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DNA Molecular Markers for Genetic Identification of Tuberose

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In this study, we developed inter simple sequence repeat molecular markers to explore the genetic variation and relationships among 23 *Polianthes tuberosa* L. varieties. In all, seven primers were selected, and 99 identifiable amplicons were obtained, among which 89 were polymorphic with a polymorphic amplicon rate of 89.9%. The amplicons fragment ranged between 100 and 2200 bp. Jaccard's coefficient was applied to calculate the similarity matrix between the varieties. The mean similarity coefficient among the 23 varieties was 0.64. *Polianthes tuberosa* 'Double' and *P. tuberosa* 'Chia–Nong Pink Single' had the farthest relationship, with a similarity coefficient of 0.35. By contrast, *P. tuberosa* 'Chia–Nong Lady Jewel' and *P. hybrida* 'NCYU Pink Lady' had the closest relationship, with a similarity coefficient of 0.8. A similarity coefficient of 0.606 was used as the basis for dividing the 23 varieties into three groups. Group (Ⅰ), with a similarity coefficient of 0.678, comprised three subgroups and an independent variety. Group (Ⅱ), with a similarity coefficient of 0.678, comprised three subgroups. Group (Ⅲ) encompassed two varieties. *P. tuberosa* 'Double' is a variation of *P. tuberosa* 'Single' in Europe. According to the dendrogram, the two varieties were classified into the same group (Ⅱ) and subgroup (Ⅱ–a), implying that they were closely related. The results revealed that *Polianthes tuberosa* L. (hereafter referred to as "tuberose") had rich genetic diversity, and the developed ISSR primers for tuberose had high discrimination ability. The study findings will facilitate subsequent tuberose genetic research and serve as a reference for further breeding and cultivation.

Key words: cultivar, genetic similarity, inter simple sequence repeat

INTRODUCTION

In recent years, plant variety protection has been increasingly emphasized. The promotion of plant variety rights has ensured more favorable plant protection for breeders, enabling them to fight for their rights when variety infringements occur. Currently, new plant varieties are mostly identified based on their appearance traits. However, to make the identification more credible, DNA molecular markers should be considered. In addition, species identification based on DNA molecular markers is faster than conventional methods as it is less affected by environmental factors (Huang *et al*., 2010).

Common molecular marker techniques for genetic identification between different varieties include random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), inter simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), and single nucleotide polymorphism (SNP), each of which has its advantages, disadvantages, scope of application, and limitations (Hao and Qu *et al*., 2009; Huang *et al*., 2010; Rafalski and Tingey, 1993). Among these methods, RAPD is the easiest to operate but has relatively low test accuracy and repeatability (Smith, 2005); AFLP requires specific equipment and high–quality DNA and analytical skills (Mueller and Wolfenbarger, 1999); SNP requires detailed nucleic acid sequencing data (Rafalski, 2002). SSR and ISSR are currently the most widely applied molecular marker techniques for genetic identification. For SSR, the DNA sequencing data around the repeated sequence must be obtained in advance. By contrast, ISSR can be applied without the requirement of genome sequencing data; has high repeatability; and is fast, simple, and inexpensive. Therefore, this method has been extensively applied in the genetic identification of various plants (Zhao *et al*., 2007) and for genetic relationship analyses, DNA polymorphism analyses, genetic marker testing, and quantitative trait loci mapping between different varieties (Bhadkaria *et al*., 2020; Hadipour *et al*., 2020).

Although RAPD and ISSR have been used in the genetic identification of tuberose, the genetic information for tuberose remains limited (Khandagale *et al*., 2014; Sarkar *et al*., 2010). We selected 23 hybridized tuberose varieties (Huang *et al*., 2001; Shen *et al*., 1997) and used tuberose genomic DNA for next–generation sequencing, wherein it was purified and mixed with the DNA samples of three main parents (i.e., *Polianthes howardii*, *P. tuberosa* 'Single', and *P. tuberosa* 'Double'). After the tuberose SSR sequencing data were preliminarily established, 23 tuberose varieties hybridized in laboratories for several years were selected for the development of a tuberose ISSR molecular marker. This molecular marker can be used to evaluate the genetic similarity between varieties and serve as a reference for resource preservation and future hybridization.

