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Genetic Divergence And Relationship Analysis Among Twenty Two Populations Of Gladiolus Cultivars By Morphological And Rapd-Pcr Tool

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ABSTRACT:
Gladiolus, the queen of flowers has revolutionized the world floriculture trade, occupying the important place in the Indian floriculture trade next to rose. However, the main emphasis is on the development of improved varieties. For making further improvement, it is essential to explore the range of variability present in the crop. Greater variability ensures better chances of selecting new improved forms. In this study, genetic variability diversity was undertaken involving twenty two diverse genotypes of Gladiolus to estimate the relative extent of genetic variability. RAPD analysis was applied in studying the diversity and genetic relationship of 22 cultivars of Gladiolus. Nine morphological traits and 125 random amplified polymorphic DNA (RAPD) markers amplified with 25 arbitrary primers were employed to discriminate between the cultivars and to evaluate the relatedness between them. A total of 125 RAPD bands (250–3000 bp) were obtained of which 111 (93.78%) were polymorphic. Number of bands generated per primer ranged from 4 (OPA-8) to 14 (OPO-4) with an average of 9 bands. . For morphological data, Euclidean distances were calculated and an unweighed pair-group method using an arithmetic average was used to construct a dendrogram. The genotypes with superior commercial characters, such as spike length, rachis length, floret diameter etc. were clustered together. At a distance of 2.65, three clusters could be obtained. The lowest distance value was observed between cultivar 'Happy End' and 'Melody' (0.84). 'Her Majesty' and 'Jester' branched out from the dendrogram showing that they are quite different from other genotypes and this result was also supported by PCA analysis This study provided some references for the classification, genetic relationship analysis and breeding selection of cultivars on molecular level, which should facilitate the production and breeding of Gladiolus in the near future. However, incongruence existed between these cluster analyses, and the relationships between some cultivars require further investigation.
Key words: Gladiolus, RAPD, Polymorphic, Genetic variability, PCR analysis, Dendrogram

INTRODUCTION:

Gladiolus is one of the most widely cultivated, economically important flowering plants. The exuberance of colourful spikes of Gladiolus is a delight in any floral bouquet and luxuriance unique colorful spikes of some high demanding Gladiolus cultivars have attained immense importance in the community of flower lovers. It occupies a pristine place in the garden for its magnificent inflorescence, wide array of colours, and fascinating varieties of shapes and sizes. It ranks fifth next to tulip (Tulipa spp), lily (Lilium spp), freesia (Freesia spp) and hippeastrum (Hippeastrum spp) among the geophytes in international florist trade (Flower Council of Holland 2008) and first in domestic bulbous flower trade. The species and varieties of it are numerous since the genus Gladiolus includes 180 species with more than 10,000 cultivars (Sinha and Roy, 2002). The numbers are rising every year through hybridization, with the aim of extending vase life, producing novel colours, floret arrangement on the spikes and to prolong the flowering period (Kumar et al., 1999). In India, about 35000 ha crop production is under bulbous ornamental plants with maximum area being under Gladiolus (12000 ha) followed by Tuberose (800 ha) (Deshraj 2001).

As the number of Gladiolus cultivars is continuously increasing, the newly constituted cultivars need to be more intensively monitored for novelty, distinctness, uniformity, stability and molecular markers useful for the protection of Plant Breeder's Rights. The absence of a scientific and valid method of classification of Gladiolus always results in a loss in production, popularizing, communicating and scientific studies, which makes it necessary to have a survey into classification and genetic relationship among the cultivars of Gladiolus. For developing improved varieties it is essential to explore the range of inability present in the crop. If the relationship among cultivars is not known, variability assessment and validation of genetic relatedness among different varieties becomes important to proceed for crop improvement programme. This would make protection of new Gladiolus cultivars more specific and effective.

Molecular approaches collectively represent a potential tool that can be applied for effective characterization of germplasm. It addresses the limitations associated with morphological and biochemical processes (Ferdousi Begum etal. 2013; Popham, 1951). A common approach for assessing levels of genetic diversity is the use of molecular markers such as randomly amplified polymorphic DNA (RAPD) (Pullaiah, 1979; Neeraj etal. 2010) RAPD technique is simple, reliable, efficient, and an economical means of cultivar identification and diversity analysis (Fan Li etal., 2012). RAPDs have previously been used successfully to assess levels of genetic diversity genotype identification in several plants including Lilium species (Hussain etal., 2012), Agave tequilana var. Azul (Pullaiah, 1979), Heliconia (Popham, 1951) and Gladiolus species (Majd etal., 2013).
At present based on our knowledge, there is not much report on the diversity analysis in Gladiolus cultivars using DNA markers, therefore our study was undertaken to assess the genetic diversity among Gladiolus population using RAPD-PCR.

