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Phylogenetic Relationships among Tea Cultivars Based on AFLP Analysis

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Several modifications of the AFLP protocol were made to simplify the analysis, i.e., the amount of the reagents for restriction digestion, ligation of adaptors, preamplification and selective amplification, the primers without labeling, and the gel staining with ethidium bromide. The phylogenetic relationships among 37 accessions of the genus *Camellia* were assessed using the modified AFLP technique and cluster analysis. The accessions, 24 Japanese tea cultivars, seven Korean wild tea varieties and six *Camellia* species closely related to tea, were clustered into five groups; Japanese tea cultivars closely related to 'Yabukita', those not closely related to 'Yabukita', Chinese tea cultivars and Korean wild tea varieties, Assam type black tea cultivars, and *Camellia* species closely related to tea. The results of this study indicated that the morphological characteristics and the origins of the tea cultivars were well reflected in the clustering of the cultivars based on the AFLP analysis, and that the AFLP technique might find its use as an efficient and effective tool for determining phylogenetic relationships among tea cultivars.

INTRODUCTION

Most plants of the genus *Camellia* are indigenous to Southeast Asia. Sealy (1958) classified the genus into 82 species and Chang and Bartholomew (1984) reported 200 species of the genus. Number of species in the genus *Camellia* is generally thought to be 135 to 300 (Linda and Parks, 2000). In addition to morphological studies, various approaches have been tried to elucidate the relationships of the species in the genus, including chromosome analysis (Kondo, 1975; Uemoto *et al.*, 1980), isozyme analysis (Wendel and Parks, 1983), pigment analysis (Sakata *et al.*, 1981), RAPD analysis (Lee *et al.*, 1995), and chloroplast DNA analysis (Lee and Nou, 1999; Prince and Parks, 2001).

Tea (*Camellia sinensis*) is commercially the most important and widely cultivated plant in the genus *Camellia*. Tea is divided into two large groups, Assam tea and China tea. Assam tea plants are not winter-hardy and have large leaves with high catechin concentration. China tea plants have small leaves with low catechin concentration and are

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relatively resistant to cold weather. Assam tea leaves are used to make fermented black tea, whereas China tea leaves are used to make semi- and non-fermented teas (Kim, 1996). It is thought that the tea cultivars of today have both the genes of Assam tea and China tea origin due to introgressions during their breeding process.

RAPD (random amplified polymorphic DNA) has been used to elucidate genetic relationships among various organisms. It has, however, its own limit due to the lack of reproducibility. Recently, AFLP (amplified fragment length polymorphism) has been successfully used to analyze genetic relationships even in species where polymorphism is low (Kang *et al.*, 1996; Mace *et al.*, 1999; Prakash *et al.*, 2002; Tsuji and Ohnishi, 2001). A genetic linkage map for Assam tea cultivars has been constructed using AFLP technique (Heckett *et al.*, 2000). The present study has been conducted to evaluate the usefulness of AFLP in analyzing the relationships among *C. sinensis* and to determine the genetic relationships among Japanese tea cultivars and Korean wild tea varieties.

MATERIALS AND METHODS

Plant material

A total of 37 accessions of the genus *Camellia* was used in this study. The origins and sources of the accessions were listed in Table 1. Twenty-four tea (*C. sinensis*) cultivars were obtained from the Tea Branch, Miyazaki Prefectural Agricultural Experiment Station, Miyazaki, Japan and seven Korean wild tea varieties were from Posung Tea Experiment Station, Korea. Six *Camellia* species closely related to *C. sinensis* were obtained from the Institute of Vegetable and Tea Science, Kagoshima, Japan.

DNA isolation and AFLP analysis

DNA was extracted from 0.1 g fresh leaf samples of all accessions using the Nucleon PhytoPure DNA extraction kit (Amersham Life Science).