MATERIALS AND METHODS

Experimental materials

We selected the 23 tuberose varieties hybridized by the flower research team in the Department of Horticulture, National Chiayi University (Table 1).

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Table 1. Twenty–three varieties of *Polianthes tuberosa* and their flower color

Code	Cultivar	Perianth/ Floral tube Color
a	*Polianthes howardii*	reddish purple/ reddish pink
b	*P. tuberosa* 'Double'	white
c	*P. tuberosa* 'Single'	white
d	*P. tuberosa* 'Chia–Nong Pink Single'	pink
e	*P. tuberosa* 'Chia–Nong White Jewel'	white
f	*P. tuberosa* 'Chia–Nong Bright Jewel'	white
g	*P. tuberosa* 'Chia–Nong Cinderella'	reddish purple
h	*P. tuberosa* 'Chia–Nong Madam Violet'	light pink, light purple
i	*P. tuberosa* 'Chia–Nong Lilac Mist'	light pink, pink
j	*P. tuberosa* 'Chia–Nong Purple Jewel'	reddish purple/pink
k	*P. tuberosa* 'Chia–Nong Sensation'	light pink, reddish purple
l	*P. hybrida* 'NCYU Passion'	light pink, reddish purple
m	*P. tuberosa* 'Chia–Nong Lady Jewel'	reddish purple
n	*P. hybrida* 'NCYU Fancy'	light pink, reddish purple
o	*P. hybrida* 'NCYU Lover'	white
p	*P. tuberosa* 'Chia–Nong Super Baby'	yellow
q	*P. tuberosa* 'Chia–Nong Queen'	reddish purple/ reddish pink
r	*P. hybrida* 'NCYU Super Gold'	yellow
s	*P. hybrida* 'NCYU Peace'	Light yellow
t	*P. hybrida* 'NCYU Peach Pink'	pink/light pink, pink
u	*P. hybrida* 'NCYU Cherry'	reddish purple
v	*P. hybrida* 'NCYU Pink Lady'	pink
w	*P. hybrida* 'NCYU Love'	reddish purple

Experimental methods

Development of the Tuberose SSR molecular marker

Genomic DNA preparation and sequencing:

To develop the tuberose SSR molecular marker, we mixed three main parents of tuberose (i.e., *Polianthes howardii*, *P. tuberosa* 'Double', and *P. tuberosa* 'Single'). The young leaves were used for genomic DNA purification, and the purified DNA was sent to Welgene Biotech for genomic DNA quality control. After the quality control process was completed, the Illumina/Solexa system was employed for DNA sequencing data analyses.

Library construction:

We cut the genomic DNA of 10 μg of DNA into 400–500 bp fragments by using a Misonix 3000 sonicator, and screened DNA fragments by using an electrophoresis analyzer (Bioanalyzer DNA 1000 chip, Agilent Technologies). Subsequently, we used 1 μg of fragmented DNA for end repair and A–tailed adaptor ligation to facilitate subsequent DNA sequence data analyses (the analyses were performed using Illumina's Truseq).

Sequencing data analyses and SSR sequence mining:

The raw FASTQ data (passing filter data) were used. Each read was deleted or partially cut off from the 3' end according to its quality value to increase the overall data quality. Subsequently, the two ends of the sequences were spliced together to obtain complete fragment sequences. These spliced reads were inspected for the existence of SSR sequences. The reads discovered from SSR sequences were designed using Primer 3.

