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GENETIC DIVERSITY OF PUMPKIN BASED ON MORPHOLOGICAL AND SSR MARKERS

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Abstract

Cucurbita pepo is an economically important plant. However, the fact that there are few high-quality varieties that have limited the development of this plant in *Cucurbita* breeding programs. Aiming to provide genetic improvement and application on breeding of *Cucurbita* breeding programs, the diversity of 64 *C. pepo* accessions was analyzed using morphological and simple sequence repeat (SSR) markers. A total of 45 morphological traits in *C. pepo* accessions presented large morphological characteristic variability, from 6.30% (for flower shape) to 70.84% (for fruit umbilicus diameter), with an average variability of 34.43%, whereas the average diversity index of all traits was 1.25, ranging from 0.2% (for seed width) to 4.8% (for seed thickness). Sixty accessions were discriminated into 2 clusters morphological and molecular markers: cluster I included 5 accessions with hull-less seeds, and cluster II included 59 accessions with shell-covered seeds. Cluster II was divided into 5 sub-clusters with different fruit and leaf shapes based on morphological data, and accessions with similar phenotypic features were grouped together. Cluster II was divided into dwarf and normal sub-clusters using SSR markers and accessions with the same origin and same geographical distribution were clustered together. There were some differences between the relationships morphological markers and SSR markers in this study. Morphological markers and SSR markers among the 64 *C. pepo* are not interchangeable methods but are complementary methods that together ensure the comprehensiveness and accuracy of analytical results.

Key words: *Cucurbita pepo*, SSR marker, Morphological marker, Genetic diversity, Cluster analysis.

Introduction

Cucurbita (2x = 2n = 40) is an important cucurbitaceous plant that is widely grown as a commercial crop across the globe. It is known to have high nutritional value and health protective properties; therefore, it has attracted an increased interest in recent years. *Cucurbita* includes five cultivated species: *Cucurbita maxima*, *Cucurbita moschata*, *Cucurbita pepo*, *Cucurbita ficifolia* and *Cucurbita mixta* (Naik *et al*., 2015). *C. pepo* is the species with the greatest economic value (Paris *et al*., 2008). The edible portions of *C. pepo,* such as the fruit, flower, leaf and seeds, are rich in sugars, fatty acids, fiber, protein, vitamins and minerals, so they can play a protective role as part of a healthy diet, in cancer prevention and in the treatment of benign prostate hyperplasia (Schmidlin & Kreuter, 2003). However, the few high-quality varieties of *C. pepo* are not enough to satisfy the huge market demand and have limitations in the development of *Cucurbita* breeding programs.

Due to their easy handling, co-dominant inheritance, and highly polymorphic nature, simple sequence repeats (SSRs) are ideal tools for broad applications in basic and applied plant biology (Rabbani *et al*., 2010; Shah *et al*., 2015). It has been reported that using SSR flanking sequences, species and genera of plants scan be clustered clearly into several genera, such as *Triticum* (Adonina *et al*., 2005), common bean (Buah *et al*., 2017), spring barley (Bengtsson *et al*., 2017), blueberry (Tailor *et al*., 2017), flax (Choudhary *et al*., 2017), cucumber (Danin-Poleg *et al*., 2001; Ritschel *et al*., 2004; Yang *et al*., 2016; Dar *et al*., 2017), melon (Zhu *et al*., 2016a), and watermelon (Zhu *et al*., 2016b). Since 48 genotypes of *C. pepo*, *C. moschata*, and *C. maxima* were classified for the first time based on 27 pairs of SSR markers in C. *pepo* (Stift *et al*., 2004), a number of SSRs have been developed for *Cucurbita*. *Cucurbita* accessions were assessed and successfully grouped into distinct clusters using SSR markers (Gong *et al*., 2008; Gong *et al*., 2012; Murovec, 2015; Sim *et al*., 2015; Wang *et al*., 2016a; Zhu *et al*., 2016b; Kazminska *et al*., 2017). However, most reports are related to *C. moschata* and *C. maxima*, and research on *C. pepo* is still lacking.

Abundant germplasm resources for *C. pepo* were collected for our research, and the resources with different geographical distributions had varying morphological characteristics. The objective of the present paper was to provide a resource foundation for *C. pepo*, and to analyse the genetic variability and phylogenetic relationships among germplasm resources of *C. pepo* using morphological and molecular markers.

Materials and Methods

Plant materials and experimental design: Sixty-four accessions of *C. pepo* from different geographical regions in China were obtained (Table 1). The seeds of the 64 accessions were germinated at 30°C in the dark for 36 h after being treated with 55°C water for 8h and then transplanted to greenhouse breeding plots at Northeast Agricultural University during the 2015 growing season. Four-leaf stage seedlings from each accession were transferred to experimental plots, and the required irrigation and fertilizer were applied. The experiment was arranged in a randomized complete block design with three replicates.

Table 1. The test materials of *C. pepo* (n=64).

