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IDENTIFICATION OF INTERSPECIFIC HYBRIDS BETWEEN LOQUAT (*ERIOBOTRYA JAPONICA* LINDL.) AND BENGAL LOQUAT (*E. BENGALENSIS* HOOK.)

YONGQING WANG[1], KUI DU[1,2*], QUNXIAN DENG[1], JIANGUO ZENG[3], WEI KANG[4], YUANJUN XU[4] AND JIAZHI ZHANG[4]

[1]College of Horticulture, Sichuan Agricultural University, Ya'an 625000, China;
[2]Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China;
[3]Bureau of Rural Area Development of Longquanyi, Chengdu 610000, China;
[4]Bureau of Agriculture, Shimian, Ya'an 625400, China
**Corresponding author: dukui@wbgcas.cn*

Abstract

Loquat (*Eriobotrya japonica* Lindl.) is an important subtropical fruit; however, loquat fruitlets are vulnerable to cold injury in winter, which significantly decreases loquat yield in most production regions. In the present study, two loquat cultivars ('Dawuxing' and '4-1-5') and one wild loquat (*E. bengalensis* Hook., Bengal loquat), were used for interspecific hybridization to produce hybrids with characteristics of spring blooming to avoid cold injury of fruitlets. Hybrid seedlings were derived from direct cross (loquat as female parent and Bengal loquat as male parent) and reciprocal cross. The authenticity of 47 hybrid seedlings was confirmed using inter-simple sequence repeat (ISSR) molecular markers; and leaf morphological characteristics of the hybrid offspring and parents were preliminarily studied and compared. The results suggested that 23 true direct cross hybrids and 12 true reciprocal cross hybrids were obtained, with hybrid authenticity rates of 100 and 50.0%, respectively. Thus, a novel method of distant hybridization for loquat breeding was developed, and with their various genetic and morphological characteristics these hybrids could be valuable germplasms for horticultural use.

Key words: Hybrids, *Eriobotrya japonica*, *Eriobotrya bengalensis*, Dawuxing.

Introduction

Loquat (*Eriobotrya japonica* Lindl., Rosaceae, Maloideae), an important economic fruit crop with high food and medical value (Lin *et al.*, 2007; Zhou *et al.*, 2011), is grown in many subtropical areas (Badenes *et al.*, 2000; Blasco *et al.*, 2014).

Eriobotrya japonica is a unique economically cultivated species in this genus. It blooms in late autumn and early winter, and the young fruits are vulnerable to suffer from low temperature in cold winter – the most serious problem in most production regions (Freihat *et al.*, 2008; Badenes *et al.*, 2013).

Some physical (e.g. hot air) and chemical methods (e.g. nitric oxide and methyl jasmonate) can enhance the cold resistance of loquat fruit (Cai *et al*., 2011; Wu *et al*., 2012; Jin *et al*., 2014), but these methods have operational difficulties and/or result in environmental pollution. Plant breeders have paid much attention to developing cold-resistant loquat cultivars, but there has been no significant progress. Cross breeding is commonly used to improve cultivar characteristics and overcome the time and labor limitations of selective breeding (Huang *et al.*, 2014). Therefore, altering the phenophase of loquat by crossing is an alternative method to overcome this issue. Bengal loquat (*E. bengalensis* Hook.) blooms in March and April and ripens in July and August in China, is considered a valuable genetic resource for breeding spring-flowering *E. japonica* cultivars which can avoid cold injury in winter.

The objective of the study is to modify loquat by interspecific hybridization in an attempt to transfer the characteristic of spring blooming and other features (e.g. seedless fruit) of Bengal loquat into loquat. The authenticity of hybrid offspring must be verified at an early stage to optimize planting time and costs. This paper reports the early identification of interspecific hybrids between *E. japonica* and *E. bengalensis* using inter-simple sequence repeat (ISSR) and the leaf morphological characteristics of offspring and parents.

