Multiple Introduction Events of Cultivated *Diospyros Kaki* L. of and Native *Diospyros* Species in Taiwan Inferred by Low Copy Nuclear Markers

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Abstract

Diospyros kaki L. is one of the most important fruits in Taiwan. Cultivated *D. kaki* cultivars are classified into four types, according to their loss of astringency in ripening fruit and their change in flesh color, namely the Pollination-Constant Non-Astringent (PCNA), the Pollination Variant Non-Astringent (PVNA), the Pollination Variant Astringent (PVA) and the Pollination Constant Astringent (PCA). Recently, persimmon breeders are paying more attention to rootstock selection for successful grafting and agricultural production. To identify suitable rootstock and to trace the origins of extant native *Diospyros* species in Taiwan, the 10 known native *Diospyros* species together with four *D. kaki* cultivars were analyzed by using two low-copy, nuclear-encoded DNA markers, ncpGS and PHYA. There was moderate to strong support for the major nodes within the phylogenetic tree, obtained from both the Maximum-likelihood or the Bayesian inference methods. The constructed *D. kaki* phylogeny using Maximum Likelihood (ML) and Bayesian Inference (BI) algorithms formed three clades. The most basal clade (clade A) comprised species distributed in the South East Asia , then species distributed in East Asia (clade B), and then *D. kaki* cultivars (clade C). The extant native Taiwan *Diospyros* species nested into clade A and B, suggesting a northward migration pattern of native Taiwan *Diospyros* species. In addition, the species *D. oldhamii* formed a sister clade with the *D. kaki* cultivars, suggesting that *D. oldhamii* may be a compatible rootstock. Lastly, *D. kaki* cultivars sampled from growers that were acquaintances formed corresponding subclades within clade C (C-1, C-2 and C-3), suggesting a high discrimination ability of the ncpGS and PHYA molecular markers used here.

Keywords: *Diospyros kaki*, ncpGS, PhyA, phylogeny, persimmon

Introduction

The *Diospyros* genus contains over 500 species, making it the largest genus in the Ebenaceae family [1]. The members of genus *Diospyros* are distributed broadly from tropical to temperate regions. Most *Diospyros* species are distributed in Asia and the Pacific, making Asia and Oceania its center of diversity [2]. Some species are well known for their edible fruits, such as *D. kaki*, *D. lotus* and *D. virginiana* [3,4], while other species are famous for their timber, like *D. ebenum*, *D. mollis* and *D. dendo*. Moreover, leaves and fruits of some *Diospyros* species have been used as medicine, e.g. *D. kaki* and *D. lotus* [5,6].

D. kaki is the most economically important and widely cultivated species in the world. Cultivars of *D. kaki* are grown in Australia, Brazil, China, Iran, Israel, Italy, Japan, New Zealand, and United States of America [7,8]. The geographical origin of the *D.*

kaki species is not yet known. However, wild *D. kaki* plants have been observed in forests in China, so *D. kaki* is believed to have originated in China [9]. According to features of the mature fruit (astringency due to tannins, presence of seeds), cultivars are classified into four types, namely 1) Pollination-Constant Non-Astringent (PCNA), 2) Pollination-Variant Non-Astringent (PVNA), 3) pollination-variant astringent (PVA) and 4) Pollination-Constant Astringent (PCA) [9] (Figure 1). Among these four types, the PCNA and PVNA types lose astringency naturally during fruit growth and become edible at maturity, while PVA and PCA types retain astringency at maturity. Further, in the PVNA cultivars, sufficient pollination can enhance the loss of astringency, but insufficient pollination will make the fruits retain astringency at maturity but may be edible when softened. In contrast, PCNA fruit consistently loses its astringency during fruit

development, regardless of pollination efficiency. PCNA-type cultivars are important for commercial production of persimmon worldwide since the fruits lose their astringency naturally and do not require post-harvest astringency processing.

