Genetic Differentiation of Portuguese Tea Plant using RAPD Markers

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Abstract. Random amplified polymorphic DNA (RAPD) analysis was used to estimate genetic similarities between Portuguese *Camellia sinensis* (L.) O. Kuntze (tea plant) accessions and those obtained from the germplasm collections from the Tea Research Foundation of Kenya and from the National Research Institute of Vegetables, Ornamental Plants, and Tea of Japan. The accessions studied are taxonomically classified as *C. sinensis*, var. *sinensis*, var. *assamica*, or ssp. *lasiocalyx*. A set of 118 ten-base arbitrary primers was tested, of which 25 produced informative, reproducible, and polymorphic banding patterns. These primers were used to amplify DNA from 71 tea plant accessions and produced a total of 282 bands, of which 195 were polymorphic. The phenotypic frequencies were calculated using Shannon's Index and employed in estimating genetic diversity within tea plant populations. Our study demonstrates that tea plant populations, including the Portuguese tea plants, show considerable genetic variability. From the UPGMA cluster analysis based on a matrix using the Jaccard coefficient, it was possible to distinguish the Portuguese tea plants from the remaining accessions. The RAPD markers discriminated the three *C. sinensis* varieties. Moreover, within each variety cluster, subclusters formed according to geographic distribution. The RAPD analysis also separated the commercially cultivated tea plants from the Taiwanese wild tea plants. The present results show that RAPD analysis constitutes a good method to estimate genetic diversity within *C. sinensis*, and to differentiate *C. sinensis* accessions according to taxonomic variety and geographical distribution.

Camellia sinensis (L.) O. Kuntze, the tea plant, is the most economically important species of the genus *Camellia* (Wachira et al., 1995). Tea is the second most consumed beverage in the world after water (Weisburger, 1997), and recent literature strongly emphasizes its value in disease prevention and as a key component of daily diets (Mukhtar and Ahmad, 1999, 2000; Weisburger, 1997).

The tea plant, originally from China, probably reached Europe through Portu-

guese and Dutch sailors in the 16th century. In Portugal, *C. sinensis* has been present since the beginning of the 19th century, both in the Archipelago of Azores (S. Miguel Island) and the mainland, where its culture is documented (Ferrão, 1992). Today it is still possible to find evidence of these plants on the mainland at "The Peak of Tea" (Alto do Chá) in the Natural Park of Sintra (Parque da Pena, Sintra) and in some botanical gardens (Jardim Botânico da Faculdade de Ciências de Lisboa and Jardim de Garcia da Horta, Lisbon). The cultivation and processing of *C. sinensis* for commercial purposes in Portugal takes place only in the Archipelago of Azores (S. Miguel Island) on a small, family-owned property, the Gorreana Tea Plantation (Plantações de Chá Gorreana) (Amaral, 2001).

The taxonomy of *C. sinensis* is based on vegetative characteristics. Although vegetative characters show a high degree of variation and plasticity, leaf aspects, such as shape, length, and margins, continue to be the basis for tea plant classification (Banerjee, 1992). By this method, Kitamura (1950) and Sealy (1958) recognized two botanical varieties of *C. sinensis* (L.), the variety China, *Camellia sinensis* var. *sinensis* (L.), and the variety Assam, *Camellia sinensis* var. *assamica* (Masters) Kitamura. Despite several proposed changes to this classification, it is common to put all the tea plants under the

name *C. sinensis* (L.) O. Kuntze, regardless of taxonomic variation. In addition, varieties China, Assam, and Cambod are often referred to as *C. sinensis*, *C. assamica*, and *C. assamica* ssp. *lasiocalyx*, respectively (Banerjee, 1992; Paul et al., 1997).