The AFLP protocol described by Vos *et al.* (1995) was employed with some modifications using the AFLP core reagent kit and the AFLP starter primer kit of GibcoBRL (Life Technologies, U.S.A.). For each sample, 125 ng of genomic DNA was digested at 37 °C for 2 h using restriction enzymes *Eco*RI and *Mse*I. Restricted DNA fragments were ligated with *Eco*RI and *Mse*I adapters using T4 DNA ligase. In pre-selective amplification, ligation mixture was amplified using primers complementary to the adapters with one additional selective 3' nucleotide. The pre-selective PCR amplification consisted of 20 cycles of denaturation at 90 °C for 30 sec, annealing at 56 °C for 60 sec and extension at 72 °C for 60 sec. Selective amplification of the pre-amplified products was carried out using 10 primer sets with three selective nucleotides for the *Eco*RI digested side and three selective nucleotides for the *Mse*I digested side. The selective amplification was carried out in a Perkin Elmer 9600 thermal cycler. The amplification was initiated by a cycle of denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec and extension at 72 °C for 60 sec. The initial cycle was followed by the same cycle for 12 times with lowered annealing temperature (0.7 °C per cycle, final temperature of 56 °C). This was followed by 23 cycles of 95 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 60 sec. Amplified DNA fragments were separated by polyacrylamide gel electrophoresis. The gel was made on a 16 cm × 16 cm glass plate, and consisted of 13% running gel (in 0.378 M Tris-HCl, pH

Table 1. Accessions used for AFLP analysis

No.	Accessions	Species	Origin and source
1	Yabukita	*C. sinensis* var. *sinensis*	Selection from seedlings, Shizuoka
2	Okumidori	*C. sinensis* var. *sinensis*	Yabukita×NN29
3	Meiryoku	*C. sinensis* var. *sinensis*	Yabukita ×Z–1
4	Saemidori	*C. sinensis* var. *sinensis*	Yabukita×Asatuyu
5	Kanayamidori	*C. sinensis* var. *sinensis*	S6×Yabukita
6	Okuyutaka	*C. sinensis* var. *sinensis*	Yutakamidori×F_1NN8 (Tamamidori ×S6)
7	Fushun	*C. sinensis* var. *sinensis*	Z–1×Kanayamidori
8	Yutakamidori	*C. sinensis* var. *sinensis*	Selection from seedlings of Asatuyu
9	Sakimidori	*C. sinensis* var. *sinensis*	NN27× ME 52
10	Unkai	*C. sinensis* var. *sinensis*	Takachiho x F_1
11	Yamanami	*C. sinensis* var. *sinensis*	Selection from Chinese seedlings
12	Fukumidori	*C. sinensis* var. *sinensis*	Yabukita× F_1 (Sayamamidori ×Yabukita)
13	Yaeho	*C. sinensis* var. *sinensis*	Selection from seedlings Yaeho, Shizuoka
14	Takachio	*C. sinensis* var. *sinensis*	Selection from seedlings, Miyazaki
15	Makinoharawase	*C. sinensis* var. *sinensis*	Selection from seedlings, Shizuoka
16	Sayamamidori	*C. sinensis* var. *sinensis*	Selection from seedlings, Uji
17	Asatuyu	*C. sinensis* var. *sinensis*	Selection from seedlings, Uji
18	Benihomare	*C. sinensis* var. *assamica*	Black tea, Selection from India seedlings
19	Hatumomiji	*C. sinensis* var. *assamica*	Black tea, Ai2×NKa05
20	Benitachiwase	*C. sinensis* var. *assamica*	Black tea, Ai2× NKa01
21	Sunmei	*C. sinensis* var. *sinensis*	Yutakamidori ×NN8
22	Uron–1	*C. sinensis* var. *sinensis*	Uron Chinese var. from Taiwan
23	Uron–2	*C. sinensis* var. *sinensis*	Uron Chinese var. from China
24	Sri Lanka	*C. sinensis* var. *assamica*	Black tea var. from Sri Lanka
25	K–1	*C. sinensis* var. *sinensis*	Collection from Hyangrimsa, Korea
26	K–2	*C. sinensis* var. *sinensis*	Collection from Seonamsa, Korea
27	K–3	*C. sinensis* var. *sinensis*	Collection from Daeheungsa, Korea
28	K–4	*C. sinensis* var. *sinensis*	Collection from Hwaumsa, Korea
29	K–5	*C. sinensis* var. *sinensis*	Collection from Sanggyesa, Korea
30	K–6	*C. sinensis* var. *sinensis*	Collection from Boriam, Korea
31	K–7	*C. sinensis* var. *sinensis*	Collection from Keumsansa, Korea
32	Rosaeflora	*C. rosaeflora*	Collection by IVT, Kagoshima, Japan
33	Oleifera	*C. oleifera*	Collection by IVT, Kagoshima, Japan
34	Miyagii	*C. miyagii*	Collection by IVT, Kagoshima, Japan
35	Japonica	*C. japonica*	Collection by IVT, Kagoshima, Japan
36	Sasanqua	*C. sasanqua*	Collection by IVT, Kagoshima, Japan
37	Hybrid–1	*C. sinensis*×*C. japonica*	Crossing line by IVT, Kagoshima, Japan

