# Analysis of genetic diversity among persimmon cultivarsusing microsatellite markers

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# Analysis of genetic diversity among persimmon cultivars using microsatellite markers

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**Abstract** In the Mediterranean area, the production of persimmon (*Diospyros kaki* Thumb) [2*n*=6*x*=90] has increased recently as an alternative to the major fruit crops. In Spain, production relies almost exclusively on the cultivar "Rojo Brillante" which accounts for 83% of the crop. A crop based on a monovarietal culture implies several commercial risks that can compromise the future of the crop. Although the species was introduced in Europe very recently, it is well adapted to the climate of southern Europe. However, the recent introduction from Japan, the mistakes on the identity of varieties in the collections due to a bad translation of variety names from Japanese, and the lack of genetic characterization of many varieties have caused difficulties for effective management of the available genetic resources. The present paper was aimed at exploring the genetic diversity among different persimmon cultivars, including those collected in the European survey as well as Japanese cultivars. Seventy-one persimmon cultivars coming from two European collections that included accessions from Japan, Italy, and Spain were analyzed using 19 polymorphic microsatellite markers. A total of 206 alleles were obtained, with a mean value of 10.8 alleles per locus. A neighbor joining dendrogram and a principal coordinate analysis arranged the cultivars according to their genetic relationships. Analysis of molecular variance revealed significant genetic variability between and within groups, 73.3% and 85.2% for astringent-type and country origin, respectively. The simple sequence repeat markers classified the persimmon cultivars according to their genetic relationship.

**Keywords** *Diospyros kaki* Thumb. · Genetic diversity · SSR markers · Cluster analysis

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## Introduction

Persimmon belongs to the genus *Diospyros* in the family Ebenaceae. Although the genus contains more than 400 species with ploidy levels ranging from diploid (2*n*=2*x*=30) to nonaploid (2*n*=9*x*=135), the most widespread cultivated species in the world is *Diospyros kaki* Thumb., which is mostly hexaploid (2*n*=6*x*=90) and originated in Eastern Asia. Since the introduction of persimmon in Spain, local varieties have been developed that reflect the influence of natural (random seedlings and bud mutations) and human selection (cross breeding). The culture of these local varieties has been limited by the astringency of the fruits; this trait is a handicap for marketing the fruits long distances. The "Rojo Brillante" cultivar, with outstanding fruit quality, along with a successful technique for removing astringency without losing fruit firmness, allowed expansion of the culture in the 1990s, especially in the area of "Ribera del Xúquer" in Valencia province (Eastern Spain). Although the origins of this variety are uncertain, it is believed to come from a bud mutation found in a local variety named "Cristalino" in the 1940s (Llácer et al. 2008). The "Rojo Brillante" cultivar currently represents 96% of the total persimmon production

in Valencia and 83% of the total Spanish production. In this context, the availability of new varieties that would avoid the risk of relying the crop on one variety would be desirable. In order to meet these goals, the "Instituto Valenciano de Investigaciones Agrarias" (IVIA, Spain) has recently started a persimmon breeding program focused on the development of new varieties with the positive agronomic features of the cultivar "Rojo Brillante" but with more diversity in ripening date, astringency, and fruit characteristics.

Germplasm collections are a source of genetic diversity to support crop improvement and botanical research as well as support conservation efforts (Greene and Morris 2001). There are only two germplasm collections in Europe. The first was established by the Department of Horticulture from the University of Florence (Italy); they have been collecting persimmon accessions for the last 30 years. The second was established at IVIA in 2000 under the framework of the European program GENRES29, aimed at the conservation of underutilized fruit tree species (Bellini and Giordani 2000) in 2000. Only partial molecular characterization of the Italian collection is available.

One of the main problems regarding diversity management of persimmon resources is the assignment of cultivar identity due to the existence of synonyms and homonyms among local varieties, misleading transliterations from Japanese, and incorrect labeling in the past. Traditionally, the method for cultivar identification was based on morphology of leaves, bud, flower, seed, and fruit characters (UPOV 2004). The main characteristics used as references for cultivar classification are the fruit astringency loss and the change in flesh color, resulting in the recognition of four groups of cultivars (Yonemori et al. 2000): pollination-constant non-astringent (PCNA), pollination-variant non-astringent (PVNA), pollination-constant astringent (PCA), and pollination-variant astringent (PVA). The limitations of phenotype-based genetic markers led to the development of DNA-based markers. Molecular markers are independent of environment and of pleiotropic and epistatic effects, providing new tools to support cultivar identification. So far, different DNA-based marker techniques such as RFLP (Kanzaki et al. 2000a; Maki et al. 2001), random amplification of polymorphic DNA (RAPD; Luo et al. 1995; Badenes et al. 2003; Yamagishi et al. 2005), and amplified fragment length polymorphism (AFLP; Kanzaki et al. 2000b; Yonemori et al. 2008a, b) have been applied to asses the genetic diversity and relationships between *Diospyros* species. Moreover, molecular markers based on PCR developed from retrotransposon sequences have also been employed (Du et al. 2009). Nevertheless, the relationships between persimmon accessions are still not completely clarified in spite of all previous efforts, probably because of the low resolution of the molecular markers previously employed in terms of polymorphic alleles found at a single locus. Although simple sequence repeat (SSR) markers have been developed for persimmon (Soriano et al. 2006), to date, no report concerning the use of SSR (also known as microsatellite) markers in genetic diversity of persimmon have been published. SSR markers evenly spaced across the genome offer an ideal tool for such purpose as they have other desirable properties of a molecular marker such as being highly polymorphic, reproducible, abundant, and co-dominant (Powell et al. 1996). However, complex ploidy levels of some species, as is the case of *D. kaki*, can make it difficult to identify the full genotype of each sample. In these cases, the presence or absence of bands has been employed as scoring system, thereby effectively employing SSRs as dominant markers (Khlestkina et al. 2004; Al-Khanjari et al. 2007).

In this paper, our aim was to asses the genetic diversity among 71 persimmon cultivars originated in Japan, Italy, and Spain using microsatellite markers from persimmon developed by Soriano et al. (2006). The use of molecular markers will provide valuable information for genebank management and will allow the broadening of the genetic resources of the species through breeding.

