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Genetic diversity in tea (*Camellia sinensis*) germplasms as revealed by ISSR markers

B Y LIU[1], L Y WANG[2], Y Y LI[3], W HE[4], J ZHOU[5], P S WANG[6] and H CHENG[7,*]

Research Centre for Tea Germplasm Improvement and Breeding, Tea Research Institute of Chinese Academy of Agricultural Science, Hangzhou, China 310 008

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ABSTRACT

To efficiently utilize, maintain and assess tea [*Camellia sinensis* (L.) O. Kuntze] germplasms, inter-simple sequence repeats (ISSR) were used to determine genetic relationships among 100 location tea germplasms. Eighteen ISSR primers generated 472 polymorphic bands (99.4% of the total) and distinguished the 100 tea germplasms. The genetic distance among the genotypes ranged from 0.167 to 0.797, with a mean of 0.501, whereas 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. The Jaccard similarity co-efficients among genotypes ranged from 0.262 to 0.810. The 100 germplasms were divided into 5 groups on the basis of unweighed pair-group method with arithmetic averages analysis (UPGMA). The largest group consisted of 30 germplasm. The other 4 groups included 67 germplasms except 3 non-clustered germplasms. The phylogenetic groups generated by ISSR markers did not correspond to their geographical origin. Principal Component analysis (PCA) of ISSR data supported UPGMA clustering results. The ISSR can be useful as DNA-based molecular markers for studying genetic diversity, phylogenetic relationships, identification of tea germplasms or cultivars, and genetic improvement and selection of crossbred parents in tea breeding programmes.

Key words: Genetic diversity, ISSR, Tea germplasm

Tea plants, belonging to the family Theaceace, genus *Camellia*, section *Thea*, usually involve one species and two or three varieties (Chen *et al*. 2006). Genetic diversity assessments both at inter- and intra-specific levels are of critical importance for collection, conservation, evaluation and utilization of tea germplasm. Some methods (Chen *et al*. 2006) have been employed to describe the phylogenetic relationships among tea plants. However, the effectiveness of these methods has been proven to be insufficient. Therefore, it appears necessary to look for more effective, stable and reliable methods to assess genetic diversity at both inter-and intra-specific levels. The ISSR is a new molecular marker technique, has more advantages than other molecular markers (Nybom 2004), and has been used to study genetic diversity of some plants (Ashok and Manoj 2007, Liu *et al*. 2008). Nevertheless, ISSR marker studies on genetic diversity of tea resources are still rare. In this study, the present investigation was aimed to measure the genetic diversity of peculiar tea germplasm lines using ISSR technique.

Based on complete information of Ph D thesis of the first author [1]Ph D Scholar (email: liubenying@mail.tricaas.com), [2,3,4,5]Research Associates, [6]Senior Scientist, Tea Research Institute of Yunnan Academy of Agricultural Science, Menghai, China 666 201, [7]Principal Scientist and Director (email: chenghao@mail.tricaas.com)

MATERIALS AND METHODS

Plant materials

A total of 100 tea germplasm lines, including 4 species and varieties, namely, 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 (Chen *et al*. 2006), from the China National Germplasm Tea Repositories (CNGTR) at the Tea Research Institute of Yunnan Academy of Agricultural Sciences (TRIYAAS), were sampled (Table 1) using ISSR analysis during 2007–08. These materials were taken at minimum intervals of 5 m from each other to avoid a confusion of each species.