Materials and Methods

Plant materials: Two loquat (*E. japonica* Lindl.) cultivars, 'Dawuxing' (yellow flesh) and '4-1-5' (white flesh), and Bengal loquat (*E. bengalensis* Hook.) were used as experimental materials in this study (Fig. 1). Annual hybrid seedlings were derived from direct crossing (loquat as female parent and Bengal loquat as male parent) and reciprocal crossing. All offspring were planted in pots and cultivated in the Research Center for Horticultural Biotechnology, Sichuan Agricultural University, China.

Genomic DNA extraction and detection: A modified version of the CTAB method (Fu *et al*., 2009) was used to extract genomic DNA and the integrity was checked by electrophoresis in 0.8% (w/v) agarose gels. The DNA was stored at -20°C before the ISSR-PCR reactions.

Primer screening and PCR amplification: Genomic DNA of the three parents was used as templates and six primers with high polymorphism (Table 1) were selected out of 20 primers screened by Wang *et al*. (2010). Primers were synthesized by Sangon Biotech (Shanghai, China) Co. Ltd. *Taq* DNA polymerase, dNTP mixture and the 2 000-bp marker were purchased from Tiangen Biotech (Beijing, China) Co. Ltd.

Fig. 1. Loquat with young fruit (left) and Bengal loquat at anthesis (right) in March.

Table 1. ISSR primers used to identify of hybrids.

No.	Primer	Sequence (5′–3′)
1.	UBC807	$(AT)_8G$
2.	UBC836	$(AG)_8GTA$
3.	UBC857	$(AC)_8GTG$
4.	UBC873	$(GACA)_4$
5.	UBC895	AGAGTTGGTAGCTCTTGATC
6.	UBC899	CATGGTGTTGGTCATTGTTCCA

PCR amplification was performed by Bio-Rad PTC-200 (USA). PCR amplification was conducted in a volume of 25 µl containing 60 ng of genomic DNA, 2.5 µl of 10×PCR buffer, 2.0 mM $MgCl_2$, 0.15 mM dNTP mixture, 0.3 µM primer and 1.5 U *Taq* DNA polymerase. Amplification reactions were performed in a thermal cycler programmed for 5 min of predenaturation at 94°C, followed by 40 cycles of denaturation at 94°C for 70 s, 72°C for 1.5 min for annealing, and was terminated with a 7-min DNA extension step at 72°C. The amplification products were stored at 4°C.

ISSR detection: A mixture of amplification products and bromophenol blue was analyzed by electrophoresis in 2.0% agarose gels. The gels were stained with 10 mg ethidium bromide (EB) for 10 min, visualized under ultraviolet light and documented by Syngene GeneGenius densitograph system (USA). The band size was determined using the DNA size marker.

Leaf morphology: Leaves of the offspring and parents were observed and described according to the descriptors and data standards for loquat *Eriobotrya* spp. (Zheng *et al.*, 2006). In addition, CANOCO 4.5 software was used to explore the generic relationship between the offspring and their parents.

Results

Screening of primers: The OD260/OD280 of DNA was 1.80–1.95, indicating high purity of extracted DNA. Agarose gel electrophoresis showed that the extraction of DNA with clear bands, in order, low transfer rate, integrity, no noticeable degradation phenomenon and the loading wells without remainders met the requirements for ISSR analysis.

The primers were selected for their ability to yield clear, parent specific, polymorphic and reproducible patterns of amplification. As a result, of the six tested ISSR primers, only UBC895 and UBC899 were optimal (Fig. 2).