MATERIALS AND METHODS

A total of 22 Gladiolus cultivars representing majority of varieties under cultivation in India were analyzed using morphological traits and selected RAPD markers. The materials were planted in randomized block design with 2 replications at Floriculture Section, Department of Horticulture and Department of Genetics and Plant Breeding of Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. During summer and winter season of 2011–2012 and 2012-2013 for morphological characterization. The experimental site is situated at 29°N latitude, 79.3°E longitude and at altitude of 243.84 metres above mean sea level in Tarai belt of Shivalik range of Himalayas. The soil of the experimental site was well drained sandy loam having pH 7.0 and temperture varies between from 30°C to 43 °C in summer, 0^{o}C to 9°C during winter months.
The name, origin and collection of place of the cultivars is given in Table 1. The two replications and each genotype had 10 plants or corms per replication. Each replication had two rows out of which six corms were selected randomly and observations were recorded.

Table 1: List of genotypes of Gladiolus with their origin and place of collection

Name	Origin	Place of collection
American Beauty	USA	NBRI, Lucknow
Candyman	USA	PAU, Ludhiana
Eurovision	USA	NBRI, Lucknow
Happy End	_	NBRI, Lucknow
Her Majesty	India	NBRI, Lucknow
Invitation	USA	PAU, Ludhiana
Jester	USA	NBRI, Lucknow
Melody	Netherlands	PAU, Ludhiana
Novalux	_	PAU, Ludhiana
Oscar	Netherlands	NBRI, Lucknow

Pink Friendship	Dutch	IARI, New Delhi
Poppy Tear	_	PAU, Ludhiana
Red Beauty	UK	PAU, Ludhiana
Rose Supreme	USA	PAU, Ludhiana
Shobha	India	IARI, New Delhi
Snowprincess	Holland	NBRI, Lucknow
Spic and Span	_	PAU, Ludhiana
Subhangini	India	GBPAUT, Pantnagar
Sylvia	Netherlands	NBRI, Lucknow
Trader Horn	_	PAU, Ludhiana
White Frienship	_	NBRI, Lucknow
White Prosperity	USA	NBRI, Lucknow

Nine morphological traits, viz flower colour, plant height, spike length, rachis length, floret diameter, number of florets/spike, number of corms/plant, number of cormels/plant and weight of corm were recorded. All the morphological characters were scored as quantitative traits except flower colour. Data for each trait were collected from 10 to 15 plants on a single day to minimize experimental error.

DNA Extraction

Genomic DNA was extracted from fresh leaf samples and samples are stored at –80°C prior to DNA extraction. Total genomic DNA was extracted as per the protocol of Saghai-Maroof et al. (1984) using 4 g finely ground leaf tissue in 20 ml of DNA extraction buffer. The quality of DNA was determined using agarose gel (1%) electrophoresis.

RAPD-PCR

The RAPD technique cosistes of peferential amplification of random sequences by PCR. Twenty-five random decamer primers were selected from 100 primers after pre-screening the commercially available kits (Operon A, B, C, D, M, O and P series) for DNA amplification. The RAPD was performed in 25 µl reaction mixture containing 1X PCR assay buffer, 2.5 mM $MgCl_2$, 20 ng template DNA, 0.2 mM dNTPs, 0.06 µM of the decamer primer and 1 U Taq DNA polymerase (all chemicals were from M/S Bangalore Genei Ltd, Bangalore). Amplifications were performed for an initial denaturation step at 94°C for 4 min., followed by 40 cycles at 94°C for 1 min. 35°C for 1 min. and 72°C for 2 min. with a final extension step at 72°C for 5 min. Separation of the amplified fragments was performed on 1.8% agarose gels, TAE 1% at 80 V during 2 h. The gels were stained with 1 µl-1 ethidium bromide for visualizing the RAPD fragments and were photographed under UV light of wave length 325 nm. DNA molecular weight marker, 1kb DNA ladder was loaded in each gel to estimate the sizes of bands. Each RAPD band was visuallised. RAPD bands sizes were designated as amplified bands, and bands were shared as diallelic characters (present = 1 and absent = 0). The binary data were used for statistical analysis. Morphological data was standardized before Euclidean morphological distances were calculated (Wen et al. 2004), and the distance matrix was subjected to Unweighed Pair Group Method with Arithmetic Average (UPGMA) to construct the dendrogram and cladogram. The robustness of the nodes of dendrogram was tested by bootstrap analyses using 1000 samplings (Pavlicek et al. 1999). The correlations among variables was subjected to 'Eigen vectors' analyses and the resulting Eigen values were projected on 3-dimensional plane to obtain a 3-dimensional plot of first 3 principal components. NTSYS-pc (numerical taxonomy and multivariate analysis system) (Rohlf 1993) progamme is used for all statistical analysis.