DNA isolation

Genomic DNA was extracted with modi cetyl trimethyl ammonium bromide (CTAB) using a modified method (Liu *et al.* 2008). Obtained DNA pellet was washed three times with 70% ethanol, vacuum-dried and dissolved in 100 µL ddH_2O, containing 5 µL of RNaseA (10 mg/mL). The quantity and quality of the DNA was checked with

Table 1 Names, stock-numbers and origin of 100 tea accessions used in the study

Sample no.	Species/ varieties	Stock-number	Origin	Sample no.	Species/varieties	Stock-number	Origin
T1	*Camellia assamica* (Masters) Chang	GPCS 0108	Hainan, China	T51	*Camellia sinensis* (L.) O.Kuntze	GPCS 0041	Guangdong, China
T2	*C. crassicolumna* Chang	GPCS 0448	Yunnan, China	T52	*C. assamica* (Masters) Chang	GPCS 2028	Yunnan, China
T3	*C.* ser. *pentastylae* Chang	GPCS 2440	Yunnan, China	T53	*C.taliensis* (W.W.Smith) Melchior	GPCS 0428	Yunnan, China
T4	*C.taliensis* (W.W.Smith) Melchior	GPCS 1610	Yunnan, China	T54	*C.* ser. *pentastylae* Chang	GPCS 1934	Yunnan, China
T5	*C. crassicolumna* Chang	GPCS 1103	Yunnan, China	T55	*C. assamica* (Masters) Chang	GPCS 2094	Yunnan, China
T6	*C.taliensis* (W.W.Smith) Melchior	GPCS 1093	Yunnan, China	T56	*C. assamica* (Masters) Chang	GPCS 0788	Yunnan, China
T7	*C. assamica* (Masters) Chang	GPCS 0597	Yunnan, China	T57	*C.* ser. *pentastylae* Chang	GPCS 1921	Yunnan, China
T8	*C. tachangensis* F.C.Zhang	GPCS 0913	Yunnan, China	T58	*C. kwangnanica* Chang et Chen	GPCS 0510	Yunnan, China
T9	*C.*ser. *pentastylae* Chang	GPCS 2061	Yunnan, China	T59	*C.* ser. *pentastylae* Chang	GPCS 1932	Yunnan, China
T10	*C. sinensis* var. *assamica* (Masters) Chang	GPCS 0940	Yunnan, China	T60	*C.* ser. *pentastylae* Chang	GPCS 1947	Yunnan, China
T11	*C.* ser. *pentastylae* Chang	GPCS 1944	Yunnan, China	T61	*C. assamica* (Masters) Chang	GPCS 1609	Yunnan, China
T12	*C. assamica* (Masters) Chang	GPCS 0683	Yunnan, China	T62	*C.* ser. *pentastylae* Chang	GPCS 1943	Yunnan, China
T13	*C. sinensis* var. *pubilimba* Chang	GPCS 0075	Guangxi, China	T63	*C. assamica* (Masters) Chang	GPCS 0944	Yunnan, China
T14	*C. sinensis* var. *assamica* (Masters) Chang	GPCS 1048	Yunnan, China	T64	*C. sinensis* (L.)O.Kuntze	GPCS 0709	Yunnan, China
T15	*C.* ser. *pentastylae* Chang	GPCS 1935	Yunnan, China	T65	*C. sinensis* (L.)O.Kuntze	GPCS 1021	Yunnan, China