ISSR molecular maker analyses:

DNA purification:

Hexadecyl trimethyl ammonium bromide, also called cetyltrimethylammonium bromide (CTAB), was used for extraction. Healthy, whole leaves from the 23 varieties were selected, cleaned, and dried. A leaf sample of 0.1 g was taken for each variety, mixed with liquid nitrogen, and ground to a powder with a mortar. The powder was placed in a 2–mL Eppendorf tube, to which 600 μL of CTAB solution was added. After a homogeneous mixture was obtained, the solution was placed in a water bath at 65°C for 30 min. Subsequently, the solution was cooled, and 4 μL of RNase A was added for reaction at 37°C for 30 min. After the reaction, mixture was cooled and the same volume of phenol/chloroform/isoamylalcohol (25: 24: 1) was added. The solution was then shaken up and down evenly and allowed to react for 10 min at room temperature. Afterward, it was centrifuged at 13,000 rpm for 10 min. The supernatant was then retrieved and placed into another 1.5–mL Eppendorf tube, to which 1/10 volume of 5 M NaCl and the same volume of isopropanol was added. The tube was shaken up and down to obtain an even mixture and then centrifuged at 13,000 rpm for 10 min. Subsequently, the

supernatant was discarded, and the white pellet that remained at the bottom of the tube was cleaned with 70% alcohol and centrifuged for 3 min. The supernatant formed was again discarded, and the pellet was cleaned with 95% alcohol. The pellet was centrifuged for 3 min, and the supernatant was discarded. After the pellet was completely air–dried, we added 40–50 μL of sterile water to dissolve it. A spectrometer was used to measure the readings of OD 260 and 280 nm and the DNA concentration. DNA was then stored in a refrigerator at –20°C for later use.

Polymerase chain reaction:

We initially selected 100 ISSR primers to obtain clear amplicons with polymorphism between varieties. The purified tuberose DNA was diluted to a concentration of 100 ng/μL. We added 1 μL of DNA, 1 μL of primer, and 5 μL of 4X Taq DNA polymerase master mix to a 0.2–mL Eppendorf tube, added sterile water to make up a volume of 10 μL, and placed the tube in a polymerase chain reaction (PCR) thermal cycler dice (TP600, TakaRa) for reaction. The reaction procedures are listed in the table below. First, the solution was allowed to react at 96°C for 30 min. In the second to fourth steps, 35 reaction cycles were conducted, after which the solution was allowed to react for 5 min at 72°C in the fifth step. After the reaction stopped, we set 16°C as the condition. The PCR products were then used for agarose gel electrophoresis. The annealing temperature was set according to the primer annealing temperature.

Agarose gel electrophoresis:

We weighed 0.8 g (2%) of agarose gel and dissolved it in 40 mL of 1× TAE buffer solution. After it was completely dissolved, 0.5 μg/mL ethidium bromide was added for staining. The agarose gel solution was mixed and poured into a dispenser, and a comb was placed in the solution. Subsequently, solidified agarose gel was placed in an electrophoresis cell, and 1× TAE buffer solution was added to the cell until the gel was completely submerged. We poured the samples into the wells in the gel. Eventually, 3 μL of DNA ladders were added, and electrophoresis was performed at 110 V for 42 min. After the electrophoresis cell was turned off, the films were removed and observed under a UV lamp, and pictures were taken with a gel imaging system.

ISSR molecular marker data analyses

On the basis of the electrophoresis results, we assigned 1 for readings with polymorphic amplicons and 0 for those without polymorphic amplicons. On the basis of Jaccard's coefficient, we applied the software NTSYSpc–2.01c to inspect the genetic similarities between each variety and the unweighted pair group method with arithmetic means (UPGMA) for cluster analyses.

RESULTS

We selected 23 tuberose (*Polianthes tuberosa* L.) varieties and developed SSR–based ISSR primers for subsequent genetic relationship identification. Initially, seven primers with clear amplified amplicons and polymorphic fragments were screened from 100 ISSR primers (Fig. 1). Furthermore, PCR analyses were conducted on all tuberose varieties, and 99 identifiable amplicons were amplified (amplicons). Among these amplicons, 89 were polymorphic. The primers used and the total polymorphism content are presented in Table 2. The total PIC was 89.9%; the average number of amplicons in each primer was 14.14, and the average number of polymorphic amplicons was 12.71; the amplicon fragment ranged between 100 and 2200 bp. The results revealed that tuberose has rich genetic diversity, and the ISSR primers developed for tuberose had a high discrimination ability.

