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Differentiation of four strains of Chinese soft-shelled turtle (*Pelodiscus sinensis*) based on high-resolution melting analysis of single nucleotide polymorphism sites in mitochondrial DNA

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ABSTRACT. The Chinese soft-shelled turtle (*Pelodiscus sinensis*) has been one of the most economically important aquatic animals in China for thousands of years, and several breeding strains have been formed. Since the morphological characteristics of some strains are similar, a rapid and accurate molecular method to differentiate between strains is required. In this study, partial sequences of mitochondrial DNA from four turtle strains, Taihu Lake Strain, Taiwan Strain, Japanese Strain, and Yellow River Strain, were amplified and sequenced based on selected strain-specific single nucleotide polymorphism (SNP) sites. The corresponding primers were designed and a high-resolution melting (HRM) technique was employed for genotyping these SNPs. The results indicated that a total of seven SNPs can be detected by HRM. Among these SNPs, one can be used for identifying the Taihu Lake Strain, one for the Japanese Strain, two for the

Taiwan Strain, and three for the Yellow River Strain. This method is rapid and convenient, which offers technical support for strain identification and selective breeding in Chinese soft-shelled turtles.

Key words: Chinese soft-shelled turtle; Mitochondrial DNA; Differentiation; Single nucleotide polymorphism; High-resolution melting curve analysis

INTRODUCTION

A single nucleotide polymorphism (SNP) is a DNA sequence variation that commonly occurs within a population (e.g., 1%) in which a single nucleotide in the genome differs between members of a biological species or paired chromosomes. Its mutation types include transition, transversion, insertion, and deletion. Since SNPs are closely related to specific diseases, biological traits, and population groups, they are regarded as promising molecular markers that are generally employed for the analysis of relevance between diseases (Polonikov et al., 2008; Wong et al., 2008; Downey et al., 2009), screening of a breeding population (Brunner et al., 2000; Jeong and Maroof, 2004), and differentiation between similar species (Mabru et al., 2004), etc. The methods of SNP typing include single-strand conformation polymorphism analysis (SSCP) (Gonen et al., 1999), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Fukuen et al., 2002), denaturing high performance liquid chromatography (DHPLC) (Wolford et al., 2000), single base extension (Steemers et al., 2006), sequencing (Griffin et al., 2000), and high-resolution melting curve analysis (HRM) (Liew et al., 2004). HRM is based on PCR amplification of mutation sites via designed PCR primers. The melting curves are analyzed under the saturated concentration condition of a fluorescent dye. The genotypes of mutation sites are determined by melting temperatures (*Tm*) of amplicons and the melting curves. Overall, HRM represents a novel high-throughput genotyping method with low cost and convenience, which has been gradually applied in fisheries research.

The Chinese soft-shelled turtle (*Pelodiscus sinensis*) has been one of the most economically important aquatic animals in China for thousands of years, and several strains have been formed with distinct breed characteristics. However, efficient methods to differentiate between strains are lacking, and this has seriously restricted the development and protection of the resources of the Chinese soft-shelled turtle. As the morphological characteristics of some strains are too similar to enable identification, a rapid and accurate molecular differentiation method is required. Current research on different strains of Chinese soft-shelled turtle is mainly based on RFLP and random amplified polymorphic DNA (RAPD) first-generation molecular markers, and second-generation single sequence repeat (SSR) molecular markers. None of the efficient third-generation molecular markers (e.g., SNPs) have been used for differentiating distinct turtle strains. Thus, in this study, we attempted to genotype and differentiate distinct strains of Chinese soft-shelled turtle using HRM analysis of SNPs in mitochondrial DNA, which would provide effective technical support for the construction and management of turtle breeding farms.

MATERIAL AND METHODS

Sampling and DNA extraction

Four distinct Chinese soft-shelled turtle strains were used in this study, Japanese Strain (JS), Taihu Lake Strain (TLS), Yellow River Strain (YRS), and Taiwan Strain (TS) (Table 1). Twenty-

five adult turtles were collected for each strain, with 5 for primer design research and the other 20 for HRM analysis. Genomic DNA was extracted from leg muscle tissue using the Takara DNA extraction kit (Takara, Dalian, China), and diluted to a concentration of 100 ng/μL. Genomic integrity was assessed by electrophoresis on a 1.5% agarose gel. Genomic DNA was stored at -20°C until required.