Previous phylogenetic studies revealed that two species distributed in Asia, *Diospyros glandulosa* (2n=2x=30) and *Diospyros oleifera* (2n=2x=30), are most likely ancestors of *D. kaki* (2n= 6x= 90) [2,7,8]. Their phylogenies also suggested that *D. kaki* originated in Asia through polyploidization. Although the cultivar development history is not yet clear, it is proposed that the PCA-type cultivars are thought to have appeared first in China, and then split into PVA-PVNA and PCNA lineages in Japan [10]. According to this hypothesis, PCNA-type accessions exhibit a single origin from a PCA-type progenitor. However, molecular evidence showed multiple origins of both PCNA and non-PCNA types [11]. For example, PCNA-type cultivars in Japan and in China formed clades respectively [11]. Non-PCNA type cultivars in Japan also exhibit paraphyly in their phylogeny [11]. To conclude, the multiple origin hypothesis of PCNA and non-PCNA cultivars in Asia is more likely than a single origin hypothesis as proposed by [10].

Due to lack of sterility of seeds, *D. kaki* is propagated via grafting of propagated scions onto wild rootstocks. The commonly used rootstocks include *D. kaki*, *D. lotus* and *D. virginiana* [12,13]. In Taiwan, the most common rootstock for *D. kaki* cultivars is *D. japonica*. It has been proposed that a closer genetic relationship between scion and rootstock is associated with higher grafting success [14].

There are 10 *Diospyros* species identified in Taiwan, including *Diospyros ferrea*, *Diospyros japonica*, *Diospyros kotoensis*, *Diospyros morrisiana*, *Diospyros philippensis*, *Diospyros eriantha*, *Diospyros vaccinioides*, *Diospyros maritima*, *Diospyros oldhamii* and *Diospyros rhombifolia* [15]. By analyzing Random Amplified polymorphic DNA (RAPD) markers, *D. kaki* clustered with *D. oldhamii* and *D. japonica*, suggesting that *D. oldhamii* and *D. japonica* may be potentially compatible rootstocks for grafting. However, the genetic relationship among *D. kaki* cultivars and other native *Diospyros* species remains unclear.

To reconstruct a phylogeny of closely related taxa or within the same species, low copy nuclear markers are preferable than commonly used non-coding cpDNA markers that have a high genetic variability [16]. For example, eight chloroplast markers, comprising over 8000 bases, were sequenced to resolve the relationship of *Diospyros* species from New Caledonian, Hawaii and islands in the Indian Ocean [17]. However, low variability and little resolution were shown among those target *Diospyros* species in their phylogeny. In contrast, two nuclear markers, chloroplast-expressed glutamine synthetase (ncpGS) and phytochrome A (*PHYA*), comprising about 1900 bases, were be able to resolve the relationship among those New Caledonian, Hawaii and Indian ocean *Diospyros* species [2]. Thus, ncpGS and *PHYA* are more satisfactory genetic markers in drawing the relationships among native *Diospyros* species and cultivars in Taiwan.

In this study, we aimed to define 1) the relationship among native *Diospyros* species in Taiwan and 2) the relationship among *D. kaki* cultivars. Two low-copy nuclear markers, ncpGS and *PHYA*, were isolated from the target species to draw the phylogenetic relationship among the native *Diospyros* species and the *D. kaki* cultivars in Taiwan.

Materials and Methods

DNA Extraction, Molecular Marker Amplification and Sequencing

Genomic DNA from 56 samples *Diospyros* species and cultivars (Table 1) was extracted from leaf samples dried in silica gel following a CTAB method (Doyle & Doyle, 1987). Two nuclear-encoded genes (*PHYA* and the chloroplast-expressed glutamine synthetase,e ncpGS) were sequenced for phylogeny reconstruction [2]. Initial PCR products of *PHYA* and *ncpGS* were obtained using primer pairs from [19,20], respectively. As these primers were not specific enough, we sliced the major PCR products from agarose gels and sequenced the major PCR products to be able to design *Diospyros*-specific *PHYA* PCR primers (phyA_F: GAGCTTGGAAAGGCTTTGTG; phyA_R: CAGCTGCCATCCCA-CATACT) and *ncpGS* PCR primers (ncpGS_F: TTCTTGGTCTGG-TAGTGGGAAT; ncpGS_R: TGCTTTTTCTAGTCTCGGTATGC).