RESULTS AND DISCUSSION

A total of 22 Gladiolus cultivars representing majority of varieties under cultivation in India (Table 2 and 3) were analyzed using morphological traits and selected RAPD markers. Genomic DNA was isolated from fresh leaf tissues and analysed for all the 9 morphological traits, wide variation was recorded. Although

ornamentals could be identified by their morphological traits, the easiest practical method of identification so far has been by comparing colour photographs of the inflorescence as in case of Heliconia (Berry and Kress 1991). On the basis of spike colour, all cultivars could be distinguished. The genotypes with superior commercial characters, such as spike length, rachis length, floret diameter etc. were clustered together. At a distance of 2.65, three clusters could be obtained. The lowest distance value was observed between cultivar 'Happy End' and 'Melody' (0.84). 'Her Majesty' and 'Jester' branched out from the dendrogram showing that they are quite different from other genotypes. Cluster I was dominated with exotic cultivars and was divided into 2 sub-clusters with variation from peachish white to pinkish red coloured genotypes. Besides this, five cultivars, namely 'White Prosperity', 'Happy End', 'White Friendship' 'Jester' and 'Red Beauty' reflected close similarity in their morphological characters. However, their closeness could not be supported by their parental relationships. The cluster II was formed by cultivars, 'Her Majesty' and 'Jester' both of them are phenotypically very different with flower colours yellow and purple, but genotypically, they out branched from rest of the cultivars. Cluster III is constituted by a major group of cultivars including, ''American Beauty', Candyman', 'Happy End', 'Melody', 'Pink Friendship, 'Subhangini', 'Red beauty', 'Trader Horn', 'Poppy Tear', 'White Friendship', 'Novalux', 'Snow Princess', 'Oscar', 'Spic and Span', 'Sylvia' and 'White Prosperity'. In cluster III, 'Spic and Span' and 'Subhangini' were found similar with respect to almost all morphological features including flower colour to some extent (Table 2 & 3).

Table 2: Morphological characters of 22 Gladiolus cultivars

Sl No.	Cultivar	Flower colour
1.	American Beauty	Light peach
2.	Candyman	Purplish red
3.	Eurovision	Red
4.	Happy End	Red with white throat
5.	Her Majesty	Purple
6.	Invitation	Pink
7.	Jester	Light yellow with red thr
8.	Melody	Peach with red throat
9.	Novalux	Yellow
10.	Oscar	Soft red
11.	Pink Friendship	Peach with white tip
12.	Poppy Tear	Purplish pink
13.	Red Beauty	Red
14.	Rose Supreme	Light peach
15.	Shobha	White with red stripes
16.	Snow Princess	Milky white
17.	Spic and Span	Off white
18.	Subhangini	White
19.	Sylvia	Red with white tip
20.	Trader Horn	Red
21.	White Friendship	White
22.	White Prosperity	White

