Pak. J. Bot., 44(6): 1989-1992, 2012.

AFLP ANALYSIS OF GENETIC VARIATION AMONG CLONED, SEED PRODUCED AND WILD *CAMELLIA SINENSIS* VAR. *ASSAMICA* TEA PLANT IN YUNNAN, CHINA

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Abstract

Genetic variation and differentiation in cloned, seed produced, and wild tea plants of *Camellia sinensis* var. *assamica* were analyzed by amplified fragment length polymorphism (AFLP) markers. Population diversity was highest in the wild and lowest in the cloned tea plants. Analysis of molecular variance revealed great variation among the groups (31.40%). Furthermore, a dendrogram constructed based on Nei's genetic distance and principal component analysis clustered the tea population into three groups. Taken together, our study demonstrated that there is wide genetic variation among cloned, seed produced and wild tea plants of var. *assamica* in Yunnan.

Introduction

Tea is one of the most popular healthy beverages in the world. The Chinese were the first to use tea as a medicine, later as a beverage, and have been cultivating tea for the past 3000 years (Eden, 1958). Tea plants (*Camellia spp.*) have been propagated sexually by seed in most tea growing countries over the past several hundred years. However, the selected elite genotypes are often asexually propagated and thus released as clonal varieties. While tea grows naturally as tall as 15 m, the bush height of cloned plants is usually maintained at 60-100 cm to facilitate harvesting the tender leaves.

Yunnan Province in China is host to most species of *Camellia*, wild and cultivated, and thus is an important germplasm conservation resource for improving tea crops by increasing genetic diversity. Several *Camellia* species have been used as tea by the local population for many hundreds of years (Chen *et al*., 2005; Ji *et al*., 2011) and some propagated by seed grow naturally over 5m, and are considered wild.

Numerous studies to evaluate genetic diversity in *Camellia* have been conducted using genetic markers. Such markers include restriction fragment length polymorphisms (RFLPs; Matsumoto *et al*., 1994), random amplification of polymorphic DNAs (RAPDs; Wachira *et al*., 1995; Kaundun *et al*., 2000), and inter simple sequence repeats (ISSRs; Lai *et al*., 2001; Ji *et al*., 2011). However, the genetic variation and differentiation of tea germplasms are not well investigated. The detailed understanding of genetic variability is important for tea breeding because it will help to develop desirable genotypes (Kaundun *et al*., 2000). Amplified fragment length polymorphism (AFLP; Vos *et al*., 1995) is highly reliable for the assessment of genetic variation among and within populations (Paul *et al*., 1997; Heidi & Andrew, 2007; Chen & Peng, 2010; Ipek *et al*., 2010; Yasmin *et al*., 2010). Another advantage of AFLP is its capability to produce multilocus fingerprints in a single analysis, significantly reducing the cost of analysis and increasing the possibility of detecting polymorphisms (Vos *et al*., 1995).

The present study used AFLP markers to determine the genetic variation and differentiation among seed produced, cloned, and wild tea (*Camellia sinensis* var. *assamica*) germplasms taken from the field in Yunnan, China.

Materials and Methods

Sample collection: Leaf tissues were collected in the field from 77 plants of var. *assamica*, representing seed produced, cloned, and wild tea plants (Table 1). Fresh leaves were dried with silica gel and stored at 4 °C until DNA extraction was performed. 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.

DNA extraction and PCR amplification: Genomic DNA was extracted from dried leaves following the cetyl trimethylammonium bromide (CTAB) protocol (Doyle, 1991). The AFLP analysis was carried out using the GibcoBRL AFLP Analysis System I (Gaithersburg, MD, USA) with minor modifications. Restriction digests of genomic DNA with EcoRI and MseI were carried out at 37°C for 4 h. Following heat inactivation of the restriction endonucleases, the genomic DNA fragments were ligated to EcoRI and MseI adapters overnight at 18°C to generate template DNA for amplification. PCR was performed in two consecutive reactions. The template DNA was first preamplified using AFLP primers, none of which contained selective nucleotides. The PCR products of the preamplification reaction were then used as templates, at 1:10 dilution, for selective amplification using 2 AFLP primers containing 3 selective nucleotides. Two primers selected from 8 primer combinations (at the selective amplification stage) were used and the primer sequences are shown in Table 2. The PCR products were run in polyacrylamide gel on a Beckman Coulter CEQ 8000 genetic analysis system.

Table 1. Populations of var. *assamica* examined in the AFLP analysis.

Code	Population (in Yunnan)	Type	Sample size (*n*)	Latitude (N)	Longitude (E)
MS	Mengsong, Menghai	Wild	15	21.93	100.06
ML	Yiwu, Mengla	Wild	11	21.48	101.56
J	Damenglong, Jinghong	Wild	15	22.00	100.79
NNS	Nannuoshan, Menghai	Wild	9	21.98	100.07
YK	Yunkang 10, Menghai	Cloned	13	21.95	100.05
MD	Menghai Daye, Menghai	Seed produced	14	21.95	100.05

Table 2. Amplified efficiency of screened primer combinations in tea plants.

