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FINGERPRINTING FOR DISCRIMINATING TEA GERMPLASM USING INTER-SIMPLE SEQUENCE REPEAT (ISSR) MARKERS

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Abstract

For the discrimination of tea germplasm at the inter-specific level, 134 tea varieties preserved in the China National Germplasm Tea Repositories (CNGTR) were analyzed using inter simple sequence repeat (ISSR) markers. Eighteen primers were chosen from 60 screened for ISSR amplification, generating 99.4% polymorphic bands. The mean Nei's gene diversity (H) and the overall mean Shannon's Information index (I) were 0.396 and 0.578, respectively, indicating a wide gene pool. Using the presence, sometimes absence of unique ISSR markers, it was possible to discriminate 32 of the genotypes tested. No single primer could discriminate all the 134 genotypes. However, UBC811 provided rich band patterns and it can discriminate 35 genotypes. The combination of two and three primers could discriminate 99 and 121 genotypes, respectively. Furthermore, the combination of band patterns or the DNA fingerprinting based on specific ISSR markers generated by UBC811, UBC835, ISSR2 and ISSR3 could discriminate all 134 genotypes tested. ISSR markers also provide a powerful tool to discriminate tea germplasm at the inter-specific level.

Introduction

Tea plants belong to the Theaceace, genus Camellia, section Thea and usually involve one species and two or three varieties, i.e., *Camellia sinensis*, *C. sinensis* var. assamica, *C. sinensis* var. pubilimba and *C. sinensis* var. kucha (Chang, 1981, 1984; Ming, 1992; Chen *et al*., 2000). The tea plants originated from southwest China, Yunnan province (Hashimoto & Takasi, 1978; Yu, 1986), and are the most commercially important species and varieties in the section. Distinct discrimination at both inter-and-intra-specific levels of tea germplasm is of critical important for collection, conservation, evaluation and utilization. Understanding the genetic background it will greatly help in selecting parents for current and long-term success of tea breeding plans.

A large number of tea germplasm and their allied species have been collected and preserved in China, Japan (Takeda, 2000), India and Kenya. The China National Germplasm Tea Repositories (CNGTR) had preserved about 3700 accessions of tea germplasm by the end of 2006. Of particular importance were the more than 200 wild tea camellias collected from southwestern China (Yu & Chen, 2001).The discrimination of tea germplasm at inter-species level has been quite difficult and somewhat inadequate. Morphological traits (Sealy, 1958; Chang, 1981, 1984; Ming, 1992; Chen *et al*., 2000), main chemical components (Du *et al*., 1990), esterase isoenzymes (Lu *et al.*, 1992) and the karyotype (Liang *et al.*, 1994) have been employed to evaluate the phylogeny and to classify tea plants and their related species. However, the effectiveness of these methods for variety identification has proven in most cases to be insufficient. Thus, it is necessary for discriminating tea germplasm to look for more effective, stable and reliable methods.

Inter-simple sequence repeat (ISSR) analysis is a polymerase chain reaction (PCR)-based technique with primers composed of microsatellite sequences with one to three selective bases anchored at the 3' or 5' ends of the primer (Zietkiewicz *et al*., 1994). The method combines higher annealing temperatures and more selective primers than random polymorphic DNA (RAPD), which enhance the reproducibility (Ge & Sun, 1999; Nybom, 2004). ISSR markers can detect considerable levels of polymorphism (Wolf & Liston, 1998; Wolf & Kephart, 1998; Bornet & Branchard, 2001; Muminovic *et al.*, 2005), especially after the combination of different primers in the same PCR reaction (Liu & Wendel, 2001), and have demonstrated their usefulness with respect to improving conservational strategies in many plant species (Esselman *et al.*, 1999; Ge *et al*., 2003; Lee *et al*., 2003; Hatcher *et al*., 2004; Xue *et al*., 2004; Jian *et al*., 2005; Tan *et al*., 2005) and determining the influence of life history traits in the evolution and dynamics of populations (Hess *et al*., 2000; Vargs & Kadereit, 2001). The applicability of ISSR-PCR for genomic fingerprinting at the inter-specific level has been indicated by Zietkiewicz *et al*., (1994). Recently, ISSRs have also been used for the investigation of genetic relationship (Yao *et al*., 2007; Lin *et al*., 2007; Liu *et al*., 2008; Ji *et al*., 2011) and identification of parentage (Liu *et al*., 2008). Nevertheless, specific DNA markers to differentiate between tea plants and their allied species are still rare. The present study aimed to generate specific DNA markers for discriminating inter-specific level tea germplasm and provide guidelines for the conservation, management, and utilization of tea resources using ISSR analysis.

Materials and Methods

Plant materials: A total of 134 tea genotypes, including four species and varieties, i.e., Ser. Quinquelocularis Chang, Ser. Pentastylae Chang, Ser. Gymnogynae Chang and Ser. Sinensis Chang and their allied species in section Thea, genus camellia, based on Chang's taxonomic systems (Chang, 1981, 1984), from the CNGTR at the Tea Research Institute, Yunnan Academy of Agricultural Sciences (TRIYAAS), were sampled (described in Table 1) using ISSR analysis during 2007 to 2008. These materials were taken at minimum intervals of 5m from each other to avoid a confusion of each species.

Table 1. Name, stock-number, germplasm type and origin of 134 tea accessions used in this study.

