Rapid development of novel microsatellite markers from *Mauremys reevesii* (Testudines: Geoemydidae) using next-generation DNA sequencing technology

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(Received 25 January 2018; accepted 10 June 2018)

**Abstract**

*Mauremys reevesii* (Gray, 1831), which belongs to *Mauremys* of Geoemydidae (Testudines), distributed in China, as well as Japan and Korea. Previous studies have developed several polymorphic microsatellite loci, but most of them were dinucleotide motifs. Here, we developed 15 polynucleotide-repeat microsatellite loci (including di-, tri, tetra- and penta-nucleotide motifs) for *M. reevesii* through Restriction-site Associated DNA tags sequencing (RAD-seq). A total of 987 microsatellite loci with flanking sequences were suitable for setting primers for polymerase chain reactions (PCR). To verify the identified SSRs, 40 primer pairs were selected for PCR detection. In total, 32 primer sets produced strong PCR products matching their expected sizes, in which species amplification tests showed that 15 were polymorphic. And the number of alleles per locus ranged from 3 to 16. The observed and expected heterozygosity per locus varied from 0.3784 to 1.000 and from 0.3995 to 0.9700, respectively. The methodology of microsatellite isolation constructed in this study is not only cost-effective and time-saving in comparison to traditional approaches, but also can be served as useful tools which benefit population genetics studies and conservation management of *M. reevesii*.

**Keywords:** *M. reevesii*, microsatellite loci, RAD-seq, dinucleotide, pentanucleotide

**Introduction**

Microsatellites are short tandem DNA repeats (Bu et al. 2011) and have been broadly used to assess genetic population structure, construct genetic linkage map and so on due to the features of highly polymorphic and co-dominant (Bu et al. 2014; Zhang et al. 2016). Traditionally, the isolation of microsatellites markers has two major approaches, the one is developed from gene library, including PIMA, FIASCO and etc, the other is the utilization of other closely related species microsatellite sequences (Liu et al. 2012). However, these methods are not only costly and time-consuming but also limited by the difficulties of de novo development in species without any genomic information. The emergence of next-generation sequencing technologies has rapidly improved the development of SSR because of its ability to generate a large amount of sequence information quickly and economically (Inoue et al. 2013; Rico et al. 2014; Hu et al. 2016).

*M. reevesii* is widely distributed in China, Japan and Korea. Because of the destruction of the habitat, overhunting and environmental pollution, the wild populations have decreased dramatically (Altherr & Freyer 2000; Spinks et al. 2004). Consequently, it has been categorized as endangered in the Chinese Red List of Threatened species. Thus, the preservation, management of genetic resources and artificial breeding required accurate genetic analysis for *M. reevesii* are important. Previous studies have been done in these areas of research population genetic structure and genetic analysis. RAPD technique was used to analyze the genetic diversity of *M. reevesii* at molecular level (Zhu et al. 2005).

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Eight novel polymorphic microsatellite loci developed by FIASCO were presented for the *M. reevesii* (Ye et al. 2009). The eight microsatellite core motifs were AC/AT, AC/GT, these microsatellites consisted of five dinucleotides and three compound motifs were unitary. In order to isolate more types of microsatellite for analyzing the genetic diversity, we reported the development of novel microsatellite primers (including di- to pentanucleotide motifs) for *M. reevesii* using RAD-seq technology (Castoe et al. 2012; Brandt et al. 2014; Nugraha et al. 2014).

**Materials and methods**

**Sample collection and DNA extraction**

Procedures involving animals and their care were consistent with NIH guidelines (NIH Pub.No. 85–23, revised 1996) and in accordance with the approval of the Committee of Anhui Normal University under approval number #20130710.

Three sexually mature turtles (*M. reevesii* (♀, ♂)) were, respectively, collected in Guangdong, Guangxi and Anhui. Total genomic DNA from three turtles was, respectively, extracted from the tail tissues by a standard phenol/chloroform procedure via proteinase K digestion. And the DNA quality was assessed on 1% agarose gel.