The PCR reactions were made up to a total volume of 25 µl, containing 13 µl of 2x Ampliqon master mix Red (Ampliqon, Denmark), 0.25 µl each primer at 2 µM, 10.5 µl ddH2O and 1 µl template DNA at 20 ng/µl. The PCR profile for amplification of both the *PHYA* and ncpGS markers was an initial denaturing for 3 min at 94ºC followed by 37 cycles of 30 s at 94ºC, 30 s at 56ºC, and 1 min at 72ºC with a final extension for 10 min at 72ºC, . All PCR products were examined by 1.0% (w/v) agarose gel electrophoresis, and then the band of the expected size was sent for sequencing by ABI 3700 automatic sequencer (manufacturer). All sequences were examined by Sequence Scanner v1.0 first and then aligned for phylogenetic reconstruction.

Phylogeny Reconstruction

The sequences of *PHYA* and ncpGS of the native *Diospyros* species in Taiwan were used as an outgroup, considering the close phylogenetic relationship of native species to cultivars. In addition, representatives of genus *Diospyros* were chosen based on [2] (Details see Table 1). The sequences were automatically aligned using MUSCLE [21] in MEGA v.6 [22]. After alignment, the *PHYA* and ncpGS datasets were concatenated to obtain all genetic information of these two nuclear markers. For both Maximum Likelihood (ML) and Bayesian Inference (BI) phylogeny, the web interface PhyML 3.0 was applied [23]. The best-fit nucleotide substitution model of each dataset was evaluated by Smart Model Selection (SMS), which was implementing in PhyML 3.0 [24]. The GTR + G model was also the best-fit model for concatenated *PHYA* and ncpGS dataset. The phylogeny is visualized in FigTree v1.4.3. [25].

Results

Relationships of *Diospyros* Species and Cultivars in Taiwan

To infer the relationships among the native Taiwan *Diospyros* species and the currently grown *Diospyros kaki* cultivars, we reconstructed the phylogeny using sequences isolated from the target species (number of samples) as well as sequences obtained from the NCBI database of other *Diospyros* species (listed in Table 1, number of dB data). Only nodes with a Maximum Likelihood (ML) and Bayesian Inference (BI) probability value over 0.7 (BI>0.7 or aLRT > 0.7) were regarded as a reliable clade. According to our criteria, the native Taiwan *Diospyros* species were distributed into two clades (Figure 2). Sequences

Table 1: Sequences of Diospyros species and D. kaki cultivars used in this study.