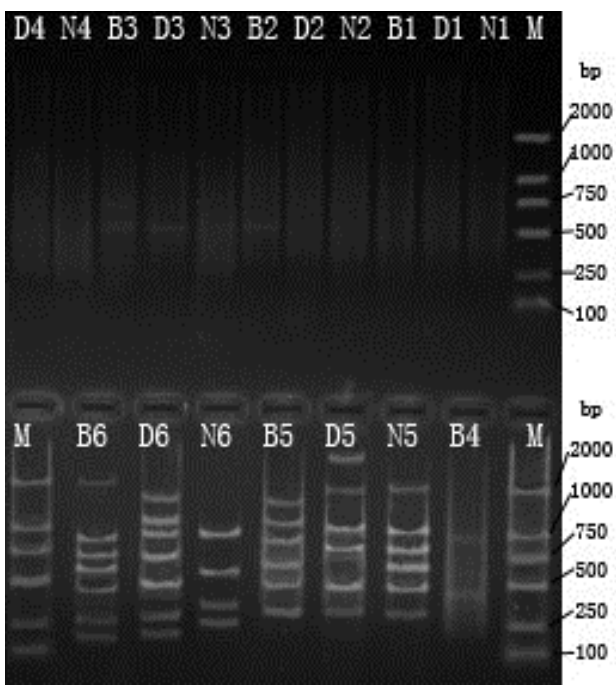

Fig. 2. Screening of primers: 'Dawuxing' loquat (D); Bengal loquat (N); '4-1-5' loquat (B); PCR amplification of parent using primer (N1–B6); and D2000 marker (M).

ISSR amplification results: UBC895 and UBC899 were used in ISSR amplification analysis of 50 materials including three parents. Polymorphisms observed between male and female parents were used as markers. There are 261 and 275 DNA bands amplified (Figs. 3-6), of which 215 and 225 DNA bands were polymorphic, with a mean of 4.3 and 4.5 polymorphic bands per material, accounting for 82.4 and 81.8% of the total number, respectively, showing good polymorphism on DNA level.

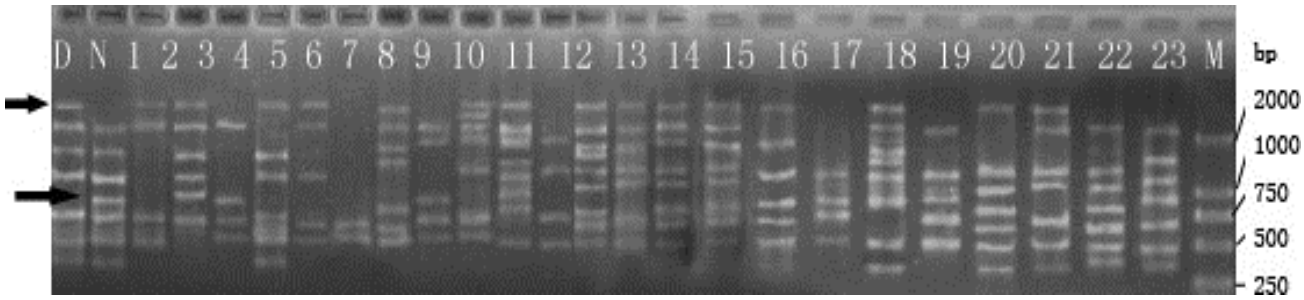

Fig. 3. ISSR amplification of DN offspring using primer UBC895: 'Dawuxing' loquat (D); Bengal loquat (N); reciprocal cross offspring (Nos 1–11); direct cross offspring (Nos 12–23); and D2000 marker (M).