## Materials and methods

### Plant materials and DNA extraction

Seventy-one cultivars of *D. kaki* Thumb. from Japan (46), Spain (14) and Italy (11) were evaluated in this study (Table 1). *Diospyros lotus* L. and *Diospyros virginiana* L. were used as reference outgroups. The plant materials were obtained from the persimmon germplasm collection at IVIA (Valencia, Spain) and the collection belonging to the Department of Horticulture in Florence University. Young, fully expanded leaves were collected from mature trees and kept at −20°C until DNA extraction. DNA was extracted according to the CTAB method of Doyle and Doyle (1987) with minor modifications (Soriano et al. 2006).

### Microsatellite analysis

All plants were screened for variation at 19 polymorphic microsatellite loci developed for *D. kaki* by Soriano et al. (2006) (Table 2). Each polymerase chain reaction was performed with three primers: the specific forward primer of each microsatellite with M13(-21) tail at its 5′ end, the sequence-specific reverse primer, and the universal fluorescent-labeled M13(-21) primer (Schuelke 2000).

PCR conditions were performed as described by Soriano et al. (2006). Allele lengths were determined using an ABI Prism 3130 Genetic Analyzer with the aid of GeneMapper software, version 4.0 (Applied Biosystems).

**Table 1** List of persimmon cultivars analyzed in this study

| Number | Cultivar | Abbreviations | Types | Origin |
|---|---|---|---|---|
| 1 | Garidells | Garide | PCA | Spain |
| 2 | Ferran 12 | Fer12 | PCA | Spain |
| 3 | Aneka | Aneka | PCA | Spain |
| 4 | Tomatero | Tomate | PCA | Spain |
| 5 | CristalinoB | CristaB | PCA | Spain |
| 6 | Reus 6 | Reus6 | PCA | Spain |
| 7 | Reus 15 | Reus15 | PVA | Spain |
| 8 | Xato Bonrepos | XatBon | PVA | Spain |
| 9 | Rojo Brillante | RojBri | PVA | Spain |
| 10 | Picudo | Picu | PVA | Spain |
| 11 | Betera 2 | Bet2 | PVA | Spain |
| 12 | Betera 3 | Bet3 | PVNA | Spain |
| 13 | La Selva | LaSel | PVNA | Spain |
| 14 | Constanti | Consta | PVNA | Spain |
| 15 | Costata | Costa | PCA | Italy |
| 16 | Lampadina | Lampa | PVNA | Italy |
| 17 | Kaki Tipo | KakTi | PVNA | Italy |
| 18 | Acerra 1 | Ace1 | PVNA | Italy |
| 19 | Acerra 4 | Ace4 | PVNA | Italy |
| 20 | Acerra 5 | Ace5 | PVNA | Italy |
| 21 | Cioccolatino | Ciocco | PVNA | Italy |
| 22 | Castellani | Caste | PVNA | Italy |
| 23 | Rispoli | Rispo | PVNA | Italy |
| 24 | Vainiglia | Vaini | PVNA | Italy |
| 25 | Mandarino | Manda | PVNA | Italy |
| 26 | Gionbo | Gion | PCA | Japan |
| 27 | Takura | Taku | PCA | Japan |
| 28 | Atago | Atago | PCA | Japan |
| 29 | *Guilbecky* | Guilbe | PCA | Japan |
| 30 | Kawabata | Kawa | PCA | Japan |
| 31 | Aizumishirazu A | AizuA | PCA | Japan |
| 32 | Fuji | Fuji | PCA | Japan |
| 33 | Hachiya | Hachi | PCA | Japan |
| 34 | Jiro | Jiro | PCNA | Japan |
| 35 | Jiro C24267 | JC24267 | PCNA | Japan |
| 36 | Ichikikey Jiro | IchJiro | PCNA | Japan |
| 37 | Maekawa Jiro | MaeJiro | PCNA | Japan |
| 38 | *Mukaku Jiro* | MukJiro | PCNA | Japan |
| 39 | O´Gosho | Ogos | PCNA | Japan |
| 40 | Fukuro Gosho | FukGos | PCNA | Japan |
| 41 | Yamato Gosho | YamGos | PCNA | Japan |
| 42 | Fujiwara Gosho | FujGos | PCNA | Japan |
| 43 | Suruga | Suru | PCNA | Japan |
| 44 | Nishijo | Nishi | PCNA | Japan |
| 45 | Midai | Midai | PCNA | Japan |
| 46 | Isahaya | Isaha | PCNA | Japan |
| 47 | Benisakigake | Benisa | PCNA | Japan |
| 48 | Fuyu | Fuyu | PCNA | Japan |
| 49 | Matsumoto Wase Fuyu | MaWaFu | PCNA | Japan |

**Table 1** (continued)

| Number | Cultivar | Abbreviations | Types | Origin |
|---|---|---|---|---|
| 50 | Cal Fuyu | CalFu | PCNA | Japan |
| 51 | Koda Gosho | KodGos | PCNA | Japan |
| 52 | Bangosho | BanGos | PCNA | Japan |
| 53 | Mikado | Mika | PCNA | Japan |
| 54 | Hana Fuyu | HanaFu | PCNA | Japan |
| 55 | Aizumishirazu B | AizuB | PVA | Japan |
| 56 | Hiratanekaki | Hiraka | PVA | Japan |
| 57 | Pakistan Seedless | PaSeed | PVA | Japan |
| 58 | Koshu Hyakume | KoHyaku | PVA | Japan |
| 59 | Hiratanenashi | Hira | PVA | Japan |
| 60 | Sugita Hiratanenashi | SuHira | PVA | Japan |
| 61 | Tonewase | TonWas | PVA | Japan |
| 62 | Tone Hiratanenashi | ToHira | PVA | Japan |
| 63 | Sugita Wase | SuWas | PVA | Japan |
| 64 | Zenjimaru | Zenji | PVNA | Japan |
| 65 | Mikatani Gosho | MikGos | PVNA | Japan |
| 66 | Agakaki | Agaka | PVNA | Japan |
| 67 | Amankaki | Amanka | PVNA | Japan |
| 68 | Amahyakume | Amahy | PVNA | Japan |
| 69 | Hyakume | Hyaku | PVNA | Japan |
| 70 | Edoichi | Edoi | PVNA | Japan |
| 71 | Hirotakaki | Hiroka | PVNA | Japan |
| *Diospyros lotus* | | *D. lotus* | Outgroup spp. | |
| *Diospyros visginiana* | | *D. virginiana* | Outgroup spp. | |

### Analysis of genotype data

For each microsatellite, the presence or absence of each single fragment was coded as 1 or 0, respectively, to generate a binary data matrix.

