GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Transferability of retrotransposon primers derived from Persimmon (*Diospyros kaki* Thunb.) across other plant species

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Genet. Mol. Res. 12 (2): 1781-1795 (2013)
Received August 30, 2012
Accepted February 15, 2013
Published June 6, 2013
DOI http://dx.doi.org/10.4238/2013.June.6.2

ABSTRACT. Retrotransposon-based molecular markers are powerful molecular tools. However, these markers are not readily available due to the difficulty in obtaining species-specific retrotransposon primers. Although recent techniques enabling the rapid isolation of retrotransposon sequences have facilitated primer development, this process nonetheless remains time-consuming and costly. Therefore, research into the transferability of retrotransposon primers developed from one plant species onto others would be of great value. The present study investigated the transferability of retrotransposon primers derived from 'Luotian-tianshi' persimmon (*Diospyros kaki* Thunb.) across other fruit crops, as well as within the genus using inter-retrotransposon amplified polymorphism molecular marker. Fourteen of the 26 retrotransposon primers tested (53.85%) produced robust and reproducible amplification products across all fruit crops tested, indicating their applicability across plant species. Four of the 13 fruit crops showed the best transferability performances: persimmon,

grape, citrus, and peach. Furthermore, similarity coefficients and UPGMA clustering indicated that these primers could further offer a potential tool for germplasm differentiation, parentage identification, genetic diversity assessment, classification, and phylogenetic studies across a variety of plant species. Transferability was further confirmed by examining published primers derived from Rosaceae, Gramineae, and Solanaceae. This study is one of the few currently available studies concerning the transferability of retrotransposon primers across plant species in general, and is the first successful study of the transferability of retrotransposon primers derived from persimmon. The primers presented here will help reduce costs for future retrotransposon primer development and therefore contribute to the popularization of retrotransposon molecular markers.

Key words: Persimmon; Retrotransposon-based molecular marker; Transferability; Inter-retrotransposon amplified polymorphism

INTRODUCTION

Retrotransposons are ubiquitous in the plant kingdom and are amongst the most variable of all genomic components, differing greatly in copy number, genomic localization, and sequence structure over relatively short evolutionary time periods. They are present in high copy numbers and are highly heterogeneous in plant genomes, and can be transmitted both vertically and horizontally, across generations and between different plant species, respectively. Retrotransposons are also mutagenic agents and their activity produces large genome rearrangements as well as sequence changes in single genes, both of which can alter patterns of gene expression and function (Krom et al., 2008; Butelli et al., 2012). Hence, retrotransposons play an important role in the structure, organization, and evolution of plant genomes (Kumar and Bennetzen, 1999). Due to these properties, retrotransposons have become the genetic markers of choice in research involving many plant species in recent decades.

Retrotransposon-based molecular markers have a number of advantages over other molecular markers, such as their abundance and dispersion throughout almost the entire length of all host chromosomes, high information content, genomic specificity, capability for tracing the frequency and history of retrotransposition, and adaptation to automation, which ensures more reliable and ideal methods for DNA fingerprinting (Kumar and Hirochika, 2001), particularly due to their powerful capacity for bud sports identification in fruit crops. Inter-retrotransposon amplified polymorphism (IRAP) (Kalendar et al., 1999), retrotransposon-microsatellite amplified polymorphism (Kalendar et al., 1999), and sequence-specific amplified polymorphism (Waugh et al., 1997) are the 3 most commonly used retrotransposon-based molecular markers. To date, they have been successfully used for fingerprinting (Venturi et al., 2006; Du et al., 2009; Castro et al., 2012), linkage analysis and mapping of agronomic traits (Manninen et al., 2000; Bernet and Asíns, 2003), and biodiversity and phylogenetic analyses (Pearce et al., 2000; Bretó et al., 2001; Sanz et al., 2007; Cornman and Arnold, 2008; Kalendar et al., 2011; Mandoulakani et al., 2012).

Unfortunately, popularization of these marker systems has been hindered because the development of retrotransposon-based marker systems for a new plant species requires the isolation, cloning, sequencing, and characterization of the retrotransposon sequence as a prerequisite to obtaining the species-specific retrotransposon primers. Therefore, except for species with whole or partial retrotransposon sequences available, development of retrotransposon primers is time-consuming and costly. Investigations about the transferability of retrotransposon primers developed from one plant species onto others would be of great value to enable the low-cost and highly efficient development of retrotransposon-based molecular markers in plant species with very little or no information on retrotransposon sequences. For those plant species with abundantly available public data of retrotransposon sequences or retrotransposon-based marker systems, transferable primers from other plant species would be useful to further increase the number of markers. Nevertheless, to our knowledge, since the initial study about the transferability of retrotransposon primers from Gramineae (barley) to banana (Teo et al., 2005), no related studies have been published. In particular, there is a clear need for a systematic investigation on the transferability of retrotransposon primers to more diverse plant species.

We previously successfully developed retrotransposon primers from 'Luotian-tianshi' persimmon (*Diospyros kaki* Thunb.) (Du et al., 2009). In the present study, we explored the transferability of these primers to a series of unrelated fruit crop species, as well as to other *Diospyros* species. In order to further corroborate the transferability of retrotransposon primers across plant species, the transferability of published retrotransposon primers from Rosaceae, Gramineae, and Solanaceae was tested on these 13 fruit crops. The information generated is expected to be of great help in increasing the popularization and application of retrotransposon-based molecular markers, as well as contributing to the number of retrotransposon primers for use in a wide variety of fruit crops.