No.	Origin	Pumpkin taxon	No.	Origin	Pumpkin taxon	No.	Origin	Pumpkin taxon
2.	Neimenggu	*C. pepo*	40.	Neimenggu	*C. pepo*	73.	Yunnan	*C. pepo*
4.	Neimenggu	*C. pepo*	42.	Neimenggu	*C. pepo*	77.	Yunnan	*C. pepo*
5.	Neimenggu	*C. pepo*	43.	Neimenggu	*C. pepo*	78.	Yunnan	*C. pepo*
7.	Neimenggu	*C. pepo*	44.	Heilongjiang	*C. pepo*	82.	Yunnan	*C. pepo*
10.	Neimenggu	*C. pepo*	45.	Heilongjiang	*C. pepo*	95.	Shanxi	*C. pepo*
12.	Neimenggu	*C. pepo*	47.	Heilongjiang	*C. pepo*	97.	Heilongjiang	*C. pepo*
13.	Neimenggu	*C. pepo*	46.	Heilongjiang	*C. pepo*	98.	Heilongjiang	*C. pepo*
14.	Neimenggu	*C. pepo*	48.	Heilongjiang	*C. pepo*	100.	Heilongjiang	*C. pepo*
17.	Neimenggu	*C. pepo*	50.	Gansu	*C. pepo*	103.	Heilongjiang	*C. pepo*
18.	Neimenggu	*C. pepo*	51.	Gansu	*C. pepo*	106.	Heilongjiang	*C. pepo*
19.	Neimenggu	*C. pepo*	52.	Heilongjiang	*C. pepo*	114.	Neimenggu	*C. pepo*
21.	Neimenggu	*C. pepo*	56.	Heilongjiang	*C. pepo*	115.	Shanxi	*C. pepo*
24.	Neimenggu	*C. pepo*	57.	Heilongjiang	*C. pepo*	116.	Neimenggu	*C. pepo*
26.	Neimenggu	*C. pepo*	58.	Heilongjiang	*C. pepo*	117.	Neimenggu	*C. pepo*
27.	Neimenggu	*C. pepo*	60.	Heilongjiang	*C. pepo*	118.	Neimenggu	*C. pepo*
28.	Neimenggu	*C. pepo*	63.	Neimenggu	*C. pepo*	120.	Neimenggu	*C. pepo*
29.	Neimenggu	*C. pepo*	64.	Yunnan	*C. pepo*	119.	Neimenggu	*C. pepo*
30.	Neimenggu	*C. pepo*	65.	Yunnan	*C. pepo*	121.	Gansu	*C. pepo*
31.	Neimenggu	*C. pepo*	66.	Yunnan	*C. pepo*	180.	Gansu	*C. pepo*
35.	Neimenggu	*C. pepo*	67.	Yunnan	*C. pepo*	181.	Gansu	*C. pepo*
36.	Neimenggu	*C. pepo*	68.	Yunnan	*C. pepo*			
39.	Neimenggu	*C. pepo*	70.	Yunnan	*C. pepo*			

Morphological characteristics: A total of 45 sets of morphological data of *C. pepo* were recorded, including quantitative and qualitative traits. The quantitative traits included fruit vertical diameter (cm), fruit longitudinal diameter (cm), fruit index, flesh thickness (cm), stalk width (cm), fruit umbilicus diameter (cm), ventricle number, single fruit weight (kg), seed length (cm), seed width (cm), seed thickness (cm), seed kernel weight(g), seed shell weight(g), hundred seed weight(g), seed number per fruit, cotyledon length (cm), cotyledon width (cm), and cotyledon index. The qualitative traits included growth habit, growth vigor, leaf shape, foliage white spotting, number of white spots per leaf, main vine section shape, flower shape, flower size, flower color, floral tube shape, sepal shape, fruit shape, stalk basal enlargement shape, flesh color, skin color, fruit speckle, characteristics of fruit skin, edge and ditch, gourd tumor quantity, gourd skin wax powder, hardness of fruit stem, seed shell, seed color, characteristics of seed beak, seed surface, seed margin, and seed margin color. The morphological traits were divided into 4 groups according to plant development: the cotyledon period, the growing period, the fruit maturation period and the seed harvest period. The morphological characteristics at different stages are listed in Table 2. The quantitative and qualitative morphological data were measured in 10 samples of seeds, fruits and plants per accession.

DNA extraction and polymerase chain reaction: Young leaves from 10 samples from each accession were collected for DNA extraction using the CTAB method (Murray & Thompson, 1980) for SSR marker analysis. A total of 300 SSR markers, including 100 markers from *C. pepo*, 75 markers from www.icugi.org, 100 markers from *C. maxima* (Wang *et al*., 2016b), and 25 markers from *C. moschata,* were chosen for polymorphism screening. The polymorphic primer sequences used in this study are shown in Table 3. PCR was carried out using 20 µl samples containing ~40 ng of genomic DNA, 1 µM of each primer, 400 µM dNTPs, 1 × reaction buffer, and 1 U of Taq DNA polymerase. PCR amplifications were performed using the following program: 5min of denaturation at 95°C, 35 cycles of 30 s at 94°C, 30 s at 49°C and 30 s at 72°C and an extension reaction at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 8% polyacrylamide gels. After electrophoresis at 200 V for 1.5 h, the gel was visualized using silver staining.

Data analysis

The quantitative morphological data were calculated in a range from 1 to 10 using the average Euclidean distance to match the cluster analysis format, and the qualitative morphological traits were evaluated according to the methods presented in Table 4. The bands of each SSR marker were scored as presence (1) or absence (0) in the 64 accessions. Clustering was carried out using the unweighted pair group method with arithmetic mean (UPGMA) in the SAHN sub-program, and principal coordinate analyses were performed on the genetic similarity matrix after eigen values and eigenvectors were computed using the Eigen of Ordination program. The statistical analyses were carried out using the NTSYSpc software package, version 2.10e.

Table 2. The morphological characters on different stage of *C. pepo*.

Stage	Traits	CV	*I*	Stage	Traits	CV	*I*
Cotyledon period	Cotyledon length	19.24	1.2	Growing period	Foliage white spotting	40.47	1
	Cotyledon width	19.80	1.5		Number of white spots per leaf	59.28	0.6
	Cotyledon index	38.71	1.5		Leaf shape	66.77	1.1
	average	25.92	1.4		Flower shape	6.3	1
					Flower size	35.99	0.7
Fruit maturation period	Fruit longitudinal diameter	25.37	1.7		Floral tube shape	41.43	0.8
	Fruit vertical diameter	32.96	0.9		Average	37.88	1
	Fruit index	39.09	1.5				
	Flesh thickness	19.21	1.8	Seed harvest period	Seed length	14.82	0.6
	Stalk width	28.14	1.1		Seed width	12.72	0.2
	Fruit umbilicus diameter	70.84	1.1		Seed thickness	16.74	4.8
	Ventricle number	15.21	1.4		Seed kernel weight	36.43	0.2
	Single fruit weight	33.60	0.9		Seed shell weight	64.77	2.9
	Fruit shape	49.71	1.7		Hundred seed weight	32.42	1.6
	Flesh color	60.62	1.6		Seed number per fruit	55.92	2
	Skin color	46.67	2		Seed shell	14.07	0.7
	Fruit speckle	61.88	1.7		Seed color	28.58	0.8
	Edge and ditch	30.72	0.4		Characteristics of seed beak	24.92	1.2
	average	35.69	1.37		Seed surface	25.85	1
					Seed margin	25.99	1.1
Growing period	Growth habit	31.07	0.9		Seed margin color	25.71	1
	Growth vigor	21.73	1.1		Average	29.15	1.55

Notes: CV: coefficient of variance, *I*: Shannon's information index

Table 3. The polymorphic primer sequence used in this study.

