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Development and Characterization of Microsatellite Markers for the Ornamented Pygmy Frog *Microhyla fissipes* and Transferability in *Microhyla*

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LL designed the study, analyzed the data, performed the experimental work and wrote the article. LC analyzed the data and revised the article. XZ and YL performed the experimental work. JJ supervised the study and reviewed the article.

Key words
***Microhyla fissipes*, Genic microsatellites, Transcriptome, Transferability, Microhyla.**

ABSTRACT

Microhyla fissipes, from family Microhylidae suborder Neobatrachia, widely distributes from western Myanmar eastward through Indochina and northward into southern China including Hainan and Taiwan. But the availability of microsatellites especially from expressed sequences is currently very limited in this species and the genus *Microhyla*. A total of 17,339 potential microsatellites were identified in 15,385 unigenes which were generated by Illumina paired-end sequencing in *M. fissipes*. Within all microsatellites, AG/CT, AAG/CTT, and AAAG/CTTT are most prevalent motif in each repeat class. We randomly selected 61 unigenes with the microsatellite to design primers and do genetic analysis in the Sichuan basin population of *M. fissipes*. Of all, 35 primer pairs (57.38%) successfully amplificated in *M. fissipes*, of which 14 (40.00%) were polymorphism. The observed and expected heterozygosity in the test population ranged from 0.02 to 0.92 and from 0.02 to 0.62, respectively. High transferability rates were detected in *M. butleri* (37.14%), *M. heymonsi* (45.71%), *M. pulchra* (57.14%) and *M. mixture* (71.43%). These results indicate that the Illumina paired-end sequencing system is of great value for identifying massive numbers of genic microsatellites in *M. fissipes* with high-efficiency. Furthermore, the described polymorphic loci in this study should be useful for population genetic and conservation genetic studies in *M. fissipes* and other closely related species from this important genus *Microhyla*.

INTRODUCTION

Microhyla fissipes is a wide spread frog in family Microhylidae of suborder Neobatrachia. They inhabits in open lowlands (lowland scrub forest, grassland, agricultural land, pastureland, and some urban areas) at altitudes below 1,400 m, and widely distributes from western Myanmar eastward through Indochina and northward into southern China including Hainan and Taiwan (Fei *et al*., 2009; Yuan *et al*., 2016). Matsui *et al*. (2011) detected that *M. ornata* (sensu lato) is a composite of three discrete lineages, *M. ornate*, *M. fissipes* and *M. okinavensis*. Yuan *et al*. (2016) found that *M. fissipes* species complex could further divided into two clades (*M. fissipes* and *M. mukhlesuri*) by Red River since late Miocene tectonic movement using DNA sequence data from both mitochondrial and nuclear genes. However genetic diversity and structure in genus *Microhyla*, especially *M. fissipes* were not well known, which is essential for management and preservation. Therefore, taxonomy and genetic studies are needed in *M. fissipes* to identify the potential cryptic species, illustrate lineage diversification, and reveal the population structure and distribution pattern by developing more molecular markers.

Microsatellites, also termed simple sequence repeats (SSRs), consist of tandemly arranged repeats of short DNA motifs (1-6 bp in length) (Zhu *et al*., 2017). Microsatellite has become one of the most attractive markers for the determination of hybridization, genetic diversity, genetic mapping, gene tagging, gene flow and molecular evolution (Cheng *et al*., 2016a; Liu *et al*., 2018). Furthermore, the conservation of microsatellite flanking regions allows using primers isolated from a particular species in another closely related one to reduce the cost of isolation of species-specific primers (Buzatti *et al*., 2016; Wang *et al*., 2018). Since the transcripts represent transcribed regions of the genome, the candidate microsatellites from the transcriptome are expected to have high cross-species transferability (Wei *et al*., 2017). Cross-species

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amplification is a prerequisite for multispecies studies, such as analyses of hybrid zones, phylogenic analysis and comparative linkage mapping. Therefore, ways to enhance the utility of markers across more species have been proposed (Dufresnes *et al*., 2014).

At present, microsatellites of *M. fissipes* were just developed from genome by FIASCO (Fast isolation by AFLP of sequences containing repeats) (Zhang *et al*., 2013). In addition, transferability in genus *Microhyla* was not tested for any microsatellite. Transcriptome analysis by next-generation sequencing (NGS) has become a powerful and convenient tool in biology, which provided a cost and time effective way for the development of microsatellite markers (Chen *et al*., 2016; Gutiérrez *et al*., 2017). Furthermore, microsatellites developed from transcriptome without noncoding DNA are thought to be more transferrable in closed related species than those from random genome sequence.