MATERIAL AND METHODS

Plant material

A total of 65 accessions, representing 13 fruit crops, were used in this study, including citrus (*Citrus reticulata*, *C. grandis*, and *Poncirus trifoliata*), grape (*Vitis vinifera*, *V. labrusca* x *V. vinifera*), kiwifruit (*Actinidia deliciosa*), plum (*Prunus domestica*, *P. salicina*), pear (*Pyrus pyrifolia*, *P. bretschneideri*, *P. communis*), peach (*Prunus persica*), loquat (*Eriobotrya japonica*), apple (*Malus pumila*), hawthorn (*Crataegus pinnatifida*), jujube (*Zizyphus jujube*), chestnut (*Castanea mollissima*), ginkgo (*Ginkgo biloba*), and persimmon (*D. kaki*, *D. lotus*, *D. oleifera*, *D. glaucifolia*, *D. tutcheri*, *D. miaoshanica*, *D. rhombifolia*) (Table 1). The above 65 accessions were all used for the assay of retrotransposon primers from 'Luotian-tianshi' persimmon (*D. kaki* Thunb.); 15 accessions selected from the above-mentioned 13 fruit crops (indicated with asterisks in Table 1) were used for the assay of published retrotransposon primers from Rosaceae, Gramineae, and Solanaceae. Except *D. tutcheri* and *D. miaoshanica* that were collected from the Wuhan Botanical Garden, Chinese Academy of Science, all plants were sampled from the Fruit Repository, Huazhong Agricultural University, Wuhan, China.

Table 1. List of plant materials used in this study.

Fruit crops	Code	Genotype	Scientific name	Fruit crops	Code	Genotype	Scientific name
Japanese persimmon	1	Hana-gosho	*Diospyros kaki* Thunb.		34	Black olympia*	*V. labrusca* L.x *V. vinifera* L.
	2	Suruga	*D. kaki* Thunb.		35	Crimson	*V. vinifera* L.
	3	Oku-gosho	*D. kaki* Thunb.		36	Minicure finger	*V. vinifera* L.
	4	Hiratanenashi	*D. kaki* Thunb.	Kiwifruit	37	Jinkui	*Actinidia deliciosa* var. *deliciosa* Chev.
	5	Maekawa-Jirou	*D. kaki* Thunb.		38	Miliang 1*	*A. deliciosa* var. *deliciosa* Chev.
	6	Jirou	*D. kaki* Thunb.	Plum	39	Black amber	*Prunus domestica* Linn.
	7	Youhou	*D. kaki* Thunb.		40	Oishi wase*	*P. salicina* Linn.
	8	Fuyuu*	*D. kaki* Thunb.	Pear	41	Huangmi	*Prunus pyrifolia* Nakai
	9	Matsumoto-wase	*D. kaki* Thunb.		42	Rosewood	*P. pyrifolia* Nakai
	10	Uenishi-wase	*D. kaki* Thunb.		43	Sanhua	*P. pyrifolia* Nakai
	11	Huashi 1	*D. kaki* Thunb.		44	Cili	*P. bretschneideri* Rehd.
	12	Yamafuji	*D. kaki* Thunb.		45	Housui*	*P. pyrifolia* Nakai
	13	Zenjimaru	*D. kaki* Thunb.		46	Shounan	*P. pyrifolia* Nakai
	14	90-1-10a	*D. kaki* Thunb.		47	Huali 1	*P. pyrifolia* Nakai
	15	90-1-10b	*D. kaki* Thunb.		48	Enoshima	*P. pyrifolia* Nakai
	16	Luotian-tianshi*	*D. kaki* Thunb.		49	Le Counte	*P. communis* Linn.
	17	Mopanshi	*D. kaki* Thunb.	Peach	50	Yanguang	*P. persica* var. *nectarina* Maxim.
Relative species of Japanese persimmon	18	Date plum	*D. lotus* Linn.*		51	Shuguang	*P. persica* var. *nectarina* Maxim.
	19	Oily persimmon	*D. oleifera* Cheng		52	Chunhua	*P. persica* Linn.
	20	Chekiang persimmon	*D. glaucifolia* Metc.		53	Sunagowase	*P. persica* Linn.
	21	Lingnanshi	*D. tutcheri* Dunn		54	Zaolupan*	*P. persica* var. *compressa* Bean.
	22	Miaoshanshi	*D. miaoshanica* S. Lee		55	Gold-mine	*P. persica* var. *nectarina* Maxim.
	23	Diamond leaf persimmon	*D. rhombifolia* Hemsl.		56	Ailihong	*P. persica* var. *densa* Mak.
Citrus	24	Zaojin satsuma	*Citrus reticulata* Blanco		57	Bitao	*P. persica* var. *duplex* Rehd.
	25	Wakiyame-wase satsuma	*C. reticulata* Blanco	Loquat	58	Dawuxing*	*Eriobotrya japonica* Linn.
	26	Zhongqiu satsuma	*C.reticulata* Blanco		59	Huabao 5	*E. japonica* Linn.
	27	Bendizao tangerine*	*C. reticulata* Blanco		60	Mogi	*E. japonica* Linn.
	28	Oota	*C. reticulata* Blanco	Apple*	61	-	*Malus pumila* Mill.
	29	HB pummelo	*C. grandis* Osbeck	Hawthorn*	62	-	*Crataegus pinnatifida* Bge.
	30	Trifoliate orange	*Poncirus trifoliate* (L.) Rafin.	Jujube*	63	-	*Zizyphus jujube* Mill.
Grape	31	Kyoho	*Vitis labrusca* Linn. x *V. vinifera* Linn.	Chestnut*	64	-	*Castanea mollissima* Blume
	32	Red fuji	*V. labrusca* L. x *V. vinifera* L.	Ginkgo*	65	-	*Ginkgo biloba* Linn.
	33	Jingya	*V. labrusca* L. x *V. vinifera* L.				