Primer name	Forward Primer	Reverse primer
comp107035	TGGAATTTGTTGGAGTCAGATG	ACGACCCCCTACAATGACAA
comp11018	GGGAGGCTACGAAGGAAAAG	CAATGGGGAAGTGGTAGGTG
comp13350	GGACGAGGAGGTTTCAACAA	AAGGAAGCAAGAACACTCGG
comp27618	TGAGCAGACCCTTGTTTTCC	GGTTGATGGGTGGAACTTTG
comp28857	GACCAACCTCTTTCCCATCA	AAGGCGTAATAGCAGCTCCA
comp40082	GGTGGTCCGGCTATCAACTA	TGACAGGGGATGAAAGGAAC
comp41315	TTTGAAGTTGTTGTGCGGTG	ACTGTTGGCCGGTAGATTTG
comp52235	GCCGATTGTTGAATATTTCG	TCATTGAGAGGGAGTCCCATA
comp52664	CACTGTTTGGCAACGGTTTA	GATCCATGTGACAACCATGC
comp64835	GTGGAGATCGTTGAAGGGAA	GAGCTTTCCACTCAGGCAAC
comp66738	CGCCTTCATTGCAACATAAA	AGTGAGGGAAAGCAAAGCAA
comp71199	CTGGGCACACTAGGGTCAAT	TTTATGCCGAGCAAACCAAT
comp71344	GCTCTGGAGGATGAAACTCG	GGCTAACCCAGAAGGAAAGG
comp71363	TGATGAGATTGAGAGCGGTG	AAGTGAATCCTTTCCGCCTT
comp71542	AAATGCTCCTACCGAAGGGT	CAGCTTGAACATGATGCCAC
comp72289	TGGTTGCTCATTGTCTTGCT	ATGCGCTATTTGCTTTCTCC
comp72586	ATCACCACCGCAGGAACTAC	GGGCATCCCAACCTTTTATT
CMTm89	ATAGGAATGTGCAGAGCTGAG	CAATATAGATACCGTTTTTCGAATC
CMTmC1	AACGTCCTTACTGGCACACC	TTCCACAAGTTGTTTTGGTCAC
CMTmC60	ATCAGGCTAAGGCCCAAACT	GCCAATGTAATCTCCCCACA
CMTm13	AAGCTCCCCAGAAACACAAT	ATTGGGGTCAGAATGAAGGT
CMTm14	TCTGCTGTCTTCATCTTTGCT	CCAGCAGACAAGCTAATGTGT
CMTm115	AAGTCCACAACATGCAAACG	TCTCTTAATTGTTTCTCCCGATCT
CMTm11	TGGAAGGATTCTCCCACAGT	TACAATTTGACGTCCGCAAG
comp73642	TTCACTGCCACTGTCAAAGC	GGACTCGTACCAGTGCCTGT
PU025208	ACAGCAGACTTTGCGAGCTT	AGAGAACCGGAAACCCAAGT
PU002959	TGAGCAGTCAATATCAACCAAAA	GAGGATTGAAGGCCATGAGA
comp98739	ATGCACGGTTGCTTGAAACT	CCAAGCAAAATATCCGCCTA
PU026252	GTCCCTTTGTTGAGCAAGGA	CTTCCAATCGGAAATGGCTA
PU024278	GGATTTGAGAGCAACCCAGA	CCCCTTTTCCCTCTCTTTTG
H39	CGTTTTCACAAAACCCTCGT	GAGAAGAGCAACGCTTTCGT
H41	TAGGTTCAACTCTCTCCCCG	TACTGGTTTTTCCAATCCGC
H65	ATCATAGTCGTCGTCGGGTC	GCCGATTCTTGAGGAACAGA
CMTp37	GTCTGGTCTTGGGGTGGTTC	AGAAACAAAGTGGCGGGTGT
CMTp57	GCCGTCAACACCAAACTCC	AGCGCTGACGGAGGTTAAAT
CMTm115	AAGTCCACAACATGCAAACG	TCTCTTAATTGTTTCTCCCGATCT
CMTm11	TGGAAGGATTCTCCCACAGT	TACAATTTGACGTCCGCAAG

Table 4. The evaluates of qualitative characters.