No.	Species and varieties	Stock-number	Germplasm type	Origin
1.	*C. assamica* (Masters) Chang	GPCS 0108	Traditional cultivar	Hainan, China
2.	*C. crassicolumna* Chang	GPCS 0448	Wild	Yunnan, China
3.	*C. ser. pentastylae* Chang	GPCS 2440	Traditional cultivar	Yunnan, China
4.	*C.taliensis* (W.W.Smith) Melchior	GPCS 1610	Wild	Yunnan, China
5.	*C. crassicolumna* Chang	GPCS 1103	Wild	Yunnan, China
6.	*C.taliensis* (W.W.Smith) Melchior	GPCS 1093	Wild	Yunnan, China
7.	*C. assamica* (Masters) Chang	GPCS 0597	Traditional cultivar	Yunnan, China
8.	*C. tachangensis* F.C.Zhang	GPCS 0913	Wild	Yunnan, China
9.	*C.ser. pentastylae* Chang	GPCS 2061	Traditional cultivar	Yunnan, China
10.	*C. sinensis var. assamica*(Masters) Chang	GPCS 0940	Traditional cultivar	Yunnan, China
11.	*C. ser. pentastylae* Chang	GPCS 1944	Traditional cultivar	Yunnan, China
12.	*C. assamica* (Masters) Chang	GPCS 0683	Traditional cultivar	Yunnan, China
13.	*C. sinensis* var. *pubilimba* Chang	GPCS 0075	Breeding line	Guangxi, China
14.	*C. multisepala* Chang et Tang	GPCS 1048	Traditional cultivar	Yunnan, China
15.	*C. ser. pentastylae* Chang	GPCS 1935	Traditional cultivar	Yunnan, China
16.	*C.taliensis* (W.W.Smith) Melchior	GPCS 1617	Wild	Yunnan, China
17.	*C. ser. pentastylae* Chang	GPCS 1936	Traditional cultivar	Yunnan, China
18.	*C. assamica* (Masters) Chang	GPCS 0933	Traditional cultivar	Yunnan, China
19.	*C. assamica* (Masters) Chang	GPCS 2013	Traditional cultivar	Yunnan, China
20.	*C. sinensis* var. *pubilimba* Chang	GPCS 2043	Traditional cultivar	Yunnan, China
21.	*C. assamica* (Masters) Chang	GPCS 1004	Traditional cultivar	Yunnan, China
22.	*C. gymnogynoides* Chang et Yu	GPCS 1026	Wild	Yunnan, China
23.	*C. ser. pentastylae* Chang	GPCS 1930	Traditional cultivar	Yunnan, China
24.	*C. assamica* (Masters) Chang	GPCS 0451	Traditional cultivar	Yunnan, China
25.	*C. ser. pentastylae* Chang	GPCS 1922	Traditional cultivar	Yunnan, China
26.	*C. assamica* (Masters) Chang	GPCS 0534	Traditional cultivar	Yunnan, China
27.	*C.ser. pentastylae* Chang	GPCS 2011	Traditional cultivar	Yunnan, China
28.	*C. assamica* (Masters) Chang	GPCS 2105	Wild	Yunnan, China
29.	*C.taliensis* (W.W.Smith) Melchior	GPCS 1068	Wild	Yunnan, China
30.	*C. assamica* (Masters) Chang	GPCS 2103	Traditional cultivar	Yunnan, China
31.	*C. assamica* (Masters) Chang	GPCS 0533	Traditional cultivar	Yunnan, China
32.	*C. sinensis* (L.)O.Kuntze	GPCS 0651	Traditional cultivar	Yunnan, China
33.	*C. ser. pentastylae* Chang	GPCS 1917	Traditional cultivar	Yunnan, China
34.	*C. assamica* (Masters) Chang	GPCS 0824	Traditional cultivar	Yunnan, China
35.	*C. assamica* (Masters) Chang	GPCS 0387	Introduction cultivar	Vietnam
36.	*C. assamica* (Masters) Chang	GPCS 0859	Traditional cultivar	Yunnan, China
37.	*C. gymnogynoides* Chang et Yu	GPCS 0465	Wild	Yunnan, China
38.	*C. sinensis* (L.)O.Kuntze	GPCS 1053	Traditional cultivar	Yunnan, China
39.	*C. yankiangcha* Chang et Wang	GPCS 0860	Traditional cultivar	Yunnan, China
40.	*C.taliensis* (W.W.Smith) Melchior	GPCS 1612	Wild	Yunnan, China
41.	*C. atrothea* Chang et Wang	GPCS 1109	Wild	Yunnan, China
42.	*C. assamica* (Masters) Chang	GPCS 0617	Traditional cultivar	Yunnan, China
43.	*C. ser. pentastylae* Chang	GPCS 2066	Traditional cultivar	Yunnan, China
44.	*C. ser. pentastylae* Chang	GPCS 1933	Traditional cultivar	Yunnan, China
45.	*C. assamica* (Masters) Chang	GPCS 1880	Introduction cultivar	Burma

Table 1. (Cont'd.).

No.	Species and varieties	Stock-number	Germplasm type	Origin
46.	*C. ser. pentastylae* Chang	GPCS 1948	Traditional cultivar	Yunnan, China
47.	*C. ser. pentastylae* Chang	GPCS 1582	Introduction cultivar	Vietnam
48.	*C. assamica* (Masters) Chang	GPCS 0938	Traditional cultivar	Yunnan, China
49.	*C. assamica* (Masters) Chang	GPCS 0983	Traditionalcultivar	Yunnan, China
50.	*C. assamica* (Masters) Chang	GPCS 1605	Breeding line	Yunnan, China
51.	*C. assamica* (Masters) Chang	GPCS 0109	Traditional cultivar	Hainan, China
52.	*C. assamica* (Masters) Chang	GPCS 0987	Traditional cultivar	Yunnan, China
53.	*C. irrawadiensis* P.K.Barua	GPCS 0594	Wild	Yunnan, China
54.	*C. assamica*(Masters) Chang	GPCS 0615	Traditional cultivar	Yunnan, China
55.	*C. ser. pentastylae* Chang	GPCS 1916	Traditional cultivar	Yunnan, China
56.	*C. assamica* (Masters) Chang	GPCS 1618	Traditional cultivar	Yunnan, China
57.	*C. assamica* (Masters) Chang	GPCS 0936	Traditional cultivar	Yunnan, China
58.	*C. assamica*(Masters) Chang	GPCS 0559	Traditional cultivar	Yunnan, China
59.	*C. Irrawadiensis* P.K. Barua	GPCS 0583	Traditional cultivar	Yunnan, China
60.	*C. yankiangcha* Chang et Wang	GPCS 1019	Other	Yunnan, China
61.	*C. ser. pentastylae* Chang	GPCS 1111	Wild	Yunnan, China
62.	*C. assamica* (Masters) Chang	GPCS 1881	Introduction cultivar	Burma
63.	*C. ser. pentastylae* Chang	GPCS 1942	Traditional cultivar	Yunnan, China
64.	*C. assamica* (Masters) Chang	GPCS 0388	Introduction cultivar	Vietnam
65.	*C. crassicolumna* Chang	GPCS 0693	Wild	Yunnan, China
66.	*C. sinensis* (L.)O.Kuntze	GPCS 0041	Traditional cultivar	Guangdong, China
67.	*C. assamica* (Masters) Chang	GPCS 2028	Traditional cultivar	Yunnan, China
68.	*C.taliensis* (W.W.Smith) Melchior	GPCS 0428	Wild	Yunnan, China
69.	*C. ser. pentastylae* Chang	GPCS 1934	Traditional cultivar	Yunnan, China
70.	*C. assamica* (Masters) Chang	GPCS 2094	Traditional cultivar	Yunnan, China
71.	*C. assamica* (Masters) Chang	GPCS 0788	Traditional cultivar	Yunnan, China
72.	*C. ser. pentastylae* Chang	GPCS 1921	Traditional cultivar	Yunnan, China
73.	*C. kwangnanica* Chang et Chen	GPCS 0510	Traditional cultivar	Yunnan, China
74.	*C. ser. pentastylae* Chang	GPCS 1932	Traditional cultivar	Yunnan, China
75.	*C. ser. pentastylae* Chang	GPCS 1947	Traditional cultivar	Yunnan, China
76.	*C. assamica* (Masters) Chang	GPCS 1609	Traditional cultivar	Yunnan, China
77.	*C. ser. pentastylae* Chang	GPCS 1943	Traditional cultivar	Yunnan, China
78.	*C. assamica* (Masters) Chang	GPCS 0944	Traditional cultivar	Yunnan, China
79.	*C. sinensis* (L.)O.Kuntze	GPCS 0709	Traditional cultivar	Yunnan, China
80.	*C. sinensis* (L.)O.Kuntze	GPCS 1021	Traditional cultivar	Yunnan, China
81.	*C. assamica* (Masters) Chang	GPCS 0587	Wild	Yunnan, China
82.	*C. sinensis* var. *pubilimba* Chang	GPCS 2337	Improved cultivar	Guangdong, China
83.	*C. assamica* (Masters) Chang	GPCS 0807	Traditional cultivar	Yunnan, China
84.	*C. assamica* (Masters) Chang	GPCS 0894	Traditional cultivar	Yunnan, China
85.	*C. ser. pentastylae* Chang	GPCS 1920	Traditional cultivar	Yunnan, China
86.	*C. assamica* (Masters) Chang	GPCS 0900	Traditional cultivar	Yunnan, China
87.	*C. assamica* (Masters) Chang	GPCS 1110	Traditional cultivar	Yunnan, China
88.	*C. assamica* (Masters) Chang	GPCS 2042	Traditional cultivar	Yunnan, China
89.	*C. ser. pentastylae* Chang	GPCS 1919	Traditional cultivar	Yunnan, China
90.	*C. polyneura* Chang et Tang	GPCS 0681	Traditional cultivar	Yunnan, China

Table 1. (Cont'd.).