In order to evaluate the informativeness of the microsatellites employed, the number of alleles per locus and the polymorphism information content (PIC) were calculated. PIC was calculated according to Weir (1990) based on allele frequencies of all of the varieties analyzed as: $\mathrm{PIC}_i = 1 - \sum P_{ij}^2$, where $P_{ij}$ is the frequency of the $j$th allele for the $i$th marker locus and summation extends over $n$ alleles.

Pairwise genetic similarities were estimated with the Dice (Sorensen) similarity coefficient $S_{ij} = 2a/(2a + b + c)$, where $a$ is the number of bands shared by $i$ and $j$, $b$ is the number of bands present in $i$ and absent in $j$, and $c$ is the number of bands present in $j$ and absent in $i$. The resulting genetic similarity matrices were used to generate a principal coordinates analysis using the NTSYSpc2.0 software package (Applied Biostatistics Inc.).

Bootstrapped distance matrices were calculated using the Phyltools 1.32 software (Buntjer 1997) and used to test the stability of the neighbor joining tree constructed with the Phylip 3.62 package (Felsenstein 2005).

To test the goodness of fit of the NJ clustering, a cophenetic value matrix was estimated using homemade scripts based on R software v.2.6.0 (R Development Core Team 2007). Then, it was compared with the original similarity matrix using the Mantel matrix correspondence test (Mantel 1967) using $h$.

The significance of the partitioning of genetic variance among cultivar types was further investigated by an analysis of molecular variance (AMOVA) using ARLEQUIN 3.0 (Excoffier et al. 2005). Also, microsatellite variation across persimmon groups acording to their origin was analyzed.

## Results

### Genetic variation of SSR markers

A total of 206 alleles resulted from the analysis of the genetic variation in 71 cultivars of persimmon by 19 SSR polymorphic markers ranging in size from 115 to 365 bp. Figure 1 shows the results obtained at loci ssrdk02. An

**Table 2** Summary of microsatellite allele data revealed by 19 microsatellite loci in 71 cultivars of *Diospyros kaki*

| Marker | Repeat motif | Primers (5′-3′) | Allele size (bp) | No. of alleles | Rare alleles | Unique alleles | PIC |
|---|---|---|---|---|---|---|---|
| ssrdk01 | $(AG)_{19}$ | F: CGGCATGAAGGAATAAGGAA<br>R: GCTCACATTCCAACCAATCA | 155–184 | 12 | 3 | 0 | 0.8926 |
| ssrdk02 | $(GA)_{17}$ | F: TTAATTTGGACACAAGTTCT<br>R: TCTCTTCAAGTCTTCTATCCT | 196–224 | 13 | 4 | 0 | 0.8662 |
| ssrdk03 | $(AG)_{16}$ | F: GGCTCTCGGTCAAATAGTAG<br>R: GGAGGTTAGAAATCCAGCTA | 158–198 | 20 | 7 | 1 (Kawabata) | 0.9275 |
| ssrdk04 | $(GA)_{17}$ | F: CATTTGAAAGCAGTCGTCCA<br>R: GCGCCAAATCATTGCTATCT | 336–365 | 9 | 3 | 0 | 0.8245 |
| ssrdk06 | $(AG)_{19}$ | F: CGGCATGAAGGAATAAGGAA<br>R: GCTCACATTCCAACCAATCA | 158–187 | 12 | 3 | 0 | 0.8901 |
| ssrdk09 | $(AG)_{13}$ | F: ATGCCTCAAGCCTGTCATTT<br>R: GACATCCCTGTCATTTGAGGA | 137–190 | 8 | 4 | 0 | 0.7527 |
| ssrdk10 | $(GA)_{15}$ | F: CGACACTGATGGTTGATAAG<br>R: CAGCTTCACCTCCTAGAGAC | 193–224 | 7 | 2 | 0 | 0.8164 |
| ssrdk14 | $(AG)_{16}$ | F: GTGAAGGAACCCCATAGAA<br>R: CCATCATCAGGTAGGAGAGA | 155–178 | 10 | 6 | 1 (Takura) | 0.8433 |
| ssrdk15 | $(GA)_{9}$ | F: AGAGAACAGAGAGGGAATAG<br>R: TTGGGATTAGTTGATTGTAG | 235–254 | 9 | 4 | 1 (Xato Bonrepós) | 0.7930 |
| ssrdk16 | $(GA)_{12}$ | F: ACTACAACGGCGGTGAGAAC<br>R: GTCCTTCACTTCCCGCATT | 134–173 | 10 | 2 | 0 | 0.8404 |
| ssrdk17 | $(GA)_{19}$ | F: GGTGTTGGGATATTAATGCT<br>R: CTGCAGATTATAGGCACAAA | 138–168 | 7 | 1 | 0 | 0.7715 |
| ssrdk25 | $(CT)_{15}$ | F: GGGGTAATATGAATTGAATC<br>R: CTCAGAGAGGAGAAGAAATAG | 229–283 | 11 | 4 | 1 (Hirotakaki) | 0.8615 |
| ssrdk26 | $(GA)_{15}$ | F: GGGAAATTAAGAGGGAAGAA<br>R: AGGAACTGGATCAGCATAAA | 152–202 | 16 | 8 | 2 (Gionbo y Reus15) | 0.8784 |
| ssrdk28 | $(GA)_{16}$ | F: CGAGCAAGTAGATGTTTATT<br>R: TCATGATGATTAAAGAGGAC | 176–199 | 7 | 3 | 0 | 0.7955 |
| ssrdk29 | $(CCTTT)_{8}$ | F: ATCATGAGATCAGAGCCGTC<br>R: CACGTTAACGTTACGGAACA | 115–150 | 3 | 1 | 0 | 0.5135 |
| ssrdk30 | $(TG)_{9}(AG)_{17}$ | F: TGGTGATCGTGGTAGTGGTT<br>R: GGCCTAATCTCTGTCCATCC | 137–275 | 13 | 4 | 2 (Takura y Reus15) | 0.8637 |
| ssrdk32 | $(GA)_{8}$ | F: TAGAGCGGGAAAGATCGAGA<br>R: TACTTGGCGAGCAGTTAGCA | 147–201 | 9 | 8 | 0 | 0.7883 |
| ssrdk36 | $(GA)_{16}$ | F: GGGAAGAACAAAGAGAACTG<br>R: ACGAAGTTGTAATCCTGAGC | 226–259 | 13 | 3 | 1 (Takura) | 0.8816 |
| ssrdk37 | $(CT)_{10}$ | F: CAAAATGAAGCCCATAAGAC<br>R: GTGAAAGTGTGGTTGGATTT | 154–211 | 17 | 3 | 0 | 0.9237 |
| Mean | | | | 10.84 | 3.89 | | 0.8276 |
| Total | | | | 206 | 74 | 9 | |

