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High resolution melting curve analysis: an efficient method for fingerprinting of hybrid rice cultivars and their parental lines

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Abstract

Rice (*Oryza sativa* L.) is one of the most important crops in the world. It is essential to develop an efficient method to build the fingerprinting database of commercial hybrid rice cultivars and their parental lines in seed market for rapid and unambiguous cultivar identification and seed purity analysis as new similar cultivars increasing yearly. The objective of this investigation were to fingerprint three commercial hybrid rice cultivars and their parental lines using SSR molecular markers to develop an efficient fingerprinting method for rice cultivar identification and seed purity analysis in agriculture production. Three methods, agarose gel electrophoresis, capillary electrophoresis and high resolution melting curve analysis were used to fingerprinting three hybrid F_1 rice cultivars and their parental lines. Out of the fourteen SSR primer pairs, nine primer pairs which produced stable, distinct and polymorphic amplifications as visualized on agarose gel were selected for capillary electrophoresis and high resolution melting curve analysis to compare their fingerprinting efficiency. Six (RM206, RM209, RM211, RM212, RM219, and RM297), four (RM72, RM209, RM212, and RM276) and six (RM190, RM206, RM211, RM212, RM219, and RM297) primer pairs were successfully used to fingerprint the three hybrid F_1 rice cultivars, YLiangYou689, ZhuLiangYou06, and QianYou1, respectively. The result suggested that capillary electrophoresis and high resolution melting curve analysis could be used as potential, efficient and valuable methods for fingerprinting hybrid rice cultivars, and high resolution melting curve analysis should be given priority compared with capillary electrophoresis for its high accuracy and high efficiency.

Keywords: Capillary electrophoresis; Cultivar identification; Fingerprinting; High resolution melting curve analysis; Rice; TP-M13-SSR.

Abbreviation: CTAB_Hexadecyl trimethyl ammonium Bromide; FAM_6-carboxy- fluorescein; HRM_High resolution melting curve analysis; HEX_hexachloro-6- carboxy-fluorescein; ISSR_inter- simple sequence repeat; SSR_simple sequence repeat; TET_tetrachloro-6-carboxy-fluorescein; TP_M13-SSR-PCR performed with three primers, a sequence-specific forward primer with universal primer M13 tail at its 5' end, a sequence-specific reverse primer, and the fluorescence-labeled universal primer M13; RFU_ relative fluorescence unit; ROX_6-carboxy-X-fluorescine.

Introduction

Rice (*Oryza sativa* L.) is one of the leading food crops in the world and staple for more than half the world's population (Kumar et al., 2011). High-quality seeds play a crucial role in rice production. However, since new cultivars normally arise from hybridizations between members of an elite group of genetically similar parents, the amount of genetic variability among newly developed cultivars and their parental lines is likely to become even smaller, which makes it more difficult for cultivar identification and hybrid seed purity analysis with morphological characteristics and isozyme electrophoresis patterns. Fingerprinting with molecular markers allows precise, objective and rapid cultivar identification and genetic purity analysis, which has been proved to be an efficient tool for crop germplasm characterization, collection and management (Zhu et al., 2012). Simple sequence repeat (SSR) markers have been widely used for plant genetic analysis because of their abundance, co-dominance inheritance, high polymorphism, reproducibility, and ease of assay by polymerase chain reaction (PCR) (Kuleung et al., 2004; Xie et al., 2011). SSR markers have been used in genetic analysis and fingerprinting of different rice accessions (Thomson et al., 2007; Lu et al., 2010; Faivre-Rampant et al., 2011). When large numbers of hybrid rice cultivars and their parental lines are involved, the fingerprinting work can be costly in terms of laboratory consumables, labor and time. As a consequence, it is essential to develop an efficient method to build the fingerprinting database of commercial hybrid rice cultivars and their parental lines in seed market for rapid and unambiguous cultivar identification and seed purity analysis. SSR fragments are most often visualized by electrophoresis on agarose or polyacrylamide gels for analysis and fingerprinting in previous studies. An elementary fingerprinting database of the forty-eight main commercial rice cultivars in Zhejiang Province of China was built using SSR markers by agarose gel-electrophoresis (Zhu et al., 2012). However, gel-electrophoresis is principally sensitive only to the fragment length. What's more, gel-electrophoresis is not only time-consuming, but may also lead to toxic pollution and problems in interpretation due to stutter bands. Except for agarose and polyacrylamide gels, capillary electrophoresis is more and more popular for SSR analysis, which affords the use of fluorescent dye labeled primers. To analyze the exact length of SSR fragments on a laser detection system, one of the SSR primer pair has to carry a fluorescent dye label, which may be

