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Analysis of the Genetic Relationships among Pollination-constant and Non-astringent (PCNA) Cultivars of Persimmon (*Diospyros kaki* Thunb.) from Japan and China using Amplified Fragment Length Polymorphism (AFLP)

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Summary

The genetic relationships among 19 pollination-constant and non-astringent (PCNA) cultivars and 14 non-PCNA cultivars of persimmon (*Diospyros kaki* Thunb.), including one Chinese PCNA cultivar 'Luo Tian Tian Shi', were analyzed by comparing 138 AFLP markers. A phylogenetic tree constructed based on the similarity indices of these AFLP markers indicated a close relationship among Japanese PCNA cultivars, but a more distant relationship with the Chinese PCNA cultivar 'Luo Tian Tian Shi'. The close relationship between PCNA cultivars native to Gifu prefecture was distinct, indicating that these cultivars developed from crosses among restricted sources in this region. The cultivars 'Gosho', 'Hana-gosho', 'Oo-gosho', and 'Yamato-gosho' showed a close relationship with some non-PCNA cultivars.

Key Words: AFLP, *Diospyros kaki*, genetic variation, phylogeny.

Introduction

Persimmon (*Diospyros kaki* Thunb.) originated in East Asia and has been cultivated for a long time in China, Korea, and Japan. Various cultivars with a wide range of variation have been developed in each country, but non-astringent type cultivars have been well developed only in Japan. China has the longest history of persimmon cultivation with more than 950 cultivars in the sub-tropical to temperate regions (Wang et al., 1997). Most indigenous cultivars of China are astringent types (Wang et al., 1997) with an exception of 'Luo Tian Tian Shi', a PCNA cultivar recently been reported by Wang (1982) that grows in Luo Tian prefecture in Hubei province. In Korea, 186 cultivars were collected between 1959-1964 and identified at Kim-Hae Horticulture Experimental Station, and all of them proved to be astringent types (Cho and Cho, 1965). Recently, a non-astringent type cultivar, 'Daean Dangam' has been reported (Kim et al., 1988), but its characteristics are identical with 'Mushiroda-gosho', a PCNA-type cultivar of Japanese origin (Yamada, unpublished). This cultivar may have been introduced from Japan.

A nation-wide survey of persimmon cultivars in Japan (Agricultural Research Station, 1912) revealed the existence of 6 PCNA-type cultivars, in contrast to 401 pollination variant and non-astringent (PVNA) type cultivars, among more than 1,000 cultivars collected from all over the country. PVNA cultivars have very wide variations, whereas PCNA types show little variation (Yamada et al., 1988; Yamada et al., 1993; Yamada et al., 1994b). Furthermore, PCNA cultivars originated in a limited area within the central part of Japan, in contrast to the wide distribution of the original PVNA cultivars (Ikeda et al., 1985; Yamada, 1993). These facts indicate that PCNA cultivars were developed uniquely in Japan and derived from limited sources with a relatively narrow genetic background.

Sugiura et al. (1990) analyzed the isozymes of 42 PCNA cultivars including bud sports, which cover almost all PCNA-type cultivars currently existing in Japan. They concluded that the 42 cultivars could be classified into 18 groups based upon the pattern of four isozymes. Among these, native cultivars were classified into 17 groups, since 'Suruga', which is derived from a breeding program, formed one group by itself. The genetic relationships among cultivars belonging to these 17 groups have not been investigated. Furthermore, the relationship between the PCNA-type cultivar of Chinese origin, 'Luo Tian Tian Shi', and those of Japanese origin has not been investigated.

Detection of the DNA polymorphism through the application of molecular techniques is an effective method for studying genetic diversity and relationships. Amplified fragment length polymorphism (AFLP) analysis is a new powerful tool for this purpose with advantages of high reproducibility and a large number of detectable loci over other molecular marker systems.

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This technique has been applied to determine the genetic relationship in coconut (Perera et al., 1998), olive (Baldoni et al., 1998), and cassava (Roa et al., 1997). In the present study, we used the AFLP technique to investigate the genetic diversity among PCNA cultivars and the genetic relationship between Japanese PCNA cultivars and 'Luo Tian Tian Shi'.

Materials and Methods

Plant materials

Nineteen PCNA and 14 non-PCNA-type cultivars were used for this study (Table 1). All of the 19 PCNA cultivars were collected from Persimmon and Grape Research Center, National Institute of Fruit Tree Science, Akitsu. The PCNA cultivars included one each from the 17 PCNA groups previously classified by isozyme analysis (Sugiura et al., 1990), one Japanese PCNA cultivar, 'Sagiyama-gosho', which was not included in that analysis, and one Chinese PCNA cultivar, 'Luo Tian Tian Shi'. Fourteen non-PCNA cultivars were collected from the Experimental Farm of Kyoto University, Kyoto. To check for the possible variation of polymorphism within a cultivar, four trees of 'Fuyu' and three trees of 'Hana-gosho' and 'Jiro' were also used.