Table 3: Morphological characters of 22 Gladiolus cultivars

Cultivar	Plant height (cm)	Spike length (cm)	Rachis length (cm)	Floret diamete (cm)	Florets spike	Corms plant	Cormel plant	Weight of Corms (g
American Beaut	83.46	60.70	48.27	10.10	8.28	1.08	9.34	20.13
Candyman	116.17	62.51	54.58	9.37	7.23	1.47	19.94	25.21
Eurovision	111.56	58.67	44.78	8.01	7.40	1.07	19.48	22.50
Happy End	117.58	64.65	46.14	8.57	12. 14	2.52	12.42	25.18
Her Majesty	103.48	55.38	45.30	9.34	7.56	1.02	11.31	16.24
Invitation	102.35	49.88	25.57	9.45	7.52	1.68	19.51	18.15
Jester	119.37	57.63	45.24	8.25	13.09	2.54	23.41	17.47
Melody	119.19	40.25	30.62	8.16	8.41	1.82	21.02	16.25
Novalux	107.45	45.67	31.85	8.38	8.25	1.20	20.38	18.51
Oscar	139.26	48.82	35.19	9.57	9.26	1.88	14.44	19.42
Pink Friendship	109.41	58.78	50.38	8.10	9.50	1.89	8.30	17.82
Poppy Tear	108.40	60.31	40.65	8.50	14.36	1.48	15.31	24.50
Red Beauty	120.11	56.37	39.74	9.31	10.12	1.39	15.72	22.16
Rose Supreme	111.41	52.34	30.35	8.23	9.50	1.07	12.11	30.29
Shobha	120.36	68.61	47.39	10.88	9.19	1.30	10.11	25.72
Snow Princess	119.56	69.20	48.17	11.76	8.04	1.46	11.43	30.95
Spic and Span	118.57	45.67	34.80	7.34	9.03	1.08	12. 19	25.26
Subhangini	120.13	48.68	28.54	8.32	8.25	0.99	20.11	20.23
Sylvia	112.34	58.81	32.58	8.16	9.23	1.89	20.14	19.19
Trader Horn	110.66	60.25	50.10	8.31	8.19	1.20	12.98	29.18
White Friendshi	120.16	57.81	37.34	9.01	12.68	1.54	19.38	30.04
White Prosperity	129.48	78.38	66.14	9.71	10.12	1.27	40.69	19.44

RAPD Analysis

The Gladiolus cultivars were characterized using morphological traits and RAPDs to estimate the genetic relationships between the selected genotypes. The utility of RAPD markers in estimating genetic variability has been demonstrated in several studies. A similar study was done in Withania somnifera by Bilal et al. (2010). 7 populations of W. coagulans from the districts of Kohat and Karak in Pakistan were analyzed by Syed (2009). Nine morphological traits and 125 random amplified polymorphic DNA (RAPD) markers amplified with 25 arbitrary primers were employed to discriminate between the cultivars and to evaluate the relatedness between them. A total of 125 RAPD bands (250–3000 bp) were obtained of which 111 (93.78%) were polymorphic. Number of bands generated per primer ranged from 4 (OPA-8) to 14 (OPO-4) with an average of 9 bands. The amplification products obtained with primer OPD-20 are illustrated in Fig. 1. Pair-wise genetic distances based on RAPD data for 22 Gladiolus cultivars ranged from 0.407 to 0.782 indicating high diversity of genetic relationships among cultivars.

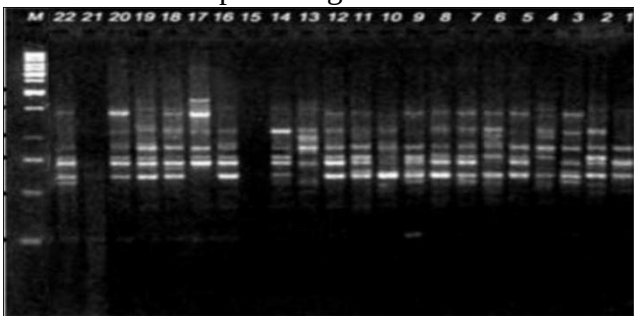

Fig 1. RAPD profile of 22 Gladiolus cultivars amplified with primer OPD-20. Refer Table 4.40 for identity code of these cultivars. Lane M, DNA marker

GENETIC RELATIONSHIPS AMONG CULTIVARS:

