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Construction of Fingerprint for *Michelia* Germplasm by Fluorescent SSR Markers

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Abstract In this study, a fingerprinting system for 16 *Michelia* germplasms was established using high-throughput genotyping of fluorescent simple sequence repeat (SSR) markers. In total, 102 alleles were detected using 13 pairs of SSR markers from closely related species, and the number of alleles per locus ranged from 5 to 11, with an average of 7.8 alleles. Observed heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC) ranged from 0.1250 to 0.5625 (mean 0.3650), 0.6703 to 0.9113 (mean 0.8099), and 0.5748 to 0.8714 (mean 0.7515), respectively. Among the four selected core primer pairs, the combinations LT106 and SGA5, LT106 and LT58, and SGA5 and MMA51 could unambiguously distinguish 16 *Michelia* germplasms. A cluster analysis showed the similarity coefficient of the 16 *Michelia* germplasms to range from 0.70 to 0.90, and different individuals from the same species clustered in the same branch. The fluorescence SSR genotyping system established in this study was efficient, rapid, and accurate; moreover, this approach provides a theoretical basis for identifying germplasms and for the protection of new varieties of the genus *Michelia*, and it provides a robust foundation for further breeding development of *Michelia* sp.
Keywords *Michelia*, Fluorescent marker, SSR, Fingerprint

The Magnoliaceae are one of the oldest angiosperm families and comprise various important flowering plants. The taxonomic and phylogenetic relationships of numerous Magnoliaceae species has been disputed due to the similarity of morphological characteristics and tissue structures, and particularly the division of genera has been an issue of disagreement (Sun and Zhou, 2004; Meng et al., 2006). The genus *Michelia* is the second largest genus of the Magnoliaceae and comprises more than 40 species indigenous to China, the phenotypes of which are considerably variable within and between species, e.g., *M. maudiae* (Chen et al., 2016) and *M. crassipes* (Wen et al., 2014). A phylogenetic classification of *Michelia* was the main focus of the study of Zhang (2007). Moreover, hybridization of various *Michelia* species is easily achieved owing to their close relationships and lack of reproductive isolation; furthermore, phenotypic variations may occur due to environmental factors, which further complicates the unambiguous identification of species (Fowler et al., 1988). Therefore, novel DNA sequence-based approaches are needed for a reliable classification of *Michelia* on species level.

Most trees of the genus *Michelia* are evergreen, show an attractive growth habit, and produce a pleasant scent, which made these trees popular for urban landscaping; furthermore, *Michelia* trees have been used as a raw material in the wood industry and for the production of medicine and oil (Ye et al., 2013). In recent years, numerous new varieties of *Michelia* have been produced following a rapid development of breeding programs in China; these new varieties include 'Yujin', 'Danxin' and 'Qinfang' (Gong et al., 2003a), 'Chuju', 'Chunyue' (Gong et al., 2003b), 'Mozi' (Liao et al., 2007), 'Wanchun' (Mao et al., 2009), 'Huahaoyueyuan' (Liu et al., 2014), 'Chunyun', 'Yuxia' (Central South University of Forestry and Technology), and 'Jinxiu' (Huang et al., 2016). However, because of the morphological similarities between varieties of *Michelia*, frequent introduction of new varieties in production systems inevitably causes undesired hybridization. Homonymies and synonymies may occur from time to time, and even variety fraud has occurred to achieve commercial advantages. This further

complicates the evaluation of genetic resources, variety selection, and popularization of *Michelia* varieties. At the same time, in view of the need for the legal protection of new plant varieties, a robust, efficient, and reliable molecular marker identification system is required, which will contribute to the protection of commercially registered varieties and can ensure further development of the *Michelia* planting industry in China.