**DNA sequencing and microsatellite discovery**

Extracted DNA was sent to the Genergy Biotechnology Company and sequenced by Restriction-site Associated DNA tags sequencing. Restriction endonuclease digestion of genomic DNA was used by *PstI*. This sequencing run yielded over 22.5 M reads, with a sequencing depth of 4.04. These reads were assembled into contigs. Based on the contigs, the potential microsatellite loci were searched for simple sequence repeats (SSRs) by MISA software, 987 loci with enough flanking sequence were selected. Then, we looked for dinucleotides motifs with at least six repeats in the consensus sequences and tri-, tetra-, penta- and hexanucleotides motifs with at least five repeats, in which 40 were chosen for primer design using Oligo7.0. The primers were designed with the following criteria: (i) GC content 40–60%; (ii) product size 150–350 bp; (iii) primer length 18–25 bp; and (iv) melting temperature 50–60°C with a maximum 2°C difference between paired primers.

**SSR markers screening**

Each pair of primers was pre-tested on eight specimens of *M. reevesii*. Total genomic DNA was extracted from tail tissues by phenol-chloroform method. PCRs were performed in a total volume of 25 μL PCR mixture containing 1 μL template DNA (30–50 ng/μL), 1U Taq DNA polymerase (TaKaRa Co., Ltd, Dalian, China), 2.5 μL 10× PCR buffer, 2 μL of 25 mM MgCl₂, 2 μL of 25 mM dNTPs, 0.5 μL of 25 mM primer (each). PCR cycling was as follows: 95°C for 5 min for pre-denaturation plus, 94°C for 50 s, and at the annealing temperature for 45 s 72°C for 60 s for 33 cycles followed by an additional extension at 72°C for 10 min. The PCR products were separated with agarose gel on an 1%.

**PCR amplification and genotyping**

An M13 tail (5′-AGGTTTTTCCCCAGTCAG-3′ or 5′-GAGCGGATAAAGATCTGAGC-3′) was added to the 5′ end of each forward primer of these loci. The M13 universal primer with the same sequence to the M13 tail was labeled with FAM, TAMRA or HEX at its 5′ end. Each pair of primers was tested on 37 specimens of *M. reevesii* (7 were collected in Anhui, 14 were collected in Guangxi and 16 were collected in Guangdong), PCR experimental system and reaction program as above.

**Microsatellite cross-species amplification**

Cross-species amplifications of 15 microsatellite loci were tested in six individuals (*Mauremys megalocephala* were collected in Anhui, *M. mutica* were collected in Guangdong, *Ocadia sinensis* were collected in Anhui, two individuals of each class) by using the same amplification conditions described above. The PCR products were visualized on 1% agarose gel.

**Data analysis**

PCR products were analyzed on an ABIPRISM 3730 Genetic Analyzer by using ROX 350 or LIZ 500 (Applied Biosystems). Genemarker (Applied Biosystems) was used to analyze the size standard. Pogene version 1.32 (Yeh et al. 1997) and Genepop 4.0 (Raymond & Rousset 1995) were also used to estimate Hardy–Weinberg equilibrium (HWE), allelic counts (*N*ₐ), observed heterozygosity (*H*ₒ) and expected heterozygosity (*H*ₑ) for the loci.

**Results and discussion**

987 contigs were obtained from RAD-seq, which contain microsatellites loci, the average size of 482 bp, with dinucleotide motif were themost frequent (45.29%), followed by tri- (9.52%) except mononucleotides (40.93%). Longer motifs like tetra- (1.11%), penta-
To prevent the screening of the same loci, 15 novel microsatellite loci were compared with previous results have been reported (Ye et al. 2009), the same sequence and primers were not found. The 15 microsatellite loci generated amplification products with 3 to 16 alleles per locus. The number of alleles per locus ranged from 3 to 16. The observed and expected heterozygosity per locus varied from 0.3784 to 1.000 and from 0.3995 to 0.9700, respectively. Compared with the other turtle species, our screening microsatellite loci high polymorphism, such as the following: Fantin et al. screened 17 microsatellite loci from Podocnemis unifilis, the scope of alleles between 3 ~ 11, the range of the observed heterozygosity and expected heterozygosity was 0.208 ~ 0.950 and 0.395 ~ 0.592, respectively (Fantin et al. 2007). Que et al. screened of 15 microsatellite loci and alleles from Pelodiscus sinensis between the range of 2~7, observed heterozygosity varied from 0.03 ~ 0.98 and expected heterozygosity ranged from 0.05 ~ 0.81 (Que et al. 2007). This implies M. reevesi has a high level of genetic diversity. Besides, there were only two loci (SLN 02, SLN 08) exhibited significant deviation from Hardy-Weinberg equilibrium (Table II). It may be caused by the non-randomized sampling, the small number of individuals or the existence of null alleles.