Primer combinations	Primer sequences	Scorable bands	Polymorphic bands	Polymorphism (%)
E-ACC/M-CTC	5'-GACTGCGTACCAATTCAAC-3' 5'-GATGAGTCCTGAGTAACTC-3'	228	205	90.14
E-ACC/M-CTA	5'-GACTGCGTACCAATTCAAC-3' 5'-GATGAGTCCTGAGTAACTA-3'	266	251	94.48
Total		494	456	92.31
Average		247	226	

Data Analysis: AFLP bands were scored as the presence or absence of binary characters. The resulting data matrix was analyzed using POPGENE v. 1.31 (Yeh *et al*., 1999) to estimate 2 genetic diversity parameters: the percentage of polymorphic loci (PPL) and the expected heterozygosity (H_E). The genetic identity and the genetic distance between the populations were computed using the model by Nei (1972). The Shannon index (H_O) was calculated as $H_O = -\sum p_i \cdot \log_2(p_i)$ (Lewontin, 1972), with p_i as the frequency of a given AFLP fragment. This matrix was used to construct a dendrogram with the unweighted pair group method (UPGMA). The distance matrix was generated with the AMOVA-PREP version 1.01. The resulting distance matrix was subjected to an analysis of molecular variance (AMOVA) using WINAMOVA version 1.55 (Excoffier *et al*., 1992).

Results and Discussion

The two primers chosen for further analysis produced a total of 494 reproducible AFLP bands at an average of 247 bands per primer in var. *assamica*, and 456 bands were polymorphic (Table 2). The PPL was 92.31% (Table 3). Based on the Hardy-Weinberg equilibrium, the average Nei's H_E was estimated to be 0.1366 and Ho was 0.2323 at the variety level (Table 3). Among 6 populations, the wild tea plants ML and NNS exhibited higher levels of genetic variability with PPLs of 59.11% and 45.95%, H_E of 0.1192 and 0.1132, and H_O of 0.2009 and 0.1813, respectively. The wild tea plants exhibited higher levels of genetic variability with a PPL of 85.02%, H_E of 0.1189, and H_O of 0.2075. The seed produced tea plants exhibited a moderate level of genetic diversity with a PPL of 35.02%, H_E of 0.0986, and H_O of 0.1531. In contrast, the cloned tea plants YK possessed the lowest genetic diversity with a PPL of 13.77%, H_E of 0.0437, and H_O of 0.0666 (Table 3). Thus these results showed that there were great genetic differences among the wild, seed produced, and cloned tea plants.

Table 3. Genetic variability within populations of var. *assamica* as revealed by AFLP.

Population	*n*	PPL	H_E (SD)	H_O (SD)
MS	15	36.44	0.0810 (0.1437)	0.1312 (0.2118)
ML	11	59.11	0.1192 (0.1398)	0.2009 (0.2090)
J	15	40.69	0.0936 (0.1521)	0.1504 (0.2231)
NNS	9	45.95	0.1132 (0.1599)	0.1813 (0.2339)
Wild tea plant	50	85.02	0.1189 (0.1366)	0.2075 (0.1941)
MD	13	35.02	0.0986 (0.1625)	0.1531 (0.2383)
YK	14	13.77	0.0437 (0.1216)	0.0666 (0.1791)
Variety level		92.31	0.1366 (0.1484)	0.2323 (0.2081)

Note: Population codes were listed in Table 1. *n*, sample size; PPL, percentage of polymorphic loci; H_E, expected heterozygosity; H_O, Shannon's diversity index; SD, standard deviation.

The coefficient of genetic differentiation between tea plants (Gst, estimated by partitioning the total gene diversity) was calculated as 0.3201, consistent with the AMOVA, which suggested that 31.4% of the total variation was partitioned among the wild, seed produced, and cloned tea plants population (Table 4). In addition, based on Nei's (1972) gene diversity index, the average genetic distance was calculated as 0.0677, with variations from 0.1674 between YK and NNS to 0.0122 between J and ML (Table 5). These data prove that there was significant differentiation among the 3 plant types.

Table 4. Analysis of molecular variance (AMOVA) for tea samples from the six populations of var. *assamica*.

Source of variation	df	MSD	Variance components	*P*-value	Percent of total variance
Among group	2	364.151	16.4919	<0.001	31.40%
Within group	74	36.025	36.025		68.60%

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

Table 5. Nei's Original Measures of Genetic distance.

