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Two EST-derived marker systems for cultivar identification in tree peony

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Abstract Tree peony (*Paeonia suffruticosa* Andrews), a woody deciduous shrub, belongs to the section *Moutan* DC. in the genus of *Paeonia* of the Paeoniaceae family. To increase the efficiency of breeding, two EST-derived marker systems were developed based on a tree peony expressed sequence tag (EST) database. Using target region amplification polymorphism (TRAP), 19 of 39 primer pairs showed good amplification for 56 accessions with amplicons ranging from 120 to 3,000 bp long, among which 99.3% were polymorphic. In contrast, 7 of 21 primer pairs demonstrated adequate amplification with clear bands for simple sequence repeats (SSRs) developed from ESTs, and a total of 33 alleles were found in 56 accessions. The similarity matrices generated by TRAP and EST-SSR markers were compared, and the Mantel test ($r = 0.57778$, $P = 0.0020$) showed a moderate correlation between the two types of molecular markers. TRAP markers were suitable for DNA fingerprinting and EST-SSR markers were more appropriate for discriminating synonyms (the same cultivars with different names due to limited information exchanged among different geographic areas). The two sets of EST-derived markers will be used further for genetic linkage map construction and quantitative trait locus detection in tree peony.

Keywords Tree Peony · TRAP · EST-SSR · Fingerprinting

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Introduction

Tree peony (*Paeonia suffruticosa* Andrews) belongs to the section *Moutan* DC., in the genus of *Paeonia* of the Paeoniaceae family. It is famous as an ornamental plant due to its variety of flower shapes and colors, for its medicinal value and as a food. Its root bark ('Dan Pi' in Chinese) is an important ingredient in Chinese traditional medicine. There are nine wild species of tree peony, *P. suffruticosa*, *P. cathayana*, *P. jishanensis*, *P. qiui*, *P. ostii*, *P. rockii*, *P. decomposita*, *P. delavayi* and *P. ludlowii* (Hong and Pan 2005a, b, 2007), which are valuable resources for traditional breeding (Cheng et al. 1998; Zhou et al. 2003). There are about 1,500 cultivars in the world, in seven geographical groups. The first four groups originate from China. They are (1) Zhongyuan, (2) Xibei, (3) Jiangnan, (4) Xinan; The last three are overseas groups: (5) Japanese, (6) American, and (7) French (Li 1999; Zhang et al. 2007). During the long breeding history, lack of communication among breeders in different regions led to confusion in the

naming of cultivars. Thus, synonyms (the identical biological taxon that has two or more different names) exist in tree peony. For instance, 'DC3' and 'PZTPH', considered as different cultivars, are actually identical (Wang 1997). Effective markers are needed to settle the problem in tree peony nomenclature and to define the cultivars.

Morphological characteristics have been used to discriminate different cultivars but its success has been limited. Molecular markers have been widely applied to cultivar identification, genetic map construction, genetic diversity assessment, and molecular *m*arker-*a*ssisted *s*election (MAS) in many horticultural plants (De Keyser et al. 2010).

To date, the genomic DNA (gDNA) marker systems used in tree peony included random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), sequence-related amplified polymorphism (SRAP) and genomic simple sequence repeat (gSSR). These gDNA marker systems have been used to study genetic diversity of tree peony species (Pei 1993; Yang et al. 2005a, b), genetic relationships among Chinese tree peony cultivars (Hosoki et al. 1997), molecular characterization of tree peony germplasm (Han et al. 2008a), classification and identification of cultivars (Su et al. 2006), characterization of bud sports (Han et al. 2008b), identification of intersectional hybrids (Hao et al. 2008), and isolation and characterization of gSSR (Wang et al. 2008).

EST-derived markers have remarkable advantages, such as easy and rapid utilization, low cost, abundant information and high transferability (Arnold et al. 2002; Kuleung et al. 2004; Chen et al. 2006). Among the EST-derived markers, target region amplification polymorphism (TRAP) uses fixed and arbitrary primers to generate markers. The fixed primer is designed from the targeted EST sequences in the EST database. The arbitrary primer is an arbitrary sequence with either an AT- or GC-rich core intended to anneal with an intron or exon, respectively (Hu and Vick 2003). TRAP markers have great advantages for genetic linkage map construction, chromosome-specific marker location and genetic diversity assessment (Alwala et al. 2006; Chen et al. 2006; Li et al. 2007). Simple sequence repeats (SSRs) consist of simple motifs of 1-6 nucleotides that are repeated in tandem up to a few dozen times per locus (Litt and Luty 1989). EST-SSR markers are microsatellite markers specifically derived from ESTs. EST-SSR markers are relatively inexpensive to develop, representative of transcribed genes with putative function, and are significantly more transferable across taxonomic boundaries than traditional genomic SSRs (Arnold et al. 2002; Chagne et al. 2004. 2004; Kuleung et al. 2004; Pashley et al. 2006).

The lack of an EST database in tree peony has limited genetic research in this species. We have constructed a cDNA library of tree peony and obtained 2,241 ESTs, among which a total of 167 SSRs was derived. A dataset of 185 putative SNPs was obtained (Shu et al. 2009). Until now, no EST-derived molecular markers were available to study herbaceous peony, which was the member of genus *Paeonia* of the family Paeoniaceae and considered as a traditional precious flower in China (Stern 1946). Thus, tree peony EST-derived SSRs could be transferred to herbaceous peony for genetic research, and to facilitate breeding.

In the present paper, the main purpose was to develop TRAP and EST-SSR markers from the tree peony EST database and to attempt to test the validity of the EST-derived markers in comparing cultivars of tree peony. The two types of EST-derived markers were used for DNA fingerprinting and identification of synonyms in tree peony. These two marker systems will be used in future studies, including characterizing genetic relationships among wild species and cultivars, specific gene locations, genetic map construction, functional mapping, and marker-assisted selection (MAS). In addition, the developed marker systems may aid in the study of evolutionary genetics and population structure, because the relationships among wild species and the systematic position of Paeoniaceae still need to be explored.

Materials and methods

Plant materials and DNA extraction

Fifty-six accessions including 8 wild species and 48 cultivars used in this study were from the Peony Germplasm Garden, Institute of Botany, the Chinese Academy of Sciences (Beijing). The information of all samples can be referred to Table 1.