Taxon	collection site	Collection codes	Accession no. for ncpGS	Accession no. for PHYA
Diospyros eriantha Champ. ex Benth.	Wufeng, Taichung△		ON351647	ON351614
Diospyros ferrea (Lour.) A.Chev.	Wufeng, Taichung△		ON351643	ON351612
Diospyros japonica Sieb.& Zucc.	Heping, Taichung△		ON351670	ON351634
Diospyros kotoensis T. Yamaz.	Wufeng, Taichung△		ON351644	none
Diospyros maritima Blume	Wufeng, Taichung△		ON351645	ON351613
Diospyros morrisiana	Zhongzheng, Taipei△		ON351678	ON351642
Diospyros oldhamii	Anle, Keelung△		ON351677	ON351641
Diospyros philippensis (Desr.) Gürke	Wufeng, Taichung△		ON351646	none
Diospyros rhombifolia Hemsl.	Wufeng, Taichung△		ON351648	ON351615
Diospyros vaccinioides Lindl.	Wufeng, Taichung△		ON351649	ON351616
Diospyros andamanica	Thailand*		**KF291447**	**KF291624**
Diospyros apiculata	Thailand*		**KF291449**	**KF291626**
Diospyros borbonica	Reunion*		KF291453	KF291630
Diospyros discolor	Thailand*		KF291473	KF291650
Diospyros dictyoneura	Thailand*		KF291471	KF291648
Diospyros diepenhorstii	Thailand*		**KF291472**	**KF291649**
Diospyros glandulosa	Thailand*		KF291492	KF291669
Diospyros kupensis	Cameroon*		KF291501	KF291678
Diospyros lotus	Living coll.Kew 1882-3501*		KF291507	KF291684
Diospyros macrocarpa	New Caledonia*		KF291509	KF291686
Diospyros mollis	Thailand*		KF291522	KF291699
Diospyros oubatchensis	New Caledonia*		KF291529	KF291706
Diospyros rhodocalyx Kurz	Thailand*		KF291567	KF291744
Diospyros samoensis	Cult. Hawaii Bot Garden*		KF291569	KF291746
Diospyros sp.(Madagascar)	Madagascar*		KF291571	KF291748
Diospyros texana Scheele	Middle America*		KF291575	KF291752
Diospyros venosa	Thailand*		KF291596	KF291773
Diospyros virginiana L.	USA*		KF291612	KF291789
Diospyros vera	Central African Republic*		KF291597	KF291774
Diospyros winitii Fletcher	Thailand*		KF291615	KF291792
Styrax officinalis	Indonesia*		KF291623	KF291800
Euclea undulata	Living coll. HBV*		KF291620	KF291797
Diospyros kaki cv. Amahyakume	Heping, Taichung△	G21	ON351663	ON351627
Diospyros kaki cv. Aoso	Heping, Taichung△	G18	ON351660	none
Diospyros kaki cv. Bull Heart	Kungkuan, Miaoli△	G08	ON351650	ON351617
Diospyros kaki cv. Diamond Bull Heart	Heping, Taichung△	G11	ON351653	ON351619
Diospyros kaki cv. Fudegaki	Dongshi, Taichung△	G09	ON351651	none
Diospyros kaki cv. Fuyu	Heping, Taichung△	G25	ON351667	ON351631
Diospyros kaki cv. Fuyu	Heping, Taichung△	G19	ON351661	ON351625
Diospyros kaki cv. Fuyu	Heping, Taichung△	G17	ON351659	ON351624
Diospyros kaki cv. Fuyu	Sinyi, Nantou△	G23	ON351665	ON351629
Diospyros kaki cv. Fuyu	Heping, Taichung△	G22	ON351664	ON351628
Diospyros kaki cv. Fuyu	Heping, Taichung△	G29	ON351671	ON351635
Diospyros kaki cv. Fuyu	Dongshi, Taichung△	G10	ON351652	ON351618
Diospyros kaki cv. Hana-gosho	Heping, Taichung△	G16	ON351658	none
Diospyros kaki cv. Hirataneanshi	Wufeng, Taichung△	G13	ON351655	ON351621
Diospyros kaki cv. Maekawa-Jirow	Sinyi, Nantou△	G24	ON351657	ON351623
Diospyros kaki cv. Maekawa-Jirow	Heping, Taichung△	G15	ON351666	ON351630
Diospyros kaki cv. Soshu	Heping, Taichung△	G14	ON351656	ON351622
Diospyros kaki cv. Syh Jou	Dongshi, Taichung△	G12	ON351654	ON351620
Diospyros kaki cv.Taishuu	Sinyi, Nantou△	G26	ON351662	ON351626
Diospyros kaki cv.Taishuu	Heping, Taichung△	G20	ON351668	ON351632
Diospyros kaki cv. Fuyu	Wufeng, Hsinchu△	C017	ON351671	ON351635
Diospyros kaki cv. Fuyu	Wufeng, Hsinchu△	C019	ON351672	ON351636
Diospyros kaki cv. Fuyu	Wufeng, Hsinchu△	J26	ON351673	ON351637
Diospyros kaki cv. Fuyu	Wufeng, Hsinchu△	G44	ON351675	ON351639
Diospyros kaki cv.Tonewase	Heping, Taichung△	G27	ON351669	ON351633

Table 2: Distribution range of native Diospyros species in Taiwan.

