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ISSR DIVERSITY AND GENETIC DIFFERENTIATION OF ANCIENT TEA (*CAMELLIA SINENSIS* VAR. *ASSAMICA*) PLANTATIONS FROM CHINA: IMPLICATIONS FOR PRECIOUS TEA GERMPLASM CONSERVATION

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Abstract

Over 10 centuries, ancient cultivated tea populations (*Camellia sinensis* var. *assamica*) are still planted merely in Yunnan province of China. Genetic diversity and differentiation were examined in 10 ancient tea plantations by using ISSR markers. The average genetic diversity within populations, estimated with Nei's genetic diversity (H_E), was approximately 0.2809, while Shannon indices (H_O) was 0.4179. The percentage of polymorphic loci (*P*) of the 10 populations ranged from 56.5% to 90.91%. We found a moderate level of genetic differentiation among population as evidenced by the coefficients of gene differentiation (*Gst*) of 0.3911 and the analysis of molecular variance (AMOVA) of 39.70%. The result could be explained by the nature of highly out crossing in the tea species as well as serious habitat fragmentation. Finally, conservation strategies were discussed to protect these ancient tea populations, including *in situ* reserve settings and *ex situ* germplasm sampling.

Introduction

Tea is the most popular non-alcoholic soft beverage throughout the world. Chinese were the first to use tea as a medicine, later as a beverage and have been doing so for the past 3000 years (Eden, 1958). The tea plant, belonging to the family Theacea, has been cultivated and consumed in China for more than 2000 years (Li, 1983). The original region or "the primary center of origin" of tea was South-East Asia, at the point of intersection between the 29°N and 98° E near the source of the Irrawaddy river at the confluence of North-East India, North Burma, South-West China, and Tibet province (Wight, 1959). Yunnan Province, located in southwestern China, is one of the most important centers of origins and genetic diversity for tea and many other cultivated crops, resulting from its particular geographical environments, complicated landforms, abundant climate conditions, and cultural diversity of numerous indigenous minority groups in this region (Zeng *et al*., 2001).

Tea plant propagation by seed in most of the tea growing countries over the past several hundred years. However, the selected elite genotypes are often asexually propagated and thus released as clonal varieties (as called clone tea). The cultivated tea species is mainly classified as three taxonomic varieties, *Camellia sinensis* var. *sinensis*

(L.) O. Kuntze with small leaf, *C. sinensis* var. *assamica* (Masters) with big leaf and *C. assamica* var. *lasiocalyx* (Planchon ex Watt) with the intermediate leaf size (Sealy, 1958). Among them, C. *sinensis* var. *assamica* is the main taxa for commercial cultivation in Yunnan (Chen, 1986; Ming, 2000). Tea cultivation in Yunnan might historically date back to the Tang Dynasty (618-907 A.D.) (Zhou, 2004). In the Song Dynasty (960-1279 A.D.) and Yuan Dynasty (1279-1368 A.D.), Pu'er County in Southwestern Yunnan already became a worldwide famous market of tea trading. Commercial tea products of *C. sinensis* var. *assamica* were shipped to other regions of Yunnan directed by local governments. Tea was one of the most important commodities for people of all ethnic groups (Chen, 1986). In the earlier period of the Qing Dynasty (1644 - 1911 A. D.), hundreds of thousands people planted *C. sinensis* var. *assamica* in "liu da cha shan" in Xishuangbanna, and the tea industry became a major one in Yunnan (Zhou, 2004). Yunnan 'Pu'er' Tea, made from fresh shoot of *C. sinensis* var. *assamica*, was one of the best-known tea products which were widely exported to Tibet and many other regions (Zhou, 2004). During 1950s to 1990s, in an attempt to replace ancient tea gardens by clone tea gardens, the planting of ancient tea gardens fell throughout the province. Fortunately, many populations were spared from destruction and grew in the southwest of the Yunnan (Long *et al*., 1997). Even in 1950s, ancient tea gardens in Yunnan occupied up to 32000 hm^2. However, it has seriously decreased to 13000 hm^2 owing to the increased deforestation and rapid exploitation and plantation of cloned tea gardens (Sha & Guo, 2005). Today, ancient tea plantations are mainly distributed in Mengla, Menghai, Changning, Jinghong, Lancang, Puer and Lincang counties of Yunnan province (Zhao, 2006; Xu *et al*., 2006).

