anderssoni	+	+	+	-	-	+	-	-	+	+	-	-
N. argentea	+	+	+	+	-	+	-	-	+	+	-	-
N. oligodontis	+	+	+	+	-	+	-	-	+	+	-	-

+ indicates successful amplification; - indicates failed amplification.

Out of 303, only 27 clones were suitable for primer design and amplification of polymorphic loci, and these primers were designed using the software primer Primer 5 (Lalitha, 2000). A total of 24 individuals were used for genotyping, and all PCR reactions were conducted in an Applied Biosystems (ABI) 2720 Thermal Cycler, which being performed with 30 cycles of a 15µl reaction volumes containing 100-200 ng genomic DNA, 7.5 µl 2×Easy*Taq* PCR Supermix (TransGen Biotech), and 1.5 pmol each of primer pairs (upstream primer fluorescently labeled with FAM, HEX or TAMRA). 30 cycles of amplification were used: denaturation at 95°C for the 30s, with annealing temperature in Table I for 30 s and extension at 72°C for 30s. PCR products were determined on an ABI PRISM 3730 genetic analyzer (Applied Biosystems) on the basis of GS500 and estimated using GENEMARKER (Version 1.85, Applied Biosystems).

Data analysis

GENEPOP version 3.4 Software (Raymond and Rousset 1995) was used to test Hardy-Weinberg equilibrium (HWE), the number of alleles at each locus (N_A), vlinkage disequilibrium (LD), polymorphic information content values, observed (Ho) and expected heterozygosity (He). Polymorphism information content (PIC) was estimated using PIC-CALC version 0.6.

RESULTS AND DISCUSSION

Out of 303 positive clones, 27 clones were utilized for primer design and amplification of polymorphic loci. The number of alleles per locus ranged from 12 to 26, the expected heterozygosity (H_E) and observed heterozygosity (H_O) values ranged from 0.902 to 0.972 and 0.750 to 0.958. Moreover, the polymorphic information content (PIC) values ranged 0.869 to 0.949 with most of the above 0.900. Although 19 polymorphic microsatellite loci from *Neosalanx* have been reported (Liu *et al*., 2015), the PIC varied from 0.258 to 0.893 with most of those lower than 0.600. Three loci were detected significant deviations from HWE after Bonferroni correction (Table I). Some reasons can cause this situation such as insufficient sample size, but no evidence is sufficient to linkage disequilibrium among pairs of loci in the samples. Eventually, 12 microsatellite loci were isolated and characterized successfully from *N. tangkahkeii* by following the PCR conditions described above. Polymorphic information of those 12 microsatellite loci was shown in Table I.

Four primitive lineages within close related *Neosalanx* and *Protosalanx* species were identified in the previously studied (Zhang *et al*., 2007b). In addition cross-species amplification and applicability were also evaluated on *Protosalanx chinensis*, *Neosalanx anderssoni*, *Neosalanx argentea* and *Neosalanx oligodontis* for these 12 polymorphic microsatellite loci, Study showed 11 polymorphic microsatellite loci in the *Relict gull* by cross-species amplification (Lin *et al*., 2018). The PCR reactions were also performed according to the previous conditions.

In conclusion, seven of these micro satellite loci were suitable for *Protosalanx chinensis*, *Neosalanx argentea* and *N. oligodontis*, and six loci for *N. anderssoni*, respectively (Table II). *N. tangkahkeii* and *P. chinensis* have been introduced into many lakes and reservoirs because of their high commercial value. But it has reported that the populations in some waters are unstable (Hu and Chen *et al*., 1998; Huang and Chang 2001; Kang *et al*., 2015). Previous studies on the molecular mechanism of invasion indicated that some fish populations have undergone genetic bottlenecks and low genetic diversity (Salmenkova, 2008), while others experienced rapid population expansion with potential genetic diversification after its introduction (Chen *et al*., 2012). In order to maintain stable high production, fertilized eggs either from native or introduced populations of icefish was frequently introduced to the various types of waters throughout China mainland except for Tibet Plantae. Thus, the genetic diversity is urgently needed to be detected whether the founder effect or the rapid population expansion occurs within and among native and introduced salangids populations.

CONCLUSION

It is concluded that microsatellite loci developed here will be a useful tool to evaluate the genetic diversity, gene

flow and the population structure of this species in the future.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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