Species	Distribution range
Diospyros eriantha	China to Nansei-shoto and W. & Central Malesia, Taiwan
Diospyros ferrea	Tropical Africa to SW. Pacific.
Diospyros japonica	S. China, Central & S. Japan to Nansei-shoto, Taiwan
Diospyros kotoensis	Taiwan
Diospyros maritima	China (Yunnan) to Nansei-shoto and N. Australia, Taiwan
Diospyros morrisiana	Central & S. Japan to SE. China, Taiwan
Diospyros oldhamii	Nansei-shoto to Central & E. Taiwan.
Diospyros philippensis	E. & S. Taiwan to E. Borneo
Diospyros rhombifolia	SE. China, Taiwan
Diospyros vaccinioides	China (Guangxi, Guangdong) to Hainan, Taiwan

of *D. philippensis* and *D. kotoensis* were grouped with published sequences from species originating in Indo-China, Indonesia and Philippines (Figure 2, Clade A; BI: 0.98, aLRT: 1), while *D. ferrea*, *D. rhombifolia*, *D. maritime*, *D. eriantha*, *D. vaccinioides*, *D. morrisiana*, *D. japonica* and *D. oldhamii* grouped together (Figure 2, Clade B; BI: 1, aLRT: 1). All *D. kaki* cultivars formed a single clade, topologically the sister clade of *D. oldhamii* (Figure 2, clade C). *D. japonica* is a Taiwan native persimmon species used as the rootstock of *D. kaki* cv. Fuyu. The test results showed that *D. kaki* cv. Fuyu was closely related to cv. Aoso. On the other hand, the native Taiwan persimmon species *D. oldhamii* is closely related to *D. kaki* cv. Fuyu, while *D. kaki* cv. Fuyu is distantly related to *D. japonica*.

According to the classification based on fruit characteristics, *D. kaki* is divided into four types: PCNA, PVNA, PVA, and PCA. The two sampled PVNA types formed a single clade, namely *D. kaki* cv. Amahyakume and *D. kaki* cv. Fudegaki. It is noteworthy that the PCNA, PVA, and PCA types of *D. kaki* did not form single clades based on fruit ripening characteristics. PCNA types were distributed among at least four subclades within clade C, within which both PCA and PVA types were also nested.

Fuyu is the most grown cultivar in Taiwan and forms three subclades within clade C, namely C-1, C-2, and C-3 (Figure 2). Fuyu samples G19, J26, C019, C017, and G44 are in subclade C-1 and were collected from Heping District, Taichung City (G19) and Wufeng Township, Hsinchu County (J26, C019, C017, and G44), respectively. Samples G23, G25, and G10 are subclades of Clade C-2 and were collected from Xinyi Township (G23 and G25), Nantou County and Dongshi District, Taichung City (G10), while G17 and G29 are in subclade C-3 and were both collected from Heping District, Taichung City, but cultivated by different farmers.

