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GENETİC ANALYSİS OF 38 DOUBLE-FLOWERED AMARYLLİS (*Hippeastrum hybridum*) CULTİVARS BASED ON SRAP MARKERS

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ABSTRACT

The genetic diversity and population structure of 38 commercial accessions of double-flowered amaryllis (*Hippeastrum hybridum*) from the Netherlands and South Africa were evaluated using sequence-related amplified polymorphism (SRAP) markers. Thirty SRAP primer pairs produced 294 loci, of which 263 (89.16%) were polymorphic. A relatively high level of genetic diversity was observed, with estimates of Nei's diversity index (*H*) and the Shannon information index (*I*) of 0.27 and 0.42 respectively. Additional genetic distance- and STRUCTURE-based analyses, clustered all accessions into two or four subgroups based mostly on origin or color. The genetic differentiation between/among countries and inferred groups was significant, with *Fst* values ranging from 0.08–0.19%. Accessions from the Netherlands showed higher genetic variation than those from South Africa. Several accessions, such as Aphrodite, are recommended for future programs employing selective hybridization with the goal of expanding the color range. The results of the present study provide appropriate information applicable to designing effective breeding programs for double-flowered amaryllis.

Key words: amaryllis, molecular markers, genetic evaluation

INTRODUCTION

Amaryllis (*Hippeastrum hybridum*) is a bulbous flowering plant in the family Amaryllidaceae. It mainly originated in two centers, namely, eastern Brazil and the Southern Andes of Peru in South America. Of all flowering bulb species, amaryllis is the easiest to bring to bloom, which makes it very popular and in demand worldwide. Amaryllis has yielded more than thousand popular hybrids or cultivars since its breeding began in 1799 [Meerow 1999]. Five types [Szilard 2013] are customarily sold: double-flowered, single-flowered (large flowering), trumpet, jumbo (mammoth), and miniature (dwarf or small flowering). Of these types, the double-flowered cultivars have significant market appeal due to their increased horticultural and ornamental value. Currently, commercial double-flowered cultivars from the Netherlands and South Africa dominate the global flower market [Meerow 2009]. Most of these cultivars come in beautiful varieties including various shades of red, white, pink and orange. There are also many striped and multicolored varieties, usually displaying shades of pink or red combined with white.

Double-flowered plants have extra petals and often contain flowers within flowers [Meyerowitz et al.

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1989]. Additional tepal-like structures in double-flowered amaryllis apparently result from transformations of both male and female reproductive structures [Bell 1977]. The origin of double-flowered amaryllis is highly complex and important for breeding. The first significant breeding of double-flowered amaryllis was reported by McCann [1937], who used pollen of an apparent diploid (n = 22) to pollinate garden hybrids (probably tetraploid, n = 44). Generally, double-flowered amaryllis can produce some pollen-containing anthers at the ends of some transformed, petal-like stamens [Meerow 1988, 2009]. Recent research has also demonstrated that the pollen germination percentage in normal, defective, and petaloid anthers of six double-flowered cultivars can reach 78–91% [Liu et al. 2015a]. In addition, double-flowered amaryllis may also be used as a female parent because of its extremely reduced ovary and small amount of stigmatic tissue [Meerow 1988, 2009]. Embryo rescue can assist in the development of hybrid embryos that might not survive to become viable plants. However, there are no reports available on this topic. Thus, cross-breeding with pollen of double-flowered cultivars/hybrids has become a common and useful method of producing double-flowered hybrids on a commercial basis [Shi et al. 2014, Liu et al. 2015b].

In fact, there are only a few dozen commercial double-flowered cultivars in the global amaryllis market. This low number may be attributed to the fact that most hybrid combinations, with the double-flowered type used as the male, do not easily produce seeds and seedlings [Bell 1977]. Shi et al. [2014] further showed that the percentages of seed set and seedling emergence among several hybrid combinations (formed by pollinating single-flowered cultivars with double-flowered types such as Blossom Peacock, Dancing Queen, Ferrari and Flaming Peacock) varied dramatically from 0 to 100%. Therefore, promising pollen parents should be identified for the successful development of new double-flowered hybrids. With respect to this goal, it is important to know the level and distribution of genetic diversity in available collections of double-flowered amaryllis germplasm. Molecular markers are very powerful tools for assessing the genetic diversity of bulbous flower plants [Wei et al. 2016, 2017b]. Sequence-related amplified polymorphism (SRAP) is a developed PCR-based molecular marker technique. It has many advantages, such as simple and quick operation, abundant polymorphisms, reliable repeatability, and low cost, etc. However, this technique is rarely used in bulbous flowers, especially in amaryllis, there is no relevant report. Here, we used SRAP markers to evaluate the genetic relationships among the 38 most common commercial double-flowered amaryllis cultivars bred in the Netherlands and South Africa. The present research will provide a theoretical basis for breeding double-flowered varieties in the future.