In recent decade, a number of molecular markers, including RFLPs (Matsumoto *et al*., 1994), AFLPs (Paul *et al*., 1997; Misra & Sen-Mandi, 2001), and RAPDs (Wachira *et al*., 1995; Kaundun *et al*., 2000; Kaundun & Park, 2002), have been applied to investigating genetic diversity and genetic differentiation in *C. sinensis*. Inter-simple sequence repeats (ISSRs) marker developed by Zietkiewicz *et al*., (1994) has also proven a suitable genetic marker for the same purpose (Gupta *et al*., 1994; Fang & Roose, 1997). In order to maintain, evaluate and utilize germplasm efficiently and effectively, it is important to investigate the extent of genetic diversity it contains (Smith & Smith, 1989; Gept, 1993; Zahid *et al*., 2009). In this study, ISSR markers were employed to analyze a total of ten ancient populations of *C. sinensis* var. *assamica*. We aim to examine levels of genetic diversity and differentiation of ancient tea plantations and to further propose the conservation strategies for *C. sinensis* var. *assamica* in Yunnan province, China.

Materials and Methods

Sample collection: Leaf tissues were collected from a total of 181 plants of *C. sinensis* var. *assamica*, representing 10 ancient populations (Table 1; Fig 1). These populations were cultivated from 1000 A. D. to 1800 A. D. according to historic records (Zhao, 2006; Xu *et al*., 2006). Fresh leaves were dried with silica gel and stored at 4°C until DNA extraction. Voucher specimens were collected from each population and deposited in the Herbarium of Biotechnology and Germplasm Resource Institute at the Yunnan Agricultural Academy of Sciences (YAAS).

Table 1. Descriptions of all sampled ancient tea populations of *C. sinensis* var. *assamica*.

Code	Population localities	Sample size	Latitude (N)	Longitude (E)
YW	Yiwu, Mengla, Yunnan	20	21°48'	101°56'
NNS	Nannuoshan, Menghai, Yunnan	14	21°98'	100°07'
MSH	Mangshui, Changning, Yunnan	19	24°82'	99°61'
DML	Damenglong, Jinghong, Yunnan	21	22°00'	100°79'
MJ	Mengjing, Lancang, Yunnan	18	22°53'	99°99'
KLS	Kunlushan, Puer, Yunnan	20	23°07'	101°03'
MS	Mengsong, Menghai, Yunnan	15	21°93'	100°06'
JM	Jingmai, Lancang, Yunnan	19	22°55'	99°97'
BD	Bangdong, Lincang, Yunnan	16	23°88'	100°09'
MH	Menghai, Yunnan	19	21°95'	100°05'