Table 1. Animal samples (II ages) from four strains of Chinese soft-shelled turtle.

Strain	Weight (g)	Sampling location
JS[a]	750 ± 53	Provincial Chinese soft-shelled turtle seed farm, Xiaoshan, Zhejiang
TLS[b]	475 ± 32	Provincial Chinese soft-shelled turtle seed farm, Deqing, Zhejiang
YRS[c]	332 ± 37	National Chinese soft-shelled turtle seed farm, Shaoxing, Zhejiang
TS[d]	436 ± 44	Chinese soft-shelled turtle farm, Deqing, Zhejiang

[a]Japanese Strain; [b]Taihu Lake Strain; [c]Yellow River Strain; [d]Taiwan Strain.

Screening of mutation sites and primer design

Partial sequences of mitochondrial DNA (mtDNA) of five samples from each strain were amplified and sequenced as described previously (Peng et al., 2005). Sequence alignment was conducted using the Clustal X v2.0 software (Larkin et al., 2007), and specific SNP sites for each strain were analyzed using the BioEdit software (Hall, 1999). Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was employed to design primers ranging in length from 19 to 25 bp, with an amplified fragment length in the range 80 to 150 bp, and an annealing temperature between 59° and 65°C.

HRM analysis and SNP genotyping

PCR amplification of the mtDNA fragments from 5 samples of each strain was conducted using a ABI 7500 Fast real-time PCR detection system (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). The PCR mixture (in a volume of 20 μL) consisted of 10 μL 2X HRM MeltDoctor Master Mix (Perkin Elmer/Applied Biosystems), 0.4 μL 10 μM primer mix (containing 10 μM forward primer and 10 μM reverse primer, Invitrogen, Guangzhou, China), 2 μL template DNA (50 ng), and 7.6 μL RNase-free water. Cycling conditions for HRM analysis were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, and finally annealing and extension at 60°C for 30 s. During the reaction, temperature varied from 60°C to 95°C, at a rate of 0.05°C/s. When the reaction finished, samples were cooled at 40°C for 1 s, and the melting curves and T_m were analyzed using the HRM 2.0 software (Applied Biosystems).

RESULTS

Screening of candidate SNP sites and primer design

MtDNA from 20 turtles (5 from each strain) was used for gene mutation screening by sequence blast. A total of 22 specific sites were obtained, and seven primer pairs that successfully yielded expected amplicons were designed (Table 2).

HRM genotyping based on SNPs

All amplicons yielded from the seven pairs of primers were useful for SNP genotyping. The T_m of amplicons from the five samples of each strain was averaged to provide the T_m value for

each strain (Table 3). The T_m of different amplicons differed by more than 0.3°C, suggesting these primers are effective for distinguishing between strains. TLS could be identified by primer 1, YRS by primers 2, 4 and 6, TS by primers 3 and 5, and JS by primer 7. The results indicate that HRM is a promising method that could be used to differentiate between these distinct turtle strains.

Table 2. Primers used in high-resolution melting assays to assess single nucleotide polymorphisms (SNPs) in four strains of Chinese soft-shelled turtle.

Primer number	Primer sequence (5'-3')	SNP locus in mitochondrial DNA (AY687385)
1	F: GCCCCTTCCACCAAACTGTCATAC R: GGGTATCTAATCCCAGTTTGTGTCT	486
2	F: GGGGCAAGTCGTAACAAGGTAAG R: GCTCAAAATCAAAATAACCCTGG	1064
3	F: TAAGTAGAGGTGAAAAGCCTAACGA R: CTGTTTCAATTTGGCTGTACCCTAA	1559
4	F: AAGCATTCTCATCAAAACGAAAAGT R: AATCCTTCCTTTCTTGTGTTTTGTA	6879
5	F: CACATTCACACCAACTACACAACTCT R: GTAGTCCTGTGAGTACGGTGGCT	8330
6	F: CGAAGCCACACTAATCCCAACA R: GAAGTAGGTTCCAGCATTTAGTCGT	10668
7	F: TATACTTCAACACCTTAATCCACCG R: CGTATCAAATTAGGTCGGTTAGGTG	13454

Table 3. Variation type and melting temperature (T_m) values of the products amplified in the mitochondrial DNA of four strains of Chinese soft-shelled turtles using the seven primers.

