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Evaluation on Genetic Relationships of Persimmons in Thailand Based on RAPD Markers

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Abstract

Genetic relationships of persimmon in Thailand were evaluated with randomly amplified polymorphic DNA (RAPD) markers. The samples in this analysis included 35 *Diospyros kaki* Thunb. accessions (20 non-astringent and 15 astringent accessions), two *D. lotus* L. accessions and one accession of *D. glandulosa* Lace. The objectives were to analyse for their genetic relationships and to identify ambiguous cultivars that were cultivated in Thailand. The RAPD-PCR analysis showed that 10 out of 16 primers resulted in 80 scorable and reproducible RAPD markers of which 78 (97.5 percent) were polymorphic. These markers range in size between 200 and 2600 bp. Genetic similarity of persimmons within *D. kaki* was greater than 0.70. *D. kaki* are as closely related to *D. lotus* (0.45) as to the *D. glandulosa* (0.44), while the similarity coefficient between *D. lotus* and the *D. glandulosa* is about 0.39. Based on the RAPD markers, genetic difference was found between cultivars 'Hyakume', 'Nightingale', 'Fuyu Wase', 'Fuyu Japan' and 'Fuyu Australia' clones collected from various places, while no genetic difference was found between cultivar 'Jiro' clones collected from Royal Project Development Center at Khun Huay Haeng and from Royal Project Development Center at Mae-Hae, similarly, cultivar 'Xichu' clones collected from Royal Project Development Center at Mae-Hae and from Royal Angkhang Agricultural Station showed genetic uniformity.

Key words: *Diospyros kaki*, *D. lotus* L., *D. glandulosa*, identification, UPGMA.

Introduction

Persimmon belongs to the family Ebenaceae and genus *Diospyros* which is distributed in tropical and subtropical regions of the world. The genus includes about 400 species, with only four species have been used commercially for the production of fruit. They are *D. kaki* Thunb. (Japanese or Chinese persimmon), *D. lotus* L. (Date Plum), *D. virginiana* L. (American persimmon) and *D. oleifera* Cheng. Among them, *D. kaki* is the most important species (Itoo, 1980).

Japanese or Chinese persimmon (*D. kaki*) consists of approximately up to 1,000 horticultural cultivars (Itoo, 1980). They are considered native to China and some cultivars with good fruit quality are cultivated in Japan, Korea, Brazil, Italy, Israel, America, New Zealand

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and Australia (Ryugo, 1988).

In Thailand, the *D. kaki* persimmon has been cultivated for more than 50 years and all cultivars were introduced from other countries. Propagation was done by grafting onto a rootstock. Common rootstocks are Date Plum (*D. lotus*) and 'Kluai Rusi' (*D. glandulosa*), 'Kluai Rusi' is indigenous to Thailand (Northern, North-Easthern and Easthern) and distributed in India, Burma and Laos (Phenglai, 1981).

Because of lengthy and wide spread cultivation in many countries, the same clones have been called differently and different clones have been called similarly. This has resulted in confusion in the evaluation of cultivars characteristics and to mislabeling of experimental materials.

Many researchers have used different techniques to identify species or cultivars and to evaluate genetic relationships in persimmons. These techniques included morphological markers (Kim and Ko, 1997a), biochemical markers such as isozyme (Tao and Sugiura, 1987; Sugiura *et al.*, 1988; Kim and Ko, 1997b) and molecular markers such as restriction fragment length polymorphisms (RFLP) (Nakamura and Kobayashi, 1994; Yonemori *et al.*, 1996; Kanzaki *et al.*, 2000), amplified fragment length polymorphisms (AFLP) (Kanzaki *et al.*, 2001), and randomly amplified polymorphic DNA (RAPD) (Luo *et al.*, 1995; Zhengrong *et al.*, 1999).