6-carboxy-fluorescein (FAM), tetrachloro-6-carboxy-fluorescein (TET), hexachloro- 6-carboxy-fluorescein (HEX) or 6-carboxy-X-fluorescine (ROX) in conventional fluorescence-labeling method, which is high cost. In order to overcome this financial burden, Schuelke (2000) put forward and demonstrated fluorescence-labeled TP-M13-SSR PCR method. In this improved method, PCR was performed with three primers, a sequence-specific forward primer with universal primer M13 tail at its 5' end, a sequence-specific reverse primer, and the fluorescence-labeled universal primer M13. Fluorescence-labeled TP-M13-SSR PCR method has the advantages of high-throughput and high accuracy, which has been successfully used for genotype identification of *Sorghum*, rice, and wheat (Zhu et al., 2011).

High resolution melting (HRM) curve analysis is a novel, closed-tube, post-PCR technique invented in 2003 (Wittwer et al., 2003) to detect DNA variation, which has been used in clinical chemistry, epidemical analysis, microorganism typing and molecular biology (Wu et al., 2008; Hofinger et al., 2009; Ganopoulos et al., 2011; Thomsen et al., 2012). In HRM experiments, the target sequence is amplified by PCR in the presence of a saturating fluorescent dye (e.g., LightCycler® 480 ResoLight Dye). HRM dye fluoresces strongly only when bound to dsDNA. This change of fluorescence during an experiment can be used both to measure the increase in DNA concentration during PCR amplification and, subsequently, to measure temperature-induced DNA dissociation during high resolution melting. After PCR in the presence of the dsDNA-binding fluorescent dye, amplifications are briefly denatured and then rapidly reannealed. If the DNA sample is heterozygous, perfectly matched hybrids (homoduplexes) and mismatched hybrids (heteroduplexes) are formed. When the temperature is slowly increased again, the dsDNA begins to melt, and the shapes of melting curves are significantly different based on the G-C content, length and sequence of the fragments (Herrmann et al., 2006). In fact, the HRM technique is so sensitive that it can even detect single base variations between homozygous samples. SSR-HRM method has the advantages of high efficiency and high accuracy, which has been successfully used for variety identification of grape and olive (Mackay et al., 2008), common bean (Ganopoulos et al., 2012), and sweet cherry (Ganopoulos et al., 2012).

Both Fluorescence-labeled TP-M13-SSR and SSR-HRM techniques have the latent capacity to develop into an automatic and high throughput method of fingerprinting for cultivar identification and seed genetic purity analysis in further study. However, there was no report on comparison of the efficiency between the two methods in fingerprinting of hybrid F_1 rice cultivars for cultivar identification and seed purity analysis. The objective of this investigation was to fingerprint three commercial hybrid F_1 rice cultivars and their parental lines using agarose gel electrophoresis, capillary electrophoresis and high resolution melting curve analysis based on SSR molecular markers, and to develop a more efficient method for fingerprinting of hybrid F_1 rice cultivars and their parental lines.

Results

SSR fragments analysis by agarose gel electrophoresis

Fourteen SSR primer pairs were initially screened against three commercial hybrid F_1 rice cultivars and their parental lines. All of the primer pairs had good amplification, and the number of fragments produced for each primer pairs was ranged from one to three. Six primer pairs, RM206, RM209, RM211 (Fig. 1), RM212, RM219, and RM297, were able to amplify complementary band patterns among YLiangYou689 and its parental lines. Four (RM72, RM209, RM212, and RM276) and seven (RM190, RM206, RM211, RM212, RM219, RM297, and RM311) primer pairs were for ZhuLiangYou06 and QianYou1, respectively.

Table 1. Names of hybrid rice cultivars and their parental lines used in this study.