T16	*C.taliensis* (W.W.Smith) Melchior	GPCS 1617	Yunnan, China	T66	*C. assamica* (Masters) Chang	GPCS 0587	Yunnan, China
T17	*C.* ser. *pentastylae* Chang	GPCS 1936	Yunnan, China	T67	*C. sinensis* var. *pubilimba* Chang	GPCS 2337	Guangdong, China
T18	*C. assamica* (Masters) Chang	GPCS 0933	Yunnan, China	T68	*C. assamica* (Masters) Chang	GPCS 0807	Yunnan, China
T19	*C. assamica* (Masters) Chang	GPCS 2013	Yunnan, China	T69	*C. assamica* (Masters) Chang	GPCS 0894	Yunnan, China
T20	*C. sinensis* var. *pubilimba* Chang	GPCS 2043	Yunnan, China	T70	*C.* ser. *pentastylae* Chang	GPCS 1920	Yunnan, China
T21	*C. assamica* (Masters) Chang	GPCS 1004	Yunnan, China	T71	*C. assamica* (Masters) Chang	GPCS 0900	Yunnan, China
T22	*C. gymnogynoides* Chang et Yu	GPCS 1026	Yunnan, China	T72	*C. assamica* (Masters) Chang	GPCS 1110	Yunnan, China
T23	*C.* ser. *pentastylae* Chang	GPCS 1930	Yunnan, China	T73	*C. assamica* (Masters) Chang	GPCS 2042	Yunnan, China
T24	*C. assamica* (Masters) Chang	GPCS 0451	Yunnan, China	T74	*C.* ser. *pentastylae* Chang	GPCS 1919	Yunnan, China
T25	*C.* ser. *pentastylae* Chang	GPCS 1922	Yunnan, China	T75	*C. polyneura* Chang et Tang	GPCS 0681	Yunnan, China
T26	*C. assamica* (Masters) Chang	GPCS 0534	Yunnan, China	T76	*C. sinensis* (L.)O.Kuntze	GPCS 0614	Yunnan, China
T27	*C.*ser. *pentastylae* Chang	GPCS 2011	Yunnan, China	T77	*C.* ser. *pentastylae* Chang	GPCS 2442	Yunnan, China
T28	*C. assamica* (Masters) Chang	GPCS 2105	Yunnan, China	T78	*C. gymnogynoides* Chang et Yu	GPCS 1591	Yunnan, China
T29	*C.taliensis* (W.W.Smith) Melchior	GPCS 1068	Yunnan, China	T79	*C. crassicolumna* Chang	GPCS 0694	Yunnan, China
T30	*C. assamica* (Masters) Chang	GPCS 2103	Yunnan, China	T80	*C. assamica* (Masters) Chang	GPCS 0570	Yunnan, China
T31	*C. assamica* (Masters) Chang	GPCS 0533	Yunnan, China	T81	*C. assamica* (Masters) Chang	GPCS 0989	Yunnan, China
T32	*C. sinensis* (L.)O.Kuntze	GPCS 0651	Yunnan, China	T82	*C. sinensis* (L.)O.Kuntze	GPCS 0424	Yunnan, China
T33	*C.* ser. *pentastylae* Chang	GPCS 1917	Yunnan, China	T83	*C. assamica* (Masters) Chang	GPCS 1101	Yunnan, China
T34	*C. assamica* (Masters) Chang	GPCS 0824	Yunnan, China	T84	*C. assamica* (Masters) Chang	GPCS 0563	Yunnan, China
T35	*C. assamica* (Masters) Chang	GPCS 0387	Vietnam	T85	*C.* ser. *pentastylae* Chang	GPCS 1927	Yunnan, China