Qualitative characters	Criteria for Pumpkin characterization
Growth habit	Normal=1; semi dwarf=2; dwarf=3
Growth vigor	Weak=1; intermediate type=2; strong=3
Leaf shape	Palmate=1; palmate and pentagonal=2; heart shaped=3; heart shaped and pentagonal=4; round leaf=5; triangular=6
Foliage white spotting	None=1; have=2
White spotting number of leaf	None=1; less=2; more=3; most=4
Main vine section shape	Five prismatic=0; round=1; oval=2
Flower shape	Cylindrical=1; conical=2
Flower size	Big=1; middle=2; small=3
Flower color	Light yellow=1; yellow=2; orange-yellow=3
Floral tube shape	Bell-shaped=1; cylindrical=2; open=3
Sepal shape	Small and thin=1; big and leafy=2
Fruit shape	Oval=1; medium elliptic=2; elliptical=3; tall round=4; oblong=5; long elliptic=6; pear-shaped=7; dumbbell-shaped=8; heart shaped=9
Stalk basal enlargement shape	Star-like=1; pentagonal=2；
Flesh color	White=1; light yellow=2; light green=3; light yellow-green=4; yellow green=5, yellow=6; orange-yellow=7
fruit speckle	None=1; strip=2; mesh=3; massive=4; streak=5
Skin color	Dark green=1; mossy green=2; orange-red=3; orange-yellow=4; yellow=5; wheat=6; light yellow=7; orange=8
characteristics of fruit skin	Flat=1; ditch=2; edge=3; shrinking=4; bulged=5
Edge and ditch	Without=0.5; shallow=1; deeper=2; deepest=3
Gourd tumor quantity	None=1; less=2; more=3; most=4
Gourd skin wax powder	None=1; less=2; more=3; most=4
hard of fruit stalk	Hard=1; soft=2
seed shell	None=1; have=2
Seed color	Without=1; White=2; off-white=3; yellowish-white=4; yellow=5
Seed surface	Without=1; smooth=2; rough=3
Characters of seed beak	None=1; obtuse=2; flat=3; flat and tilt=4
Seed margin	Flat=1; ridgy=2; narrow=3
Seed margin color	Light=1; similar to seed coat=2; dark=3

Results

Morphological characterization: Most morphological traits of the 64 accessions of *C. pepo* presented large variability, from 6.30% to 70.84% (Tables 5, 6), and 8 traits, including main vine section shape, flower color, sepal shape, stalk basal enlargement shape, characteristics of fruit skin, gourd tumor quantity, gourd skin wax powders, and hardness of the fruit stem, had similar phenotypic features. The average variation coefficient of all varying morphological traits was 34.43%. The largest variation coefficient was 70.84% (for fruit umbilicus diameter), followed by 64.77% (for seed shell weight), and the smallest variation coefficient was 12.72% (for seed width) among the quantitative traits. The highest variation coefficient was 66.77% (for leaf shape), followed by 61.88% (for fruit speckle) and 60.62% (for flesh color), and the lowest variation coefficient was 6.30% (for flower shape seed width) among the qualitative traits. The average variation coefficient of the quantitative traits (32.33%) was slightly lower than the average variation coefficient of the qualitative traits (36.72%).

All morphological traits were divided into 4 groups according to plant development (Table 2). Cotyledon index (CV = 38.71%), fruit umbilicus diameter (CV = 70.84%), leaf shape (CV = 66.77%) and seed shell weight (CV = 64.77%) had the highest variation coefficients during the cotyledon period, fruit maturation period, growing period and seed harvest period, respectively. As Table 2 shows, the accessions had the richest genetic diversity during the growing period (average CV = 37.88%) among the 4 periods, followed by the fruit maturation period (average CV = 35.69%), the seed harvest period (average CV = 29.15%) and the cotyledon period (average CV = 25.92%).

Based on Shannon's information index, the diversity index of the 64 accessions was calculated (Tables 5, 6). The average Shannon's information index of all varying traits was 1.25, ranging from 0.2% to 4.8%. For qualitative traits, the highest diversity index was observed for seed thickness (with a value of 4.8), and a high diversity index was calculated for seed shell weight, with a value of 2.9.The lowest diversity index was observed for seed width, with a value of 0.2. The diversity index of qualitative traits was the highest for skin color, with a value of 2, followed by fruit shape and fruit speckle (with a value of 1.7), and the lowest value was observed for edge and ditch, with a value of 0.4.

The average diversity index of the quantitative traits (1.49) was much higher than the average diversity index of the qualitative traits (1.07) (Tables 5, 6). Based on a data analysis of the 4 periods, cotyledon index (I = 1.5), skin color (I = 2), leaf shape (I = 1.1) and seed thickness (I = 4.8) (Table 2) had the highest diversity indexes during the cotyledon period, fruit maturation period, growing period and seed harvest period, respectively, and the morphological traits of 64 accessions showed high diversity during the seed harvest period (average I = 1.55) (Table 2).

Table 5. The basic statistical data of quantitative traits.

Traits	Maximum	Minimum	Mean	Deviation	(CV %)	*I*
Fruit longitudinal diameter /cm	20.5	7	12.13	3.08	25.37	1.7
Fruit vertical diameter /cm	38	9	22.64	7.46	32.96	0.9
Fruit index	4.14	0.69	2.03	0.92	45.08	1.5
Flesh thickness/cm	3	1	2.14	0.41	19.21	1.8
Stalk width /cm	4.5	0.5	2.70	0.76	28.14	1.1
Fruit umbilicus diameter/cm	3	0.3	1.08	0.76	70.84	1.1
Ventricle number	4	2	2.98	0.45	15.21	1.4
Single fruit weight /kg	3.8	0.6	1.78	0.60	33.60	0.9
Seed length/cm	2.37	1.13	1.53	0.23	14.82	0.6
Seed width/cm	1.26	0.64	0.87	0.11	12.72	0.2
Seed thickness/cm	0.36	0.14	0.25	0.04	16.74	4.8
Seed kernel weight/g	2	0.39	0.97	0.35	36.43	0.2
Seed shell weight/g	1.16	0	0.31	0.20	64.77	2.9
Hundred seed weight/g	22.9	4.11	11.53	3.74	32.42	1.6
Seed number per fruit	909	33	259.64	145.19	55.92	2
Cotyledon length/cm	2.8	1.2	1.92	0.37	19.24	1.2
Cotyledon width/cm	0.7	1.7	1.15	0.23	19.80	1.5
Cotyledon index	1.18	2. 83	5.48	2.12	38.71	1.5
Mean	56.93	3.73	18.40	9.28	32.33	1.49

Notes: CV: coefficient of variance, *I*: Shannon's information index.

Table 6. The basic statistical data of qualitative traits.

