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ESTABLISHMENT OF DNA FINGERPRINTING IN CLONAL TEA IMPROVED CULTIVARS FROM YUNNAN OF CHINA USING ISSR MARKERS

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Abstract

In this study, DNA fingerprints were constructed by using ISSR markers for 20 clonal improved varieties developed by two breeding institutes in Yunnan province. Seven core ISSR primers were selected from 15 primers. A total of 110 bands were generated by PAGE with seven core primers, 93 of which were polymorphic bands, the percentage of polymorphic band (PPB) was 84.54%, and the mean value of polymorphism information content (PIC) reached 0.417; the genetic similarity coefficient of the cultivars was 0.574-0.854. The two primers, UBC835 and ISSR2, had high PIC values, and could be used to distinguish all cultivars, presenting the most efficient single primers. Among the all of primer combinations from the seven core primers, the three combinations, UBC835/UBC811, UBC835/ISSR2, and UBC835/ISSR3 showed lower similar coefficients, and more efficient in identifying the 20 improved varieties than the other primer combinations. Then these three primer combinations were further scored in 15 traditional cultivars. The results showed that UBC835/ISSR2 was the optimal primer combination, which could be used to distinguish each material among the 20 clonal improved varieties and 15 traditional cultivals. Finally, the DNA fingerprints of the 20 clonal improved varieties were constructed based on country and region code, breeding institute, core primer name and ISSR marker data. The established fingerprints could provide reliable scientific base for the protection of intellectual property right for these clonal improved varieties, and the important molecular information contained in these fingerprints would be useful for the authenticity identification and genetic relationship analysis of tea varieties.

Key words: DNA fingerprinting, Clonal tea improved cultivar, ISSR.

Introduction

Tea tree is a perennial crop, the revenue can be earned for a long period when the improved varieties of tea tree were planted, but it will take over a decade to breed a new variety. Due to technical limitations, the authentic identification (evaluation) for the varieties are mainly based on morphological characteristics and biochemical compositions, which are affected by environmental conditions, cultivation practices and development phase with low reliability. As a result, the fake or controversial varieties mixed in the extended tea improved variety would be difficult to effectively supervise and arbitrate due to lack of effective species identification methods. Thus it is an urgent need to establish a set of steady, reliable, and easily accessibility identification methods and technical regulations on tea tree clonal varieties to promote the extension process of improved varieties in Yunnan tea gardens and effectively protect intellectual property rights of new tea varieties. Bracingly, with the development of DNA marker technology and improvement of testing technique, it is possible to quickly and accurately identify varieties at the DNA level, which is not affected by environmental conditions (Gao *et al*., 2009). DNA markers identification has been currently incorporated into DUS tests by the international union for the protection of new varieties of plants (UPOV). In China, variety identification at DNA level is also an important measure for variety quality monitoring, and provide a theoretical foundation and legal basis for variety protection (Wang *et al*., 2007).

Molecular marker technology is one of the most effective methods to identify crop varieties. This technology has many advantages, including the capacity to reflect genome variation in different varieties, genetic stability, elite polymorphism, and the steady results unaffected by environmental conditions. Recently, many types of molecular markers have been applied in tea resources research, and showed an outstanding practicability (Chen *et al*., 2002; Chen *et al*., 2005; Li *et al*., 2001; Liang *et al*., 2000; Lee *et al*., 1995; Wachira *et al*., 1997; Chen *et al*., 1998; Paul *et al*., 1997; Chen *et al*., 2005; Wachira *et al*., 2001; Huang *et al*., 2004; Matsumoto *et al*., 2002; Huang *et al*., 2008; Chen *et al*., 2009). While ISSR maker is similar to RAPD in the technical principle and has the most advantages of RAPD, the ISSR amplification results are more steady owing to the longer primer (16-25 bp), higher annealing temperature, and have been reported in many tea tree resource studies on genetic diversity, genetic relationships, fingerprints and molecular identification (Ziekiewca *et al*., 1994; Liu *et al*., 2009; Liu *et al*., 2010; Yao *et al*., 2007; Ji *et al*., 2009; Liu *et al*., 2008; Liu *et al*., 2006). However, DNA fingerprinting by ISSR markers in Yunnan clonal tea varietis has not been carried out, although there are mature technologies. Thus in this study, fingerprints for 20 varieties developed by tea tree breeding institution in Yunnan province were were constructed by using ISSR markers, to provide a reliable scientific basis for the molecular identification and the intellectual property of these clonal improved varieties.