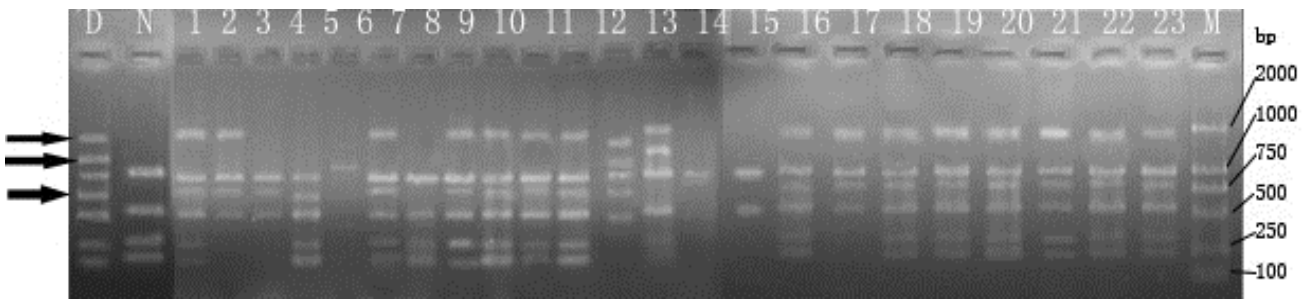

Fig. 4. ISSR amplification of DN offspring using primer UBC899: 'Dawuxing' loquat (D); Bengal loquat (N); reciprocal cross offspring (Nos 1–11); direct cross offspring (Nos 12–23); and D2000 marker (M).

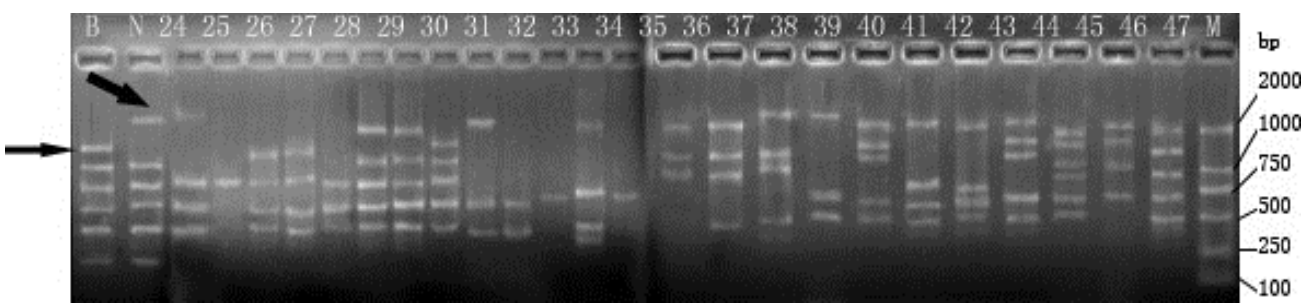

Fig. 5. ISSR amplification of BN offspring using primer UBC895: '4-1-5' loquat (B); Bengal loquat (N); reciprocal cross offspring (Nos 24–36); direct cross offspring (Nos 37–47); and D2000 marker (M).

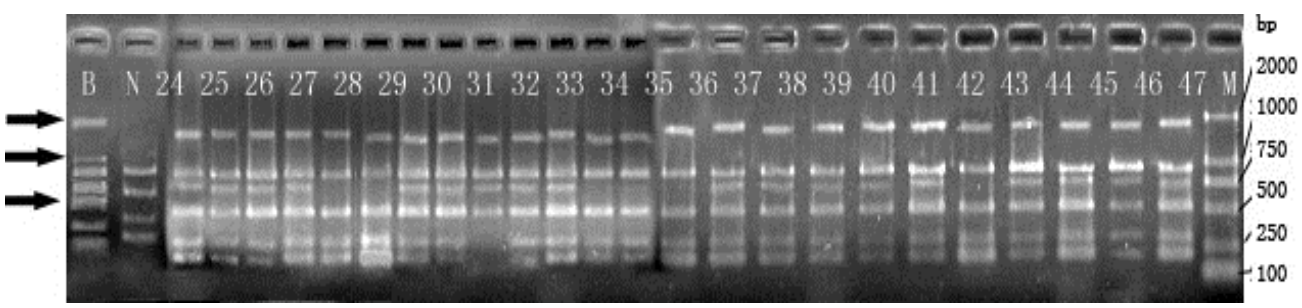

Fig. 6. ISSR amplification of BN offspring using primer UBC899: '4-1-5' loquat (B); Bengal loquat (N); reciprocal cross offspring (Nos 24–36); direct cross offspring (Nos 37–47); and D2000 marker (M).