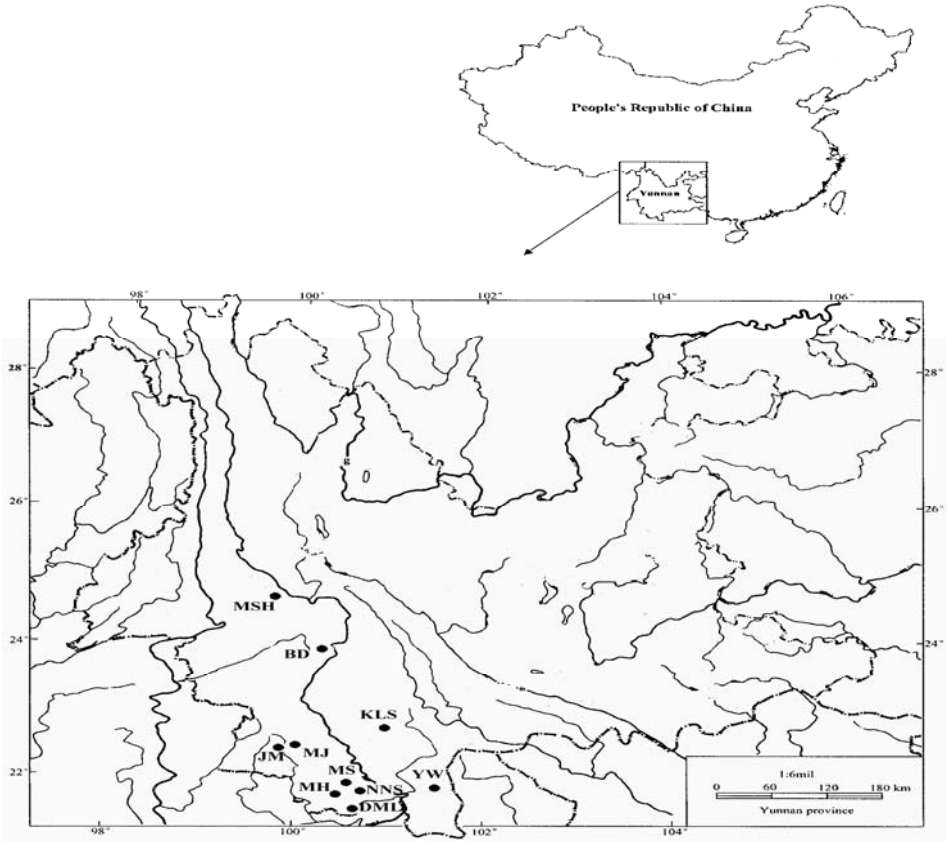

Fig. 1. Distribution of the 10 ancient tea populations of *sinensis* var. *assamica* in Southwestern Yunnan.

DNA extraction and PCR amplification: Genomic DNA was extracted following the CTAB protocol (Doyle, 1991). Nuclear DNA was then PCR-amplified using ISSR primers obtained from the University of British Columbia. Following an initial screen of 100 primers, 19 primers were selected for further analyses (Table 2). PCR reactions were carried out in a total volume of 20 ul consisting of 20 ng of template DNA, 2.0 ul 10×PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 2% formamide, 1uM primer, 1 unit of Taq polymerase and double-distilled water. PCR was performed with an MJ Research (Waltham, MA, USA) PTC200-well thermal cycler with a hot bonnet. Amplicons were electrophoretically separated in 2% agarose gels buffered with 0.5×TAE. DL2000 (Takara Biotech Co., Ltd) was used as a size marker.

Table 2. Number of amplification products generated with 19 ISSR primers in this study.

Primer	Sequence, 5' to 3'	No. of Scorable bands	No. of polymorphic bands	Polymorphism (%)
810	GAGAGAGAGAGAGAGAT	10	8	80.0
811	GAGAGAGAGAGAGAGAC	9	7	77.8
813	CTCTCTCTCTCTCTCTT	8	7	87.5
815	CTCTCTCTCTCTCTCTG	9	8	89.9
835	AGAGAGAGAGAGAGAGYC	8	7	87.5
836	AGAGAGAGAGAGAGAGYA	7	5	71.4
840	GAGAGAGAGAGAGAGAYT	10	8	80.0
842	GAGAGAGAGAGAGAGAYG	7	6	85.7
843	CTCTCTCTCTCTCTCTRA	5	4	80.0
844	CTCTCTCTCTCTCTCTRC	11	9	81.8
845	CTCTCTCTCTCTCTCTRG	8	7	87.5
847	CACACACACACACACARA	7	5	71.4
855	ACACACACACACACACYT	6	4	66.7
856	ACACACACACACACACYC	5	4	80.0
857	ACACACACACACACACYG	8	7	87.5
871	TATTATTATTATTAT	13	10	76.9
873	GACAGACAGACAGACA	8	5	62.5
879	CTTCACTTCACTTCA	9	8	88.9
880	GGAGAGGAGAGGAGA	7	6	85.7
Total		**155**	**125**	

R = (A, G); Y = (C, T)

A negative control reaction in which the template DNA was replaced by water was performed along with every PCR amplification to ensure the absence of contamination. DNA fragments were then identified by image analysis software for gel documentation (Gene Tools Analysis Software Version 3.0; Syngene; Beacon House, Nuffield Road, Cambridge, England) following staining with Ethidium bromide. Only those bands that showed consistent amplification were further analyzed. Smeared and weak bands were excluded.