MATERIALS AND METHODS

Plant material. A total of 38 cultivars or hybrids of double-flowered amaryllis (29 from the Netherlands and 9 from South Africa) were employed in this study (Tab. 1). All accessions were grown under the same conditions and fertilized monthly at the Bulb and Perennial Flowers Genebank Collection, Vegetable Research Center, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. Tender leaves were collected in the spring and stored at -80°C until they were used for DNA extraction.

DNA extraction and storage. Genomic DNA was extracted from the leaves of the 38 accessions using the cetyl triethylammonium bromide (CTAB) method [Wang et al. 2018]. Approximately 0.2 g of leaf tissue was first ground into a powder in liquid nitrogen and transferred from the mortar to a tube. Then, 1.0 ml of CTAB extraction buffer was carefully added to the tube and incubated at 65°C for 30 min. Genomic DNA was centrifuged at 10 000 rpm for 8 min. The DNA was washed using 70% ethyl alcohol and air dried. DNA quality and concentration were evaluated using 1.0% (w/v) agarose gel electrophoresis and spectrophotometry, respectively. DNA samples were diluted to 10 mmol/µl and stored at 4°C for further use.

SRAP-PCR. The SRAP technique [Li and Quiros 2001] is a PCR-based marker system employing a combination of two primers, specifically, a forward primer of 17 bases and a reverse primer of 18 bases, which preferentially amplify open reading frames (ORFs). In the current study, all SRAP primer pairs were synthesized by Shanghai Bioengineering Technology Co. Ltd. (Tab. 2). A total of 783 combinations were finally obtained based on 27 forward

Table 1. The 38 double-flowered amaryllis accessions used for SRAP analyses

Accessions	Color	Country	Accessions	Color	Country
Pretty Nymph	pink/white	Netherlands	Aphrodite	white/pink	Netherlands
Sunny Nymph	red/white	Netherlands	Blossom Peacock	white/red	Netherlands
Nymph	white/red	Netherlands	Dancing Queen	white/red	Netherlands
Marilyn	white/green	Netherlands	Double Dragon	red	Netherlands
Exotic Nymph	white/pink	Netherlands	Lady Jane	red/white	Netherlands
Double King	red	Netherlands	Exotic Peacock	red/white	Netherlands
Double Dream	pink	Netherlands	Red Peacock	red	Netherlands
Diva	red	Netherlands	Flaming Peacock	white/red	Netherlands
Benito	red	Netherlands	Jewel	white	Netherlands
Ch1655	pink	Netherlands	Alfresco	white	Netherlands
Double Delight	red	Netherlands	BB115	pink	South Africa
Splash	red/white	Netherlands	Harlequin	white/red	South Africa
Red Nymph	red	Netherlands	Ragtime	red	South Africa
Sweet Nymph	pink	Netherlands	Ballerina	red	South Africa
Arctic Nymph	white	Netherlands	Rozetta	pink	South Africa
Double Record	white/red	Netherlands	First Love	pink/white	South Africa
Cherry Nymph	red	Netherlands	Zombie	pink/white	South Africa
Pasadena	red/white	Netherlands	Fanfare	red	South Africa
Elvas	white/pink	Netherlands	Vegas	red/white	South Africa

primers and 29 reverse primers. Five varieties were randomly selected to identify the polymorphism of the SRAP primer combinations.

Each 25 µl SRAP-PCR amplification system contained 2.5 µl of 10 × PCR buffer, 3.5 µl of $MgCl_2$ (10 mmol/µl), 0.5 µl of dNTPs (0.2 mmol/µl), 1.1 µl of forward primer (0.2 mmol/µl), 1.1 µl of reverse primer (0.2 mmol/µl), 2.0 µl of genomic DNA (10 mmol/µl), 0.6 µl of Taq DNA polymerase (0.06 U/µl), and 13.7 µl of double-distilled water. The PCR amplification was executed with a GeneAmp PCR System 9700 (Applied Biosystems) thermocycler with the following steps: predenaturation at 94°C for 5 min, followed by 5 successive cycles of denaturation at 94°C for 50 s, annealing at 35°C for 50 s, and extension at 72°C for 60 s; 25 successive cycles of denaturation at 94°C for 50 s, annealing 50°C for 50 s, and extension at 72°C for 60 s; a final extension at 72°C for 10 min; and storage at 10°C. The whole process used negative controls to control for contamination. We performed gel electrophoresis and silver staining as described in previous works on calla lily [Wei et al. 2012, 2016, 2017a, b]. Eight percent nondenaturing polyacrylamide gel electrophoresis (PAGE) was used to separate PCR products with Tris/borate/EDTA (TBE) buffer at 150 V for one hour. The primer pair combinations that produced clear, unique and polymorphic bands were screened for all SRAP-PCR amplification samples.