In this study, genic microsatellites were isolated and characterized from the transcriptome of *M. fissipes* dorsal muscle, and successfully screened in the Sichuan basin population of 83 individuals. Then cross-species transferability was tested in *M. butleri*, *M. heymonsi*, *M. pulchra* and *M. mixture* from *Microhyla* genus. The new developed microsatellite markers in this study could enrich the current resource of molecular markers and would facilitate genetic and molecular population studies in *M. fissipes* and other congeneric species.

Table I.- Samples used in this study.

Species	Voucher No.	Population	Location	GPS position
M. fissipes	20110802001-020	Sichuan basin (SB)	Anxian, Sichuan	31°21′15.876″N, 104°11′32.100″E
	20120707001-018	SB	Jianyang, Sichuan	30°11′1.824″N, 104°10′13.259″E
	20110963-983	SB	Nanjiang, Sichuan	32°12′37.476″N, 106°29′35.628″E
	20110908001-024	SB	Pujiang, Sichuan	30°6′54.720″N, 103°18′10.152″E
M. butleri	2013063101		Liangping, Chongqing	30°40'17.574"N, 107°47'11.214"E
	HN052		Chengmai, Hainan	19°41'39.06"E, 110°0'43.55"N
	HN700		Baisha, Hainan	19°6'36.66"E, 109°32'33.04"N
	2013063717		Qijiang, Chongqing	30°46'2.784"E, 108°20'5.388"N
	2013063811		Shizhu, Chongqing	30°1'45.516"E, 108°20'5.388"N
	151363		Huizhou, Guangdong	23°18'20.700"N, 114°24'2.556"E
	151337		Luoding, Guangdong	22°44'53.412"N, 111°35'7.944"E
	2013063703		Qijiang, Chongqing	30°46'2.784"E, 108°20'5.388"N
M. pulchra	HN025		Haikou, Hainan	19°58'53.940"N, 110°14'38.890"E
	HN092		Danzhou, Hainan	19°42'48.500"N, 109°17'1.040"E
	GX364		Congzuo, Guangxi	22°26'7.539"N, 107°21'55.270"E
	GX425		Ningming, Guangxi	21°48'38.380"N, 107°18'18.930"E
	GX516		Yulin, Guangxi	22°38'12.600"N, 111°41'42.370"E
	HNNU0606043		Wuzhishan, Hainan	18°46'0.849"N, 109°30'21.360"E
	HNNU0606040		Wuzhishan, Hainan	18°46'0.849"N, 109°30'21.360"E
	151338		Luoding, Guangdong	22°44'53.412"N, 111°35'7.944"E
M. heymonsi	Jiangkou069		Tongren, Guizhou	27°34'44.299"N, 109°35'47.500"E
	Hekou011		Hekou, Yunnan	22°30'48.766"N, 103°56'56.421"E
	LYLa0017		Jinhua, Zhejiang	28°35'27.600" N, 120°19'28.920"E
	LYLa0018		Jinhua, Zhejiang	28°35'27.600"N, 111°41'42.370"E
	151340		Luoding, Guangdong	22°44'53.412"N, 111°35'7.944"E
	20160046		Zhijin, Guizhou	26°38'32.928"N, 105°47'8.124"E
	141431		Congzuo, Guangxi	22°26'7.54″N, 107°21'55.270"E
	HS		Huangshan, Anhui	30°08′06.260″N, 118°14′27.680″E
M. mixtura	2013051805-12		Wanyuan, Sichuan	31°53'36.42"N, 107°56'18.456"E

MATERIALS AND METHODS

Transcriptome sequencing and microsatellite mining

Dorsal muscle tissues from *M. fissipes* were collected for transcriptome sequencing. Then 12 libraries of the dorsal muscle of *M. fissipes* produced about 120 G clean data, with average of 10 G of one library. The entire sequencing data generated in the study have been deposited in the Gene Expression Omnibus (GEO) database under the accession numbers GSE108552. After quality control, de novo transcriptome assembly and gene annotation, the MIcroSAtellite (MISA, http://pgrc.ipk-gatersleben.de.sci-hub.org/misa/) Perl script was employed to identify microsatellites in unigenes. In this study, genic microsatellites were considered to contain motifs of two to six nucleotides with a minimum of five contiguous repeat units. Based on the assembled unigene sequences containing the microsatellite, the primer pairs for the flanking sequences of each unique microsatellite were designed using primer premier version 5.00 (PREMIER Biosoft International, Palo Alto, CA).

All experiments were performed according to the guideline for the care and use of laboratory animals in China and proved by the Experimental Animal Care and Use Ethics Committee of the Chengdu Institute of Biology, Chengdu, China (Permit Number: 1603).