Traits	Maximum	Minimum	Mean	Deviation	(CV %)	*I*
Growth habit	3	1	2.50	0.78	31.07	0.9
Growth vigor	3	1	2.64	0.57	21.73	1.1
Leaf shape	6	1	2.61	1.74	66.77	1.1
Foliage white spotting	2	1	1.28	0.52	40.47	1
Number of white spots per leaf	4	1	1.44	0.85	59.28	0.6
Flower shape	2	1	1.98	0.13	6.30	1
Flower size	3	1	1.48	0.53	35.99	0.7
Floral tube shape	3	1	2.19	0.91	41.43	0.8
Fruit shape	8	1	3.55	1.76	49.71	1.7
Flesh color	7	1	2.20	1.34	60.62	1.6
Skin color	9	1	4.81	2.25	46.67	2
Fruit speckle	4	1	1.98	1.23	61.88	1.7
Edge and ditch	2	1	1.14	0.35	30.72	0.4
Seed shell	2	1	1.92	0.27	14.07	0.7
Seed color	4	1	3.47	0.99	28.58	0.8
Characteristics of seed beak	3	1	2.94	0.73	24.92	1.2
Seed surface	2	1	2.48	0.64	25.85	1
Seed margin	2	1	2.47	0.64	25.99	1.1
Seed margin color	3	1	3.53	0.91	25.71	1
Mean	3.79	1	2.45	0.90	36.72	1.07

Notes: CV: coefficient of variance, *I*: Shannon's information index

Using NTSYS software, the similarity coefficient of all morphological traits among the 64 accessions ranged from 0.19 to 0.76. As shown in Fig. 1, clusters or sub-clusters were clearly defined according to morphological markers. All accessions were grouped into 2 clusters, with a similarity coefficient of 0.253. Five accessions (44, 45, 46, 47 and 48) were included in cluster I, and 59 accessions were included in cluster II. Cluster I was characterized by hull-less seeds, and cluster II was characterized by shell-covered seeds and was composed of 5 sub-clusters with a similarity coefficient of 0.33. Sub-cluster II-I included 1 accession (13) that was semi-dwarf, with yellow fruit skin, oblong fruit, palmate and pentagonal leaves with mostly white spotting, cylindrical flowers, and a bell-shaped floral tube. Sub-cluster II-II included 2 accessions (95 and 26) that were semi-dwarf, had dark green fruit skin, oval fruits with no wax powder, flat fruit skin with massive speckling, palmate leaves with no white spotting, conical flowers and cylindrical floral tubes. Sub-cluster II-III included 2 accessions (97 and 118) with similar fruit size, tall round fruit with no fruit speckle, heart-shaped leaves with less white spotting, large conical flowers, and open floral tubes. Sub-cluster II-IV included 12 accessions that had

no white leaf spotting, were semi-dwarf or dwarf and had conical flowers and heart-shaped leaves, except for 2 accessions with triangular leaves. Sub-cluster II-V included 42 accessions that were normal dwarfs. In sub-cluster II-V, accessions (36, 51 and 106) with mossy green fruit skin with speckles and less white foliage spotting clustered together. Accessions 10, 17 and 19, which both had tall round fruit without fruit speckle, a large flower size and palmate and pentagonal leaves, clustered together. Accessions 14, 64, 78, 82, 180 and 181 which had yellowish-white seeds with a flat seed beak clustered together. Accessions 7, 12, 24, 39, 43, 63, 66 and 115 clustered together and had similar fruit weights, tall round fruit with light yellow skin, no foliage white spotting, bell-shaped floral tubes. Accessions 5, 18, 30, 31, 40 and 65 with middle to small flowers were clustered together. Accessions 2, 4, 27, 29, 42 and 103 had white colored fruit flesh, yellowish-white seeds with a similar seed size, yellow fruit skin, and white flesh were also clustered together.