Data analysis. Amplified fragments were scored as present (1) or absent (0) based on particular mobilities on nondenaturing polyacrylamide gels for all samples. The "1, 0" matrix was used to assess the level of genetic diversity in POPGENE version 1.32. The genetic diversity parameters, including the total number of loci (*N*), the number of polymorphic loci (*Np*), the percentage of polymorphic loci (*Pp*), the effective number of alleles (*Ne*), Nei's diversity index (*H*), and the Shannon information index (*I*), were acquired at both the genetic population and country level. A neighbor-joining (NJ) dendrogram was constructed to

Table 2. SRAP primer sequences used to analyze the 38 double-flowered amaryllis accessions

Forward primer	Sequence (5' to 3')	Reverse primer	Sequence (5' to 3')
Me01	TGAGTACAAACCGGATA	Em01	GACTGCGTACGAATTATT
Me03	TGAGTACAAACCGGAAT	Em03	GACTGCGTACGAATTGAC
Me04	TGAGTACAAACCGGACC	Em04	GACTGCGTAcGAATTTGA
Me05	TGAGTACAAACCGGAAG	Em05	GACTGCGTACGAATTAAC
Me06	TGAGTACAAACCGGTAG	Em06	GACTGCGTACGAATTGCA
Me07	TGAGTACAAACCGGTTG	Em07	GACTGCGTACGAATTATG
Me08	TGAGTACAAACCGGTGT	Em08	GACTGCGTACGAATTAGC
Me09	TGAGTACAAACCGGTCA	Em09	GACTGCGTACGAATTACG
Me10	TGAGTACAAACCGGTAA	Em10	GACTGCGTACGAATTCAA
Me11	TGAGTACAAACCGGTCC	Em11	GACTGCGTACGAATTTCC
Me12	TGAGTACAAACCGGTGA	Em12	GACTGCGTACGAATTGTC
Me13	TGAGTACAAACCGGCAT	Em13	GACTGCGTACGAATTGGT
Me14	TGAGTACAAACCGGTCT	Em14	GACTGCGTACGAATTCAG
Me15	TGAGTACAAACCGGAAA	Em15	GACTGCGTACGAATTCTG
Me16	TGAGTACAAACCGGCTA	Em16	GACTGCGTACGAATTCGC
Me17	TGAGTACAAACCGGAAC	Em17	GACTGCGTACGAATTCCA
Me18	TGAGTACAAACCGGTGC	Em18	GACTGCGTACGAATTCAT
Me19	TGAGTACAAACCGGCAG	Em19	GACTGCGTACGAATTCCT
Me20	TGAGTACAAACCGGACA	Em20	GACTGCGTACGAATTTCA
Me21	TGAGTACAAACCGGACT	Em21	GACTGCGTACGAATTTCG
Me22	TGAGTACAAACCGGGTA	Em22	GACTGCGTACGAATTGAG
Me23	TGAGTACAAACCGGACG	Em23	GACTGCGTACGAATTCAC
Me24	TGAGTACAAACCGGAGA	Em24	GACTGCGTACGAATTGCC
Me25	TGAGTACAAACCGGGAC	Em25	GACTGCGTACGAATTCTA
Me26	TGAGTACAAACCGGAGG	Em26	GACTGCGTACGAATTCTT
Me27	TGAGTACAAACCGGGGT	Em27	GACTGCGTACGAATTCTC
		Em28	GACTGCGTACGAATTCGA
		Em29	GACTGCGTACGAATTCGG

Table 3. Characteristics of SRAP primers in 38 double-flowered amaryllis accessions

Primer pairs	*N*	*Np*	*Pp* (%)	*Ne*	*H*	*I*
Me03/Em11	8	8	100.0%	1.504	0.298	0.455
Me02/Em14	6	6	100.0%	1.500	0.297	0.452
Me03/Em09	7	7	100.0%	1.523	0.320	0.490
Me01/Em09	14	13	92.9%	1.496	0.301	0.459
Me05/Em03	6	6	100.0%	1.580	0.349	0.528
Me03/Em13	6	6	100.0%	1.467	0.294	0.455
Me07/Em05	9	8	88.9%	1.513	0.297	0.445
Me12/Em02	7	7	100.0%	1.177	0.140	0.255
Me18/Em10	8	7	87.5%	1.393	0.240	0.374
Me15/Em08	11	11	100.0%	1.487	0.305	0.472
Me13/Em08	11	11	100.0%	1.481	0.290	0.445
Me19Em14	10	9	90.0%	1.361	0.231	0.367
Me06/Em07	14	14	100.0%	1.433	0.268	0.420
Me18/Em09	16	16	100.0%	1.600	0.346	0.516
Me02/Em03	12	8	66.7%	1.413	0.231	0.340
Me06/Em11	9	9	100.0%	1.724	0.397	0.578
Me03/Em04	16	15	93.8%	1.353	0.222	0.355
Me04/Em05	12	12	100.0%	1.596	0.353	0.528
Me15/Em04	10	5	50.0%	1.390	0.214	0.308
Me04/Em04	6	4	66.7%	1.356	0.218	0.335
Me06/Em05	10	9	90.0%	1.669	0.364	0.526
Me09/Em11	6	4	66.7%	1.226	0.153	0.249
Me04/Em11	9	8	88.9%	1.349	0.208	0.330
Me01/Em04	8	6	75.0%	1.468	0.268	0.399
Me13/Em06	13	7	53.9%	1.221	0.142	0.225
Me04/Em02	17	17	100.0%	1.617	0.358	0.534
Me13/EM01	10	9	90.0%	1.615	0.343	0.501
Me05/Em11	10	9	90.0%	1.457	0.289	0.445
Me17/Em15	7	5	83.3%	1.275	0.192	0.315
Me08/Em12	7	7	100.0%	1.333	0.232	0.377
Total	294	263	89.2%	43.577	8.158	12.475
Average	9.84	8.77	88.4%	1.453	0.272	0.416