Genetic diversity in *C. sinensis* has been estimated using primarily morphological and agronomic characters. Recently, several biochemical and molecular techniques have been employed as an attempt to overcome problems caused by the environmental influences on vegetative characters, which make it difficult to identify distinct taxonomic groups. Magoma et al. (2000) used total green leaf catechins and the ratio of dihydroxylated to trihydroxylated catechins as biochemical markers to determine genetic variation in Kenyan tea plant germplasm. Using RFLP markers, Matsumoto et al. (1994) distinguished Assam hybrids and Japanese green tea plant cultivars with high and low catechin content and grouped Japanese green tea plants at the cultivar level. Paul et al. (1997) employed the AFLP technique to study genetic variation between and within populations of *C. sinensis*. Wachira et al. (1995) used RAPD markers to estimate the level of genetic diversity in cultivated tea and, in a later study (1997), used RAPD markers and PCR amplification of chloroplast and mitochondrial sequences to assess the relationships among cultivated tea and some wild species in the genus *Camellia*.

In the present study, we used random amplified polymorphic DNA (RAPD) analysis to estimate genetic similarities among Portuguese *C. sinensis* var. *sinensis* accessions and those from China, Japan, and Kenya; *C. sinensis* var. *assamica* accessions from India, Burma, Vietnam, Taiwan, and Kenya; *C. assamica* ssp. *lasiocalyx* accessions from Cambodia; and a Kenyan accession of *Camellia irrawadiensis*, a wild species related to the tea plant. Among the Portuguese accessions studied are the commercial cultivars from Azores, and wild tea plants from the mainland, not used for tea production.

Materials and Methods

Plant material. Silica-dried leaves of *C. sinensis* were obtained from germplasm collections of the Tea Research Foundation of Kenya (TRFK) and the National Research Institute of Vegetables, Ornamental Plants, and Tea of Japan (NIVOT). Fresh leaves from the Portuguese accessions were obtained from two botanical gardens (Jardim Botânico da Universidade de Lisboa, and Jardim de Garcia da Horta, Lisbon), from a natural park (Parque da Pena, Sintra), from the Gorreana Tea Plantation (S. Miguel Island, Azores), and also from a private collection (O'Porto). The varieties studied include 37 accessions of Assam teas, 24 Chinese teas, 2 Cambodian teas, 1 Assam hybrid, 6 Portuguese accessions of unknown variety, and 1 accession of *Camellia irrawadiensis* (Table 1).

DNA isolation. DNA was extracted from fresh tea leaves with the DNeasy Plant Kit (Qiagen, Valencia, Calif.) and from silica-

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dried leaf material using the method Doyle and Doyle (1987) modified by Weising (1995). DNA concentration was determined using the Mini-Fluorometer TKO 100 (Hoefer Scientific Instruments, San Francisco) with the calf thymus DNA standard. Samples were diluted to 1 ng·μL⁻¹ with 1/10 TE buffer (Sambrook et al., 1989) and allowed a working life of 6 weeks. New stocks were made up at the end of this period or following exhaustion of stock.

RAPD assay. Operon 10-base oligonucleotide primers kits A, B, X, V, W, and also 10 and 8 primers from kits AC and AK, respectively (Qiagen Operon, Alameda, Calif.), were screened for DNA polymorphism. In total, 118 primers were tested, and the 25 informative polymorphic primers were chosen for analysis (Table 2). PCR conditions, including the concentration of template DNA, primer, dNTPs, magnesium chloride, and *Taq* DNA polymerase were optimized to generate RAPD profiles of high intensity and sharp bands with clear background. The 25-μL reaction mixture contained 2.5 ng of template DNA, 100 μM each of dATP, dCTP, dGTP, and dTTP (Perkin-Elmer, Foster City, Calif.), 5 μM primer, 0.25 U *Taq* DNA polymerase (Qiagen), 2.5 mM MgCl₂ (Qiagen), and 1X *Taq* polymerase buffer (Qiagen). Amplifications were performed in a Thermal Cycler (PTC-100 Programmable Thermal Controller, M.J. Research, Waltham, Mass.) programmed for an initial denaturing period of 94 °C for 3 min and then 45 cycles, each consisting of 92 °C for 1 min, 35 °C for 1.5 min, 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. Samples were stored at 4 °C until analysis. Fragments generated by amplification were separated according to size by electrophoresis on 2.0% (w/v) agarose gels run in 1X TAE buffer for 4 h at 100 V, stained with ethidium bromide, and visualized by illumination with ultraviolet light. A 100-bp DNA Ladder (Invitrogen Life Technologies, Carlsbad, Calif.) was used as the size marker. Negative controls (where the DNA sample was replaced with water) and duplicated DNA samples were included in all amplification reactions. Moreover, DNA samples from different plants of the same accession were amplified in the same PCR reaction and all the amplification reactions were repeated three times to ascertain reproducibility of bands.