Genomic DNA was isolated from young leaves according to the modified CTAB method of Han et al. (2008a). DNA concentration was estimated on a 1% agarose gel stained with ethidium bromide, in comparison with λDNA (TaKaRa Biotechnology (Dalian)). DNA was diluted to 30 ng/μL with 1×TE buffer, and stored at −20°C.

Primer design

An EST database of tree peony was constructed in our laboratory, and parts of these data were submitted to NCBI EST database (NCBI: dbEST_Id, FE527971-FE530174) (Shu et al. 2009). The primers were designed with DNAman 2.0 software. TRAP primers included 13 fixed primers and 3 arbitrary primers (Table 2). Seven primers screened from 21 EST-SSR primers were obtained for analysis (Table 3).

Table 1 Origin and flower characteristics of 56 accessions of tree peony used in this study

Accession	Abbreviation	Subsection/Cutivar-group	Flower color[a]	Flower form[b]
P. qiui		Subsect. *Vaginatae*	Light pink	Single
P. jishanensis		Subsect. *Vaginatae*	White	Single
P. rockii		Subsect. *Vaginatae*	White	Single
P. ostii		Subsect. *Vaginatae*	White	Single
P. lutea		Subsect. *Delavayanae*	Yellow	Single
P. delavayi		Subsect. *Delavayanae*	Purplish red	Single
P. potaninii		Subsect. *Delavayanae*	Purplish red	Single
P. ludlowii		Subsect. *Delavayanae*	Yellow	Single
Souvenir de Maxime Cornu	SdMC	French	Yellow	Rose
Golden Isles	GI	American	Yellow	Chrysanthemum
High Noon	HN	American	Yellow	Chrysanthemum
Kohka Mon	KM	Japanese	Purple	Chrysanthemum
Taiyoh	Ty	Japanese	Red	Chrysanthemum
Shima Nishiki	SN	Japanese	Purplish red and pink	Chrysanthemum
Hakuoh Jishi	HJ	Japanese	White	Chrysanthemum
Hohki	Hk	Japanese	Red	Lotus
Kan Zakura Jishi	KZJs	Japanese	Pink	Chrysanthemum
Hatsu Garasu	HG	Japanese	Black	Single
Hong Guan Yu Pei	HGYP	Xibei	Reddish purple	Rose
Zhong Chuan Fen	ZCF	Xibei	Pinkish purple	Single
Hua He Shang	HHS	Xibei	Blackish purple	Single
Huo Yan	HY	Xibei	Red	Lotus
Ou Duan Si Lian	ODSL	Xibei	Pink	Anemone
Xi Qing	XQ	Xibei	Reddish purple	Lotus
Da Ban Fen	DBF	Xibei	White	Lotus
Qing Luo	QL	Jiangnan	Reddish purple	Chrysanthemum
Feng Dan	FD	Jiangnan	White	Single
Dian Dan 2	DD2	Xinan	Purplish red	Single
Dian Chong 3	DC3	Xinan	Purplish red	Rose
Peng Zhou Tai Ping Hong	PZTPH	Xinan	Purplish red	Rose
Yan Zhi Tu	YZT	Zhongyuan	Pinkish red	Lotus
Xue Lian	XL	Zhongyuan	White	Lotus
Hei Hua Kui	HHK	Zhongyuan	Blackish purple	Lotus
Liu Li Guan Zhu	LLGZ	Zhongyuan	White	Crown
Xiao Ye Zi	XYZ	Zhongyuan	Reddish purple	Chrysanthemum
Guan Shi Mo Yu	GSMY	Zhongyuan	Blackish purple	Crown
Hu Lan	HL	Zhongyuan	Bluish pink	Lotus
Fu Gui Hong	FGH	Zhongyuan	Purplish red	Rose
Qing Long Xi Tao Hua	QLXTH	Zhongyuan	Pink	Crown
Lan Yue	LY	Zhongyuan	Bluish pink	Crown proliferate-flower
Xiu Qun Fang	XQF	Zhongyuan	Red	Rose
Hong He	HH	Zhongyuan	Light purplish red	Lotus
Shui Jing Bai	SJB	Zhongyuan	Pinkish	Lotus
Gu Cheng Chun Se	GCCS	Zhongyuan	Yellowish	Crown
Shao Nv Qun	SNQ	Zhongyuan	Pinkish red	Rose
Qing Long Zhen Bao	QLZB	Zhongyuan	Purplish red	Hundred proliferate-flower
Huang Li	HLi	Zhongyuan	Yellowish	Single
Kui Hua Hong	KHH	Zhongyuan	Red	Chrysanthemum

Table 1 continued

Accession	Abbreviation	Subsection/Cutivar-group	Flower color[a]	Flower form[b]
Si Rong Hong	SRH	Zhongyuan	Light purplish red	Crown
Wu Cai Die	WCD	Zhongyuan	Purplish	Chrysanthemum
Luo Yang Hong	LYH	Zhongyuan	Purplish red	Rose
Shou An Hong	SAH	Zhongyuan	Dark purplish red	Crown
Dou Lv	DL	Zhongyuan	Yellowish green	Crown
Yao Huang	YH	Zhongyuan	Yellow	Crown
Er Qiao	EQ	Zhongyuan	Pink and purplish red	Rose
Jin Pao Hong	JPH	Zhongyuan	Purplish red	Rose

[a] The non-blotch color of petals if they have blotch at the base of petals

[b] In tree peony, stamen or carpel turning to petal and petal increasing naturally which are two important ways to the formation of flower form. Single flower, Single indicates that the flower consists of 1–3 whorls of petals and has normal stamens and carpels; As the increasing of flower petals, while the flower looks like lotus form (4–5 whorls of petals, normal stamens and carpels), Chrysanthemum form (6–8 whorls of petals, petals gradually become smaller from outside to inside, partial stamen petaloid, and normal carpels), Rose form (more than 9 whorls of petals, petals gradually become smaller from outside to inside, most of stamen petaloid, and normal carpels), Crown form (2–5 whorls of petals outside are bigger, inside petals are smaller), Anemone form (the outer petals are wide and unfold arranging in 2–3 whorls, most stamen petaloid, the petaloid petals are smaller than outer petals, and normal carpels), Globular form (more than hundred petals, all stamens are highly petaloid and the petaloids are similar to outer petals in shape and size, and normal carpels, the whole flower is buxom and full like globular) and Crown-proliferation form, the demonstration of flower shapes can be referred [Hao et al. (2008); Han et al.(2008a); Li (1999)]