Primer number	Product size (bp)	Variation type	T_m values of amplified products (°C)			
			Taihu Lake Strain	Taiwan Strain	Japanese Strain	Yellow River Strain
1	118	T/C	75.80	75.43	75.43	75.43
2	126	C/T	77.54	77.54	77.54	77.08
3	149	C/A	75.33	74.93	75.33	75.33
4	136	C/T	78.39	78.39	78.39	77.92
5	83	T/C	74.30	74.78	74.30	74.30
6	82	A/G	73.00	73.00	73.00	73.46
7	96	T/C	76.33	76.33	76.93	76.33

Application of HRM genotyping for breed identification

A total of 80 Chinese soft-shelled turtles with unknown background were selected from the four farms and were identified using the HRM method, with the results suggesting an accuracy as high as 100% (Figures 1 to 4).

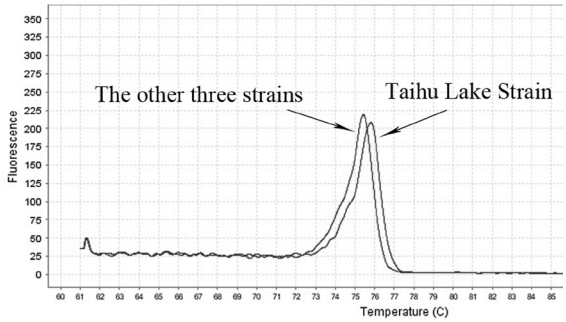

Figure 1. Single nucleotide polymorphism genotyping result for locus 486 in the mitochondrial DNA of four Chinese soft-shelled turtle strains using primer 1.

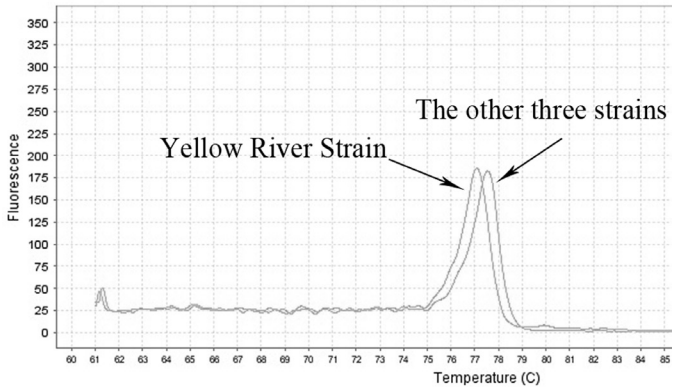

Figure 2. Single nucleotide polymorphism genotyping result for locus 1064 in the mitochondrial DNA of four Chinese soft-shelled turtle strains using primer 2.

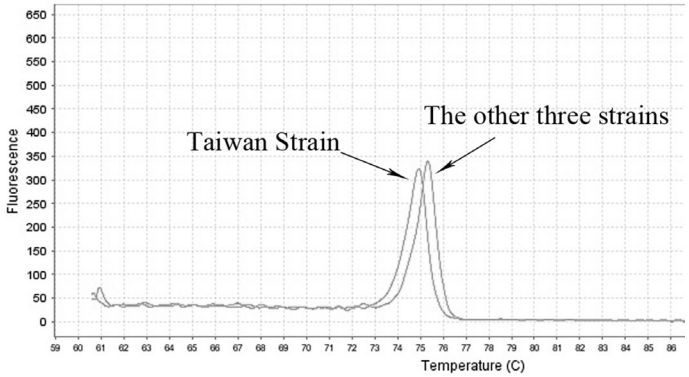

Figure 3. Single nucleotide polymorphism genotyping result for locus 1559 in the mitochondrial DNA of four Chinese soft-shelled turtle strains using primer 3.