Discussion

By sampling all known native *Diospyros* species and the most widely grown *D. kaki* cultivars in Taiwan, we reconstructed a comprehensive phylogeny which allowed the inference of two potential dispersal routes of native *Diospyros* species and indicted that multiple introduction events of cultivated *D. kaki* cultivars had occurred. Native Taiwan *Diospyros* species formed two distinct clades, including the South East Asia clade and the East Asia clade (Figure 2 & Table 2). In addition, the East Asia clade nested within the South East Asia clades. Extant native *Diospyros* species in Taiwan within these two clades may hint that these species in Taiwan entered their current habitat through two colonization routes. The evidence showing that the East Asia clades nested within the South East Asia clade indicates that a dispersal route from Indo-China through the Asian continent to Taiwan is likely. A second dispersal route would have been through the Philippine Archipelago to Taiwan. The Quaternary, glacial and inter-glacial periods, caused by climatic oscillation, lead to repeated lowering and raising of sea levels in East Asia [26,27]. During a glacial period, submerged sea floor may have be exposed due to lower sea levels, forming land bridges connecting isolated landmass [28]. Land bridges provide opportunity allowing terrestrial plant species to migrate between the Asian mainlands and adjacent islands, like Taiwan and Japan. For example, a haplotype (H9) of *Quercus variabilis* was identified from both the Asia continent and some adjacent islands (including Taiwan and Japan), suggesting plant migration through land bridges [29]. The phylogeny of some herbaceous plants also support landbridging. *Lysionotus pauciflorus* exhibits similar distribution pattern to *Q. variabilis*, inhabiting the Asia continent and adjacent islands [30]. and a, few individuals from both Asia and adjacent islands formed a monophyletic clade with a calculated time of divergence during the maximal glacial period [30]. In our study, *Diospyros* species belonging to the East Asia clade (including *D. maritima*, *D. eriantha*, *D. vaccinioides*, *D. morrisiana*, *D. japonica* and *D. oldhamii*) were distributed in both the Asia continent and the adjacent islands of Taiwan and Japan), suggesting migration through exposed East Asia seafloor likely. The aforementioned second possible dispersal route through the Philippine archipelago to Taiwan has no historical geological event (glacial-interglacial period) that connected the Philippine archipelago and Taiwan [31], making dispersal from

Figure 1: Four types of *Diospyros kaki* cultivars based on fruit classification. (a). *Diospyros kaki* cv. Fuyu, PCNA type. Fruit will be sweet whether hard or soft (b). *Diospyros kaki* cv. Fudegaki, PVNA. Unseeded fruit must soften after harvest to be sweet (c). *Diospyros kaki* cv. Hirataneanshi, PVA. Fruit remains astringent, except near seeds. (d). *Diospyros kaki* cv. Bull Heart, PCA. Fruit must become soft to be edible.
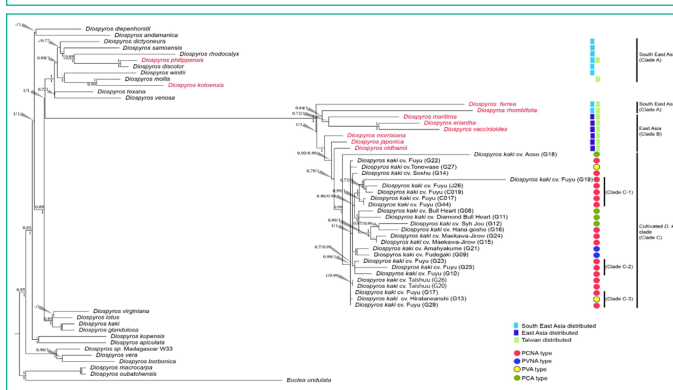

Figure 2: Phylogeny inferred from *PHYA* and ncpGS sequences. Numbers above a branch represent posterior probability estimated using Bayesian Inference (BI) algorithm; Numbers below a branch represent the value estimated using Maximum Likelihood (ML) algorithm. Species name colored in red represent native *Diospyros* species distributed in Taiwan. Light blue squares denote *Diospyros* species distributed in South East Asia; Blue squares denote *Diospyros* species distributed in East Asia; Green squares denote *Diospyros* species distributed in Taiwan. Red circle represents PCNA-type cultivars; green circle represents PCA-type cultivars; blue represents PVNA-cultivars; and yellow represents PVA-type cultivars.

the Philippine archipelago through a land bridge to Taiwan unlikely. One possible scenario is through human activity. In South East Asia, Austronesian-speaking people migrate among islands using boats. To build a reliable boat, solid timber is necessary. Austronesian-speaking people in Taiwan, like the Tao people in Lanyu, use timber from *D. philippensis* to build a boat [32]. Austronesian-speaking people carrying necessary and useful plants with them is traceable. For example, paper mulberry (*Broussonetia papyrifera*) was carried by Austronesian-speaking people for its bark [33,34]. Molecular phylogeny conducted using cpDNA of *B. papyrifera* offers genetic evidence supporting the transport of this species by Austronesian-speaking people [35]. the migratory transport of *D. philippensis* by Austronesian-speaking people requires further study.