Materials and Methods

Materials: Twenty clonal tea improved varieties were selected for fingerprinting, among which 19 varieties have been registered by the nation and province, and one variety ("Zijuan") with the national protection right of new plant variety (2005) had been registered in Yunnan province (Dian Tea Registration Number: 2014009). A total of 35 varieties (20 clonal improved varieties plus another 15 local tea varieties) were selected to test the efficiency of core primer combinations. The leaf tissue materials of all varieties were collected from China National Germplasm Tea Repositories (CNGTR) and the Tea Research Institute of Yunnan Academy of Agricultural Sciences (TRIYAAS) (Table 1). The samples were rapidly frozen, processed, and then stored at −80°C until extraction of DNA.

Table 1. The name, type and provenance of 35 tea varieties from Yunnan Province.

No.	Variety name	Provenance	Level of variety certification	Breeding institution or donor institution
1.	Yunkang10	Menghai, Yunnan	National Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
2.	Yunkang14	Menghai, Yunnan	National Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
3.	Yunkang27	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
4.	Yunkang37	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
5.	73-8	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
6.	Yunkang48	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
7.	Yunkang50	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
8.	Yunkang43	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
9.	Changyebaihao	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
10.	Foxiang1	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
11.	Foxiang2	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
12.	Foxiang3	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
13.	Foxiang4	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
14.	Foxiang5	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
15.	73-11	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
16.	76-38	Menghai, Yunnan	Provincial Improved Tea Variety	Tea Research Institute of Yunnan Academy of Agricultural Sciences
17.	Zijuan	Menghai, Yunnan	National union for Protection of Plant Variety Resources	Tea Research Institute of Yunnan Academy of Agricultural Sciences
18.	Yunmei	Puer, Yunnan	Provincial Improved Tea Variety	Tea Seed Farm in Si Mao of Yunnan
19.	Yungui	Puer, Yunnan	Provincial Improved Tea Variety	Tea Seed Farm in Si Mao of Yunnan
20.	Aifeng	Puer, Yunnan	Provincial Improved Tea Variety	Tea Seed Farm in Si Mao of Yunnan
21.	Manmianliuye	Longchuan, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
22.	Dazhelangdayecha	Tengchong, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
23.	Mengkedaye	Zhenyuan, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
24.	Mengkudaheicha	Shuangjiang, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
25.	Yibanghongyecha	Mengla, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
26.	Jiulongdayecha	Luoping, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
27.	Niuhongcha	lüchun, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
28.	Shuangbaizhencha	Shuangbai, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
29.	Luojiehongyecha	Jiangcheng, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
30.	Mengsongdayecha	Menghai, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
31.	Wenshanxiaojiecha	Wenshan, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
32.	Xishelubaiyacha	Chuxiong, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
33.	Longkoucha	Jinping, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
34.	Piangugangcha	Lushui, Yunnan	Local Cultivar	China National Germplasm Tea Repositories
35.	Changlü1	Mouding, Yunnan	Local Cultivar	China National Germplasm Tea Repositories

DNA isolation: DNA from leaves was extracted as described by Rohlf (2000).

ISSR amplification: According to the existing ISSR-PCR reaction system (Rohlf, 2000), 15 pairs of primers were synthesized by Shanghai biological engineering technology services company (Liu *et al*., 2010). Reactions were performed on a programmable peltier thermocycler PTC 200. The components of 10 μL of PCR reaction mixture contained 1.0 μL DNA (40 ng/μL template DNA), 0.4 μL of 10 μmol/L primer, 1.0 μL of 10× PCR reaction buffer, 0.8 μL of 25 mmol/L Mg^{2+}, 0.2 μL of 10 nmol/L dNTPs, 0.1 μL of 5 U *Taq* DNA polymerase. Amplification protocol included initial denaturation for 5 min at 94°C, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 30s at 52°C-60°C with respective Tm values of the selected primers, and 2 min elongation at 72°C. Final elongation was performed for 7 min at 72°C and hold at 4°C.