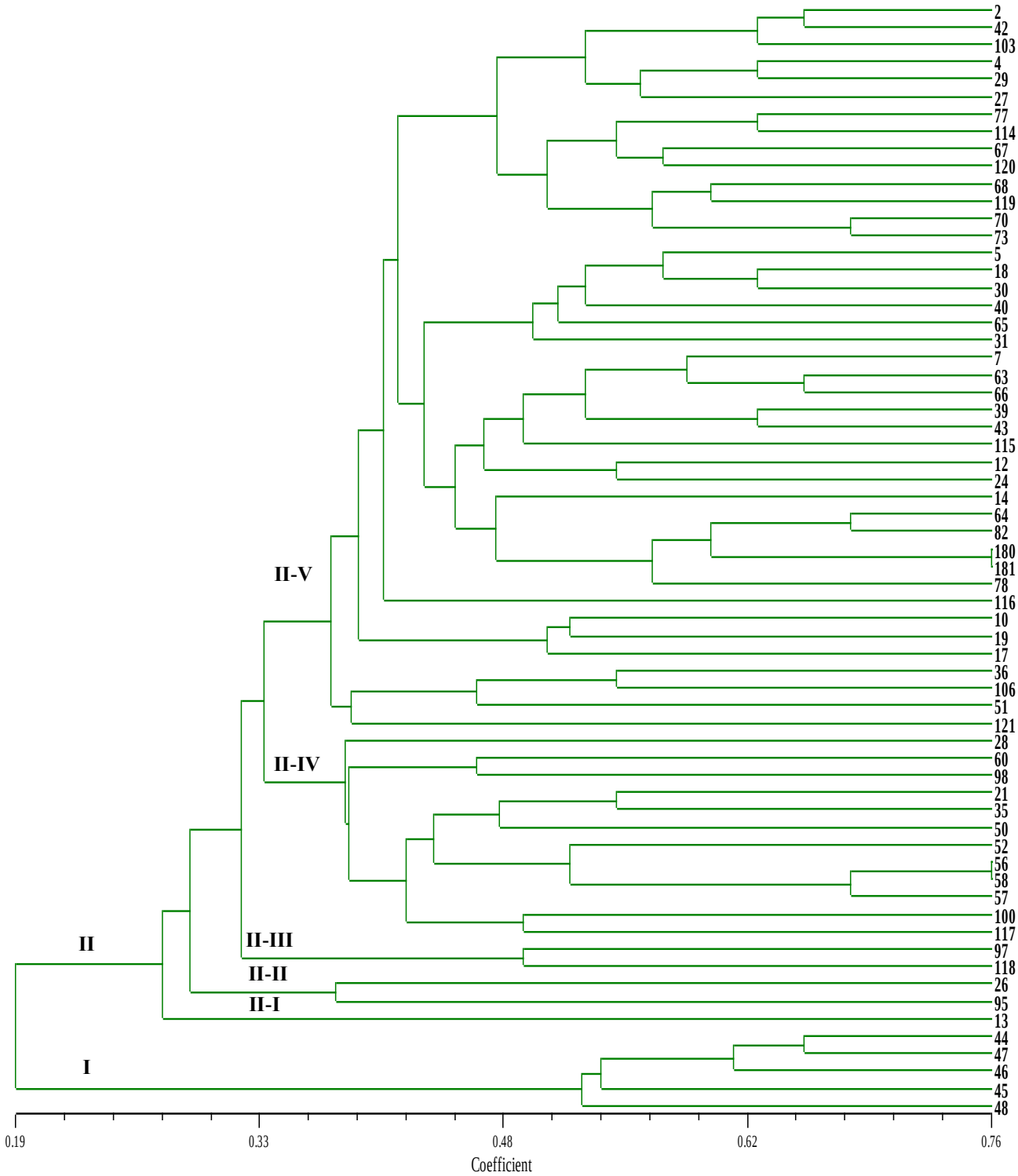

Fig. 1. Cluster analysis of morphological markers. The accessions correspond with the designations listed in Table 1.

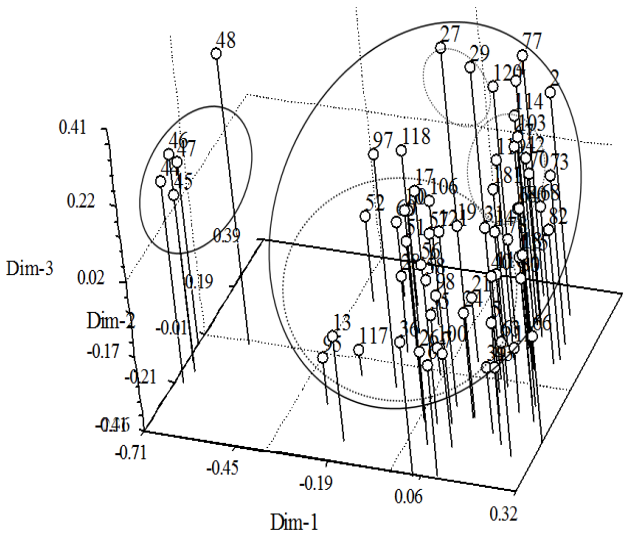

Fig. 2. Distribution of principal components in 3-dimension coordinate on morphological markers. The accessions correspond with the designations listed in Table 1.

The principal components of 64 accessions were analyzed according to the genetic similarity coefficient (Fig. 2). The variance contribution rates of the1st, 2nd and 3rd main coordinates were 21.16%, 13.49% and 7.93%, respectively, and the accumulated value of the variance contribution rate was 42.58%. The 1st, 2nd and 3rd main coordinates of the principal component analysis (PCA), 64 accessions separated clearly into two groups: a hull-less seeds group and a shell-covered seeds group. The results of the PCA are basically consistent with the results obtained from the cluster analysis.

Molecular characterization using SSR markers: Six accessions revealing different morphotypes were screened with 300 pairs of SSR primers, and 35 primer pairs produced clear bands with high polymorphism and good stability. The 35 polymorphic SSR markers were used to fingerprint 64 accessions, resulting in 145 differential amplification products, and each pair was amplified between 2 and 5 fragments, with an average of 4.12 bands. Based on Shannon's information index, the diversity index of the 64 accessions ranged from 0.2 to 3.8, with an average of 0.89. H39 showed the highest diversity index, whereas P18 had the lowest diversity index (Table 7).

The bands of each SSR marker were scored as presence (1) or absence (0). The similarity coefficients of the 64 accessions ranged from 0.73 to 1, and all accessions were clearly discriminated into 2 clusters, with a similarity coefficient of 0.732 using UPGMA analysis (Fig. 3). Cluster I included 5 accessions with hull-less seeds, and cluster II was composed of 2 sub-clusters that included 59 accessions with shell-covered seeds. Sub-cluster II-I included 6 accessions that were dwarf and shared the same parent. In sub-cluster II-II, accession 18, 19, 27, 103, 106, 114, 116, 118 and 119 originated from the same parent and showed short genetic distances; 10 accessions (64, 65, 66, 67, 68, 70, 73, 77, 78, 82) originating from the Yunnan region clustered together. Accessions 2, 5, 7, 21, 28, 39 and 43, which had dark green long round fruit and originated from the Neimenggu region were also clustered together. In principal coordinate analysis, the different accessions clustered together in terms of seed shell, growth habit, geographical origin and genetic relationship, successively. According to the results of the PCA, the variance contribution rates of the1st, 2nd and 3rd main coordinates were 10.61%, 8.77% and 7.41%, respectively, and the accumulated value of the variance contribution rates was 26.79% (Fig. 4). Constructed based on the 1st, 2nd and 3rd main coordinates of the PCA, the clustering of the 64 accessions was very messy, and the accessions could not be classified clearly.

Comparison between morphological characterization and molecular characterization: Using NTSYS software, Mantel tests between the similarity matrix of morphological characterization and the similarity matrix of molecular characterization were analyzed, and the correlation value was 0.4647. The results showed that there is moderate consistency between the genetic relationships among *Cucurbita* accessions revealed by SSR and the relationships assessed based on morphological markers.

