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PATERNITY ANALYSIS AND SNP DEVELOPMENT FOR *OSMANTHUS FRAGRANS* 'PUCHENG DANGUI' USING SLAF-SEQ

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Abstract

Osmanthus fragrans 'Pucheng Dangui' is a new ornamental and urban-greening plant variety developed through long-term natural and artificial selection. Urban and rural horticultural industries in Pucheng County, Fujian Province, China, have developed rapidly and many nurseries now grow *O. fragrans* 'Pucheng Dangui' by cuttings. We developed a paternity test and SNP markers for *O. fragrans* 'Pucheng Dangui' using SLAF-seq to identify the fine variety *O. fragrans* 'Pucheng Dangui' based on molecular research. The genome sequence of *Fraxinus excelsior* was used as the reference to predict restriction enzyme digestion for *O. fragrans* 'Pucheng Dangui'. Restriction enzyme digestion fragments between 400 and 480 bp length were defined as SLAF tags digested by the enzyme Hpy166II+EcoRV-HF®. A total of 413,743 SLAF tags was identified, of which 113,061 polymorphic SLAF tags and 353,073 population SNPs were derived. Analysis of the phylogenetic tree, the population admixture structure, the admixture K value cross validation error rate, and the PCA clustering figure showed that all 29 plant samples belonged to the same population, with no significant differences detected between samples. This comprehensive analysis of DNA polymorphisms provided valuable insights into the different individuals or populations of *O. fragrans* 'Pucheng Dangui' and it provided producers and vendors with confidence in the source of their plant material. This results provides a reliable method for researchers to identify *O. fragrans* 'Pucheng Dangui' from other *O. fragrans*species or varieties by the molecular approach. The identification of intraspecific genetic variations also provides the basis for further development of individual plant analysis, identification and introgression breeding in other species.

Key words: Paternity test; SNP; *Osmanthus fragrans* 'Pucheng Dangui'; SLAF-seq.

Abbreviations: SLAF-seq: Specific locus amplified fragment sequencing; PCA: Principal components analysis; BMK: Biomarker Technologies; SNP: Single nucleotide polymorphism; BWA: Burrows-Wheeler aligner; GATK: Genome analysis toolkit.

Introduction

Sweet osmanthus, *Osmanthus fragrans* (Thunb.) Lour. (Oleaceae), an evergreen shrub or tree, it is regarded as one of the top ten traditional flowering trees in China (Xiang & Liu, 2004; Xiang & Liu, 2008). 'Pucheng Dangui' is a new ornamental, floricultural and urban-greening variety of *O. fragrans* that has been developed through long-term natural and artificial selection. This variety has an excellent reputation in the horticulture industry because of its attractive evergreen foliage, profuse flowering, unusual scarlet flowers, large petals and long-lasting fragrance (Yang, 2012). Its flowers are also used as edible jams, scented tea and herbal tea. Many nurseries in Pucheng County, Fujian Province, are mass-producing 'Pucheng Dangui' to meet rapidly growing demand from flower markets and urban greening projects across China. The long history of *O. fragrans* cultivation in Pucheng means that some vendors suspect that 'Pucheng Dangui' nursery stock might have been mixed with other *Osmanthus* species or varieties. Previous studies on *O. fragrans* have focused on varietal classification, flowering times, environmental interactions, cultivation physiology and reproduction (Peng & Ji, 2004; Chen & Jiang, 2006).

SLAF-seq (Specific Length Amplified Fragment Sequencing) (Sun *et al*., 2013) is a highly automated high-throughput sequencing technique based on bioinformatics that performs double-ended sequencing of specific restriction fragments. By setting the specific pre-enzyme digestion solution to obtain specific fragment length, the specific fragment sequencing and the sequencing method repeatability are high, which can generate large amounts of information in the genome in a short time so as to realize the precise positioning, the candidate functions and the fragment distribution density in the genome. By using this technique the developed markers have high density, good consistency, and more effective markers. The high-throughput molecular marker development costs lower than that of conventional markers. For eample, a genetic delineation of *Pogostemon cablin* was revealed by SLAF-sequencing technique (Huang *et al*., 2016).

However, no method is reported for verifying the identity of *O. fragrans* varieties using DNA-based markers. We employed specific locus amplified fragment sequencing (SLAF-seq) (Sun *et al*., 2013) in this study to identify single nucleotide polymorphisms (SNPs) that we could use to verify the identity of putative *O. fragrans* 'Pucheng Dangui' plants.