Table 2 Primer sequences used in TRAP analysis

Primer	Type	Sequence (5′–3′)
Fix1	Fixed	CCAAGAAGTCGGGAAGG
Fix2	Fixed	ATTTGGCGAAACACCC
Fix4	Fixed	GAGCAACAATGGCGTCTA
Fix5	Fixed	GACGGGAAGGAAGCAA
Fix6	Fixed	TCCCTGCTGCTTTCCG
Fix7	Fixed	GGCTGACAAAGGCGACAC
Fix8	Fixed	GCACAAGGCACAGTAT
Fix9	Fixed	GAAGACCACCACCGAGAC
Fix10	Fixed	AGGGCAAGACGCCAGAA
Fix11	Fixed	AGGCATTGTTCCGTTCA
Fix12	Fixed	TTGTTGTGCCCGTTGC
Fix13	Fixed	TCGCTTCCTTTGCTGC
Fix14	Fixed	GGCGACGGCTGTATCAC
RP1	Arbitrary	GAC TGC GTA CG AATT CTG
RP2	Arbitrary	GAC TGC GTA CG AATT GAC
RP3	Arbitrary	GAC TGC GTA CG AATT TGC

TRAP analysis

Thirty-nine different primer combinations were employed using 13 fixed and 3 arbitrary primers. Each 15-μL PCR reaction mixture consisted of 60 ng of genomic DNA, 333 μM of each dNTP, 2 mM of Mg^{2+}, 0.4 μM of each primer, 1.5 units of EasyTaq DNA Polymerase (TransGen Biotechnology, Beijing, China) and 1×Easy Taq Buffer. Samples were subjected to the following thermal profile for amplification in the Biometra TGradient 96 cycler (Biometra, Göttingen, Germany): 2 min of denaturation at 94°C, five cycles of three steps: 45 s of denaturation at 94°C, 45 s of annealing at 40°C and 1 min of elongation at 72°C. In the following 30 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 7 min at 72°C.

Separation of amplified fragments was accomplished on 8.7% polyacrylamide gels [acrylamide–bisacrylamide (29:1), 1×TBE] at 300 V for 2 h. Gels were stained with ethidium bromide (4×10^{-4} mg/ml) for 20 min, and photographed using a Transilluminator (BINTA 2020D, China).

EST-SSR analysis

The PCR reaction mixtures (20 μL total volumes) consisted of 90 ng of genomic DNA, 0.5 μM each of both primers and 1 × EasyTaq PCR SuperMix (TransGen Biotech, China). The amplification was carried out in a Biometra Tgradient thermocycler programmed for 5 min at 94°C for denaturing, 35 cycles of 30 s at 94°C, 30 s at 55.4°C, 1 min at 72°C, and then a final elongation step for 10 min at 72°C.

The amplified products were mixed with 2× denaturing gel loading buffer, and then fractionated on a 4% denaturing polyacrylamide gel using a vertical gel apparatus (JY-CX2B, Beijing Liuyi Instrument Factory, China). Electrophoresis was conducted at 75 W for 40 min and at 1,500 V for 90 min. DNA fragments were visualized by silver staining (Bassam et al. 1991).

Table 3 Primer sequences and information used in EST-SSR analysis

Primer	EST name	Forward primer (5′-3′)	Reverse primer (5′–3′)	Repeat motif
5 F, R[a]	EST-36	CGTTCAATCTATGTCGTACC	TGCTCATCTACTACAACAGG	$(ACC)_5$
7-1 F, R	EST-4E2	GGAGAAGAAAGTTGGAGAG	TGGTTTGTCTCGTGAGTC	$(AG)_4$
7-2 F, R	EST-4E2	ATCAAAGCAGAACAGAGG	AAGATATTGCACGCTAGG	$(GAT)_9$
8 F, R	EST-4H4	GGAACCAAAGTGGAAATTAG	AGGAAACATAGACAGCCC	$(AG)_7$
10 F, R	EST-6C12	CATGTCTCTCTCTCTCAACC	CATCGAGTAGGCATGTTC	$(TC)_6$
11 F, R	EST-5D11	ATCAGAAACACACAACTCAG	GGCCTTCTTTAAGTAACCT	$(GA)_{11}$
14 F, R	EST-9E1	CCACAGATACAGAAGAAAGA	GGACGCACATTAGGATTA	$(GA)_5$
17 F, R	EST-9H10	ACAACAGAGAGAGAGAGAGG	TGCAGTATTAGCTACCACG	$(GA)_6$, $(GA)_5$
18 F, R[a]	EST-6D6	GACAAACAGAAGAGAACCA	CACGTCCCACTAACTAGC	$(AG)_8$
21 F, R[a]	EST-15C6	GTGGAGGATGCAAGATGT	CATTAAGCGAAACTCAAACC	$(TG)_5$
25 F, R	EST-21D5	AGAGAGAGAGAGAGAGGAGAG	CTAGTTGAAACCGTATTCG	$(AG)_9$
28 F, R	EST-23D9	AATGTAATGTCGGCAACG	CCATGACGATCGATCAAT	$(TC)_{11}$
29 F, R	EST-24G1	TGATGCTGATGGTAATGG	CCCAGATAGGTTCACAGG	$(GGT)_5$
34 F, R	EST-29B4	AGTAAGGAGGAGGAGGAGGC	GTTTGGGTGCATTTGGAG	$(AGG)_5$
37 F, R[a]	EST-31D11	TCATCTATCCTTTCTGAAGC	GAGAGCACTGCATCACTG	$(CT)_8$
42 F, R[a]	EST-33F4	TTTAAGAATGAGGCTCGC	ATGGCTGCAACAACAGTT	$(CT)_{17}$
44 F, R[a]	EST-34B7	TCTGAAGCCACATCTACC	ATAGAGAGAGAGAGAGCACTG	$(CT)_7$
48 F, R	EST-1C11	TGTAGTGCCCTCATAAGAG	CTCATCTTTCACTCGACC	$(AG)_7$
50 F, R[a]	EST-1E8	AGAAGAGTAACATGCGCC	AAGACCTCCACTGCAGAT	$(CT)_{10}$
51 F, R	EST-1F4	TCGATTCCGCTTTCTGGT	TCAGCTGCCCATTTACCC	$(AGC)_5$
56 F, R	EST-38G10	AAGACAAGAAGAAGGAGC	GGTCTGGAACCTTAGTAGT	$(AGA)_5$