No.	Species and varieties	Stock-number	Germplasm type	Origin
91.	*C. sinensis* (L.)O.Kuntze	GPCS 0614	Traditional cultivar	Yunnan, China
92.	*C. ser. pentastylae* Chang	GPCS 2442	Traditional cultivar	Yunnan, China
93.	*C. gymnogynoides* Chang et Yu	GPCS 1591	Breeding line	Yunnan, China
94.	*C. crassicolumna* Chang	GPCS 0694	Wild	Yunnan, China
95.	*C. assamica* (Masters) Chang	GPCS 0570	Traditional cultivar	Yunnan, China
96.	*C. assamica* (Masters) Chang	GPCS 0989	Wild	Yunnan, China
97.	*C. sinensis* (L.)O.Kuntze	GPCS 0424	Traditional cultivar	Yunnan, China
98.	*C. assamica* (Masters) Chang	GPCS 1101	Traditional cultivar	Yunnan, China
99.	*C. assamica* (Masters) Chang	GPCS 0563	Traditional cultivar	Yunnan, China
100.	*C. ser. pentastylae* Chang	GPCS 1927	Traditional cultivar	Yunnan, China
101.	*C. assamica* (Masters) Chang	GPCS1130	Wild	Yunnan, China
102.	*C.arborescens* Chang and Yu	GPCS1024	Wild	Yunnan, China
103.	*C. Irrawadiensis* P.K. Barua	GPCS0494	Wild	Yunnan, China
104.	*C.atrothea* Chang and Wang	GPCS0447	Wild	Yunnan, China
105.	*C. sinensis* (L.)O.Kuntze	GPCS2025	Wild	Yunnan, China
106.	*C.arborescens* Chang and Yu	GPCS0997	Wild	Yunnan, China
107.	*C.taliensis* (W.W.Smith) Melchior	GPCS1077	Wild	Yunnan, China
108.	*C.taliensis* (W.W.Smith) Melchior	GPCS0907	Wild	Yunnan, China
109.	*C.taliensis* (W.W.Smith) Melchior	GPCS1133	Wild	Yunnan, China
110.	*C. assamica* (Masters) Chang	GPCS 0895	Wild	Yunnan, China
111.	*C.atrothea* Chang and Wang	GPCS0861	Wild	Yunnan, China
112.	*C. Irrawadiensis* P.K. Barua	GPCS0435	Wild	Yunnan, China
113.	*C. Irrawadiensis* P.K. Barua	GPCS0609	Wild	Yunnan, China
114.	*C.atrothea* Chang and Wang	GPCS0968	Wild	Yunnan, China
115.	*C.taliensis* (W.W.Smith) Melchior	GPCS0418	Wild	Yunnan, China
116.	*C.taliensis* (W.W.Smith) Melchior	GPCS2090	Wild	Yunnan, China
117.	*C. assamica* (Masters) Chang	GPCS1114	Wild	Yunnan, China
118.	*C. dehungensis*(Chang et Chen) Ming	GPCS0707	Wild	Yunnan, China
119.	*C.taliensis* (W.W.Smith) Melchior	GPCS0579	Wild	Yunnan, China
120.	*C.taliensis* (W.W.Smith) Melchior	GPCS0782	Wild	Yunnan, China
121.	*C.gymnogyna* Chang	GPCS0415	Wild	Yunnan, China
122.	*C.sinensis* var.*kucha* Chang and Wang	GPCS0538	Wild	Yunnan, China
123.	*C. tachangensis* F.C.Zhang	GPCS 0491	Wild	Yunnan, China
124.	*C.kwangnanica* Chang and Chen	GPCS 0912	Wild	Yunnan, China
125.	*C.taliensis* (W.W.Smith) Melchior	GPCS0576	Wild	Yunnan, China
126.	*C.taliensis* (W.W.Smith) Melchior	GPCS1588	Wild	Yunnan, China
127.	*C.taliensis* (W.W.Smith) Melchior	GPCS0667	Wild	Yunnan, China
128.	*C.taliensis* (W.W.Smith) Melchior	GPCS0664	Wild	Yunnan, China
129.	*C.taliensis* (W.W.Smith) Melchior	GPCS1124	Wild	Yunnan, China
130.	*C.taliensis* (W.W.Smith) Melchior	GPCS0487	Wild	Yunnan, China
131.	*C.taliensis* (W.W.Smith) Melchior	GPCS0638	Wild	Yunnan, China
132.	*C. sinensis* (L.)O.Kuntze	GPCS0548	Wild	Yunnan, China
133.	*C. sinensis* (L.)O.Kuntze	GPCS0536	Wild	Yunnan, China
134.	*C.taliensis* (W.W.Smith) Melchior	GPCS1132	Wild	Yunnan, China

GPCS, the code of the China National Germplasm Tea Repositories (CNGTR)

DNA isolation: Genomic DNA was extracted with cetryl trimethyl ammonium bromide (CTAB) using the modified protocol. Obtained DNA pellet was washed three times with 70% ethanol, vacuum dried and dissolved in 100µL ddH_2O, After treating with 5µL of RNaseA (10 mg/mL), the quantity and quality of the DNA was checked with spectrophotometer (Genesys 10UV, USA) and agarose gel (0.8%) electrophoresis. Absorbance ratio between 260 and 280nm was computed and the quality of the genomic DNA was confirmed. The DNA stock, which was stored at -20°C, was then diluted to 20ng/µL with sterile MilliQ water for downstream applications.

ISSR amplification, separation and visualization: ISSR primers (synthesized by Shanghai Bioasia Technology Co. Ltd., China) were synthesized based on di- and tri-nucleotide repeats (GA, GT, CT and GTC) as a core sequence with a T_m value range of 40.0-58.0. Screening was carried out with 60 primers. Out of which 18 primers, which gave clear banding pattern were used for confirmatory studies (Table 2). PCR reactions were carried out in a volume of 10µL including 40ng of total DNA, 10 × PCR buffer (200 mmol /L Tris-HCl pH 8.4, 500 mmol/L KCl), 2.0 mmol/L $MgCl_2$, 0.2mmol/L of each dNTP, 4pmol/L of each primer, and 0.5U of Taq DNA polymerase. The optimum annealing temperature was determined for each primer. Amplification was carried out in a programmable peltier thermo cycler PTC 200 (MJ Research, USA). Amplification protocol includes initial denaturation for 5 min at 94°C followed by 39 successive cycles of 60 s denaturation at 94°C, annealing for 30 s at respective Tm values of the selected primers and 2 min elongation at 72°C. Final elongation was performed for 10 min at 72°C. Amplifications were checked by separating on 6% polymeric acrylamide gel electrophoresis for 4 h at a constant temperature of 150 V with 1 × TBE buffer (100mmol/L Trisborate, pH 8.0, 2 mmol/L EDTA), in 2000 ml of distilled water) running buffer. Finally the gel was silver-stained, visualized under ultraviolet light, photographed and documented. The analysis was performed for all the samples at least three times with each selected primers. Molecular sizes of the amplified fragments were roughly estimated using a 3000 bps ladder marker (Shanghai Bioasia Technology Co. Ltd., China).