Hybrid Cultivar	Female line	Male line
YLiangYou689	Y58S	R689
ZhuLiangYou06	Zhu1S	06EZ11
QianYou1	QianJiang1A	R7954

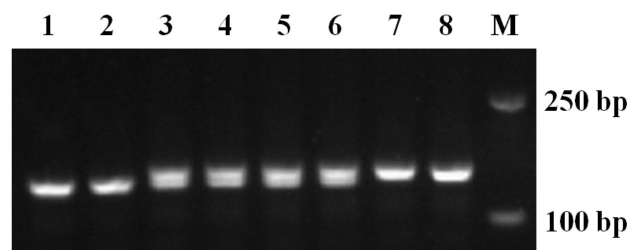

Fig 1. Amplification results of primer pair RM211 from YLiangYou689 and their parental lines. M is DL 2000 DNA molecular marker. Lanes 1~2 represent female line (Y58S), lanes 3~6 represent hybrid rice cultivar (YLiangYou689), and lanes 7~8 represent male line (R689).

SSR fragments analysis by capillary electrophoresis (SSR-CE)

Based on the agarose gel electrophoresis results above, six (RM206, RM209, RM211, RM212, RM219 and RM297), four (RM72, RM209, RM212, and RM276) and six (RM190, RM206, RM211, RM212, RM219, and RM297) sets of FAM-labeled TP-M13-SSR primers (Table 3) were selected for capillary electrophoresis analysis to analyze the exact length of the amplified complementary bands for precise fingerprinting of the three commercial hybrid F_1 rice cultivars, YLiangYou689, ZhuLiangYou06, and QianYou1 as well as their parental lines, respectively. The sizes of the SSR fragments detected ranged from 116 bp to 234 bp, and the variation detected between the alleles was ranged from 6 bp (RM72) to 42 bp (RM206) (Table 3). Hybrids were identified by the number and size of the SSR fragments. An initial fingerprinting database was built for three hybrid F_1 rice cultivars (YLiangYou689, ZhuLiangYou06, and QianYou1) and their parental lines.

SSR fragments analysis by high resolution melting curve analysis (SSR-HRM)

Based on the results of agarose gel electrophoresis and capillary electrophoresis analysis above, six (RM206, RM209, RM211, RM212, RM219 and RM297), four (RM72, RM209, RM212, and RM276) and six (RM190, RM206, RM211, RM212, RM219, and RM297) primer pairs were selected for high resolution melting curve analysis for precise fingerprinting of the three commercial hybrid F_1 rice cultivars, YLiangYou689, ZhuLiangYou06, and QianYou1 as well as their parental lines, respectively. Materials having different amplification fragments were able to be identified by the shape of melting curves, which is the principle of HRM for

Table 2. Sequences of SSR primer pairs used in this study.

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
RM297	TCTTTGGAGGCGAGCTGAG	CGAAGGGTACATCTGCTTAG
RM71	CTAGAGGCGAAAACGAGATG	GGGTGGGCGAGGTAATAATG
RM274	CCTCGCTTATGAGAGCTTCG	CTTCTCCATCACTCCCATGG
RM190	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG
RM336	CTTACAGAGAAACGGCATCG	GCTGGTTTGTTTCAGGTTCG
RM72	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG
RM219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
RM311	TGGTAGTATAGGTACTAAACAT	TCCTATACACATACAAACATAC
RM209	ATATGAGTTGCTGTCGTGCG	CAACTTGCATCCTCCCCTCC
RM19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA
RM 206	CGTTCCATCGATCCGTATGG	CCCATGCGTTTAACTATTCT
RM 212	CACCCATTTGTCTCTCATTATG	CCACTTTCAGCTACTACCAG
RM211	CCGATCTCATCAACCAACTG	CTTCACGAGGATCTCAAAGG
RM276	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA

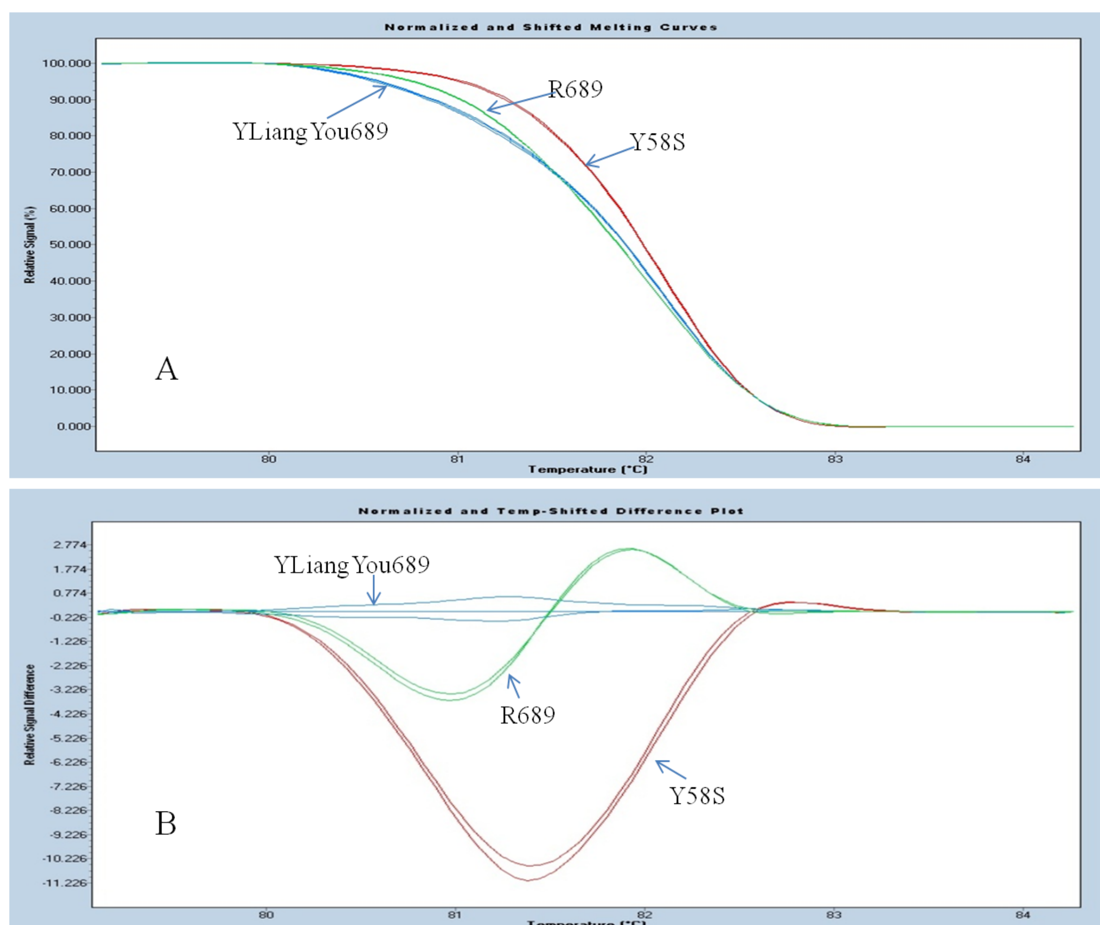

Fig 2. HRM analysis of amplifications from YLiangYou689 and their parental lines by RM211. Temperature-shift melting curves (A) and difference plots (B) for the identification of YLiangYou689 (Blue curves, amplification sizes 162:172 bp), Y58S (Red curves, amplification size 162 bp) and R689 (Green curves, amplification size 172 bp) at SSR locus RM211.

fingerprinting and cultivar identification. Significantly different curves were achieved based on the different amplifications of hybrid F_1 cultivar and its parental lines. For example, based on SSR primer pair RM211, hybrid rice cultivar YLiangYou689 (YF) had two amplification fragments, and the sizes of them were 162 bp and 172 bp. However, its female line (YM) had one amplification fragment (162 bp), and male line (YD) also had only one amplification fragment (172 bp) (Table 3). As the results, hybrid F_1 rice cultivar YLiangYou689 and its parental lines had significantly different curve (Fig.2), which could be used for precise and fast fingerprinting and cultivar identification.

Discussion

Fingerprinting of three hybrid rice cultivars and their parental lines

Agarose and polyacrylamide gel electrophoresis were traditional methods to analyze SSR fragments. In this study, agarose gel electrophoresis was selected for initial analysis of the amplifications because of its simple, affordable technology, time saving and low toxic with the use of Gel-Red dye. The agarose gel electrophoresis results showed that SSR molecular markers were valuable for fingerprinting and genetic analysis

Table 3. Analysis of polymorphic loci produced from FAM-labeled TP-M13-SSR primers.