(Continued . . .)

(Table 1 Concluded)

Sample no.	Species/ varieties	Stock-number	Origin	Sample no.	Species/varieties	Stock-number	Origin
T36	*C. assamica* (Masters) Chang	GPCS 0859	Yunnan, China	T86	*C. assamica*(Masters) Chang	GPCS 0559	Yunnan, China
T37	*C. gymnogynoides* Chang et Yu	GPCS 0465	Yunnan, China	T87	*C. Irrawadiensis* P.K. Barua	GPCS 0583	Yunnan, China
T38	*C. sinensis* (L.)O.Kuntze	GPCS 1053	Yunnan, China	T88	*C. yankiangcha* Chang et Wang	GPCS 1019	Yunnan, China
T39	*C. yankiangcha* Chang et Wang	GPCS 0860	Yunnan, China	T89	*C.* ser. *pentastylae* Chang	GPCS 1111	Yunnan, China
T40	*C.taliensis* (W.W.Smith) Melchior	GPCS 1612	Yunnan, China	T90	*C. assamica* (Masters) Chang	GPCS 1881	Burma
T41	*C. atrothea* Chang et Wang	GPCS 1109	Yunnan, China	T91	*C.* ser. *pentastylae* Chang	GPCS 1942	Yunnan, China
T42	*C. assamica* (Masters) Chang	GPCS 0617	Yunnan, China	T92	*C. assamica* (Masters) Chang	GPCS 0388	Vietnam
T43	*C.* ser. *pentastylae* Chang	GPCS 2066	Yunnan, China	T93	*C. crassicolumna* Chang	GPCS 0693	Yunnan, China
T44	*C.* ser. *pentastylae* Chang	GPCS 1933	Yunnan, China	T94	*C. assamica* (Masters) Chang	GPCS 0109	Hainan, China
T45	*C. assamica* (Masters) Chang	GPCS 1880	Burma	T95	*C. assamica* (Masters) Chang	GPCS 0987	Yunnan, China
T46	*C.* ser. *pentastylae* Chang	GPCS 1948	Yunnan, China	T96	*C. irrawadiensis* P.K.Barua	GPCS 0594	Yunnan, China
T47	*C.* ser. *pentastylae* Chang	GPCS 1582	Vietnam	T97	*C. assamica*(Masters) Chang	GPCS 0615	Yunnan, China
T48	*C. assamica* (Masters) Chang	GPCS 0938	Yunnan, China	T98	*C.* ser. *pentastylae* Chang	GPCS 1916	Yunnan, China
T49	*C. assamica* (Masters) Chang	GPCS 0983	Yunnan, China	T99	*C. assamica* (Masters) Chang	GPCS 1618	Yunnan, China
T50	*C. assamica* (Masters) Chang	GPCS 1605	Yunnan, China	T100	*C. assamica* (Masters) Chang	GPCS 0936	Yunnan, China

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

spectrophotometer and agarose gel (0.8%) electrophoresis. Absorbance ratio between 260 and 280 nm was computed and the quality of the genomic DNA was confirmed. The DNA stock, which was stored at –20°, was then diluted to 20 ng/μL.

PCR amplification and electrophoresis

Screening was carried out with 60 ISSR primers, out of which 18 primers, generating clear banding patterns, were used for confirmatory studies (Fig 1, Table 2). PCR reactions were carried out in a volume of 10 μL including 20 ng template genomic DNA, 1 × PCR buffer (200 mmol/L Tris-HCl *p*H 8.4, 500 mmol/L KCl), 2.0 mmol/L $MgCl_2$, 0.2 mmol/L each of dNTP, 0.4 μmol/L primer, and 0.5 U 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. Amplification protocol includes initial denaturation for 5 min. at 94 followed by 39 successive cycles of 60 s denaturation at 94, annealing for 30 s at respective Tm values of the selected primers and 2 min. elongation at 72. Final elongation was performed for 7 min at 72. Amplification products were resolved on 6% polymeric acrylamide gel electrophoresis for 4 hr at a constant voltage of 150 V with 1 × TBE running buffer. Finally, the gel was silver-stained, visualized under ultraviolet light, photographed and documented. The experiment was repeated at least three times.

Data collection and analysis

Polymorphic bands were scored qualitatively as either presence ("1") or absence ("0"). These data were used to calculate the genetic distance among tea accessions and the genetic similarity matrix was constructed. This matrix was subjected to unweighed pair-group method with arithmetical averages analysis (UPGMA) to generate the dendrogram. Principal component analysis (PCA) was done based on the genetic distance. These analyses were done using the NTSYS-pc 2.10 package. The Nei's gene diversity (H) and the mean Shannon's Information index (I) were analyzed using Popgene software, version 3.5.