[a] The primers in bold were selected primers for phylogenetic analysis

Data analysis

Amplified fragments were scored as present (1) or absent (0). Genetic similarities between cultivars were measured by the Dice similarity coefficient based on the proportion of shared alleles (Dice 1945; Nei and Li 1979). A Neighbor-joining (NJ) tree was constructed using the PAUP 4.0b10 program (Swofford 1998). The species *P. ludlowii* was used as an outgroup in the NJ tree based on previous phylogenetic hypotheses (Hong and Pan 2005a). NJ trees were constructed using distance matrics. The robustness of the dendrograms was tested by bootstrap analysis with 1,000 replications using PAUP 4.0b10. A Principal Component Analysis (PCA) was performed based on the 0/1 matrix using the Multi-Variate Statistical Package (mvsp) ver. 3.1. A Mantel test was performed to estimate the similarity of cluster analysis based on TRAP and EST-SSR marker systems using NTSYSpc-2.02 (1,000 permutations) (Rohlf 2000; Mantel 1967).

Results and discussion

TRAP analysis and its application to DNA fingerprinting

Primers were excluded from the study if their banding patterns of amplification were faint or if they failed to amplify consistently in all accessions. Two primer combinations and 15 tree peony accessions were randomly selected for confirmation of the reproducibility of TRAP markers (ESM_1–3, ESM_4). Nineteen primer combinations were selected. The number of fragments detected by an individual primer combination ranged from 8 (Fix14 + RP1) to 23 (Fix4 + RP3) with an average of 14.5. The bands ranged in size from 120 to 3,100 bp long (Fix1 + RP1), most bands were in the range of 120 to 1,000 bp in length. A total of 276 bands were obtained, among which 274 (99.3%) were polymorphic (Table 4). Amplification by primer pair (Fix6 + RP2), 14 bands were obtained, among which 13 were polymorphic, and one monomorphic band (290 bp) existed in all accessions. Meanwhile, using primer pair (Fix10 + RP2), 17 bands were amplified, among which 16 were polymorphic, and one monomorphic band existed in all accessions, and the polymorphic rate was 92.9% (Fix6 + RP2) and 94.1% (Fix10 + RP2), respectively (Table 4). In general, tree peony is identified by morphological characters. Many cultivars have similar flower color, flower form and leaf shape. In this study, we attempted to construct DNA fingerprints by TRAP markers. These marker system resulted in a high level of polymorphism and distinct fingerprints. The primer combination Fix4 + RP1 could distinguish 42 accessions (7 wild accessions and 35 cultivars) (Fig. 1).

Table 4 Total number of bands, number of polymorphic bands, and polymorphism rate for each of the 19 selected TRAP primer combinations

Primer combination	Bands observed	Polymorphic bands	Polymorphism rate
Fix1 + RP1	14	14	100
Fix4 + RP1	19	19	100
Fix5 + RP1	12	12	100
Fix6 + RP1	14	14	100
Fix7 + RP1	11	11	100
Fix8 + RP1	14	14	100
Fix9 + RP1	15	15	100
Fix11 + RP1	17	17	100
Fix13 + RP1	13	13	100
Fix14 + RP1	8	8	100
Fix1 + RP2	10	10	100
Fix5 + RP2	16	16	100
Fix6 + RP2	14	13	92.9
Fix10 + RP2	17	16	94.1
Fix12 + RP2	13	13	100
Fix13 + RP2	16	16	100
Fix4 + RP3	23	23	100
Fix5 + RP3	15	15	100
Fix10 + RP3	15	15	100
Total	276	274	99.3
Average	14.5	14.4	99.3

Nineteen distinct fragments ranging from 120 to 910 bp long were amplified. There were a total of 208 bands in the 42 accessions, and 2 to 8 bands per accession. Each accession had its own distinctive fingerprint. For example, 'EQ' (41) had amplicons with lengths of 900, 500, 350, 290 and 130 bp, while 'JPH' (42) had amplicon with the lengths of 950, 500, 290, 220 and 130 bp.

A NJ tree was constructed based on TRAP data (Fig. 2). These wild accessions in Subsect. *Delavayanae* (*P. ludlowii*, *P. delavayi*, *P. potaninii* and *P. lutea*) were found to span the extremes of the dendrogram. *P. delavayi* and *P. potaninii* were clustered in one branch with a 67% bootstrap value. In this study, *P. lutea* and *P.rockii* were cluster together with a cultivar ('XQ') which was congruent with the hypothesis that *P. lutea* was the ancestor of cultivars with yellow flowers (Cheng et al. 1998). These cultivars grouped closely with wild accessions in Subsect. *Vaginatae* (*P. rockii*, *P. qiui*, *P. jishanensis* and *P. ostii*). *P. rockii* and 'XQ' (Xibei cultivar) clustered into one branch with a 95% bootstrap value. According to a previous study (Zhou et al. 2003), *P. jishanensis*, *P. rockii* and *P. ostii* were the ancestors of the Zhongyuan cultivars, however, *P. rockii* and *P. ostii* were the main ancestors of the Xibei and Jiangnan cultivars, respectively. In this study, the relationship of cultivars characterized by TRAP data generally agreed on the division of cultivars. Cultivars grouped mainly in three clusters. In cluster I, 6 Xibei cultivars were distributed in 1 subcluster supported by a 77% bootstrap value. Twenty-two Zhongyuan cultivars and 2 Xinan cultivars ('DC3' and 'PZTPH') formed into cluster II, which separated into several subclusters. A significant relationship was found between 'DC3' and 'PZTPH', and the branch was supported with bootstrap value of 100%. In cluster II, 'QLXTH' and 'HH' formed a