The molecular marker technology is based on DNA and cannot be affected by the environment; moreover, this approach which is simple, efficient, and provides accurate and reliable results, has been widely used in various fields of genetics and genomics. Among molecular markers, simple sequence repeats (SSR) have been proven considerably useful as genetic markers owing to substantial genome coverage, hyper-variability, co-dominant inheritance, high interspecies transferability, and good reproducibility (Kalia et al., 2011). This approach has been successfully applied for phylogenetic analyses and germplasm identification of several woody species, e.g., *Populus* sp. (Jia et al., 2015; Liu et al., 2016; Ou et al., 2017), *Eucalyptus* sp. (Li et al., 2011), *Pinus massoniana* (Shen et al., 2015), *Ginkgo biloba* (Wang et al., 2017), *Camellia oleifera* (Zhou et al., 2017), *Amygdalus persica* (Wang et al., 2016), *Osmanthus fragrans* (Duan et al., 2014), *Tamarix chinensis* (Ye et al., 2015), and *Litchi chinensis* (Madhou et al., 2013). However, the molecular biological study of *Michelia* in China commenced relatively late, and only one scientific report has been published so far (Wen et al., 2014). Therefore, EST-SSR primers for *M. maudiae* and *Liriodendron tulipifera* were chosen and screened for interspecies transferability and detection of polymorphisms, which were subsequently labeled using fluorescent probe and used to establish a fingerprinting system for 16 *Michelia* germplasms. The objective of our study was to establish an efficient, rapid, and accurate genotyping system for the genus *Michelia*, to provide a scientific theoretical basis for the identification of germplasm resources and for the legal protection of new varieties, and to provide reference for future breeding of *Michelia*.

1 Results and Analyses

1.1 Cross-species amplification

A set of 34 SSR primer pairs was selected to evaluate interspecific transferability in eight *Michelia* species: *M. chapensis*, *M. maudiae*, *M. Jinxiu*, *M. wilsonii*, *M. foveolata*, *M. platypetala*, *M. compressa*, and *M. macclurei*. Out of ten primer pairs for *M. maudiae*, only one primer pair failed to show amplification from *M. platypetala* samples, whereas the other nine primer pairs showed successful amplification from samples of all eight species, which indicated high interspecific transferability. Using the 24 *L. tulipifera* primer pairs, amplification efficiency ranged from 21% in *M. wilsonii* and *M. macclurei* to 38% in *M. maudiae* (Table 1). Therefore, nine primer pairs of *M. maudiae* and four primer pairs of *L. tulipifera* were selected and labeled with fluorescent probes for *Michelia* genotyping and fingerprinting.

1.2 Polymorphism evaluation

A total of 13 EST-SSRs were used to assess polymorphisms in 16 *Michelia* germplasms, which produced 102 alleles, and the number of alleles per locus ranged from 5 to 11 with an average of 4.8 alleles. The highest number of alleles (n=11) was amplified using SGA5, MMA72, and LT106, and the lowest number (n=5) was produced using

Table 1 Cross-species amplification of the 34 SSR primers

Species	10 Primers from *Michelia*		24 Primers from *Liriodendron*		Total
	No. of successful	Percentage	No. of successful	Percentage	
M. chapensis	10	100%	8	33%	18
M. maudiae	10	100%	9	38%	19
M. maudiae 'jinxiu'	10	100%	7	29%	17
M. wilsonii	10	100%	5	21%	15
M. foveolata	10	100%	8	33%	18
M. platypetala	9	90%	6	25%	15
M. compressa	10	100%	6	25%	16
M. macclueei	10	100%	5	21%	15

SGA15, MMA19, LT47, and LT186. Observed heterozygosity (Ho) ranged from 0.1250 to 0.5625, with an average of 0.3650, and expected heterozygosity (He) ranged from 0.6703 to 0.9113, with an average of 0.8099. Polymorphic information content (PIC) varied from 0.5748 to 0.8714, with an average of 0.7515. The highest PIC (0.8714) was produced using SGA5 and the lowest PIC (0.5748) was produced using LT186 (Table 2). Partial genotyping markers of *Michelia* germplasms using SGA5 is shown in Figure 1.

1.3 Fingerprinting of *Michelia*

Four core primer pairs were selected from 13 SSR primer pairs to establish a fingerprinting system for *Michelia* germplasms by further analyzing the allelic fragments of each locus (Table 3). Ten *Michelia* germplasms could be distinguished using the primer pair LT106, and eight *Michelia* germplasm could be distinguished using the primer pair SGA5. All 16 *Michelia* germplasms could be identified using a combination of the primers LT106 and SGA5 or a combination of the primers LT106 and LT58, or SGA5 and MMA51. Therefore, the four selected core primer pairs can be used to distinguish the germplasms examined here and also for reciprocal validation by using different combinations of primers.