RAPD-PCR technique was initially applied by Welsh and McClelland (1990) and Williams *et al.* (1990). Because of its quick, convenient and relatively cheap method the technique was used in many fields. RAPD markers have been used successfully for estimating genetic relationships in several fruit crops such as almond (Bartolozzi *et al.*, 1998), guava (Prakash *et al.*, 2002), persimmon (Luo *et al.*, 1995; Zhengrong *et al.*, 1999) and plum (Boonprakop *et al.*, 2001), for identifying and classifying of cultivars such as apple (Koller *et al.*, 1993), apricot (Takeda *et al.*, 1998), grapevine (Ulanousky *et al.*, 2002), mume (Shimada *et al.*, 1994) and peach (Lu *et al.*, 1996). In addition, RAPD markers are reliable enough to detect differences between the genetically close accessions such as bud-sport mutations of almond (Bartolozzi *et al.*, 1998), progenitors of diploid plum (Boonprakob *et al.*, 2001) and synonymies and hononymies of grapevines (Ulanovsky *et al.*, 2002)

The objectives of this study were to estimate genetic relationships of persimmons based on RAPD markers and to evaluate the application of RAPD-PCR technique as a DNA marker for identification of ambiguous persimmon cultivars in Thailand.

Materials and Methods

Plant materials

Thirty eight accessions of persimmon from various places were included in this study. These consisted of 35 *D. kaki* Thunb. accessions of which 20 were non-astringent and 15 were astringent types. Two *D. lotus* L. accessions and one *D. glandulosa* Lace accession were also analyzed (Table 1).

DNA extraction

The procedure was modified from that of Doyle and Doyle (1987) for use in a 1.5 ml microcentrifuge tube with 50 mg of young fresh leaf tissue (Boonprakob *et al.*, 2001). The DNA concentration was quantified by visual comparison with Ezload Precision Molecular Mass Standard (Bio-Rad, U.S.A.) on a 0.8% (w/v) agarose gel (Promega, U.S.A.) in 0.5X TBE buffer (Sambrook *et al.*, 1989). The DNA stock was then diluted to a concentration of 2.5 $ng.\mu l^{-1}$ with sterile Reverse Osmosis H_2O as a working stock.

Table 1 Persimmon accessions used in genetic relationships study

Accession No.	Cultivar	Fruit type*	Station**	Code	Origin
	Matsumoto Wase Fuyu	PCNA	KW	Mat. Fuyu-KW	Bud mutant of Fuyu
	Giant Fuyu	PCNA	AK	Giant Fuyu-AK	Japan
	Fuyu U.S.A.	PCNA	MH	Fuyu U.S.A.-MH	U.S.A.
	Fuyu Taiwan	PCNA	AK	Fuyu Taiwan-AK	Taiwan
	Fuyu Wase	PCNA	DP	Fuyu Wase-DP	Japan
	Fuyu Wase	PCNA	KW	Fuyu Wase-KW	Japan
	Fuyu Wase	PCNA	AK	Fuyu Wase-AK	Japan
	Fuyu Japan	PCNA	MH	Fuyu Japan-MH	Japan
	Fuyu Japan	PCNA	KW	Fuyu Japan-KW	Japan
	Fuyu Japan	PCNA	AK	Fuyu Japan-AK	Japan
	Fuyu Australia	PCNA	DP	Fuyu Aust.-DP	Australia
	Fuyu Australia	PCNA	KW	Fuyu Aust.-KW	Australia
	Fuyu Australia	PCNA	AK	Fuyu Aust.-AK	Australia
14	Xichu	PCA	KH	Xichu-KH	Taiwan
	Xichu	PCA	MH	Xichu-MH	Taiwan
	Xichu	PCA	DP	Xichu-DP	Taiwan
	Xichu	PCA	AK	Xichu-AK	Taiwan
	Hiratanenashi	PVA	AK	Hirata.-AK	Japan
19	Nightingale	PCA	MH	Nightingale-MH	Japan
20	Nightingale	PCA	DP	Nightingale-DP	Japan
21	Nightingale	PCA	AK	Nightingale-AK	Japan
22	Angsai	PCA	DP	Angsai-DP	Japan
23	Tone Wase	PVA	AK	Tone Wase-AK	Bud mutant of Hiratanenashi
24	*D. lotus*	PCA	MH	*D. lotus*-MH	Taiwan
25	*D. lotus*	PCA	AK	*D. lotus*-AK	Taiwan
26	*D. glandulosa*	PCA	AK	*D. glandulosa*-AK	Thailand
27	Hyakume	PVA	DP	Hyakume-DP	Japan
28	Hyakume	PVA	AK	Hyakume-AK	Japan
29	Amahyakume	PVNA	AK	Amahyakume-AK	Japan
30	Jiro	PCNA	KH	Jiro-KH	Japan
31	Jiro	PCNA	MH	Jiro-MH	Japan
32	Jiro	PCNA	AK	Jiro-AK	Japan
33	Mikado	PCNA	AK	Mikado-AK	Japan
34	Fujiwara Gosho	PCNA	AK	Fujiwara.-AK	Japan
35	Aizu Mishirazu	PVA	AK	Aizumi.-AK	Japan
36	Anzai	PVNA	AK	Anzai-AK	Japan
37	Kuramitsu	PCA	AK	Kuramitsu-AK	Japan
38	Tamopan	PCA	AK	Tamopan-AK	Japan