DNA extraction and PCR amplification

Samples (n=83) of *M. fissipes* from the Sichuan basin collected by Zhang *et al*. (2013) (Table I) were used for primer validation and population analysis. Total genomic DNA was extracted from muscle tissues (preserved in 95% ethanol) using TIANamp Genomic DNA Kit (TIANGEN Biotech Co., Ltd.). PCR amplifications were carried out in 12 µl volume containing 30-50 ng genomic DNA, 0.3 µM for each primer, and 6 µl EasyTaq PCR SuperMix (TransGen Biotech Co., Ltd.). The amplification programmed the following conditions: an initial denaturation at 94 °C for 3 min; 35 cycles including denaturation at 94 °C for 35 s, annealing at the proper temperature (Table II) for 40 s and elongation at 72 °C for 40 s, and a final elongation at 72 °C for 5 min. PCR products were visualized by electrophoresis on 1.5% agarose gels. DL 2000 Markers (Qingke Biotech Co., Ltd.) were tested for amplification success and specificity in *M. fissipes*. Then the developed primers were amplified on 6 samples randomly selected from 83 samples for the polymorphism analysis. PCR products were size-fractionated on 10% polyacrilamide gels and visualized by silver staining for primer and polymorphism validation. pBR322 DNA/*Msp*I molecular weight marker (TIANGEN Biotech Co., Ltd.) was used as size standard to identify alleles. For the primers that could successfully yield clear and polymorphism target products, the forward primer of each pair was labeled with one of the fluorescent dye FAM, HEX or ROX (Table II), and ran the PCR-products on an ABI-3730XL sequencer by Sangon Biotech Co. Ltd. (Shanghai, China). For checking the microsatellite sequence, PCR product was ligated into pMD 18-T vector (TaKaRa, Tokyo, Japan) and sequenced using an automated DNA sequencer ABI 3730XL.

Statistical analysis

The electropherograms of microsatellites were scored with GeneMarker v1.85 (SoftGenetics LLC). The microsatellite loci diversity was estimated using PopGene version 1.32 (Yeh *et al*., 2000) which included the following parameters: the number of alleles (N_a), the observed heterozygosity (H_o) and expected heterozygosity (H_e). Exact tests for the Hardy-Weinberg equilibrium (HWE) were also performed by using arlequin version 3.0 (Excoffier *et al*., 2005). Sequential Bonferroni correction was applied to adjust the results for multiple simultaneous comparisons (Rice, 1989). Polymorphism information content (PIC), were calculated with CERVUS v3.0 (Kalinowski *et al*., 2007).

Cross-species amplification

Each eight individuals of *M. butleri*, *M. heymonsi* *M. pulchra*, and *M. mixtura* were used for cross-species amplification analysis (Table I). All 35 genic microsatellites which could be successfully amplified in *M. fissipes* were used to evaluate cross-species transferability and polymorphisms in a panel consisting of 8 randomly selected varieties of four related species, respectively. PCR amplification, size-fractionated and sequencing is the same as above.

RESULTS AND DISCUSSION

Detection of genic microsatellites

A total of 17,339 potential microsatellites were identified in 15,385 unigenes, of which, 13,798 unigenes contained only one microsatellite, and 1,587 contained more than one microsatellite. In addition, 1,039 were present in compound form (with adjacent tandem simple repeats of a different sequence).

Genic microsatellites with six tandem repeats (40.50%) were the most common, followed by five (25.81%), seven (17.28%) and eight (7.92%) tandem repeats, whereas the remaining tandem repeats accounted for less than 10% (Table III). Furthermore, five types of microsatellites, from dinucleotide to hexanucleotide repeats among the unigenes were detected in this study. Dinucleotide repeats accounting for 65.5% (11356/17339) were the most abundant type, followed by tri- nucleotide repeats accounting for 28.8% (4990/17339), the phenomenon was

similar to previous reports on other vertebrate (Deng *et al*., 2014; Li *et al*., 2012; Mohindra *et al*., 2012). Within the types of dinucleotide repeat microsatellites, AG/CT is most prevalent repeat type, followed by AT/AT and AC/GT, while the CG/GC motif nearly absent (Supplementary Table I). The bias towards AG and against CG repeats has been demonstrated across eukaryotes (Tóth *et al*., 2000). It may be due to the methylation of cytosine, which increases its chance of mutation to thymine by deamination (Mohindra *et al*., 2012), while the extremely low frequency of CG/GC dinucleotide repeats were also detected in genomes. Among the types of trinucleotide and tetranucleotide repeat microsatellites, the three of ten kinds AGG/CCT, AGC/GCT, and AAT/ATT together accounted for 61.1%, while the two of 29 kinds AAAG/CTTT and AGAT/ATCT together accounted for 47.1%, indicating that they are respectively most prevalent motif in its repeat type (Supplementary Table I). The bias above was also detected in other vertebrates (Chen *et al*., 2016; Sathyanarayana *et al*., 2017; Zhang *et al*., 2004).

Table II.- Characterization of the 14 polymorphic microsatellite loci developed for *Microhyla fissipes*.