Range of fragment size, allele number, rare alleles (freq < 0.02), alleles exclusive for one accession (name between parentheses), and PIC

average of 10.8 alleles per locus was obtained, ranging from three alleles (ssrdk29) to 20 alleles (ssrdk03) (Table 2). Rare and unique alleles were also observed in the analysis. The number of rare alleles (frequency < 0.02) varied from one (ssrdk17 and ssrdk29) to eight (ssrdk26 and ssrdk32), with a total of 74 and an average value of 3.89 (Table 2). Moreover, nine unique alleles, i.e., amplified in just one accession, were found at seven marker loci (Table 2).

The PIC results for each marker confirmed their utility to show differences between the samples analyzed in this study (Table 2). PIC values ranged from 0.513 (ssrdk29) to 0.927 (ssrdk03), with an average value of 0.827. Fifteen of the 19 markers were highly polymorphic, having a PIC value equal to or higher than 0.78. The PIC values of a locus were associated with the number of alleles detected; for instance, the highest PIC value corresponded to the ssrdk03 with 20 alleles and the lowest corresponded to the ssrdk29 with three alleles, which corresponded to a locus amplifying penta-nucleotide repeat motifs.

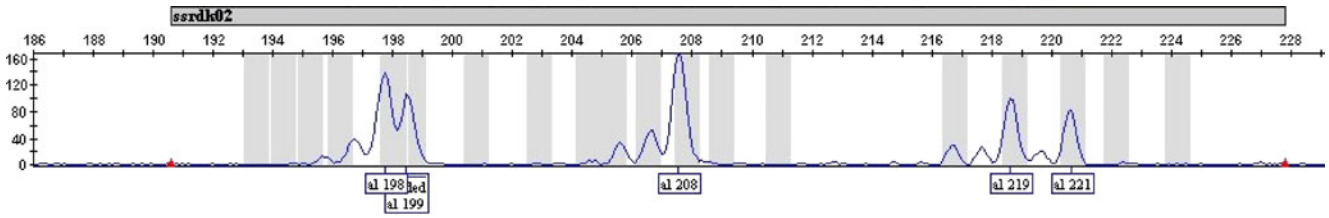


**Fig. 1** Electropherogram obtained with the SSR ssrdk02

## Genetic diversity within and between astringent types of persimmon

The number of polymorphic alleles varied between groups according to the astringency status groups PCNA, PCA, PVA, and PVNA (Table 3). Nine unique alleles were found in PCA cultivars (five), PVA cultivars (three), and PVNA cultivars (one). PVNA revealed the highest number of rare alleles (35), followed by PVA (29), PCNA (25), and PCA (19).

The gene diversity (*h*; Nei 1973) and PIC value of the four persimmon groups ranged from 0.210 (*h*) and 0.86 (PIC) at PVA cultivars to 0.155 (*h*) and 0.77 (PIC) at PVNA cultivars (Table 3). Nei's (1972) genetic distance values varied from 0.0395 (PCNA vs. PCA) to 0.0868 (PCNA vs. PVNA; Table 4). Based on AMOVA, 26.69% of the total variation resided between persimmon types and 73.31% was present within groups (Table 5), which indicated that the type of astrincency is not a trait that determined the diversity into persimmon.

Another AMOVA analysis was made grouping the accessions by their geographic origin. In this case, 14.85% of the total variation resided between country of origin and 85.15% was present within groups (Table 5), which agrees with the fact that persimmon spread to non-Asian countries is very recent because not much diversity has been originated in European countries.

## Cluster and principal component analysis

Based on microsatellite data, genetic distances among persimmon accessions were used to generate a neighbor joining cladogram (Fig. 2). The cophenetic correlation between the original similarity matrix and those given by the clustering process ($r$=0.93) indicates quite a good fit, being statistically significant at the 1% level according to Lapointe and Legendre (1992). The cladograme showed six major clades in general agreement with their geographic origins (Spain, Italy, and Japan) and astringent type of cultivar (PCNA, PVNA, PCA, or PVA). Clader 1 (C1) consisted of Japanese PCNA genotypes and contained three highly supported subclusters (bootstrap >70%). The first subcluster (C1a) included "Fuyu," "Matsumoto Wase Fuyu" (the early ripening bud sport of "Fuyu"), "Isahaya" (a large fruited selection of "Fuyu"), and "Benisakigake." The second one (C1b) included "Jiro" and different bud sports of it, "Ichikikey Jiro," "Mukaku Jiro," "Maekawa Jiro," and the clonal selection "Jiro C24267." The third one (C1c) included "Cal Fuyu," a Californian cultivar PCNA introduced from Japan, but not releated to "Fuyu" (Parfitt et al. 1991), "Bangosho," and "Kodagosho," and close to them appear "Midai," "Suruga," and "Fujiwara Gosho," but without a high support. Cluster 2 (C2) consisted mainly of PCA cultivars with the exception of the PVA cultivar "Reus 15" from Spain. Almost all accessions of this cluster form a