Table 2. Inter-simple sequence repeat(ISSR) primers used in this study, core sequences, attached bases, number of polymorphic bands, mean Shannon Weaver diversity indices (H) and Shannon's Information İndex (I) obtained by each primer.

Primer	Core sequence	Attached bases	Polymorphic bands	*H*	*I* (av. values)
S807*	$(AG)_8$	+T	23	0.3993	0.5835
S808*	$(AG)_8$	+C	24	0.3693	0.5420
S810*	$(GA)_8$	+T	28	0.4339	0.6230
S811*	$(GA)_8$	+C	29	0.4284	0.6151
S826*	$(AC)_8$	+C	25	0.3738	0.5500
S834*	$(AG)_8$	+YT	30	0.4152	0.6006
S835*	$(AG)_8$	+YC	33	0.4015	0.5877
S836*	$(AG)_8$	+YA	25	0.4138	0.5991
S840*	$(GA)_8$	+YT	23	0.3181	0.4792
S841*	$(GA)_8$	+YC	25	0.4104	0.5969
S842*	$(GA)_8$	+YG	27	0.4105	0.5983
S856*	$(AC)_8$	+YA	22	0.3491	0.5284
S890*	$(GT)_7$	VHV+	26	0.4259	0.6141
ISSR2[a]	$(AC)_8$	+T	31	0.4031	0.5887
ISSR3[a]	$(TC)_8$	+AGG	23	0.3151	0.4709
ISSR4[a]	$(GA)_8$	+CTT	26	0.4278	0.6155
ISSR5[a]	$(AG)_8$	+CTA	28	0.4023	0.5881
ISSR8[a]	$(TC)_8$	+AGT	22	0.3808	0.5594

*Primer set 13, University of British Columbia (Canada).

[a]Liu *et al*., (2008) Y= Pyrimidine, V =Non- T (i.e. A, C or G), H=Non- G (i.e. A, C or T).

Data recording and analysis: Scanned gel image was analyzed using DNA marker ladder (Shanghai Bioasia Technology Co. Ltd., China) for fragment length calibration. Only distinct, reproducible, well-resolved amplified fragments were scored manually for the band presence (1) and absence (0) for each of the ISSR markers using the primer and its band size. For example, UBC835-400bp represented the 400bp marker of the primer UBC835. Unique, specific ISSR markers, band patterns and DNA fingerprinting were employed to discriminate the inter-specific level tea germplasm.

Results and Discussion

Genetic variability revealed through ISSR markers: The tea species and varieties investigated, especially the allied wild ones, were mainly collected from the original centre of tea plants in Yunnan province (Table 1), and nominated by a well-known Theaceace taxonomist at Sun Yat-sen University, China (Chang, 1981, 1984). They were direct offspring from the type specimen plants in the type locality, using cutting propagation and *ex situ* preserved in the CNGTR at the TRIYAAS. Most of the

wild species have very small populations, and some even have one huge plant for one species (Yu, 1986). The tea species and varieties show wide morphological variations in tree height, tree habit, leaf size and shape, particularly in the flower and fruit characters. They have 5-15 petals, a 3- or 5-locule ovary with or without pubescence, (2) 3-7(7) splttings of style, flat or round capsules with various sizes of central axis, thick or thin pericarp and different kinds of seed shape (Chen *et al*., 2000). In present study, 18 ISSR primers generated a total of 475 bands with 99.4% polymorphism and the number of polymorphism bands generated by a primer ranged between 22 (UBC856 and $(TC)_8$ AGT) and 33(UBC835), with an average of 26.4 bands per primer. The size of the bands ranged from 200 to 3000 bp. According to the amplification results, there were 3 bands that existed in all the test germplasm, showing that these tested germplasm had similar genetic base or originated from a common ancestor.

The analyses of variance showed significant differences among the eighteen primers in the Nei's gene diversity (*H*) and the mean Shannon's Information index (*I*) (Table 2). The former ranged from 0.315 for primer P17 to 0.434 for primer P03. The PIC values varied from 0.479 for primer P09 to 0.623 for primer P03. This indicated that these tested germplasm had relatively high genetic polymorphism. In previous tea germplasm DNA polymorphism studies, the number of scorable bands generated varied from 2 to 17 per primer in RAPD analysis (Kaundun *et al*., 2000; Chen *et al*., 2002; Shao *et al*., 2003; Huang *et al*., 2004; Rajan & Swati, 2004; Huang *et al*., 2006; Yao *et al*., 2007). Moreover, these studies revealed polymorphism less than 95%. However, our results showed a wider range of polymorphism which could be attributed to the greater resolution of diversity with ISSR than with RAPD markers and also to the differences in the genotypes included, indicating that the tea species and varieties investigated had a wide gene pool. The reason for this result was mainly that Yunnan province was the origin center of tea germplasm and the distribution center of species diversity (Wight, 1959; Zeng *et al*., 2004; Chen *et al*., 2005).

According to amplification results, there were 3 bands that existed in all the test *germplasm*, showing that these test germplasm had similarly genetic base or originated from a common ancestor. As shown in the Table 2, the analyses of variance showed significant differences among the eighteen primers in Shannon Weaver diversity index (H) and the mean Shannon's Information index (I).The former ranged from 0.315 for primer P17 to 0.434 for primer P03. The PIC values varied from 0.479 for primer P09 to 0.623 for primer P03. This indicated that these test germplasm had relatively high genetic polymorphism.

Specific markers to discriminate tea germplasm at the inter-specific level: The detected variability allowed the discrimination of plant germplasm at inter-specific level using various independent methods, i.e., unique ISSR markers, specific band patterns, a combination of band patterns provided by different primers (Belaj *et al*., 2001) and DNA fingerprinting (Jia *et al*., 2000; Conner & Wood, 2001). Table 3 provided data for the discrimination of tea germplasm by unique ISSR markers. The presence of 4 unique markers and the absence of 9 unique markers obtained from 12 primers made it possible to discriminate 13 inter-specific germplasm, i.e., GPCS1935, GPCS2043, GPCS0534, GPCS1068, GPCS1109, GPCS0424, GPCS0548, GPCS0997, GPCS0447, GPCS0576, GPCS1077, GPCS0861 and GPCS1130. GPCS1026 and GPCS1132 could be differentiated by the presence of 2 different specific DNA markers, respectively. GPCS0579 and GPCS0609 could be differentiated by the absence of 2 different specific DNA markers, respectively. Similarly, GPCS1618, GPCS0583, GPCS1021 and GPCS0694 could be differentiated by the presence and the absence of one specific DNA markers, respectively. Some primers provided plentiful band patterns and allowed discrimination of the germplasm. For example, UBC835 gave 90 band patterns, 67 being specific. It was possible to discriminate 67 tea germplasm (Table 4, Fig.1-4) using the specific band patterns.