Data analysis. Each polymorphic band was labelled based upon the primer code and approximate size. For example, OPV02-1550 represented the 1550-bp band amplified by primer OPV02. Bands were scored as present (1) or absent (0). Ambiguities (reactions that failed) were scored as missing data. The degree of polymorphism was quantified using Shannon's Index of phenotypic diversity, defined as:

$$H = -\sum p_i \log_2 p_i$$

where " p_i " is the frequency of phenotype "i," or the frequency of each band amplified by a given primer (Zar, 1999). Averaging H over all primers allows calculation of diversity within populations (H_0) for each population or

Table 1. *Camellia sinensis* accessions used in RAPD analysis

Genotype number	Accession code	Species and variety	Collection	Source
1	P1	<i>C. sinensis</i> var. <i>assamica</i>	Azores	Portugal
2	P2	<i>C. sinensis</i> (hybrid)	Azores	Portugal
3	P3	<i>C. sinensis</i> var. <i>sinensis</i>	Azores	Portugal
4	P4	<i>C. sinensis</i> (var. unknown)	Sintra	Portugal
5	P5	<i>C. sinensis</i> (var. unknown)	O'Porto	Portugal
6	P6	<i>C. sinensis</i> (var. unknown)	O'Porto	Portugal
7	P7	<i>C. sinensis</i> (var. unknown)	Garcia da Horta	Portugal
8	P8	<i>C. sinensis</i> (var. unknown)	Garcia da Horta	Portugal
9	P9	<i>C. sinensis</i> (var. unknown)	Botanical Garden	Portugal
10	AK450	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
11	AK533	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
12	AK552	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
13	AK329	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
14	AK744	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
15	AK547	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
16	AK251	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
17	AK127	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
18	AK757	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
19	AK293	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
20	AK31	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
21	AK562	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
22	AK433	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
23	Ai2	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
24	Ai66	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
25	Ai104	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
26	Ai142	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
27	Stock 173	<i>C. sinensis</i> var. <i>assamica</i>	India	NIVOT
28	BUM3	<i>C. sinensis</i> var. <i>assamica</i>	Burma	NIVOT
29	BUM5	<i>C. sinensis</i> var. <i>assamica</i>	Burma	NIVOT
30	BUM6	<i>C. sinensis</i> var. <i>assamica</i>	Burma	NIVOT
31	Shan1-3	<i>C. sinensis</i> var. <i>assamica</i>	Vietnam	NIVOT
32	Shan1-5	<i>C. sinensis</i> var. <i>assamica</i>	Vietnam	NIVOT
33	Shan21	<i>C. sinensis</i> var. <i>assamica</i>	Vietnam	NIVOT
34	T-Yamacha75	<i>C. sinensis</i> var. <i>assamica</i>	Taiwan	NIVOT
35	T-Yamacha80	<i>C. sinensis</i> var. <i>assamica</i>	Taiwan	NIVOT
36	T-Yamacha84	<i>C. sinensis</i> var. <i>assamica</i>	Taiwan	NIVOT
37	T-Yamacha88	<i>C. sinensis</i> var. <i>assamica</i>	Taiwan	NIVOT
38	CK9	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
39	CK10	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
40	CK11	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
41	CK12	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
42	CK13	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
43	CK15	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
44	CK16	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
45	CK17	<i>C. sinensis</i> var. <i>sinensis</i>	China	NIVOT
46	Yabukita	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
47	Tamamidori	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
48	Sayamamidori	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
49	Miyoshi	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
50	Asatsuyu	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
51	Yamatomidori	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
52	Benibanacha	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
53	ZAI 80-10	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
54	ZAI 80-19	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
55	ZAI 80-20	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
56	ZAI 80-25	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
57	ZAI 80-37	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
58	ZAI 81-13	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
59	ZAI 81-27	<i>C. sinensis</i> var. <i>sinensis</i>	Japan	NIVOT
60	317/28	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
61	330/83	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
62	C12	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
63	D99/10	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
64	SFS 150	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
65	S 15/10	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
66	TN 14-3	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
67	6/8	<i>C. sinensis</i> var. <i>assamica</i>	Kenya	TRFK
68	K-Purple	<i>C. sinensis</i> var. <i>sinensis</i>	Kenya	TRFK
69	301/6	<i>C. sinensis</i> ssp. <i>lasiocalyx</i>	Cambodia	TRFK
70	301/4	<i>C. sinensis</i> ssp. <i>lasiocalyx</i>	Cambodia	TRFK
71	91/1	<i>C. irrawadiensis</i>	Kenya	TRFK

