Pakistan J. Zool., vol. 46(4), pp. 1147-1151, 2014.

Development of Microsatellite Markers for *Leptobotia elongata* (Cypriniformes: Cobitidae) Using 454 Sequencing and Cross-species Amplification

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Abstract.- Thirty microsatellite markers were developed from *Leptobotia elongata* through 454 sequencing and 27 were polymorphic among 24 individuals, with an average of 9.67 alleles per locus, and the mean observed and expected heterozygosities were 0.81 and 0.79, respectively. Polymorphism information content (PIC) ranged from 0.416 to 0.933. In addition, cross-species amplification was tested for all 30 loci in four other loaches of Cobitidae (*Leptobotia microphthalma*, *Botia superciliaris*), and Nemacheilidae (*Oreias dabryi*, *Triplophysa stenura*). The results showed a significant high level of transferability (63%–97%), which means the possibility of these markers applying to other related species. The microsatellite loci described here would be useful in conservation of *L. elongata* and possibly some other species.

Keywords: *Leptobotia elongate*, microsatellite markers, next-generation sequencing, cross-species amplification, polymorphism information content.

INTRODUCTION

Leptobotia elongata (Cypriniformes: Cobitidae) is an important fish species endemic to China with high ornamental and edible value. It is distributed in the main streams and tributaries along the middle and upper reaches of the Yangtze River (Ding, 1994). Recently, this species has become endangered as a consequence of a sharp decrease in the population size due to overfishing, water pollution, and the loss of habitats following completion of dams along the middle and upper Yangtze River (Yue and Chen, 1998). Thus, it was listed as vulnerable in the China Red Data Book of Endangered Animals (Yue and Chen, 1998). It is essential to study its genetic diversity and population structure in order to make some protection measures for the wild resources and provide some appropriate data for artificial propagation and breeding. So it is required to develop molecular markers used to population and conservation genetic studies for this endangered species.

The traditional process of developing genomic microsatellite markers is time consumption due to the preparation of genomic libraries and the subsequent sequencing of a large number of clones that potentially contain microsatellite regions (Squirrell *et al.*, 2003). Nevertheless, the next-generation sequencing platforms, such as the 454 GS-FLX platform (Roche Applied Science), facilitate high-throughput genome sequencing and provide a much more efficient and cost-effective method for the acquisition of microsatellite genetic markers in large quantities, in those organisms for which adequate databases are not currently available (Abdelkrim *et al*., 2009; Yan *et al*., 2012).

Here, polymorphic microsatellite loci of *L. elongata* were developed based on the 454 sequencing to offer important genetic resources for the conservation of the endangered species *L. elongata* and they were tested in four related species to facilitate future work on the other related species.

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0030-9923/2014/0004-1147 $ 8.00/0

MATERIALS AND METHODS

Sample collection and 454 sequencing

Fins of *L. elongata* collected from Yibin range of the Yangtze River were sent to run 454 sequencing. Other samples of *L. elongata* were collected from Luzhou range of the Yangtze River and Chongqing range of the Jialing River, respectively. *Oreias dabryi* were collected from upper reaches of the Baoxing River. *Triplophysa stenura* were collected from the Ouqu River. *L. microphthalma* and *Botia superciliaris* were collected from Jiajiang range of the Qingyi River. Total genomic DNA was extracted from the fin tissue using TIANamp marine animals DNA kit (TIANGEN, China).

Microsatellite discovery and primer screening

The raw sequences for *L. elongata* were assembled into contigs using Newbler 2.6. Misa. pl was used to screen microsatellites. Finally, the microsatellite loci used for the polymorphism screening were selected to increase the probability of polymorphism. The primers were designed using the online software PRIMER 5.0.

DNA amplification, genotyping and cross-species amplification

The total PCR reaction volume was 25μL contained 1μL genomic DNA (50 ng/lL). Each forward primer was labeled with fluorescent dyes (FAM, TAMRA or HEX) for electrophoresis on an ABI Prism 377 Genetic Analyzer (Applied Biosystems). All of the developed microsatellite loci in *L. elongata* were assessed for cross-species amplification in four related species (*L. microphthalma*, *B. superciliaris*, *O. dabryi*, and *T. stenura*).

Data analysis

CERVUS 3.0.3 software (Kalinowski *et al.*, 2007) was used to determine the number of alleles (N_A), polymorphic information content (PIC), observed and expected heterozygosities (H_O and H_E). Exact tests were implemented in GENEPOP 3.3 (Raymond and Rousset, 1995; Zaigham *et al*., 2012) for assessment of deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between the loci. The presence of null alleles was assessed at a 95% confidence interval using MICRO-CHECKER 2.2.3 (Oosterhout *et al*., 2004).