Table 2 Polymorphisms of the thirteen SSRs in 16 individuals of *Michelia*

Locus	No. of allele	Length of allele	H_o	H_e	PIC
SGA5	11	209-247	0.4375	0.9113	0.8714
SGA15	5	157-183	0.1250	0.7036	0.6271
MMA10	8	189-221	0.1875	0.8367	0.7868
MMA19	5	201-229	0.4667	0.7471	0.6706
MMA36	10	268-294	0.2000	0.9011	0.8577
MMA37	9	206-238	0.5000	0.8768	0.8215
MMA51	8	298-330	0.5625	0.8427	0.7926
MMA67	8	138-156	0.3750	0.6976	0.6371
MMA72	11	210-250	0.2000	0.8782	0.8331
LT47	5	276-310	0.4615	0.8000	0.7312
LT58	6	217-236	0.5000	0.7540	0.6970
LT106	11	276-310	0.5625	0.9093	0.8692
LT186	5	227-303	0.1667	0.6703	0.5748
Total	102				
Mean	7.8		0.3650	0.8099	0.7515

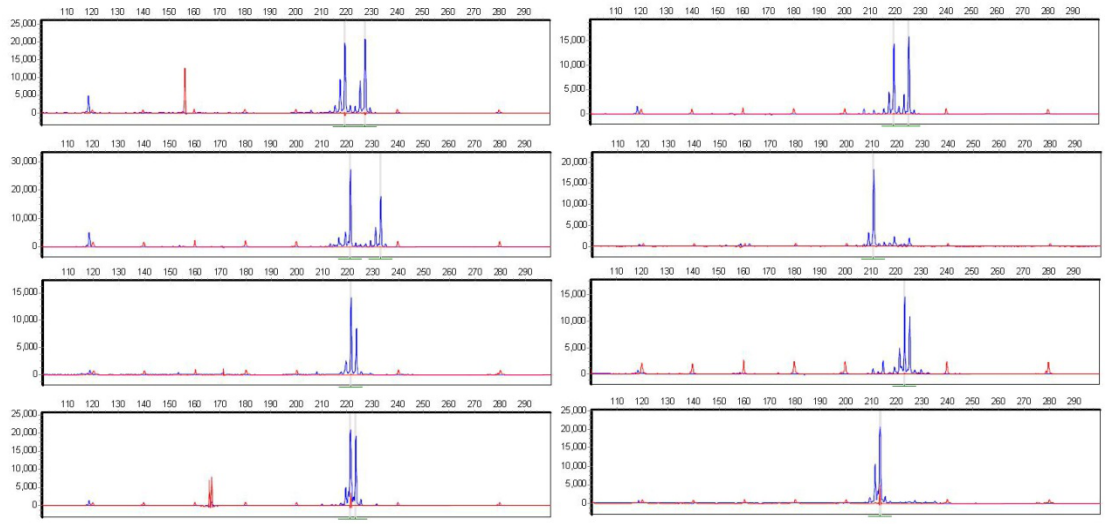

Figure 1 Genotyping by SGA5 marker for partial *Michelia* germplasms

Table 3 Fingerprint map of 16 materials of *Michelia*

Individuals	Core Primer			
	SGA5	MMA51	LT58	LT106
M. chapensis-1	219/227	298/324	221/233	308
M. chapensis -2	213	324/330	221	298/310
M. chapensis -3	219/227	326	221/233	296/308
M. chapensis -4	219	326	221/230	296/308
M. chapensis -5	211	326	221	298
M. maudiae-1	221/233	298/304	230	278
M. maudiae-2	221	298/300	230/236	286/300
M. maudiae-3	219/225	298/300	230	280
M. maudiae-4	209	298/300	230	276/280
M. maudiae-5	221/223	298/300	230/236	286
M. maudiae '*Jinxiu*'	219/225	300/324	230/236	282/296
M. wilsonii	211	306	227	280
M. foveolata	223	320	224/230	286/294
M. platypetala	231/247	298	227/230	286/294
M. compressa	213	300	224	294
M. macclueei	225	298/304	230	282

1.4 Cluster analysis

Genetic similarity coefficients were calculated and clustered based on UPGMA using the 13 fluorescently amplified SSR markers (Figure 2). The genetic similarity coefficient of 16 *Michelia* germplasms varied from 0.70 to 0.90, and a similarity coefficient of 0.77 produced five groups.

Group I consisted of five individuals (1, 2, 3, 4, and 5), all of which were *M. chapensis*. Individuals 3 and 4 were most closely related and produced the highest genetic similarity coefficient (0.90). Individual 2 was slightly divergent from the other four individuals. Group II included *M. wilsonii* (12), *M. compressa* (15), and *M. macclurei* (16). Group III comprised five *M. maudiae* individuals (6-10) and one *M. maudiae* cv. Jinxiu individual (11). However, the variety 'Jinxiu' was a superior individual originating from a natural variation of *M. maudiae*. Group IV comprised only *M. platypetala* (14), which was closely related to *M. maudiae*. Group V contained *M. foveolata* (13), which was most divergent from all other individuals.