Table 2. Primers used for the detection of polymorphism in *Camellia sinensis* and the number of amplification products obtained.

Primer	Sequence (3'-5')	Total no. of reproducible bands	Total no. of polymorphic bands
OPAC-03	CACTGGCCCA	7	5
OPAC-11	CCTGGGTGAC	13	10
OPW-01	CTCAGTGTCC	8	6
OPW-02	ACCCCGCCAA	13	9
OPW-05	GGCGGATAAG	12	4
OPW-10	TCGCATCCCT	12	8
OPW-11	CTGATGCGTG	14	10
OPW-16	CAGCCTACCA	9	6
OPW-17	GTCCTGGGTT	13	10
OPW-19	CAAAGCGCTC	14	12
OPAK-17	CAGCGGTCAC	13	10
OPAK-18	ACCCGGAAC	7	6
OPA-05	AGGGGTCTTG	4	4
OPA-07	GAAACGGGTG	14	10
OPA-08	GTGACGTAGG	12	7
OPA-10	GTGATCGCAG	13	10
OPA-11	CAATCGCCGT	13	7
OPA-18	AGGTGACCGT	12	10
OPB-07	GGTGACGCAG	10	8
OPB-08	GTCCACACGG	9	6
OPB-12	CCTTGACGCA	12	8
OPV-02	AGTCACTCCC	14	8
OPV-10	GGACCTGCTG	11	7
OPV-12	ACCCCCCACT	12	7
OPV-14	AGATCCCGCC	11	7
Total	---	282	195

Fig. 1. Agarose-gel revealing bands amplified with primer OPB-08. L: 100-bp DNA ladder; arrows: two polymorphic bands for Portuguese accessions; lanes 1–12: genotype numbers according to Table 1. The repeated lane numbers (1–4) indicate duplicated DNA samples. The reaction in lane 2 failed in this set of assays.

group. The average diversity for all populations (H_{pop}) is calculated as the mean of individual H_0 values. The total diversity (H_{tot}) over “n” populations is calculated from phenotypic frequencies in all populations considered together (all the 71 accessions analyzed). The proportion of diversity within a population, obtained by dividing H_{pop}/H_{tot} , was compared to diversity between populations ($1 - H_{pop}/H_{tot}$) (Kaundun et al., 2000). Using NTSYS-pc (version 2.1, Exeter Software, Setauket, N.Y.) software, the Jaccard and Dice similarity coefficients were used to estimate the similarity among all 71 genotypes with 195 characters. The phenograms were constructed with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering procedure. The cophenetic correlation values were determined to test the goodness of fit of the data clustering. Data ordination was performed by Principal Coordinate Analysis (PCOORDA), and a Minimum Spanning Tree (MST) was computed and superimposed on PCOORDA.

Results

Due to the reported sensitivity of the RAPD methodology to experimental conditions (Edwards, 1998; Sala et al., 1998), PCR amplifications were carried out under optimal standardized conditions and only highly reproducible bands were scored. From a total of 118 primers used to amplify tea plant DNA, 25 (21%) produced polymorphic and reproducible bands. A total of 282 bands were produced, of which 195 (69%) were scored for the analysis because they were reproducibly polymorphic. The number of polymorphic bands detected for each primer varied from 4 (primers OPW-05 and OPA-05) to 12 (primer OPW-19) with an average of 7.8 (Table 2). The amplification products obtained for primer OPB-08 are shown in Fig. 1.