To confirm the hybrid authenticity, only the primers that amplified male parent-specific bands of each progeny were considered (Bianco *et al.*, 2011; Huang *et al.*, 2014). In the hybrid combination of 'Dawuxing' loquat × Bengal loquat, primer UBC895 amplified one specific band of both parents, and 19 of 23 offspring were true hybrids (except for Nos 3, 6, 9 and 11) and hybrid authenticity rate was 82.6% (Fig. 3). Primer UBC899 amplified three specific bands from 'Dawuxing' loquat, nine of 11 progenies were true hybrids (except for Nos 5 and 7) and with hybrid authenticity rate of 81.8% (Fig. 4). Thus, five of 11 offspring were identified as true hybrids by both primers.

Similarly, in the hybrid combination of '4-1-5' loquat × Bengal loquat, primer UBC895 amplified a specific band of both parents and, of 24 identified offspring, 18 were true hybrids (except for Nos 24, 25, 28, 33, 34 and 36) (Fig. 5). Primer UBC899 amplified three specific bands from '4-1-5' loquat with all offspring identified as true hybrids (Fig. 6). Only seven of 13 offspring were identified as true hybrids by both primers.

Table 2. The foliar characteristics and given values of offspring from loquat and Bengal loquat

No.	Color of upper side	Leaf shape	Shape of underside	Luster of upper side	Shape of leaf tip	Shape of leaf base	Shape of leaf margin	Color of underside
1.	Dark green 4	Lanceolate 1	Flat 1	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Involute 2	Offwhite 1
2.	Green 3	Elliptic 2	Rugose 3	Light lustrous 1	Pungent 2	Narrowly cuneate 1	Revolute 3	Isabelline 2
3.	Kelly 1	Lanceolate 1	Rugose 3	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Revolute 3	Offwhite 1
4.	Dark green 4	Elliptic 2	Rugulose 2	Lustrous 2	Acuminate 3	Cuneate 2	Revolute 3	Isabelline 2
5.	Dark green 4	Lanceolate 1	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Revolute 3	Isabelline 2
6.	Green 3	Elliptic 2	Rugulose 2	Lustrous 2	Pungent 2	Narrowly cuneate 1	Involute 2	Isabelline 2
7.	Green 3	Elliptic 2	Rugose 3	Light lustrous 1	Pungent 2	Narrowly cuneate 1	Flat 1	Isabelline 2
8.	Dark green 4	Lanceolate 1	Flat 1	Lusterless 0	Pungent 2	Narrowly cuneate 1	Involute 2	Isabelline 2
9.	Green 3	Elliptic 2	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Flat 1	Offwhite 1
10.	Dark green 4	Elliptic 2	Rugulose 2	Lustrous 2	Acuminate 3	Cuneate 2	Flat 1	Isabelline 2
11.	Dark green 4	Elliptic 2	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Flat 1	Offwhite 1
12.	Kelly 1	Elliptic 2	Rugulose 2	Lustrous 2	Pungent 2	Narrowly cuneate 1	Flat 1	Grayish brown 3
13.	Green 3	Elliptic 2	Rugulose 2	Light lustrous 1	Broadly acute 1	Narrowly cuneate 1	Flat 1	Isabelline 2
14.	Green 3	Elliptic 2	Rugose 3	Light lustrous 1	Pungent 2	Cuneate 2	Revolute 3	Grayish brown 3
15.	Dark green 4	Elliptic 2	Rugulose 2	Lustrous 2	Broadly acute 1	Narrowly cuneate 1	Flat 1	Isabelline 2
16.	Kelly 1	Elliptic 2	Rugose 3	Light lustrous 1	Broadly acute 1	Cuneate 2	Flat 1	Isabelline 2
17.	Green 3	Obovoid 3	Rugulose 2	Light lustrous 1	Broadly acute 1	Narrowly cuneate 1	Flat 1	Isabelline 2
18.	Green 3	Elliptic 2	Rugulose 2	Lustrous 2	Broadly acute 1	Narrowly cuneate 1	Flat 1	Offwhite 1
19.	Kelly 1	Lanceolate 1	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Revolute 3	Isabelline 2