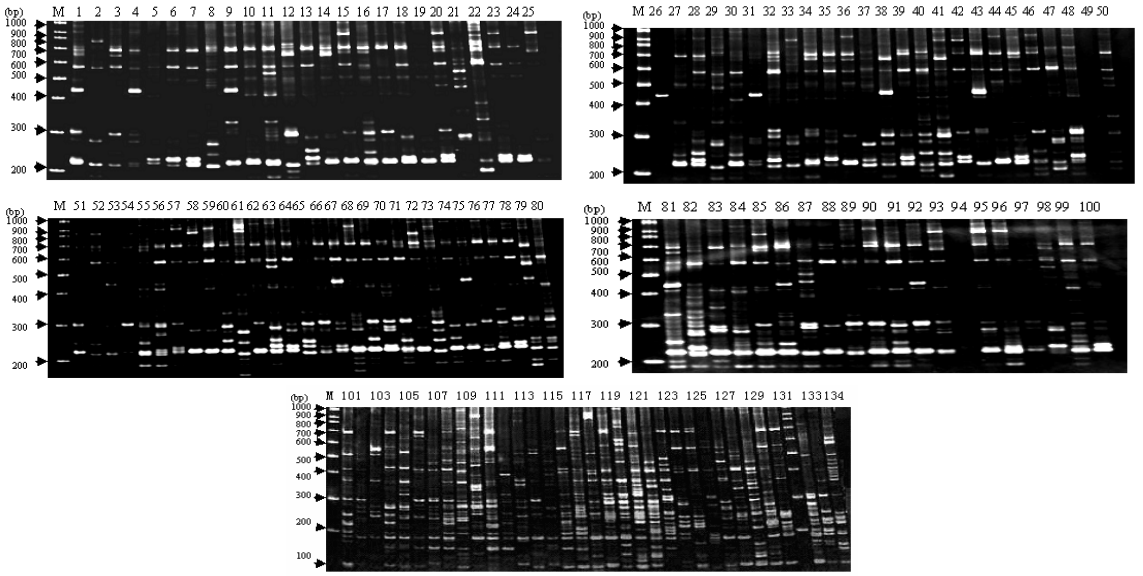

Fig. 1. ISSR analysis of 134 tea germplasm lines by using primer S835 and Lane 1-134 for tea germplasm lines listed in Table 1, M = 1kb DNA ladder.

Table 3. Specific markers that can be used for discrimination of cultivars.

Stock number of discriminate varieties	specific markers	criteria
GPCS1935	S811-200bp	presence
GPCS2043	ISSR3-300bp	absence
GPCS1026	S826-400bp, S808-600bp	presence
GPCS0534	S826-400bp	presence
GPCS1068	S808-300bp	absence
GPCS1109	S856-300bp	presence
GPCS1618	S826-300bp	presence
	S826-700bp	absence
GPCS0583	ISSR5-300bp	absence
	ISSR5-1000bp	presence
GPCS1021	ISSR5-300bp	presence
	ISSR5-400bp	absence
GPCS0694	S835-400bp	absence
	S826-300	presence
GPCS0424	S807-300bp	absence
GPCS1132	S841-500bp , S836-400bp	presence
GPCS0579	S841-200bp , S836-300bp	absence
GPCS0548	ISSR5-900bp	absence
GPCS0997	S810-400bp	absence
GPCS0609	ISSR4-700bp , ISSR3-500bp	absence
GPCS0447	ISSR4-600bp	absence
GPCS0576	ISSR3-600bp	absence
GPCS1077	S811-400bp	absence
GPCS0861	S841-1200bp	presence
GPCS1130	S841-300bp	absence

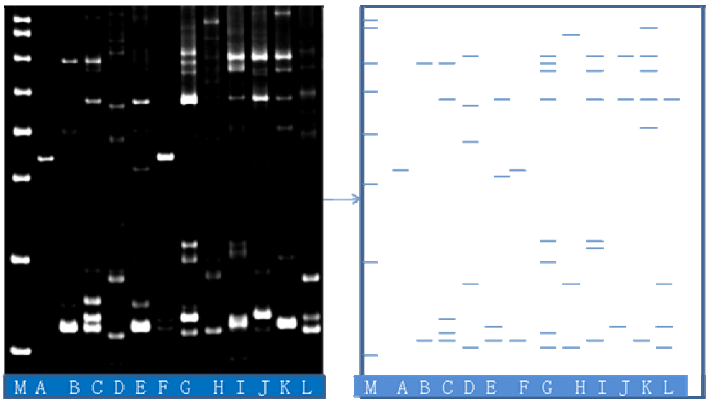

Fig. 2. ISSR amplification and schematic of the band patterns generated from UBC835. The letters at the bottom correspond to the band patterns. The left first lane is DNA marker.

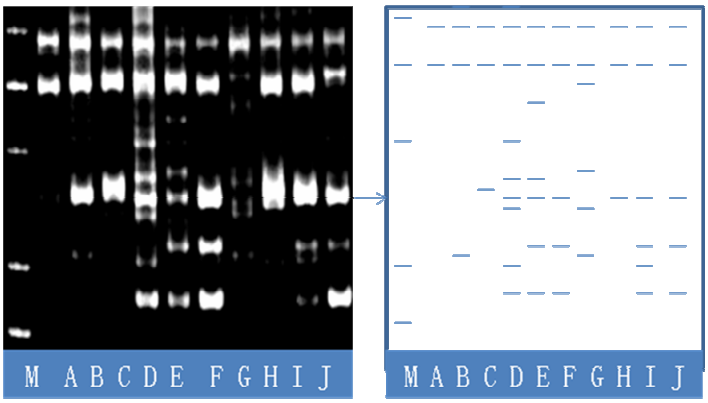

Fig. 3. ISSR amplification and schematic of the band patterns generated from $(GA)_8$CTT. The letters at the bottom correspond to the band patterns. The left first lane is DNA marker.

Table 4. The origin of Tea germplasm, ISSR band patterns and their identification capacity.