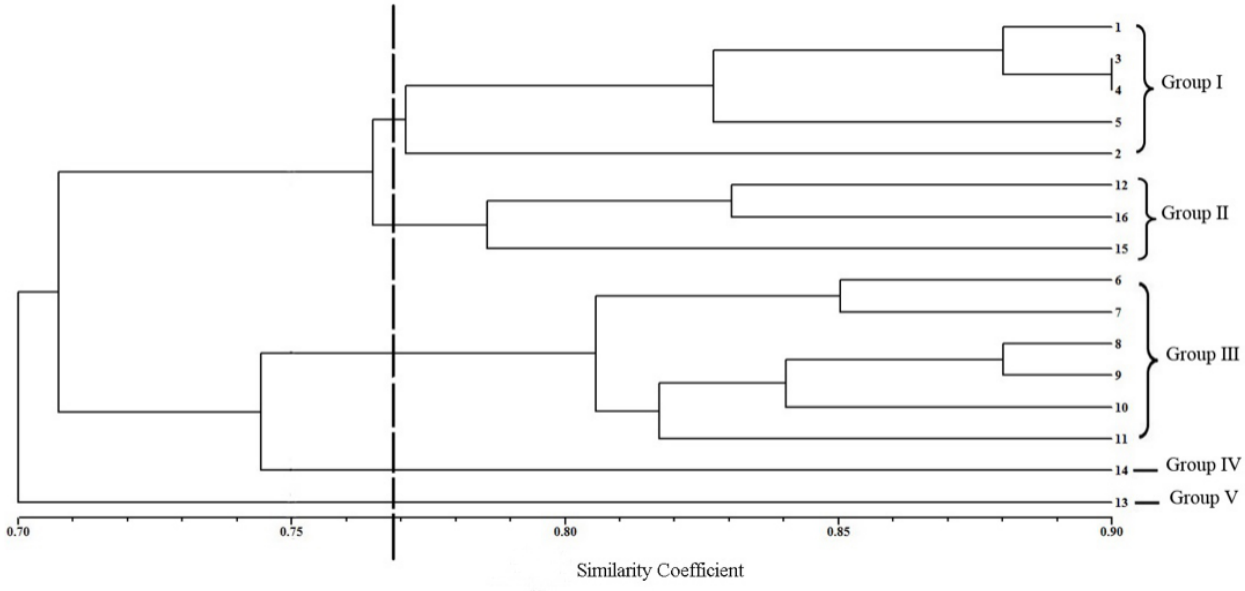

Figure 2 Dendrogram of 16 *Michelia* individuals based on SSR data

2 Discussion

SSR markers are widely distributed in eukaryotic and prokaryote genomes. Compared with genomic SSR, EST-SSR markers located in a transcription region are typically less polymorphic than genomic SSR (He, 2010), therefore they tend to be more conserved and thus are more compatible between species (Xu et al., 2006; Yadav et al., 2011), genera (Yadav et al., 2011; Li and Sun, 2012), and families (Decroocq et al., 2003). A fingerprinting system for 16 *Michelia* germplasms was successfully established using EST-SSR markers of *M. maudiae* and *L. tulipifera*. In this study, the transferability of EST-SSRs of *Liriodendron* and *Michelia* was 21-38%, which was lower than that observed by Li et al. (2011; 24-71%) and lower than that of *Liriodendron* and *Magnolia* (54%). However, the transferability of EST-SSRs is generally affected by the proportion of conserved SSR-flanking sequences (Cordeiro et al., 2001), the position of the primer sequences in the EST (Li et al., 2011), genome size, life span, and the type of mating system (Ueno et al., 2009).