20.	Green 3	Lanceolate 1	Rugose 3	Lustrous 2	Broadly acute 1	Broadly cuneate 3	Involute 2	Grayish brown 3
21.	Kelly 1	Elliptic 2	Rugulose 2	Lusterless 0	Broadly acute 1	Narrowly cuneate 1	Revolute 3	Isabelline 2
22.	Light green 2	Lanceolate 1	Rugose 3	Lustrous 2	Broadly acute 1	Narrowly cuneate 1	Flat 1	Isabelline 2
23.	Green 3	Obovoid 3	Rugose 3	Lustrous 2	Acuminate 3	Narrowly cuneate 1	Flat 1	Grayish brown 3
24.	Kelly 1	Elliptic 2	Rugulose 2	Lustrous 2	Acuminate 3	Narrowly cuneate 1	Flat 1	Isabelline 2
25.	Green 3	Elliptic 2	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Flat 1	Grayish brown 3
26.	Green 3	Elliptic 2	Rugose 3	Lustrous 2	Acuminate 3	Narrowly cuneate 1	Revolute 3	Isabelline 2
27.	Dark green 4	Lanceolate 1	Rugose 3	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Revolute 3	Offwhite 1
28.	Green 3	Lanceolate 1	Rugose 3	Lusterless 0	Acuminate 3	Narrowly cuneate 1	Flat 1	Isabelline 2
29.	Kelly 1	Lanceolate 1	Rugose 3	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Involute 2	Isabelline 2
30.	Green 3	Lanceolate 1	Rugose 3	Lusterless 0	Acuminate 3	Narrowly cuneate 1	Revolute 3	Isabelline 2
31.	Dark green 4	Elliptic 2	Rugose 3	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Flat 1	Offwhite 1
32.	Kelly 1	Lanceolate 1	Rugulose 2	Lustrous 2	Acuminate 3	Narrowly cuneate 1	Flat 1	Isabelline 2
33.	Green 3	Elliptic 2	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Flat 1	Isabelline 2
34.	Green 3	Lanceolate 1	Rugulose 2	Lustrous 2	Acuminate 3	Narrowly cuneate 1	Flat 1	Grayish brown 3
35.	Green 3	Elliptic 2	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Flat 1	Grayish brown 3
36.	Green 3	Lanceolate 1	Rugulose 2	Light lustrous 1	Acuminate 3	Narrowly cuneate 1	Involute 2	Isabelline 2
37.	Dark green 4	Elliptic 2	Flat 1	Lusterless 0	Acuminate 3	Broadly cuneate 3	Flat 1	Isabelline 2
38.	Dark green 4	Elliptic 2	Rugulose 2	Lusterless 0	Acuminate 3	Broadly cuneate 3	Revolute 3	Isabelline 2
39.	Dark green 4	Elliptic 2	Rugulose 2	Lusterless 0	Pungent 2	Broadly cuneate 3	Involute 2	Isabelline 2
40.	Dark green 4	Lanceolate 1	Flat 1	Lusterless 0	Acuminate 3	Broadly cuneate 3	Involute 2	Offwhite 1
41.	Dark green 4	Lanceolate 1	Rugose 3	Lusterless 0	Acuminate 3	Narrowly cuneate 1	Revolute 3	Isabelline 2
42.	Dark green 4	Elliptic 2	Flat 1	Lusterless 0	Broadly acute 1	Broadly cuneate 3	Flat 1	Isabelline 2
43.	Green 3	Lanceolate 1	Rugulose 2	Light lustrous 1	Acuminate 3	Broadly cuneate 3	Revolute 3	Offwhite 1
44.	Dark green 4	Lanceolate 1	Rugulose 2	Lusterless 0	Acuminate 3	Narrowly cuneate 1	Involute 2	Grayish brown 3
45.	Dark green 4	Elliptic 2	Rugulose 2	Light lustrous 1	Broadly acute 1	Broadly cuneate 3	Flat 1	Isabelline 2
46.	Dark green 4	Elliptic 2	Flat 1	Lusterless 0	Acuminate 3	Broadly cuneate 3	Involute 2	Isabelline 2
47.	Dark green 4	Elliptic 2	Rugulose 2	Lusterless 0	Broadly acute 1	Cuneate 2	Revolute 3	Isabelline 2
48.	Dark green 4	Lanceolate 1	Rugulose 2	Lustrous 2	Acuminate 3	Narrowly cuneate 1	Revolute 3	Grayish brown 3
49.	Green 3	Elliptic 2	Flat 1	Lusterless 0	Pungent 2	Broadly cuneate 3	Involute 2	Offwhite 1
50.	Dark green 4	Lanceolate 1	Rugose 3	Light lustrous 1	Acuminate 3	Cuneate 2	Flat 1	Isabelline 2