Data analysis: ISSR bands were scored as presence or absence of binary characters. The resulting data matrix was analysed using POPGENE v. 1.31 (Yeh *et al*., 1999) to estimate two genetic diversity parameters: the percentage of polymorphic loci (*P*) and the expected heterozygosity (H_E). Two genetic diversity measures (total gene diversity, H_T, and the coefficient of gene differentiation, G_{ST}) were determined based on the model described by Nei (1973). The genetic identity (*I*) and the genetic distance (*D*) between populations were computed using the model by Nei (1972). Gene-flow estimates (*Nm*) were estimated as $Nm = (1 \pm G_{ST})/4\ G_{ST}$ (Slatkin & Barton, 1989). The Shannon index was calculated as $H_O = -\sum pilog_2\ pi$ (Lewontin, 1972), in which *pi* is the frequency of a given ISSR fragment. Shannon's index of phenotypic diversity was used to measure the total diversity (H_{SP}) as well as the mean intra-population diversity (H_{POP}). The proportion of diversity between populations was then calculated as $(H_{SP}\text{-}H_{POP})/\ H_{POP}$. This matrix was used to construct a dendrogram using the unweighted pair group method (UPGMA). The distance matrix was generated with the AMOVA-PREP version 1.01 (Miller, 1998). The resulting distance matrix was subjected to an analysis of molecular variance using WINAMOVA version 1.55 (Excoffier *et al*., 1992).

In order to test correlations between genetic distances (*D*) and geographical distances (in km) amongst the populations, a Mantel test was performed with Tools for Population Genetic Analysis (Miller, 1997) (computing 5000 permutations).

Results

The 19 primers chosen for further analysis produced a total of 155 reproducible ISSR bands at an average of 8.2 bands per primer in *C. sinensis* var. *assamica*. In total 125 were polymorphic (Table 2). The average percentage of polymorphic loci (*P*) was 79.30%, ranging from 56.57% (MJ) to 90.91% (DML) (Table 3). Assuming Hardy-Weinberg equilibrium, the average Nei's genetic diversity (H_E) was estimated to be 0.2809 within populations and 0.461 at the variety level, respectively. Shannon's index (*Ho*) ranged from 0.3011 to 0.5099, with an average of 0.4179 at the population level and 0.6526 at the variety level, respectively (Table 3). Among the ten populations, DML and MS exhibited higher levels of genetic variability with PPL of 90.91 and 89.90%, H_E of 0.3252 and 0.3473, and H_O of 0.4826 and 0.5099, respectively, while the population MJ possessed the lowest genetic diversity with PPL of 56.57, H_E of 0.2021, and H_O of 0.3011, respectively (Table 3).

In this study, moderate differentiation was detected among 10 ancient tea plantations of *C. sinensis* var. *assamica*. The coefficient of genetic differentiation between populations (G_{ST}, estimated by partitioning of the total gene diversity) was 0.3911 (Table 4). The finding is consistent with the result of genetic structure predicted by Shannon's diversity index analysis, which showed that 35.96% of the total variation was partitioned between populations (Table 4). The analysis of molecular variance (AMOVA) further indicated that 39.7% of the total variation was partitioned between populations (Table 5). Genetic identity (*I*) between populations varied from 0.6224 to 0.7858 with an average of 0.73. The value was the lowest between populations MJ and KLS ($I = 0.6224$), while it was the highest between populations DML and MS ($I = 0.7858$) (Table 6). The UPGMA dendrogram (Fig. 2) shows that there is no significant correlation between genetic distance and geographical distance. Mantel test also failed to detect significant correlation between levels of genetic differentiation and geographical distances ($r = -0.0613$, $P<0.001$), indicating that levels of genetic differentiation does not fit with their spatial distances. In addition, the extent of gene-flow between populations (*Nm*) was estimated to be 0.3893 individuals per generation, indicating a rather low migration rate between populations (Table 4).