Discussion

The morphological characterization and evaluation of *C. moschata* accessions was performed to estimate genetic diversity, and the results demonstrated that the simultaneous analysis of qualitative and quantitative data is feasible and increased our understanding of the variation among accessions (Rebeca *et al*., 2016; Nagar *et al*., 2017). In our study, a wide range of fruit and seed characteristics across 45 morphological traits revealed high diversity and variation, particularly in terms of fruit speckle, flesh color, seed number per fruit and seed shell weight. Some characteristics showed minor differences, such as main vine section shape, flower color, sepal shape, stalk basal enlargement shape, characteristics of the fruit skin, gourd tumor quantity, gourd skin wax powders, and the hardness of fruit stems. The data revealed that accessions from the same region show diversity in most morphological traits. We also found that accessions from the same region had similar growth habits. The accessions from Neimenggu had semi-dwarf or dwarf vines, the accessions from Gansu and Yunnan showed dwarf vines, and most accessions from Heilongjiang had normal vines. This pattern might be related to many years of artificial selection in *C. moschata*.

Within *Cucurbita* species, there exists high heterosis for early fruit maturity, increased fruit size, increased fruit number per plant and increased seed number (Ferriol & Picó, 2008); thus, selecting the most distant parental germplasm is extremely important for hybrid breeding in *C. pepo*. The collection of 64 accessions from different regions was divided into two distinct clusters according to morphological markers. Cluster II was discriminated into 5 sub-clusters that differed in terms of fruit shape, fruit color and seed surface. According to the morphological marker clustering results, a potential source of variability could be selected for in breeding programs that aim to improve fruit traits.

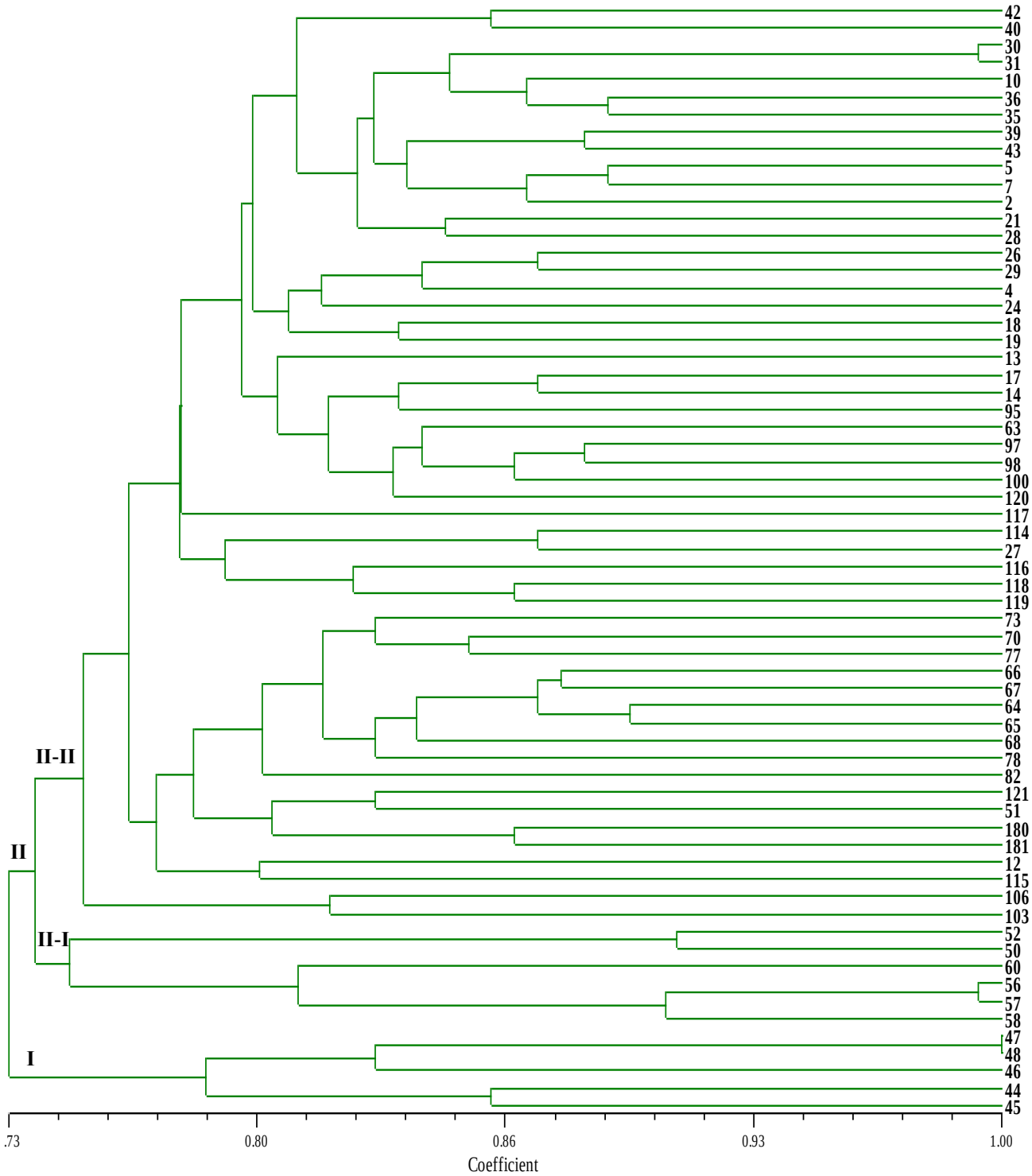

Fig. 3. Dendrogram of resulting from a UPGMA cluster analysis based on SSR markers. The accessions correspond with the designations listed in Table 1.

We further focused on the relationships between the variation coefficient and the diversity index and found no correlation between these factors. The coefficients of variation of fruit umbilicus diameter and seed shell weight were the highest among the morphological traits, but the diversity indexes for these traits were below average. The seed thickness diversity index was the highest, but the variation coefficient for seed thickness was low. Differences between the variation coefficient and the diversity index are caused by many factors. The coefficient of variation reflects the range of variation and explains the genetic variation by means of deviations from the average, whereas the diversity index describes the frequency distribution and rank difference for a trait. Furthermore, to be amenable to the cluster analysis format, the morphological quantitative data were converted to a range from 1 to 10, which might affect the frequency distribution results. Therefore, these two aspects were both considered to obtain a better understanding of the genetic diversity.