* PCNA : pollination constant and non-astringent, PVNA : pollination variant and non-astringent, PCA : pollination constant and astringent, PVA : pollination variant and astringent

** KH Royal Project Development Center at Khun Huay Haeng, MH : Royal Project Development Center at Mae-Hae, DP : Doi Pui Research Station, KW : Royal Project Development Center at Khun Wang, AK : Royal Angkhang Agricultural Station

RAPD amplification

The PCR reaction contained 12.5 µl of a mixture of 1X reaction buffer [Tris -Cl, KCl, $(NH_4)SO_4$, 1.5 mM $MgCl_2$] pH 8.7 at 20° C (QIAGEN GmbH, Germany), 0.33 µM of oligonucleotide decamer-primer (Operon Technology, U.S.A.), 1 unit of *Taq* DNA polymerase (QIAGEN GmbH, Germany), 200 µM of each dNTP (dATP, dCTP, dTTP and dGTP) (Promega, U.S.A.) and 6 to 7 ng genomic DNA. The PCR reaction was performed in an Omni-Gene Thermal Cycler (Hybaid, U.K.) under the following conditions: 1 min at 92° C, 1 min at 35° C and 2 min at 72° C for 41 cycles.

Amplifications for each DNA sample and primer were repeated three times independently in order to score only RAPD markers that were reproducible and consistent. Ambiguous or inconsistent RAPD markers in any run such as those present in one amplification but absent in another were not included in the analysis.

Gel electrophoresis

After completion of the PCR, 1.5 µl bromophenol blue was added to each PCR product. PCR products were size-seperated on a 1.5% (w/v) agarose gel by electrophoresis in 0.5X TBE buffer (Sambrook *et al.*, 1989). The horizontal electrophoresis system (Mupid 2, Cosmo Bio Co; LTB, Japan) was used with an agarose gel cast into a size of 6.0 × 11.0 × 0.5 cm (W×L×H).

Electrophoresis was run at a constant voltage at 50 V until the bromophenol blue migrated to the other end of the gel. The gel was then stained with 1 $\mu g.ml^{-1}$ ethidium bromide solution for 20 min and destained in water for 10-20 min on a shaker.

The size of RAPD markers was estimated by comparing the bands to a 100 bp DNA ladder standard marker (GibcoBRL, U.S.A.) included in the gel. The gel was photographed on a UV transillaminator using Fluorescent Table Combi-Light (ETS VILBER-LOURMAT, France). Detection of markers was done with photocaptMW 99.03 program (ETS VILBER-LOURMAT, France). Each amplified RAPD marker was identified by size of base pairs (bp).

Data analysis

Scoring of RAPD markers was based on reproducibility and consistency in fragment amplification of replicated assays. A binary matrix was prepared with 1 for presence and 0 for absence of each marker. The genetic similarity matrix among accessions was calculated with the SIMQUAL procedure (Sokal and Sneath, 1963) using Dice similarity coefficients (Sneath and Sokal, 1973) and is written as

$$\text{Similarity coefficient } (C_{jk}) = \frac{2a}{2a + b + c}$$

in which ***a*** is the number of RAPD markers present in both **j** and **k** accessions
b is the number of RAPD markers present only in **j** accession
c is the number of RAPD markers present only in **k** accession

Cluster analysis using the unweighted pair-group method with arithmetic average (UPGMA) (Sokal and Michener, 1958) with the SAHN procedure (Sneath and Sokal, 1973) based on the similarity coefficients of RAPD data were performed to construct a dendrogram representing genetic relationships among persimmon accessions. The SIMQUAL and SAHN procedures are the packaging of NTSYS-pc 2.00 program (Rohlf, 1997).