**Table 3** Estimates of diversity in the different astringent types of persimmon based on microsatellite (*h* = Nei (1973) gene diversity)

| Group | Non-PCNA | | | | PCNA | Total |
|---|---|---|---|---|---|---|
| | PCA | PVA | PVNA | All non-PCNA | | |
| No. of accessions | 15 | 14 | 21 | 50 | 21 | 71 |
| No. of amplified alleles | 184 | 174 | 160 | 206 | 171 | 206 |
| No. of polymorphic alleles | 178 | 161 | 147 | 202 | 162 | 203 |
| Average no. of alleles/marker | 9.68 | 9.16 | 8.42 | 10.84 | 9.00 | 10.84 |
| No. of rare alleles | 19 | 29 | 35 | 46 | 25 | 74 |
| No. of unique alleles | 5 | 3 | 1 | 9 | 0 | 9 |
| h (Nei 1973) | 0.207 | 0.210 | 0.155 | 0.217 | 0.190 | 0.224 |
| Average PIC value | 0.81 | 0.86 | 0.77 | 0.82 | 0.80 | 0.83 |

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

**Table 4** Pairwise Nei (1972)) genetic distance between the different astringent types of persimmon

| | PVNA | PCA | PVA |
|---|---|---|---|
| PVNA | – | – | – |
| PCA | 0.0769 | – | – |
| PVA | 0.0509 | 0.0465 | – |
| PCNA | 0.0868 | 0.0395 | 0.0606 |

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

highly supported group (C2a, 81%), including four Spanish cultivars ("Tomatero," "Aneka," "Reus 6," and "Ferran 12") and two Japanese cultivars ("Guilbecky" and "Aizumishirazu A"). Cluster 3 (C3) consisted mainly of Japanese PCA cultivars and close to them two cultivars, "Costata" from Italy and "Garidells" from Spain, appeared grouped with a 100% bootstrap value. Cluster 4 (C4) comprised Japanese cultivars and divided into two highly significant subclusters, PCNA cultivars (C4a), "Hana Fuyu," "Mikado, "and "O'Gosho" (94%) and non-PCNA cultivars (C4b), "Koshu Hyakume" (PVA), "Hachiya" (PCA), and "Fuji" (PCA; 100%). Cluster 5 (C5), strongly supported with a bootstrap value of 100%, consisted of PVA cultivars from Japan, "Hiratanenashi," and different bud sports of this cultivar, "Sugita Hiratanenashi," "Tone Hiratanenashi," and "Hiratanekaki," and two early ripening bud sports, "Tonewase" and "Sugita Wase." Finally, cluster 6 (C6) consisted of non-PCNA cultivars, with the exception of the PCNA "Fukuro Gosho," and contained the majority of European genotypes analyzed in this study. This cluster was further divided into four subclusters highly supported. The first one (C6a) included three Italian PVNA cultivars, "Lampadina," "Acerra 4," and "Cioccolatino" at a bootstrap probability of 100%. The second one (C6b) included two PCA cultivars, "Atago" from Japan and "Cristalino B" from Spain, and three PVA cultivars, "Pakistan Seedless" from Japan and "Picudo" and "Rojo Brillante" from Spain (100%). The third one (C6c) included PVNA cultivars, "Amankaki," "Amahyakume," "Hyakume," and "Edoichi" from Japan, "Kaki Tipo," "Castellani," and "Acerra 1" from Italy and "Betera 3," "La Selva," and "Constanti" from Spain (100%).

**Table 5** AMOVA considering variation between and within two structure models: astringent types of persimmon and country of origin

| Structure model | *df* | Among-group component (%) | Within-group component (%) |
|---|---|---|---|
| Astringency type | 3 | 26.69 | 73.31 |
| Country origin | 2 | 14.85 | 85.15 |

Finally, the fourth one (C6d) included PVNA cultivars, "Rispoli," "Mandarino," "Vainiglia," and "Acerra 5" from Italy and "Zenjimaru" from Japan (100%).

Microsatellite data were subjected to a principal component analysis in order to obtain an alternative view of the relationships between the accessions (Fig. 3). As expected, this analysis agrees roughly with the neighbor joining dendrogram.

The first three principal components accounted for 36.52% of the total variance. It agrees with the fact that the loci are unlinked and assorting independently. Some degree of grouping by types of persimmon and origin was revealed in the principal component analysis (Fig. 3a, b). Almost all PCNA accessions, except "Yamato Gosho" and "Fukuro Gosho," appeared together without subgroups; only "Jiro" and different bud sports of this cultivar appear closer (subcluster C1b). The rest of the persimmon types appeared more structured. Some Spanish and one Japanese PCA accessions formed a compact group separated from the rest by the second component (C2a). The rest of PCA accessions appeared more dispersed and mixed with other types of persimmon.

PVNA accessions formed four subgroups. The first was formed by the majority of the accessions (C6c) and appeared separated from the rest by the first component. Close to them appeared one Japanese PCNA accession ("Fukuro Gosho"). The second one consisted of four Italian and one Japanese accessions (C6d). The third subgroup, formed by three PVNA Italian accessions (C6a), was intermingled with other PCA and PVA accessions from Spain and Japan (C6b). The last subgroup was formed by three Japanese accessions ("Agakaki," "Mikatani Gosho," and "Hirotakaki") and was close to the PCNA group.

Most of the PVA accessions ("Hiratanenashi" and different bud sports of this cultivar) formed a group clearly separated by the third component (C5).

## Discussion

SSR markers have been used to evaluate the levels of genetic diversity among 71 persimmon cultivars from Japan, Italy, and Spain. The 19 SSR polymorphic markers used were highly informative and revealed an average of 10.8 alleles per locus. One characteristic of previous persimmon genetic diversity studies was the high proportion of unclassified variation found among the cultivars. In contrast, the SSRs have allowed classification of the majority of persimmon cultivars into moderately to well-supported (bootstrap > 50%) clusters and sub-clusters.