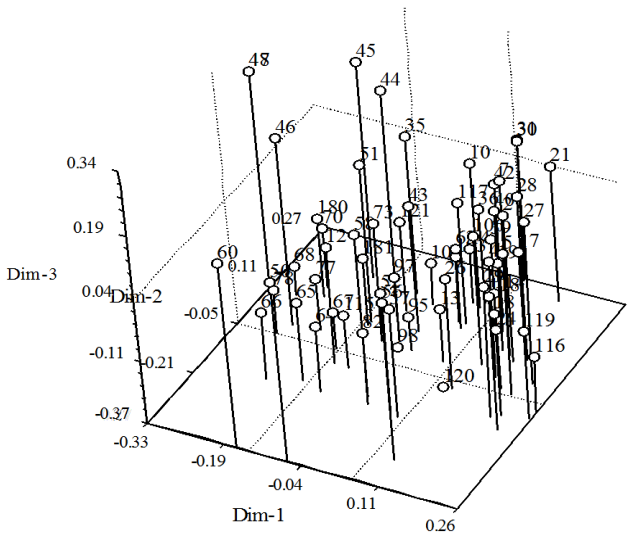

Fig. 4. Distribution of principal components in 3-dimension coordinate on morphological marker. The accessions correspond with the designations listed in Table 1.

Table 7. Polymorphism information content of SSR primer.

Primer name	H′	Primer name	H′
comp107035	0.8	comp98739	0.8
comp11018	0.9	PU002959	1.7
comp13350	0.7	PU024278	0.2
comp27618	0.2	PU025208	0.2
comp28857	1.1	PU026252	1.5
comp40082	1.1	H39	3.8
comp41315	1.1	H41	0.9
comp52235	0.8	H65	0.2
comp52664	0.7	CMTp37	0.9
comp64835	0.4	CMTp57	1.4
comp66738	0.3	CMTm115	0.2
comp71199	1.1	CMTm11	1.3
comp71344	1.1	CMTm13	0.8
comp71363	1.1	CMTm14	0.4
comp71542	0.9	CMTm89	1.4
comp72289	0.6	CMTmC1	0.4
comp72586	1	CMTmC60	1
comp73642	0.2	Mean	0.89

Notes: H′: diversity index

The genetic diversity of *Cucurbita* accessions from different geographical origins was identified using molecular markers such as RAPD (Ferriol *et al*., 2003; Zhao *et al*., 2017), SSR (Kong *et al*., 2014; Murovec, 2015; Sim *et al*., 2015; Kazminska *et al*., 2017), and HFO-TAG (Harry *et al*., 2015; Paris *et al*., 2015), and the results were valuable for further germplasm characterization in different species and for taxonomical identification within the genus *Cucurbita*. In our study, 64 genetic resources were clearly distinguished, and the status of biological classification and genetic relationships among them were identified using 35 highly polymorphic SSR primers. With morphological and molecular markers, 64 accessions were divided into 2 clusters. Cluster I included 5 accessions with hull-less seeds, and cluster II included 59 accessions with shell-covered seeds. The hull-less pumpkins had wider genetic variation than the shell-covered species. The 64 accessions in this study came from various geographical origins in China: 32 from Neimenggu, 15 from Heilongjiang, 10 from Yunnan, 5 from Gansu and 2 from Shanxi. The 10 accessions from Yunnan and the accessions from Neimenggu with dark green fruit were clearly delineated by the SSR analysis in our study. These results were consistent with previous findings by Liu *et al*., (2013) and Wu *et al*., (2011), who separated the accessions of *Cucurbita* using molecular markers into sub-clusters that reflected geographical origin.

Based on previous research, 47 accessions of rootstock-used pumpkin were collected, and the genetic diversity and relationships were analyzed with 63 phenotypes and 40 polymorphic SSR markers. Finally, 47 germplasm was divided into 3 groups using SSR primers, which was similar to the results of the morphological study (Li *et al*., 2014). However, there were also some differences among the relationships of *Cucurbita* accessions revealed by molecular marker and also by morphological marker analysis. The genetic diversity and relationships among *C. moschata* were analyzed by combining morphological characteristics and the molecular markers RAPD (Cai, 2006), SSR (Zheng *et al*., 2016), SRAP and AFLP (Ferriol *et al*., 2004). Clustering based on molecular markers had a low correlation with clustering based on morphological characteristics. In our study, Cluster II was divided into 5 sub-clusters with different fruit and leaf shapes based on morphological data, and accessions with similar phenotypic features were grouped together. Cluster II was discriminated into dwarf and normal sub-clusters with SSR markers, and accessions of same origin and similar geographical distribution were clustered together. The results obtained using morphological markers were mildly to moderately correlated with the molecular marker results (r=0.4647). There were some differences between the relationships of the 64 *C. pepo* accessions revealed by SSR markers and the relationships revealed by morphological markers. The reasons for this phenomenon are complex. The morphological characteristics correspond to the plant phenotypes which are affected by both innate factors and environmental factors, whereas SSR molecular markers indicate differences in DNA sequence (Rabbani *et al*., 2010). Moreover, during data collection, morphological marker data can be disturbed by anthropogenic activities or other environmental factors. The SSR marker polymorphisms had low coverage in the complete genomes. Therefore, morphological markers and SSR markers are not interchangeable; rather, they are complementary and together ensure the comprehensiveness and accuracy of analytical results.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Declaration

The experiments comply with the current laws of the country in which we were performed.

Author contribution statement: Y Wang performed experiment of PCR identification, preparing the manuscript. Y Wang contributed to using data analysis. W Xu contributed to growing plants. C Wang contributed to collecting phenotypic characteristics. C Cui contributed to providing experimental material. S Qu, the corresponding author, oversaw all activities related to the project implementation and manuscript development. All the authors reviewed and approved this manuscript.

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