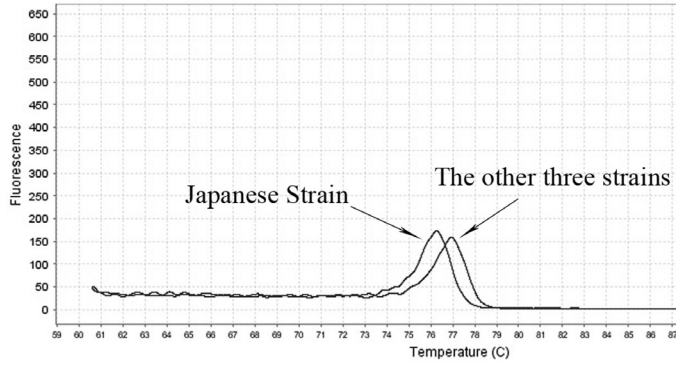

Figure 4. Single nucleotide polymorphism genotyping result for locus 13454 in the mitochondrial DNA of four Chinese soft-shelled turtle strains using primer 7.

DISCUSSION

Currently, commonly-used SNP genotyping techniques included SSCP (Gonen et al., 1999), PCR-RFLP (Fukuen et al., 2002), DHPLC (Wolford et al., 2000), single base extension (Steemers et al., 2006), sequencing (Griffin et al., 2000), and HRM (Liew et al., 2004). Among these, the dideoxynucleotide sequencing method is the most direct and accurate method and is regarded as the gold-standard for SNP genotyping; unfortunately, it is time-consuming, inconvenient, and expensive. Compared with other methods, HRM represents a high-throughput, low-cost, easily-conducted, and time-efficient one-step genotyping method. Moreover, its accuracy and reliability can be greatly enhanced by positive control. In this study, HRM analysis was employed to differentiate between distinct strains of Chinese soft-shelled turtles via PCR amplification using specific primers. As shown in Figures 1 to 4, this genotyping method was effective.

MtDNA is widely employed in studies of genetic differentiation owing to its high mutation rate (Stoneking and Soodyall, 1996) and maternal hereditary traits, which can reflect genetic characteristics, population differentiation, and evolutionary relationships among species (Cann et al., 1984). Until now, studies on the mitochondrial markers of Chinese soft-shelled turtles have mainly focused on Cyt *b* and 12S rRNA. Through comparative analysis of the results of Cyt *b* and 12S rRNA sequences of Chinese soft-shelled turtles, sand turtles (*P. axenaria*), and wattle-necked soft-shell turtles (*Palea steindachneri*), Chen et al., (2005, 2006) have concluded that these three species can be effectively identified using the PCR-RFLP method. A comparative analysis of Cyt *b* from five strains of Chinese soft-shelled turtles has been conducted by Zhang et al., (2008), which demonstrated that unique haplotype patterns could be identified in the JS. Conclusively, Cyt *b* served as an identification marker for the JS; however, specific differentiation markers in other strains remain unknown. By comparative analysis of partial 12S rRNA sequences of two turtle strains, i.e., Qingxi Wubie and JS, Xu et al. (2012) confirmed that the two strains shared no identical haplotype patterns, suggesting that 12S rRNA could serve as an identification marker for these two strains. However, there are still no reports to date on specific markers for identification of distinct turtle strains.

As shown in previous studies, mtDNA SNP markers are practical for identifying turtle strains (Mabru et al., 2004). This study aimed to detect specific SNP markers in mtDNA of four turtle strains with similar morphological resemblance. The specific loci of the four strains were detected using sequence analysis, and the targeted SNPs were genotyped using the HRM method. As shown in Tables 2 and 3, locus 468 can be used to identify TLS by using primer 1, loci 1559 and 8330 are applicable to TS by using primer 3 and primer 5, locus 13454 to JS by using primer 7, and loci 1064, 6879, and 10668 to YRS by using primer 2, 4 and 6. To test the reliability of the identification method, a blind test was carried out using turtles belonging to the four strains, and the results (Figures 1 to 4) showed 100% accuracy compared to the background information for the turtles. No nucleotide variants were detected within these loci indicating that the HRM method has a high accuracy in identification of Chinese soft-shelled turtle strains. Moreover, compared to nuclear DNA, mtDNA has no recombinant hybrid, leading to the absence of double peaks in the melting curve, which benefits the direct visual representation of genotyping results.

In conclusion, HRM genotyping based on specific mtDNA SNPs in this study can effectively identify four strains of Chinese soft-shelled turtle. This study greatly enhances the ability to differentiate between distinct strains of Chinese soft-shelled turtles compared to morphological identification, and provides further technical support for identifying fake Chinese soft-shelled turtles in circulation in the market and guiding the selective breeding of turtles.

Conflicts of interest

The authors declare no conflict of interest.

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