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Morphological and Molecular Analyses of Reciprocal Hybrids between 'Slim Whitman' and 'Pinza', Two *Narcissus pseudonarcissus* Cultivars

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Abstract. Narcissus pseudonarcissus*, also known as daffodil, is a world-famous ornamental flower. In this study, for the first time, cross-pollinations between two widely cultivated *N. pseudonarcissus* varieties 'Slim Whitman' and 'Pinza' were performed. After eight consecutive years of cultivation, 27 reciprocal hybrids with different genotypes survived; 15 hybrids in 'Slim Whitman' × 'Pinza' and 12 in 'Pinza' × 'Slim Whitman'. Twenty ornamental and agronomic characters were observed to evaluate the pattern and extent of genetic variability of the hybrids and relatedness with their parents. The hybrids showed great variation in most morphological characters compared with the parents, especially in leaf and flower characters. Hybrids SP03, SP04, SP05, SP12, SP13, PS04, PS06, PS07, PS08, and PS11 had evident growth advantage in some aspects compared with both parents. Of these hybrids, SP04 got novel flowers with white petals and an yellow-orange corona, and had the potential to become a new popular *N. pseudonarcissus* cultivar. Hybrids SP01, SP03, SP05, PS04, PS06, and PS07 also possessed a great ornamental value. Using cluster analysis based on morphological traits and random amplified polymorphic DNA (RAPD) molecular markers, genetic relationships among the reciprocal cross hybrids and their parents were further analyzed. The 27 reciprocal hybrids and their parents grouped into divergent clusters, showing that there was rich genetic variation among the hybrids tested. This study will pave the way for hybridization breeding programs of *N. pseudonarcissus*.

Narcissus pseudonarcissus, a flowering bulbous plant of the Amaryllidaceae family, is a typical Mediterranean genus of geophyte, with unique flower shape (with cup-shaped or crown-like corona) and outstanding flower color (Fernandes, 1968). In recent years, growing attention has been focused on *N. pseudonarcissus*, owing mainly to its horticultural success and popularity and the abundant alkaloids in bulbs with important biological activities (Bastida et al., 2006).

There are thousands of cultivars of *Narcissus* registered during the past century. Among them, many are natural hybrids or natural interspecific hybrids. As the members of the genus are so popular in cultivation and have been hybridized over such a long period, a complex range of hybrid forms occur with unknown state of the parents' genetic characteristics. It is now difficult or impossible to elucidate the exact parents for most existing hybrids (Fernández, 1984; Marques et al., 2010; Ribeiro et al., 2007). Therefore, artificial hybridization with distinct breeding objective on *N. pseudonarcissus* is still relatively backward development for the following reasons:

1) The chromosome number is usually $2n = 14$ or 28 (Philp, 1934) for the species *N. pseudonarcissus*, but there is considerable variation throughout the genus (Darlington and Janaki, 1945), and aneuploidy and polyploidy are quite common. In addition to some diploids, most types face the certain problem of fertility decrease in various degrees or even sterility (Brandham, 1992). 2) *N. pseudonarcissus*, as a perennial bulbous geophyte, mainly reproduces by developing vegetative daughter bulblets. The seedling plant is usually very small during the first 4 years and only has vegetative growth and first flowers in the fifth or sixth season of growth if survived from insect visitation every year (Caldwell and Wallace, 1955). This makes breeding quite inefficient, expensive, and time-consuming. 3) Breeding is also being impeded because of the asynchronous nature of flowering in *N. pseudonarcissus*. To conduct successful crosses, it is necessary to select genotypes on their individual values and to plant them in a way that will enable flowering at almost the same time. 4) The cultivars chosen as parents are always not homozygous in most genetic loci. Genetic segregation will be comprehensive in the unbeknown genetic background and molecular breeding still has a far way to go. Moreover, low fertility and disease also affect the obtaining of artificial hybrids. All those mentioned previously hindered the efficiency of crosses to obtain new *N. pseudonarcissus* cultivars with desirable traits. Although hybridization, polyploidization, and adaptive radiation occur within the genus along with evolutionary processes, there were no artificial genetic populations with clear genetic background, and no F1 hybrids generation has been reported as far as we know.

Over the past decades, molecular markers, especially RAPD markers, have been extensively used with considerable success in several horticultural plants, including lemon (Deng et al., 1995), fig (Galderisi et al., 1999), strawberry (Hokanson et al., 2000), pecan (Conner and Wood, 2000), and apricot (Badenes et al., 2000) for germplasm characterization, population structure analysis, and assessment of genetic diversity. Despite the economic and ecological importance of *N. pseudonarcissus*, little genomic information is available for this genus, and molecular markers have only recently been used in genetic studies. In addition, both basic genetic researches related to population studies and prebreeding programs of daffodil remain scarce for most *Narcissus* species. RAPD was used to analyze the genetic structure of populations of *N. pseudonarcissus* L. at the regional level (Guy et al., 2010) and the genetic variations of *Narcissus tazetta* var. *chinensis* (Chen et al., 2002). There were no reports of molecular markers used in some specific genetic populations in *Narcissus*.

Narcissus pseudonarcissus cultivar Slim Whitman was bred by the Netherlands breeder D.P. de Graaf and first flowered at 1978. 'Pinza' was registered in 1962, and it was popular and won a lot of awards since then (http://daffseek.org/ and http://apps.rhs.org.uk). They are famous and widely cultivated varieties all the time for their attractive flowers: 'Slim Whitman' with white perianth and light yellow corona, 'Pinza' with yellow perianth and yellow-orange corona. They are both large-cupped daffodils and possess the same basic chromosome numbers ($2n = 28$) (Brandham, 1992). Most importantly, they are fertile, which means they can be used as parents in cross breeding. In this study, we did reciprocal crosses between 'Slim Whitman' and 'Pinza', aiming to select individuals with novel flower color or flower shape in the F1 generation. The morphological and molecular differences of the reciprocal cross hybrids were further analyzed to elucidate the inheritance patterns on different traits and to facilitate the breeding development of new market-oriented daffodil flowers.

Materials and Methods

Hybridizations. Reciprocal crosses between cultivated *N. pseudonarcissus* 'Slim

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Whitman' and 'Pinza' were attempted to produce hybrids. The reciprocal cross breeding was performed in Mar. 2008 with temperature and relative humidity ranging from 20 to 25 °C and 50% to 80%, respectively, always from 8:30 to 10:30 AM, a period in which the flowers remain open and the stigmas are receptive. The flower buds of genitors in pre-anthesis stage were protected with white paper bags 1 d before the hybridization and the stamens of the female parent were emasculated. The paper bags were placed over the buttons the floral peduncle, avoiding contamination with undesired pollen. On the following morning, the anthers were collected and scrubbed over the stigmas of the female parent. After the artificial pollination, the flowers were labeled and protected with paper bags again for 10 d. The seeds resulting from such hybridizations were protected with nylon nets until they were ripe.