Locus	GenBank accession No.	Primer sequences (5'-3')	Repeat motif	Allele size (bp)	*Ta* (°C)	Fluorescent dye	BLASTX top hit description (species)*	E-value
Mic1	MH175502	F: TTGGACGTAAGTTGGGAGT R: CATGAGAAAGAGGCTGTGAA	$(CAT_G)5$	202	52	FAM	unknown	
Mic3	MH175503	F: GGAAGGGATTGGATTGGC R: CGACAGAGGCGGGTAGAT	$(GA_G)6$	234	58	HEX	unknown	
Mic6	MH175504	F: AATCCCCGTCCTTCTCCG R: AGTCATGGGTCTGGCGTCTC	$(A_G)8$	257	58	HEX	unknown	
Mic9	MH175505	F: GGAGGGAAGTGACAGAGG R: CCAGGGGAGATGAAGAAG	$(AGCC)_5$ $(CCT)_6$	237	58	HEX	homeobox C8 (*Xenopus tropicalis*)	0
Mic10	MH175506	F: ACCGTGCTGCCTCGCCTTCC R: CTTGCCCAGGTTCAGGTAGCCG	$(TGC)_5$	163	65	ROX	subtilisin-like kinase SPC4 (*Xenopus tropicalis*)	0
Mic12	MH175507	F: CACCGCTGTATCCCTCCG R: GCCAGTCACGGCTCCCTA	$(CT)_6$	233	65	ROX	nuclear receptor coactivator 2 (*Xenopus tropicalis*)	9E-18
Mic15	MH175508	F:GGGTGCTGAAGGACCTTATA R:AACTCCTGGTGTACTTATTCTCAG	$(TG_G)5$	134	65	ROX	Probable cation-transporting ATPase 13A4 (*Gallus gallus*)	0
Mic24	MH175509	F: AAAGCAAGTATGACCAAGC R: GGACAAAAGTGAGGAGCA	$(GCA_)5$	244	60	FAM	uncharacterized protein LOC105947883 (*Xenopus tropicalis*)	8.8E-27
Mic26	MH175510	F: GTTGGAAAGTGAAATCGGC R: AGCCCTGTTGCCTGTCTTA	$(CAT_A)5$	244	60	FAM	serine/threonine-protein kinase Nek5 isoform X3 (*Xenopus tropicalis*)	4.2E-42
Mic27	MH175511	F:TGGATGCTACGAATGGAGAC R:CCAAAGAGGAGCCAATAAGG	$(AT_)7$	244	65	ROX	lipoma HMGIC fusion partner-like 4 (*Xenopus tropicalis*)	3.78E-106
Mic41	MH175512	F: CGCACTATCACAGCCCGACC R:CCGGAGAAGAACTCGCCATG	$(CA_G)5$	139	68	ROX	Angiomotin (*Xenopus tropicalis*)	0
Mic44	MH175513	F: GACTCGCTGCTTCGGCTCT R: GTGTTTTGGGGTTGAGGGG	$(GC_A)6$	212	58	HEX	unknown	
Mic45	MH175514	F: TCTAACAGATGGAGGAGTGG R: ATTGGTGCTTCAGAGTCATT	$(GAGC_)6$	254	58	FAM	unknown	
Mic51	MH175515	F: CAGTGCCCTGCCAAAGAG R: CAGATCCGAGCCAATCCA	$(GC_T)6$	299	58	FAM	Cullin-associated NEDD8-dissociated protein 1 (*Columba livia*)	8E-23

* Putative functional annotation by the NCBI nr database search.

Table III.- Frequency of SSRs based on repeat types in *M. fissipes* transcriptome.

Repeat type	5	6	7	8	9	10	11	12	>12	Total	Percentage (%)
Dinucleotide	0	5926	2612	1356	706	530	220	6	0	11356	65.49
Trinucleotide	3560	1030	382	17	0	1	0	0	0	4990	28.78
Tetranucleotide	893	66	1	0	1	0	0	0	1	962	5.55
Pentanucleotide	15	1	0	0	3	0	0	0	0	19	0.11
Hexanucleotide	8	0	1	0	0	0	3	0	0	12	0.07
Total	4476	7023	2996	1373	710	531	223	6	1	17339	1
Percentage (%)	25.81	40.50	17.28	7.92	4.09	3.06	1.29	0.03	*		

* indicated the value is 5.76735E-05.

Table IV.- Genetic diversity revealed by 14 microsatellites in *M. fissipes* population.