N – number of loci; *Np* – number of polymorphic loci; *Pp* – percentage of polymorphic loci; *Ne* – effective number of alleles; *H* – Nei's diversity index; *I* – Shannon information index

examine the genetic relationships among all accessions using the software packages PowerMarker version 3.25 [Liu and Muse 2005] and MEGA 4 [Tamura et al. 2007]. The population structure of the 38 samples was also analyzed to determine group and individual distributions by STRUCTURE 2.3 [Pritchard et al. 2000]. The length of the burn-in period and number of Markov chain Monte Carlo (MCMC) iterations after the burn-in were set to 10 000 and 500 000, respectively. Algorithm simulation was performed ten times for each K, which ranged from 1 to 10. The optimal *K*-value for the data set was assessed using Δ*K* as described by Evanno et al. [2005]. In addition, analysis of molecular variance (AMOVA) among countries and inferred populations was performed using GenAlEx 6.5, with 1000 permutations used to test variance components.

RESULTS

SRAP loci. Thirty SRAP primer combinations yielded clear, high-stability polymorphic loci (Fig. 1); the total number of total loci (*N*), the number of polymorphic loci (*Np*), the percentage of polymorphic loci (*Pp*), the effective number of alleles (*Ne*), Nei's diversity index (*H*), and the Shannon information index (*I*) are shown in Table 3. Amplification of the thirty SRAP primers across the 38 amaryllis accessions generated 294 loci, of which 263 (89.16%) were polymorphic. The total number of loci scored per primer combination ranged from 6 (Me2/Eml, Me5/Em3, Me3/Em1, Me4/Em4, and Me9/Em1) to 17 (Me4/Em2), with an average of 9.84 loci per primer combination.

Among these primer pairs, Me15/Em4 generated the lowest percentage of polymorphic loci (50.0%), and 14 primer pairs (Me3/Em1, Me2/Em1, Me3/Em9, Me5/Em3, Me3/Em1, Me12/Em2, Me15/Em8, Me13/Em8, Me6/Em7, Me18/Em9, Me6/Em11, Me4/Em5, Me4/Em2, and Me8/Em2) yielded 100% polymorphic loci. The effective number of alleles (*Ne*) ranged from 1.177 for Me12/Em2 to 1.724 for Me6/Em11. Estimates of Nei's diversity index (*H*) and the Shannon information index (*I*) averaged 0.272 and 0.416 across all SRAP loci, respectively (Tab. 3).

Clustering and population structure of 38 double-flowered amaryllis accessions. Notably, the accessions can be described on the basis of their origin or color (Fig. 2). All of the accessions in Cluster II are from the Netherlands. This sub cluster forms two main groups. The first group comprises 7 accessions, mainly with pink or red/white bicolored flowers, such as Pretty Nymph and Double Record. The second group includes 13 accessions, mainly with red or pink flowers, such as Double King and Double Dream. Similarly, Cluster I comprises 12 accessions, 9 of which are from South Africa and 3 of which are from

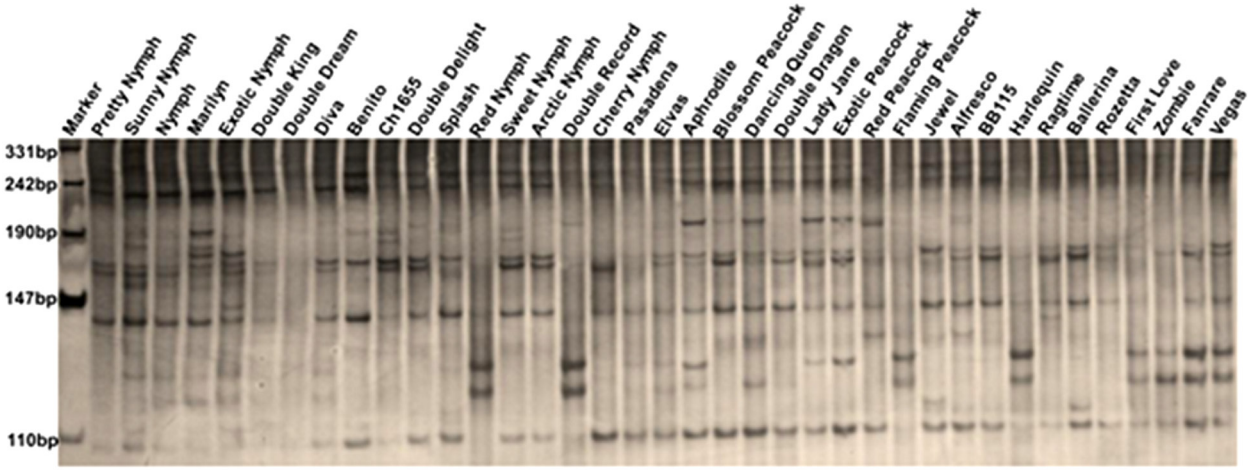

Fig. 1. Amplification profiles of 38 double-flowered amaryllis accessions with primer combination Me6 and Em7