DNA extraction and AFLP analysis

Total DNA was extracted from 5 g fresh leaf samples by the CTAB method of Doyle and Doyle (1985) followed by additional phenol extraction and PEG (MW8000) precipitation for DNA purification. The AFLP reaction was performed according to the manufacturer's instructions for the AFLP Analysis System I (Life Technologies, USA). Briefly, 250 ng of total DNA was digested with 2.5 units of *Eco*RI and *Mse*I. Then, the DNA fragments were ligated to *Eco*RI and *Mse*I adapters. Preselective and selective amplifications were performed with two primers based on the sequence of *Eco*RI and *Mse*I adapters including one and

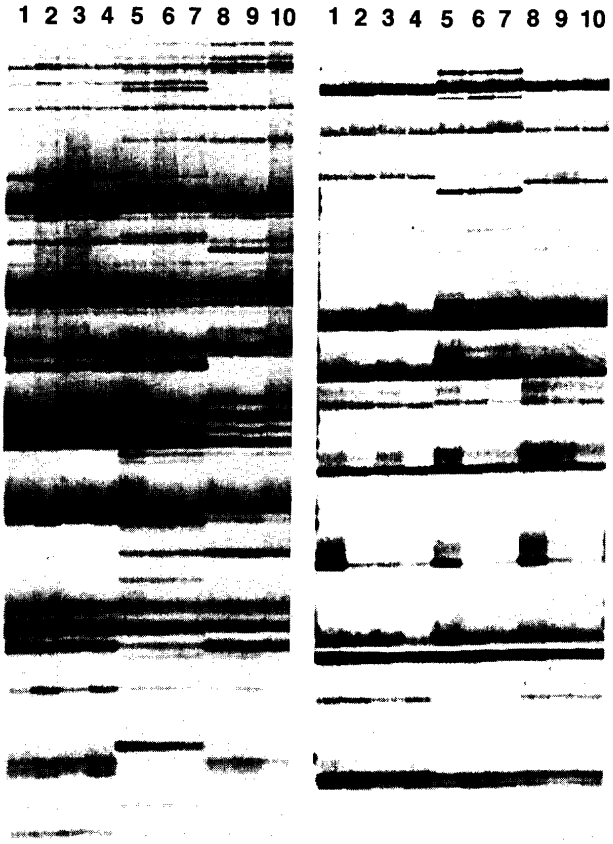

Fig. 1. Gel showing AFLP analysis of individual trees of three cultivars using the primer combinations E-ACC/M-CTA (left) and E-ACG/M-CTA (right). Lane 1-4: 'Fuyu', 5-7: 'Hana-gosho', 8-10: 'Jiro'.

Table 1. Nineteen PCNA type cultivars and 14 non-PCNA cultivars used in this study.

No.	Cultivar	Type	Origin	No.	Cultivar	Type	Origin
1	Oku-gosho	PCNA	Gifu	18	Fujiwara-gosho	PCNA	Nara
2	Haze-gosho	PCNA	Gifu	19	Luo Tian Tian Shi	PCNA	China
3	Tokuda-gosho	PCNA	Gifu	20	Saijo	PCA	Hiroshima
4	Tenjin-gosho	PCNA	Gifu	21	Tamopan	PCA	China
5	Sagiyama-gosho	PCNA	Gifu	22	Shougatsu	PVNA	Fukuoka
6	Mushiroda-gosho	PCNA	Gifu	23	Ama-hyakume	PVNA	Unknown
7	Mizu-gosho	PCNA	Gifu	24	Koshu-hyakume	PVA	Unknown
8	Fuyu	PCNA	Gifu	25	Monpei	PVA	Ishikawa
9	Hana-gosho	PCNA	Tottori	26	Atago	PCA	Ehime
10	Yoshimoto-gosho	PCNA	Nara	27	Yokono	PCA	Yamaguchi
11	Gosho	PCNA	Nara	28	Kyara	PVNA	Saga
12	Ikutomi	PCNA	Nara	29	Kikuhira	PVA	Hyogo
13	Jiro	PCNA	Shizuoka	30	Amayotsumizo	PVNA	Shizuoka
14	Mikado	PCNA	Unkown	31	Sakushumishirazu	PVA	Okayama
15	Midai	PCNA	Yamanashi	32	Touhachi	PVNA	Shizuoka
16	Yamato-gosho	PCNA	Nara or Hyogo	33	Nishimura-wase	PVNA	Shiga
17	Oo-gosho	PCNA	Hyogo				