Field cultivation and morphological description of the hybrids. The parents and reciprocal cross hybrids (adult bulbs with maximum perimeter of more than 13 cm) were grown in the horticultural farm of Shanghai Jiao Tong University, Shanghai, China. These daffodil bulbs were planted and grown under identical conditions, including fertilization, irrigation, and disease prevention methods.

In the years 2015 and 2016, the following morphological characteristics were observed for the description about the hybrids and parents: 1) phenophase: days from sowing date to blooming; days from sowing date to withered period; 2) vegetative growth: leaf length, leaf width, and leaf thickness; stem diameter and plant height; 3) flower shape: flower diameter; corona diameter; and length and width of the petal; 4) flower color: the color of the petal, the base of the corona, and the rim of the corona were measured by using CIE 1976 $L^*a^*b^*$ (CIELAB), respectively, which contained L^*, a^*, and b^* parameters to describe all aspects of the color (Schmitzer et al., 2012).

The morphological characters (leaf length, width, and thickness; stem diameter; flower shape and color; and growth period traits) of the hybrids (five plants), 'Slim Whitman' and 'Pinza' (20 plants) were examined. Leaf length was measured from the base of the largest leave midrib up to the tip, whereas leaf width and thickness was measured at maximum breadth and thickness using the same leaves. Stem diameter was measured from the base of the plant using a vernier caliper. Flower diameter, corona diameter, petal length, and width were measured at the maximum part. The Royal Horticultural Society Color Chart (RHSCC) was used for the description of color parameters of fresh petal and corona. The differences of sample chromaticity were measured using a portable colorimeter which was based on the CIELAB system and contained L^*, a^*, and b^* parameters to describe all aspects of the color (Schmitzer et al., 2012).

Molecular analysis. Genomic DNA was extracted from young leaves of 'Slim Whitman', 'Pinza', and the hybrids using the cetyl trimethylammonium bromide (CTAB) method (Doyle, 1990) with some modifications. To be brief, 1.0 g well-grinded leaves was mixed with 500 µL of prewarmed 2× CTAB buffer and incubated at 58 °C for 30 min. Then 750 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the mixture, vortexed, and centrifuged at 12,000 relative centrifugal force (RCF) for 15 min. Precooled isopropanol was added to the supernatant and incubated for 20 min without shaking. Then, the mixture was centrifuged at 8000 RCF for 2 min and the DNA pellet was washed using 70% ethanol and resuspended in 100 µL sterile distilled water and stored at –20 °C before use. The samples of each genomic DNA were separated using electrophoresis in 1.0% agarose gel to estimate the concentration and integrity of the extracted DNA.

The RAPD primers (Table 1) were custom synthesized and used for the molecular analysis of the parents and their F1 hybrids. The polymerase chain reaction amplification was performed in a reaction volume of 20 µL

Table 1. Sequence of the 10 random amplified polymorphic DNA primers and the number of specific bands.

Primer	Sequence 5′-3′	Number of polymorphic bands
S6	5′-TGCTCTGCCC-3′	8
S24	5′-AATCGGGCTG-3′	7
S64	5′-CCGCATCTAC-3′	2
S71	5′-AAAGCTGCGG-3′	2
S89	5′-CTGACGTCAC-3′	4
S103	5′-AGACGTCCAC-3′	5
S127	5′-CCGATATCCC-3′	4
S295	5′-AGTCGCCCTT-3′	5
S303	5′-TGGCGCAGTG-3′	2
S417	5′-TCAGTCCGGG-3′	4

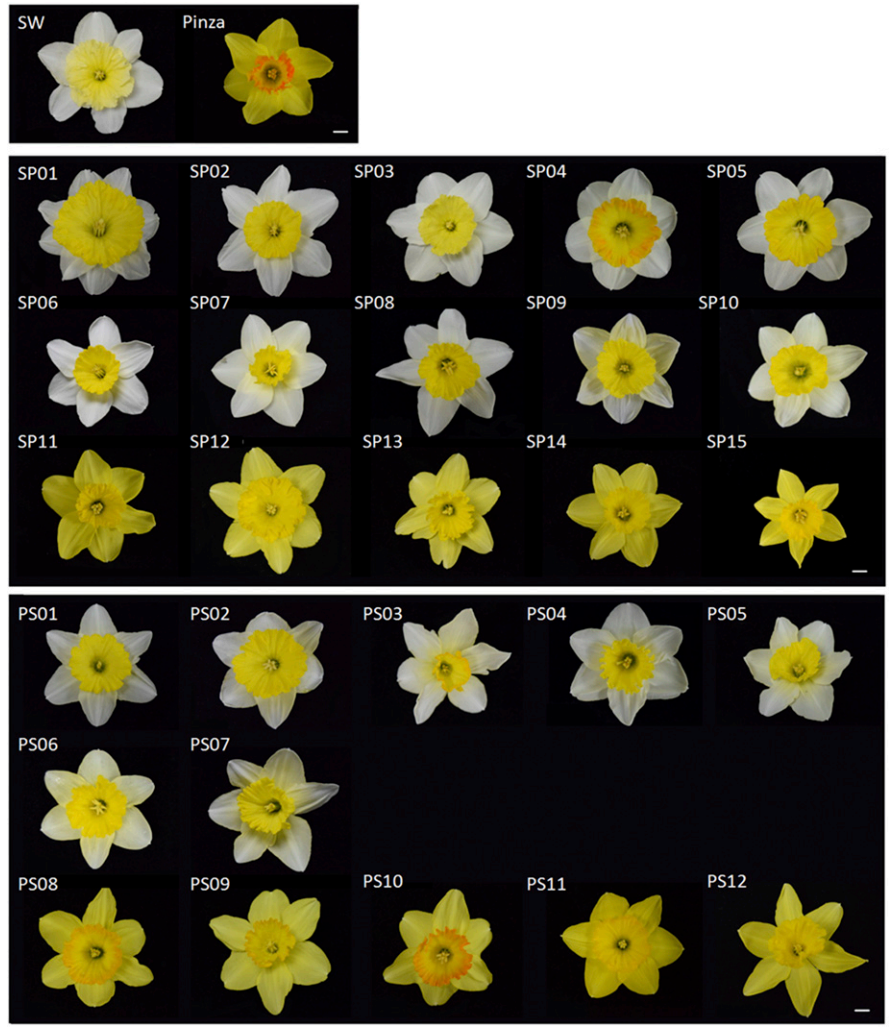

Fig. 1. Parents and hybrids obtained out of the crossing.

containing 0.2 μM of each primer, 10 μL Premix Taq (*TaKaRa Taq* 1.25 U/25 μL, dNTP mixture 0.4 mM, *Taq* buffer, 3 mM Mg^{2+}; Takara Biomedical Technology, Beijing, China), and 30 ng of genomic DNA. The amplifications followed the program: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 1 min, at 35 °C for 30 s, and at 72 °C for 2 min; and a final extension at 72 °C for 10 min. After the amplification, the samples were run on 1.2% agarose gel containing 0.5 $\mu g \cdot mL^{-1}$ of ethidium bromide. The electrophoretic separation took ≈1 h at 80 V.