Results and Discussion

RAPD analysis

Sixteen primers (OPB-01 to 16) were Screened with selected persimmon DNA samples. Ten primers resulted in successful PCR, yielding informative RAPD markers (Table 2, Figure 1 illustrates the results using primer

Table 2 Primer and RAPD markers used for genetic relationship of persimmons

Primer code	No. of RAPD marker	Marker fragment size*
OPB-01	5	OPB-01-700, 750, 800, 1200 and 1400
OPB-02	8	OPB-02-400, 800, 1000, 1400, 2000, 2200, 2400 and 2500
OPB-04	8	OPB-04-800, 900, 1000, 1300, 1800, 2000, 2200 and 2500
OPB-06	7	OPB-06-400, 500, 600, 700, 800, 900, and 1300
OPB-07	7	OPB-07-300, 400, 700, 1300, 1600, 2200, and 2400
OPB-08	8	OPB-08-600, 700, 800, 900, 1100, 1200, 1700 and 2600
OPB-10	11	OPB-10-300, 350, 600, 650, 700, 800, 1100, 1200, 1250, 1300 and 1500
OPB-11	9	OPB-11-200, 600, 700, 800, 1100, 1200, 1300, 1700 and 2200
OPB-12	8	OPB-12-400, 500, 600, 700, 900, 1000, 1300 and 1800
OPB-15	9	OPB-15-900, 1000, 1100, 1200, 1300, 1400, 2000, 2400 and 2500

* coding of marker fragment size : OPB-01-700 indicated the primer code and a size of marker in base pair.

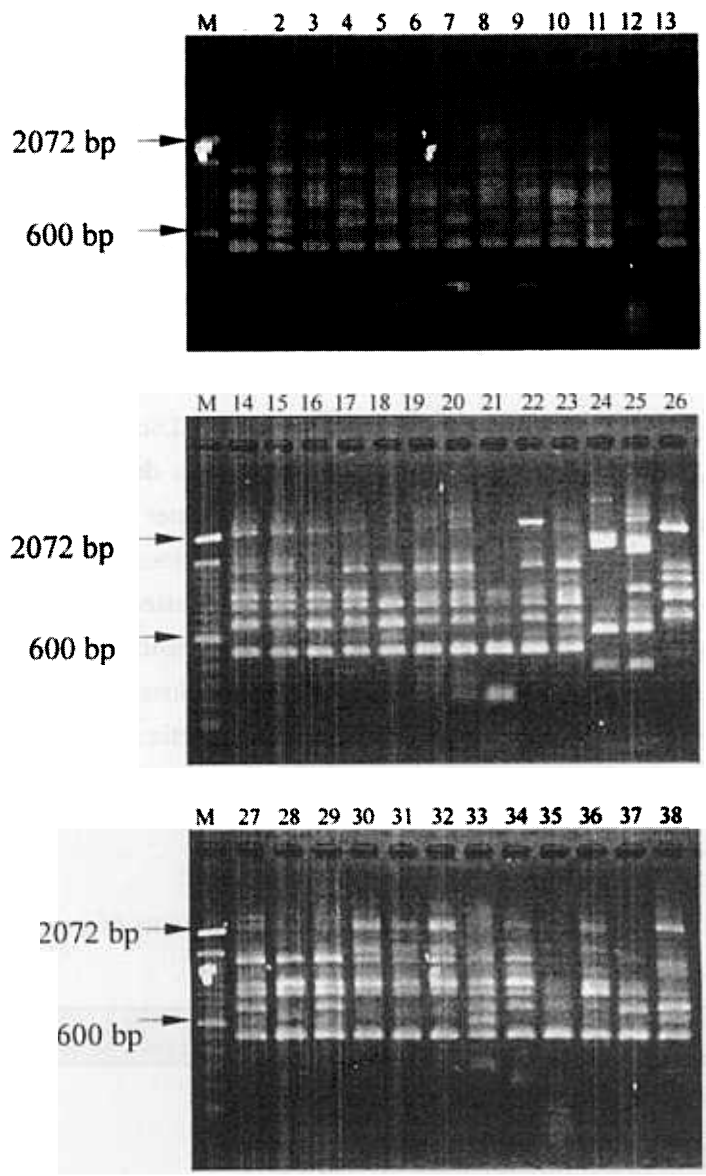

Figure 1 RAPD patterns obtained with primer OPB-12, M is a standard marker and lane numbers refer to the accession members in Table 1.