The 15 primers could produce stable and clear bands in three species (M. megacephala, M. mutica, O. sinensis). Hereby, the results of partial cross-species amplification of PCR products in 1% agarose gel electrophoresis are shown in Figure 1, and the genotyping was exhibited in Figure 2. It revealed that the microsatellite flanking sequences of the turtles were highly conserved, which suggested that the 15 microsatellite loci in this study were conservative in Geoemydidae and could be also applied to the research of some species of Geoemydidae in genetic diversity (Baggiano et al. 2011).

RAD-seq was utilized to identify SSR markers in eggplant in the previous study (Barchi et al. 2011), the

Table I. Frequency of simple sequence repeats (SSRs) in Mauremys reevesi.

| Motif length | 4–5 | 6–7 | 8–9 | 10–11 | 12–13 | 14–15 | 16–17 | 18–19 | >20 | Total number | % |
|--------------|-----|-----|-----|-------|-------|-------|-------|-------|-----|--------------|---|
| Mono-        |     |     |     |       |       |       |       |       |     | 404          | 40.93 |
| Di-          | 302 | 75  | 28  | 11    | 7     | 6     | 5     | 13    |     | 447          | 45.29 |
| Tri-         | 80  | 12  | 2   |       |       |       |       |       |     | 94           | 9.52  |
| Tetra-       | 8   | 1   | 1   |       |       |       |       |       |     | 11           | 1.11  |
| Penta-       | 5   | 1   | 2   |       |       |       |       |       |     | 8            | 0.81  |
| Hexa-        | 4   | 1   |     |       |       |       |       |       |     | 5            | 0.51  |
| Compound     |     |     |     |       |       |       |       |       | 18 | 18           | 1.82  |
| Total        | 97  | 317 | 80  | 322   | 100   | 21    | 12    | 6     | 14  | 987          | 100   |
| %            | 9.83| 32.1| 8.11| 32.62 | 10.13 | 2.13  | 1.22  | 0.61  | 1.42|              |       |
sequences generated nearly 2,000 putative SSRs, and primer pairs were designed to amplify 1,155 loci. In this study, we obtained a smaller number of 987 target contigs with a reason that a low shearing efficiency of PstI enzyme in DNA of turtles. RAD-Seq methodology took advantage of one restriction enzyme and random shearing to generate genomic fragments, came with high levels of DNA loss and little control over the sequenced fragments, mainly for organisms without a reference genome (Hohenlohe et al. 2010). RAD-seq technology has made progress and developed, double digest RAD-seq (two kinds of enzymes were utilized in the methodology) has arisen (Bonatelli et al. 2015), it can overcome these shortcomings and increased the sequencing of the same genomic regions across individuals. This will be a new way for us to screen microsatellite in the future.