Unlike traditionally used marker such as RAPD, ISSR, and AFLP, SSRs are co-dominant, which means that homozygous or heterozygous loci can be distinguished and the complete genetic information can be extracted (Song et al, 2012). Moreover, owing to hyper-variability, closely related species and even hybrid progeny can be accurately identified. For example, in species related to *Osmanthus fragrans* (Duan et al., 2014), *Populus* (Jia et al., 2015), *Ginkgo biloba* (Wang et al., 2017), or *Camellia sinensis* (Chen et al., 2017), and in hybrid progeny of *Juglans regia* (Pollegioni et al., 2009), *Ziziphus jujuba* (Sun et al., 2015), *Myrica rubra* (Wang et al., 2015) and other woody plants, fingerprinting systems were successfully established using SSR markers. Compared with traditional high-resolution agarose gel electrophoresis and polyacrylamide gel electrophoresis, the technique of fluorescence SSR capillary electrophoresis genotyping based on high-throughput sequencing are easy to use, less time consuming, highly efficient, cost-effective, highly accurate and reproducible (Liesebach et al., 2010; Fariaet al., 2011). To test large numbers of individuals, multiple PCRs can be employed to save costs and increase throughput (Drašnarová et al., 2014; Wu et al., 2016). The fingerprinting system established in this study may help distinguish eight closely related species by using different primer combinations and can also be used to identify different individuals of *M. chapensis* and *M. maudiae*species, emphasizing the high efficiency and accuracy of SSR markers.

The classification of genera and species of the Magnoliaceae has traditionally been an issue of disagreement, including the phylogenetic classification of *Michelia* (Zhang, 2007). The phylogenetic history and divergence of the genus *Michelia* has been widely discussed, and at present, there are two taxonomic systems, one suggested by Liu et al. (1996) and the other by Chen and Nooteboom (1993). In the current study, eight species of *Michelia* were assigned to five groups. In line with Li (2008; 2013), group I (containing five *M. chapensis* individuals) and group II (*M. wilsonii*, *M. compressa* and *M. macclurei*) formed a branch, which indicated that *M. chapensis* was closely related to the other three species. Group III consisted of five *M. maudiae* individuals and one *M. maudiae* cv. Jinxiu individual, which also suggested that *Michelia* 'Jinxiu' was a superior individual originating from a natural variation of *M. maudiae*. The groups IV and V were *M. foveolata* and *M. platypetala*, respectively, indicating considerably divergent phylogeny from other species, whereas Wen et al. (2014) suggested *M. platypetala* and *M. maudiae* to be closely related; however, *M. maudiae* and *M. chapensis* formed two separate branches in their study, which is in line with our results. The phenotypes of numerous *Michelia* species vary depending on their habitat, including *M. foveolata* (Zhang, 2007), and a different number or type of markers and different sample numbers may also lead to discrepancies in taxonomic classifications. Therefore, in order to accurately classify the genus *Michelia*, multiple criteria such as morphology, cytology, and molecular biology should be applied.

3 Materials and Methods

3.1 Plant material and DNA isolation

A total of 16 individuals of *Michelia*, including five *M. chapensis,* five *M. maudiae*, one 'Jinxiu' *Michelia*, one *M. wilsonii*, one *M. foveolata*, one *M. platypetala*, one *M. compressa*, and one *M. macclurei* were selected from a

Michelia nursery at the Jiangsu Academy of Forestry (Nanjing, Jiangsu Province. China; Table 4). From each individual, young leaves were collected, immediately frozen using liquid nitrogen, and stored at -70°C. A commercially available plant DNA extraction kit (DP305; Tiangen Biochemistry Technology Co. Ltd, Beijing) was used to isolate total DNA, according to the manufacturer's instructions. DNA integrity was assessed by 0.8% agarose gel electrophoresis. Purity and DNA concentration were determined using a UV spectrophotometer, and the samples were stored at -20°C.

3.2 Primer screening and transferability detection

Out of 49 SSR markers derived from *M. maudiae* (Sun et al., 2010) and *L. tulipifera* (Yang et al., 2012), 34 markers with high transferability were selected for cross-species amplification. Genomic DNA of individuals 1, 6, 11, 12, 13, 14, 15, and 16 was used as templates for preliminary primer screening after which 13 SSR markers were selected for fingerprinting (Table 5). All markers were labeled with fluorescent groups at the 5' end as mentioned above and were synthesized by Shanghai Lingen Biotechnology Co., Ltd.

PCR reactions of 10 μL contained 5 μL PCR mix, 3.4 μL ddH_2O, 0.3 μL forward primer, 0.3 μL reverse primer, and about 1 μL DNA template. The reaction was performed using the following thermocycling program: 94 °C for 5 min, followed by 35 cycles of 94 °C for 50 s, 56 °C for 50 s, and 72 °C for 90 s, and a final extension step at 72 °C for 7 min. PCR products were visualized by electrophoresis using 1.5% agarose gels.