A wide range of analysis methods (UPGMA, bootstrapping and PCA) was used to provide detailed insights into the genetic relationships. UPGMA tree based on RAPD data (Fig 2) reflected three major clusters, which is supported by UPGMA tree on morphological traits (Fig 3) shown by the cladogram. In both the cluster analyses, 'Her Majesty' and 'Jester' branched out from the dendrogram as well from cladogram, confirming that they are quite different from the other genotypes. However, at molecular level, 'American Beauty' and 'Candyman' clustered together (J= 0.74), indicating a close genetic relationship. Nevertheless, 'Candyman' has purplish red florets which is different from 'American Beauty' with light peach florets. Thus, the genetic closeness could not be supported by their parental relationships. This could have happened due to highly heterozygous nature of this crop. Cluster I of both the dendrogram and cladogram was formed by almost same group of cultivars. A further comparison of morphological characters indicated that all the cultivars in this group had spikes of more than 40 cm with medium sized florets (>8.0 cm) and colours ranging from light-pink to peach or red to purple. However, out of 16 genotypes in cluster III, genetic closeness of only 5 ('Happy End', 'Red Beauty', 'Shobha', 'Subhangini' and 'White Friendship') was supported by their phenotypic expressions. Furthermore, cultivar pairs 'American Beauty' and 'Candyman', 'White Friendship' and 'Poppy Tear', 'Oscar and Spic and Span', 'Sylvia' and 'White Prosperity', 'Novalux' and 'Snow Princess, 'Red Beauty' and 'Trader Horn', 'Subhangini' and 'Pink Friendship', 'Happy End' and 'Melody' were very close in both the dendrogram as well as cladogram indicating that they are quite similar to each other. Due to the lack of pedigree information of many of these cultivars the above said results could not be supported by their parental relationships. However, considering the highly heterozygous nature of the crop and consistency of morphological data with RAPD data, it can be concluded that both morphological traits and RAPD markers were suitable for analyzing the genetic relationships between Gladiolus cultivars to some extent, which could further be helpful in deciding the parents for effective breeding programme.