**Table II. Details for 15 polymorphic microsatellites isolated from *Mauremys reevesii.***

| Locus  | Primer sequences(5'-3')          | Repeat motif | Size range (bp) | Sample size (°C) | Sample size | NA  | HO     | HE     | p          |
|--------|----------------------------------|--------------|-----------------|------------------|-------------|-----|--------|--------|------------|
| SLN01  | F: CATGTCTGTGCTATCATTG          | (GT)10       | 165-185         | 37 52.9          | 37          | 13  | 0.6216 | 0.8634 | 0.5372     |
|        | R: CAGAATAACACGCACGGGTC         |              |                 |                  |             |     |        |        |            |
| SLN02  | F: TTAGCCCCATCATGCTGCT          | (CA)13       | 255-265         | 37 58             | 37          | 7   | 1      | 0.6801 | 0.0000*    |
|        | R: GATTTCAATAGTGCCAAGGGTT       |              |                 |                  |             |     |        |        |            |
| SLN03  | F: GCACACTTTATATTACCTA          | (TG)10       | 216-290         | 37 57.5           | 37          | 15  | 0.8919 | 0.9185 | 0.4881     |
|        | R: TAAATGCTCTAGGGCACC           |              |                 |                  |             |     |        |        |            |
| SLN04  | F: GAGGAGCTGACACCTACCA          | (TG)11       | 194-228         | 37 57.9           | 37          | 10  | 0.9189 | 0.7967 | 0.4234     |
|        | R: CTGTTGACCTGTCATGCCCA         |              |                 |                  |             |     |        |        |            |
| SLN05  | F: GAGGAGCTGACACCTACCA          | (GT)17       | 284-322         | 37 57.6           | 37          | 16  | 0.8378 | 0.8649 | 0.9327     |
|        | R: CTGTTGACCTGTCATGCCCA         |              |                 |                  |             |     |        |        |            |
| SLN06  | F: TGCTTGGTTTCCCCCTATGCTT       | (AC)11       | 260-280         | 37 52.5           | 37          | 12  | 0.7838 | 0.8497 | 0.9868     |
|        | R: TCGCGGCTGCAACACACCAC         |              |                 |                  |             |     |        |        |            |
| SLN07  | F: CAAAAATGGGTCTGGGTCG          | (AGG)5       | 267-276         | 37 62.8           | 37          | 7   | 0.5676 | 0.5298 | 0.223      |
|        | R: TCTGGAGGAGCAGGGCAAG          |              |                 |                  |             |     |        |        |            |
| SLN08  | F: CAGACGACATACACAGCAAGGAG      | (CCTTG)9     | 140-150         | 37 61.2           | 37          | 3   | 0.973  | 0.622  | 0.0000*    |
|        | R: CACCCACCGCAAAGCAT            |              |                 |                  |             |     |        |        |            |
| SLN09  | F: AAGACGACCCACAGACAGACCT       | (AGC)5       | 240-278         | 37 59.9           | 37          | 10  | 0.9459 | 0.8563 | 0.0268     |
|        | R: AGGACACCCTTCCTCCACGCCGT      |              |                 |                  |             |     |        |        |            |
| SLN10  | F: TTCTCCCTCCTCCTGAAACC         | (TTG)5       | 245-275         | 37 50.1           | 37          | 14  | 0.8108 | 0.97   | 1.0000     |
|        | R: RGTCCTCATATGGCTTCCC          |              |                 |                  |             |     |        |        |            |
| SLN11  | F: AGACCGTTTTCCTGCTTATACG       | (TG)6        | 261-281         | 37 58.3           | 37          | 6   | 0.7568 | 0.7353 | 0.5643     |
|        | R: GTGACTCTGCCTTGGACCA          |              |                 |                  |             |     |        |        |            |
| SLN12  | F: AAGTGTTCAGCTCTACC            | (ACCA)5      | 191-199         | 37 50             | 37          | 3   | 0.4054 | 0.3995 | 0.7714     |
|        | R: GCCCCTTAAATGTTTTAC           |              |                 |                  |             |     |        |        |            |
| SLN13  | F: CTTCCGGGCGGCCAAAGCCT         | (AGC)5       | 343-371         | 37 60.7           | 37          | 6   | 0.6757 | 0.7212 | 0.1126     |
|        | R: CCCCTCTATGGGCCCCTGGCCT       |              |                 |                  |             |     |        |        |            |
| SLN14  | F:CCCCCCCTTTCCGTTTTTGGCCTT      | (GAAG)6      | 217-228         | 37 57.6           | 37          | 8   | 0.3874 | 0.475  | 0.2752     |
|        | R: ACTTTTCCCCCCCCGTGCTT         |              |                 |                  |             |     |        |        |            |
| SLN15  | F: CTTTTTGCTTTCTGGCTGGGTCTC     | (CTG)10      | 396-411         | 37 50             | 37          | 7   | 0.5946 | 0.5568 | 0.4383     |
|        | R: CCTATTACGCCCCTCAGACACCAC    |              |                 |                  |             |     |        |        |            |

NA, number of alleles; HO, observed heterozygosity; HE, expected heterozygosity; p, p-value from exact tests for Hardy–Weinberg equilibrium (HWE).