PCR amplified products were resolved on 6% polymacrylamide gel for 4 h at a constant voltage of 150 V with 0.5×TBE running buffer. Finally, the gel was silver-stained, visualized under ultraviolet light, photographed, and documented. The experiment was repeated twice or three times.

Data collection and analysis: Each band of map was as a molecular marker depending on the ISSR amplification bands through artificial reading belt way. All amplified fragments from each primer were arranged by the order of molecular size. DNA bands were scored qualitatively as either presence ("1") or absence ("0"). . Data matrix (1, 0) was established depending on different primers amplified bands. Primers were identified by using the percentage of polymorphic bands (PPB), the genetic similarity (GS), the polymorphism information content (PIC) and the rate of distinguishing variety by cluster (RDVC). The PPB was estimated by $PPB = a/(a+b)$, and the GS was estimated by $GS_{ij} = a/(a+b+c)$, where a is the total number of common bands for the i^{th} and j^{th} lines, and b and c are the numbers of unique bands for the ith and the jth lines, respectively. The PIC was estimated by $PIC = 1-\Sigma P_i^2$, where Pi is the frequency at the ith allelic locus. The RDVC was estimated by $RDVC = (N-N_i)/N$, where N_i is unable to distinguish between varieties, N is the total number of specie varieties. GS and unweighted pair group method analysis (UPGMA) were carried out using the NTSYS-pc2.10 package.

Results

Rate of distinguishing efficiency of core primers analysis: Selecting appropriate primers is one of the key factors for DNA fingerprinting. The core primers of DNA fingerprinting should have more alleles, richer polymorphism, higher rate of distinguishing, more steady amplified bands and easier to make statistical analysis. 15 ISSR primers with high allelic loci quantity, rich polymorphism and steady amplified bands were selected in this study, among which seven ISSR excellent primers were used as core primers to establish DNA fingerprints for tea tree varieties (Tables 2, 3). The gel map of ISSR primer amplification was shown in Fig. 1. A total 110 bands were amplified by using seven core primers, of which 93 (84.54%) were polymorphic, 12 were variety-specific bands (each variety with a unique band), the highest variety similarity coefficient was 0.854, which could fully effectively distinguish all Yunnan clonal improved varieties. The higher maximum similarity coefficient among varieties, and lower RDVC indicated lower distinguishing rate of primers. As a single primer was considered, the primer UBC835 with the lowest maximum variety similarity coefficient of 0.693 and RDVC of 100%, could completely distinguish all Yunnan clonal improved varieties, and was scored as the most effective core primer, which, followed by the primer ISSR2. The highest maximum variety similarity coefficient from the primer UBC808 was 1.000 and RDVC was 82%, which indicated that UBC808 was the worst primer with the lowest distinguishing efficiency.

Thirty five tea varieties were divided into two major groups A and B (Fig. 2). Group A was composed of 19 lines in three subgroups at average genetic similarity coefficient of 0.72. Subgroup I contained 10 lines such as"Yunkang10", "Yunkang14", "Foxiang1", "Foxiang5", "Foxiang3", "Foxiang4", "Foxiang2", "73-11", "Changyebaihao "and "Yunkang 48". Subgroup II included "Yunkang43", "Yunkang27" and "Yunkang37". Subgroup III involved "Aifeng", "Yungui" and "Yunmei". In addition, "73-8", "76-38", "Zijuan" and "Yunkang50" were classified as a solitary group. Group B constituted 15 local varieties, which were completely separated from the clonal improved varieties. Genetic similarity coefficient among all 35 accessions ranged from 0.693 to 0.854 with an average of 0.465. The level of genetic diversity among species was relatively lower.