Materials and Methods

Plant samples: Leaves of 29 *O. fragrans* ´Pucheng Dangui′ plants growing in Pucheng County, China, were harvested for genome extraction (Table 1).

SLAF tags and population SNPs: The SLAF-seq technique (Sun *et al*., 2013) developed by Biomarker Technologies was used to develop molecular makers for the 29 samples. The closely related species, *Fraxinus excelsior* L. (Oleaceae), was selected to provide the reference to predict enzyme digestion. Its genome is 842 Mb, with GC content 34.46% (https://www.ncbi.nlm.nih.gov/genome/?term=European%20ash). Enzyme digestion prediction software developed by BMK was used to select an optimal digestion protocol. The selection principles were as follows: (a) the rate of repeat sequences in the enzyme digested fragments was as low as possible; (b) the distribution of the enzyme digested fragments was as uniform as possible; (c) the consistent degree of coincidence between the enzyme digested fragment length and the target experimental system (Davey *et al*., 2013); and (d) the number of final enzyme digested fragments (SLAF tags) satisfied the predicted tag number.

The qualifying genomic DNA was digested by the restriction enzymes according to the optimal enzyme digestion protocol. 'A' was added to the tail of the restriction enzyme digestion fragments (SLAF tags) that were produced, which were then transferred to a Dual-index (Kozich *et al*., 2013) sequencing adapter, followed by PCR amplification, purification, sample mixing and gel cutting to obtain target fragments. Library quality inspection was performed prior to Illumina HiSeq sequencing. *Oryza sativa* L. was used as the control to evaluate the accuracy of restriction enzyme digestion. Restriction enzyme digestion fragments between 400 and 480 bp length were defined as SLAF tags digested by the enzyme Hpy166II+EcoRV-HF®.

The raw data for each sample's reads were identified by the Software Dual-index. The reads were filtered before the assessment of sequencing quality and data size. The digestion efficiency of enzyme Hpy166II+EcoRV-HF® was evaluated against the control data to judge experimental accuracy and validity. The SALF-seq SNP makers were developed by bioinformatic analysis, and representative high-quality SNPs were used to perform population analysis. The reads from the same enzyme clustered together were regarded as the same SLAF tags. The SLAF tags vary between different samples, meaning polymorphism SLAF tags.

Phylogenetic analysis: A phylogenetic tree was constructed using the software MEGA5 (Tamura *et al*., 2011) to express the relationship among sampled trees. The analysis employed the neighbor-joining method with considering SNPs with 50% missing data and MAF 0.05%.

Population admixture structure analysis: Population structure was analyzed using the software, Admixture (Alexander *et al*., 2009), considering SNPs filtered by integrity degree 0.5 and secondary genotype frequency 0.05. The K value was assumed as 1-10 to construct clustering figures. The optimal population number was inferred according to the valley value by using the admixture K value cross validation error rate.

Table 1. Leaf samples of *Osmanthus fragrans* ´Pucheng Dangui′ harvested in Pucheng County, Fujian Province, China.

S.No.	BMK-ID	Nursery name	Sampling site
1.	al	Parent tree: Jiulong Gui, Pucheng County	Linjiang 000-1
2.	ab	Jinyuan Landscape Flowers and Seedlings Professional Cooperative of Pucheng County	Dongfeng Reservoir
3.	ac	Minyuan Seedlings Professional Cooperative of Pucheng County	Zhang Ancestral Hall
4.	ad	Hebin Dangui Farmer Professional Cooperative of Pucheng County	Liantang
5.	ae	Heshun Seedlings and Greening Company of Pucheng County	Maxi
6.	af	Jinhui Seedlings Professional Cooperative of Pucheng County	Taiping Village
7.	ag	Chaoxin Dangui Seedlings Professional Cooperative of Pucheng County	End of Sanyuan Reservoir
8.	ah	Forestry Nursery of Pucheng County	Shipaixia
9.	ai	Bafang Seedlings and Greening Company of Pucheng County	Xixia
10.	aj	Xuli Seedlings Professional Cooperative of Pucheng County	Luchuwu
11.	ak	Dawu Dangui Seedlings Professional Cooperative of Pucheng County	Linjiang
12.	aa	Luye Dangui Company of Pucheng County	Pushu Bridge
13.	am	Liangxing Ecological Farm of Pucheng County	Dazhuang 001-2
14.	an	Minhong Seedlings and Greening Company of Pucheng County	Highway entrance
15.	ao	Guangda Dangui Cooperative of Pucheng County	Linjiang
16.	ap	Xinyuanjia Greening Company of Pucheng County	Xiaxia
17.	aq	Yongsheng Seedlings Professional Cooperative of Pucheng County	Yule
18.	ar	Fugui Greening Engineering Company of Pucheng County	Qiaotou
19.	as	Jianbin Seedlings Professional Cooperative of Pucheng County	Guancuo 002-1
20.	at	Kaisheng Dangui Seedlings Professional Cooperative of Pucheng County	Guancuo 002-2
21.	au	Baimiao Nursery of Pucheng County	Linjiang
22.	av	Liangxing Ecological Farm of Pucheng County	Dazhuang 001-1
23.	aw	Zhangshan Seedlings Professional Cooperative of Pucheng County	Linjiang
24.	ax	Wangke Seedlings and Greening Company of Pucheng County	Dongyuan
25.	ay	Lucheng Dangui Professional Cooperative of Pucheng County	Sanyuan
26.	az	Juguan Dangui Seedlings Professional Cooperative of Pucheng County	Shipaixia Shegong Temple
27.	ba	Shengyong Greening Seedlings Professional Cooperative of Pucheng County	Sanyuan Village
28.	bb	Chenyang Seedlings Professional Cooperative of Pucheng County	Xixia Pig Farm
29.	bc	Luzhiyuan Dangui Professional Cooperative of Pucheng County	Hongshan Bridge