*Indicates departures from HWE (p < 0.05).

**Figure 1.** The PCR products of cross-species amplification (SLN15 was chosen, M: Marker, 1 ~ 6: PCR products, 1 ~ 2 *Mauremys megaloecephala*, 3 ~ 4 *M. mutica*, 5 ~ 6 *Ocadia sinensis*).
the first time to develop polynucleotide-repeat microsatellite markers in *M. reevesii* by using RAD-sequencing technology.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This research was supported by the National Natural Science Foundation of China (NSFC, No. 31372198 and 30970351), the Research Fund of the Key Laboratory of Biotic Environment and Ecological Safety of Anhui province.

**References**

Alther S, Freyer D. 2000. Asian turtles are threatened by extinction. Turtle and Tortoise Newsletter 1:7–11.

Baggiano O, Schmidt DJ, Hughes JM. 2011. Nine microsatellite markers for the Australian side-necked turtle *Chelodina expansa* (Chelidae) and cross species amplifications. Marine Genomics 4:297. DOI:10.1016/j.margen.2011.08.002.

Barchi L, Lanteri S, Portis E, Acquadro A, Valè G, Toppino L, Rotino GL. 2011. Identification of SNP and SSR markers in eggplant using RAD tag sequencing. BMC Genomics 12:1–9. DOI:10.1186/1471-2164-12-304.

Bonaventura IAS, Carstens BC, Moraes EM. 2015. Using next generation RAD sequencing to isolate multispecies microsatellites for *Philochloeris* (Cactaceae). Plos One 10:e0142602. DOI:10.1371/journal.pone.0142602.

Brandt JR, Groot PJVCD, Zhao K, Dyck MG, Boag PT, Roca AL. 2014. Development of nineteen polymorphic microsatellite loci in the threatened polar bear (*Ursus maritimus*) using next generation sequencing. Conservation Genetics Resources 6:59–61. DOI:10.1007/s12686-013-0003-9.

Bu X, Liu L, Nie L. 2014. Genetic diversity and population differentiation of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) in three geographical populations. Biochemical Systematics & Ecology 54:279–284. DOI:10.1016/j.bse.2014.02.022.

Bu XJ, Liu L, Wang L, Nie LW. 2011. Isolation and characterization of 21 novel polymorphic microsatellite loci in the Chinese soft-shelled turtle *Pelodiscus sinensis*. Genetics & Molecular Research 10:1006–1010. DOI:10.4238/vol10-2gm1119.

Carleton KL, Steelman JT, Lee BV, Garnhart N, Kidd M, Kocher TD. 2002. Rapid isolation of CA microsatellites from the tilapia genome. Animal Genetics 33:140–144. DOI:10.1046/j.1365-2052.2002.00817.x.

Castoe TA, Poole AW, Koning APJD, Jones KL, Tombback DF, Oyler-McCance SJ, Fike JA, Lance SL, Stiecher JW, Smith EN, Pollock DD. 2012. Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. Plos One 7:e30953. DOI:10.1371/journal.pone.0030953.

Fattini C, Carvalho C, Hrbek T, Sites Jr JW, Monieló LAS, Astolfilo-Filho S, Farias IP. 2007. Microsatellite DNA markers for *Podocnemis unifilis*, the endangered yellow-spotted amazon river turtle. Molecular Ecology Resources 7:1235–1238.

Fernando P, Vidya TNC, Melnick DJ. 2001. Isolation and characterization of tri- and tetranucleotide microsatellite loci in the Asian elephant, *Elephas maximus*. Molecular Ecology Notes 1:232–233. DOI:10.1046/j.1471-8278.2001.00082.x.

Haberl M, Tautz D. 1999. Tri- and tetranucleotide microsatellite loci in honey bees (*Apis mellifera*)-a step towards quantitative genotyping. Molecular Ecology 8:1358–1360.