Data analysis. The 20 morphometric characters were measured and further used to evaluate for the genetic diversity among the reciprocal hybrids and their parents. Data on morphological characters were standardized using the YBAR option of the Stand program from the NTSYS-pc 2.1 software (Rohlf, 2002).

Amplified bands generated from RAPD were scored based on the presence (1) or absence (0) of bands for each primer and cluster analysis was performed using NTSYS-pc version 2.1 (Rohlf, 2002). The cluster analysis was done using unweighted pair group method with arithmetic mean (UPGMA) analysis and dendrograms were constructed using the SAHN program.

Results and Discussion

Hybridizations. Seeds were obtained from both 'Slim Whitman' and 'Pinza' in the reciprocal cross in 2008. Afterward, the seedling hybrids plants, which were very small during the first to the fourth seasons, only had foliage leaves emerging from the bulbs. In the fifth and sixth year of growth, 2014 and 2015, the hybrids normally flowered. After years of cultivation, till maturity, 27 reciprocal hybrids with different genotypes survived: 15 hybrids in 'Slim Whitman' × 'Pinza' and 12 in 'Pinza' × 'Slim Whitman'. The 15 hybrids used 'Slim Whitman' as the female parent and 'Pinza' as pollen donor received specific numbers from SP01 to SP15. The 12 hybrids obtained from the pollination of 'Pinza' with pollens from 'Slim Whitman' were named as PS01 to PS12 (Fig. 1).

The breeding work of *N. pseudonarcissus* is quite different from that of the other crops. They are bulbous perennial plants that reproduce by bulblets rather than seeds. It is difficult to get seeds by crossing. Although some *Narcissus* varieties are hybrids on the market, they are usually not selected and bred by a large F1 population. This is the largest F1 population ever reported, and each hybrid seedling represents a unique genotype. The respective offsprings were consecutively planted for bulb propagation for 8 years, and daughter bulblets of the hybrids were developed very rapidly and afford a very efficient method of vegetative reproduction since 2009.

Morphological characteristics of F1 hybrids. In the present investigation, 20 important ornamental morphological and agronomic characters had been measured to evaluate the genetic variability of the hybrids and relatedness with their parents (Tables 2 and 3). The reciprocal populations exhibited wide phenotypic variation. For phenophase, hybrids SP01, SP03, SP06, and PS01 bloomed earlier than 'Slim Whitman', hybrids SP05, SP08, PS10, and PS12 took more days to bloom than 'Pinza', whereas the others were between the parents. Hybrids SP04, SP10, SP11, PS08, and PS10 presented a longer whole growth period. SP01, SP02, SP03, etc., withered earlier than 'Slim Whitman'.

For vegetative growth, the width, length, and thickness of leaf ranged from 1.20 to 2.07 cm, 16.80 to 34.00 cm, and 0.81 to 1.97 mm among the hybrids, respectively. The F1 hybrids also showed great variability in the stem diameter, ranging from 5.20 to 11.40 mm. There was also obvious difference in the range of plant height, which varies from 24.30 to 44.30 cm. Most hybrids were superior to their parents on vegetative growth with larger leaves, taller plant height, and stronger stems, and there was not a single morphological trait that showed strict intermediacy. The leaf width of SP03, SP05, SP13, and PS07 were no less than 2.0 cm, obviously wider than any of the parents (1.50 and 1.27 cm). The leaf length of SP02, SP03, SP06, SP13, PS02, PS03, PS04, PS06, and PS07 were more than 25 cm, whereas the mean leaf length of 'Slim Whitman' and 'Pinza' were 20.34 and 20.18 cm, respectively. Plant height of the parents were no more than 34 cm, whereas in the hybrids, some even reached 40 cm. Thicker stems also occurred in the reciprocal hybrids, such as SP04, SP05, SP13, PS04, and PS07. In general, hybrids SP03, SP04, SP05, SP12, SP13, PS04, PS06, PS07, PS08, and PS11 had evident growth

Table 2. The characteristics of phenophase and vegetative growth in the parents and the reciprocal hybrids.