No.	Species and varieties	S811	S835	ISSR2	ISSR3	S811	S811 S835	S811 S835 ISSR2	S811 S835 ISSR2 ISSR3
1.	*C. assamica* (Masters) Chang	A[1]	A	A	A	+[2]	+	+	+
2.	*C. crassicolumna* Chang	B	B	B	B	-[3]	-	+	+
3.	*C. ser. pentastylae* Chang	C	C	C	C	-	-	+	+
4.	*C. taliensis* (W.W.Smith) Melchior	B	D	D	D	-	+	+	+
5.	*C. crassicolumna* Chang	D	E	E	E	+	+	+	+
6.	*C. taliensis* (W.W.Smith) Melchior	C	B	F	F	-	-	+	+
7.	*C. assamica* (Masters) Chang	E	B	G	G	+	+	+	+
8.	*C. tachangensis* F.C.Zhang	F	F	H	H	+	+	+	+
9.	*C. ser. pentastylae* Chang	G	G	I	I	-	+	+	+
10.	*C.sinensis* var. *Assamica* (Masters) Chang	G	H	J	G	-	+	+	+
11.	*C. ser. pentastylae* Chang	G	I	K	C	-	+	+	+
12.	*C. assamica* (Masters) Chang	H	B	I	J	+	+	+	+
13.	*C. sinensis* var. *pubilimba* Chang	I	J	L	K	+	+	+	+
14.	*C. multisepala* Chang et Tang	J	C	L	L	+	+	+	+
15.	*C. ser. pentastylae* Chang	K	C	M	L	+	+	+	+
16.	*C. taliensis* (W.W.Smith) Melchior	G	K	I	L	-	+	+	+
17.	*C. ser. pentastylae* Chang	C	C	I	M	-	-	+	+
18.	*C. assamica* (Masters) Chang	C	C	N	L	-	-	+	+
19.	*C. assamica* (Masters) Chang	L	L	L	L	-	+	+	+
20.	*C. sinensis* var. *pubilimba* Chang	L	D	M	N	-	+	+	+
21.	*C. assamica* (Masters) Chang	M	M	O	O	+	+	+	+
22.	*C. gymnogynoides* Chang et Yu	N	B	P	P	+	+	+	+
23.	*C. ser. pentastylae* Chang	L	N	Q	C	-	+	+	+
24.	*C. assamica* (Masters) Chang	O	O	R	L	+	+	+	+
25.	*C. ser. pentastylae* Chang	L	P	M	L	-	+	+	+
26.	*C. assamica* (Masters) Chang	P	Q	S	L	-	+	+	+
27.	*C. ser. pentastylae* Chang	Q	R	L	C	+	+	+	+
28.	*C. assamica* (Masters) Chang	L	S	L	C	-	+	+	+
29.	*C. taliensis* (W.W.Smith) Melchior	P	T	Q	L	-	+	+	+
30.	*C. assamica* (Masters) Chang	R	U	T	L	+	+	+	+
31.	*C. assamica* (Masters) Chang	S	V	U	Q	+	+	+	+
32.	*C. sinensis* (L.)O.Kuntze	T	W	V	R	+	+	+	+
33.	*C. ser. pentastylae* Chang	U	X	W	Q	+	+	+	+
34.	*C. assamica* (Masters) Chang	V	W	X	S	-	-	+	+
35.	*C. assamica* (Masters) Chang	W	W	Y	S	+	+	+	+
36.	*C. assamica* (Masters) Chang	V	W	Z	T	-	-	+	+
37.	*C. gymnogynoides* Chang et Yu	X	W	U	U	+	+	+	+
38.	*C. sinensis* (L.)O.Kuntze	V	Y	AB	V	-	+	+	+
39.	*C. yankiangcha* Chang et Wang	V	Z	AC	W	-	+	+	+
40.	*C. taliensis* (W.W.Smith) Melchior	Y	W	AC	W	+	+	+	+
41.	*C. atrothea* Chang et Wang	Z	W	AD	X	-	-	-	+
42.	*C. assamica* (Masters) Chang	AB	AB	AC	W	-	+	+	+
43.	*C. ser. pentastylae* Chang	AB	AC	AE	W	-	+	+	+

44.	*C. ser. pentastylae* Chang	Z	AD	AF	W	-	+	+	+
45.	*C. assamica* (Masters) Chang	AB	AE	AG	W	-	+	+	+

Table 4. (Cont'd.).

No.	Species and varieties	S811	S835	ISSR2	ISSR3	S811	S811 S835	S811 S835 ISSR2	S811 S835 ISSR2 ISSR3
46.	*C. ser. pentastylae* Chang	AC	W	AH	Y	+	+	+	+
47.	*C. ser. pentastylae* Chang	AD	AF	AI	W	+	+	+	+
48.	*C. assamica* (Masters) Chang	AE	AG	AJ	Z	+	+	+	+
49.	*C. assamica* (Masters) Chang	AF	AH	S	S	-	+	+	+
50.	*C. assamica* (Masters) Chang	AF	AI	AJ	AB	-	+	+	+
51.	*C. assamica* (Masters) Chang	AG	AJ	AK	AC	-	+	+	+
52.	*C. assamica* (Masters) Chang	AG	AK	AL	AD	-	+	+	+
53.	*C. irrawadiensis* P.K.Barua	AG	AL	AM	AC	-	+	+	+
54.	*C. assamica*(Masters) Chang	AG	AM	AN	AE	-	+	+	+
55.	*C. ser. pentastylae* Chang	AG	AN	AL	AD	-	-	-	+
56.	*C. assamica* (Masters) Chang	AG	AO	AK	AF	-	+	+	+
57.	*C. assamica* (Masters) Chang	AG	AP	AL	AD	-	+	+	+
58.	*C. assamica*(Masters) Chang	AH	AQ	AO	AD	-	+	+	+
59.	*C. Irrawadiensis* P.K. Barua	AG	AR	AP	AD	-	+	+	+
60.	*C. yankiangcha* Chang et Wang	AG	AN	AQ	AG	-	-	+	+
61.	*C. ser. pentastylae* Chang	AG	AS	AR	AG	-	+	+	+
62.	*C. assamica* (Masters) Chang	AG	AT	AS	AD	-	-	+	+
63.	*C. ser. pentastylae* Chang	AI	AN	AK	AH	+	+	+	+
64.	*C. assamica* (Masters) Chang	AH	AU	AT	SI	-	+	+	+
65.	*C. crassicolumna* Chang	AG	AT	AU	AJ	-	-	+	+
66.	*C. sinensis* (L.) O. Kuntze	AJ	AT	AV	AK	+	+	+	+
67.	*C. assamica* (Masters) Chang	AG	AN	AW	AL	-	-	+	+
68.	*C.taliensis* (W.W.Smith) Melchior	AG	AV	AR	AM	-	+	+	+
69.	*C. ser. pentastylae* Chang	AG	AW	AX	AN	-	+	+	+
70.	*C. assamica* (Masters) Chang	AG	AX	AY	AM	-	+	+	+
71.	*C. assamica* (Masters) Chang	AG	AY	AZ	AD	-	+	+	+
72.	*C. ser. pentastylae* Chang	AG	AZ	BA	AN	-	+	+	+
73.	*C. kwangnanica* Chang et Chen	AG	BA	BB	AO	-	+	+	+
74.	*C. ser. pentastylae* Chang	AG	BB	BC	AM	-	+	+	+
75.	*C. ser. pentastylae* Chang	AG	BC	BD	AP	-	+	+	+
76.	*C. assamica* (Masters) Chang	AG	BD	BE	AM	-	+	+	+
77.	*C. ser. pentastylae* Chang	AG	BE	S	AM	-	+	+	+
78.	*C. assamica* (Masters) Chang	AG	BF	BE	AM	-	+	+	+
79.	*C. sinensis* (L.) O. Kuntze	AG	BG	BF	AQ	-	+	+	+
80.	*C. sinensis* (L.) O. Kuntze	AG	BH	BG	AR	-	+	+	+
81.	*C. assamica* (Masters) Chang	AK	BI	BH	AD	-	+	+	+
82.	*C. sinensis* var. *pubilimba* Chang	AK	BJ	BI	AS	-	+	+	+
83.	*C. assamica* (Masters) Chang	AK	BK	BJ	AD	-	+	+	+
84.	*C. assamica* (Masters) Chang	AL	BL	BK	AD	+	+	+	+
85.	*C. ser. Pentastylae* Chang	AK	BM	BJ	AD	-	+	+	+
86.	*C. assamica* (Masters) Chang	AK	BN	BL	AT	-	+	+	+
87.	*C. assamica* (Masters) Chang	AK	BO	BM	AU	-	+	+	+
88.	*C. assamica* (Masters) Chang	AK	BP	BN	AV	-	+	+	+
89.	*C. ser. pentastylae* Chang	AM	BQ	BO	AW	+	+	+	+
90.	*C. polyneura* Chang et Tang	AG	BR	BP	AD	-	+	+	+

Table 4. (Cont'd.).