Discussion

In this study, we detected abundant genetic diversity within ancient tea plantations of *C. sinensis* var. *assamica* in Yunnan. ISSR analysis, as expected, detected much higher levels of genetic diversity ($H_O = 0.4179$) than allozyme loci reported previously in *C. sinensis* var. *assamica* (*Ho*=0.176) and *C. sinensis* var. *sinensis* (*Ho*= 0.191) (Chen *et al*., 2005). Genetic diversity was evaluated in *C. sinensis* var. *assamica* and *C. sinensis* var. *sinensis* by using RAPDs (Chen *et al*., 1998; Kaundun & Park, 2002; Luo *et al*., 2004) and SSRs (Kaundun and Matsumoto, 2002). However, it is difficult to directly compare the above-mentioned estimates of genetic variation with our ISSR data. Comparisons of genetic diversity previously estimated in *C. sinensis* with ISSR technique (Lai *et al*., 2001; Devarumath *et al*., 2002; Mondal, 2002) apparently suggest high genetic variation harbored in ancient tea populations of *C. sinensis* var. *assamica* in Yunnan.

Table 3. Genetic variability within populations of *C. sinensis* var. *assamica* as revealed by the ISSR analysis.

Populations	*N*	*P*	H_E (s.d.)	H_O (s.d.)
YW	20	81.82	0.3063 (0.1897)	0.4501 (0.2609)
NNS	14	80.81	0.2975 (0.1782)	0.4423 (0.2483)
BD	19	69.70	0.2489 (0.1946)	0.3720 (0.2751)
DML	21	90.91	0.3252 (0.1691)	0.4826 (0.2239)
MJ	18	56.57	0.2021 (0.2033)	0.3011 (0.2908)
KLS	20	84.85	0.2432 (0.1853)	0.3734 (0.2509)
MS	15	89.90	0.3473 (0.1596)	0.5099 (0.2155)
JM	19	80.81	0.2875 (0.1931)	0.4260 (0.2646)
MSH	16	74.75	0.2794 (0.1997)	0.4113 (0.2777)
MH	19	82.83	0.2713 (0.1818)	0.4104 (0.2477)
Population level	18.1	79.30	0.2809 (0.1854)	0.4179 (0.2554)
Variety level	181	100	0.4610 (0.0507)	0.6526 (0.0542)

N, sample size; *P*, percentage of polymorphic loci; H_E, Nei's genetic diversity; H_O, Shannon's diversity index

Table 4. Analyses of Nei's gene diversity and Shannon's diversity index in ancient tea populations of *C. sinensis* var. *assamica*.

	Ht	*Hs*	*Gst*	*Nm*	*Hsp*	*Hpop*	(*Hsp*-*Hpop*)/*Hsp*
Mean	0.4613	0.2809	0.3911	0.3893	0.6526	0.4179	0.3596
Standard deviation	0.0026	0.0040			0.0542	0.2554	

Ht, Total gene diversity; *Hs*, Gene diversity within populations; *Gst*, Coefficient of gene differentiation; *Nm*, Gene flow, *Nm*= (1-*Gst*)/4*Gst;* (*Hsp*-*Hpop*)/*Hsp*, Shannon's diversity index analysis; *Hpop*, Shannon indices (*Ho*) at the population level; *Hsp,* Shannon indices (*Ho*) at the species level.

Table 5. Analysis of molecular variance (AMOVA) for 181 tea individuals sampled from the ten populations of *C. sinensis* var. *assamica*.

Source of variation	d. f.	MSD	Variance components	*P*-value	Percent of total variance
Among populations	9	167.912	8.98420	<0.001	39.70
Within populations	162	13.649	13.64875		60.30

Statistics include degrees of freedom (d. f.), mean squared deviations (MSDs), variance component estimates, the probability (*P*) of obtaining a more extreme component estimate by chance alone after 1000 permutations and the percentage of total variance contributed by each component.

Table 6. Measures of Nei's genetic identity and genetic distance between the ten populations of *C. sinensis* var. *assamica*.