three additional selective nucleotides at the 3' end of each primer, respectively. After selective amplification, amplified fragments were electrophoresed in a 6% polyacrylamide sequencing gel (acrylamide-bisacrylamide 29:1) and then transferred to a Biodyne B membrane (Pall BioSupport, USA). The membranes were hybridized with AFLP Non-Radioactive Probe (Life Technologies, USA) according to the manufacturer's instructions. Then, the membrane was washed and incubated with CDP-Star (Boehringer Mannheim, Germany) at 37℃ for a few minutes and exposed to X-ray film for 3-7 hours. Some primer combinations which produce an appropriate number of amplified fragments were selected and used for phylogenetic analysis.

Data analysis

A binary matrix reflecting the presence (1) or absence (0) of each AFLP-band was generated for each cultivar. After similarity indices (Nei and Li, 1979) were calculated based on the number of shared bands, the neighbor-joining (NJ) method (Saitou and Nei, 1987) was used with PHYLIP (Felsenstein, 1993) to construct the phylogenetic tree.

Results and Discussion

Tests using several primer combinations revealed 15-50 bands per primer combination. No polymorphisms were observed among four individual trees of 'Fuyu' or among the three individual trees of 'Hana-gosho' and 'Jiro' (Fig. 1). These results confirmed the high reproducibility and the efficiency of AFLP analysis for DNA fingerprinting. Using several cultivars, 12 primer combinations were tested and five primer combinations (E-ACC/M-CTA, E-AGC/M-CTA, E-AGC/M-CAC, E-AGC/M-CAG and E-ACG/M-CAG) were selected for phylogenetic analysis, based upon the number of scorable strong bands and the level of polymorphisms. An example of the level of polymorphism detected with the primer combination E-ACC/M-CTA is shown in Fig. 2. In total, 138 polymorphic bands were scored and the similarity indices were calculated based on each of these bands.

Thirty-three persimmon cultivars used in this study were divided into three groups in an unrooted tree constructed by the neighbor-joining method (Fig. 3). Two Chinese cultivars, 'Luo Tian Tian Shi' and 'Tamopan', and nine Japanese non-PCNA cultivars formed one group. This group did not form a tightly connected cluster and distances among these cultivars were relatively far. This supported the wide genetic diversity of cultivars in this group. 'Luo Tian Tian Shi' and 'Tamopan' were genetically distant from Japanese cultivars, indicating that these Chinese cultivars have a distant genetic relationship with Japanese cultivars. In other word, 'Luo Tian Tian Shi', which is the only PCNA cultivar of Chinese origin, did not seem to have contributed to the development of Japanese PCNA