Efficiency analysis of core primer combinations: Although part of single core primers could fully distinguish all Yunnan tea varieties, as the highest maximum variety similarity coefficient was up to 0.854, the rate of distinguishing of single primer would significantly decreased with the increasing of varieties number. Therefore, it is necessary to use multiple primer pairs for variety identification. Seven primers were pair-wise combined to sort out the effective primer combination. The results showed that 20 out of the all 21 primer combinations could completely distinguish 20 clonal improved varieties (Table 4), except for the primer combination UBC808-ISSR4 with the maximum variety similarity coefficient of 1.000 and RDVC of 95%, which could not distinguish two varieties and was scored as an inefficient primer combination. Among the effective primer combinations, UBC835-ISSR2 was the most effective primer combination with maximum similarity coefficient of 0.693 and RDVC of 100%, which followed by UBC835-UBC811 and UBC835-ISSR3. On the whole, the primer UBC835 showed a better performance in the all primer combinations to distinguish than other single primers, and followed by ISSR2, UBC811 and ISSR3. As the all varieties tested could be effectively distinguished by the combination of two primer pairs, DNA fingerprints of Yunnan tea varieties in this study were constructed by using the molecular data derived from pair-wise primer combinations.

Table 2. Sequences and traits of core ISSR primers.

Primer name	Primer sequence	T_m	T_a	GS (%)	MW(ug/umole)
UBC835	$(AG)_8$YC	56.16	57	52.78	5642.50
UBC808	$(AG)_8$C	54.59	54	52.94	5366.54
UBC811	$(GA)_8$C	54.59	55	52.94	5366.54
ISSR2	$(AG)_8$CTA	55.41	52	47.37	5983.94
ISSR3	$(GA)_8$CTT	55.41	58	47.37	5974.92
ISSR4	$(TC)_8$AGT	55.41	55	47.37	5631.57
ISSR5	$(TC)_8$AGG	57.56	56	52.63	5656.59

Tm: melting temperature; Ta: annealing temperature; GS: genetic similarity; MW: molecular weight

Table 3. Efficiency of core ISSR primers.

Core primers	Total bands	PB	PPB (%)	Specific band	PIC	The highest GS between cultivars	RDVC (%)
UBC835	18	15	83.3	3	0.693	0.854	100
UBC808	17	13	76.5	2	0.688	1.000	82
UBC811	13	11	84.6	1	0.673	1.000	85
ISSR2	17	15	88.2	2	0.688	0.819	92
ISSR3	14	12	85.7	1	0.647	1.000	83
ISSR4	16	14	87.5	1	0.647	1.000	85
ISSR5	15	13	86.7	2	0.611	1.000	83
Average	15.7	13.2	84.54	1.6	0.664	0.953	87.1

PB: polymorphic band; PPB: percentage of polymorphic bands; PIC: polymorphism information content; GS: genetic similarity; RDVC: rate of distinguishing cultivars by cluster.

Table 4. Efficiency of core primer combinations.

Core primers	Total bands	PB	PPB (%)	Specific bands	The highest GS between cultivars	RDVC (%)
UBC835/UBC808	35	28	80.0	5	0.812	100
UBC835/UBC811	31	26	83.9	4	0.709	100
UBC835/ISSR2	35	30	85.7	5	0.693	100
UBC835/ISSR3	32	27	84.4	4	0.709	100
UBC835/ISSR4	34	29	85.3	4	0.809	100
UBC835/ISSR5	33	28	84.8	5	0.745	100
UBC808/UBC811	30	24	80.0	3	0.818	100
UBC808/ISSR2	34	28	82.4	4	0.854	100
UBC808/ISSR3	31	25	80.6	3	0.736	100
UBC808/ISSR4	33	27	81.8	4	1.000	95
UBC808/ISSR5	32	26	81.2	4	0.763	100
UBC811/ISSR2	30	26	86.7	3	0.745	100
UBC811/ISSR3	27	23	85.2	2	0.718	100
UBC811/ISSR4	29	25	86.2	2	0.827	100
UBC811/ISSR5	28	24	85.7	3	0.754	100
ISSR2/ISSR3	31	27	87.1	3	0.782	100
ISSR2/ISSR4	33	29	87.9	3	0.800	100
ISSR2/ISSR5	32	28	87.5	4	0.773	100
ISSR3/ISSR4	30	26	86.7	2	0.836	100
ISSR3/ISSR5	29	25	86.2	3	0.827	100
ISSR4/ISSR5	31	27	87.1	3	0.818	100