	Na	H_o	H_e	PIC	P
Mic1	3	0.17	0.16	0.152	0.884
Mic3	3	0.20	0.18	0.168	0.827
Mic6	4	0.31	0.30	0.284	0.056
Mic9	4	0.39	0.37	0.339	0.712
Mic10	3	0.57	0.52	0.444	0.141
Mic12	2	0.08	0.08	0.078	0.711
Mic15	2	0.02	0.02	0.024	0.938
Mic24	3	0.63	0.47	0.366	0.010*
Mic26	2	0.39	0.31	0.263	0.033*
Mic27	3	0.10	0.12	0.111	0.088
Mic41	2	0.27	0.39	0.314	0.003
Mic44	3	0.92	0.59	0.505	p<0.01**
Mic45	3	0.70	0.62	0.537	0.139
Mic51	2	0.14	0.13	0.125	0.498

Na, numbers of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; PIC, polymorphism information content; P, P-value for deviation from Hardy-Weinberg equilibrium (*, p<0.05; **, p<0.01).

Genic microsatellite validation

Of the 61 primer pairs selected, 35 (57.38%) successfully amplified in *M. fissipes*, 14 primer pairs were polymorphisms in these 83 samples. Thus, other microsatellites obtained from our transcriptome data can provide a larger pool for mining more polymorphic loci. And polymorphism could not be correlated to the number of microsatellite repeats and the type of repeat in our study, since microsatellite polymorphisms are positively correlated with the number of repeats in other specie (Ellegren, 2004; Kayser *et al*., 2004; Mun *et al*., 2006; Zhang *et al*., 2014). Furthermore, cloning and sequencing of microsatellite size variant amplicons revealed that the variation in the number of repeats was the source of microsatellite fragment polymorphism. The amplification failure of some primers may be caused by the location of the primers across splice sites, the presence of large intron between primers, chimeric primers, or poor quality sequences.

To confirm the 14 polymorphic genic microsatellites developed in this study, we performed a validation in *M. fissipes* population from Sichuan basin. In total, the number of alleles per locus varied from 2 to 4. The observed heterozygosity (H_o) ranged from 0.02 to 0.92, with average 0.349, while the expected heterozygosity (H_e) was from 0.02 to 0.62, with average 0.304 (Table IV). The PIC is an important parameter for microsatellite polymorphisms, loci was classified as highly informative (PIC > 0.5), reasonably informative (0.25 < PIC < 0.5), or slightly informative (PIC < 0.25) (Botstein *et al*., 1980; Zhang *et al*., 2013). According to this standard, 2 of the 14 microsatellite loci in this study are highly informative, while 6 are reasonably informative. The average PIC values of 14 loci in the population were 0.265±0.162. Microsatellites from the transcriptome were less polymorphic than genomic microsatellites, but possessed potential polymorphisms. Deviation from the Hardy-Weinberg equilibrium (HWE) (p < 0.01) was evident at one locus (Mic44) (Table IV). Generally, it could be due to the limited sample size, size homoplasy, or the presence of null alleles (Olivatti *et al*., 2011). No significant evidence for linkage disequilibrium existed in any comparisons by location or by locus after Bonferroni correction (p < 0.01). Thus, analyses showed that genic microsatellite markers described here were validated and could be used in future genetic studies.

Functional annotation

To assess the putative functional determination of unigenes containing polymorphism microsatellites, BLASTX searches were performed against the GenBank non-redundant protein database (nr) with a threshold E value cutoff of 1E-5. Out of the 14 unigene with polymorphism microsatellites, 9 had significant hits for their putative identities including 8 annotated for specific

functions (Table II). Homeobox C8 (HOXC8) belongs to the 39-member HOX family, which plays an important role in morphogenesis in all multicellular organisms, and it plays a role in the regulation of cartilage differentiation and the tumorigenesis of various cancer types (Cheng *et al*., 2016b). Higher polymorphism of Mic9 among all loci indicated that polymorphism of homebox C8 may somehow affected their morphologic characteristics for adaptation. On the other hand, non-annotated unigenes with microsatellites could be due to the sequences being incomplete, too short or the novel protein. However, the unannotated unigenes are still valuable sources of microsatellite markers, and will likely be identified when they cluster with additional transcripts produced in the future. Therefore, being parts of genes, genic microsatellites are more useful as molecular markers, as they represent variation in the expressed part of the genome.

Cross-species transferability in genus Microhyla

One drawback of microsatellites is their high species-specificity, which resulted in low cross-amplification success (Hyun *et al*., 2017). Nevertheless, genic microsatellite loci, being derived from coding region of the genome, are expected to be more conserved and more cross transferable as expectation. For 35 genics markers successfully amplified in *M. fissipes*, 30 of these successfully amplified in at least one species and 10 successfully amplified in all four species. Especially, 13 (37.14%), 16 (45.71%), 20 (57.14%) and 25 (71.43%) amplification succeeded in *M. butleri*, *M. pulchra*, *M. heymonsi* and *M. mixtura*, respectively (Table V). The high cross-species amplification of microsatellite from *M. fissipes* indicated that the sequences containing microsatellite loci were conserved across species of the *Microhyla* genus. The extent of cross-transferability of a marker system determines its suitability in other genetic studies. Species from *Microhyla* are non-model organisms, lack genome sequence and maker information. These cross-species transferable markers indicated their potential utilization in these four related species. The amplification success rate of genic marker depended on their phylogenetic and the evolutionary relationship (Matsui *et al*., 2011). Interestingly, the closer of the specie to *M. fissipes* in phylogenetic, the higher rate of cross transferability was detected.