*Accessions selected were used for the assay of published retrotransposon primers from Rosaceae, Gramineae, and Solanaceae.

Genomic DNA extraction

Genomic DNA was isolated from young leaves by the CTAB-based extraction method, although protocols differed somewhat depending on the particular fruit crop tested. DNA isolation of persimmon, apple, pear, and hawthorn followed the methods of Doyle and Doyle (1987); isolation of citrus, grape, loquat, kiwifruit, jujube, chestnut, and ginkgo DNA followed the methods of Cheng et al. (2003); plum and peach DNA isolation followed the protocol of Cheng (2007). Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of 10 ng/μL before use.

Source of retrotransposon primers

The retrotransposon primers derived from 'Luotian-tianshi' persimmon were proprietary primers developed by our laboratory (Du et al., 2009), which will henceforth be referred to as 'persimmon retrotransposon primer'. The sequences, lengths, annealing temperatures, and accession numbers of corresponding sequences that formed the basis of primer design are shown in Table 2. The names, sequences, and sources of the retrotransposon primers published from Rosaceae, Gramineae, and Solanaceae are shown in Table 3.

Table 2. Retrotransposon primer sequences and characteristics used in this study.

Primers[a]	Sequence (5'-3')	Length (bp)	Tm (°C)	GenBank accession No.
rtdk14-f1	TTCATTGTCGTGTTTCGTAGGCG	23	60.2	EU068711
rtdk12-f2	CATAGGAGGCTGTTGAGCAGAGTG	24	63.7	EU068709
rtdk20-f3	CTATCTTGCCTCCTTCTGCTTTC	23	60.2	EU068717
rtdk4-f4	TTGAAGAAAATAACAACAGATA	22	51.2	EU068701
rtdk13-f5	CATCATCTGTTACTCTTGTGCTT	23	59.7	EU068710
rtdk15-f6	TGAGTGGTGATGTAAGAGAGTA	22	56.5	EU068712
rtdk7-f7	GGAGACTTTACTATTGAGCC	20	58.0	EU068704
rtdk16-f8	GCTGTCCCCTTCCCCTGTTT	20	64.0	EU068713
rtdk14-r1	CATTGGGTCCATCAGTTTCC	20	57.8	EU068711
rtdk12-r2	CTCTATTCTTGTATCCATCACCAC	24	58.6	EU068709
rtdk20-r3	ACTAATCTACTACCGTTTGGCTA	23	57.1	EU068717
rtdk24-r4	CAAGGAACTACTCTAATAGCAAT	23	55.3	EU068721
rtdk13-r5	ACAAGAGTAACAGATGATGATTC	23	55.3	EU068710
rtdk13-r6	GCATTGGGTCCATCAGTTTC	20	60.0	EU068710
rtdk15-r7	GTAAAAACCTGGAGTAAGAAAG	22	64.7	EU068712
rtdk2-r8	GAGTAAGTTGGCTCA ATA GT	20	56.0	EU068699
rtdk16-r9	CAACACGAAATACGGCTACG	20	60.0	EU068713
rtdk16-r10	CTGCGACTTCACCAAGCCATAAA	23	60.6	EU068713
rtdk11-p1	GCTTGAGGGGGAGTGTTGAGTT	22	61.9	EU068708
rtdk1-p2	GTGGTCTGATTTGAGGGGGAATGTAG	26	63.6	EU068698
rtdk4-p3	GAAGGGAGGTCTAAACTGAGGAAA	24	60.3	EU068701
rtdk10-p4	GGTCCATCTTGAGGGGGAGTAA	22	62.1	EU068707
rtdk5-p5	TGCTAAGAACAAAATACCTCATAG	24	57.3	EU068702
rtdk2-p6	TGAGGGAGGCCTTACTATTGAG	22	60.3	EU068699
rtdk5-p7	AACAAAATACCTCATAGAGTGGC	23	59.3	EU068702
rtdk17-p8	GAGGGGAAACTCAGTGACTTAGG	23	62.4	EU068714

[a]Retrotransposon primers according to Du et al. (2009). Tm = melting temperature.

Table 3. Retrotransposon primers and their sources used in IRAP-PCR.

Primer	Sequence (5'-3')	Source	Reference
LTR1	GCAATTTCGTTCATTCACAG	Apple	Venturi et al. (2006)
LTR2	GTGCAAATGGTTACGTCACT	Apple	Venturi et al. (2006)
LTR6149	CTCGCTCGCCCACTACATCAACCGCGT TTATT	Barley	Teo et al. (2005)
LTR6150	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA	Barley	Teo et al. (2005)
5'LTR1	TTGCCTCTAGGGCATATTTCCAACA	Barley	Teo et al. (2005)
5'LTR2	ATCATTCCCTCTAGGGCATAATTC	Barley	Teo et al. (2005)
3'LTR	TGTTTCCCATGCGACGTTCCCCAACA	Barley	Teo et al. (2005)
Sukkula	GATAGGGTCGCATCTTGGGCGTGAC	Barley	Teo et al. (2005)
Nikita	CGCATTTGTTCAAGCCTAAACC	Tobacco	Teo et al. (2005)
Tnt-1	TGATGATGTCCATCTCATT	Tobacco	Tam et al. (2005)
Tnt1-OL16	TTCCCACCTCACTACAATATCGC	Tobacco	Tam et al. (2005)
ToRTL1	CCCTGGGTTTGTTTCATCTGC	Tobacco	Tam et al. (2005)
Bare-1	TGTTGGAATTATGCCCTAG	Barley	Waugh et al. (1997)