We further employed NTSYS–pc, a software package, for analysis and Jaccard's coefficient to calculate the similarity matrix of the varieties (Table 3). The maximum similarity was 1, and larger values indicated a closer relationship. The similarity coefficients of the 23 varieties ranged between 0.35 and 0.8 (Table 3), with a mean similarity coefficient of 0.64. *Polianthes tuberosa* 'Double' and *P. tuberosa* 'Chia–Nong Pink Single' had the farthest relationship, with a similarity coefficient of 0.35, whereas *P. tuberosa* 'Chia–Nong Lady Jewel' and *P. hybrida* 'NCYU Pink Lady' had the closest relationship, with a similarity coefficient of 0.8. We conducted UPGMA cluster analyses to illustrate a genetic dendrogram and divided 23 tuberose varieties into three groups based on

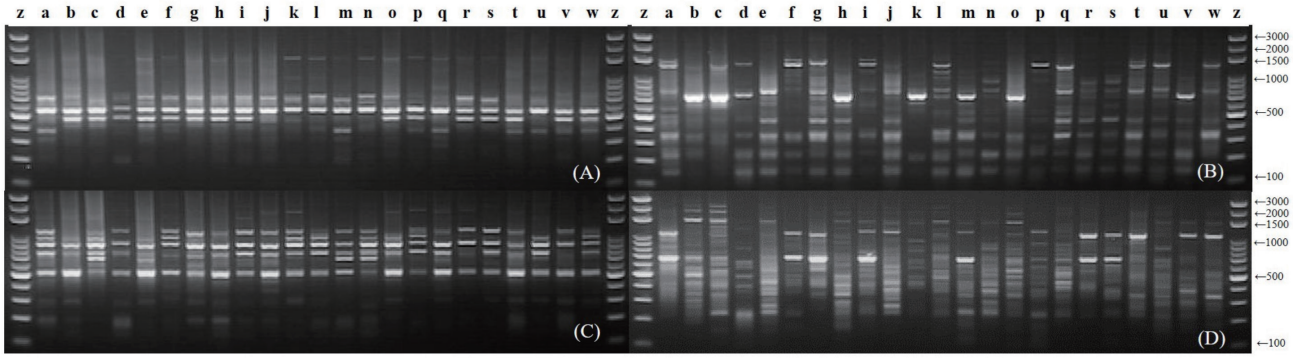

Fig. 1. Inter simple sequence repeat (ISSR) analysis amplicons of electrophoresis gram in 23 varieties of *Polianthes tuberosa* using P9 (A), P27 (B), P21 (C), and P32 (D) primer.

Table 2. Polymorphic inter simple sequence repeat (ISSR) primer sequences and their information of DNA fragments amplified which analyzed of tuberose

Primer	primer (5'–3')	Annealing temperature (°C)	Size distribution of amplicons (bp)	Total no. of amplicons	Polymorphic amplicons no.	Polymorphic information content (%)
P9	CCCGATTGGCATGATCTAGG	69.5	375–1400	7	6	85.7
P21	GGACGCTGGACCACATAGC	71	200–1250	14	12	85.7
P27	CATGTGTGCCTCATTTAGAGCC	71	150–1500	20	19	95.0
P32	AGCGGTCAGCGACACTGG	72	220–2200	20	19	95.0
P50	CGTACACCGAGCCACATACC	71	100–1750	13	11	84.6
P70	ACTTAAAATTCTGATGATGCCCC	64	200–1100	10	7	70.0
P72	CTAGGGCTGACTCCGAATCC	67	225–2000	15	15	100.0
Total				99	89	89.9
Average				14.14	12.71	