RESULTS AND DISCUSSION

The number of alleles per locus ranged from 4 to 22, and the observed and expected heterozygosities ranged from 0.375 to 1.000 and from 0.467 to 0.957, respectively; the PIC values ranged from 0.416 to 0.933 with an average of 0.75 (Table I). Six loci (LS38/41/55/56/62/66) deviated from HWE, suggesting the possibility of null alleles (Hedgecock *et al*., 2004). Fifteen loci (LS60/66, 51/56/62, 06/35/54, 43/65, 38/41/46/55/57, $P < 0.05$, Table I) observed in *L. elongata* were in LD ($P< 0.05$, Table I), which means these markers should be used with caution. Compared to microsatellite markers which have been developed (Liu *et al*., 2013), these markers include not only tetra-nucleotide repeats but also di- and tri- nucleotide repeats and most tetra-nucleotide loci exhibited higher genetic polymorphism than di- and tri-nucleotide loci. Nevertheless, the repeatability of di- and tri-nucleotide loci were better than tetra-nucleotide loci. So we choose which type of repeats, according to own actual situation.

The cross-species amplification of 30 microsatellite loci showed high success rates, 96.7% (29/30) for *L. microphthalma*, 93.3% (28/30) for *B. superciliaris*, 83.3% (25/30) for *O. dabryi*, and 63.3% (19/30) for *T. stenura*. We found multiple alleles in 18 of 29 loci (62.1%) for *L. microphthalma*, 11 of 28 (39.3%) for *B. superciliaris*, 8 of 25 (32.0%) for *O. dabryi* and 4 of 19 (21.1%) for *T. stenura*. We can see that most loci of *L. elongata* showed monomorphic in other species, except for *L. microphthalma*, through cross-species amplification, that might because of limited samples. So if these markers were used in the future, we must enlarge the number of samples to make sure their polymorphism. Although the numbers of genera and samples for each genus are limited, these results suggest the possibility that the polymorphic markers developed in *L. elongata* can be used in other fish species through cross-species amplification. The markers described herein would offer important genetic resources for the

Table I.- **Characteristics of microsatellite loci in *Leptobotia elongata* and cross-species amplification in other loaches**

Locus	Repeat motif	Primer sequence (5'–3')	*L. elongata* (N=24)						Cross-species amplification (N=5)			
			Tm (°C)	N_A	H_O	H_E	PIC	GenBank Accession No.	Lm	Bs	Ts	Od
LS46[#5]	(ATA)12	F□CAATGGTCCTACAGGCTTCA R□GGTCTCCTGTGGTGGTCATT	62	8	0.708	0.698	0.657	KF440369	P	P	M	P
LS41[#5]	(TCAC)10	F□TGAAGGACAAAAGATGACT R□TGAGTGAGTGACTGAATGAG	63	10	1.000*	0.778	0.737	KF440370	M	P	F	P
LS43[#4]	(TCTT)14	F□ATCTAAACCACATTTCTCTCA R□GGACAATGATTTGAAGGAT	57	13	0.833	0.918	0.890	KF440371	P	M	F	P
LS67	(TG)13	F□GATGAGATGGTGCTTGGTGA R□TGCAACATGAGTCATCGAAA	62	13	0.792	0.885	0.854	KF440372	P	M	M	M
LS65[#4]	(AC)12	F□TGTTTCCTGCACCAATCAGA: R□TGTTTCCTGCACCAATCAGA	58	7	0.792	0.752	0.700	KF440373	P	M	F	F
LS53	(TCA)11	F□CCCGTATATGGCAGGAAGAA R□GTGTGGATGGACATGGATGA	61	6	0.667	0.697	0.630	KF440374	M	M	M	M
LS61	(AC)13	F□CATCTGCCCTGATGGAAAGT R□AGTTCATCTGGCCCTGTGAT	66	5	0.708	0.696	0.626	KF440375	M	P	M	M
LS06[#3]	(TG)13	F□CTCATGGACAGACTGGCAGA R□CTGAACACCACCTTCACTGG	62	10	0.792	0.826	0.784	KF440376	P	M	M	M
LS66[#1]	(GA)13	F□CGCTAGCAGTCTGTTGCTGT R□TGCACGCAGCATTATTTCTC	62	7	0.417*	0.702	0.641	KF440377	M	F	F	M
LS47	(ATA)10	F□CGAAGGAAACACTCTGGTCTG R□CAGAAATGCCCATTCGTACA	62	9	0.875	0.840	0.800	KF440378	M	P	M	M
LS07	(CA)21	F□TGGAGCACAACTCTGTTGTCA R□TCATGACAGACTGGAGAGAGTGA	62	10	0.833	0.856	0.820	KF440379	P	P	F	F
LS51[#2]	(TAT)11	F□ATGCTCTGGGTGATCTGGTC R□CTCAGGCTGGAACACAGAC	62	5	0.625	0.716	0.660	KF440380	P	M	M	P
LS56[#2]	(TAG)10	F□TCCAAGGTTGCTTTCCAAAC R□TCAGACATGCCTCCTGAAGA	61	5	0.958*	0.785	0.732	KF440381	M	M	F	M
LS55[#5]	(TTA)12	F□TTTGTAAGGATTTTTAGGGGTT R□GATTGGGCTGGGTCTGAGTA	61	8	0.458*	0.784	0.735	KF440382	M	M	M	M
LS63	(TG)13	F□TGTGGCCATAGCATTTGTGT R□CTGAACACCACCTTCACTGG	66	9	0.792	0.815	0.770	KF440383	P	M	M	M
LS52	(ATA)10	F□AAAAGTGAGTGGCGATGAGG R□TGACAATATTACAACAGCATCAAC	62	4	0.375	0.467	0.416	KF440384	P	M	P	M
LS54[#3]	(TAT)11	F□TTTCGCCTCATGCTGAGATT R□ACCACCAAACTCCACACCAT	61	13	0.792	0.807	0.771	KF440385	P	P	M	P
LS28	(ATCT)11	F□TTGGGCTTGTGTAGCACTTG R□AATTCACGTTTTATTGGTCACA	64	14	0.833	0.922	0.895	KF440386	P	P	F	M
LS60[#1]	(AC)16	F□AATCACACTTTGGGCCAACT R□CATGCTGCAGTGATTTGTCA	63	12	0.958	0.886	0.853	KF440387	P	P	M	M