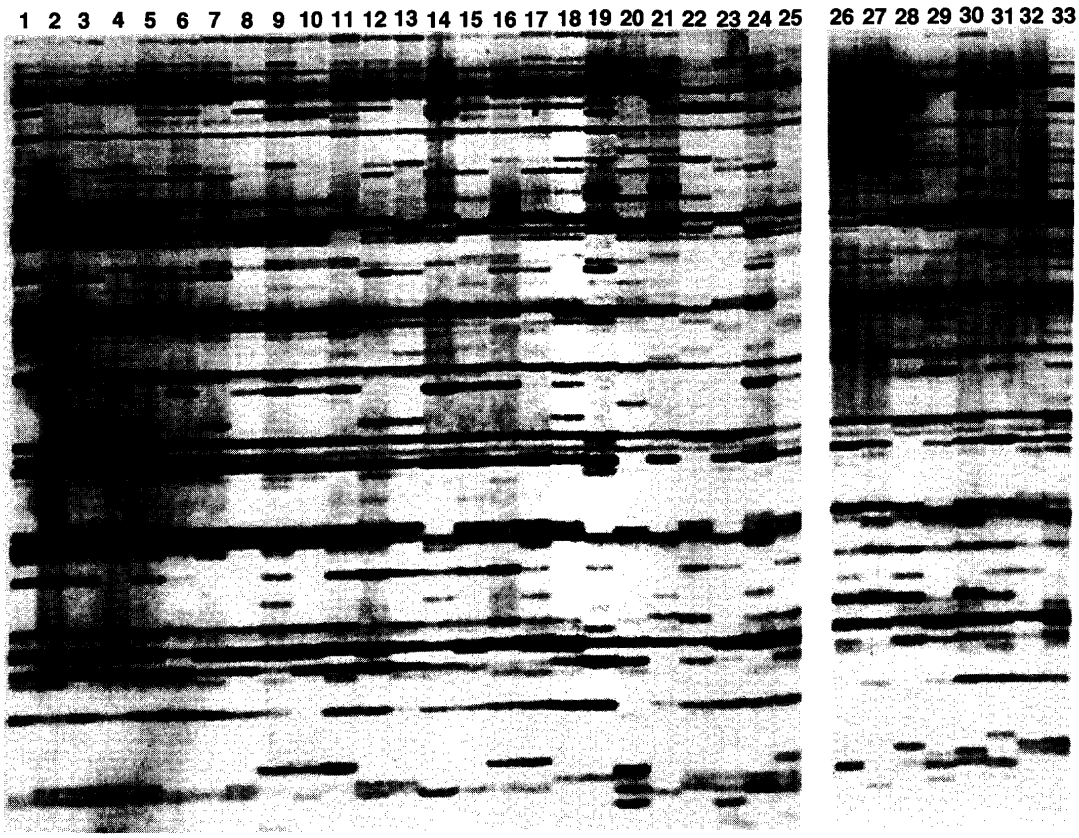

Fig. 2. Gel showing AFLP analysis of 33 persimmon cultivars using the primer combination E-ACC/M-CTA. Lane assignments are identical to the cultivar number in Table 1.

cultivars.

The second group consisted of 14 Japanese indigenous PCNA cultivars and formed a subgroup of 12 cultivars except for 'Fujiwara-gosho' and 'Mizu-gosho'. This subgroup showed a close relationship among themselves. Seven of the 12 PCNA cultivars in this subgroup ('Oku-gosho', 'Tokuda-gosho', 'Sagiyama-gosho', 'Haze-gosho', 'Tenjin-gosho', 'Mushiroda-gosho', and 'Fuyu') originated in Gifu prefecture. Considering the concentration of PCNA cultivars of Gifu origin and the close relationship among them, PCNA cultivars in this group may have developed from the crosses made with the restricted sources of germplasm in this region. Five other cultivars in this group ('Ikutomi', 'Jiro', 'Midai', 'Yoshimoto-gosho', and 'Mikado') may also have been derived from these seven cultivars of Gifu origin or from the same sources as these cultivars. Two cultivars, 'Fujiwara-gosho' and 'Mizu-gosho', formed another subgroup, indicating that these cultivars may have a slightly different genetic background as compared to the Gifu-native group.

Four PCNA cultivars ('Hana-gosho', 'Gosho', 'Oo-gosho', and 'Yamato-gosho') made another group together with 4 non-PCNA cultivars ('Monpei', 'Sakushu-mishirazu', 'Atago', and 'Koshu-hyakume'), and were separated from the 14 PCNA cultivars in the second group. 'Gosho' is considered to be the oldest PCNA cultivar and may be the ancestor of all PCNA cultivars existing in Japan (Kikuchi, 1948; Yamada et al., 1988). The relatively distant relationship between 'Gosho' and the 14 PCNA cultivars in the second group would indicate that 'Gosho' and the other three PCNA cultivars in this group may have a different genetic background from the PCNA cultivars of Gifu origin.

PCNA cultivars of Japanese origin show a small phenotypic variation. When Yamada et al. (1993) analyzed 27 fruit characters of 16 PCNA, including 'Luo Tian Tian Shi', and 18 non-PCNA cultivars using the principal component analysis, each type of cultivars were clearly grouped on the first and second principal component plane. Japanese PCNA cultivars have specific morphological features such as flat fruit shape, depressed calyx, crinkles at fruit bottom, in addition to late fruit ripening and fruit cracking. However, fruit characteristics of 'Luo Tian Tian Shi' are different from those of Japanese PCNA cultivars. The present result that 'Luo Tian Tian Shi' is distantly related to PCNA cultivars of Japanese origin is in agreement with the morphological characteristics. The relationship among Japanese PCNA cultivars could not be determined by morphological characters. 'Hana-gosho', 'Gosho', 'Oo-gosho', and 'Yamato-gosho' resemble each other, but no specific characteristics which distinguish these four cultivars from cultivars of Gifu origin were observed. In the present study, it is indicated that the genetic relationship among Japanese PCNA cultivars is relevant to their geographical origins.