RESULTS AND DISCUSSION

Genetic variability revealed throuth ISSR markers

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*. 2006). In the present study, 18 ISSR primers generated a total of 475 bands with 99.4% polymorphism and the number of polymorphic bands generated by a primer ranged between 22 (UBC856) and $(TC)_8$ AGT) and 33 (UBC835), with an average of 26.4

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

Code	Primer	Core sequence	Attached bases	polymorphic bands	*H*	*I* (av. values)
P01	UBC807*	$(AG)_8$	+ T	23	0.3993	0.5835
P02	UBC808*	$(AG)_8$	+ C	24	0.3693	0.5420
P03	UBC810*	$(aGA)_8$	+ T	28	0.4339	0.6230
P04	UBC811*	$(GA)_8$	+ C	29	0.4284	0.6151
P05	UBC826*	$(AC)_8$	+ C	25	0.3738	0.5500
P06	UBC834*	$(AG)_8$	+ YT	30	0.4152	0.6006
P07	UBC835*	$(AG)_8$	+ YC	33	0.4015	0.5877
P08	UBC836*	$(AG)_8$	+ YA	25	0.4138	0.5991
P09	UBC840*	$(GA)_8$	+ YT	23	0.3181	0.4792
P10	UBC841*	$(GA)_8$	+ YC	25	0.4104	0.5969
P11	UBC842*	$(GA)_8$	+ YG	27	0.4105	0.5983
P12	UBC856*	$(AC)_8$	+ YA	22	0.3491	0.5284
P13	UBC890*	$(GT)_7$	VHV +	26	0.4259	0.6141
P14	(AC)8T[a]	$(AC)_8$	+ T	31	0.4031	0.5887
P15	(TC)8AGG[a]	$(TC)_8$	+ AGG	23	0.3151	0.4709
P16	(GA)8CTT[a]	$(GA)_8$	+ CTT	26	0.4278	0.6155
P17	(AG)8CTA[a]	$(AG)_8$	+ CTA	28	0.4023	0.5881
P18	(TC)8AGT[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)

bands/primer (Table 2). The size of the bands ranged from 200 to 3 000 bp. Three of the total bands were amplified in all the germplasm lines. As shown in Table 2, the analyses of variance showed significant differences among the 18 primers in the Nei's gene diversity (*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 the tested germplasm had relatively high genetic polymorphism. In previous studies, RAPD was used to fingerprint tea germplasm (Shao *et al*. 2003, Chen *et al*. 2006).

The Jaccard similarity co-efficient among the 100 materials ranged from 0.262 between the most distant (dissimilar) germplasm GPCS0424 and GPCS1026 to 0.810 between the closest (most similar) germplasm GPCS0563 and GPCS1927, with an average of 0.526. Across all genotypes, the genetic distance ranged 0.167 (between GPCS0563 and GPCS1927) to 0.797 (between GPCS1048 and GPCS0807), with an average of 0.500. The amplification results also showed that the 18 ISSR primers enabled the separation of all the 100 test germplasm lines. Examination of the band differences between even the most similar (Jaccard similarity co-efficient = 0.810) germplasm GPCS0563 and GPCS1927 (Fig 2) indicated that they differ in 29 (=6%) of the 475 bands or fragments. The Jaccard similarity co-efficient and genetic distance in present study were higher than those in earlier studies (Rajan and Swati 2004, Huang *et al*. 2006, Yao *et al*. 2007), 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 germplasms and the distribution center of species diversity (Zeng *et al*. 2004, Chen *et al*. 2005).

Cluster analysis

The dendrogram (Fig 2) using UPGMA based on the Jaccard similarity co-efficient divided the 100 germplasms into 2 clusters (I, II), including 5 groups: a group of 28 germplasms (A); a group of 18 germplasms (B); one of 2 germplasms (C), i e GPCS0983 and GPCS1605; one of 30 germplasms (D); one of 19 germplasms (E). Otherwise, 3 germplasms remained solitary without grouping (Jaccard similarity co-efficient = 0.535), of which the most distinct germplasm was GPCS0424 from Yunnan Province in China, standing solitary without grouping with any others. For the main part, the 4 groups mostly consisted of tea resources from Yunnan Province in China, but also included other germplasms from Burma, Vietnam and other regions of China (Guangdong, Guangxi and Hainan Provinces).The remaining tea germplasms, including the group C and 3 non-clustered germplasms, consisted of a heterogeneous collection of germplasms. The clustering results exhibited the complicated genetic backgroud of test germplasms, indicating that tea resources from Yunnan Provinces had a wide heredity base.