Code	MS	ML	J	NNS	YK	MD
MS	****	0.0327	0.0221	0.0925	0.1180	0.0435
ML		****	0.0122	0.0520	0.1126	0.0256
J			****	0.0572	0.1089	0.0245
NNS				****	0.1674	0.0686
YK					****	0.0771
MD						****

A dendrogram constructed on the basis of Nei's genetic distances and the UPGMA method showed three main clusters: Group I contained three wild tea plants (ML, J and MS) and one seed produced tea plant (MD); Group II included one wild tea plant (MD); and Group III included one cloned tea plant (YK; Fig. 1). The above data were further analyzed on an individual basis using a principal component analysis (PCA). The first plane of PCA accounted for only 18.6% of total variability and showed a pattern similar to that observed with the cluster analysis (Fig. 2). Taken together, these results provide further support for the existence of wide genetic variation among cloned, seed produced, and wild tea plants.

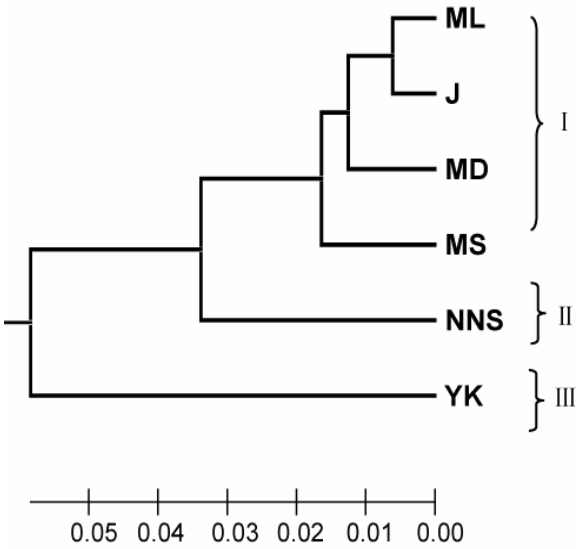

Fig. 1. Dendrogram based on Nei's (1972) genetic distance: method = UPGMA.

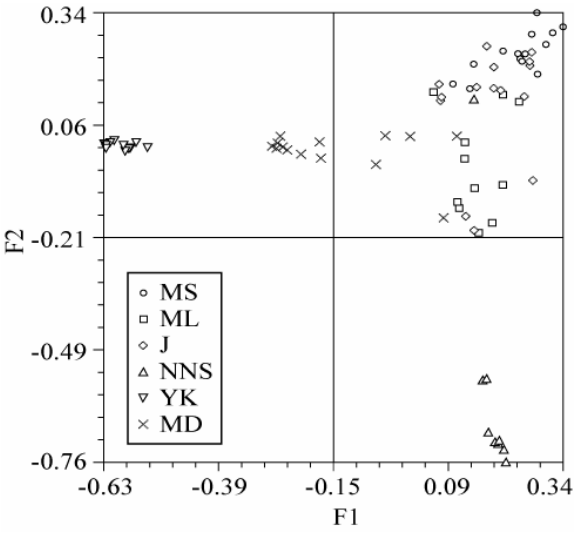

Fig. 2. Two dimensional map of principal components analysis based on AFLP characters. Variance explained: 18.6% (F1) and 14.1% (F2).

It has been reported that extensive planting of a few clones will cause the erosion of genetic diversity, and in other studies a lower level of genetic variability was found in cloned tea (Balasaravanan *et al*., 2003; Mishra & Sen-Mandi, 2001; Yao *et al*., 2007). Consistent with this, in our study a great genetic variation was found within tea plants of var. *assamica* in Yunnan, but the genetic diversity among cloned tea plants was lower than that among the seed produced and wild plants. The cloned tea plant had a low genetic diversity (PPL = 13.77%), while the wild was high (PPL = 85.02%). Consistent with our results, Lai *et al*., (2001) earlier reported that the genetic diversity of native wild tea was higher than that of cultivated cloned tea in Taiwan. In early report, the great variation of morphological, biochemistry characters was found within and among tea plant germplasm of Yunnna, China (Chen *et al*., 2004; Chen *et al*., 2005; Li *et al*., 2010). Compare with new cultivars, the relatively primitive tea tree contain greater amounts of compounds ECG and EC and lower amounts of compounds EGCG and EGC (Li *et al*., 2010). Unlike most of the commercial tea cultivars, the wilds teas are resistant to cold and common diseases affecting the tea species (Kaundun & Park, 2002). Taken together, these studies indicate that wild tea is genetically highly variable and a large proportion of valuable tea germplasm may have been lost during the commercial planting of vegetatively propagated plants (Balasaravanan *et al*., 2003). Detailed understanding of the genetic relationship between tea plants will be of utmost importance for the development of new variants. In this regard, by demonstrating the higher genetic diversity in wild var. *assamica* tea plants our study suggests that future improvements and breeding programs will benefit from the germplasm of wild tea plants from Yunan, China.

Acknowledgements

This work was supported by the Key Nature Science Foundation of Yunnan Province (2006C0012Z).

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(Received for publication 30 April 2011)