Species	Days from sowing to blooming	Days from sowing to wilting	Leaf width (cm)	Leaf length (cm)	Leaf thickness (mm)	Stem diam (mm)	Plant ht (cm)
Slim Whitman	116 ± 1.00 e	150 ± 1.00 c	1.50 ± 0.03 g	20.34 ± 0.32 k	1.79 ± 0.06 b	9.72 ± 0.65 cde	33.97 ± 1.26 hi
Pinza	126 ± 1.15 b	155 ± 1.73 b	1.27 ± 0.02 k	20.18 ± 0.24 k	1.39 ± 0.03 fgh	8.82 ± 0.35 fgh	33.60 ± 2.03 i
SP01	113 ± 1.00 f	145 ± 1.73 d	1.80 ± 0.03 d	19.43 ± 0.53 k	0.81 ± 0.02 l	8.83 ± 0.09 fgh	28.67 ± 0.95 l
SP02	116 ± 1.53 e	145 ± 1.00 d	1.60 ± 0.03 f	30.50 ± 0.56 b	1.60 ± 0.04 cd	9.07 ± 0.16 efg	38.03 ± 0.95 d
SP03	113 ± 1.00 f	145 ± 1.73 d	2.00 ± 0.04 b	27.30 ± 0.78 cd	1.81 ± 0.05 b	8.40 ± 0.58 ghi	38.10 ± 0.91 d
SP04	126 ± 2.00 b	160 ± 1.73 a	1.50 ± 0.03 g	23.20 ± 0.64 i	1.80 ± 0.04 b	10.70 ± 0.62 ab	40.10 ± 0.55 c
SP05	130 ± 2.00 a	155 ± 1.15 b	2.00 ± 0.05 b	24.20 ± 0.66 ghi	1.74 ± 0.04 b	11.40 ± 0.74 a	41.90 ± 0.26 b
SP06	113 ± 1.00 f	145 ± 1.00 d	1.50 ± 0.03 g	27.90 ± 0.93 c	1.19 ± 0.02 j	6.10 ± 0.66 l	35.50 ± 0.62 fg
SP07	119 ± 1.00 d	145 ± 1.73 d	1.70 ± 0.04 e	19.70 ± 1.00 k	1.48 ± 0.02 e	8.70 ± 0.29 ghi	37.10 ± 0.98 de
SP08	130 ± 0.57 a	150 ± 1.73 c	1.50 ± 0.02 g	20.00 ± 0.35 k	1.42 ± 0.06 efgh	6.10 ± 0.22 l	33.40 ± 0.34 i
SP09	122 ± 1.00 c	155 ± 1.00 b	1.50 ± 0.03 g	24.83 ± 0.62 fgh	1.46 ± 0.03 ef	8.90 ± 0.36 fgh	40.53 ± 0.59 bc
SP10	126 ± 0.57 b	160 ± 2.64 a	1.70 ± 0.04 e	19.90 ± 0.23 k	1.67 ± 0.03 c	7.20 ± 0.14 k	31.40 ± 1.00 j
SP11	126 ± 0.57 b	160 ± 3.61 a	1.60 ± 0.03 f	20.47 ± 0.47 k	1.01 ± 0.03 k	8.35 ± 0.21 ghi	33.30 ± 1.02 i
SP12	116 ± 1.15 e	145 ± 1.00 d	1.80 ± 0.05 d	24.60 ± 1.02 fgh	1.57 ± 0.05 d	9.60 ± 0.34 efg	37.77 ± 0.63 d
SP13	116 ± 1.15 e	145 ± 0.57 d	2.07 ± 0.05 a	25.07 ± 0.35 fg	1.38 ± 0.02 gh	10.47 ± 0.86 bc	39.77 ± 0.66 c
SP14	116 ± 1.15 e	145 ± 0.57 d	1.60 ± 0.02 f	19.40 ± 0.26 k	1.23 ± 0.03 ij	7.30 ± 0.29 jk	23.40 ± 0.39 m
SP15	119 ± 0.57 d	145 ± 3.61 d	1.40 ± 0.03 hi	20.50 ± 0.22 k	1.48 ± 0.04 e	5.20 ± 0.22 m	24.30 ± 0.26 m
PS01	113 ± 0.57 f	145 ± 1.00 d	1.35 ± 0.02 j	23.80 ± 0.31 hi	1.27 ± 0.03 i	7.97 ± 0.37 ijk	35.83 ± 0.62 ef
PS02	119 ± 1.00 d	150 ± 2.00 c	1.45 ± 0.02 gh	26.30 ± 1.06 de	1.35 ± 0.03 h	9.00 ± 0.71 efg	42.00 ± 0.89 b
PS03	122 ± 1.15 c	155 ± 1.00 b	1.20 ± 0.02 l	34.00 ± 1.25 a	1.65 ± 0.05 c	7.90 ± 0.38 ijk	44.30 ± 1.06 a
PS04	126 ± 2.00 b	155 ± 0.57 b	1.90 ± 0.05 c	26.30 ± 0.68 de	1.74 ± 0.04 b	10.00 ± 0.26 bcd	41.90 ± 1.06 b
PS05	116 ± 2.00 e	145 ± 0.57 d	1.60 ± 0.04 f	24.60 ± 0.38 fgh	1.48 ± 0.03 e	8.10 ± 0.28 hij	37.50 ± 0.63 d
PS06	119 ± 1.15 d	150 ± 3.61 c	1.82 ± 0.04 d	25.30 ± 0.61 efg	1.47 ± 0.04 e	9.80 ± 0.64 bcd	40.55 ± 1.32 bc
PS07	116 ± 1.00 e	145 ± 0.57 d	2.00 ± 0.04 b	25.60 ± 0.34 ef	1.58 ± 0.04 d	10.60 ± 0.28 b	34.20 ± 0.85 ghi
PS08	126 ± 2.00 b	160 ± 2.00 a	1.58 ± 0.02 f	21.63 ± 0.33 j	1.17 ± 0.02 j	9.00 ± 0.35 efg	35.15 ± 0.32 fgh
PS09	116 ± 1.00 e	145 ± 0.57 d	1.40 ± 0.03 ij	23.90 ± 0.65 hi	1.62 ± 0.05 cd	7.30 ± 0.25 jk	28.70 ± 0.45 l
PS10	130 ± 2.00 a	160 ± 1.73 a	1.70 ± 0.04 e	17.90 ± 0.78 l	1.43 ± 0.03 efg	8.60 ± 0.11 ghi	29.80 ± 0.47 kl
PS11	126 ± 2.00 b	150 ± 2.64 c	1.60 ± 0.03 f	23.70 ± 0.39 hi	1.49 ± 0.03 e	9.10 ± 0.36 efg	28.90 ± 0.68 l
PS12	130 ± 1.53 a	155 ± 1.00 b	1.70 ± 0.04 e	16.80 ± 0.59 m	1.97 ± 0.08 a	7.50 ± 0.42 jk	31.10 ± 0.47 jk

Table 3. The flower shape and flower color parameters of parents and the reciprocal hybrids.