Populations	YW	NNS	BD	DML	MJ	KLS	MS	JM	MSH	MH
YW	****	0.7721	0.7658	0.7508	0.6996	0.7611	0.7736	0.7543	0.7329	0.7768
NNS	0.2587	****	0.7422	0.7846	0.6881	0.7091	0.7475	0.7511	0.7353	0.7031
BD	0.2668	0.2981	****	0.7118	0.7015	0.6660	0.7611	0.6789	0.7092	0.6787
DML	0.2866	0.2426	0.3399	****	0.6783	0.7081	0.7858	0.7501	0.7540	0.7840
MJ	0.3573	0.3738	0.3545	0.3881	****	0.6224	0.7729	0.6741	0.7186	0.6314
KLS	0.2730	0.3438	0.4065	0.3452	0.4742	****	0.6992	0.7112	0.6594	0.6910
MS	0.2567	0.2911	0.2730	0.2410	0.2576	0.3579	****	0.7410	0.7289	0.7281
JM	0.2820	0.2862	0.3873	0.2875	0.3944	0.3408	0.2997	****	0.7093	0.7059
MSH	0.3108	0.3075	0.3436	0.2823	0.3304	0.4165	0.3162	0.3434	****	0.7280
MH	0.2526	0.3522	0.3876	0.2433	0.4598	0.3697	0.3173	0.3483	0.3174	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

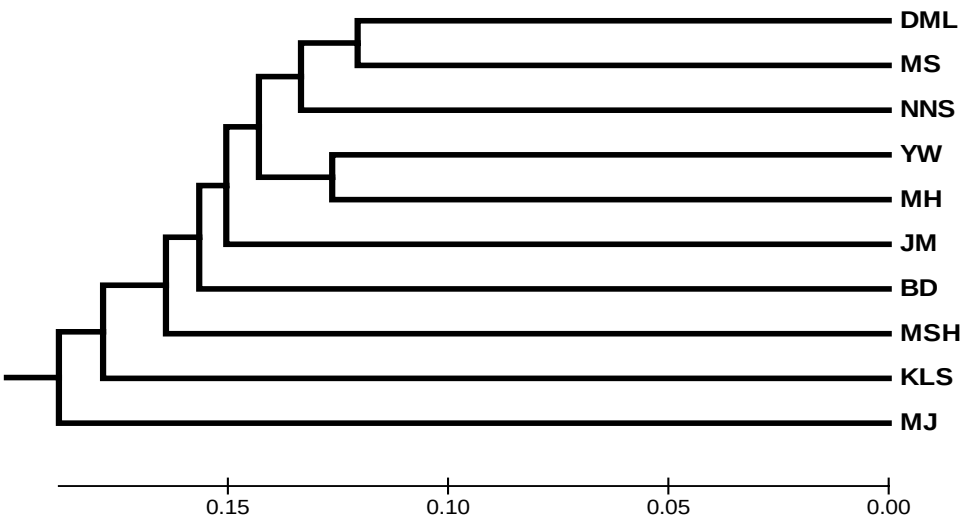

Fig. 2. Dendrogram Based Nei's (1978) Genetic distance: Method = UPGMA.

In recent years, numerous studies revealed high levels of genetic variability in rare or narrow endemic species (Richter *et al*., 1994; Smith & Pham, 1996; Kang *et al*., 2000; Zawko *et al*., 2001; Xue *et al*., 2006). There are several possible explanations for abundant genetic diversity detected in ancient tea plantations of *C. sinensis* var. *assamica*. First, a great number of populations of *C. sinensis* var. *assamica* occur in an extensive geographical range. The relatively high genetic diversity might result from large-sized populations widely grown in the Ming and Qing Dynasty. Second, Yunnan is one of the most important centers of tea origin and domestication, which in return could help to maintain a relatively extensive genepool for the species. Finally, since the *Camellia* species are highly outcrossing, many wild relatives naturally occurred among ancient tea gardens in Yunnan (Chen, 1986; Ming, 2000), possibly having contributed to high genetic variation found in *C. sinensis* var. *assamica*.