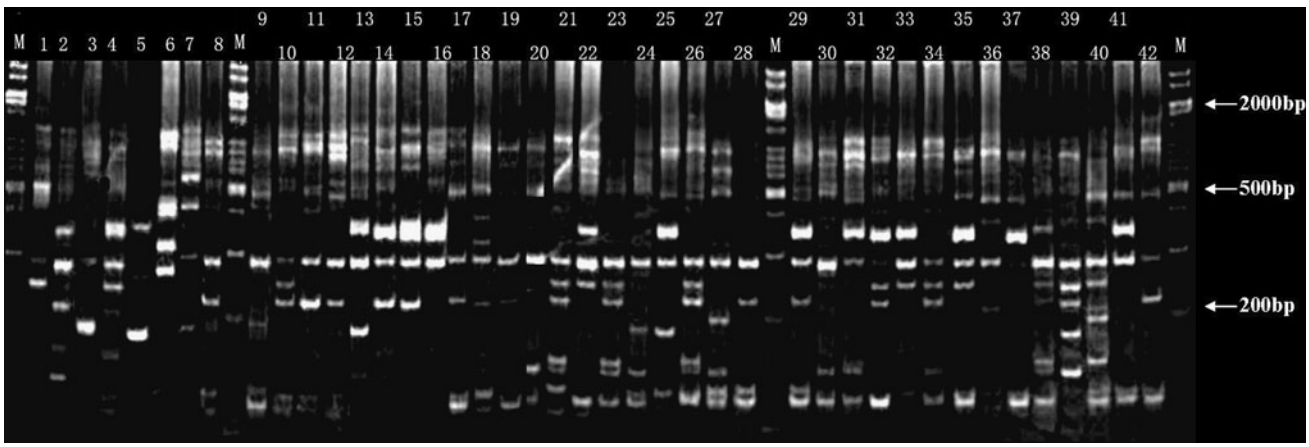

Fig. 1 Electrophoretic results displaying TRAP fingerprints detected in 42 tree peony accessions using primer combination Fix4 + RP1. The DNA samples were fractionated in 8.7% polyacrylamide gels stained with ethidium bromide. Each accession had its own distinctive fingerprint that made it different from other accessions. 'EQ' (41) had the amplicons with the length of 900, 500, 350, 290 and 130 bp, while 'JPH' (42) got the amplicons with the length of 950, 500, 290, 220 and 130 bp. The length of amplicons at different position can be used as fingerprints for discrimination of cultivars. *Lanes* from *left* to *right*: 1 *P. ostii*, 2 *P. lutea*, 3 *P. delavayi*, 4 *P. jishanensis*, 5 *P. rockii*, 6 *P. potaninii*, 7 *P. ludlowii*, 8 'HN', 9 'SdMC', 10 'HJ', 11 'Hk', 12 'GI', 13 'ZCF', 14 'HHS', 15 'HY', 16 'ODSL', 17 'QL', 18 'FD', 19 'DD2', 20 'PZTPH', 21 'YZT', 22 'XL', 23 'HHK', 24 'LLGZ', 25 'XYZ', 26 'GSMY', 27 'HL', 28 'LY', 29 'XQF', 30 'SJB', 31 'GCCS', 32 'SNQ', 33 'QLZB', 34 'HLi', 35 'SRH', 36 'WCD', 37 'LYH', 38 'SAH', 39 'DL', 40 'YH', 41 'EQ', 42 'JPH'