In conclusion, the narrow genetic diversity and close

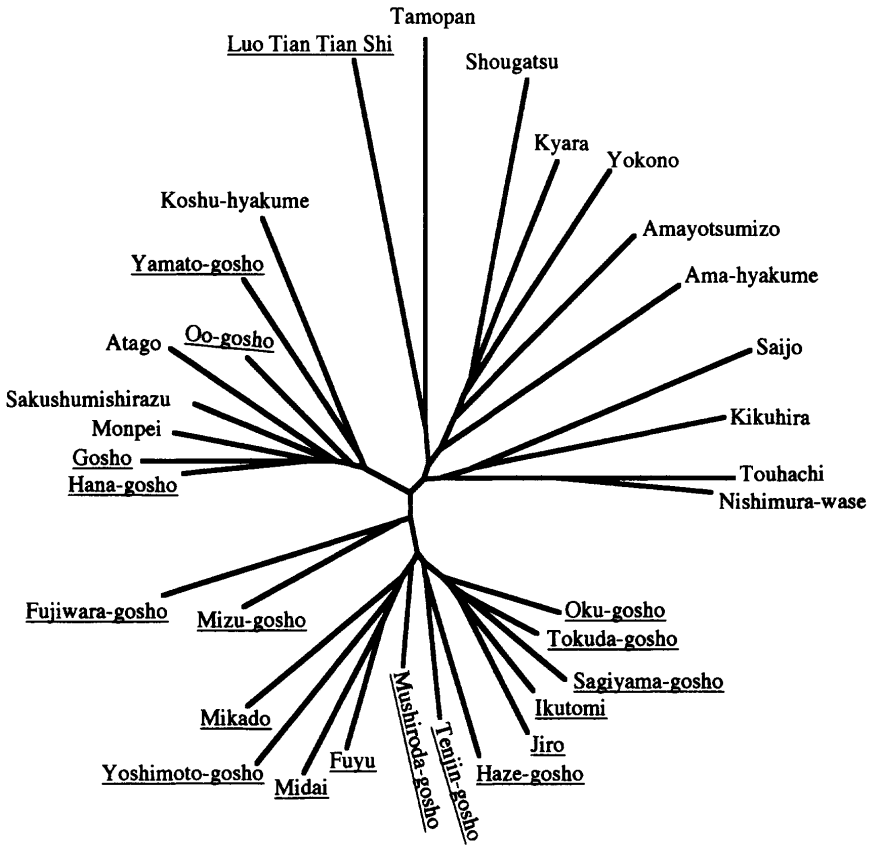

Fig. 3. Unrooted phylogenetic tree of 33 persimmon cultivars based on AFLP analysis using five primer combinations. PCNA cultivars are underlined.

relationship among Japanese PCNA cultivars was confirmed in this study. However, one Chinese PCNA cultivar 'Luo Tian Tian Shi' was found to be distantly related to Japanese PCNA cultivars. This would indicate an independent occurrence of this PCNA cultivar in China. Among Japanese PCNA cultivars, the close genetic relationships among Gifu-native cultivars was conspicuous. The narrow genetic diversity among Japanese PCNA cultivars sometimes causes inbreeding depression in progenies obtained from crosses among PCNA genotypes. This problem has been observed in the recent breeding program aimed at developing new PCNA cultivars (Yamada, 1993; Yamada et al., 1994a). To produce new PCNA cultivars with good quality, non-PCNA type germplasm should be included in breeding programs as this extends the genetic background of breeding populations.

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AFLP法を用いた日本および中国原産の完全甘ガキ品種群の遺伝的類縁関係の解析

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摘　要

完全甘ガキ品種群は遺伝的変異が小さく，比較的新しい時期に日本において独自に発達してきたと考えられているが，一方で，中国原産の完全甘ガキ‘羅田甜柿’と日本の完全甘ガキ品種群との関係は明確ではない．これらの完全甘ガキ品種群の類縁関係を明らかにするため，‘羅田甜柿’を含めた完全甘ガキ19品種と非完全甘ガキ14品種についてAFLP法を用いた解析を行った．多型を示した138のAFLPマーカーの類似性に基づき系統樹を作成したところ，日本の完全甘ガキ品種群は比較的近縁関係にあることが確認された．一方，‘羅田甜柿’は日本の品種とは離れた関係にあることが示され，日本の完全甘ガキ品種群の成立に‘羅田甜柿’は関与していないことが示唆された．日本の完全甘ガキ品種群の中では岐阜県原産の品種の近縁性が顕著であり，これらの品種の起源はこの地方の限られた在来品種に由来していることが示唆された．また，日本の完全甘ガキの起源であると考えられている‘御所’は岐阜原産の完全甘ガキ品種群とは異なるクラスターに属し，岐阜原産の品種群とは異なる遺伝的背景を持つことが示唆された．