Furthermore, 6, 10, 12, and 13 were polymorphic with a banding pattern that could be clearly resolved in *M. butleri*, *M. pulchra*, *M. heymonsi* and *M. mixtura*, respectively. Reusing microsatellite markers might be tedious in amphibians, where cross amplification success was unpredictable and unexpectedly low, presumably because of their genome size and complexity and the relatively low number of potentially amplifying loci (Dufresnes *et al*., 2014). Our results indicated that development of genic microsatellite from transcriptome would not only be a useful system for identifying massive numbers of microsatellites in the specific species, but also be high-efficiency in their related species of the same genus in amphibian.

Table V.- Transferability (Tr) of the *M. fissipes* microsatellites to related species.

Species	No. successful ampl.	Tr (%)	No. of polym.	Polym. (%)
M. butleri	13	37.14	6	46.15
M. pulchra	16	45.71	10	62.50
M. heymonsi	20	57.14	12	60.00
M. mixtura	25	71.43	13	52.00

ampl., amplification; Tr, transferability; polym., polymorphism.

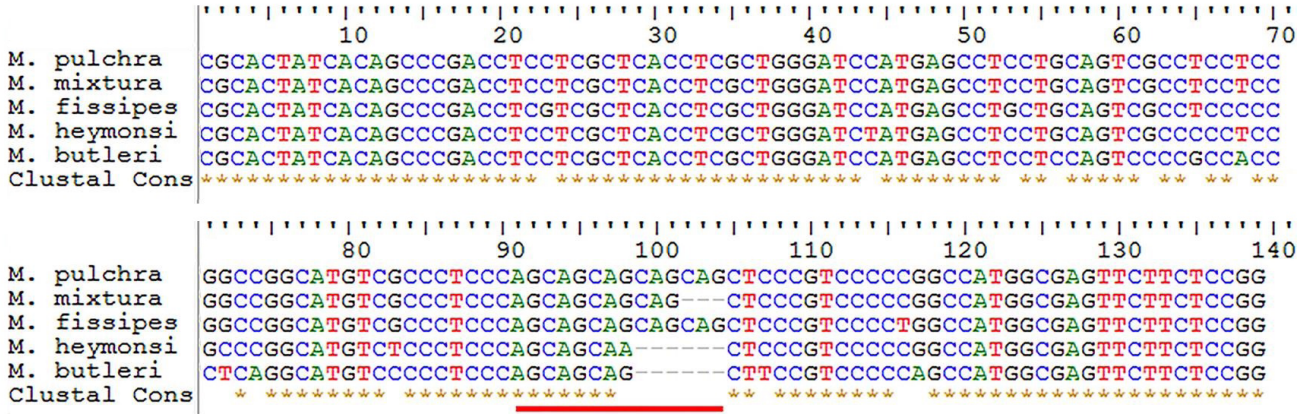

Fig. 1. Alignment of the nucleotide sequences of the microsatellite species-specific alleles at locus Mic41 amplified from 5 species of genus *Microhyla*. Asterisk represents the consensus.

Then, PCR amplification of Mic41 in five *Microhyla* species was sequenced to check the cross species conservation and transferability. Multiple alignments of nucleotide sequences of Mic41 in five species were conducted and the motif in different repeat number is shown in Figure 1.

CONCLUSIONS

We firstly used transcriptome to efficiently develop 14 microsatellite loci and provide massive microsatellites resources for *M. fissipes*. Genic microsatellite markers described here were validated and could be used in future studies (*i.e.,* population genetics, behavioral ecology, *etc*.). Furthermore, transferability of these microsatellite loci was effective for the 4 congeneric species.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1881.1889

Statement of conflict of interest

We declare that we have no conflict of interest.

REFERENCES

Botstein, D., White, R.I. and Skolnick, M., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.,* **32**: 314-331.

Buzatti, R.S.O., Chicata, F.S.L. and Lovato, M.B., 2016. Transferability of microsatellite markers across six *Dalbergia* (Fabaceae) species and their characterization for *Dalbergia miscolobium*. *Biochem. Syst. Ecol.,* **69**: 161-165. https://doi.org/10.1016/j.bse.2016.07.017

Chen, X., Li, J., Xiao, S. and Liu, X., 2016. *De novo* assembly and characterization of foot transcriptome and microsatellite marker development for *Paphia textile*. *Gene,* **576**: 537-543. https://doi.org/10.1016/j.gene.2015.11.001