1–24: from Tea Branch, Miyazaki Prefectural Agricultural Experiment Station, Miyazaki, Japan.
25–31: from Posung Tea Experiment Station, Korea.
32–37: from Institute of Vegetable and Tea Science, Kagoshima, Japan.

8.8, 11 cm in length) and 5% stacking gel (in 0.133 M Tris–HCl, pH 6.8, 1.5 cm in length). Electrophoresis was performed for 3 h at 150 W constant power with Tris–Glycine buffer (25 mM Tris and 192 mM Glycine, pH 8.3). Gels were stained with ethidium bromide for 10 min, and photographed under UV.

Data analysis

For each accession, a binary matrix reflecting specific AFLP–band presence (1) or

absence (0) was generated. Only clear bands were scored from the gel photograph magnified using Photoshop program (version 6.0). Cluster analysis and dendrogram generation was performed by the unweighted pair group method using arithmetic mean (UPGMA) clustering method with the NTSYS package (Tateno *et al.*, 1982).

RESULTS AND DISCUSSION

Modification of AFLP analysis methodology

Several modifications of the methodology were made in this study to simplify the analysis. The modifications did not cause any significant change in the results (Figs. 1–3). First, the amount of the reagents for restriction digestion, ligation of adapters, preamplification and selective AFLP amplification were reduced to half of the amount recom-

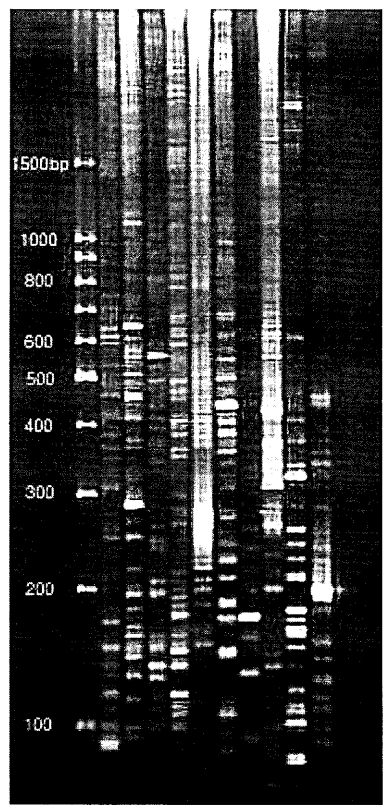

Fig. 1. AFLP patterns of tea (Yabukita) detected with ten primer combinations. Primer combinations were from left to right: E+AAC/M+CTT, E+AAG/M+CAG, E+ACT/M+CAG, E+AGC/M+CTG, E+ACA/M+CAC, E+AGG/M+CAC, E+AGG/M+CTT, E+AGC/M+CTT, E+ACG/M+CTA, E+ACT/M+CAT (E and M indicate *Eco*RI and *Mse*I, respectively). The numbers on the left side indicate the sizes of the DNA fragments in base pairs.