In this study, a diverse sample of cultivars has been chosen in relation to their astringency; even if the low genetic diversity among PCNA-type cultivars has been

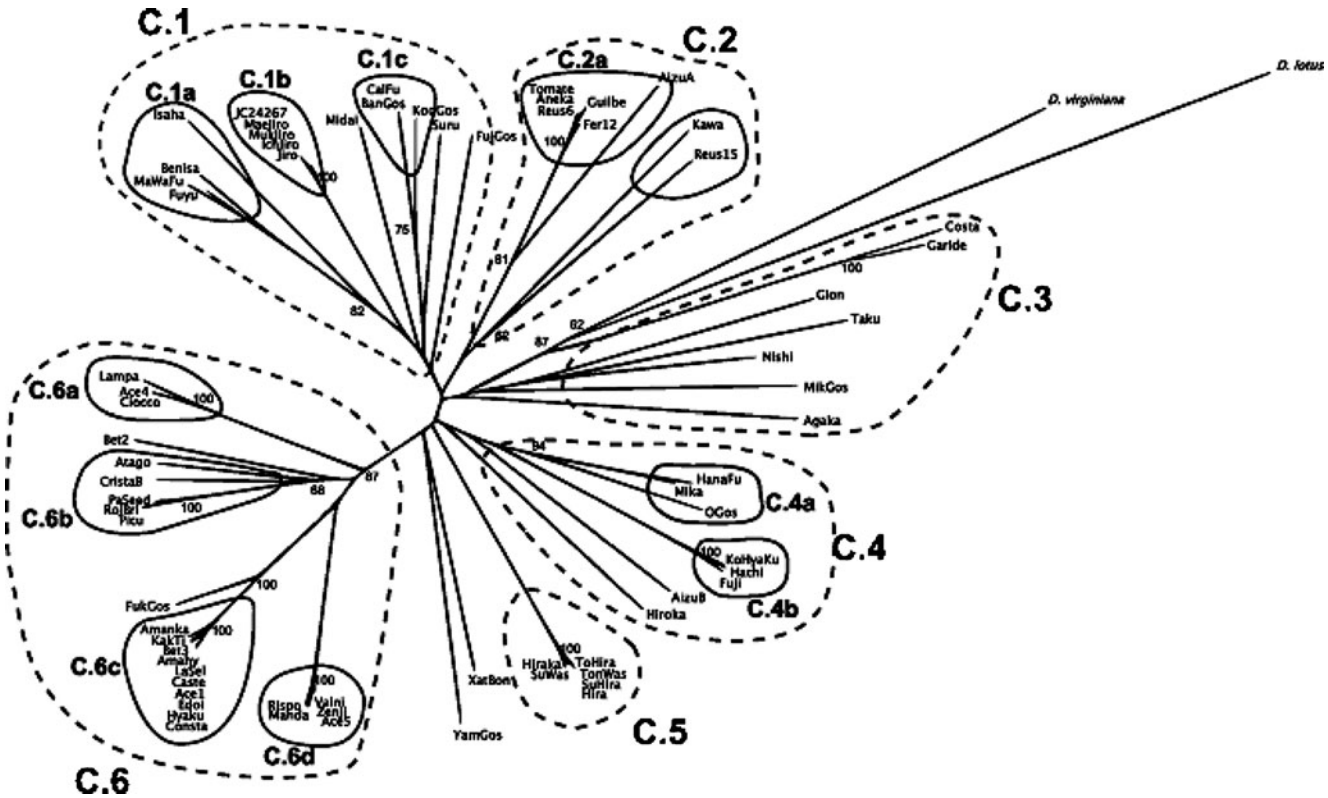


**Fig. 2** Cladrogram obtained by neighbor joining analysis for 71 persimmon cultivars based on SSR markers. Bootstrap values >50% are shown in the tree

confirmed previously (Kanzaki et al. 2000b), the gene diversity in all different type groups is very similar, from 0.210 (*h*) in PVA cultivars to 0.155 (*h*) in PVNA cultivars. Rare (frequency < 0.02) and unique alleles were observed in the study. These alleles can be considered markers of interest to separately identify cultivars. This is a major issue in non-Asian countries where the introduction of persimmon varieties has lead to a high number of misidentified cultivars. The SSRs used in this study have allowed identification of several cultivars, among which "Kawabata" (PCA), "Takura" (PCA), "Gionbo" (PCA), "Xató de Bonrepós" (PVA), "Reus15" (PVA), and "Hirotakaki" (PVNA), with unique alleles.

Values of Nei's genetic distance between the astringent types groups ranged from 0.0395 to 0.0868. The highest genetic distance corresponded to PCNA and PVNA for the cultivars analyzed. In general, the PVNA cultivars show a wide range of genetic variation in morphological characters being classified as the most diverse group against the very narrow genetic variability of the PCNA-type group (Yamada et al. 1994).

In addition, AMOVA analysis determined that the highest percentage of the total genetic diversity was distributed within group (astringent-type or country origin), although a significant percentage of the diversity is attributed to differences between groups. This conclusion is similar to that expressed by Yonemori et al. (2008a) in a genetic diversity study with AFLPs on a similar set of persimmon varieties, and the authors indicated that this may be due to a high level of selection and/or the large number of characters scored.

Genetic similarities obtained from SSR data were used to create a cladogram, indicating an association between the SSRs patterns and the astringent-type and/or origin of cultivars. Cluster analysis placed most of the Japanese PCNA types together, showing a high level of genetic relatedness. Similar results were obtained by Yonemori et al. (2008a); the authors studied a total of 61 accessions, including five PCNA, and four were grouped together and one grouped with other astringency types. However, in another study Yonemori et al (2008b) analyzed a larger number of persimmon accessions including Japanese, Korean, and Chinese by AFLP markers. The results obtained showed a unique clade of PCNA Japanese cultivars, suggesting an independent evolution, although the authors did not present the bootstrap value that support the clades. In the present study, we obtained most of the PCNA accessions grouped in the clade C.1 (15 accessions out of 21). A subgroup is included in C.4.a, closely related to other Japanese cultivars. Although the clades seem apart in the figure, looking at the genetic distances are not as apart. The explanation of these close genetic distances