The phenotypic frequencies detected with the 25 primers were calculated and used in estimating genetic diversity (H) within each tea plant group (Table 3). The Portuguese tea plants exhibited high variability (2.35), equal to that of the Indian group. The low variability

Table 3. Estimates of genetic diversity (H) within *C. sinensis* groups^z, averaged over all 195 polymorphic bands.

Group	All pop. (a)	Portugal (a)	India (a)	Burma (a)	Vietnam (a)	Taiwan (a)	China (a)	Japan (a)	Kenya (a)	Cambodia (a, b)	var. <i>assamica</i> (b)	var. <i>sinensis</i> (b)
No. of accessions per group	71	9	18	3	3	4	8	14	10	2	37	24
H range per primer	1.67 to 3.46	0.85 to 3.22	1.38 to 3.09	0.81 to 2.87	0.00 to 2.86	0.00 to 2.60	0.92 to 3.10	0.00 to 3.12	1.00 to 2.89	0.00 to 2.32	1.41 to 3.21	2.18 to 3.29
H average	2.7	2.35	2.35	1.7	1.51	1.52	1.83	1.87	2.06	1.30	2.56	2.18

^zGroups were constructed based on the geographical origin (a) and on the taxonomic variety (b). All pop. = all 71 tea plant accessions considered together.

Table 4. Partitioning of the overall genetic diversity (H) within and between tea plant groups.^z

	H_{tot}	H_{pop}	H_{pop}/H_{tot}	$(H_{tot} - H_{pop})/H_{tot}$
Range per primer	1.67 to 3.46	1.16 to 2.45	0.58 to 0.89	0.11 to 0.42
Average	2.70	1.93	0.72	0.28

^z H_{tot} = total diversity (all 71 accessions considered together); H_{pop} = average diversity for all populations (i.e., for all the *C. sinensis* groups studied); H_{pop}/H_{tot} = proportion of diversity within populations; $(H_{tot} - H_{pop})/H_{tot}$ = diversity between populations.

Fig. 2. Phenogram derived from 71 *Camellia sinensis* accessions and 195 RAPD markers using the Jaccard similarity coefficient and UPGMA cluster analysis. Asterisks show accessions where morphological features and RAPD markers give incongruent classifications (see text for details). 1 to 71: genotype number according to Table 1.

Fig. 3. Minimal-spanning tree (MST) superimposed on the principal coordinate analysis (PCOORDA) of *Camellia sinensis* based on RAPD markers. 1 to 71: genotype number according to Table 1. (▽) Taiwanese accessions; (▼) Portuguese accessions; (◆) Cambodian accessions; (△) Kenyan accessions; (▲) Vietnamese accessions; (✦) Burmese accessions; (✧) Indian accessions; (○) Chinese accessions; (●) Japanese accessions.

found for the accessions from Taiwan, Vietnam, and Burma could be due to the small size of those populations, which affects H values. When the genotypes were grouped according to the variety, the diversity indices ranged from 1.30 (*C. assamica* ssp. *lasiocalyx*) to 2.56 (*C. sinensis* var. *assamica*). Shannon's Index, based on phenotypic frequency, was then used to partition diversity within and between these tea plant groups (Table 4); 72% of variability was found within groups, and only 28% between groups. The distribution of variability between populations varied from 11% to 42% with different primers.

Measures of similarity based on both the Jaccard and Dice coefficients and UPGMA cluster analysis were used to construct phenograms. The cophenetic correlation values obtained for each phenogram were 0.87 and 0.84, respectively. The cophenetic correlation value can be used as a measure of goodness of fit for a cluster analysis, and the values obtained for both clusters can be interpreted as a good fit ($r = 0.99$ between both phenograms). Because it has a higher correlation coefficient, the phenogram obtained with the Jaccard similarity coefficient (Fig. 2) was used for the analysis.