No.	Species and varieties	S811	S835	ISSR2	ISSR3	S811	S811 S835	S811 S835 ISSR2	S811 S835 ISSR2 ISSR3
91.	*C. sinensis* (L.)O.Kuntze	AG	BS	S	AX	-	-	-	+
92.	*C. ser. pentastylae* Chang	AG	BT	S	AD	-	+	+	+
93.	*C. gymnogynoides* Chang et Yu	AG	BS	S	AY	-	-	-	+
94.	*C. crassicolumna* Chang	AG	BU	BQ	AZ	-	+	+	+
95.	*C. assamica* (Masters) Chang	AG	BW	BR	AD	-	+	+	+
96.	*C. assamica* (Masters) Chang	AK	BS	S	BA	-	-	-	+
97.	*C. sinensis* (L.)O.Kuntze	AK	BS	S	BB	-	-	-	+
98.	*C. assamica* (Masters) Chang	AK	BX	S	BC	-	+	+	+
99.	*C. assamica* (Masters) Chang	AK	BY	S	BC	-	+	+	+
100.	*C. ser. pentastylae* Chang	AK	BS	S	BD	-	-	+	+
101.	*C. assamica* (Masters) Chang	AL	BT	BS	BE	+	+	+	+
102.	*C.arborescens* Chang and Yu	AM	BU	BT	BF	-	+	+	+
103.	*C. irrawadiensis* P.K. Barua	AN	BV	BU	BG	-	-	-	+
104.	*C.atrothea* Chang and Wang	AO	BV	BV	BH	-	-	-	+
105.	*C. sinensis* (L.) O. Kuntze	AP	BW	BW	BI	-	+	+	+
106.	*C. arborescens* Chang and Yu	AQ	BX	BX	BI	-	+	+	+
107.	*C. taliensis* (W.W. Smith) Melchior	AR	BY	BY	BI	+	+	+	+
108.	*C. taliensis* (W.W. Smith) Melchior	AS	BV	BZ	BJ	-	-	+	+
109.	*C. taliensis* (W.W. Smith) Melchior	AS	BZ	CA	BI	+	+	+	+
110.	*C. assamica* (Masters) Chang	AT	BX	BT	BK	-	-	-	+
111.	*C. atrothea* Chang and Wang	AT	BV	BV	BL	-	-	-	+
112.	*C. ırrawadiensis* P.K. Barua	AT	BU	CB	BF	-	-	+	+
113.	*C. ırrawadiensis* P.K. Barua	AT	CA	CC	BG	+	+	+	+
114.	*C. atrothea* Chang and Wang	AU	BX	CD	BF	-	+	+	+
115.	*C. taliensis* (W.W. Smith) Melchior	AV	BU	CE	BM	-	+	+	+
116.	*C. taliensis* (W.W. Smith) Melchior	AW	CB	BY	BN	+	+	+	+
117.	*C. assamica* (Masters) Chang	AW	CC	BU	BO	-	-	-	+
118.	*C. dehungensis* (Chang et Chen) Ming	AX	BW	CF	BI	-	+	+	+
119.	*C. taliensis* (W.W. Smith) Melchior	AW	BU	CG	BP	-	-	+	+
120.	*C. taliensis* (W.W. Smith) Melchior	AY	CD	BU	BQ	+	+	+	+
121.	*C. gymnogyna* Chang	AT	CE	CH	BR	-	-	+	+
122.	*C. sinensis* var. *kucha* Chang and Wang	AT	CC	CI	BS	-	-	-	+
123.	*C. tachangensis* F.C. Zhang	AW	CE	CJ	BK	-	-	+	+
124.	*C. kwangnanica* Chang and Chen	AZ	BU	CC	BT	-	+	+	+
125.	*C. taliensis* (W.W. Smith) Melchior	AN	CF	CK	BF	+	+	+	+
126.	*C. taliensis* (W.W. Smith) Melchior	AT	CG	CL	BU	+	+	+	+
127.	*C. taliensis* (W.W. Smith) Melchior	AT	CA	CM	BK	-	-	+	+
128.	*C. taliensis* (W.W. Smith) Melchior	AW	CH	CN	BK	+	+	+	+
129.	*C. taliensis* (W.W. Smith) Melchior	AT	CE	CM	BV	-	-	-	+
130.	*C. taliensis* (W.W. Smith) Melchior	AT	BW	CO	BM	-	-	+	+
131.	*C. taliensis* (W.W. Smith) Melchior	AT	BU	CP	BW	-	-	+	+
132.	*C. sinensis* (L.) O. Kuntze	BA	BU	CQ	BX	-	+	+	+
133.	*C. sinensis* (L.) O. Kuntze	AW	BU	CR	BY	-	-	+	+
134.	*C. taliensis* (W.W. Smith) Melchior	BB	BU	CS	BZ	-	+	+	+
	Total	**54**	**90**	**97**	**76**	**35**	**99**	**121**	**134**

Note: Letters represent the band patterns; "+"represent the germplasm could be discriminated by the primer(s); "-" represent the germplasm could not be discriminated by the primer(s).

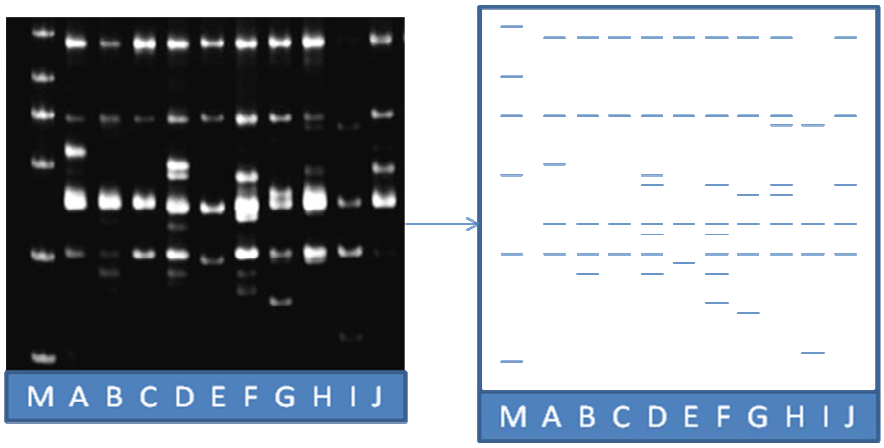

Fig. 4. ISSR amplification and schematic of the band patterns generated from $(GA)_8$CAT. The letters at the bottom correspond to the band patterns. The left first lane is DNA marker.