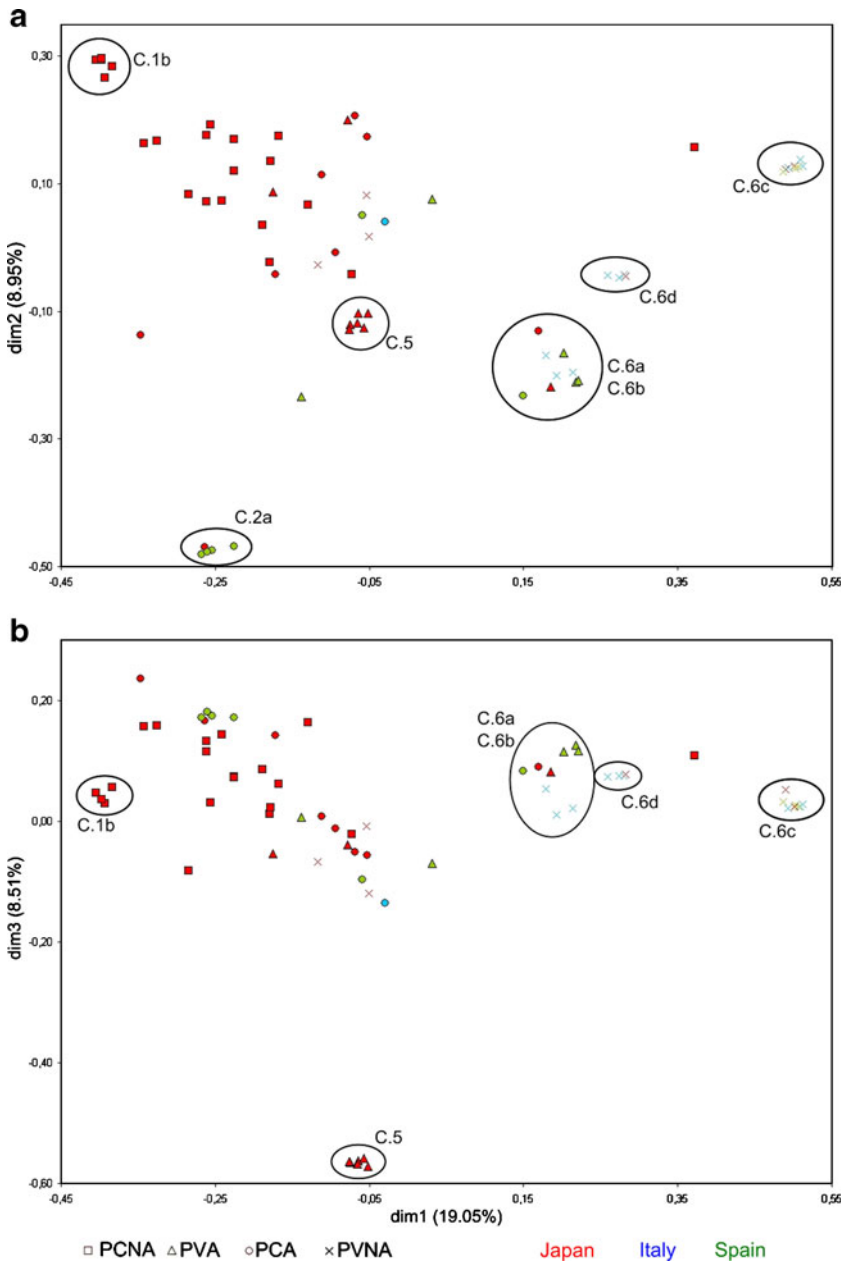


**Fig. 3** Scatter plot of 71 persimmon cultivars estimated with 19 SSR markers using the genetic similarity matrix. **a** Based on first and second components of principal coordinate analysis. **b** Based on first and third components of principal coordinate analysis. *Squares* indicate PCNA cultivars, *crosses* indicate PVNA cultivars, *circles* indicate PCA cultivars, and *triangles* indicate PVA cultivars

obtained in the present study could be that all accessions come from Japanese origin, including the Italian and Spanish cultivars that according to the records trace back to a Japanese introduction in the Mediterranean basin. As a result, the distances among clades are very small, but with a high support by bootstrap analysis. On the other hand, Du et al. (2009) studied 28 accessions which included 13 PCNA from China and five from Japan. The grouping according to the genetic distance obtained did not fit in the astringency type; it fits the geographical origin (Chinese and Japanese). Taking all facts into account, the results obtained in our study do not disagree with the very narrow genetic diversity into Japanese PCNA accessions. Additionally, all cultivars studied might share a Japanese origin which would explain the genetic distances and distribution of clades obtained. According to Yonemori et al. (2008a, b), the Italian and Spanish cultivars share a common gene pool. These authors obtained the placement of several Japanese cultivars within the European group, suggesting that European cultivars were developed from Japanese germplasm relatively recently.

In terms of cultivar identification, in the PCNA group, 19 SSR markers allowed us to discriminate related genotypes such as different cultivars and their bud sports. Among the genotypes studied, it is known that both "Matsumoto Wase Fuyu," an early ripening cultivar (Yonemori et al. 2000), and "Isahaya," a large fruited variety from Nagasaki patented in 1986, are mutations of

"Fuyu." Furthermore, "Jiro" and its bud sports ("Ichikikey Jiro," "Mukaku Jiro," "Maekawa Jiro," and the clonal selection "Jiro C24267") are clustered together. This suggests that the SSR analysis could be useful to solve cases of homonyms and synonyms, which are known to exist in the Spanish collection where cultivars selected in different places were named differently, although it is possible that they could be the same cultivar (Badenes et al. 2003). In previous studies of persimmon genetic diversity using two different molecular markers, isozymes (Romero et al. 2002) and RAPDs (Badenes et al. 2003), several groups shared the same isozyme pattern and the same RAPD marker profile, making it impossible to distinguish among closely related genotypes with those markers.