Accession number 25 (Ai104, a variety from India) with one asterisk in Fig. 2, was distinct from the other individuals sampled in its geographical region. The Taiwanese tea accessions were grouped together by coefficients of similarity ranging from 0.54 to 0.77, but separated from the other accessions by a coefficient of similarity of 0.25. Two other major clusters emerged, consisting of the Portuguese tea plant accessions, with similarities ranging from 0.31 to 0.64, and a similar larger group, divided into three branches, consisting of the Indian, Chinese, and Cambodian varieties (Fig. 2). Moreover, each variety subgroup clustered according to geographical origin. The *C. sinensis* var. *assamica* accessions formed clusters from India, Burma, Vietnam, and Kenya, and the *C. sinensis* var. *sinensis* formed clusters from China and Japan.

Principal coordinate analysis (PCOORDA) was carried out to further discriminate the tea plant accessions. A minimum-spanning tree (MST) was computed and superimposed on the PCOORDA to help interpret the cluster analysis. As referred previously, the accession Ai104 (genotype number 25), *C. sinensis* var. *assamica* tea plant from India, was clustered

separately from the remaining var. *assamica* accessions by the UPGMA analysis; with the ordination analysis, it was possible to ascertain its position in that group (Fig. 3). From this analysis it is also evident that the Indian, Chinese, and Cambodian varieties are clearly differentiated. Moreover, it was possible to cluster based on geographical distribution. The accessions from Japan and China formed two distinct subgroups within the var. *sinensis*. The same differentiation occurred within the var. *assamica* group, where the Kenyan accessions were clustered separately from the Indian accessions. The separation of the Vietnamese and Burmese accessions is apparent, in spite of the low number of accessions sampled. The Portuguese accessions were also unequivocally distinguished as a distant, separate group, as well as the wild tea plant accessions from Taiwan.

Discussion

The present study is the first report on the genetic relationships of Portuguese *Camellia sinensis* accessions. This work not only shows that RAPD analysis constitutes a good method

to estimate genetic diversity in this economically important species, but also that it can offer valuable information about the potential taxonomic and geographic origin of unstudied accessions. DNA from different tea plant populations, amplified with arbitrary primers, exhibited a high degree of polymorphism. Our results demonstrate that the nine Portuguese tea accessions analyzed showed considerable diversity (2.35) despite the low number of accessions sampled. This high variability may reflect a relatively large genetic diversity in the *C. sinensis* plantations where these accessions originated. From all the accessions studied, the Vietnamese and Taiwanese tea plants exhibited the lowest diversity; one possible explanation is that the number of accessions studied was low and probably not representative of the available diversity of *C. sinensis*. The low diversity might also be the result of intensive selection and crossing from a genetically limited stock.

When the overall genetic diversity was partitioned, on average 72% was distributed within and 28% between tea plant groups. These values are in agreement with results previously described (Kaundun et al., 2000; Wachira et al., 1995). *Camellia sinensis* is an outcrossing, highly heterogeneous plant, and consequently most of the variation exhibited is within populations (Paul et al., 1997).

The placement of tea plant varieties in the phenogram is consistent with the present taxonomy of *C. sinensis*. However, the accession Yamatomidori (genotype number 51 from Japan, highlighted by two asterisks in Fig. 2), did not conform to its assumed taxonomic classification. *Camellia sinensis* classification has been based mostly on highly variable morphological characters. It is possible that this accession is a hybrid with morphological characters that more closely resemble *C. sinensis* var. *sinensis*, but which, genetically, is more related to *C. sinensis* var. *assamica* tea plants. Previous reports have also succeeded in genetically differentiating *C. sinensis* accessions at the variety level. Matsumoto et al. (1994) reported that phenylalanine ammonia-lyase (PAL) RFLPs allowed the differentiation of Assam hybrids and Japanese tea plants with high and low catechin content and succeeded in grouping Japanese accessions at the cultivar level. Wachira et al. (1995) and Paul et al. (1997), using RAPD and AFLP analysis, respectively, were able to distinguish commercial tea plant clones. Our results confirm that RAPD analysis is a valuable tool to study genetic diversity among *C. sinensis* varieties, enabling us to determine the genetic origin of the Portuguese (unknown) accessions.