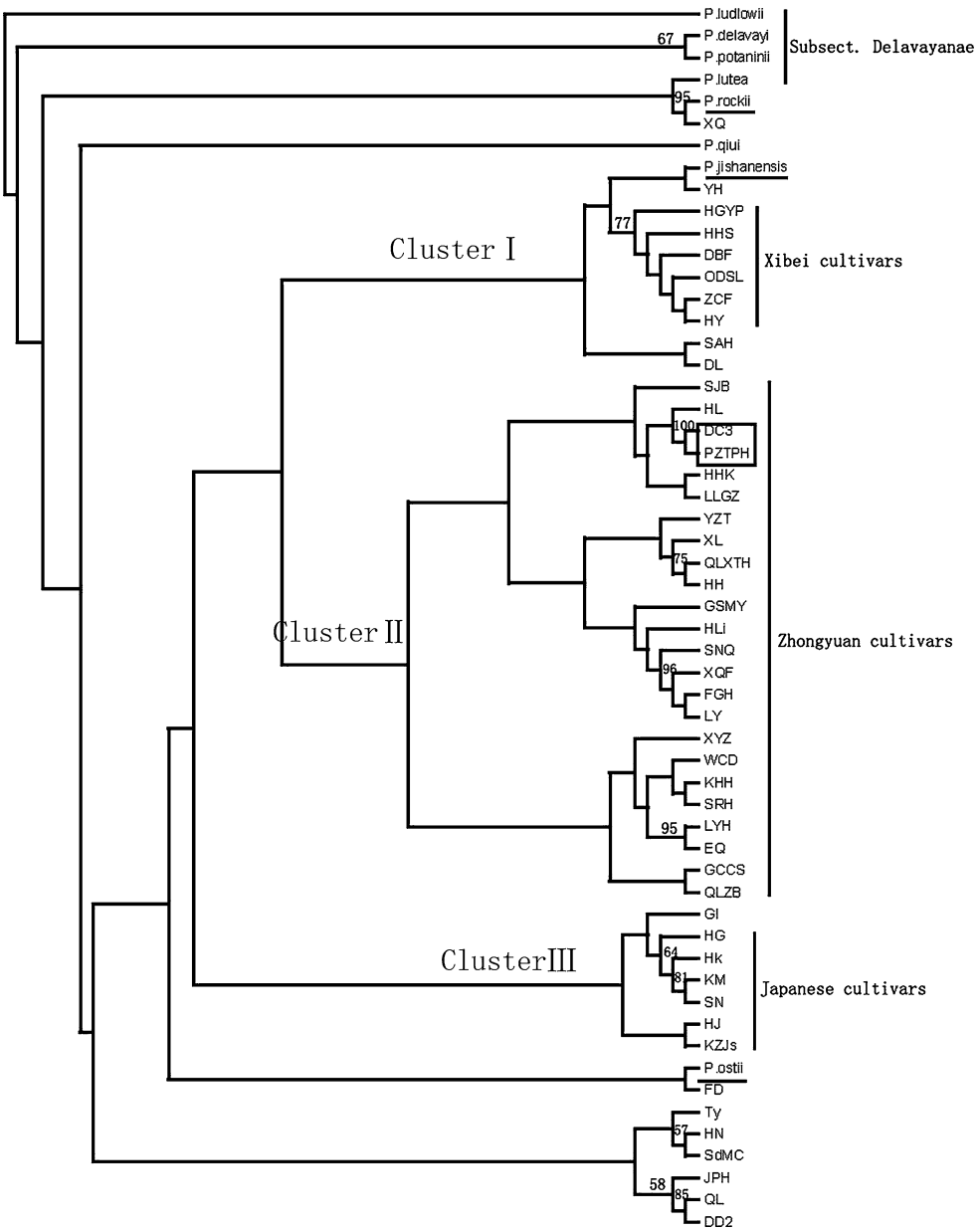

Fig. 2 The NJ dendrogram of 56 tree peony accessions based on TRAP markers obtained with 19 primer combinations. *Numbers* above branches are bootstrap values over 50. NJ tree was constructed using the PAUP 4.0b10 program. *P. ludlowii* was used as an outgroup. Cultivars grouped mainly in three clusters, Clusters I, II and III which mostly included Xibei, Zhongyuan and Japanese cultivars, respectively. The information of the short name for each accession is referenced in Table 1. The cultivars clustered together from the same cultivar group or wild accessions from the same subsection were indicated in the tree by *vertical lines*. Three important ancestors of cultivars, *P. jishanensis*, *P. rockii* and *P. ostii* were indicated by *underlines*. 'DC3' is a synonym of 'PZTPH', both are indicated by a *rectangle*

branch with a 75% bootstrap value. 'XQF', 'FGH' and 'LY' grouped together with a 96% bootstrap value. 'EQ' is a bud sport, originated from 'LYH', and famous for its secondary colors (Wang 1997). 'EQ' and 'LYH' clustered together with a high bootstrap value of 95%, which confirmed a close genetic relationship and demonstrated the reliability of TRAP marker system. Six Japanese cultivars and one American cultivar ('GI') formed cluster III, among which, 'KM' and 'SN' formed a branch with a 81% bootstrap value, and then grouped together with 'Hk' with 64% bootstrap value. There was some incongruence in a few branches, at the bottom of the dendrogram, 'HN' (an American cultivar) and 'SdMC' (a French cultivar) formed a branch with 57% bootstrap value; 'QL' (a Jiangnan cultivar) and 'DD2' (a Xinan cultivar) formed a branch with 85% bootstrap value, and then grouped into one cluster with 'JPH' (a Zhongyuan cultivar) with 58% bootstrap value. The incongruence between the dendrogram and the division of cultivars might be due to the limited number of individuals in the American (2), French (1), Jiangnan (2) and Xinan (3) cultivar groups used in this study, or potentially indicated that germplasm resources were exchanged among these cultivar groups. Most bootstrap values were below 50% by NJ clustering analysis, which indicated that tree peony has a complex genetic background owing to natural hybridization, anthropochory (dispersed by human beings), and germplasm exchange among cultivar groups (Wang 1997).

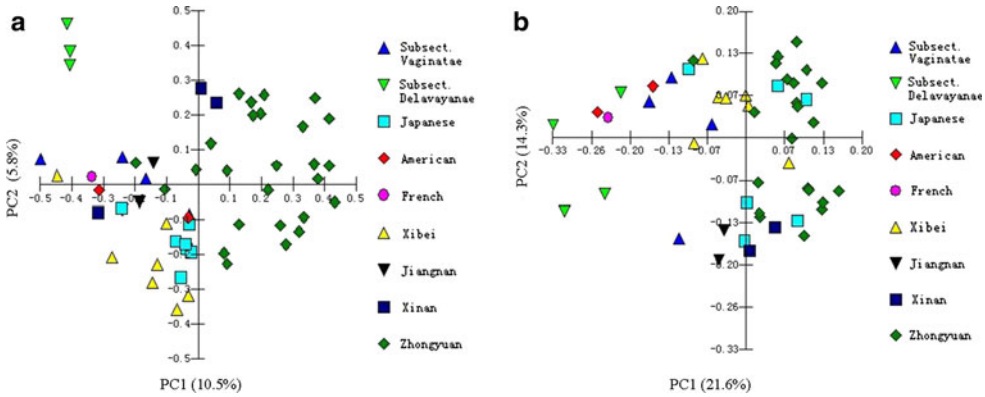

Fig. 3 Principal component analysis of 56 tree peony accessions based on TRAP (**a**) and EST-SSR markers (**b**). PC1 and PC2 indicated the first and second principal component, respectively. Cultivar groups or subsections were used to illustrate the distribution of 56 accessions in the two dimensions. The results of the PCA generally agreed on the division of cultivars, especially in Fig. 2. Compared with wild accessions in subsect. *Delavayanae*, the wild accessions in subsect. *Vaginatae* distributed closely with cultivars. The short names Subsect. Vaginatae, Subsect. Delavayanae, Japanese, American, French, Xibei, Jiangnan, Xinan and Zhongyuan indicated the origin of species or cultivars

Table 5 Total number of alleles, number of polymorphic alleles, and percentage polymorphism for each of 7 EST-SSR primer pairs

Primer pairs[a]	Forward primer (5′–3′)	Reverse primer (5′–3′)	Alleles	Polymorphic alleles[a]	Percentage polymorphism
5 F, R	CGTTCAATCTATGTCGTACC	TGCTCATCTACTACAACAGG	5	5	100
18 F, R	GACAAACAGAAGAGAACCA	CACGTCCCACTAACTAGC	5	5	100
21 F, R	GTGGAGGATGCAAGATGT	CATTAAGCGAAACTCAAACC	4	4	100
37 F, R	TCATCTATCCTTTCTGAAGC	GAGAGCACTGCATCACTG	3	3	100
42 F, R	TTTAAGAATGAGGCTCGC	ATGGCTGCAACAACAGTT	5	5	100
44 F, R	TCTGAAGCCACATCTACC	ATAGAGAGAGAGAGAGCACTG	4	4	100
50 F, R	AGAAGAGTAACATGCGCC	AAGACCTCCACTGCAGAT	7	7	100
Total			33	33	100
Average			4.7	4.7	100

[a] One allele can be considered polymorphic if it is absent in at least one sample

Relationships among 56 accessions of tree peony were further illustrated by PCA based on the 0/1 matrix, using the Multi-Variate Statistical Package (mvsp) ver. 3.1. PCA supported the results of clustering analysis, the first two principal components (PC1 and PC2) accounted for 10.5 and 5.8% of the total variation, respectively (Fig. 3a). It can be easily recognized that cultivars from the same group distributed together, while wild accessions with close relationships with cultivars distributed together with cultivars by PCA analysis (Fig. 3a). The results of PCA based on TRAP data generally agreed on the division of cultivars based on geographic distribution, and indicated the close relationship among them. However, wild accessions in Subsect. *Delavayanae* were distantly related to cultivars (except one accession) and distributed sparsely.