Cheng, J., Zhao, Z., Li, B., Qin, C., Wu, Z., Trejo-Saavedra, D.L., Luo, X., Cui, J., Rivera-Bustamante, R.F., Li, S. and Hu, K., 2016a. A comprehensive characterization of simple sequence repeats in pepper genomes provides valuable resources for marker development in *Capsicum*. *Scient. Rep.,* **6**: 18919. https://doi.org/10.1038/srep18919

Cheng, L., Wei, X., Zhao, K., Wu, F., Lu, W., Tong, S. and Yu, G., 2016b. The predictive potential and oncogenic effects of HOXC8 expression on osteosarcoma. *Tumor. Biol.,* **37**: 14961-14967. https://doi.org/10.1007/s13277-016-5384-4

Deng, Y., Lei, Q., Tian, Q., Xie, S., Du, X., Li, J., Wang, L. and Xiong, Y., 2014. *De novo* assembly, gene annotation, and simple sequence repeat marker development using Illumina paired-end transcriptome sequences in the pearl oyster *Pinctada maxima*. *Biosci. Biotech. Biochem.,* **78**: 1685-1692. https://doi.org/10.1080/09168451.2014.936351

Dufresnes, C., Brelsford, A., Béziers, P. and Perrin, N., 2014. Stronger transferability but lower variability in transcriptomic-than in anonymous microsatellites: Evidence from Hylid frogs. *Mol. Ecol. Resour.,* **14**: 716-725. https://doi.org/10.1111/1755-0998.12215

Ellegren, H., 2004. Microsatellites: Simple sequences with complex evolution. *Nat. Rev. Genet*., **5**: 435. https://doi.org/10.1038/nrg1348

Excoffier, L., Laval, G. and Schneider, S., 2005. Arlequin (version 30): An integrated software package for population genetics data analysis. *Evol. Bioinform. Online*, **1**: 47-50. https://doi.org/10.1177/117693430500100003

Fei, L., Hu, S., Ye, C. and Huang, Y., 2009. *Fauna of China*. Amphibians Science Press, Beijing.

Gutiérrez, E.G., Hernández, C.G., León-Paniagua, L.S., Martínez, M.N. and Ortega, J., 2017. Isolation and characterization of microsatellite markers for Sturnira parvidens and cross-species amplification in *Sturnira* species. *PeerJ.,* **5**: e3367. https://doi.org/10.7717/peerj.3367

Hyun, Y.S., Song, H.Y., Wool, J.Y., Oh, S., Kim, M.S. and An, H.S., 2017. Isolation and characterization of 20 polynucleotide microsatellite markers in a vulnerable Korean snail, Ellobium chinense, using 454 pyrosequencing. *Genes Genom.,* **39**: 155-160. https://doi.org/10.1007/s13258-016-0482-7

Kalinowski, S.T., Taper, M.L. and Marshall, T.C., 2007. Revising how the computer program cervus accommodates genotyping error increases success

in paternity assignment. *Mol. Ecol.,* **16**: 1099-1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x

Kayser, M., Kittler, R., Erler, A., Hedman, M., Lee, A.C., Mohyuddin, A., Mehdi, S.Q., Rosser, Z., Stoneking, M., Jobling, M.A., Sajantila, A. and Tyler-Smith, C., 2004. A comprehensive survey of human Y-chromosomal microsatellites. *Am. J. Hum. Genet.,* **74**: 1183-1197. https://doi.org/10.1086/421531

Li, D., Deng, Z., Qin, B., Liu, X. and Men, Z., 2012. *De novo* assembly and characterization of bark transcriptome using Illumina sequencing and development of EST-SSR markers in rubber tree (*Hevea brasiliensis* Muell Arg). *BMC Genomics,* **13**: 192. https://doi.org/10.1186/1471-2164-13-192

Liu, H.X., Ye, Y.W., Li, Y., Liu, X.L., Xiong, D.M. and Wang, L.X., 2018. Genetic Characterization of the endangered *Brachymystax lenok tsinlingensis* (Salmonidae), populations from tsinling mountains in china using microsatellite markers. *Pakistan J. Zool.,* **50**: 743-749. https://doi.org/10.17582/journal.pjz/2018.50.2.743.749

Matsui, M., Hamidy, A., Belabut, D.M., Ahmad, N., Panha, S., Sudin, A., Khonsue, W., Oh, H.S., Yong, H.S., Jiang, J.P. and Nishikawa, K., 2011. Systematic relationships of Oriental tiny frogs of the family *Microhylidae* (Amphibia, Anura) as revealed by mtDNA genealogy. *Mol. Phylogenet. Evol.,* **61**: 167-176. https://doi.org/10.1016/j.ympev.2011.05.015

Mohindra, V., Singh, A., Barman, A.S., Tripathi, R., Sood, N. and Lal, K.K., 2012. Development of EST derived SSRs and SNPs as a genomic resource in Indian catfish, *Clarias batrachus*. *Mol. Biol. Rep.,* **39**: 5921-5931. https://doi.org/10.1007/s11033-011-1404-z