Our study also showed that RAPD markers enabled discrimination among tea plant varieties from different geographical origins. It was possible to separate *C. sinensis* var. *assamica* tea plants originated from India from those from Vietnam, Burma, and Kenya, and to separate the *C. sinensis* var. *sinensis* accessions from China from those from Japan. The Portuguese accessions, taxonomically falling within the var. *assamica*, were also differentiated from the remaining Assam tea plants. Furthermore,

the Portuguese tea plants were clustered further distant from the Taiwanese plants than from the Indian, Japanese, and Chinese plants. These findings are in agreement with historical data showing that the Portuguese tea plants (both from the Gorreana Plantation in Azores and from the botanical gardens in the mainland) had originated from tea seeds brought mainly from China, but also from Japan and India. This geographical differentiation may be a result of interactions of climatic and/or edaphic factors and, presumably, also due to selections made on the tea plant populations. The formation of subgroups within the China-type group, based on the geographic distribution, was reported earlier using AFLP markers, but the Assam clones, both from India and Kenya, still clustered closely (Paul et al., 1997). Semagn et al. (2000), using RAPD analysis, were also able to differentiate lowland and central populations of *Phytolacca dodecandra* (Endod) from those of highlands regions. Those authors explained the variation in RAPD markers mostly by the combination of the different eco-geographical variables. In our study, the observed genetic differentiation among tea-cultivating regions suggests that accessions could possibly be assigned to their regions of origin according to their genotypes. Thus, RAPD markers may be useful in determining the geographic origin of accessions of unknown background.

Interestingly, the accessions from Taiwan formed a group distinct from the remaining commercially cultivated tea plants of the var. *assamica*. Those wild tea accessions are distributed in the mountainous areas in central Taiwan and are not commercially cultivated. They are generally classified in the group of *C. sinensis* var. *assamica* because the mature leaf is large (>10 cm), they lack cold hardiness, and levels of chemical components, such as caffeine and catechins, are high. However, Taiwan wild tea plants are also phenotypically similar to var. *sinensis* in many traits, including a lack of leaf hairs on young leaves, a glabrous ovary, and coffee-fragrance leaves (Y. Takeda, NIVOT, Japan, personal communication). Thus, it is not unexpected that accessions from Taiwan were clustered separately from the remaining *C. sinensis* var. *assamica* accessions.

Camellia irrawadiensis, a wild relative of tea characterized by the presence of brick-red young shoots and numerous pellucid punctation on the leaves (Banerjee, 1992), was included in this study primarily for its suspected contribution to the genetic pool of *C. sinensis*. RAPD markers cluster this wild accession (accession 91/1) next to the tea plants originated from Kenya, with 38% similarity. Wachira et al. (1997) found that *C. irrawadiensis* showed 76% similarity with *C. sinensis* var. *sinensis* accession K-Purple, a commercial tea accession from Kenya that also contains characteristic red anthocyanin pigments. Those authors postulated that the cultivar K-Purple was probably a natural hybrid between *C. sinensis* var. *sinensis* and *C. irrawadiensis*. Our RAPD analysis supported this relationship, although the similarity between the previous accessions was lower (41%).

Furthermore, as stated before, the accession K-Purple clustered together with Assam tea plants from Kenya. Nevertheless, hybridization between different *C. sinensis* taxa is a very common phenomenon, which sometimes makes it difficult to assign a clone to a particular varietal taxon (Visser et al., 1969).

The PCORDA supported the results obtained from the UPGMA analysis and was useful in locating artifacts, like the case of the accession Ai104. In the phenogram, this accession was clustered separately from the var. *assamica* group but was correctly grouped by the ordination analysis. Nevertheless, the PCORDA analysis of the accession Yamatomidori, where morphological features and RAPD profiles gave distinct classifications, conforms to UPGMA analysis. From the ordination analysis it was still possible to distinguish *C. sinensis* at the variety level and differentiate varieties according to their geographical origin.

In conclusion, RAPD markers are phenetically informative and represent a suitable method to estimate genetic variability in *Camellia sinensis*. RAPD data provides a high degree of resolution at the variety level and strongly suggests that genetic variability corresponds to taxonomic variety as well as geographical distribution.