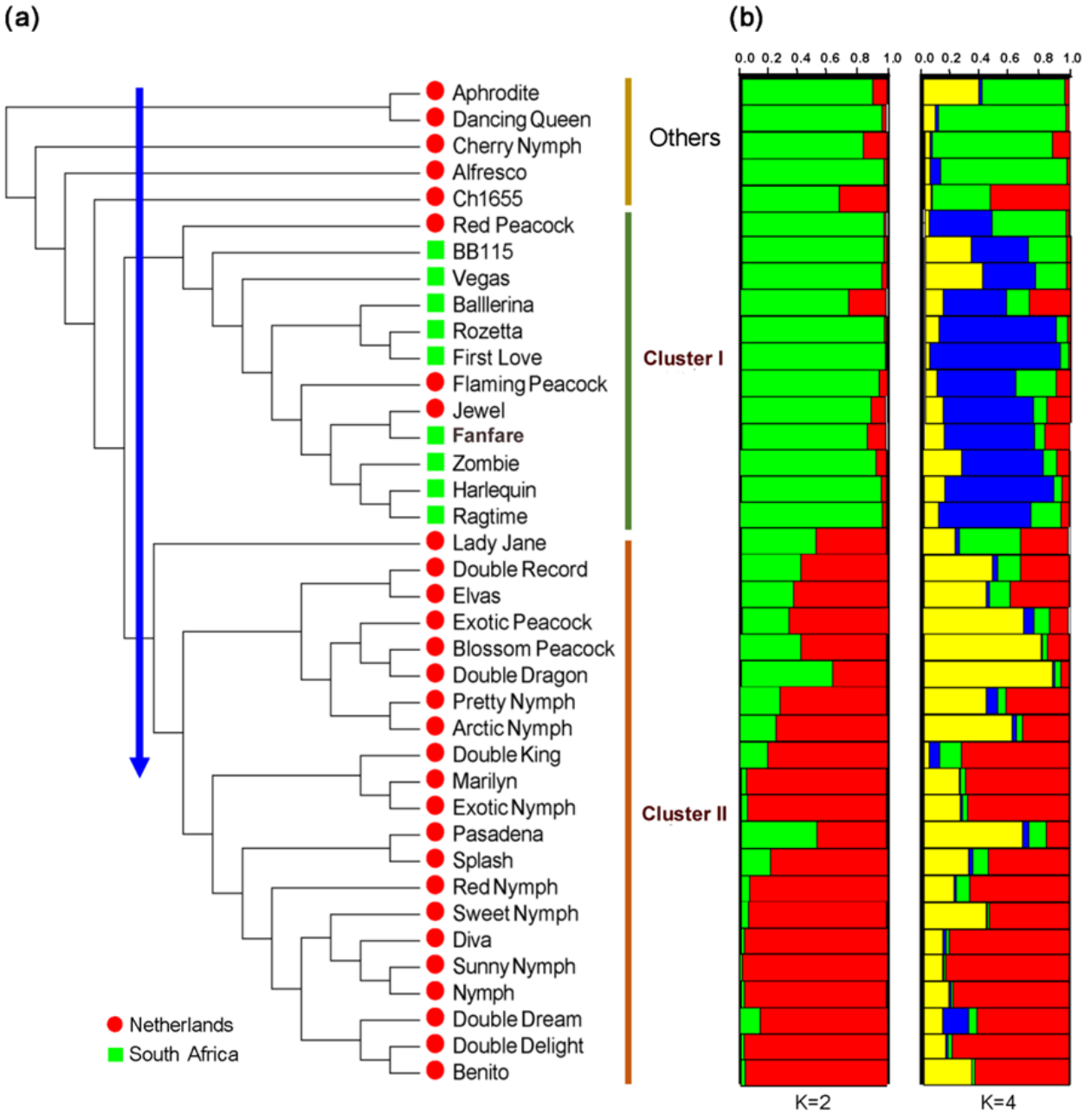

Fig. 2. NJ phylogenetic tree aligned with a structural analysis of 38 amaryllis accessions from the Netherlands and South Africa. (a) NJ tree based on Nei's distance of 30 SRAP markers showing two major groups, namely, Class I (12 accessions) and Class II (21 accessions), and others (5 accessions); (b) clustering of the accessions based on multilocus analysis in STRUCTURE. The two best models (K = 2 and K = 4) on the basis of Evanno's delta. Each individual accession is represented by a vertical line divided into K colored bars

the Netherlands. In contrast to Jewel (white), six accessions, including Red Peacock and Ragtime, have red or pink flowers, while the other five have bicolored flowers, such as Flaming Peacock and First Love. In addition, there are a few other varieties, all of which are from the Netherlands. The color in these groups is complex, even though the group contains only six accessions. The flowers of Aphrodite and Dancing Queen are bicolored (red or pink/white), while the flowers of Ch1655, Cherry Nymph and Alfresco are pink, red and white, respectively.