Taxonomy and genetic affinity are prerequisites for grafting affinity [15]. The greater the genetic distance between the rootstock and the scion, the lesser the chance of forming a successful graft union [36,37]. To achieve a successful grafting combination, the genetic distance between the two ranks as intraclonal > interclonal > intraspecific > interspecific > intrageneric > intergeneric > intrafamilial [38]. An autografted combi-

nation between rootstock and scion indicates close similarity in their taxonomy and a good affinity. If the rootstock and scion are different species but belong to the same genus, the graft is more or less compatible. While graft combinations are generally rarely compatible, those between families are incompatible and unsuccessful [39]. *D. lotus* was used as the rootstock for cultivating persimmon in California, whose grafting incompatibility resulted in dwarfism and came into bearing early of persimmon, and declined within a few years. On the other hand, *D. virginiana*, native to the United States, is an ideal rootstock for persimmon. Compared with *D. lotus* and *D. virginiana*, the parent species of cultivated persimmon, *D. kaki* is even more compatible with grafting [40].

The relationships of 19 kiwifruit species were analyzed through the chloroplast genome. The greater the pairwise genetic distance between the rootstock (*Actinidia deliciosa*) and the xenografted kiwifruit, the lower the survival rate of the scion [15]. In this study, the sequencing of two marker genes confirmed that *D. kaki*, *D. japonica*, cultivar Aoso, and *D. oldhamii* belong to the same genus. It also confirmed that *D. kaki* and cultivar Aoso are the same species. Considering their close kinship, *D. oldhamii* and *D. kaki* may show a better affinity after grafting, and *D. oldhamii* should be tested for agricultural use as a rootstock in the Taiwan persimmon industry.

It is hypothesized that PCA-type cultivars of *D. kaki* are first in China, and then split into the PVA-PVNA type and PCNA lineages in Japan [10]. However, this is not supported by recent molecular studies [7,9,11]. An extensive analysis of the genetic diversity of 146 *D. kaki* cultivars, obtained from China, Korea and Japan, was surveyed with 496 distinct AFLP markers [1]. By using clustering algorithm, these 146 cultivars were assigned into three clusters corresponding to geological regions. In addition, relationships among the studied *D. kaki* cultivars did not follow horticultural classification [11]. According to molecular data, the primary character separating them appears to be their origin instead of their astringency level. In our study, extant *D. kaki* cultivars in Taiwan exhibit polyphyly (Figure 2), similar to previous studies [7,9,11,41]. Unlike PCNA-type cultivars forming a monophyletic clade in previous studies, PCNA-type cultivars in Taiwan formed at least three sub-clades (Figure 2). These three sub-clades may suggest at least three origins of Taiwanese PCNA-type *D. kaki* cultivars. One possible reason behind multiple origins may associate with human behavior, e.g. scion sharing among farmers, since Grafting is the only way to grow cv. Fuyu. In subclade C-1, G19 was collected from Heping, Taichung, but others (including C019, C017 and G44) were collected 100 km away in Wufeng, Hsinchu. After consulting local farmers, samples collected from Wufeng originated from farmers in Heping. Scion sharing could also be found in the C-2 and C-3 sub-clades. Within the C-2 sub-clade, samples G10 (Dongshi, Taichung), G23 and G25 (Sinyi, Nantou) are close to each other. Lastly, G17 and G29 were collected from Heping, Taichung and are grown by acquaintances. To conclude, sequencing of the genes *ncpGS* and *PhyA* is able to identify the origin of *D. kaki* cv. Fuyu cultivars in Taiwan.

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