Locus	Repeat motif	Primer sequence (5'–3')	*L. elongata* (N=24)						Cross-species amplification (N=5)			
			Tm (°C)	N_A	H_O	H_E	PIC	GenBank Accession No.	Lm	Bs	Ts	Od
LS27	(ATCT)17	F□AAATGGAAAGTTCAAGAAAA R□GCTGGGTAAGCATGAGCAGT	64	22	0.958	0.957	0.933	KF440388	P	P	F	P
LS57[#5]	(TAT)11	F□ACAGCCTAATGGTTGCCTTG R□GGCCAGATATCAGACCTCCA	61	4	0.750	0.731	0.666	KF440389	P	M	F	M
LS49	(ATT)11	F□TCAAAACAGCAGCTCTGGAA R□CAGCCCAACATTTATTTCAGG	62	5	0.667	0.671	0.608	KF440390	P	M	P	M
LS33	(TTTC)13	F□CCCACACAGCATGGAATTTA R□TCAGTCCGAGCATTTGAGTG	63	16	0.958	0.937	0.912	KF440391	P	P	P	P
LS38[#5]	(TATC)12	F□AGATTTTTATGGGCAAACCTG R□GAGATAGATGGGGAGAGAGAGG	63	16	0.542*	0.902	0.874	KF440392	P	M	F	F
LS62[#2]	(AT)13	F□TCACACTAAAACGCCCAACA R□ATGGCCTGCAGCATTAAATC	66	7	0.667*	0.843	0.803	KF440393	M	M	F	M
LS35[#3]	(ATCT)31	F□TCTCAGGCTCAAACTGGACA R□TGAGCAAATCATTACCGTCCT	62	17	0.792	0.898	0.869	KF440394	P	P	F	M
LS48	(ATA)10	F□AAACTGAAGCCGGTTTGAATA R□AGACCCTGCAGAACCAGAGA	62	6	0.625	0.663	0.614	KF440395	M	M	P	P
LS12	(AT)10	F□CATACTCCCAACGGCTGAAT R□CATGGGGGCATCTGTCTAAA	63	1	0.00	0.00	M		M	M	M	M
LS58	(AT)14	F□CACTGCTTTGCTGCATGATT R□TTGTGCCACGTGTTTAAGGA	66	1	0.00	0.00	M		M	M	M	M
LS59	(TC)12	F□CCACGTGACCTGATTCCTTT R□TGGTCCACAGCTCATTCTACA	63	1	0.00	0.00	M		F	F	F	F

Loci with[#1], [#2], [#3], [#4], [#5] are in linkage disequilibrium, 'N_A' the number of alleles, 'H_O' observed heterozygosity, 'H_E'expected heterozygosity, and * significant deviation from Hardy-Weinberg equilibrium ($P< 0.05$). 'PIC' polymorphism information content, 'P' polymorphic, 'M' monomorphic, 'F' failed to amplify. The three monomorphic loci screening in *L. elongata* were shown on the last three rows in the table. Species abbreviations: *Oreias dabryi* 'Od', *Botia superciliaris* 'Bs', *Triplophysa stenura* 'Ts', and *L. microphthalma* 'Lm'

conservation of *L. elongata*, and facilitate future work on the other related species as well.

ACKNOWLEDGMENTS

The work was supported by the Yalong River Hydropower Development Company, Ltd. (No. 12H0856) and the Program for New Century Excellent Talents in University (No. NCET-11-0347). We would like to thank Chaochao Yan for their help in experimental procedures and data analysis.

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(Received 6 May 2014, revised 18 May 2014)