Admixture model-based simulations embedded in STRUCTURE 2.3 (Pritchard et al. 2000) were also implemented to analyze the population structure of the 38 amaryllis accessions by varying K from 1 to 10. An ad hoc measure (ΔK) was calculated to determine the optimal number of sub clusters. The highest ΔK values were observed for $K = 2$ ($\Delta K = 28.375$) and followed by $K = 4$ ($\Delta K = 11.802$). These indicated that the 38 amaryllis accessions were classified into two and four subtle subgroups, respectively (Fig. 2b).

The genetic component of each accession is illustrated using a bar plot to enable a simple comparison with the NJ tree (Fig. 2). The population partitioning of the 38 double-flowered amaryllis accessions was almost compatible with the NJ clustering results.

Genetic diversity and differentiation within countries and inferred groups of 38 double-flowered amaryllis accessions. The allele number (*Na*), Nei's diversity index (*H*), and Shannon's information index (*I*) were calculated to evaluate the genetic diversity in the countries and inferred groups. As shown in Table 4, relatively large differences in genetic diversity were observed at the country scale. The Netherlands exhibited higher values (*Na* = 1.871, *Ne* = 1.454, *H* = 0.271, and *I* = 0.414) than South Africa (*Na* = 1.643, *Ne* = 1.378, *H* = 0.225, and *I* = 0.338). The reason for this difference might be that the Netherlands was represented by more accessions than South Africa in this study. The pattern was the same for the two inferred subgroups. Based on Q-values of ≥0.60 in the STRUCTURE analysis, the inferred G1 and G2 groups for *K* = 2 contained 18 and 16 accessions, respectively, while the G1, G2, G3 and G4 groups for *K* = 4 comprised 3, 8, 5 and 10 accessions, respectively (Tab. 4). G1 exhibited a larger value for allele number, Nei's diversity index and the Shannon information index (*Na* = 1.840, *Ne* = 1.458, *H* = 0.274,

Table 4. Genetic diversity in different double-flowered amaryllis populations

Source		*Nd*	*N*	*Np*	*Pp* (%)	*Na*	*Ne*	*H*	*I*
Country	Netherlands	29	294	256	87.07	1.871	1.454	0.271	0.414
	South Africa	9	294	189	64.29	1.643	1.378	0.225	0.338
Model-based population (*K* = 2)	G1	18	294	247	84.01	1.840	1.458	0.274	0.416
	G2	16	294	204	69.39	1.694	1.376	0.225	0.342
Model-based population (*K* = 4)	G1	3	294	160	54.42	1.544	1.435	0.242	0.346
	G2	8	294	168	57.14	1.571	1.354	0.208	0.310
	G3	5	294	130	44.22	1.442	1.279	0.167	0.248
	G4	10	294	165	56.12	1.561	1.336	0.199	0.298

Nd – number of individuals; *N* – number of loci; *Np* – number of polymorphic loci; *Pp* – percentage of polymorphic loci; *Ne* – effective number of alleles; *H* – Nei's diversity index; *I* – Shannon information index

Table 5. Analysis of molecular variance (AMOVA) of different subpopulations of double-flowered amaryllis

Source		*df*	*SS*	*MS*	*EV*	*PV*	*Fst*
Country	between countries	1	90.633	90.633	3.659	8.3%	0.083
	within populations	36	1453.525	40.376	40.376	91.7%	
	total	37	1544.158		44.034	100%	
Model-based population ($K = 2$)	among groups	1	135.915	135.915	5.710	12.7%	0.127
	within populations	32	1253.674	39.177	39.177	87.3%	
	total	33	1389.588		44.887	100%	
Model-based population ($K = 4$)	among groups	3	258.679	86.226	8.395	19.4%	0.194
	within populations	22	765.167	34.780	34.780	80.6%	
	total	25	1023.846		43.175	100%	

df – degrees of freedom; *SS* – sum of squares; *MS* – mean of squared observations; *EV* – estimated variance; *PV* – percentage of variance; *Fst* – fixation index

I = 0.416) than G1 (Na = 1.694, Ne = 1.376, H = 0.225, I = 0.342). However, this result was not observed for the four inferred groups. Despite G1 and G2 having only three and 8 accessions, respectively, they showed the highest abundance and evenness of all double-flowered amaryllis accessions (Na = 1.544, Ne = 1.435, H = 0.242, and I = 0.346 and Na = 1.571, Ne = 1.354, H = 0.208, and I = 0.310, respectively), suggesting that their breeding origin was complex.

Significant differentiation ($P < 0.01$, Tab. 5) was detected between/among the countries/inferred groups by AMOVA. The percentage of variance (*PV*) among groups ranged from 8.3–19.4%. The result was the same for genetic differentiation (*Fst*). This consistency indicates that the total genetic diversity can be attributed to the differences within countries and inferred subgroups.