3.3 Genotyping system establishment

The PCR reaction volume for genotyping was 15 μL (Table 6). The reaction was performed under the following thermocycling conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, and a final extension step at 72 °C for 3 min. The PCR products were visualized by agarose gel electrophoresis; after this, the products were diluted 10-fold and mixed with the internal standard of ROX 500. The denaturation reactions of a volume of 10 μL contained 8.25 μL HiDi, 0.25 μL ROX 500, and 1.5 μL diluted PCR product. After 5 min of denaturation at 95 °C, the reaction was immediately placed on ice for 3 min and then sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

3.4 Data analyses

The number of alleles (Na), He, Ho, and PIC were calculated for each EST-SSR locus using MSA software.

Table 4 Experimental materials

Individuals code	Species	Collection sites
1	*M. chapensis*-1	*Michelia* germplasm nursery of Jiangsu Academy of Forestry
2	*M. chapensis* -2	
3	*M. chapensis* -3	
4	*M. chapensis* -4	
5	*M. chapensis* -5	
6	*M. maudiae*-1	
7	*M. maudiae*-2	
8	*M. maudiae*-3	
9	*M. maudiae*-4	
10	*M. maudiae*-5	
11	*M. maudiae* '*Jinxiu*'	
12	*M. wilsonii*	
13	*M. foveolata*	
14	*M. platypetala*	
15	*M. compressa*	
16	*M. macclueei*	

Table 5 Details of 13 SSR primers

Code	Name	Primer sequence	Motif	Expectation fragment (bp)
1	SGA5	F: GAGATGAGTCACCGCCTGTT R: ATTCAGTTGCACGGCTCTCT	$(AG)_{15}$	234
2	SGA15	F: CTGACGTAACCCGACCTGAT R: CCTGACTTGATCCCACCACT	$(GA)_{14}$	171
3	MMA10	F: TCCACCCCTTTCTCTCCTTT R: AGCCTCCGGATGAGTCCTAT	$(TC)_{10}$	197
4	MMA19	F: GAAATTGGAGAAATCGACTG R: CCCTCTCTTACGCCTCTC	$(AG)_{6}$… $(AG)_{5}$	211
5	MMA36	F: CAATATGGGTTCTTCGGGTTT R: TCCACACTGGTTTTTGGTGA	$(AG)_{12}$	260
6	MMA37	F: TAGGATGGTCCCACCTTGTT R: CAAAACCGTTGAGGCAAAAC	$(TC)_{18}$	222
7	MMA51	F: CGATGCAGCCTAAAAAGAGC R: CGATCATCTCTCCCGTCACT	$(GA)_{10}$	298
8	MMA67	F: CACGAATCCAAGGAAAGGAA R: CGTCCGTCTCAGAACCTCTC	$(TC)_{8}$	147
9	MMA72	F: TTTTCCACCCCTCTCGAATA R: CCATTATGCTGGGGTGTCTT	$(GA)_{14}$	222
10	LT47	F: TCCATCCCTATCTCTGCCAT R: GGCGTTTGTCCATGTTTGTA	$(TG)_{13}$	278
11	LT58	F: GTACTGCCATCAAGGCCAAT R: CAACACTTTCGCATGCAACT	$(CTT)_{10}$	236
12	LT106	F: ATGCAGTGGCCATACATCAG R: GCACAGCAGAACAAAGGTCA	$(CT)_{17}$	237
13	LT186	F: TGCCCACATGTGCTATGTTT R: CCAACCCAACTGCTTTTGTT	$(GA)_{17}$	241

Table 6 PCR reaction system for genotyping

Code	Item	Volume
1	$MgCl_2$ (25mmol/L)	1.5 μL
2	DNA	1 μL
3	10×buffer	1.5 μL
4	Forward Primer	0.15 μL
5	Reverse Primer	0.15 μL
6	DNTP (10mmol/L)	0.3 μL
7	Taq Polymerase (5u/μL)	0.3 μL
8	ddH_2O	10.1 μL
9	Total	15 μL

Alleles were transformed to a 0/1 matrix according to the fragments, and SM similarity coefficients were calculated. The software NTSYS-pcversion 2.1 was used to cluster the 16 individuals of *Michelia* using a UPGMA method.

Authors' Contributions

JZY and ZJW designed and performed the experiments of this study. ZJW analyzed the data. DQQ and HLB participated in the experiment design and data analyses. HXD conceived this study and supervised experimental design, data analyses, manuscript writing, and revisions. All authors read and approved the final manuscript.

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