PB: polymorphic band; PPB: percentage of polymorphic bands; PIC: polymorphism information content; GS: genetic similarity; RDVC: rate of distinguishing cultivars by cluster.

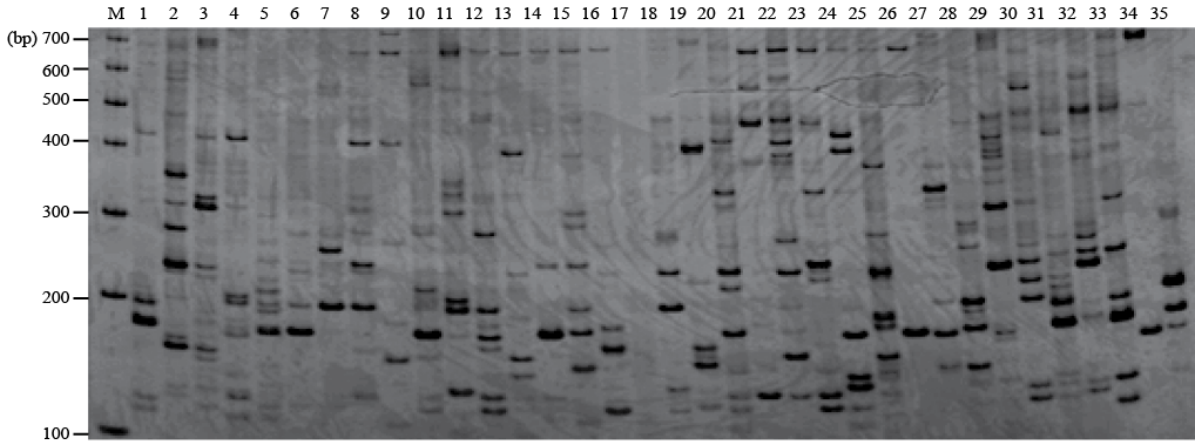

Fig. 1. Gel map of ISSR amplification of primer for Yunnan tea cultivars
The numbers for each lane correspond with the numbers for cultivar names listed in Table 1.