Test of transferability

The tests for retrotransposon primer transferability were conducted using an IRAP retrotransposon molecular marker. IRAP-PCR amplifications were performed as described by Kalendar et al. (1999), with minor modifications. Amplification reactions were carried out in 20-µL volumes containing 0.25 mM dNTPs, 0.4 µM primer, 50 ng genomic DNA, 2.0 mM $MgCl_2$, 1X PCR buffer, and 1 U *Taq* DNA polymerase (Beijing Sun-Biotech Co. Ltd., China). The mixture was overlain with mineral oil. The amplifications were carried out in a Biometra Tgradient thermal cycler (Goettingen, German) using the following amplification profile: 1 cycle at 95°C for 5 min; 1 cycle at 95°C for 1 min; 56°C for 1 min; ramp +0.6°C/s to 72°C; 44 cycles of 72°C for 2 min; 1 cycle at 72°C for 8 min; and a pause for 4°C. At least 2 PCR amplifications were conducted for each sample to ensure the reproducibility of the produced bands. Products were separated on 2% agarose gel at a constant 8 V/cm for 5 to 6 h using 0.5X Tris-borate-EDTA buffer, pH 8.3, which were then stained with ethidium bromide and photographed in a SYNGENE Automated Gel Documentation System (Cambridge, USA). The resulting total numbers of the retrotransposon primers tested were 26 and 13, from persimmon and the Rosaceae, Gramineae, and Solanaceae groups, respectively.

Band scoring and data analysis

The well-resolved and consistently reproducible IRAP bands were scored as 1 (presence) or 0 (absence) in a binary matrix for each primer. The Dice similarity coefficient was calculated using the SIMQUAL module, and cluster analysis was performed to construct dendrograms by using the unweighted pair-group method with arithmetic averages (UPGMA) and the SAHN clustering program. All analyses were performed with the NTSYS-pc 2.10e software (Rohlf, 2000).

RESULTS

Transferability of persimmon retrotransposon primers

Overall, of the 26 persimmon retrotransposon primers tested, 14 produced good amplifications across all 65 accessions of the 13 fruit crops, accounting for 53.85% of the total number of primers. Only one primer (rtdk4-f4) was unable to amplify across all 65 accessions. The amplifications of persimmon retrotransposon primers in the 13 fruit crops are summarized in Table 4.

Table 4. Transferability of retrotransposon primers from 'Luotian-tianshi' persimmon (*Diospyros kaki* Thunb.) in the fruit crops examined.

Primer	Persimmon				Citrus				Grape				Peach			
	TB	PB	Pi (%)	EN	TB	PB	Pi (%)	EN	TB	PB	Pi (%)	EN	TB	PB	Pi (%)	EN
rtdk11-p1	19	19	100	23	9	8	88.89	7	10	4	40	6	7	1	12.50	8
rtdk1-p2	12	12	100	23	8	6	75	7	8	3	37.50	6	2	0	0	8
rtdk4-p3	19	19	100	23	16	12	75	7	11	4	36.36	6	13	0	0	8
rtdk10-p4	13	12	92.31	23	12	10	83.33	7	6	0	0	6	10	0	0	8
rtdk5-p5	15	15	100	23	8	7	87.50	7	6	1	16.67	6	4	0	0	8
rtdk2-p6	17	10	58.82	23	11	2	18.18	7	8	1	12.59	6	5	1	20	8
rtdk5-p7	18	19	95	23	10	9	90	7	9	4	44.44	6	6	1	16.67	8
rtdk17-p8	16	13	81.25	23	8	7	87.50	7	4	2	50	6	6	0	0	8
rtdk14-f1	11	11	100	23	5	4	80	7	7	5	71.43	6	4	2	50	8
rtdk12-f2	16	16	100	23	13	8	61.54	7	7	2	28.57	6	9	2	22.22	8
rtdk20-f3	3	1	81.25	23	3	2	66.67	7	7	4	57.14	6	3	1	33.33	8
rtdk4-f4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
rtdk13-f5	5	5	100	23	7	6	85.71	7	6	4	66.67	6	3	0	0	8
rtdk15-f6	12	11	91.67	23	10	7	70	7	6	3	50	6	5	0	0	8
rtdk7-f7	13	12	92.31	23	5	3	60	7	7	1	14.20	6	5	1	20	8
rtdk16-f8	14	11	78.57	23	11	9	81.82	7	12	3	25	6	7	2	28.57	8
rtdk14-r1	10	10	100	23	14	9	64.29	7	8	0	0	6	4	1	25	8
rtdk12-r2	3	2	66.67	23	5	3	60	7	4	0	0	6	3	1	33.33	8
rtdk20-r3	0	0	0	0	9	8	88.89	7	5	2	40	6	0	0	0	8
rtdk24-r4	-	-	-	23	2	1	50	7	4	1	25	6	1	0	0	8
rtdk13-r5	4	4	100	23	1	0	0	7	2	0	0	6	3	0	0	8
rtdk13-r6	8	7	87.50	23	8	4	50	7	6	3	50	6	2	1	50	8
rtdk15-r7	-	-	-	23	6	3	50	7	4	2	50	6	3	1	33.33	8
rtdk2-r8	7	7	100	23	4	3	75	7	2	0	0	6	2	0	0	8
rtdk16-r9	14	13	92.86	23	5	2	40	7	9	6	66.67	6	5	2	40	8
rtdk16-r10	13	13	100	23	8	3	37.50	7	9	3	33.33	6	5	2	40	8
Total	262	240	91.60		197	136	69.04		167	58	34.73		117	19	19.01	
Average	11.9	10.91		23	7.9	5.4		7	6.7	2.3		6	4.88	0.79		8
Number of amplifiable primers	22				25				25				24			
Number of polymorphic primers	22				24				20				13			
Number of polymorphic primers/ total number of amplifiable primers (%)	100				96				80				54.17			
Number of amplifiable primers/ total number of primers tested (%)	84.62				96.15				96.15				92.31			

TB, PB, Pi, EN represent total bands, polymorphic bands, percentage of polymorphic bands, and number of effectively amplified genotypes, respectively.