Principle component analysis: Principal component analysis (PCA) was performed using the software EIGENSOFT (de Hoon *et al*., 2004), considering SNPs with 50% missing data and MAF 0.05%.

Results

SLAF tags and population SNPs: Restriction enzyme digestion fragments between 400 and 480 bp length were defined as SLAF tags and 300,000 SLAF tags were predicted. The efficiency of Hpy166II+EcoRV-HF® in this study was 94.08%, and 149.03M reads were obtained. A total of 413,743 SLAF tags was identified by bioinformatic analysis, of which 113,061 polymorphic SLAF tags and 353,073 population SNPs were obtained (Tables 2 and 3). The highest depth sequence was taken as the reference and the reads were aligned to the reference genome sequence by BWA (Li & Durbin, 2009). SNPs were developed by both GATK (McKenna *et al*., 2010) and Samtools (Li *et al*., 2009). SNPs from the intersection dataset were selected as the reliable target SNP marker dataset (Tables 2 and 3).

Phylogenetic analysis: The phylogenetic tree showed no significant diversity among samples and the confidence coefficient was very low from the beginning; i.e. we can consider it as reasonable that all samples were from the one population. Thus, the sampled genotypes of *O. fragrans* 'Pucheng Dangui' appeared to be pure, lacking mixture with other varieties or species (Fig. 1).

Population admixture structure analysis: The population structure analysis provided individual lineage source and composition information (Figs 2 and 3). Admixture clustering indicated that all samples were displayed in one color and the population structure was evident as one population when K=1 (i.e. when the 29 samples were assumed as one population) whereas not all samples were in one color and the population structure was not evident as one population when K=2-10. Thus, we can again infer that the 29 samples belonged to the same population. The K value cross validation error rate was lowest when K=1, also indicating that it is reasonable that the 29 samples belonged to one population.

Principle component analysis: Five samples, aa, ac, ad, ae and as, did not cluster tightly with the principal population (Fig. 4 and Table 4), but they were near to it. We also regarded all 29 samples were in the same population, and so we attribute these five points to SNP variations or sequencing errors.

Table 2. Polymorphic SLAF tags obtained from *Osmanthus fragrans* ´Pucheng Dangui′.

Sample ID	BMK ID	SLAF number	Total depth	Average depth
1.	al	335,938	6,833,143	20.34
2.	ab	219,547	4,485,117	20.43
3.	ac	298,081	4,699,379	15.77
4.	ad	305,380	5,506,847	18.03
5.	ae	310,406	4,377,959	14.10
6.	af	296,252	4,062,859	13.71
7.	ag	317,290	3,656,122	11.52
8.	ah	314,826	3,068,319	9.75
9.	ai	325,143	3,876,400	11.92
10.	aj	295,620	3,306,564	11.19
11.	ak	311,209	3,613,809	11.61
12.	aa	305,903	4,815,682	15.74
13.	am	241,321	3,250,349	13.47
14.	an	284,710	3,705,890	13.02
15.	ao	268,876	3,948,591	14.69
16.	ap	310,456	4,049,145	13.04
17.	aq	313,906	4,337,773	13.82
18.	ar	301,831	3,855,525	12.77
19.	as	279,315	3,745,459	13.41
20.	at	308,184	4,071,378	13.21
21.	au	300,827	5,214,468	17.33
22.	av	295,843	4,494,601	15.19
23.	aw	290,348	4,470,182	15.40
24.	ax	285,175	4,233,033	14.84
25.	ay	250,117	4,856,766	19.42
26.	az	283,825	3,823,932	13.47
27.	ba	265,528	5,329,339	20.07
28.	bb	273,254	3,824,122	13.99
29.	bc	279,898	4,794,519	17.13
Total	**Total**	**413,743**	**124,307,272**	**300.45**