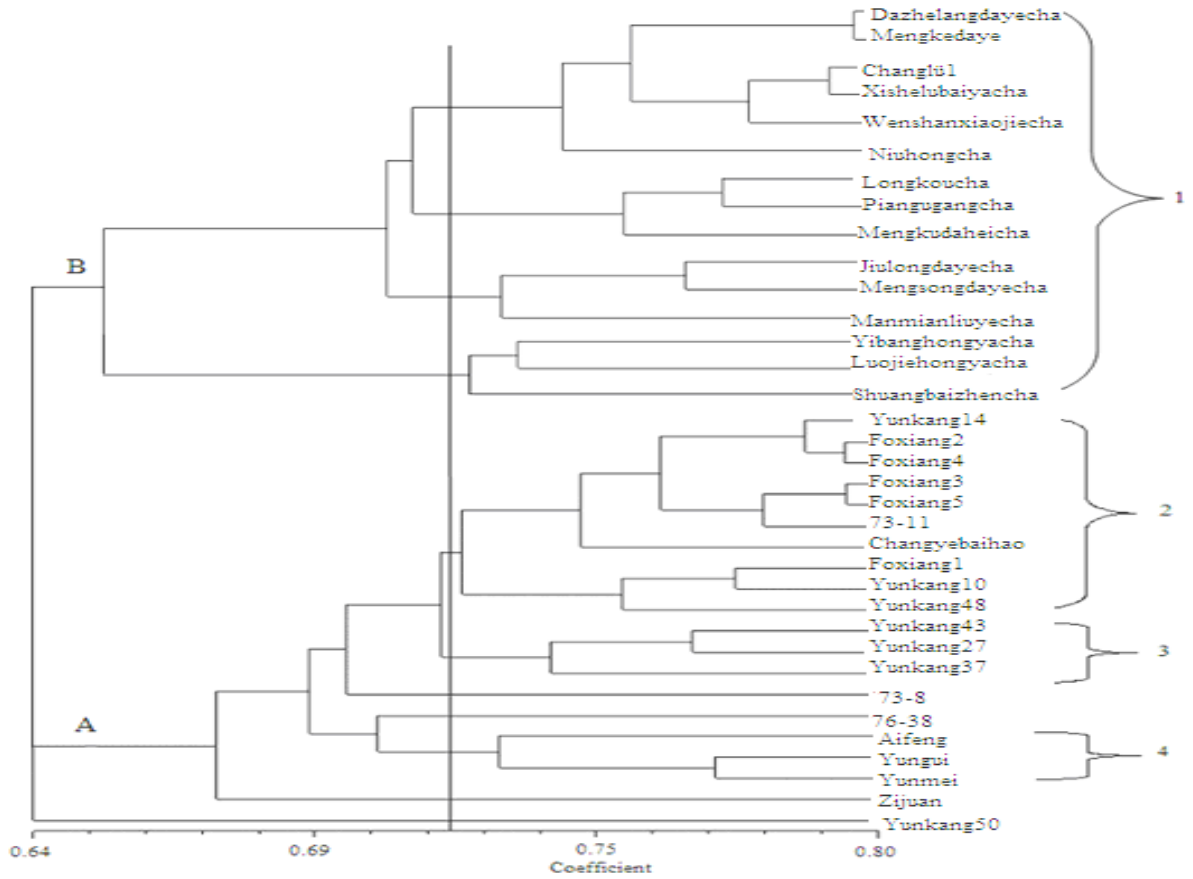

Fig. 2. Dendrogram of 35 tea cultivars resulting from UPGMA analysis based on Dice′s similarity coefficient calculated from the ISSR data

The verification of efficient primer combinations: To increase the practicability of the constructed molecular fingerprints, similarity and cluster analysis were carried out in 35 tea varieties (15Yunnan local varieties and 20 Yunnan clonal improved varieties) to evaluate distinguishing efficiency of the top three primer combinations (Table 5). The results showed that the all three primer combinations had the RDVC of 100% for the all 35 varieties, and could effectively distinguish the all tested varieties. When the primer combination UBC835/ISSR2 was used, the maximum variety similarity coefficient among 35 cultivars was the lowest of 0.693. Varieties cluster of 0.693 was the most similar between “Foxiang2” and “Foxiang4”, followed by varieties cluster of 0.718 between “Foxiang3” and “Foxiang5”. When the primer combinations

UBC835/UBC811 and UBC835/ISSR3 were used the both maximum similarity coefficients were 0.709, which seems suggest an equivalent distinguishing efficiency for the both primer combinations. However, the rate of distinguishing for UBC835 / ISSR2 was best according to different primers combination of clustering relations, which was constructed molecular fingerprint identification of Yunnan clonal improved varieties as the best primer combination. UBC835 / UBC811 were exhibited the most similar clustering relations for clonal improved varieties. For instance, the similar clustering relations of "Foxiang2" and "Foxiang4", "Foxiang3"and "Foxiang5", "Yunkang10" and "Foxiang1" were 0.709, 0.745 and 0.773, respectively. The similarity of *Menghai grandifoliate* tea with most similar to local variety was 0.667. So UBC835/ UBC811 could not only effectively distinguish Yunnan clonal varieties, but also Yunnan clonal varieties and local varieties. The similarity coefficient of UBC835/ISSR3 was lower than UBC835/ UBC811. For instance, the similar cluster relations of "Yunkang10" and "Foxiang1", "Yunkang14" and"Foxiang2" were 0.709 and 0.700, respectively.