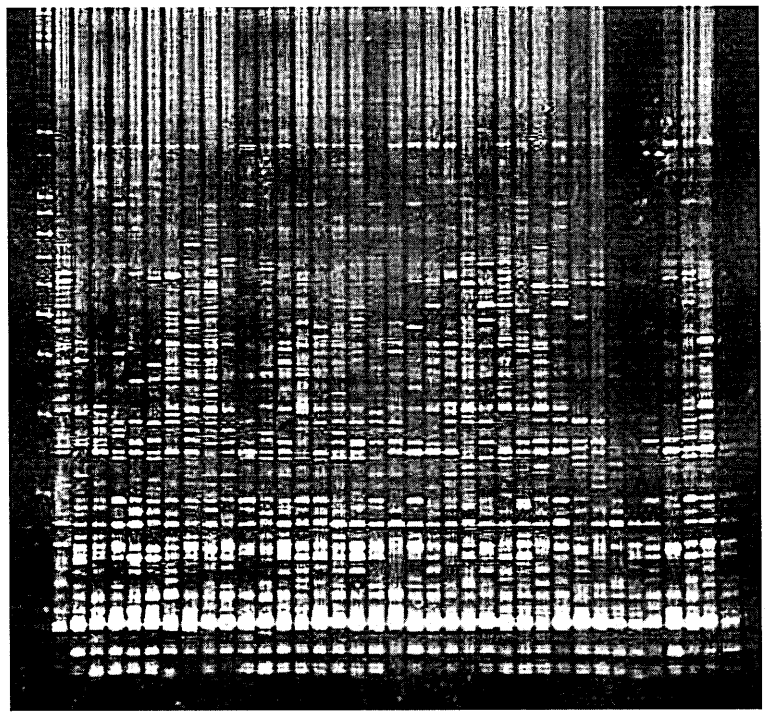

Fig. 2. AFLP patterns of the 37 accessions detected with the E+AAC/M+CTT primer combination. The accessions were 1 to 37 (from left to right) as appeared in Table 1. The left-most lane was DNA size markers.

mended by the supplier (GibcoBRL, Life Technologies, U.S.A.). For example, 125 ng DNA, and 1.25 U each of *Eco*RI and *Mse*I were used for restriction digestion (250 ng DNA, and 2.5 U each of *Eco*RI and *Mse*I were recommended by the supplier). Second, the procedure could be simplified by using unlabeled primer and by staining the gel with ethidium bromide. The supplier recommends labeling the primer with P^{32} prior to selective amplification and autoradiographing the gel. Alternatively, some researchers silver-stained the gel without labeling the primer. Third, the gel length was reduced to 11 cm (made on a 16 cm×16 cm glass plate) and the gel concentration was increased to 13% polyacrylamide. By this, the electrophoresis could be completed in a shorter time and the gel could be handled more easily and safely. The difficulty in scoring due to the shortened distances between the bands could be circumvented by magnifying the image of the photographed gel using a image processing program. In this study, Photoshop version 6.0 was used for the purpose.

AFLP pattern and extent of polymorphism

Sixty-four AFLP primer combinations were tested for their applicability in tea analy-