DISCUSSION

Molecular markers, including random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers, have been used to estimate the genetic diversity and relationships among different *Hippeastrum* species and cultivars [Chakrabarty et al. 2007, Zhang et al. 2012, Phuong et al. 2014, Wang et al. 2018]. In this study, we used SRAP markers to provide insight into the pattern of genetic variation in double-flowered *Hippeastrum* cultivars from the Netherlands and South Africa. SRAP technology involves the preferential random amplification of coding regions in the genome [Li et al. 2001]. The information derived from these markers is more concordant with the morphological variability and evolutionary history of morphotypes than that derived from other types of molecular markers [Ferriol et al. 2003].

In our investigation, thirty combinations of primer pairs produced a relatively large number of polymorphic bands (263) among 38 accessions, which may indicate a high level of genetic diversity within the double-flowered germplasm of amaryllis. The number of polymorphic loci (88.4%) across the SRAP markers was higher than that detected in previous studies focused on RAPD markers in 25 accessions [72.6%, Phuong et al. 2014] but lower than that

detected with ISSR [92.4%; Zhang et al. 2012] and SSR [93.9%; Wang et al. 2018] markers, for which 62 and 104 accessions were used, respectively. This difference may be attributed to the number of amaryllis germplasms employed for genetic assessment. However, it is surprising that the average estimates of *H* (Nei's diversity index) and *I* (Shannon information index) in our research were higher (H = 0.272; I = 0.416) than those reported in the abovementioned SSR study (H = 0.264; I = 0.407), despite those in the latter being estimated with more accessions [Wang et al. 2018]. The genetic parameters *H* and *I*, which account for both the abundance and evenness of the species or hybrids present, are commonly used to characterize the germplasm diversity in a community. These features could explain why more genetic heterogeneity was detected in our 38 cultivated *Hippeastrum* accessions. In addition, a recent study verified relatively high genetic diversity and at least five subgroups in 104 amaryllis cultivars based on 21 SSR markers [Wang et al. 2018]. However, complex patterns were also verified in present double-flowered amaryllis panel (K = 2 or K = 4), although only 38 accessions were included. This may be due to several factors, such as the use of wild species and polyploid commercial varieties in current breeding programs [Bell 1977, Khaleel et al. 1991, Meerow 1988, 2009, Liu et al. 2015b].

A previous study pointed out that the center of amaryllis breeding shifted from the Netherlands to South Africa [Meerow et al. 1988]. However, in our study, only a limited number of South African double-flowered accessions (9) were collected; more were collected from the Netherlands (29). This discrepancy in sample size may explain why the South African accessions showed lower genetic diversity than the Dutch ones. In addition, we also showed that most of the 38 accessions could be clustered on the basis of their country of origin in genetic distance-based analyses. The accessions from the Netherlands tended to fall into Cluster II, while the South African ones tended to fall into Cluster I. The breeding of double-flowered cultivars seems to be conducted only at a small scale, although such cultivars have significant market appeal in flower catalogs.

Bell [1977] summarized that the rewards are not sufficient to encourage much activity in this area of breeding. This lack of activity is probably due to most hybrid combinations, such as those created using a double-flowered type as the male, do not easily produce seeds and seedlings [Bell 1977, Shi et al. 2014]. Therefore, it is important to find interesting combinations of polyploid hybrid or species germplasm that can be used in double-flowered amaryllis breeding programs. The Dutch accessions, such as Aphrodite, Dancing Queen, Cherry Nymph, Alfresco and Ch1655, were distantly genetically separated from the others in the NJ phylogenetic tree. Previous observations of the inheritance of doubleness indicated that it was dominant in amaryllis [Bell et al. 1977]. Selective hybridization with these hybrids should therefore be a priority in the future, which would enable us to plan breeding programs with the maximum output of genetic diversity. Moreover, we strongly recommend that this program focus on novel attributes, such as flower color, because a narrow color range (only red, pink and/or white) is currently available in the dominant commercial accessions. Finally, as Bell et al. [1977] previously stated, the lottery of genetic recombination is unchanged during crossbreeding, but our present results indicate the importance of strategies that minimize the randomness of the input in this breeding system.

CONCLUSIONS

In present study, SRAP markers were demonstrated to be effective and reliable in evaluating the genetic diversity and population structure of 38 double-flowered amaryllis accessions. The results of this study provide valuable information for double-flowered amaryllis germplasm collection and genetic improvement.

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REFERENCES

Bell, W.D. (1977). Double flowered amaryllis. Proc. Fla. State Hort. Soc., 90, 121–122.

Chakrabarty, D., Gupta, V.N., Datta, S.K. (2007). Varietal identifcation and assessment of genetic relationships in *Hippeastrumusing* RAPD markers. Plant Biotech. Rep., 1, 211–217, DOI: 10.1007/s11816-007-0034-3.

Evanno, G., Regnaut, S., Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mole. Eco., 14, 2611–2220, DOI: 10.1111/j.1365-294X.2005.02553.x.

Ferriol, M., Pico, B., Nuez, F. (2003). Genetic diversity of a germplasm collection of Cucurbita pepo using SRAP and AFLP markers. Theor. Appl. Genet., 107, 271–282, DOI: 10.1007/s00122-003-1242-z.