Table 3. Quantity of population SNPs obtained from *Osmanthus fragrans* ´Pucheng Dangui′.

Sample ID	BMK-ID	Total SNP	SNP number	Heterozygous loci ratio (%)	Integrity ratio (%)
1.	al	353,073	299,740	69.21	84.89
2.	ab	353,073	197,200	57.25	55.85
3.	ac	353,073	281,573	58.32	79.74
4.	ad	353,073	263,955	64.69	74.75
5.	ae	353,073	304,492	62.75	86.24
6.	af	353,073	272,826	56.94	77.27
7.	ag	353,073	278,077	62.04	78.75
8.	ah	353,073	281,527	60.67	79.73
9.	ai	353,073	280,876	66.61	79.55
10.	aj	353,073	256,968	57.11	72.78
11.	ak	353,073	283,547	60.09	80.30
12.	aa	353,073	304,227	61.92	86.16
13.	am	353,073	204,229	50.78	57.84
14.	an	353,073	255,421	54.31	72.34
15.	ao	353,073	229,473	53.36	64.99
16.	ap	353,073	285,010	58.80	80.72
17.	aq	353,073	271,349	62.73	76.85
18.	ar	353,073	253,551	60.98	71.81
19.	as	353,073	246,373	26.00	69.77
20.	at	353,073	262,365	61.17	74.30
21.	au	353,073	265,223	59.82	75.11
22.	av	353,073	252,960	60.63	71.64
23.	aw	353,073	259,276	56.09	73.43
24.	ax	353,073	249,363	56.41	70.62
25.	ay	353,073	223,578	55.00	63.32
26.	az	353,073	270,185	54.98	76.52
27.	ba	353,073	241,665	56.23	68.44
28.	bb	353,073	238,883	55.90	67.65
29.	bc	353,073	240,433	58.97	68.09

Table 4. PCA data obtained from *Osmanthus fragrans* ´Pucheng Dangui′.

Sample	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
aa	-0.9734	-0.0026	-0.0017	-0.0553	0.0286	0.0515	-0.0827	-0.0215	-0.0116	-0.0162
ab	0.0366	-0.0164	-0.0027	0.0675	-0.0096	-0.0470	0.1146	0.0397	-0.0321	0.0277
ac	0.1098	0.0001	-0.7491	-0.4636	0.0858	0.2252	-0.2702	-0.1259	0.0124	0.0187
ad	0.0924	-0.0617	0.1198	0.5733	0.2122	0.3585	-0.4832	-0.2368	-0.0161	-0.1113
ae	0.1065	-0.0648	0.6457	-0.6413	0.0297	0.1818	-0.2331	-0.0758	-0.0182	0.0434
af	0.0360	-0.0491	-0.0091	0.0073	0.1456	-0.8052	-0.4269	-0.1590	-0.0305	0.1043
ag	0.0457	-0.0447	-0.0177	-0.0263	-0.0938	-0.0215	0.0287	0.2685	-0.3486	-0.2116
ah	0.0225	-0.0365	-0.0082	0.0608	-0.0489	0.1445	-0.1221	0.3218	0.1021	0.1969
ai	0.0305	-0.0469	-0.0045	0.0286	-0.0562	0.0012	-0.0227	0.0407	-0.0301	-0.0044
aj	0.0264	-0.0286	-0.0219	0.0033	-0.3285	-0.1650	-0.0063	0.2418	-0.2328	-0.5275
ak	0.0333	-0.0338	0.0121	0.0232	-0.2929	0.0696	-0.0043	-0.0313	0.1404	0.0756
al	0.0502	-0.0181	0.0126	-0.0150	-0.0914	-0.0013	-0.0429	0.0183	-0.0007	-0.0182
am	0.0096	-0.0170	-0.0034	0.0491	-0.0189	-0.0249	0.0724	0.0236	-0.0953	0.0975
an	0.0140	-0.0297	-0.0145	0.0833	-0.3573	0.1394	0.1953	-0.5305	-0.3560	0.2205
ao	0.0188	-0.0297	-0.0277	0.0332	-0.1204	-0.0927	0.0629	0.2066	0.0048	0.2907
ap	0.0096	-0.0493	-0.0068	0.0286	-0.2785	-0.0596	0.0687	-0.0893	0.7314	-0.1592
aq	0.0314	-0.0303	-0.0181	0.0576	-0.0881	0.0344	0.0183	0.1665	-0.1818	0.1679
ar	0.0274	-0.0141	0.0275	0.0512	-0.1959	0.0098	-0.0568	-0.0199	0.0709	0.1090
as	0.0337	0.9783	0.0480	0.0117	0.0210	-0.0040	0.0008	0.0004	0.0181	-0.0114