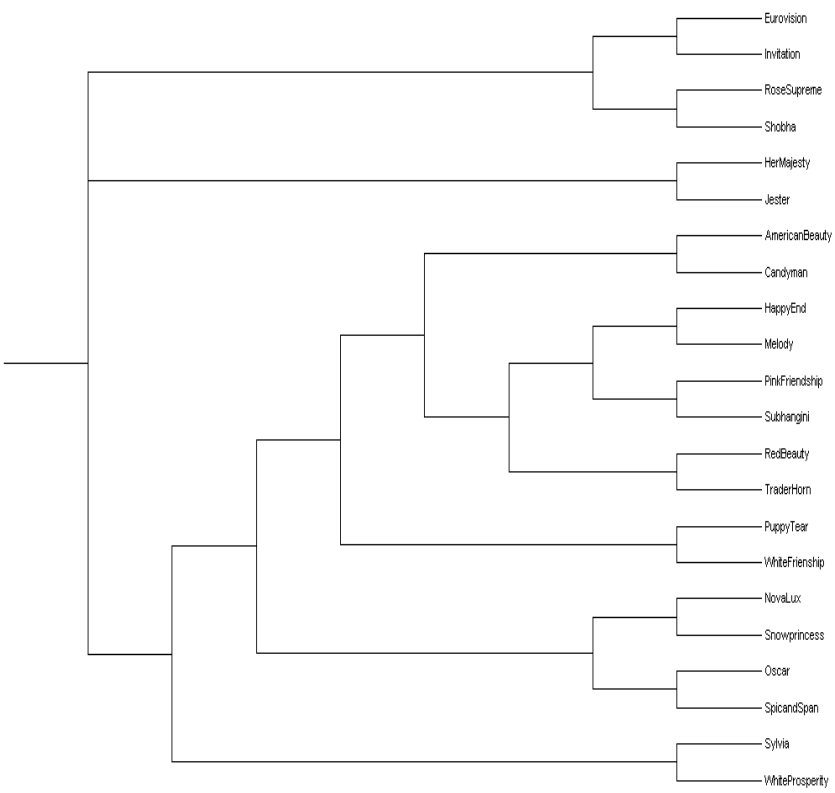

Fig 2 UPGMA dendrogram of 22 Gladiolus cultivars

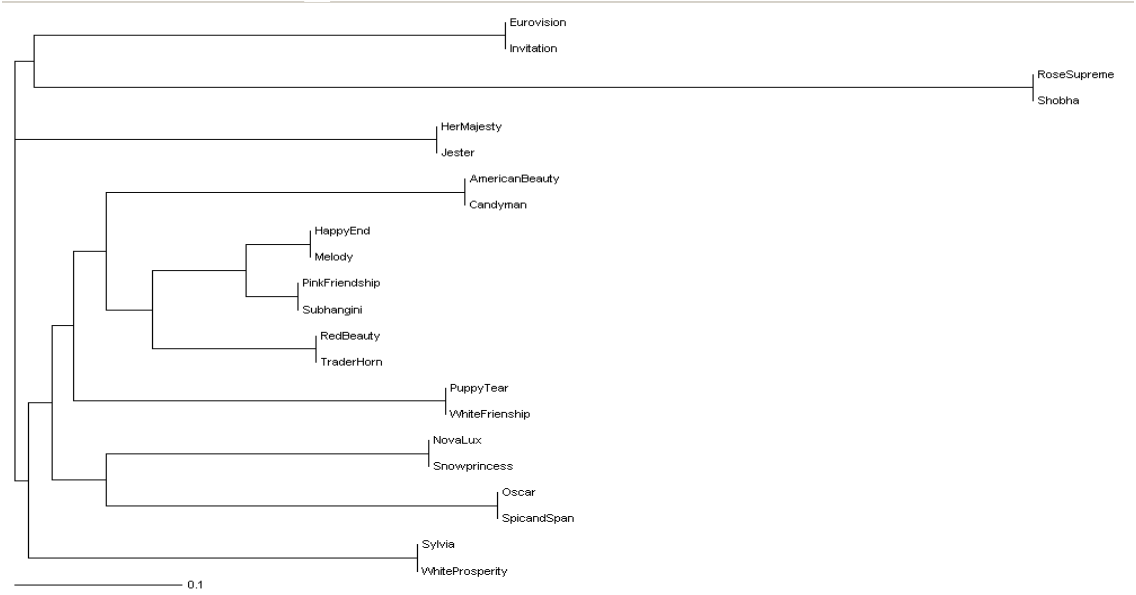

Fig 3 Cladogram of 22 Gladiolus cultivars

MORPHOLOGICAL VS MOLECULAR DATA

The genetic diversity between the 22 studied species are presented in fig 2 and 3. Genetic diversity generally is the result of long-term evolution and represents the evolutionary potential of a species. Surviving in a harsh environment, a species is subject to change in some aspects and accumulats more genetic variation in order to adapt itself to various environmental pressure. Breeders more often select a genotype based on commercial characters. That is why, all the genotypes with superior commercial characters clustered together in morphology-based dendrogram. In RAPD-based dendrogram, cluster III was again divided into two sub-clusters. The first sub-cluster was dominated with soft coloured genotypes, whereas second sub-cluster was found to be dominated with red to purple colour range. Although the parentages of these genotypes are morphologically quite different, they are found to be closely related at genotypic level. Here, the necessity of molecular tools in broadening the genetic bases for effective breeding programme could be realized. Two morphologically distinct genotypes might not have distinct genetic constitution. Thus, for creation of any new variety, selection of parents based on morphology should always be supplemented with an efficient molecular marker.

On the basis of morphological and molecular tool some controversy arises during genetic relationship study between the selected genotypes. Here, the reliability of morphological traits could also be illustrated as phenotypes are the expressions of genotypes nevertheless it is affected by many factors. In contrast to this, the genotype pair ‘Pink Friendship’ and ‘Rose Supreme’ was found very close for phenotypes but placed on different branches of RAPD-based dendrograms. These differences might have arisen due to several reasons, but the most important is that the genetic (or structural) origin of each RAPD marker is different, while morphological expression (phenotype) is conditioned by the state of the plant, agricultural management and environmental conditions. Thus, phenotypic differences are not necessarily concordant with the number of underlying gene mutations, and differences in phenotypic characters do not necessarily reflect different genetic events (Bachmann 1992). On the other hand, it is possible that the dominant nature of RAPD markers and limitation of RAPD technique to detect polymorphism in cases of heterozygosity (Parker et al. 1998), may account for the inconsistencies between the analyses. The discrepancy cases between DNA-based and morphology based classification also have been documented in many plants (Persson et al. 2000, Wang and Bao 2005). In future propective, further more research is needed to ensure the exactness of evaluation, only variables with strong genetic control should be quantified in morphological traits analysis and other more effective molecular markers such as AFLP, SSR or ISSR should be used (Wang and Bao 2005).

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