As shown in Table 4, in the case of persimmon, except 4 primers (rtdk20-f3, rtdk20-r3, rtdk24-r4, and rtdk15-r7) that either failed to amplify or did not produce discrete reproducible banding patterns, 22 other primers could successfully generate robust amplification bands across all the 23 accessions tested, representing 7 species of persimmon. This result suggests that retrotransposon primers developed from 'Luotian-tianshi' persimmon could be transferable among members of the same species and among different species within the same genus. In persimmon, the average transfer rate of retrotransposon primers (ratio of the number of amplifiable primers to the total number of tested primers) was 84.62%, and the average efficient transfer rate (ratio of the number of polymorphic primers to the total number of amplifiable primers) was 100%.

In the case of the other 12 fruit crops tested, the majority of persimmon retrotransposon primers also resulted in good amplifications, indicating that persimmon retrotransposon primers could be transferable across unrelated fruit families. The transferability of persimmon retrotransposon primers varied among the other unrelated fruit crops tested. As shown in Table 4, the best transferability of persimmon retrotransposon primers was observed in citrus, grape, and peach, with average transfer rates of 96.15, 96.15, and 92.31%, respectively, and average efficient transfer rates of 96, 80, and 54.17%, respectively. Moreover, in these 3 fruit crops, the transferable primers could amplify all accessions of their respective amplifiable fruit crops. The transferability of persimmon retrotransposon primers in kiwifruit, plum, and loquat was inferior to that in these last 3 fruit crops. The average transfer rates of primers were 84.62% for kiwifruit, 76.92% for plum, and 80.77% for loquat, and the average efficient transfer rates were 77.27, 60.0, and 47.62%, respectively. In these 3 fruit crops, not all of the transferable primers were able to amplify all accessions of their respective amplifiable fruit crops. A moderate level of transferability (88.46%) was observed in pear, in which again not all accessions were amplified. However, primers in pear were more general than those in kiwifruit, plum, and loquat. With respect to the remaining 5 fruit crops (only one accession was assayed per fruit crop), the best and the worst transferability were observed in ginkgo (92.31%) and in chestnut (65.38%), respectively. The transferability in apple was intermediate (88.46%). Representative patterns with 2 primers (rtkd5-p7 and rtdk12-f2) are presented in Figures 1 and 2, respectively.

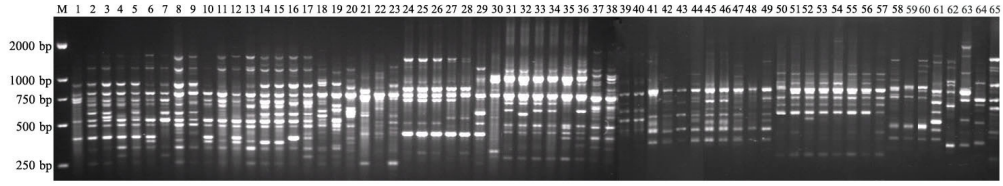

Figure 1. Inter-retrotransposon amplified polymorphism profile of rtdk5-p7 retrotransposon primer derived from 'Luotian-tianshi' persimmon (*Diospyros kaki*) on 65 accessions belonging to 13 different fruit crops. *Lanes 1-65* = accessions listed in Table 1. *Lane M* = DL 2000 molecular marker.

In summary, the persimmon retrotransposon primers showed good transferabilities in 5 (persimmon, citrus, grape, peach, and pear) of the 13 crop species tested. Moreover, since the number of accessions of these 5 species tested was relatively high in this study, we further analyzed the biological meaning of these transferable primers with respect to these 5 fruit crops.

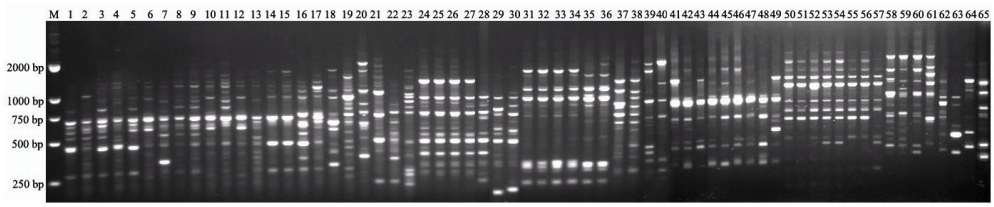

Figure 2. Inter-retrotransposon amplified polymorphism profile of rtdk12-f2 retrotransposon primer derived from 'Luotian-tianshi' persimmon (*Diospyros kaki*) on 65 accessions belonging to 13 different fruit crops. *Lanes 1-65* = accessions listed in Table 1. *Lane M* = DL 2000 molecular marker.

Polymorphism analysis of transferable persimmon retrotransposon primers in persimmon, citrus, grape, peach, and pear

Across all 5 fruit crops (persimmon, citrus, grape, peach, and pear), the total number of amplified bands resulting from transferable persimmon retrotransposon primers that could amplify all accessions of their respective fruit crops ranged from 117 (in peach) to 262 (in persimmon). The total number of polymorphic bands amplified ranged from 19 (in peach) to 240 (in persimmon). The average range of the percentage of polymorphic bands was from 16.24 to 91.60%. The average total number of bands and the average total number of polymorphic bands for each primer varied from 5 to 12, and from 1 to 11, respectively. Except 2 individuals ('90-1-10a' and '90-1-10b') from the same persimmon cultivar, all accessions of each fruit crop, including bud sports and other highly genetically similar individuals, were distinguishable by unique IRAP profiles generated by multiple transferable primers.