Table 3. Genetic similarity matrix of the 23 tuberose varieties analyzed using inter simple sequence repeat primers

a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w
1																						
0.57	1																					
0.57	0.75	1																				
0.64	0.35	0.39	1																			
0.67	0.67	0.58	0.49	1																		
0.71	0.48	0.55	0.64	0.56	1																	
0.76	0.58	0.65	0.52	0.69	0.63	1																
0.64	0.64	0.62	0.55	0.72	0.57	0.79	1															
0.63	0.54	0.58	0.61	0.53	0.70	0.62	0.58	1														
0.67	0.67	0.58	0.52	0.78	0.56	0.71	0.72	0.66	1													
0.56	0.54	0.56	0.52	0.55	0.56	0.55	0.56	0.48	0.57	1												
0.63	0.47	0.65	0.56	0.57	0.61	0.71	0.63	0.60	0.57	0.62	1											
0.67	0.47	0.56	0.67	0.64	0.61	0.60	0.56	0.73	0.66	0.53	0.64	1										
0.61	0.52	0.54	0.63	0.62	0.63	0.66	0.63	0.66	0.71	0.62	0.66	0.71	1									
0.61	0.65	0.65	0.49	0.69	0.52	0.69	0.67	0.57	0.66	0.62	0.64	0.57	0.64	1								
0.56	0.40	0.47	0.61	0.55	0.58	0.60	0.54	0.62	0.53	0.51	0.60	0.69	0.64	0.53	1							
0.65	0.63	0.63	0.45	0.62	0.56	0.73	0.65	0.64	0.71	0.55	0.62	0.57	0.60	0.64	0.62	1						
0.62	0.53	0.55	0.60	0.52	0.62	0.67	0.62	0.70	0.65	0.56	0.58	0.65	0.70	0.65	0.61	0.70	1					
0.58	0.47	0.52	0.63	0.51	0.63	0.62	0.61	0.69	0.60	0.57	0.60	0.66	0.75	0.62	0.64	0.62	0.76	1				
0.65	0.58	0.52	0.54	0.62	0.58	0.66	0.70	0.64	0.71	0.46	0.55	0.55	0.66	0.66	0.51	0.69	0.67	0.66	1			
0.64	0.48	0.48	0.62	0.56	0.57	0.63	0.64	0.67	0.65	0.54	0.61	0.65	0.74	0.54	0.61	0.63	0.62	0.58	0.61	1		
0.67	0.43	0.43	0.72	0.60	0.58	0.60	0.63	0.66	0.64	0.53	0.55	0.80	0.71	0.55	0.66	0.57	0.65	0.69	0.62	0.76	1	
0.58	0.47	0.54	0.56	0.55	0.63	0.57	0.61	0.66	0.62	0.48	0.62	0.64	0.66	0.57	0.66	0.69	0.67	0.66	0.66	0.79	0.69	1

code: Code areas those listed in the table of materials and methods.

a similarity coefficient of 0.606 (Fig. 2). The first group (I) was classified into three subgroups and one independent variety (i.e., I–a, I–b, I–c, and *P. tuberosa* 'Chia–Nong Super Baby'). I–a comprised *P. howardii*, *P. tuberosa* 'Chia–Nong Bright Jewel', and *P. tuberosa* 'Chia–Nong Pink Single'; I–b encompassed *P. hybrida* 'NCYU Fancy', *P. hybrida* 'NCYU Super Gold', and *P. hybrida* 'NCYU Peace'; and I–c comprised *P. tuberosa* 'Chia–Nong Lilac Mist', *P. tuberosa* 'Chia–Nong Lady Jewel', *P. hybrida* 'NCYU Pink Lady', *P. hybrida* 'NCYU Cherry', and *P. hybrida* 'NCYU Love'. The second group (Ⅱ) was categorized into three subgroups (i.e., Ⅱ–a, Ⅱ–b, and Ⅱ–c) according to a similarity coefficient of 0.678. Ⅱ–a comprised *P. tuberosa* 'Double' and *P. tuberosa* 'Single'; Ⅱ–b comprised *P. tuberosa* 'Chia–Nong White Jewel', *P. tuberosa* 'Chia–Nong Purple Jewel', *P. tuberosa* 'Chia–Nong Cinderella', *P. tuberosa* 'Chia–Nong Madam Violet', and *P. hybrida* 'NCYU Lover'; and Ⅱ–c