NB: PC1: first principal component; PC2: second principal component; PC3: third principal component; so on

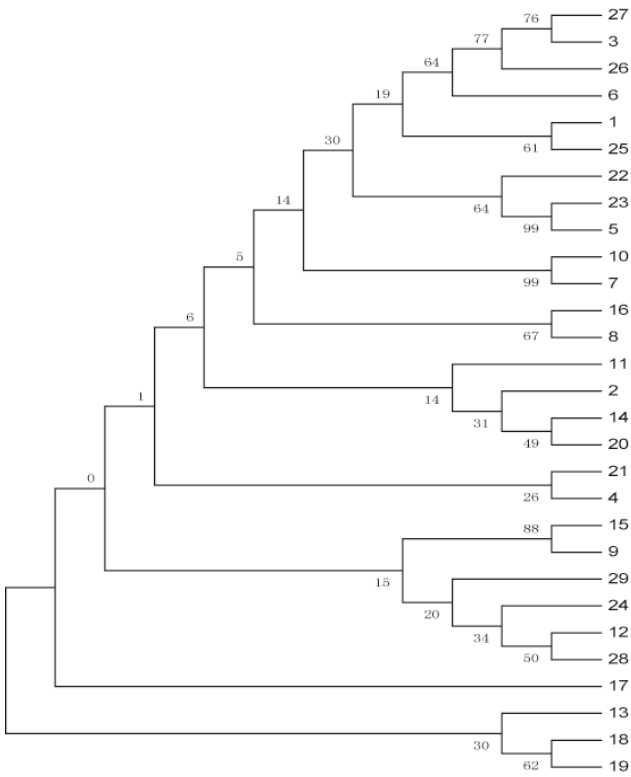

Fig. 1. A phylogenetic tree for *Osmanthus fragrans* samples constructed by MEGA5.

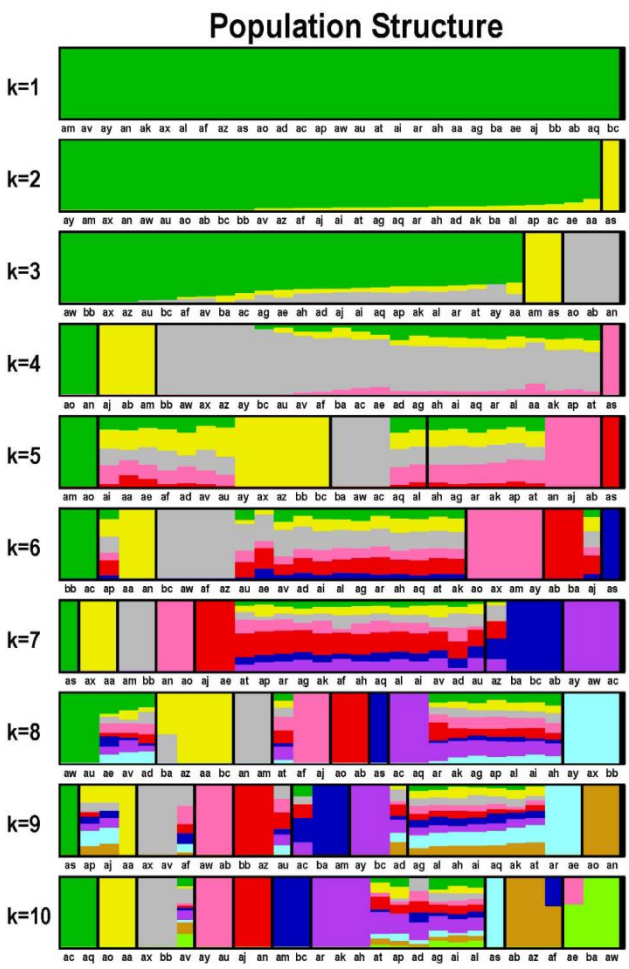

Fig. 2. The admixture figure to the corresponding K value of *Osmanthus fragrans* samples.