Nevertheless, examination of the distribution of the test germplasms among the clusters (Fig 2) did not result in comprehensively distinct clustering patterns based on their geographical origin. Although part of species and varieties

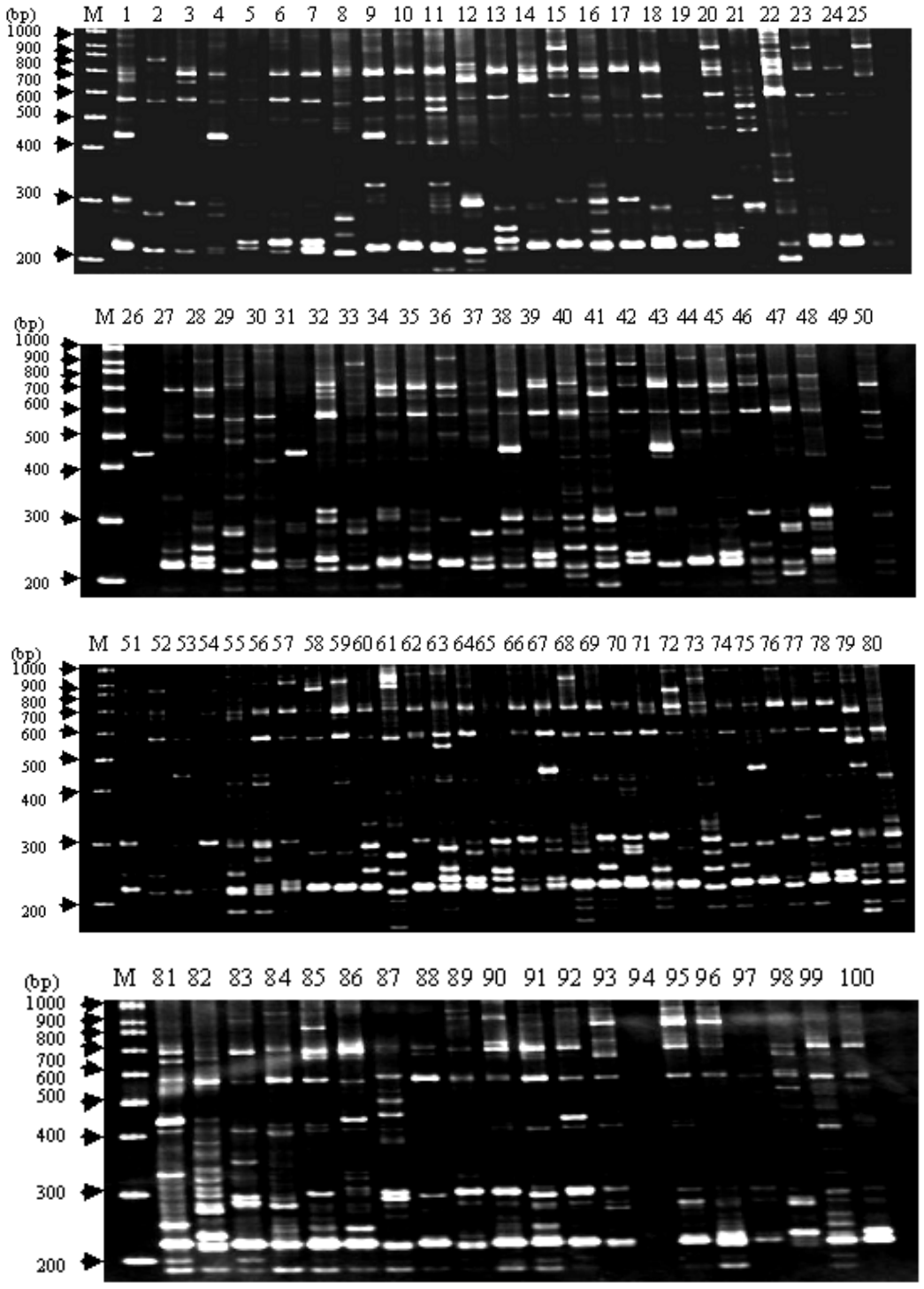

Fig 1 ISSR analysis of 100 tea germplasm lines by using primer S835 and Lane 1-100 for tea germplasm lines listed in Table 1, M = 1kb DNA ladder