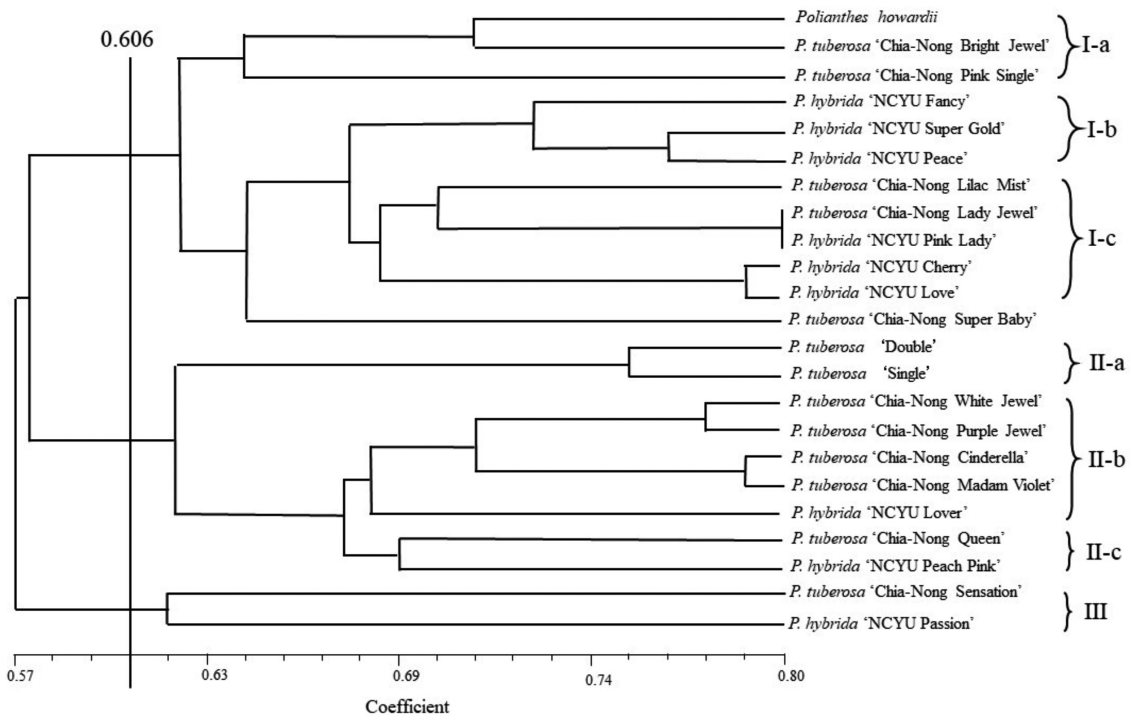

Fig. 2. Dendrogram grouping of the genetic similarity identified with inter simple sequence repeat (ISSR) DNA markers am.

comprised *P. tuberosa* 'Chia–Nong Queen' and *P. hybrida* 'NCYU Peach Pink'. The third group (Ⅲ) comprised *P. tuberosa* 'Chia–Nong Sensation' and *P. hybrida* 'NCYU Passion'. We analyzed the genetic relationships and similarities between the varieties by using the said dendrogram and similarity matrix.

DISCUSSION

We selected *Polianthes howardii* I, *P. tuberosa* 'Single', and *P. tuberosa* 'Double' as the parents to breed varieties through hybridization. The mixture of the leaves of the three parents was sent to a biotechnology company and an SSR primer was produced. The results revealed that polymorphic amplicons with SSR molecular markers could be formed at multiple annealing temperatures. The discovered amplicons were mostly those from the same fragment or fragments of the same variety with different sizes. This could be because of the complex genetic background of the three tuberose parents, which hindered the primer from identifying the correct amplicons. Therefore, we further compared the results obtained after addition of a forward primer only, a reverse primer only, and a forward and a reverse primer jointly. The results implied that when the SSR primer was applied, the amplicon could be obtained only after addition of one of the forward and reverse primers; they did not have to be added simultaneously. Hence, we used an ISSR molecular marker for testing rather than an SSR molecular marker. Bharti *et al*. (2016) used RAPD and ISSR molecular markers to categorize the genetic diversity of different tuberose varieties. They discovered that ISSR molecular markers yielded a higher level of polymorphism, with a genetic similarity ranging from 20.8% to 91%, indicating that the tuberose varieties had genetic diversity. Furthermore, we compared the results of a commercially available ISSR primer (UBC Set#9, 801–900) with those of the primers developed in the present study through sequencing and noted that the amplicons obtained using the commercially available ISSR primer were lighter in color and not polymorphic. In some circumstances, no amplicons were discovered (data not shown). By contrast, by using the developed ISSR primer, clear amplicons with polymorphism could be obtained for classification of the 23 varieties.