Species	Flower diam (cm)	Corona diam (cm)	Petal length (cm)	Petal width (cm)	Flower color on the petal			Flower color on the base of corona			Flower color on the rim of corona		
					*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
Slim Whitman	9.37 ± 0.39 bcd	5.17 ± 0.05 b	4.03 ± 0.04 cd	3.93 ± 0.04 a	48.62	–1.08	8.41	62.14	1.65	52.52	62.14	1.65	52.52
Pinza	8.10 ± 0.25 ghi	2.85 ± 0.03 o	3.35 ± 0.03 j	2.90 ± 0.05 fg	67.01	3.77	66.02	57.45	28.94	67.77	49.60	43.31	58.08
SP01	8.93 ± 0.33 cdef	5.40 ± 0.06 a	3.75 ± 0.03 fgh	3.83 ± 0.03 a	68.60	–2.01	14.17	68.97	8.24	73.75	68.97	8.24	73.75
SP02	9.48 ± 0.14 abc	4.25 ± 0.06 f	3.93 ± 0.05 cdef	3.63 ± 0.06 b	60.45	–0.98	6.93	62.34	6.58	54.10	62.34	6.58	54.10
SP03	9.50 ± 0.42 abc	4.30 ± 0.04 f	4.40 ± 0.05 a	3.90 ± 0.04 a	51.30	–0.96	8.10	56.76	18.21	56.68	56.76	18.21	56.68
SP04	8.00 ± 0.24 hi	3.90 ± 0.03 i	3.10 ± 0.04 k	3.40 ± 0.03 cde	69.53	–1.36	8.77	56.16	19.85	55.54	59.73	26.50	71.17
SP05	9.30 ± 0.31 bcde	4.70 ± 0.02 d	4.10 ± 0.05 bc	3.40 ± 0.04 cde	70.81	–1.63	10.62	62.54	21.50	69.51	62.54	21.50	69.51
SP06	9.10 ± 0.16 bcdef	3.60 ± 0.02 k	3.90 ± 0.03 def	3.50 ± 0.03 bcd	74.64	–1.76	10.33	64.53	11.95	68.26	64.53	11.95	68.26
SP07	7.80 ± 0.08 i	4.00 ± 0.05 h	3.10 ± 0.04 k	3.00 ± 0.03 f	63.45	–1.30	19.95	57.10	18.22	57.21	57.10	18.22	57.21
SP08	9.00 ± 0.47 bcdef	4.00 ± 0.05 h	4.40 ± 0.05 a	3.00 ± 0.06 de	73.14	–1.43	10.68	63.45	13.14	66.85	63.45	13.14	66.85
SP09	8.55 ± 0.38 fgh	3.75 ± 0.03 j	3.65 ± 0.06 ghi	3.40 ± 0.03 cde	72.76	–2.59	19.53	60.96	16.94	63.87	60.96	16.94	63.87
SP10	8.60 ± 0.36 fgh	3.40 ± 0.05 l	3.50 ± 0.03 ij	2.60 ± 0.05 i	70.80	–2.97	19.43	62.71	19.95	68.21	62.71	19.95	68.21
SP11	9.30 ± 0.45 bcde	3.60 ± 0.06 k	3.90 ± 0.04 def	3.47 ± 0.04 bcd	71.42	–1.72	66.19	59.99	26.14	67.24	59.99	26.14	67.24
SP12	9.40 ± 0.43 abcd	4.90 ± 0.05 c	3.80 ± 0.04 fg	3.30 ± 0.05 de	44.60	9.01	34.95	56.88	30.36	61.56	56.88	30.36	61.56
SP13	9.33 ± 0.37 bcde	4.63 ± 0.05 de	4.23 ± 0.04 ab	3.20 ± 0.05 e	45.63	7.45	35.29	57.47	24.83	60.42	57.47	24.83	60.42
SP14	7.80 ± 0.29 i	3.10 ± 0.04 m	4.00 ± 0.05 cde	3.20 ± 0.06 e	66.65	–0.06	60.27	58.91	27.60	64.93	58.91	27.60	64.93
SP15	6.80 ± 0.09 j	3.00 ± 0.03 n	3.00 ± 0.04 k	1.90 ± 0.03 j	67.57	–0.48	60.77	59.17	26.89	65.13	59.17	26.89	65.13
PS01	8.64 ± 0.17 fg	4.00 ± 0.04 h	3.92 ± 0.03 cdef	3.24 ± 0.04 e	32.86	–0.91	7.99	62.62	2.48	47.05	62.62	2.48	47.05
PS02	8.60 ± 0.39 fgh	4.60 ± 0.05 e	3.50 ± 0.05 ij	3.20 ± 0.04 e	61.18	–2.17	27.43	56.70	22.23	57.61	56.70	22.23	57.61
PS03	9.50 ± 0.22 abc	2.90 ± 0.04 o	4.00 ± 0.04 cde	2.70 ± 0.03 hi	70.07	–1.73	16.77	64.77	2.64	61.40	64.77	2.64	61.40
PS04	8.90 ± 0.49 cdef	3.60 ± 0.04 k	3.60 ± 0.05 hi	3.60 ± 0.05 bc	68.16	–2.18	14.37	58.06	20.75	59.63	58.06	20.75	59.63
PS05	8.70 ± 0.25 efg	3.40 ± 0.05 l	4.00 ± 0.04 cde	3.20 ± 0.05 e	69.31	–2.55	16.88	59.15	19.65	61.31	59.15	19.65	61.31
PS06	7.60 ± 0.33 i	4.00 ± 0.05 h	3.60 ± 0.04 hi	2.90 ± 0.06 fg	70.47	–2.32	19.46	51.37	32.35	49.86	51.37	32.35	49.86
PS07	8.75 ± 0.28 def	3.56 ± 0.03 k	3.75 ± 0.05 fgh	3.32 ± 0.04 de	70.17	–3.10	20.84	62.10	17.46	66.26	62.10	17.46	66.26
PS08	9.10 ± 0.27 bcdef	4.10 ± 0.04 g	3.83 ± 0.05 efg	2.83 ± 0.04 fgh	68.83	–1.64	57.02	59.33	27.65	66.42	51.62	40.24	61.21
PS09	9.60 ± 0.24 ab	3.40 ± 0.04 l	4.30 ± 0.03 a	3.40 ± 0.05 cde	64.11	–0.41	51.11	58.23	22.85	60.86	58.23	22.85	60.86
PS10	8.95 ± 0.23 bcdef	4.05 ± 0.05 gh	3.85 ± 0.05 hi	3.20 ± 0.06 e	67.15	–2.35	54.63	55.54	33.84	63.32	53.74	36.84	64.35
PS11	8.80 ± 0.43 def	4.10 ± 0.06 g	3.70 ± 0.04 gh	3.30 ± 0.04 de	70.91	4.16	71.62	54.58	33.31	58.42	54.58	33.31	58.42
PS12	10.00 ± 0.51 a	4.00 ± 0.04 h	4.30 ± 0.03 a	2.80 ± 0.05 gh	70.04	–1.62	62.67	57.34	33.06	62.95	57.34	33.06	62.95

RHSCC = Royal Horticultural Society Color Chart; *L** = lightness; *a**, *b** = chromatic components.

advantage compared with both parents in many aspects, which can be considered as a result of heterosis. Such variability may be useful for breeding programs with different results.

Flower shape and flower color are important traits for ornamental plants. 'Slim Whitman' had large flowers that measure 9.37 cm in diameter and 5.17 cm in corona diameter. 'Pinza' had slightly smaller flowers that measure 8.10 cm in diameter and 2.85 cm in corona diameter. When compared with the parents, a notable floral variation was observed in the hybrid progeny. Hybrids SP03, SP12, PS03, PS09, and PS12 got larger flowers than their parents. SP01 had a greater corona than 'Slim Whitman', and all the other reciprocal individuals had larger coronas than 'Pinza'.

In the meantime, a significant genetic variance on the flower color was observed. 'Slim Whitman' had ivory white petals and light yellow corona; 'Pinza' had vivid yellow petals and a corona vivid orange-yellow at the base and orange at the rim. The detailed chromatic parameters of the parents are displayed in Table 4. In the reciprocal populations, chromatic parameters of the petals were in great variation. The *L** valued ranged from 32.86 to 74.64, *a** valued from –3.10 to 9.01, and *b** valued from 6.93 to 71.62 (Table 3). In the two-dimensional quadrant plot generated from CIELAB parameters *a** and *b**, all the dots were distributed in the first and second quadrants. The ones close to the origin represented for white and the ones away from the origin represented for different extent yellow (Fig. 2). Based on the two-dimensional distribution, we grouped hybrids SP01–SP10 and PS01–PS07 as the "white petal group," and hybrids SP11–SP15 and PS08–PS12 as the "yellow petal group," which were consistent with the RHSCC value and visual description. However, the corona color did not have obvious separation in the hybrid individuals except that SP04, PS08, and PS10 got an orange circle at the rim of corona like their parent 'Pinza'. It is worth mentioning that hybrid SP04 had white petals and yellow-orange corona, which was not observed in either parent and rarely seen in the *N. pseudonarcissus*.