Genetic relatedness and cluster analysis revealed by transferable persimmon retrotransposon primers in persimmon, citrus, grape, peach, and pear

Based on the IRAP data generated by transferable persimmon retrotransposon primers, Dice similarity coefficients were calculated and cluster analysis was performed using the UPGMA method for persimmon, citrus, grape, peach, and pear. Dendrograms of different fruit crops revealed by UPGMA cluster analysis are shown in Figure 3.

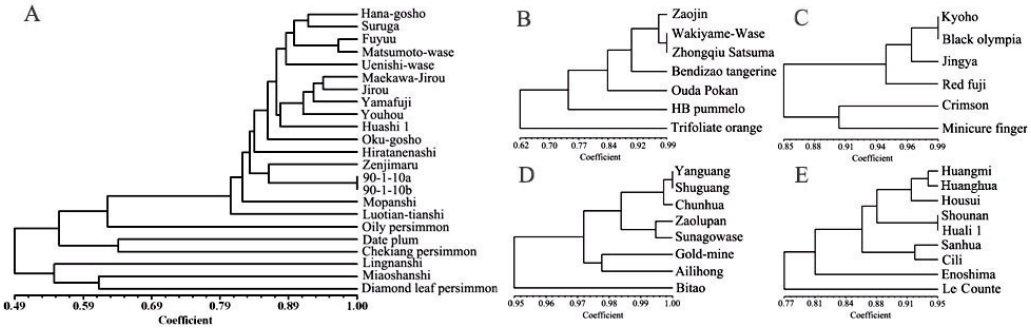

Figure 3. Phenetic relationships among the 23 accessions of persimmon (**A**), the 7 accessions of citrus (**B**), the 6 accessions of grape (**C**), the 8 accessions of peach (**D**), and the 9 accessions of pear (**E**).

At a similarity level of 0.63, all 23 accessions were divided into 2 main clusters: 17 accessions within the persimmon species (*D. kaki* Thunb.) were grouped into a cluster; 6 related species (*D. lotus*, *D. oleifera*, *D. glaucifolia*, *D. tutcheri*, *D. miaoshanica*, and *D. rhombifolia*) formed the other cluster. At a similarity level of 0.83, the cluster including all accessions of persimmon could be further separated into 2 subgroups: 1 consisted of all Japanese-native genotypes, and the other consisted of all Chinese-native genotypes, which were generally in line with their geographical origins. Consistent with their actual relationships, neither of the 2 pairs of bud sports could be grouped well in the dendrogram. Individuals from the same cultivar ('90-1-10a' and '90-1-10b') were grouped together in the dendrogram with no genetic differences detected. 'Matsumoto-wase' and 'Uenishi-wase' are the bud sports from 'Fuyuu' and 'Matsumoto-wase', respectively. 'Maekawa-Jirou' is the bud sport from 'Jirou'. 'Hana-gosho' and 'Oku-gosho' are the parents of 'Youhou'. 'Fuyuu' and 'Jirou' are the parents of 'Suruga'. In the dendrogram, 'Youhou' was clustered with its female parent, 'Hana-gosho', and 'Suruga' was adjacent to its male parent 'Jirou'. 'Hiratanenashi', a Japanese native pollination-variant and non-astringent type persimmon, which is also one of the rarest and most precious nonaploid cultivars of persimmon, exhibited a relatively distant relationship with the other Japanese-native genotypes of persimmon. In contrast, 'Hiratanenashi' was clustered directly with '90-1-10', a newly discovered and genetically unique Chinese-native pollination-variant and non-astringent type of persimmon, suggesting these are likely related. Among the related species, *D. oleifera*, *D. lotus*, and *D. glaucifolia* displayed relatively close genetic relationships with *D. kaki*; however, *D. rhombifolia* was the most distantly related to *D. kaki*. This result was supported by the results of previous studies (Kanzaki et al., 2000; Guo et al., 2006; Yonemori et al., 2008).

Figure 3B shows the phenetic relationships among the 7 accessions of citrus. The lowest mean similarity coefficient (0.62) was detected between trifoliate orange and the other accessions. Moreover, in the dendrogram, trifoliate orange was clearly separated from the rest of the accessions. The above results suggested a distant relationship between trifoliate orange and the other accessions, which is congruent with their actual taxonomic relationship, as they are classified into different genera. A greater mean genetic similarity (0.89) was detected among the genus *Citrus*, in which 'Zaojing', 'Wakiyame-wase satsuma', 'Zhongqiu satsuma', 'Bendizao tangerine', and 'Oota' all belong to *Citrus reticulata*. These accessions formed a group at a similarity level of 0.84, suggesting their close genetic relatedness. However, 'HB pummelo' fell outside of this group, indicating the relatively distant relationship between 'HB pummelo' and these 5 accessions, in agreement with their taxonomic classification of belonging to different species. 'Zaojing', 'Wakiyame-wase satsuma', and 'Zhongqiu satsuma' are the superior lines from 'Mandarin Oranges'. Among them, the closest genetic relationship was observed between 'Wakiyame-wase satsuma' and 'Zhongqiu satsuma' (0.99). In all the accessions of *C. reticulata* tested, 'Oota' showed the most genetic differences with the other accessions, likely because it was one of the superior lines selected from 'Penggan Tangerines'.

Phenetic relationships among the 6 accessions of grape are shown in Figure 3C. At the 0.85 similarity level, the accessions were grouped into 2 distinct clusters that are consistent with the traditional taxonomic classification of species: 4 accessions, namely, 'Kyoho', 'Black olympia', Jingya', and 'Red fuji', fell into a *V. labrusca* x *V. vinifera* cluster, and the other 2 accessions fell into a *V. vinifera* cluster. In the *V. labrusca* x *V. vinifera* cluster, 'Kyoho' is thought to be one of the parents of 'Black olympia', and 'Jingya' is the seedling

variation from 'Black olympia'; therefore, these 3 accessions were grouped together first, and then with 'Red fuju'.