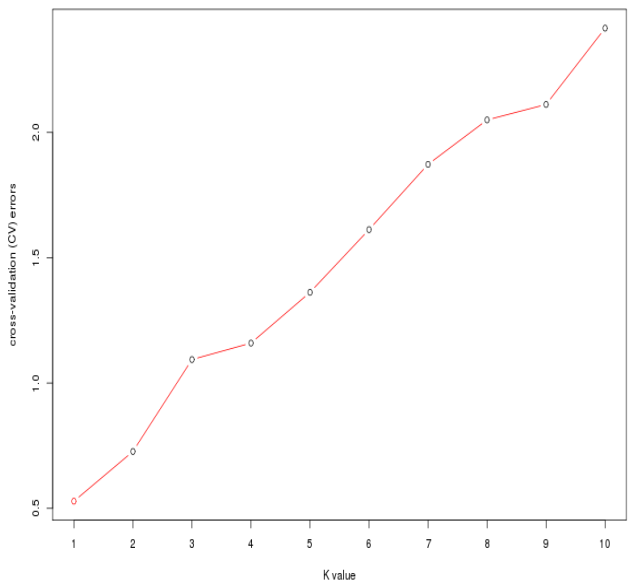

Fig. 3. The figure of K value cross validation error rate of *Osmanthus fragrans* samples.

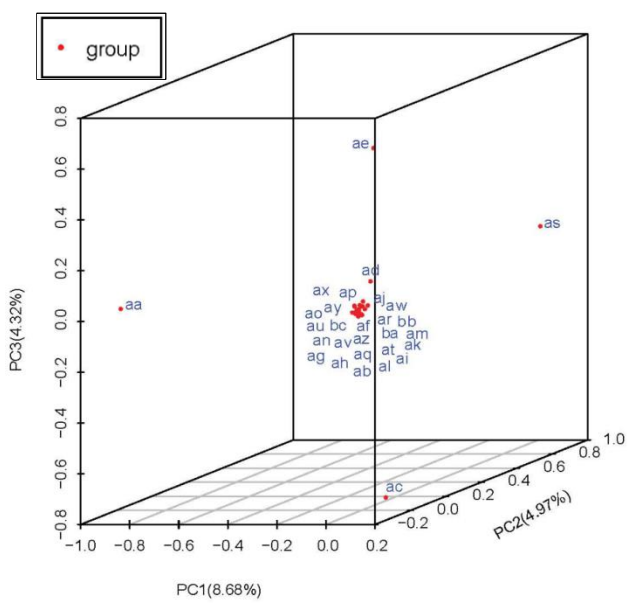

Fig. 4. Principal component analysis (PCA) clustering among *Osmanthus fragrans* samples. PC1: first principal component; PC2: second principal component; PC3: third principal component.

Discussion

The SLAF-seq strategy combines locus-specific amplification and high-throughput sequencing to effect *de novo* SNP discovery and large-scale genotyping to help us understanding more about the genetic delineation of *O. fragrans* ‘Pucheng Dangui’ variety.

The phylogenetic tree, population admixture structure, admixture K value cross validation error rates and the PCA all showed that the 29 samples of putative *O. fragrans* ‘Pucheng Dangui’ belonged to the same population, with no significant differences between samples. Furthermore, our findings reveal that no significant level of introgression occurred from one of *O. fragrans* ‘Pucheng Dangui’ into another, which may be

responsible for these varieties being closely related to the mother tree (Fig. 2). That is in contrast to the results of genetic diversity and selection in apple (Ma *et al*., 2017).

This confirmed the identity of the samples as *O. fragrans* 'Pucheng Dangui', providing confidence for producers and vendors who aim to cultivate or distribute seedlings of this valuable variety across China.

Comprehensive analysis of DNA polymorphisms also provided valuable insights into the different individuals of *O. fragrans* ´Pucheng Dangui′. Importantly, the identification of numerous intraspecific genetic variations provides the basis for further development of individual plant analysis, identification and introgression breeding. For example, five samples, aa, ac, ad, ae and as, did not cluster tightly with the principal population, but they were near to the principal population, which attributes these five points to SNP variations or sequencing errors. We can study on the five individuals morphology variation in the future.

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