The graceful flower shape, distinctive color, and vegetative growth advantage made SP04 of a great ornamental value and could become a new potential popular cultivar of *Narcissus*. Hybrids SP01, SP03, SP05, PS04, PS06, PS07, etc., were also potential assortment and unique germplasm resources, for their good performance at vegetative growth and flower shape. Although proportions of flower color are observed, the genetic rule for flower color is still unknown probably because of the unknown parents' genetic background, the complexity of the genetic control of flower color, or both. Further genetic studies, such as molecular markers, will help improve our

Table 4. The colors of perianths and coronas and color parameters in the parents.

		RHSCC	L^*	a^*	b^*
Slim Whitman	Perianths	157B	48.62 ± 5.19	–1.08 ± 0.27	8.41 ± 0. 91
	Corona	6A	62.14 ± 3.36	1.65 ± 0.65	52.52 ± 3.43
Pinza	Perianths	12A	67.01 ± 1.11	3.77 ± 2.25	66.02 ± 0.78
	Corona base	12A	57.45 ± 1.00	28.94 ± 1.77	67.77 ± 1.39
	Corona rim	28B	49.60 ± 0.40	43.31 ± 0.78	58.08 ± 0.53

RHSCC = Royal Horticultural Society Color Chart. L^* = lightness; a^*, b^* = chromatic components.

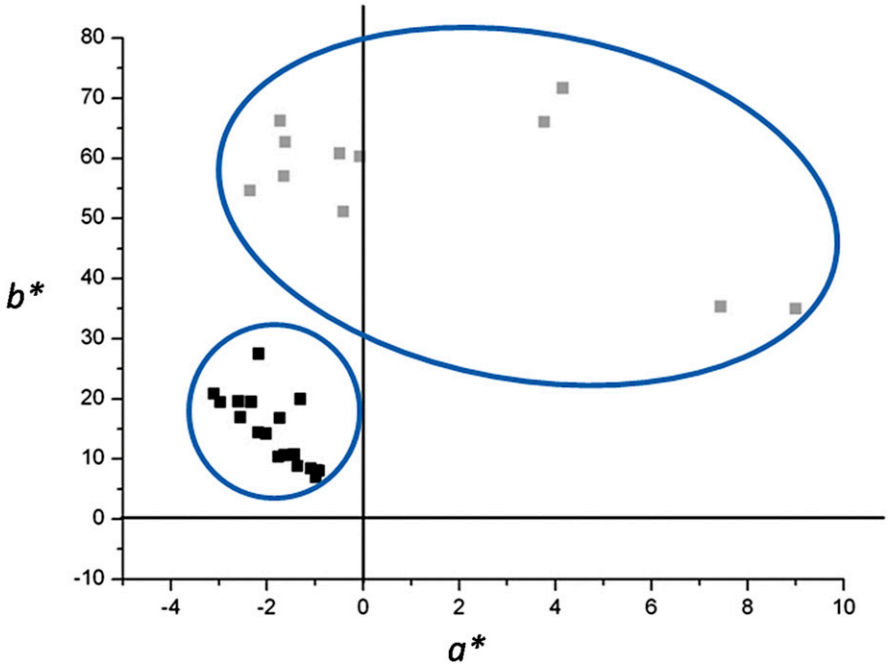

Fig. 2. Flower color distribution of *Narcissus* cultivars in coordinate systems of bivariate (a* and b*).

understanding of the genetics of *N. pseudonarcissus* flower color remarkably.

There was no evident imparity distribution in any traits in the reciprocal populations. Also, in our study, we did not find obvious linkage between the morphological traits. It may be not enough to give a conclusion that maternal effects, pollination effect, or both did not play a major role, or unilateral incongruity was not existed in this cross combination because the individuals in F1 population is relatively limited; however, there was a possibility. To verify this, multiple crosses between the two cultivars are needed in further study.

In a word, expression of traits in the reciprocal hybrids was not always intermediate between the parents but may instead be either parental-like or extreme, depending on the genetic control of the phenotypes. As the parents were heterozygous in most genetic loci, the reciprocal populations exhibited wide phenotypic variation. The abundant genetic variation generated in the hybrid progeny indicated that cross breeding is a powerful way in the *N. pseudonarcissus* breeding.

Divergence based on morphological characteristics. A dendrogram was generated based on the aforementioned 20 morphological traits to reveal the likely relationships among 'Slim Whitman', 'Pinza', and the hybrids (Fig. 3). The populations were clustered into three main groups. Group I comprised four white petal populations, including 'Slim Whitman', SP02, SP03, and PS01. Group II included eight populations with yellow petals, which were 'Pinza', PS11, SP11, PS08, PS10, PS12, SP14, and PS09. The 11 hybrids in Group III appeared to be relatively differentiated from their parents, and a similar subgroup situation appeared on hybrids SP12, SP13, PS06, PS03, SP15, and SP01.

Although some *Narcissus* varieties are hybrids on the market, they are usually not selected and bred from a large F1 population. This is the largest F1 population ever reported, and each hybrid seedling represents for a unique genotype. This result reinforced that F1 hybrid progeny was ideal populations for the selection of new ornamental individuals. As the *N. pseudonarcissus* varieties chosen as parents are always not homozygous in most genetic loci, abundant genetic variations occur in the F1 generation. There is no doubt that understanding genetic segregation in F1 generation would help to select desired traits of interest. Breeders can use a routine cross breeding method to get a great deal of new *N. pseudonarcissus* varieties.

Divergence based on RAPD markers. The 10 RAPD primers screened for the DNA amplification of 'Slim Whitman' and 'Pinza', and the reciprocal hybrids generated a total of 113 stable and unambiguous bands. Overall, 38% (43 bands) were polymorphic between 'Slim Whitman' and 'Pinza'. The 43 useful polymorphism bands were scored and used for analysis in the reciprocal cross population (Fig. 4). Based on the dendrogram generated through the UPGMA method, most of the genotypes could be organized into two main clusters. Group I comprised 23 hybrids with different genotypes and 'Slim Whitman'. Group II included SP11, PS06, PS10, and 'Pinza'. SP04 lay apart from the others, which was consistent with its distinct phenotype. The results were in good agreement with divergence based on morphological characteristics.