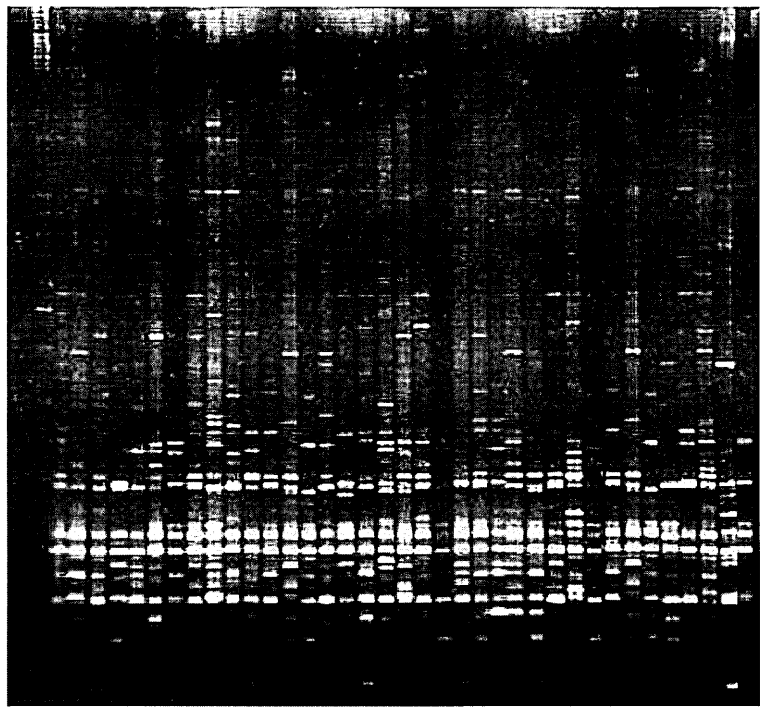

Fig. 3. AFLP patterns of the 37 accessions detected with the E+ACT/M+CAG primer combination. The accessions were 1 to 37 (from left to right) as appeared in Table 1. The left-most lane was DNA size markers.

sis using 'Yabukita' as a test material, and 10 primer combinations were selected for further study based on the number and clarity of the bands appeared on the gel (Table 2).

With the 10 primer combinations and the 37 accessions, a total of 386 bands were detected on the gel, and the number of bands per primer combination ranged from 28 with E+ACT/M+CAG to 49 with E+AAC/M+CTT with an average of 38.6 bands per primer combination (Table 3). Most of the amplified fragments were 50–600 base pairs in size (Figs. 1–3). Of the 386 bands, 209 (54.1%) were found to be polymorphic, and the number of polymorphic bands per primer combination ranged from 14 with E+AGC/M+CTT to 34 with E+AAG/M+CAG with an average of 20.9 bands per primer combination. Compared to the result of RAPD analysis of tea accessions (Lee *et al.*, 1996), the AFLP analysis resulted in more bands per primer and a higher percentage of polymorphic bands. In the RAPD analysis, an average of 10.6 bands per primer set was detected and the ratio of polymorphic bands was 18.9%. These results suggested that AFLP might be a useful tool for the identification and classification of *Camellia* species and tea cultivars. The ratio of polymorphic AFLP bands obtained with the tea accessions was found to be higher than those obtained with other plants. The ratios of polymorphic bands were 36%

Table 2. Applicability of primer combinations for AFLP analysis of tea cultivars

*Eco*RI primer	*Mse*I primer							
	M+CAA	M+CAC	M+CAG	M+CAT	M+CTA	M+CTC	M+CTG	M+CTT
E+AAC	–	+	+	+	+	+	–	+
E+AAG	+	+	+	++	++	+	–	–
E+ACA	+	–	+	+	–	+	–	+
E+ACC	–	–	+	–	+	+	–	++
E+ACG	++	+	+	+	–	+	–	++
E+ACT	++	+	+	+	+	–	–	+
E+AGC	–	+	++	–	++	+	–	+
E+AGG	–	–	++	+	–	+	–	++

[1] Applicability was tested using Yabukita as a test material, and judged based on the number and clarity of bands. –, bad; +, good; ++, excellent.

[2] E+3 and M+3. 3' end-selective nucleotides of the primers complementary to the *Eco*RI- and *Mse*I-adapter, respectively.