Khaleel, T.F., Haven, S., Gilg, T. (1991). Karyomorphology of *Amaryllis* hybrids. Cytologia, 56, 31–41, DOI: 10.1508/cytologia.56.31.

Li, G., Quiros, C.F. (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor. Appl. Genet., 103, 455–461, DOI: 10.1007/s001220100570.

Liu, K.J., Muse, S.V. (2005). PowerMarker: An integrated analysis environment for genetic marker analysis. Bioinformatics, 21, 2128–2129, DOI: 10.1093/bioinformatics/bti282.

Liu, M.C., An, C.H., Yeh, D.M. (2015a). Anther type and pollen germination of double-flowered amaryllis (*Hippeastrum* hybridum) cultivars. Seeds Nurs., 17, 35–46.

Liu, M.C., Yeh, D.M. (2015b). 'TSS. No.1-Pink Pearl': A double-flowered and fragrant amaryllis cultivar. Hortscience, 50, 1588–1590, DOI: 10.21273/HORTSCI.50.10.1588.

McCann, J.J. (1937). New double hybrid amaryllis. Herbertia, 4, 185–186.

Meerow, A. (1999). Amaryllis and Alstroemeria: old crops, new potential. University of Florida, Institute of Food and Agricultural Sciences. Fort Lauderdale Research and Education Center.

Meerow, A.W. (1988). New trends in amaryllis (*Hippeastrum*) breeding. Proc. Fla. State Hort. Soc., 101, 285–288.

Meerow, A.W. (2009). Tilting at windmills: 20 years of *Hippeastrum* breeding. Isr. J. Plant Sci., 57, 303–313.

Meyerowitz, E.M., Smyth, D.R., Bowman, J.L. (1989). Abnormal flowers and pattern formation in floral development. Development, 106, 209–217, DOI: 10.1242/dev.106.2.209.

Phuong, P.T.M., Isshiki, S., Miyajima, I. (2014). Genetic variation of *Hippeastrum* accessions in Vietnam. J. Fac. Agri. Kyushu Univ., 59, 235–241, DOI: 10.5109/1467623.

Pritchard, J.K., Stephens, M., Donnelly, P. (2000). Inference of population structure using multilocus genotype data. Genetics, 155, 945–959, DOI: 10.1093/genetics/155.2.945.

Shi, F.R., Xue, J.Q., Mu, D., Wang, S.L., Zhang, X.X. (2014). Studies on differences of hybridization efficiency and related reasons of 17 *Hippeastrum* cultivars. Acta Hortic. Sinica, 41, 553–563, DOI: 10.16420/j.issn.0513-353x.2014.03.017.

Szilard, P. (2013). Getting your *Hippeastrum*(Amaryllis) to bloom. Trop. Plant Soc., Denver, 2/10a.

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599, DOI: 10.1093/molbev/msm092.

Wang, Y., Chen, D.F., He, X.F., Shen, J.X., Xiong, M., Wang, X., Zhou, D., Wei Z.Z. (2018). Revealing the complex genetic structure of cultivated amaryllis (*Hippeastrum* hybridum) using transcriptome-derived microsatellite markers. Sci. Rep., 8, 10645, DOI: 10.1038/s41598-018-28809-9.

Wei, Z.Z., Luo, L.B., Zhang, H.L., Xiong, M., Wang, X., Zhou, D. (2012). Identification and characterization of 43 novel polymorphic EST-SSR markers for arum lily, *Zantedeschia aethiopica* (Araceae). Am. J. Bot., 99, e493–497, DOI: 10.3732/ajb.1200228.

Wei, Z.Z., Sun, Z.Z., Cui, B.B., Zhang, Q.X., Xiong, M., Wang, X., Zhou, D. (2016). Transcriptome analysis of colored calla lily (*Zantedeschia rehmannii* Engl.) by Illumina sequencing: *de novo* assembly, annotation and EST-SSR marker development. PeerJ, 4, e2378, DOI: 10.7717/peerj.2378.

Wei, Z.Z., Sun, Z.Z., Xiong, M., Wang, X., Zhou, D. (2017a). Characterization and cross-species transferability of EST-SSRs from the illumina-based *de novo* transcriptome sequencing of arum lily (*Zantedeschia aethiopica*). Acta Hortic., 1171, 175–186, DOI: 10.17660/ActaHortic.2017.1171.24.

Wei, Z.Z., Zhang, H.L., Wang, Y., Li, Y.L., Xiong, M., Wang, X., Zhou, D. (2017b). Assessing genetic diversity and population diferentiation of colored calla lily (*Zantedeschia* hybrid) for an efcient breeding program. Genes, 8, 168, DOI: 10.3390-genes8060168.

Zhang, L., Xu, Y.C., Cheng, H.Z., Zhou, Y.Z. (2012). Genetic relationship analysis and fngerprint construction of 62 cultivars of *Hippeastrum* spp. based on ISSR marker. J. Plant Resour. Environ., 21, 48–54.