OPB-12). The 10 primer amplified 80 reproducible and unambiguous RAPD markers which ranged in size between 200 to 2600 bp (Table 2). This result was comparable with other fruit crops such as plum which amplified fragments between 340 to 4000 bp (Boonprakob *et al.*, 2001), peach 400 to 2200 bp (Lu *et al.*, 1996) and almond 260 to 1900 bp (Bartolozzi *et al.*, 1998). Seventy eight markers (97.5%) were polymorphic, and among them 16 markers (20%) were unique.The unique markers were found in all species with 9 markers (11.25%) in *D. glandulosa*, 5 markers (6.25%) in *D. lotus* and 2 markers (2.5%) in *D. kaki*.

The high number of unique markers in *D. glandulosa* was due to only one sample analyzed in this study. The 2 unique markers observed in *D. kaki* clones were found in 'Fuyu Australia-KW' and 'Tamopan-AK'. These markers (OPB-06-600 and OPB-10-650) could correctly identify 'Fuyu Australia-KW' and 'Tamopan-AK', respectively.

Genetic relationships among the persimmon species

The average Dice similarity coefficients within and between persimmon species indicated that similarities within species were higher than those between species (Table 3). Genetic similarity of persimmon (*D. kaki*) in Thailand based RAPD markers was greater than 0.70 (Figure 2). It was observed that *D. kaki* accessions were as closely related to *D. lotus* accessions (similarity coefficient 0.45) as they were to *D. glandulosa* accession (similarity coefficient 0.44), while the similarity coefficient between *D.lotus* and *D. glandulosa* is about 0.39 (Table 3).

The range in genetic similarity within *D. kaki* species in Thailand was between 0.70 to 1.00 (Figure 2) with an average is about 0.80 (Table 3). The range in genetic relationship of persimmon in Japan was between 0.49 to 0.93 (Sugiura *et al.*, 1988). This indicated a much wider genetic variation of persimmon in Japan where it has been cultivated for several hundred years. The genetic relationship between 'Aizu Mishirazu-AK' and 'Kuramitsu-AK' was the lowest at about 0.70, followed by a group of 'Matsumoto Wase Fuyu-KW', 'Nightingale-AK' and 'Angsai-DP' at about 0.79 (Figure 2). The genetic similarity of 17 accessions (50%) within *D. kaki* species was greater than 0.90 indicating a very narrow genetic variation of cultivated persimmon.

Cluster analysis using the UPGMA method based on the Dice similarity coefficients of RAPD data yields a dendrogram that can be clustered into one cluster (Figure 2). The cluster included all 35 accessions of *D. kaki*. The genetic similarity of this cluster was between 0.70 to 1.00, while the two accessions of *D. lotus* and one accession of *D. glandulosa* could not be grouped to any cluster. The genetic similarity within *D. lotus* is about 0.65 (Figure 2).

Table 3 Average and standard error of Dice similarity coefficients of persimmon accessions within and between species

Species	*D. kaki*	*D. lotus*	*D. glandulosa*
D. kaki	0.80 ± 0.13		
D. lotus	0.45 ± 0.07	0.65	
D. glandulosa	0.44 ± 0.04	0.39 ± 0.16	Na