Table 3. Primer combinations used and the numbers of detected and polymorphic bands

*Eco*RI+3	*Mse*I+3	Number of detected bands	Number of polymorphic bands	Percentage of polymorphism
AAC	CTT	49	31	63.3
AAG	CAG	46	34	73.9
ACT	CAG	28	16	57.1
AGC	CTG	35	17	48.6
ACA	CAC	39	16	41.0
AGG	CAC	44	20	45.5
AGG	CTT	37	19	51.4
AGC	CTT	29	14	48.3
ACG	CTA	42	22	52.4
ACT	CAT	37	20	54.1
Total		386	209	54.1

with soybean (Maughan *et al.*, 1996) and 33.2% with rice (Subudhi *et al.*, 1998).

Cluster analysis

Cluster analysis was performed with similarity coefficient matrices calculated from AFLP markers to generate a dendrogram. The similarity coefficients ranged from 0.55 to 1.0. In the dendrogram, the 37 accessions which consisted of 31 tea cultivars (*C. sinensis*) and six *Camellia* species closely related to *C. sinensis* were clustered into five large groups (Fig. 4). The first group was mainly composed of 'Yabukita' and the cultivars derived from 'Yabukita', the second group Japanese tea cultivars not related to 'Yabukita', the third Chinese cultivars and Korean wild tea varieties, the fourth Assam type black tea cultivars, and the fifth group was composed of six *Camellia* species closely related to *C. sinensis*.

Either paternal or maternal parent of the accessions of the first group was 'Yabukita', with exceptions of 'Makinoharawase' and the Korean tea variety collected from Seonamsa,