Establishment of DNA fingerprints for the clonal tea improved varieties: To further improve the practicality and reference value of DNA fingerprint, the DNA fingerprint code needs contain not only molecular data, but also the important information for varieties. Thus in this study, four groups of code with corresponding abbreviations were selected to construct the DNA fingerprint identification card for the clonal tea improved varieties. The group I, II, III and IV represented the country and region code of the improved variety, breeding institution, core primer name and ISSR marker data, respectively. The ordinal combination of the four groups of code composed an unique DNA fingerprint for each Yunnan clonal tea improved variety (Table 6). It was easily convenient to get relevant data for variety identification through the above information.

Table 5. Efficiency analysis of the best primer combinations.

Core primers	Total bands	PB	PPB (%)	Specific bands	The highest GS between cultivars	RDVC (%)
UBC835/UBC811	31	26	83.9	4	0.709	100
UBC835/ISSR2	35	30	85.7	5	0.693	100
UBC835/ISSR3	32	27	84.4	4	0.709	100

PB: polymorphic band; PPB: percentage of polymorphic bands; PIC: polymorphism information content; GS: genetic similarity; RDVC: rate of distinguishing cultivars by cluster

Table 6. DNA fingerprint of 20 clonal tea cultivars from Yunnan Province.

Cultivar name	Country and region code + breeding institution + core primer name + ISSR marker data
Yunkang10	CYZ—TRIYAAS—UBC835-10111111110111111-ISSR2-1000011001 110111
Yunkang14	CYZ—TRIYAAS—UBC835-10011111110111111-ISSR2-1000011011 111111
Yunkang27	CYZ—TRIYAAS—UBC835-10011001101111111-ISSR2-10001 10111111111
Yunkang37	CYZ—TRIYAAS—UBC835-10011010000111111-ISSR2-1000111111111111
73-8	CYZ—TRIYAAS—UBC835-10011111000011101-ISSR2-1001110101111111
Yunkang48	CYZ—TRIYAAS—UBC835-10011111100111101-ISSR2-10001 11101111111
Yunkang50	CYZ—TRIYAAS—UBC835-10011111100110101-ISSR2-10111 11101111111
Yunkang43	CYZ—TRIYAAS—UBC835-00000000100011101-ISSR2-1000111101111111
Changyebaihao	CYZ—TRIYAAS—UBC835-00010111001111101-ISSR2-10111 11001111111
Foxiang1	CYZ—TRIYAAS—UBC835-00010111010111101-ISSR2-1000111001111111
Foxiang2	CYZ—TRIYAAS—UBC835-00010111111111111-ISSR2-1101111101111111
Foxiang3	CYZ—TRIYAAS—UBC835-0100000011100 001-ISSR2-000000000 1111111
Foxiang4	CYZ—TRIYAAS—UBC835-10111111111011001-ISSR2-0111110101111111
Foxiang5	CYZ—TRIYAAS—UBC835-10111111100111011-ISSR2-0111111101111111
73-11	CYZ—TRIYAAS—UBC835-01111111101111111-ISSR2-0110010101111111
76-38	CYZ—TRIYAAS—UBC835-00000001001011101-ISSR2-0000010101111111
Zijuan	CYZ—TRIYAAS—UBC835-10111111111101101-ISSR2-0011111001111111
Yunmei	CYZ—TSFSMY—UBC835-10111101000000110-ISSR2-0000010101111111
Yungui	CYZ—TSFSMY—UBC835-10101111000110 11-ISSR2-0000011101111111
Aifeng	CYZ—TSFSMY—UBC835-10000101100111101-ISSR2-0000010101 111111

Note: CYZ (Chinese Yunnan Zone), TRIYAAS (Tea Research Institute, Yunnan Academy of Agricultural Science), TSFSMY (Tea Seed Farm in Si Mao of Yunnan)