Note: Reciprocal cross offspring (‘Dawuxing’ loquat as male and Bengal loquat as female parent, Nos 1–11);direct cross offspring (‘Dawuxing’ loquat as female and Bengal loquat as male parent, Nos 12–23); reciprocal cross offspring (‘4-1-5’ loquat as male and Bengal loquat as female parent, Nos 24–36); direct cross offspring (‘4-1-5’ loquat as female and Bengal loquat as male parent, Nos 37–47); ‘Dawuxing’ loquat (No. 48); bengal loquat (No. 49); and ‘4-1-5’ loquat (No.50). The values of offspring were set according to the descriptors and data standards for loquat (*Eriobotrya* spp.) (Zheng *et al*., 2006)

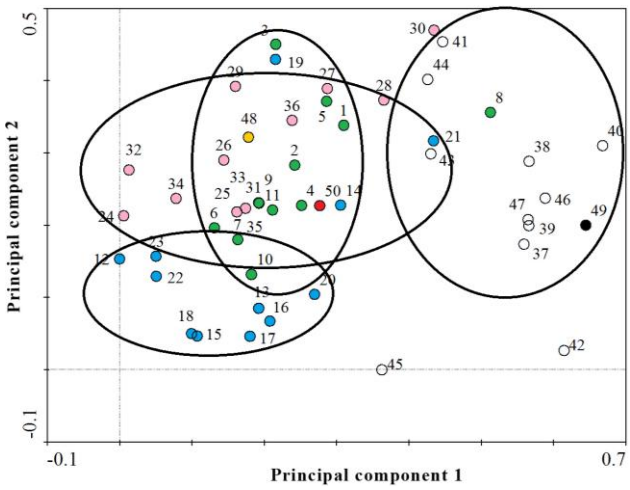

Fig. 7. Principal component analysis of leaf morphology of loquat, bengal loquat and their offspring: reciprocal cross offspring ('Dawuxing' loquat as male and Bengal loquat as female parent, Nos 1–11, green); direct cross offspring ('Dawuxing' loquat as female and Bengal loquat as male parent, Nos 12–23, blue); reciprocal cross offspring ('4-1-5' loquat as male and Bengal loquat as female parent, Nos 24–36, pink); direct cross offspring ('4-1-5' loquat as female and Bengal loquat as male parent, Nos 37–47, white); 'Dawuxing' loquat (No. 48, yellow); Bengal loquat (No. 49, black); and '4-1-5' loquat (No. 50, red).

Moreover, 12 and five true hybrids were obtained in the direct and reciprocal crosses between 'Dawuxing' loquat and Bengal loquat, with hybrid authenticity rates of 100 and 45.5%, respectively. 11 and seven true hybrids in the direct and reciprocal crosses between '4-1-5' loquat and Bengal loquat, with hybrid authenticity rates of 100 and 63.6%, respectively.

In total, 23 true direct cross hybrids and 12 true reciprocal cross hybrids of loquat and Bengal loquat were obtained, with hybrid authenticity rates of 100 and 50.0%, respectively.

Leaf morphology: To confirm the hybrids, eight morphological characters including color, shape and luster were examined (Table 2). Hybrids were clearly divided into four groups by principal component analysis (60.6% of explanation rate), indicating a closed genetic relationship between most hybrids and their parents (Fig. 7).