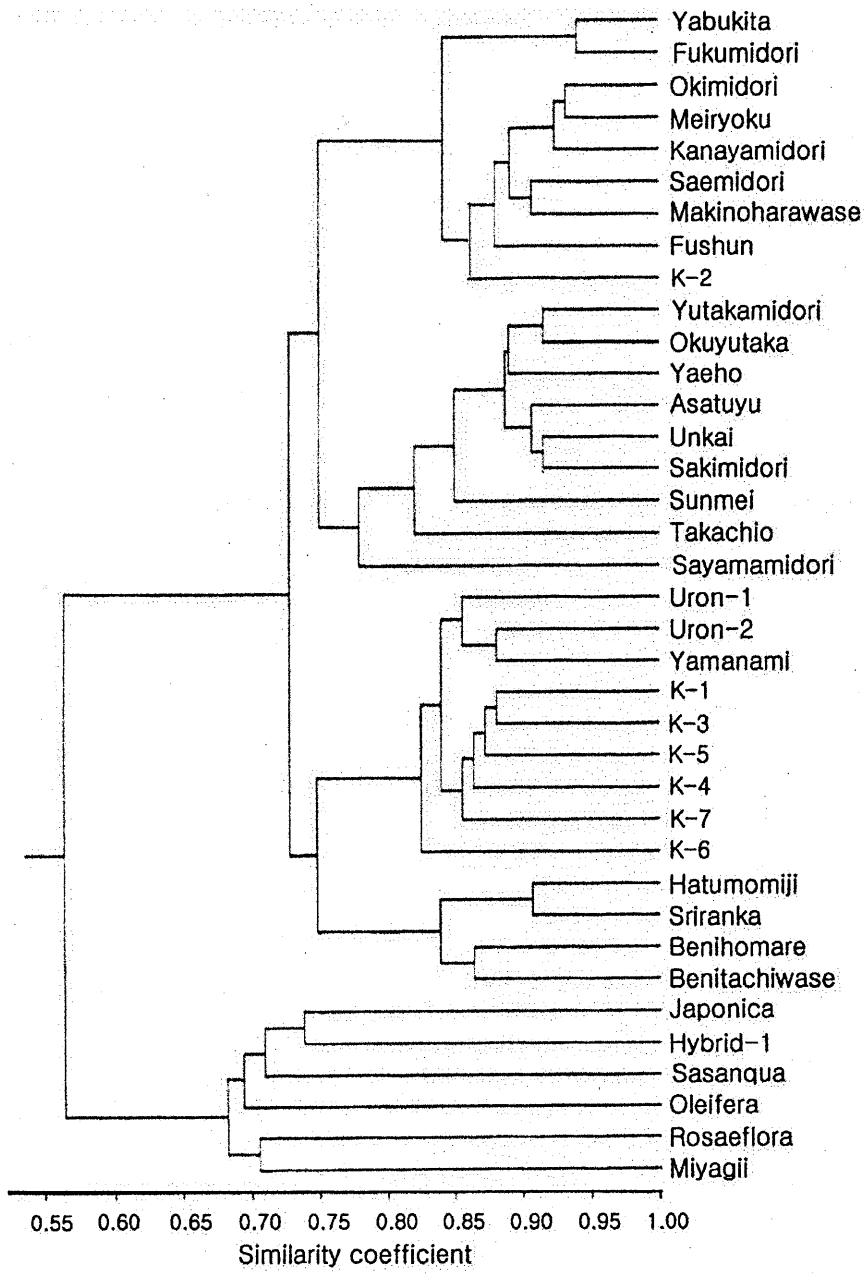

Fig. 4. Dendrogram of the 37 accessions constructed from the AFLP data. The accessions are listed in Table 1.

Korea. The paternal parent of 'Fushun' (Z–1×'Kanayamidori'), 'Kanayamidori' (S–6×'Yabukita') is an offspring of 'Yabukita' (Yamaguchi *et al.*, 1992). Among the accessions of the first group, 'Yabukita' and 'Fukumidori' showed highest similarity with an index of 0.94, reflecting the fact that 'Fukumidori' is a progeny of 'Yabukita'×F_1 ('Sayamamidori'×'Yabukita'). The presence of the Korean tea variety collected from Seonamsa (K–2) in the first group indicated that introgressions might have occurred between Korean wild tea varieties and 'Yabukita' when Japanese tea cultivars were introduced to Korea. Some Japanese tea cultivars and some Korean wild tea varieties also belonged to the same group in the RAPD analysis (Lee *et al.*, 1996). 'Makinoharawase' was selected from the seedlings obtained in Shizuoka, where 'Yabukita' originated from. The result of this study indicated that 'Makinoharawase' might also be produced by introgression with 'Yabukita'.

The second group was composed of nine accessions not related to 'Yabukita'. The accessions in this group were either naturally selected from seedlings or artificially bred. Among the accessions of the second group, 'Yutakamidori' and 'Okuyutaka', and 'Unkai' and 'Sakimidori' showed highest similarity with an index of 0.91. 'Okuyutaka' is an offspring of 'Yutakamidori'. 'Sayamamidori' showed lowest similarity to other accessions of the group. 'Sayamamidori' is a naturally selected cultivar and its origin is not clearly known. It was suggested that 'Sayamamidori' might have gone through less introgression with other tea varieties.

The third group was composed of nine accessions of Chinese tea cultivars and Korean wild tea varieties. A Chinese tea cultivar, 'Uron–2', was found to be more closely related to 'Yamanami' than another Chinese cultivar, 'Uron–1'. 'Yamanami' was bred from a Chinese seedling. The presence of several Korean wild tea varieties and Chinese tea cultivars in the same group might indicate that many Korean tea varieties have been introduced from China. The low similarity among the accessions of this group might reflect the fact that the accessions were not artificially bred varieties but naturally collected varieties from wild.

The fourth group is composed of four accessions of Assam type black tea varieties. 'Hatumomiji' was closely related to the variety collected from Sri Lanka, and 'Benihomare' to 'Benitachiwase'. The fifth group showed low similarity to other groups and is composed of six accessions of *Camellia* species closely related to *C. sinensis*. Japonica was closely related to Hybrid–1, a hybrid of *Camellia japonica* and *C. sinensis*. Japonica and Hybrid–1 were related to Sasanqua (*Camellia sasanqua*).

The results of this study indicated that the morphological characteristics and the origins of the tea cultivars were well reflected in the clustering of the cultivars based on the AFLP analysis, and that the AFLP technique might find its use as an efficient and effective tool for determining genetic relationships among tea cultivars.

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