	Fingerprinting based on SSR fragment length (bp) analysis								
Primer Name	YM	YF	YD	ZM	ZF	ZD	QM	QF	QD
RM72	—	—	—	180	180/186	186	—	—	—
RM190	—	—	—	—	—	—	144	144/128	128
RM206	188	146/188	146	—	—	—	166	146/166	146
RM209	154	146/154	146	154	154/170	170	—	—	—
RM211	162	162/172	172	—	—	—	162	162/172	172
RM212	156	134/156	134	156	132/156	132	134	134/154	154
RM219	224	224/240	240	—	—	—	234	234/214	214
RM276	—	—	—	116	116/126	126	—	—	—
RM297	170	170/178	178	—	—	—	178	170/178	170

Note: YM: Y58S (Female line of YLiangYou689); YF: YLiangYou689; YD: R689 (Male line of YLiangYou689); ZM: Zhu1S (Female line of ZhuLiangYou06); ZF: ZhuLiangYou06; ZD: 06EZ11 (Male line of ZhuLiangYou06); QM: QianJiang1A (Female line of QianYou1); QF: QianYou1; QD: R7954 (Male line of QianYou1).

of rice because of its high polymorphism. Based on the agarose gel electrophoresis results, nine SSR primer pairs which produce stable, distinct and polymorphic amplifications were selected for capillary electrophoresis and high resolution melting curve analysis to compare their efficiency in fingerprinting of rice hybrid cultivars for cultivar identification and seed purity analysis. Capillary electrophoresis has been successfully applied for fingerprinting and cultivar identification (Zhu et al., 2011), which was reported to be a potential and valuable method for fingerprinting and identification of closely related or low genetic diversity germplasm because of its high sensitiveness and accuracy. A fingerprinting database was initially built for YLiangYou689, ZhuLiangYou06, and QianYou1, which was able to be used for further cultivar identification and seed purity analysis. Compared with agarose gel electrophoresis, capillary electrophoresis was more high-throughput and apt to precisely fingerprint hybrid rice cultivars for quick cultivar identification and seed purity analysis. There were only several reports on plant variety identification based on SSR molecular markers by high resolution melting curve analysis up to now (Mackay et al., 2008; Ganopoulos et al., 2011; Ganopoulos et al., 2012). However, high resolution melting curve analysis technique showed a potential application for plant genotyping. In this study, we used a universal cycling protocol of touchdown PCR to allow all tested SSRs to be amplified and analyzed within the same PCR reaction run. Touchdown and fast PCR protocol, together with high resolution melting curve analysis steps, permitted the fingerprinting completed within 90 minutes from start of amplification. What's more, the experiments showed high repetitions. The result showed that high resolution melting curve analysis was an efficient, high-resolution and valuable fingerprinting method for hybrid rice cultivars.

Comparison between SSR-CE and SSR-HRM analysis

This is the first attempt to compare the efficiency between capillary electrophoresis and high resolution melting curve analysis in fingerprinting hybrid rice cultivars and their parental lines based on SSR molecular markers.

As one of the most popular molecular markers, SSR has great potentiality in automatic, fast, and accurate fingerprinting for cultivar identification and seed purity test of hybrid rice together with capillary electrophoresis or high resolution melting curve analysis. Compared with agarose or polyacrylamide gel electrophoresis, both capillary electrophoresis and high resolution melting curve analysis have the advantages of high accuracy, high-throughout, high efficiency, and no-touch of toxic reagents. Compared with capillary electrophoresis, high resolution melting curve analysis was more accuracy and immediacy. Capillary electrophoresis detected variation sensitively only to the fragment length, requires at least one additional analysis step following PCR. While high resolution melting curve analysis was able to distinct the differences in G-C content or fragment sequence among amplifications with the same length. What's more, the result of high resolution melting curve analysis was showed with significantly different curves based on different amplifications, while the problem of stutter peaks also occurs in capillary electrophoresis which is still quite costly offside routine analysis with well-known organisms and markers. High resolution melting curve analysis was more efficiency compared with capillary electrophoresis. High resolution melting curve analysis were able to complete analysis of 384 PCR products within 90 minutes from start of amplification in the same machine without any other treatment. However, capillary electrophoresis could analyze 384 PCR products within 4 ~ 5 hours from start of amplification, needing PCR products dilution, mixing, transferring to laser detection machine, and pretreatment before capillary electrophoresis.

Conclusion

Based on the result of this investigation, high resolution melting curve analysis is an efficient and potential method in fingerprinting of hybrid F_1 rice cultivars and their parental lines for cultivar identification and seed purity analysis in further study.