Mun, J.H., Kim, D.J., Choi, H.K., Gish, J., Debellé, F., Mudge, J., Denny, R., Endré, G., Saurat, O., Dudez, A.M., Kiss, G.B., Roe, B., Young, N.D. and Cook, D.R., 2006. Distribution of microsatellites in the genome of medicago truncatula: a resource of genetic markers that integrate genetic and physical maps. *Genetics,* **172**: 2541-2555. https://doi.org/10.1534/genetics.105.054791

Olivatti, A.M., Boni, T.A., Silva-Júnior, N.J., Resende, L.V., Gouveia, F.O. and Telles, M.P.C., 2011. Heterologous amplification and characterization of microsatellite markers in the Neotropical fish *Leporinus friderici*. *Genet. Mol. Res.,* **10**: 1403-1408. https://doi.org/10.4238/vol10-3gmr1020

Rice, W.R., 1989. Analyzing tables of statistical tests. *Evolution,* **43**: 223-225. https://doi.org/10.1111/j.1558-5646.1989.tb04220.x

Sathyanarayana, N., Pittala, R.K., Tripathi, P.K., Chopra, R., Singh, H.R., Belamkar, V., Bhardwaj, P.K., Doyle, J.J. and Egan, A.N., 2017. Transcriptomic resources for the medicinal legume *Mucuna pruriens*: *de novo* transcriptome assembly, annotation, identification and validation of EST-SSR markers. *BMC Genomics,* **18**: 409. https://doi.org/10.1186/s12864-017-3780-9

Tóth, G., Gáspári, Z. and Jurka, J., 2000. Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Res.,* **10**: 967-981. https://doi.org/10.1101/gr.10.7.967

Wang, L., Gong, Y., Chen, K., Wang, H.T. and Lyu X.G., 2018. Microsatellite loci identified by cross-species amplifications in the globally vulnerable relict gull, *Larus relictus*. *Pakistan J. Zool.,* **50**: 791-793. https://doi.org/10.17582/journal.pjz/2018.50.2.sc8

Wei, Z., Zhang, H., Wang, Y., Li, Y., Xiong, M., Wang, X. and Zhou, D., 2017. Assessing genetic diversity and population differentiation of colored calla lily (Zantedeschia Hybrid) for an efficient breeding program. *Genes,* **8**: 168. https://doi.org/10.3390/genes8060168

Yeh, F., Yang, R., Boyle, T., Ye, Z. and Xi, J., 2000. PopGene32, *Microsoft Windows-based freeware for population genetic analysis*, Version 132. Available at: https://www.scienceopen.com/document?vid=2d45ad78-b140-4b66-b80f-2c9f513ec997 (Accessed on 19 September, 2018).

Yuan, Z.Y., Suwannapoom, C., Yan, F., Poyarkov, J.N.A., Nguyen, S.N., Chen, H.M., Chomdej, S., Murphy, R.W. and Che, J., 2016. Red River barrier and Pleistocene climatic fluctuations shaped the genetic structure of *Microhyla fissipes* complex (Anura: Microhylidae) in southern China and Indochina. *Curr. Zool.,* **62**: 531-543. https://doi.org/10.1093/cz/zow042

Zhang, J., Wang, J. and Jiang, J.P., 2013. Development and characterization of 15 polymorphic microsatellite markers for a high adaptive and wide-range frog (*Microhyla fissipes*). *Asian Herpetol. Res.,* **4**: 5.

Zhang, L., Yuan, D., Yu, S., Li, Z., Cao, Y., Miao, Z., Qian, H. and Tang, K., 2004. Preference of simple sequence repeats in coding and non-coding regions of *Arabidopsis thaliana*. *Bioinformatics,* **20**: 1081-1086. https://doi.org/10.1093/bioinformatics/bth043

Zhang, S., Tang, C., Zhao, Q., Li, J., Yang, L., Qie, L.,

Fan, X., Li, L., Zhang, N., Zhao, M., Liu, X., Chai, Y., Zhang, X., Wang, H., Li, Y., Li, W., Zhi, H., Jia, G. and Diao, X., 2014. Development of highly polymorphic simple sequence repeat markers using genome-wide microsatellite variant analysis in Foxtail millet (*Setaria italica* (L) P Beauv). *BMC Genomics*, **15**: 78. https://doi.org/10.1186/1471-2164-15-78

Zhu, W.C., Wei, Q.G., Xue, S.Y., Zhang, H.X., Lv, T.S. and Zhang, H.H., 2017. Isolation and characterization of microsatellite markers for the sable, *Martes Zibellina* (Mammalia: Mustelidae). *Pakistan J. Zool.,* **49**: 1909-1012. https://doi.org/10.17582/journal.pjz/2017.49.5.sc1