Based on the 23 primers selected according to the ISSR primer, 41 sweet potato varieties and strains could be divided into two groups. The first group comprised the germplasm introduced from China in the early times or the purified varieties serving as parents of the hybrids. The second group comprised varieties introduced from Japan or the purified varieties serving as the parent of the hybrids. The genetic similarity of the groups was 12%, implying that the ISSR primer could divide varieties with closer genetic relationship into the same group (Chien *et al*., 2009). The genetic similarity of the 23 tuberose varieties selected in this study ranged between 0.35 and 0.8. We illustrated a dendrogram with a genetic similarity of 0.606 as the basis and through a UPGMA cluster analysis (Fig. 2). The results showed that *P. howardii* was classified into a different group from the remaining two parent varieties. Studies have indicated that *P. tuberosa* 'Double' is a natural variation of *P. tuberosa* 'Single' discovered in Europe. *P. tuberosa* 'Double' 2n=50 (10L+40S) or 2n=54 (10L+44S) results from *P. tuberosa* 'Single' 2n=60 (10L+50S), with reduced

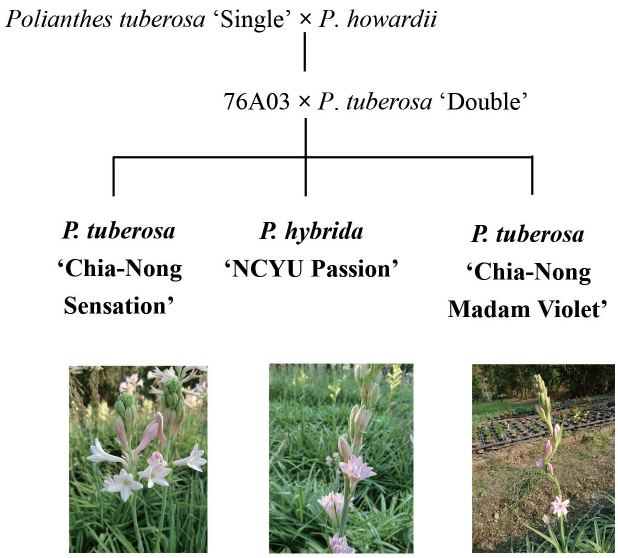

Fig. 3. Pedigree of breeding lines.

chromosomes because of abnormal mitosis or meiosis (Trueblood, 1973; Barba–Gonzalez *et al*., 2012); therefore, the two varieties are speculated to be closely related. According to the dendrogram, they were classified into the same group (Ⅱ) and even the same subgroup (Ⅱ–a). *P. howardii*, however, has 58 chromosomes and is a Mexican native variety (Barba–Gonzalez *et al*., 2012). It was classified into the first group (Ⅰ), different from the other two parents. In addition, *P. tuberosa* 'Chia–Nong Sensation' and *P. hybrida* 'NCYU Passion' in the third group (Ⅲ) had the same parents. Although *P. tuberosa* 'Chia–Nong Madam Violet' had the same parents with the said two varieties (Fig. 3), it was not categorized into the third group because the flower color was more similar between *P. tuberosa* 'Chia–Nong Sensation' and *P. hybrida* 'NCYU Passion'. Accordingly, the developed ISSR primers could clarify the genetic relationships of the tuberose varieties.