'DC3' and 'PZTPH' are actually the same cultivar. Many identical fragments were amplified in the two accessions (ESM_3). Ninety-one bands were observed in 'DC3' and 'PZTPH', while 73 bands were identical between them, and 18 bands were different. The possible reason for this was due to unspecific amplification. However, the high bootstrap values in the NJ trees supported the conclusion that two accessions were the same cultivar. Some studies indicated that using TRAP primers amplified fragments from regions other than the targeted region could generate the unspecific amplification. Hu et al. (2005) found that only 1% of amplified TRAP fragments had similar sequence with the targeted EST sequence. Yang et al. (2005a, b) could not construct a saturated map of wheat with TRAP markers. They suggested that TRAP primers amplified fragments from other regions besides the expected region. Li et al. (2007) found that only 15.5% of the 310 TRAP fragments were located on the same chromosome, which was used to generate fixed primers. A low

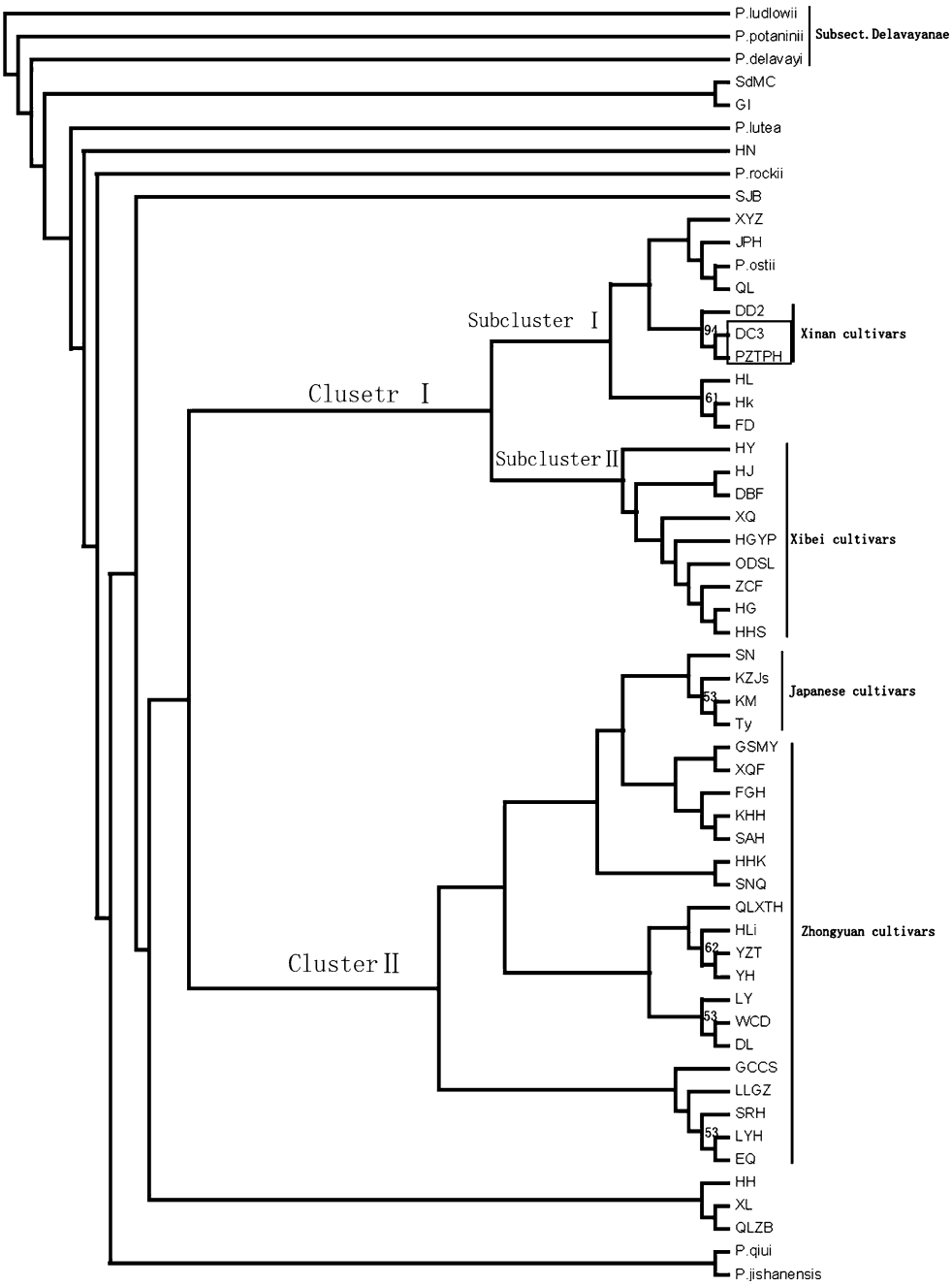

Fig. 4 The NJ dendrogram of 56 tree peony accessions based on EST-SSR markers obtained with seven primer pairs. *Numbers* above branches are bootstrap values over 50. NJ tree was constructed using the PAUP 4.0b10 program. *P. ludlowii* was used as an outgroup. Cultivars mainly formed two clusters, Clusters I and II. Two subclusters were identified in Cluster I which mainly included Xibei and Xinan cultivars. Cluster II contained Zhongyuan and Japanese cultivars. The information of the short name for each accession can be referenced in Table 1. The cultivars clustered together from the same cultivar group or wild accessions from the same subsection were indicated in the tree by *vertical line*. 'DC3' as synonym of 'PZTPH' were both indicated by a *rectangle*

annealing temperature (35–40°C) during the first cycles of PCR amplification allows mismatching between the target region and primers, and results in amplicons from multiple genomic regions. In our study, discrepancies of bands in 'DC3' and 'PZTPH' may be due to the above reason.