There is a high consistence between the divergence based on morphological characteristics and RAPD markers. SP02, SP03, and PS01 show a high similarity with 'Slim Whitman' and are classified into a cluster in both dendrograms. SP11 and PS10 are the most similar with 'Pinza', both morphologically and genetically. SP04 lay apart from 'Slim Whitman' and 'Pinza' in both dendrograms. SP05, SP15, PS04, and PS05 are always not close with 'Slim Whitman' or 'Pinza' (Fig. 5). These results suggest that the 43 polymorphism bands are sufficient to provide inferences on genetic divergence and relationships. There is rich genetic variation among the hybrids tested and that the RAPD technique has a wide prospective use in hybrid genetic analysis of daffodils.

Assessment of morphological and genetic diversity provides efficient and effective tools for detecting the relationships in genetic variation among populations. Many studies have reported on comparisons of molecular and morphological data. Some have found high associations between molecular and morphological markers in ornamental plants. Morphological character and sequence-related amplified polymorphism (SRAP) analyses of two hybrids between *Hibiscus dasycalyx* and *Hibiscus* 'Moy Grande' were preformed, and the hybrids showed hybrid vigor, and intermediate flower and leaf morphology compared with the parent plants and a higher genetic similarity coefficient with the female parent (*H. dasycalyx*) than with the male parent (*H.* 'Moy Grande') (Yu et al., 2016). Diversity analysis based on agromorphological traits and microsatellite-based markers were also used in global germplasm collections of roselle (*Hibiscus sabdariffa* L.). It turns out that employed agromorphological traits and molecular markers have complemented each other and imparted greater resolution to understand the genetic diversity of roselle plant genetic resources (Sharma et al., 2016). In *Passiflora*, cytogenetic, molecular, and morphological analyses were compared

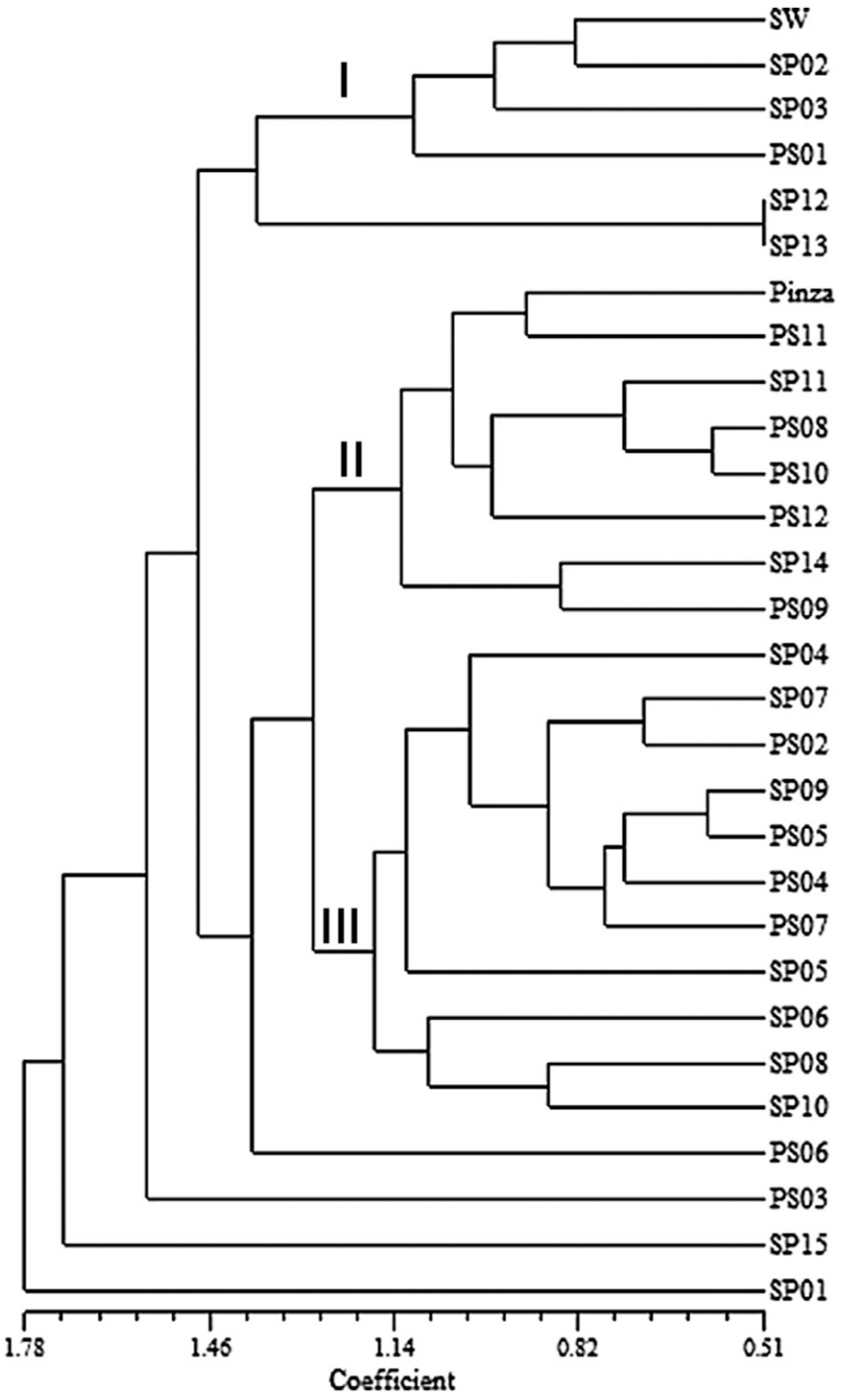

Fig. 3. Dendrogram generated based on the 20 morphological traits in the hybrids and their parents using unweighted pair group method with arithmetic mean analysis.

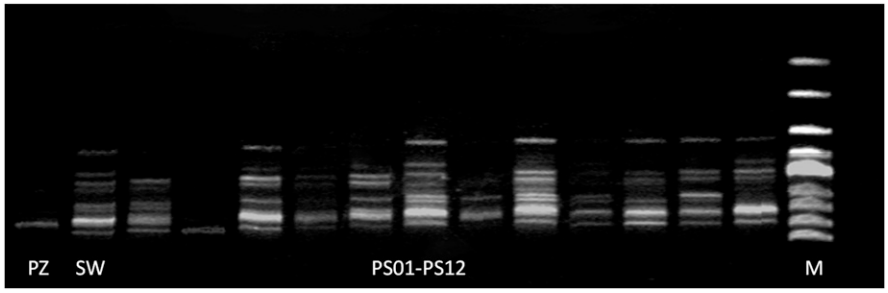

Fig. 4. Bands amplified with the primer pairs S103.

with those of *Passiflora capsularis* L. and *Passiflora rubra* L., and it showed that molecular and morphological analysis were powerful tools for the categorization of the species (Amorim et al., 2014). However, the reports on *Narcissus* family are very limited. For the first time, this study revealed that morphological and molecular markers may be successfully used for determining genetic diversity and relationships in *N. pseudonarcissus* genotypes and have great significance in designing breeding strategies.