Materials and Methods

Plant materials

Seeds of three commercial hybrid F_1 rice cultivars and their parental lines (Table 1) used in this study were acquired from Zhejiang Nongke Seed Industry Co., LTD., China.

Genomic DNA extraction

Each genomic DNA sample was extracted from 30 young seedlings for each material according to a modified CTAB method (Zhu et al., 2010). The concentrations and quality of the obtained genomic DNA samples were estimated on an ultramicrospectrophotometer ND-2000 (Nanodrop, USA). Finally, all the genomic DNA samples were diluted to a final concentration of 10 ng•μL^{-1} with 1×TE buffer (10 mM Tris-HC1, pH 8.0; 1 mM EDTA) and stored at -20 ^{o}C for further use.

SSR primer pairs

A total of fourteen SSR primer pairs (Table 2) were used in this study. Sequence information of all the primers were obtained from the SSR panel in the rice Gramene database (http://www.gramene.org/markers/microsat/ssr.html). Primers were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Primer labeling method was following Zhu et al (2011), and the universal primer M13 was labeled with fluorescence dye 6-carboxyfluorescein (FAM) at its 5' head.

SSR-PCR amplification and agarose gel electrophoresis analysis

SSR polymerase chain reaction (PCR) amplification was conducted in a 10 µL volume containing 40 ng of genomic DNA, 1 U *Taq* DNA polymerase, 2.0 mM $MgCl^2$, 0.20 mM dNTPs and 0.2 µM of each primer. The PCR protocol consisted of an initial denaturation at 95 ^oC for 5 min, followed by 40 cycles of 94 ^oC for 45 s, annealing for 45 s at 58 ^oC, 72 ^oC for 1 min, and a final extension step of 72 ^oC for 10 min. All PCR reactions were carried out in a thermal cycler C1000 (Bio-Rad, USA). PCR products were separated on 3 % agarose gels, stained with GelRed^{TM} (Biotium, USA) and photographed under UV light using Image Lab^{TM} software Version 2.0.1 (Bio-Rad, USA).

Fluorescence-labeled TP-M13-SSR analysis

TP-M13-SSR PCR amplification was conducted in a 20 µL volume containing 40 ng of genomic DNA, 1 U *Taq* DNA polymerase, 2.0 mM $MgCl^2$, 0.20 mM dNTPs, 0.25 µM reverse primer, 0.05 µM M13-forward primer, 0.20 µM FAM-labeled M13 primer. The PCR protocol consisted of an initial denaturation at 94 ^oC for 4 min, followed by 40 cycles of 94 ^oC for 45 s, annealing for 45 s at 58 ^oC, 72 ^oC for 1 min, and a final extension step of 72 ^oC for 10 min. All PCR reactions were carried out in a thermal cycler C1000 (Bio-Rad, USA). PCR products were detected using Mega BACE 1000 DNA Sequencer (Pharmacia, USA).

SSR-HRM (Simple sequence repeat-high resolution melting curve) analysis

SSR-HRM PCR amplification was conducted in a 10 µL volume containing 20 ng of genomic DNA, 2.0 mM $MgCl^2$, 5 µL LightCycler® 480 High Resolution Melting Master Mix with LightCycler® 480 ResoLight Dye (Roche, Switzerland), 0.15 µM reverse primer, 0.15 µM forward primer, and made up to 10 µL with PCR-grade water. The HRM reaction procedure and melting analysis were performed as follows: a 10-min initial denaturation followed by 55 cycles of denaturation at 95 °C for 15 s, annealing from 62 °C to 53 °C for 15 s and extension at 72 °C for 20 s. The annealing temperature decreased in subsequent cycles by 1.0 °C per cycle after the first 62 °C annealing step to 53 °C. The amplification procedure was immediately followed by the high-resolution melting steps: 95 °C for 1 min, cooling to 40 °C for 1 min, and then the temperature was raised to 60 °C. Subsequently, the temperature was raised to 95 °C with 20 fluorescent acquisitions per degree Celsius at this step. The HRM curve was acquired and analyzed on 96-well plates using the LightCycler® 480 Ⅱ real-time PCR system (Roche, Switzerland). After the verification of robust amplification curves and the presence of a specific melting peak for the microsatellite amplification, the melting curve stage was further analyzed with the GeneScanning software module on the LightCycler® 480 instrument.

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