Na is not available

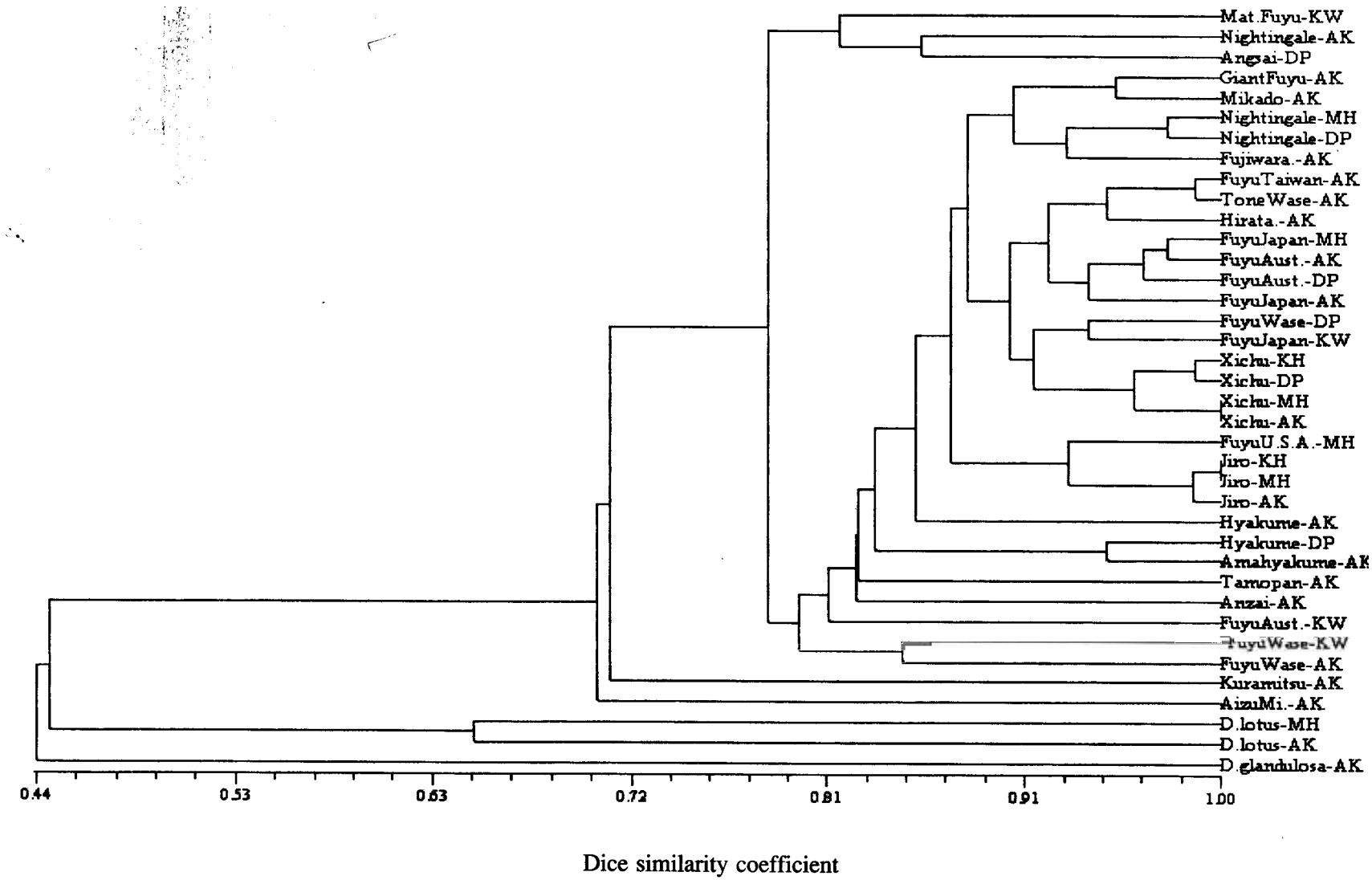

Figure 2 Dendrogram of 38 persimmon accessions using UPGMA method on Dice similarity of 80 RAPD markers.

Genetic relationships among fruit type of persimmon cultivars within *D. kaki* species

The four types of fruit were 1) pollination constant and non-astringent (PCNA), 2) pollination variant and non-astringent (PVNA), 3) pollination constant and astringent (PCA) and 4) pollination variant and astringent (PVA). The genetic control of these traits is both qualitative and quantitative. PCNA showed qualitative variation and was recessive, while other fruit types showed quantitative variation (Ikeda *et al.*, 1985).

The genetic similarity between astringent type persimmon cultivars were between 0.79 to 0.84 and similarity within a type was greater than those between types (Table 4). Within the same type of fruit the similarities were 0.86 among PCNA, 0.83 among PVNA, 0.83 among PCA and 0.80 among PVA (Table 4). Cluster analysis based on 80 RAPD markers could not distinguish accessions between types of fruit (Figure 2), while the RFLP markers could distinguish PCNA cultivars of Japanese origin from non-PCNA ones (Kanzaki *et al.*, 2000).

Genetic relationships among the cultivars 'Fuyu', 'Jiro', 'Hyakume', 'Xichu' and 'Nightingale'

In Thailand, the cultivars 'Fuyu', 'Jiro', 'Hyakume', 'Xichu' and 'Nightingale' are important for commercial production. These cultivars were repeatedly introduced from many countries and propagated to various places, leading to confusion in the investigation of the cultivar's characteristics and to mislabelling of experimental materials.