that originated in the same geographical areas was grouped together, e g GPCS 1103 and GPCS 1093, the majority of test resources from same geographical areas were not grouped together. For example, GPCS 0597 did not group with GPCS 0987, and they both were from Tengchong county of Yunnan province. In contrast to this, some germplasms from the different geographical areas were grouped together. For instance, GPCS 0108 from Hainan province grouped with GPCS 0448 from Yunnan province. Similar results were reported by Huang *et al*. (2006) and Liu *et al*. (2008).

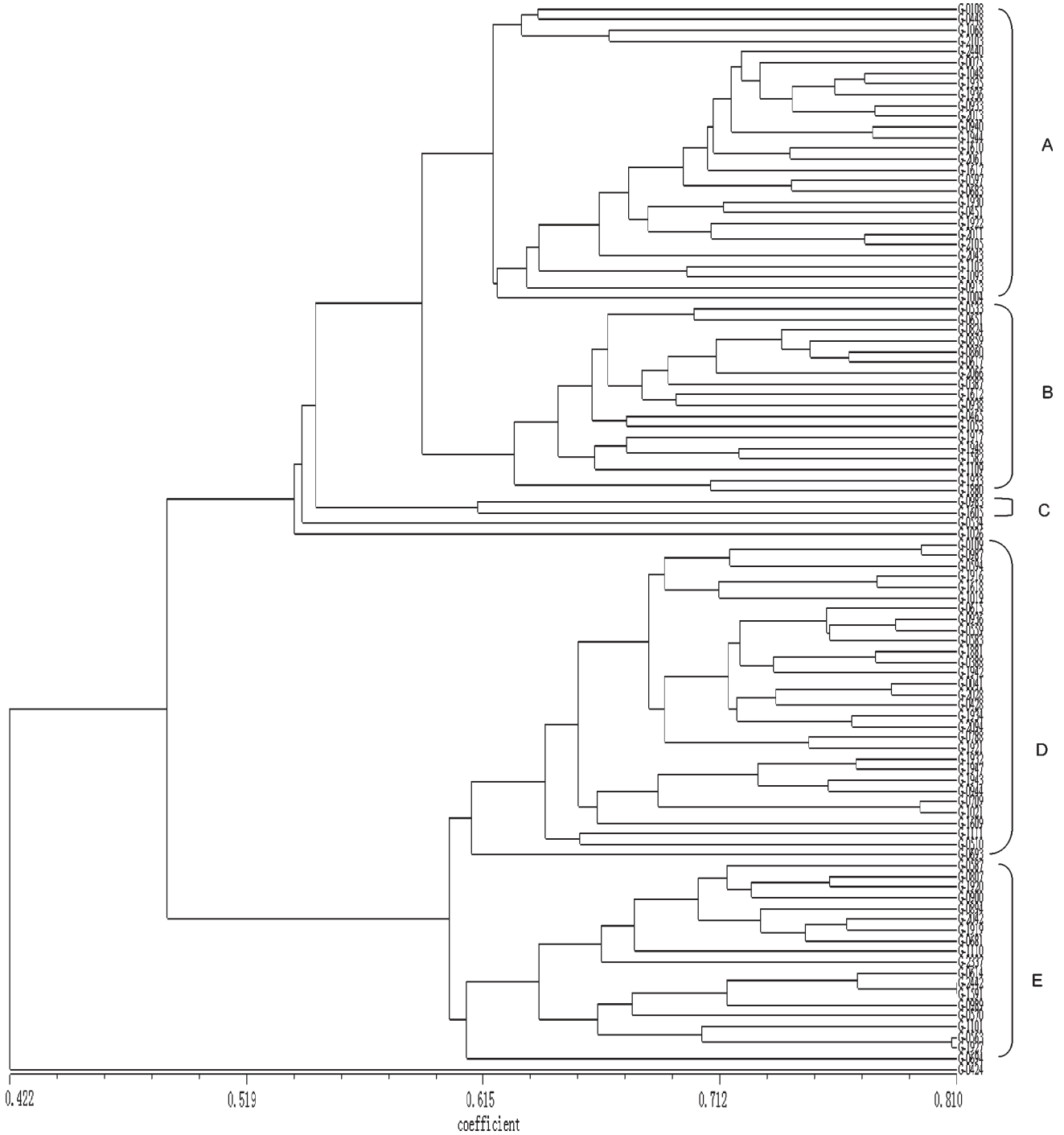

Fig 2 UPGMA phenogram of 100 tea germplasms using Jaccard similiarity of ISSR band profiles and G denotes GPCS

However, the absence of clustering showing trends of grouping based on origin accords with the findings of Chen *et al*. (2006). For example, 20 germplasms belonging to Ser. *Pentasylae* were grouped together in cluster I, 31 germplasms belonging to Ser. *Sinensis* were grouped together in cluster II, but GPCS1026 belonging to Ser. *Gymnogynae* stood solitary without grouping the others. On the other hand, the clustering result also showed a few differences with traditional taxonomy (Chen *et al*. 2006), e g GPCS0465 belonging to Ser. *Gymnogynae* grouped with GPCS1053 belonging to Ser. *Sinensis*, which was not in agreement with the traditional taxonomy. This clustering analysis showed the complexity among test tea gremplasms. A plausible explanation for this result is that they have rather complex