According to the fragments obtained using ISSR, Deng *et al*. (2006) divided the *Lycoris longituba* varieties with the same flower color into the same group to indicate a closer relationship. As shown in the dendrogram, varieties with a similar flower color were categorized into the same subgroup (Fig. 2). In Subgroup Ⅰ–b in Group Ⅰ, two of three varieties had yellow flowers, namely *P. hybrida* 'NCYU Super Gold' and *P. hybrida* 'NCYU Peace'. *P. tuberosa* 'Chia–Nong Super Baby', the variety dependent from the subgroups in Group Ⅰ, also had yellow flowers. All varieties with yellow flowers were classified into Group Ⅰ. All five varieties in Subgroup Ⅰ–c had pink and reddish purple flowers. *P. tuberosa* 'Chia–Nong Lilac Mist' and *P. hybrida* 'NCYU Pink Lady' had pink flowers, whereas *P. tuberosa* 'Chia–Nong Lady Jewel', *P. hybrida* 'NCYU Cherry', and *P. hybrida* 'NCYU Love' had reddish purple flowers. The two varieties in Subgroup Ⅱ–a had white flowers, and those in Subgroup Ⅱ–c had pink and reddish purple flowers. *P. tuberosa* 'Chia–Nong Sensation' and *P. hybrida* 'NCYU Passion', the two varieties in Group Ⅲ, had light pink and reddish purple flowers. Except for Subgroups Ⅰ–a and Ⅱ–b, the varieties in the remaining subgroups had white, pink, and reddish purple flowers. The classification using the developed ISSR primers seemed to be associated with colors despite exceptions (i.e., Subgroups Ⅰ–a and Ⅱ–b). Therefore, color–controlling primers should be developed to identify the genes that control colors. Sirohi *et al*. (2017) indicated that the genotype displayed in the tuberose dendrogram does not match with the variety phenotype. Varieties with different genotypes were mixed together (e.g., single–flowering, semi–double flowering, and double flowering). Bharti *et al*. (2016) could not perfectly group the tuberose varieties according to their flowering types (i.e., single– and double–flowering groups) despite using an ISSR molecular marker. In the present study, we also failed to classify the tuberose varieties into single–, semi–double, and double–flowering groups, because the ISSR primers that controlled the number of petals were not discovered.

We used the fragmented amplicon obtained using the seven ISSR primers to construct a dendrogram according to the UPGMA cluster analytical results. A similarity coefficient of 0.606 was used as a basis to divide the varieties into three groups. Group Ⅰ comprised 12 varieties in total and was further divided into three subgroups and an independent variety. Group Ⅱ was divided into three subgroups and comprised nine varieties. Group Ⅲ encompassed two varieties. The results revealed that tuberose had diverse germplasm resources. To identify the trait control genes, future studies should conduct sequencing analyses on a single variety and then identify the target gene amplicons through molecular marker techniques. Furthermore, the genetic relationship of tuberose obtained using molecular markers in this study could serve as a reference for further breeding operations.

AUTHOR CONTRIBUTIONS

1. Chien–Na KAO, worked on experimental process of the genetic relationship of tuberose. Primers were selected and identifiable amplicons were obtained and then it was analyzed genetic identification of relationships among 23 *Polianthes tuberosa* L. varieties. This was conducive to understanding the genetic relationship among 23 *Polianthes tuberosa* L. varieties and was served as a reference for further breeding and cultivation.
2. Wen–Lii HUANG, conceived the experiment of DNA molecular markers for genetic identification of tuberose and proposed amendments and suggestions to the research content.
3. Wen–Cheng LIN, established the tuberose SSR sequencing data, and the phylogenetic classification of the different species of tuberose was preliminarily grouped. Modifications and suggestions were made to the experiment method.
4. Ikuo Miyajima, provided practical study suggestions on analyze relationships among tuberose and revised

the research content and experiment data.

5. Kuang–Liang Huang, integrated the previous experimental of tuberose breeding studies, and conducted related study in the laboratory.

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