Discussion

At present, seed production and management institutions are not yet standardized in China. The problem occurs frequently to species introduced are either confusion or fraud, which greatly damaged variety patent of owners and economic interests of farmers. Thus it is particularly important to carry out identification of crop varieties resources (Liang *et al*., 2001a & b). The traditional identification method is simple, economical and fast, but it is mainly based on phenotypic traits easily affected by the environment (She *et al*., 2003), which would lead to a higher rate of identification error, and make the identification more difficult with the increase of the similarity between varieties. As DNA is less affected by environment, has high polymorphism and stability, DNA technology has became the most effective tool for crop variety identification (Xin *et al*., 2005; Wang *et al*., 2003).

A variety of molecular markers or DNA fingerprinting techniques are currently in use for species identification. Different molecular markers have unique advantages and disadvantages, so the first problem in DNA fingerprinting is to select appropriate molecular markers or combine different markers. Many successful experiences and examples have shown that DNA fingerprinting established by using molecular markers is an effective tool for crop variety identification (Liu *et al*., 2004; Zhao *et al*., 2003; Wang *et al*., 2005). RAPD, AFLP, RFLP and ISSR markers have been applied on tea tree resource researches, such as genetic diversity, phylogenetic analysis and germplasm resources identification (Chen *et al*., 2002; Li *et al*., 2001; Liang *et al*., 2000 ; Lee *et al*., 1995; Wachira *et al*., 1997; Chen *et al*., 1998; Paul *et al*., 1997; Wachira *et al*., 2001; Huang *et al*., 2004; Matsumoto *et al*., 2002; Huang *et al*., 2008; Chen *et al*., 2009; Ziekiewca *et al*., 1994; Yao *et al*., 2007; Ji *et al*., 2009). In the past, RAPD markers had been mostly used for molecular identification,but the accuracy of its results was discontented due to the short random primers (10bp), low annealing temperature, non-specific amplification, low repeatability and poor stability. ISSR markers has both the commonality of RAPD and the most advantages of AFLP and RFLP, and thanks to the longer primers (16-25bp) and higher annealing temperature, the amplification results are more stable while polymorphism and reproducibility better than RAPD markers (Ziekiewca *et al*., 1994). Owing to the simple and rapid operation, high reproducibility, rich polymorphism information and relatively low cost, ISSR markers have been widely used in tea varieties resources (Ziekiewca *et al*., 1994; Yao *et al*., 2007; Ji *et al*., 2009; Liu *et al*., 2006), and the practicability and feasibility of ISSR markers in the tea tree identification has also been verified (Liu *et al*., 2009; Liu *et al*., 2008).

Conclusion

In this study, we chose most polymorphic ISSR primers screened by Liu *et al.* (2008; 2009; 2010) to estimate polymorphism information content and proportion of polymorphic bands simultaneously. The maximum variety similarity coefficient and variety distinguishing rate by cluster analysis was evaluated on the basis of primers efficiency. The results showed that seven ISSR primers (UBC808, UBC811, UBC835, ISSR2, ISSR3, ISSR4, ISSR5) could generate maps with high rate of distinguishing and easy to count, suggesting that these core primers were stable and reliable as the previously screened ISSR primers (Liu *et al*., 2008; Liu *et al*., 2009; Liu *et al*., 2010). The efficiency evaluation of pair-wise primer combinations suggested that the primer combination UBC835-ISSR2 had the lowest maximum variety similarity coefficient and the best distinguishing efficiency, and could be selected as critical core primers in the DNA fingerprints construction for 20 Yunnan clonal tea tree improved varieties. The DNA fingerprint of 20 clonal tea tree improved varieties was set up by drawing the format of national identity card, orderly integrating different kinds of countries, breeding institutes, primer names and molecular data together to form a unique identification code for each variety (Table 6). The successful application of ISSR markers on the identification of Yunnan clonal improved tea varieties would be beneficial to the register of clonal improved varieties, updating of new varieties, full using of germplasm resources, and provide an important scientific basis to promote the healthy development of Yunnan tea varieties project.

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