Hohenlohe PA, Basham S, Etter PD, Stiffler N, Johnson EA, Cresko WA. 2010. Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. PLoS Genet 6:e1000862. DOI:10.1371/journal.pgen.1000862.

Hu Z, Zhang T, Gao XX, Wang Y, Zhang Q, Zhou HJ, Zhao G-F, Wang M-L, Woeste KE, Zhao P. 2016. De novo assembly and characterization of the leaf, bud, and fruit transcriptome from the vulnerable tree *Fagulus mandshurica* for the development of 20 new microsatellite markers using illumina sequencing. Molecular Genetics and Genomics 291:849–862. DOI:10.1007/s00438-016-1251-7.

Inoue K, Lang BK, Berg DJ. 2013. Development and characterization of 20 polymorphic microsatellite markers for the Texas hornshell, *Popoiaea popeii* (Bivalvia: Unionidae), through next-generation sequencing. Conservation Genetics Resources 5:195–198. DOI:10.1007/s12686-012-9766-7.

Liu L, Nie L, Bu X, Xia X, Huang Z, Jing W, Jiang Y, Wang L. 2012. Isolation and characterization of ten novel polymorphic microsatellites loci in the Chinese pond turtle (*Mauremys reevesii*) and cross-species amplification in other Cryptodira species. Microporous & Mesoporous Materials 154:185–191.

Lü Z, Li H, Liu L, Cui W, Hu X, Wang C. 2013. Rapid development of microsatellite markers from the large yellow croaker (*Pseudosciaena crocea*) using next generation DNA sequencing technology. Biochemical Systematics & Ecology 51:314–319. DOI:10.1016/j.bse.2013.09.019.
Nugraha MFI, Pouyaud L, Carman O, Kadarusman Widjastuti U, Avarre JC. 2014. Development of twelve novel polymorphic microsatellite DNA markers for the Boeseman’s rainbowfish (Melanotaenia boesemani) and tests for their cross-utility in 21 rainbowfish species from west Papua (Indonesia). European Journal of Wildlife Research 60:941–946. DOI: 10.1007/s10344-014-0868-2.

Que Y, Zhu B, Rosenthal H, Chang J. 2007. Isolation and characterization of microsatellites in Chinese soft-shelled turtle, Pelodiscus sinensis. Molecular Ecology Notes 7:1265–1267. DOI: 10.1111/men.2007.7.issue-6.

Raymond M, Rousset F. 1995. An exact test for population differentiation. Evolution 49:1280–1283. DOI: 10.1111/evo.1995.49.issue-6.

Rico Y, Paetkau D, Harris LR, Sayers J, Ethier D, Kyle CJ. 2014. Development of nuclear microsatellite markers for American badger subspecies (Taxidea taxus, spp.) using next generation sequencing. Conservation Genetics Resources 6:715–717. DOI: 10.1007/s12686-014-0195-7.

Spinks PQ, Shaffer HB, Iverson JB, Mccord WP. 2004. Phylogenetic hypotheses for the turtle family Geoemydidae. Molecular Phylogenetics & Evolution 32:164–182. DOI: 10.1016/j.ympev.2003.12.015.

Ye R, Zheng R, Wang L, Du W. 2009. Polymorphic microsatellite loci in the Chinese pond turtle (Chinemys reevesii). Conservation Genetics 10:1045–1048. DOI: 10.1007/s10592-008-9684-0.

Yeh FC, Yang RC, Boyle TB, Ye ZH, Mao JX. 1997. POPGENE, the user-friendly shareware for population genetic analysis. Canada, University of Alberta: Molecular Biology and Biotechnology Centre 10:295–301.

Zhang HR, Niu SF, Wu RX, Zhai Y, Tian LT. 2016. Development and characterization of 26 polymorphic microsatellite markers in Lateolabrax maculatus, and cross-species amplification for the phylogenetically related taxa. Biochemical Systematics & Ecology 66:326–330. DOI: 10.1016/j.bse.2016.05.008.

Zhu XP, He-Jun DU, Zhou L, Ming-You LI, Gui JF. 2005. Genetic diversity analysis of Chinese three-keeled pond turtle (Chinemys reevesii) by RAPD. Acta Hydrobiologica Sinica 29:3215–3220.