The average genetic similarity among the cultivars 'Fuyu', 'Jiro', 'Hyakume', 'Xichu' and 'Nightingale were between 0.80 to 0.87 (Table 5). The average genetic similarity within cultivars 'Fuyu' consisting of 13 accessions (numbers 1 to 13, Table 1) was 0.86 (Table 5) and of them

Table 4 Average and standard error of Dice similarity coefficients of persimmons within and between member of accessions of each fruit type

Fruit type*	PCNA	PVNA	PCA	PVA
PCNA	0.86 ± 0.06			
PVNA	0.79 ± 0.06	0.83		
PCA	0.83 ± 0.07	0.83 ± 0.04	0.83 ± 0.09	
PVA	0.82 ± 0.10	0.84 ± 0.06	0.82 ± 0.08	0.80 ± 0.09

*Refer to Table 1

Table 5 Average and standard error of Dice similarity coefficients of persimmons within and between 'Fuyu', 'Jiro', 'Hyakume', 'Xichu' and 'Nightingale' cultivars

Cultivar	Fuyu	Jiro	Hyakume	Xichu	Nightingale
Fuyu	0.86 ± 0.06				
Jiro	0.84 ± 0.07	0.99 ± 0.01			
Hyakume	0.83 ± 0.04	0.83 ± 0.03	0.82		
Xichu	0.87 ± 0.05	0.87 ± 0.01	0.87 ± 0.02	0.97 ± 0.02	
Nightingale	0.82 ± 0.05	0.84 ± 0.08	0.83 ± 0.02	0.85 ± 0.04	0.84 ± 0.12

had the same DNA fingerprint. In addition, 'Fuyu Taiwan-AK'which was closely clustered with 'Tone Wase-AK' (Figure 2) might have been mislabeled in the field. The result showed that these introduced 'Fuyu' cultivars were not identical clone; therefore, any experiment dealing with cultivars 'Fuyu' will need to specify which clone was used.

The genetic similarity within cultivars 'Hyakume' consisting of two accessions (number 27 to 28, Table 1) was 0.82 (Table 5). The genetic dissimilarity indicated that mislabeling of these materials had taken place.

The average genetic similarity within cultivars 'Jiro' consisting of three accessions (number 30 to 32, Table 1) was 0.99 (Table 5). Two accessions ('Jiro-KH' and 'Jiro-MH') were identical (Figure 2), while the other, 'Jiro-AK', differed with one RAPD marker.

The average genetic similarity within cultivars 'Xichu' consisting of four accessions (number 14 to 17, Table 1) was 0.97 (Table 5). There was no genetic difference of 'Xichu' accessions collected from MH and AK (Figure 2). This cultivar was introduced from Taiwan as seedlings; therefore, the difference observed would be a result of segregation among seedlings.

The average genetic similarity coefficients within cultivars 'Nightingale' consisting of three accessions (number 19 to 21, Table 1) was 0.84 (Table 5). 'Nightingale-AK' was clustered apart from 'Nightingale-MH' and 'Nightingale-DP' (Figure 2). This raised a suspicion on mislabeling of the materials. Authentic sample will be needed for reanalysis in order to verify the correct accession.

The observed genetic similarity coefficient within cultivars 'Fuyu', 'Hyakume', 'Jiro', 'Xichu' and 'Nightingale' accessions proved that different genotypes existed. This result confirmed with the report in a Royal Project Foundation newsletter describing phenotypic differences of fruit characters such as size and calyx among identical cultivar names in various places. Therefore, the reported phenotypic differences among identical cultivar names were results of not only environmental variation but also different genotype.

From this study RAPD markers were enough reliable to detect differences between the genetically close accessions of persimmon which agreed with other works in other fruit crops such as almond (Bartolozzi *et al.*, 1998), plum (Boonprakob *et al.*, 2001), grapevine (Ulanovsky *et al.*, 2002).

Conclusions

Genetic relationships of persimmon within *D. kaki* in Thailand showed similarity coefficient greater than 0.70. *D. kaki* related to *D. lotus* (0.45) as much as to *D. glandulosa* (0.44), while *D. lotus* related to *D. glandulosa* about 0.39. There was genetic difference of cultivars 'Fuyu', 'Hyakume' and 'Nightingale' collected from all places and no genetic difference of cultivar 'Jiro' collected from KH and cultivar 'Xichu' collected MH and AK.

RAPD markers are enough reliable mean to detect difference between the genetically close accessions of persimmon.

This result could be very useful for persimmon production and research in Thailand by properly selecting the superior clone for bud-wood propagation and precisely identifying experimental materials.

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