In the present study, considerable genetic differentiation was detected among 10 ancient populations of *C. sinensis* var. *assamica* from Yunnan Province, China. The Nei's genetic diversity index analysis (G_{ST} = 0.3911), Shannon's diversity index analysis ((*Hsp*-*Hpop*)/*Hsp* = 0.3596) as well as the AMOVA analysis (0.397) of the ISSR data generated similar estimates of genetic differentiation among these ancient populations, showing that 35-40% of the total variation was partitioned among populations.

Our investigation of genetic structure of ancient tea populations of *C. sinensis* var. *assamica* shows a larger genetic differentiation than results obtained in previous studies. For example, Wachira *et al*., (1997) examined 38 tea clones of China, Assamica and Cambod (*Camellia assamica* ssp., lasiocalyx Planchon ex Watt) using RAPD markers and estimated that 30% of the total diversity resided among population. Paul *et al*., (1997) reported genetic diversity and differentiation of Indian and Kenyan tea populations using AFLP markers. They estimated that only 21% of the total diversity was distributed between populations of Indian and Kenyan tea. Kaundun & Park (2002) studied genetic structure of six populations of Korean tea (*C. sinensis* var. *sinensis*) using RAPD markers. The AMOVA analysis showed that only 16% of the total diversity resided among populations. However, Lai *et al*., (2001) investigated genetic relationships of Taiwan tea using RAPD and ISSR markers. The AMOVA analysis revealed that the percentage of variance attributed to the difference within groups were 55.89% based on ISSR data. The finding is relatively larger than our estimation. It is likely that the estimated difference of

genetic differentiation in the species may vary with different molecular markers. Therefore, our data suggest a moderate level of genetic differentiation among ancient populations of *C. sinensis* var. *assamica*.

The major aim of biodiversity conservation is to preserve the species through maintaining genetic diversity as much as possible. Knowledge of genetic variation between and within populations of rare and endangered species is extremely useful for making appropriate management strategies directed towards their conservation (Milligan *et al*., 1994). The well-known Pu'er tea, made from ancient tea plants in Yunnan Province, enjoys a price 10-100 times higher than clone tea. For this reason, ancient tea populations of *C. sinensis* var. *assamica* have become endangered due to over-picking driven by the economic incentives (Sha & Guo, 2005). Unlike most of the clone tea cultivars, seedling tea population was found in great variation (Hamid *et al*., 2006) which show cold tolerance and are resistant to common diseases affecting the tea species, and thus they constitute valuable gene resources for local and international tea improvement programs in the future. Although efforts have been made in the tea germplasm conservation (Wachira *et al*., 2001), there is still an urgent need to take effective ways to protect this species against further loss of genetic diversity in Yunnan. Based on the finding of moderate level of genetic differentiation of ancient tea plantations of *C. sinensis* var. *assamica*, the preservation of any one population would be insufficient to conserve the majority of variation resided in ancient tea plantations. Therefore, the *in situ* priority should be given to protecting populations with large genetic variation, allowing them to propagate and increase in size through natural regeneration.

Hybridization among different taxa of *Camellia* may cause genetic introgression to the archetypal genetic resource in the primitive tea gardens (Takeda, 1990). Such genetic introgression is frequently observed in local varieties of other crops (Levin, 2002). Cultivated tea is selected and bred from parental materials largely based on the yield, quality and resistance to biotic and abiotic stress. As a consequence, the widespread cultivation of clone tea can diminish genetic diversity if care is not taken to use clones of diverse origins. We thus suggest that *in situ* conservation should make strict exclusion of clone tea.

Considering abundant genetic diversity resided within ancient tea populations in Yunnan, *ex situ* germplasm collection should have sufficient sample size from each population. Wei *et al*., (2005) compared levels of genetic diversity of *ex situ* populations with natural populations of *C. nitidssima* using ISSR markers. They found that 20 individuals grown in germplasm nursery have already maintained most genetic diversity of the species. Therefore, it is recommended that sample size of *ex situ* conservation of ancient tea plantations of *C. sinensis* var. *assamica* should not be fewer than 20 individuals per population. Since at least 35% genetic diversity distributed among populations, collections of tea germplasm should be sampled from extensive geographic origins.

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