In the identification of tree peony, DNA fingerprinting by TRAP markers is more reliable and efficient than that of morphology. Therefore, it is a better method for assessing and identifying germplasm of tree peony. In this study, 56 accessions could be identified by several primer combinations. DNA fingerprinting of all tree peony germplasm can be figured out with more primer combinations and combined with phenotype in future, which can provide scientific guidance for establishing genetic relationships and protection of the intellectual property rights of new cultivars.

EST-SSR analysis and its application

Among 56 tree peony accessions, the number of alleles per locus varied from 3 (37 F, R) to 7 (50 F, R), with an average of 4.7. The summary of EST-SSR markers produced by the 7 primer pairs across all 56 accessions was given in Table 5. A total of 33 alleles were generated, and

the polymorphism rate per primer combination was 100% (Table 5). The high level of polymorphism indicated a complex genetic background in tree peony.

The cultivars used in this paper were reproduced through root cuttings. In order to determine if the sampled cultivars are closely related to the wild accessions, the NJ tree was constructed based on EST-SSR data (Fig. 4). The results showed that *P. ludlowii*, *P. potaninii* and *P. delavayi* were distributed at the extreme of the dendrogram. *P. lutea* clustered closely with 'HN', 'GI' (American cultivars with yellow flowers) and 'SdMC' (French cultivar with yellow flowers), which indicated that *P. lutea* had a close relationship among cultivars with yellow flowers. Four accessions in Subsect. *Vaginatae* (*P. rockii*, *P. qiui*, *P. jishanensis* and *P. ostii*) were grouped closely with specific cultivars. The results based on ESR-SSR data were similar to that of the traditional division of cultivars. Cultivars grouped mainly in two clusters. Cluster I was separated into two subclusters. In subcluster I, 'DD2', 'DC3' and 'PZTPH' (Xinan cultivars) distributed in one group. 'DC3' and 'PZTPH' formed a branch with a 94% bootstrap value. 'Hk' (a Japanese cultivar) and 'FD' (a Jiangnan cultivar) formed a branch with a 61% bootstrap value. Subcluster I also included three Zhongyuan ('XYZ', 'JPH' and 'HL'), two Jiangnan ('QL' and 'FD'), one Japanese cultivar ('Hk') and one wild species (*P. ostii*). The result indicated that there was a close relationship between *P. ostii* and specific cultivars. Nine Xibei cultivars formed subcluster II. Four Japanese cultivars ('SN', 'KZJs', 'KM' and 'Ty') and 19 Zhongyuan cultivars grouped into cluster II. 'KM' and 'Ty' formed into a branch with a 53% bootstrap value. The ancestors of the Japanese cultivars were from China, especially from the Zhongyuan and Xibei cultivar groups. The clustering confirms the close relationship between Japanese and Chinese cultivars. 'YZT' and 'YH', 'WCD' and 'DL', 'LYH' and 'EQ' formed a branch with bootstrap values of 62, 53 and 53%, respectively. Although cultivars in some groups had the same ancestors, their genetic background was complicated by frequent gene flow (Li 1999; Wang 1997), which may explain the low bootstrap values in our NJ trees in this study. 'DC3' and 'PZTPH' appear to be the same cultivar (although with different names), based on their identical band patterns in the profiles of EST-SSR markers (ESM_2), and nine alleles were shared between them. EST-SSR markers were apt for defining synonyms.

In comparison, the results of PCA based EST-SSR analysis showed that Zhongyuan, Japanese and Xibei cultivars were closely grouped, which were surrounded by Jiangnan, Xinan cultivars and wild accessions in Subsect. *Vaginatae* (Fig. 3b). In contrast, wild accessions in Subsect. *Delavayanae*, American and French cultivars grouped together, but they were far away from other cultivars. The first two principal components (PC1 and PC2) explained 21.6 and 14.3% of the total variation, respectively (Fig. 3b). In EST-SSR markers, the number of primer pairs (7 pairs) and the amplified fragments (33 alleles) were not enough to analyze 56 accessions of tree peony. This might be a reason for the disagreement between the results based on EST-SSR data and the division of cultivars, and those based on the TRAP data. The use of EST-SSR marker system was more difficult in developing and screening primer pairs than that of TRAP marker system in this study, so the better solution is to develop more EST-SSR markers for future studies, because 167 EST-SSRs were obtained from the EST database of tree peony.

The similarity matrices generated by TRAP and EST-SSR markers were compared, and the Mantel test showed that the correlation value (r) was 0.57778 at $P = 0.0020$ (Jaccard's coefficient), using NTSYSpc-2.02 (1,000 permutations). This indicated a moderate correlation between the data generated by the two marker systems, possibly due to an insufficient number of EST-SSR markers.

The conserved nature of coding sequences compared to non-coding genomic DNA determines the higher levels of transferability of EST-derived SSRs as compared with genomic DNA-derived SSRs. EST-derived SSRs have a high degree of transferability to related species in several taxa or genera (Thiel et al. 2003; Cordeiro et al. 2001; Gupta et al. 2003), in contrast with genomic SSRs which show less efficient cross amplification (Peakall et al. 1998).

Conclusion

The EST database in our laboratory provided the possibility to develop TRAP and EST-SSR marker systems in tree peony. This is the first attempt to use EST-derived markers to facilitate germplasm characterization in tree peony. Cluster analysis by NJ methods showed that these analyses generally supported the division of cultivars and demonstrated these two marker systems were applicable and reliable. PCA revealed similar results as that of analysis by NJ. TRAP markers were suitable for DNA fingerprinting, and EST-SSR markers were more appropriate for discriminating synonyms. EST-derived markers in tree peony should be useful for genetic linkage map construction, quantitative trait locus (QTL) detection, gene tagging, linkage between markers and genes for important traits, and finally will facilitate marker-assisted selection (MAS).

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