Although it is a challenging work of *Narcissus* cross breeding, we successfully got a hybrid population with 27 different genotypes for the first time after hybridization and 8 years of cultivation. Furthermore, the 27 hybrids differ from one another on phenotypes, which provided a lot of new germplasm resources for *Narcissus* breeding. Hybrids often display superior performance (heterosis) in many vegetative and reproductive traits along with variation in gene expression, heterozygote advantage exhibited in some loci, or counteracting of deleterious recessive mutations that may have accumulated in each parent (Rosas et al., 2009). Reciprocal hybrid plants may also show differences in many aspects because cytoplasmic genes or the interaction of cytoplasmic genes with nuclear genes may have influence on the performance of the reciprocal hybrids (Sarah et al., 2008). In our study, several reciprocal hybrids showed hybrid vigor compared with their parents, meanwhile paternal effect or female parent effect was not observed in the hybrid populations (Fig. 1), which is probably because their parents are not homozygous.

Different diversity parameters and cluster analysis employed in this study most convincingly demonstrated that hybridization is an efficient way to obtain plentiful variation in *N. Pseudonarcissus* breeding.

Conclusion

'Slim Whitman' and 'Pinza' are widely cultivated daffodil varieties with different genetic backgrounds. Reciprocal cross-pollinations between 'Slim Whitman' and 'Pinza' were performed expecting that hybrids with diverse phenotypes could be produced. After eight consecutive years of work, we successfully obtained a reciprocal cross hybrid population containing 27 genetically different hybrids. Twenty morphological characteristics of the reciprocal cross hybrids were observed and analyzed. The 27 hybrids integrated the morphological characteristics of the two parents. They showed great variation in morphological characters compared with the parents, especially in leaf and flower characters. In general, several hybrids showed growth advantages over 'Slim Whitman' and 'Pinza'. Hybrids SP04 has great ornamental value and could

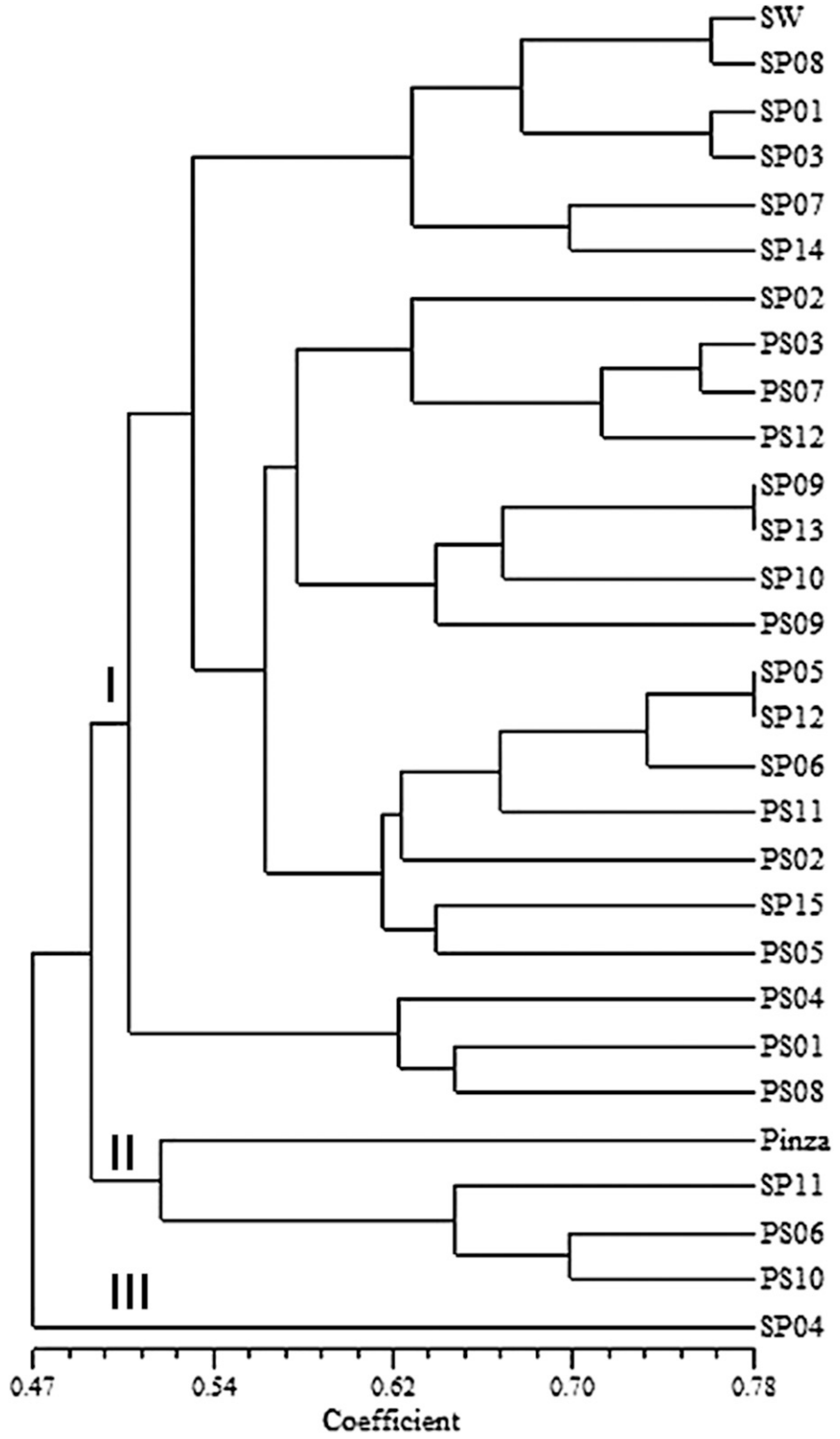

Fig. 5. Unweighted pair group method with arithmetic mean dendrogram of the hybrids and their parents generated based on random amplified polymorphic DNA data.

become a potential new popular *Narcissus* cultivar. Hybrids SP01, SP03, SP05, PS04, PS06, and PS07 also showed growth advantage and produced novel flowers. The reciprocal hybrids and their parents were valuable germplasm resources for genetic analysis and breeding.

Cluster analysis based on morphological traits and RAPD markers suggested that there was rich genetic variation among the hybrids tested and that the RAPD technique had wide prospective use in hybrid genetic analysis of daffodils. Results derived from this study would be highly useful in *Narcissus* breeding programs.

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