genetic background. For example, their geographical isolation may cause the distinctness in their clustering behaviour. In addition, it was possibly caused because of discontinuous variation from long-term hybridization, cross-pollinating and natural environment effect. The principal components analysis (PCA) results are depicted in Fig 3. The first three components of PCA accounted for 40.66% of total variation, and each of them from the first to the third PC explained about 24.32%, 12.63% and 3.71% of the overall variation among the test genotypes. According to PCA results, the 100 genotypes were well separated into 4 main groups, but GPCS 0424 and GPCS 1026 were different from the other germplasms. The PCA for ISSR data also well supported their UPGMA clustering (Fig 2).

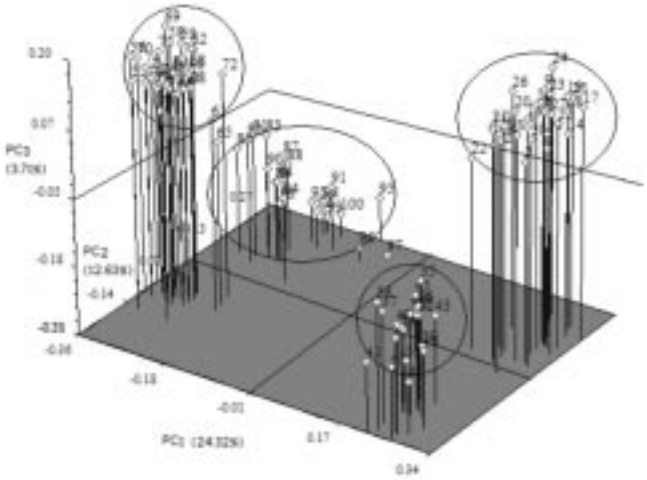

Fig.3 Patterns of relationship among 100 tea germplasms as revealed by Principal Component Analysis based on ISSR data

Thus, ISSR markers would be promising for the assessment of genetic diversity and phylogenetic relationship, the identification of cultivars or varieties, core collection constructing and for genome mapping in tea plants. The use of different ISSR primers for PCR amplification and various germplasms materials would enable the realization of potential of the technique in discerning variation in the species.

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