Discussion

Plant breeders have created abundant new germplasm resources and varieties by introduction (Badenes *et al.*, 2013), mutation (Prederi, 2001; Wang *et al.*, 2007) and intraspecific hybridization (Huang *et al.*, 1999) in loquat. However, the cold resistance of loquat fruitlets has not been significantly enhanced. In addition, the available genetic resources of this species have been increasingly exhausted (Yang *et al.*, 2006). As a result, the methods previously used cannor meet the demand of domestic and international markets. The genus *Eriobotrya* has 34 species (Yang *et al.*, 2005), containing rich germplasm resources. Interspecific hybridization is the most effective approach to genetic improvement in loquat, but has not yet been reported. Use of loquat (*E. japonica*) and Bengal loquat (*E. bengalensis*) for loquat breeding in the present study is the first reported case.

Fluorescence detection revealed that the pollen tubes formed 6 h after pollination and about 90% of the pollen germinated on the stigma; the pollen tubes sent papilla through the top of stigma, reached the middle of stigma at 24 h after pollination and about three-quarters of them reached the bottom of stigma by 48 h after pollination (data not shown). That indicated that loquat and Bengal loquat had good compatibility in both direct and reciprocal crosses. Hence, hybrids between loquat and Bengal loquat were obtained.

The rapid and accurate identification of hybrid authenticity is important. With the advantages of time- and resource-saving, less labor-consumption and more precision, DNA-based molecular markers provide a powerful tool for hybrid identification (Li *et al.*, 2014; Huang *et al.*, 2014). ISSR molecular markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence anchored at the 3′- or 5′- end by 2–4 arbitrary, often degenerate, nucleotides. The amplification products were separated on non-denaturing polyacrylamide gels and detected by staining. ISSR has evolved rapidly, does not require prior knowledge of DNA sequence for primer design and banding profiles are very repeatable on duplicate samples (Fang & Roose, 1997; Rout *et al*., 2009; Mirbahar *et al*., 2016). ISSR has proved very effective and accurate for plant hybrid identification (Dongre & Parkhi, 2005; Liu *et al.*, 2007; Gradzielewska *et al.*, 2012; Khajudparn *et al.*, 2012; Khan *et al.*, 2013) and determining genetic relationships among loquat germplasm (Srivastava *et al*., 2007; Xie *et al.*, 2007; Luo *et al.*, 2011; Hu *et al.*, 2015) . Thus, we used this technique for hybrid early identification. The results suggested that ISSR could be used to identify the authenticity of hybrid offspring in loquat, which further provided a practical method for rapid identification and early selection of the hybrid offspring among *Eriobotrya* spp.

Because of their long juvenile period, loquat trees derived from seedlings usually do not bear fruit until 3–5 years after planting. Traditional identification of hybrids by morphological methods is ambiguous, tedious, and time-consuming, and is easily affected by environment and developmental stage (Lin *et al*., 2010; Khan *et al*., 2013; Huang *et al*., 2014). However, morphological identification still plays a role in supporting and verifying the results of molecular identification.

The leaf morphology of most hybrids was similar to their parents (Fig. 7). Combining the results of ISSR and leaf morphology detection showed that most offspring were true hybrids, with satisfactory hybrid rates. Because of the absence of male parent-specific bands, the remaining progenies were identified as false hybrids, which may have resulted from contamination during emasculation or pollination. These interspecific hybrids were the first obtained for the genus *Eriobotrya*. It should be noted that some offspring showed remarked genetic and apparent differences and may differ in horticultural traits. They enriched the germplasm diversity and could be valuable for future research. If the hybrids integrated the merits (e.g. spring-blooming) of loquat and Bengal loquat, this will be beneficial in reducing the possible cold injury of loquat fruitlets and improve loquat production, and could even enlarge the cultivated area of loquat. Therefore, florescence observations and determination of cold-resistant genes will be our future focus of study.

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