The non-PCNA Japanese cultivars appeared more dispersed. In some cases, they clustered with only Japanese clusters, as C5 (PVA cultivars), and in other cases, they associated with Spanish cultivars (C2a with all PCA types and C6b with PVA and PCA types), Italian cultivars (C6d with all PVNA cultivars), or cultivars from both Italy and Spain (C6c with all PVNA types). Spanish cultivars "Cristalino B," "Rojo Brillante," and "Picudo" cluster with Japanese cultivars "Atago" and "Pakistan Seedless" and Spanish cultivars "Tomatero," "Aneka," "Reus6," and "Ferrán12" with the Japanese "Guilbecky" and "Aizumishirazu A." This confirms, as was reported previously (Bellini and Giordani 2005), that the origin of certain European cultivars are closely associated with Japanese genotypes, even though the genus *Diospyros* is believed to have originated in China. It could indicate that some cultivar groups come from common Japanese progenitors or that Japanese cultivars were used in their development (Yonemori et al. 2000). The close relationship between some Italian and Spanish cultivars, such "Kaki Tipo" and "Constantí," could be due to one of the paths of the persimmon to Spain from Asia was through Italy.

At present, the collection at the IVIA (Valencia) contains 71 cultivars of the species *D. kaki*, and this number will be increased in the next years. Germplasm conservation strategies will be based on information about genotypes within and between groups, with the aim of preserving and increasing the biodiversity. The results of this study will allow developing strategies for managing persimmon germplasm. For example, taking into account that the main breeding objective in the program is to develop cultivars with diversity in ripening date, astringency, and fruit characteristics, it could be possible to choose suitable parental material for carrying out a crossbreeding program. For instance, to choose parents with the fruit traits desired but as diverged as possible is a better strategy that can be pursued if the diversity among cultivars is known.

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## References

Al-Khanjari S, Hammer K, Buerkert A, Röder MS (2007) Molecular diversity of Omani wheat revealed by microsatellites: I. Tetraploid landraces. Genet Resour Crop Evol 54:1407–1417

Badenes ML, Garcés A, Romero C, Romero M, Clavé J, Rovira M, Llácer G (2003) Genetic diversity of introduced and local Spanish persimmon cultivars revealed by RAPD markers. Genet Res Crop Evol 50:579–585

Bellini E, Giordani E (2000) Conservation of under utilised fruit tree species in Europe. Acta Hort 522:165–173

Bellini E, Giordani E (2005) Germplasm and breeding of persimmon in Europe. In: Park YM, Kang SM (eds) Proceeding of 3rd International Symposium on Persimmon. Acta Hort 685:65–75

Buntjer JB (1997) Phylogenetic computer tools (PhylTools), version 1.32 for Windows. Wageningen: Laboratory of Plant Breeding, Wageningen University, Wageningen

Doyle JJ, Doyle JL (1987) A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Du XY, Zhang QL, Luo ZR (2009) Development of retrotransposon primers and their utilization for germplasm identification in *Diospyros* spp. (Ebenaceae). Tree Genet Genom 5:235–245

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinf Online 1:47–50

Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle

Greene SL, Morris JB (2001) The case for multiple-use plant germplasm collections and a strategy for implementation. Crop Sci 41(3):886–892

Kanzaki S, Yonemori K, Sato A, Yamada M, Sugiura A (2000a) Evaluation of RFLP analysis for discriminating PCNA genotype in some persimmon cultivars. J Jpn Soc Hort Sci 69:702–704

Kanzaki S, Yonemori K, Sato A, Yamada M, Sugiura A (2000b) 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). J Jpn Soc Hort Sci 69:665–670

Khlestkina EK, Röder MS, Efremova TT, Börner A, Shumny VK (2004) The genetic diversity of old and modern Siberian varieties of common spring wheat as determined by microsatellite markers. Plant Breed 123:122–127

Lapointe FJ, Legendre P (1992) Statistical significance of the matrix correlation coefficient for comparing independent phylogenetic trees. Syst Biol 41:378–384

Luo ZR, Yonemori K, Sugiura A (1995) Evaluation of RAPD analysis for cultivar identification of persimmons. J Jpn Soc Hort Sci 64:535–541

Llácer G, Martínez-Calvo J, Naval M, Badenes ML (2008) From germplasm to fruit export: the case of 'Rojo Brillante' persimmon. Adv Hort Sci 22(4):281–285

Maki S, Oyama K, Kurahashi T, Nakahira T, Kawabata T, Yamada T (2001) RFLP analysis for cultivar identification of persimmons. Sci Hortic 91:407–412

Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27:209–220

Nei M (1972) Genetic distance between populations. Am Nat 106:283–292

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323

Parfitt DE, Yonemori K, Ryugo K, Sugiura A (1991) Isozyme identification of Japanese persimmons (*Diospyros kaki* L.): comparison of cultivars in California and Japan. Fruit Var J 45 (2):107–113

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 2:225–238

Romero M, Clavè J, Rovira M (2002) Characterization of persimmon (*Diospyros kaki* L.f.) cultivars from Spain. First Mediterranean Symposium on Persimmon, Faenza, Italy, 23–24 Nov. 2001. Options-Mediterraneennes-Serie-A-Seminaires-Mediterraneens 51:71–74

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234

Soriano JM, Pecchioli S, Romero C, Vilanova S, Llácer G, Badenes ML (2006) Development of microsatellite markers in polyploid persimmon (*Diospyros kaki* Lf) from an enriched genomic library. Mol Ecol Notes 6:368–370

Weir BS (1990) Genetic data analysis. Methods for discrete genetic data. Sinauer Associates, Suderland

Yamada M, Yamane H, Ukai Y (1994) Genetic analysis of Japanese persimmon fruit weight. J Am Soc Hort Sci 119: 1298–1302

Yamagishi M, Matsumoto S, Nakatsuka A, Itamura H (2005) Identification of persimmon (*Diospyros kaki*) cultivars and phenetic relationships between *Diospyros* species by more effective RAPD analysis. Sci Hortic 105:283–290

Yonemori K, Sugiura A, Yamada M (2000) Persimmon genetics and breeding. In: Janick C (ed) Plant breeding reviews, vol 19. Wiley, New York, pp 191–225

Yonemori K, Honsho C, Kitajima A, Aradhya M, Giordani E, Bellini E, Parfitt DE (2008a) Relationship of European persimmon (*Diospyros kaki* Thunb.) cultivars to Asian cultivars, characterized using AFLPs. Gen Res Crop Evol 55:81–89

Yonemori K, Kanzaki S, Honsho C, Akagi T, Parfitt DE (2008b) Phylogeny and cultivar development of *Diospyrus kaki*: a survey based on molecular analysis. Adv Hort Sci 22(4):261–268