No single primer could discriminate all 134 germplasm. However, the band pattern combination of different primers offered an effective method to discriminate them. It was possible to establish aminimum number of primers with great capacity to discriminate all the germplasm. The combination of band patterns of two primers, UBC811 and UBC835 or $(AG)_8$CTA, could discriminate 99 species and varieties, respectively. The combination of band patterns of three primers, UBC811, UBC835 and $(AG)_8$CTA, UBC811, UBC835, $(AG)_8$CTA and $(GA)_8$CTT, which provided a large number of band patterns, could discriminate all the 134 inter-specific level germplasm studied (Table 4; Fig.1-3).

By the combination of different band patterns of the primers, forty-two reproducible and potentially reliable ISSR markers from the 472 polymorphic bands were selected to construct DNA fingerprinting (Fig. 5). These bands were from UBC811, UBC835, $(AG)_8$ CTA and $(GA)_8$ CTT, respectively. In the DNA fingerprinting, each tea germplasm had its exclusive fingerprinting (Fig. 5) and could be easily discriminated from one another. Previously, morphological characters (Sealy, 1958; Chang, 1981, 1984; Ming, 1992; Chen *et al*., 2000), chemical components (Du *et al*., 1992), esterase isozymes (Lu *et al*., 1992) and karyotype (Liang *et al*., 1994) were used to discriminate tea plants and their wild allied species. However, they were found not to be reproducible because of developing stages, growing environments, cultivation conditions and even experimental error.

There are many successful examples for using ISSR markers to discriminate plant germplasm. Evaluation and identification of germplasm using ISSR markers are playing an important role in studies of genetics and breeding. The DNA fingerprinting analysis also provides a good method for the discrimination of germplasm at the inter-specific level (Jia *et al*., 2000; Conner & Wood, 2001). The present results also showed that there were some independent and different ways to discriminate tea germplasm at the inter-specific level using specific ISSR markers. Thirteen genotypes could be discriminated using specific ISSR markers and eight using specific band patterns. It was easy to discriminate all the 134 tea germplasm at the species and variety level using the band pattern combination or DNA fingerprinting constructed by 42 ISSR bands generated with four primers. These putative variety-specific ISSR markers could be transformed to sequence characterized amplification regions (SCARs) after sequencing and designing primer pairs to develop specific markers for tea germplasm. Thus, ISSR analysis not only reveals high genetic polymorphism among tea plants and their allied species in the section Thea genus *Camellia* (Yao *et al*., 2007; Liu *et al*., 2008), but also provides a practical method and an effective approach to differentiate tea germplasm at the inter-specific level. Consequently, it will help us to further understand tea germplasm and select the parents for tea breeding.

Conclusion

This is the first report for the discrimination of tea germplasm using inter simple sequence repeat (ISSR) markers. In this study, these tea germplasm from Yunnan province have relatively high genetic polymorphism, which provides a proof that Yunnan province was the origin center of tea germplasm. In addition, by using three various independent methods, ISSR markers can successfully discriminate tea germplasm tested, which will be helpful to conserve, management, and utilize tea resources.

Acknowledgements

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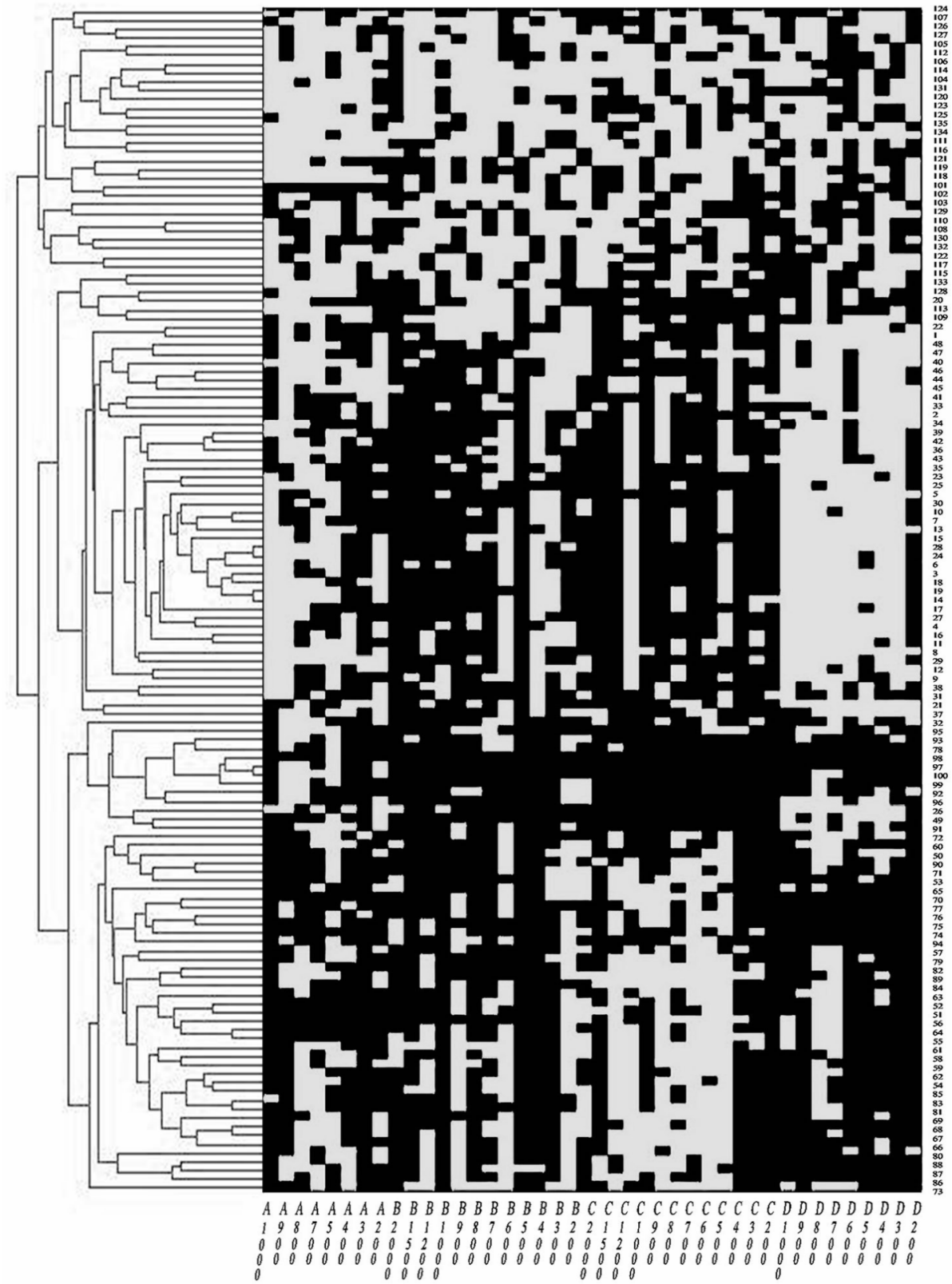

Fig.5. DNA Fingerprinting among 134 tea germplasms based on ISSR markers

Note: The number at the bottom are 42 specific ISSR markers from UBC811 (A), UBC835 (B), $(AG)_8$ CTA (C) and $(GA)_8$CTT (D), respectively. The numbers at right are serial number of 134 germplasms (see Table 1 for detail). Black blocks represent the presence of bands.

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