Figure 3D represents the phenetic relationships among the 8 accessions of peach. An extremely high genetic similarity coefficient (range: 0.94 to 0.99; mean: 0.97) was observed, indicating a narrow genetic diversity among these accessions tested. At a 0.95 similarity level, 'Bitao' formed a distinct monophyletic clade on its own (cluster 1). The remaining accessions grouped into cluster 2, which could be further divided into 3 subgroups: 'Yanguang', 'Shuguang', and 'Chunhua' in the first subgroup; 'Zaolupan' and 'Sunagowase' in the second subgroup; 'Goldmine' and 'Ailihong' in the third subgroup. In general, these results showed no clear-cut genetic differences between *P. persica* and its variations.

Phenetic relationships among the 9 accessions of pear are illustrated in Figure 3E. At a similarity level of 0.77, 'Le Counte' individually formed a cluster and the remaining accessions grouped together in the other cluster. This was expected since 'Le Counte' belongs to *P. bretschneideri* x *P. communis*, whereas the other accessions tested belong to *P. pyrifolia* or *P. bretschneideri*. Within the *P. pyrifolia* species, 'Huanghua' is the offspring of 'Huangmi' and 'Sanhua', and 'Huali 1' is the offspring of 'Shounan' and 'Enoshima'. In the dendrogram, both 'Huanghua' and 'Huali 1' also grouped with their respective female parents. These results showed good concordance with their actual pedigree relationships. 'Cili' is a cultivar of *P. bretschneideri*, but it was clustered with 'Sanhua' (*P. pyrifolia*). Moreover, with respect to the entire distribution pattern of the accessions, 'Cili' was positioned among accessions of sand pear (*P. pyrifolia*), which may indicate a certain close relationship between *P. bretschneideri* and *P. pyrifolia*.

Transferability of published retrotransposon primers derived from Rosaceae, Gramineae, and Solanaceae to 13 fruit crops

Transferability of the retrotransposon primers published from Rosaceae, Gramineae, and Solanaceae to the genus *Diospyros* was tested on 3 representative genotypes (*D. kaki* 'Luotian-tianshi', *D. kaki* 'Mopanshi', and *D. lotus*). The results showed that each of the 13 primers tested could successfully generate amplification patterns (Figure 4), indicating a good transferability of these published primers to the genus *Diospyros*. Unique fingerprints of the 3 genotypes analyzed were produced by each primer. The amplification characteristic, reflected by several aspects such as size, distribution, abundance, and polymorphism of amplifiable bands, varied depending on the different primers. For example, the bands resulting from both LTR6149 and 6150 primers were centralized in the higher molecular weight range (850 to 2000 bp), whereas the bands generated by the remaining primers (except for Tnt-1) were distributed evenly, with a medium size range (200 to 2000 bp). With respect to the abundance and polymorphism of the bands, primer Tnt-1 generated the lowest number of total and polymorphic bands, whereas, 5'LTR2 primer produced the highest number of total and polymorphic bands. The good transferability of these published primers to the genus *Diospyros* has been further confirmed by testing them on the larger scale of the germplasm (Du XY, Zeng M, Wang YB, Xiong YT, et al., unpublished data).

These 33 retrotransposon primers published were used to amplify the other 12 fruit crops. Successful transferability of these primers was also observed. Representative banding profiles generated by the 6 primers published (LTR1, LTR2, LTR6149, LTR6150, 5'LTR1, and 5'LTR2) are shown in Figure 5.

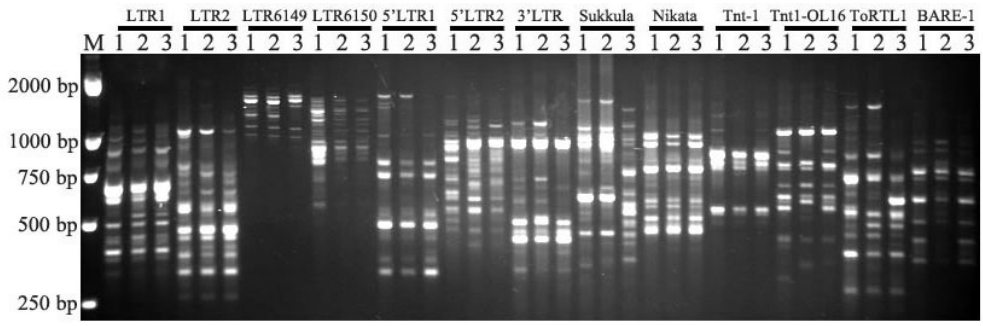

Figure 4. Inter-retrotransposon amplified polymorphism profile performed on persimmon (*Diospyros* spp) using 13 retrotransposon primers. *Lane M* = DL 2000 marker; *lane 1* = 'Fuyuu' persimmon (*D. kaki*); *lane 2* = 'Luotian-tianshi' persimmon (*D. kaki*); *lane 3* = *D. Lotus* L.