Literature Cited

- Amaral, J.D. 2001. O livro do chá. Temas e Debates Lda, Lisbon.
- Banerjee, B. 1992. Botanical classification of tea, p. 26–48. In: K.C. Wilson and M.N. Clifford (eds.). Tea—Cultivation to consumption. Chapman & Hall, London.
- Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bul. 19:11–15.
- Edwards, K.J. 1998. Randomly amplified polymorphic DNAs (RAPDs), p. 171–175. In: A. Karp, P.G. Isaac, and D.S. Ingram (eds.). Molecular tools for screening biodiversity. Chapman & Hall, London.
- Ferrão, J.M. 1992. A Aventura das Plantas e os Descobrimientos Portugueses. Inapa, Lisbon.
- Kaundun S.S., A. Zhyvoloup, and Y-G. Park. 2000. Evaluation of the genetic diversity among elite tea (*Camellia sinensis* var. *sinensis*) accessions using RAPD markers. Euphytica 115:7–16.
- Kitamura, S. 1950. On tea and camellias. Acta Phytax. and Geobot. 14:56–63.
- Magoma, G.N., F.N. Wachira, M. Obanda, M. Imbunga, and S.G. Agong. 2000. The use of catechins as biochemical markers in diversity studies of tea (*Camellia sinensis*). Genet. Resour. Crop Evolution 47:104–114.
- Matsumoto, S., A. Takeuchi, M. Hayatsu, and S. Kondo. 1994. Molecular cloning of phenylalanine ammonia-lyase cDNA and classification of varieties and cultivars of tea plants (*Camellia sinensis*) using the tea PAL cDNA probe. Theor. Appl. Genet. 89:671–675.
- Mukhtar, H. and N. Ahmad. 1999. Green tea in chemoprevention of cancer. Toxicol. Sci. 52: 111–117.
- Mukhtar, H. and N. Ahmad. 2000. Tea polyphenols—Prevention of cancer and optimizing health. Amer. J. Clin. Nutr. 71:1698S–1702S.
- Paul, S., F.N. Wachira, W. Powell, and R. Waugh. 1997. Diversity and genetic differentiation among populations of Indian and Kenyan tea [*Camellia sinensis* (L.) O. Kuntze] revealed

- by AFLP markers. *Theor. Appl. Genet.* 94: 253–263.
- Sala, F., G. Wang, S. Castiglione, and S. Bisoffi. 1998. RAPD fingerprinting as a tool for taxonomic studies in the genus *Populus*, p. 447–455. In: A. Karp, P.G. Isaac, and D.S. Ingram (eds.). *Molecular tools for screening biodiversity*. Chapman & Hall, London.
- Sambrook, E.F., F. Fritsh, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor, New York.
- Sealy, J.R. 1958. A revision of the genus *Camellia*. Royal Hort. Soc., London.
- Semagn, K., A. Bjornstad, B. Stedje, and E. Bekele. 2000. Comparison of multivariate methods for the analysis of genetic resources and adaptation in *Phytolacca dodecandra* using RAPD. *Theor. Appl. Genet.* 101:1145–1154.
- Visser, T. 1969. Tea, *Camellia sinensis* (L.) O. Kuntze, p. 459–493. In: F.P. Ferwarda and F. Wit (eds.). *Outlines of perennial crop breeding in tropics*. H Veenman & NV Zonen, Wageningen, The Netherlands.
- Wachira, F.N., W. Powell, and R. Waugh. 1997. An assessment of genetic diversity among *Camellia sinensis* L. (cultivated tea) and its wild relatives based on randomly amplified polymorphic DNA and organelle-specific STS. *Heredity* 78:603–611.
- Wachira, F.N., R. Waugh, C.A. Haxkett, and W. Powell. 1995. Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. *Genome* 38:201–210.
- Weisburger, J.H. 1997. Tea and health: A historical perspective. *Cancer Lett.* 114:315–317.
- Weising, H. 1995. *DNA fingerprinting in plant and fungi*. CRC Press, London.
- Zar, J.H. 1999. *Biostatistical analysis*, 4th ed. Prentice Hall, New Jersey.