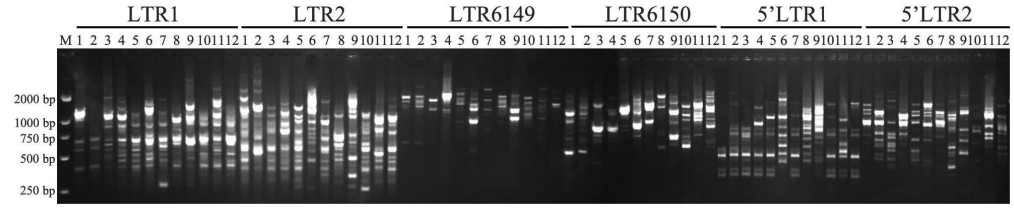

Figure 5. Inter-retrotransposon amplified polymorphism profile of 6 retrotransposon primers on 12 accessions belonging to 12 different fruit crops. *Lane 1* = Bendizao tangerine (*Citrus reticulata*); *lane 2* = chestnut (*Castanea mollissima*); *lane 3* = Black olympia (*Vitis labrusca* x *V. vinifera*); *lane 4* = Miliang 1 (*Actinidia deliciosa* var. *deliciosa*); *lane 5* = Oishi-wase (*Prunus salicina*); *lane 6* = Housui (*P. pyrifolia*); *lane 7* = Zaolupan (*P. persica* var. *compressa*); *lane 8* = Dawuxing (*Eriobotrya japonica*); *lane 9* = apple (*Malus pumila*); *lane 10* = jujube (*Zizyphus jujube*); *lane 11* = ginkgo (*Ginkgo biloba*); *lane 12* = hawthorn (*Crataegus pinnatifida*); *lane M* = DL 2000 marker.

DISCUSSION

It is generally believed that the development of a retrotransposon marker system into a plant species requires retrotransposon primers that are native to the particular species. Moreover, in a review of studies that applied retrotransposon molecular markers for characterizing germplasm, we found that almost all of the retrotransposon primers used in PCRs were designed based on the sequences of retrotransposons native to the species tested (Baumel et al., 2002; Bernet and Asíns, 2003; Labra et al., 2004; Guo et al., 2006; Antonius-Klemola et al., 2006; Bousios et al., 2007). However, Schulman (2007) pointed out that retrotransposon primers could sometimes be used between plant species. In addition, Teo et al. (2005) successfully used retrotransposon primers derived from the family Gramineae for the identification and characterization of banana cultivars and classification of Musa genome constitutions. In the present study, our results showed good transferability of retrotransposon primers derived from 'Luotian-tianshi' persimmon to other unrelated plant species, as well as to different individuals within the same species of persimmon and to different species within the same genus, *Diospyros*. These results suggested that retrotransposon primers could be transferred not only across the plant species from which they were developed, but also across genetically distant plant species, which is consistent with

conclusions drawn from the aforementioned previous studies, particularly supporting results of Teo et al. (2005), Schulman (2007), and Kalendar et al. (2011).

Two different individuals from the same cultivar of the persimmon ('90-1-10a' and '90-1-10b') showed highly consistent banding patterns with either of the retrotransposon primers, indicating good reproducibility of the IRAP molecular marker. Two pairs of bud sports of *D. kaki*, several of the superior lines from 'Mandarin Oranges', as well as some genotypes of known pedigree relationships in grape, peach, pear, and persimmon were all grouped well, suggesting the reliability of cluster results based on IRAP analysis. These patterns further indicated that these transferable persimmon retrotransposon primers could be applied to pedigree studies in these fruit crops. The broad potential of persimmon retrotransposon primers for future analyses of classification and phylogenetic relationship was further made evident by the clear-cut separation between persimmon and its several related species, the unambiguous clusters that were congruent with geographic origins within the persimmon species, and groupings of citrus, grape, and pear that were consistent with the genetic relationships or taxonomic classifications reported in these crops. In addition, we detected high similarity among peach and its variations, supporting the results of Cheng (2007). The IRAP molecular data generated by these persimmon retrotransposon primers revealed genotypes of high genetic similarity in peach, bud sports in persimmon, and different superior lines from 'Mandarin Oranges', which could all be distinguished well, suggesting that the transferable persimmon retrotransposon primers tested might offer a potential primer resource for variation identification, especially in bud sports.

In the present study, most of plant materials that were used in the investigation on the transferability of persimmon retrotransposon primers were developed from woody trees, which all belong to fruit crops. In contrast, the materials used for the experiment on the transferability of published retrotransposon primers were all fruit crops themselves. Therefore, although these 2 experiments were examined on 2 types of materials with a great degree of genetic discrepancy, consistent results were obtained nevertheless. Thus, our results provide strong evidence for the transferability of retrotransposon primers across different plant species.

In conclusion, this is the first general study that examined retrotransposon primer transferability across a range of widely diverse plant species. Although the numbers of plant species selected and accessions tested per plant species were low, and representative samples used were also limited, we were able to conclude unambiguously that retrotransposon primers could be transferable across plant species, as well as across individuals within the same species or within the same genus. Our results not only provide evidence for the transferability of retrotransposon primers but also offer a series of universal retrotransposon primers that could be utilized directly for genetic analyses of plant species, which will avoid the need to adopt the complex procedures for the development of retrotransposon primers. Moreover, this study showed that in the case of a new plant species for which retrotransposon primers are relatively scarce or hard to develop because of a lack of retrotransposon sequence information, published retrotransposon primers and massive retrotransposon sequences from other plant species in public databases would be efficient and applicable resources for developing retrotransposon molecular markers. Our results therefore offer a cost-effective and highly efficient method for obtaining retrotransposon primers, and may also be of great help in the popularization and application of retrotransposon-based molecular markers, as well as in increasing the number of retrotransposon primers available for a wide variety of crops.

ACKNOWLEDGMENTS

Research supported by the Natural Science Foundation of China (#31000896), the National Natural Science Foundation of Innovation Group Projects (#30921002), the Research Fund for the Doctoral Program of Higher Education of China (#2011014611001800), the National Science Foundation of Jiangxi Province of China (#20114BAB214005), and the Innovation Foundation of the Jiangxi Academy of Agricultural Sciences (Doctor's Science Project Foundation #2009 Dr-8).

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