Genetic Variation of Hippeastrum Accessions in Vietnam

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Genetic Variation of *Hippeastrum* Accessions in Vietnam

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The present study was conducted to clarify the distribution and genetic diversity of *Hippeastrum* accessions cultivated in Vietnam. Twenty-five *Hippeastrum* accessions collected from 17 provinces in the northern, central, and southern regions of Vietnam were included. Results of floral observation and RAPD analysis indicate that there are four distinct groups of *Hippeastrum* being grown in Vietnam. These include *cam dai* (*H. puniceum* (Lam.) Kuntze syn. *H. equestre* Herbert), *đỏ dai* (*H. x ‘Johnsonii’*), *đỏ cam dai* (*H. striatum* Lamarc), and the hybrid cultivars. In the RAPD analysis result, 25 primers produced a total of 230 distinct bands, of which 167 were polymorphic (about 72.6%). The number of polymorphisms per primer ranged from 1 to 13, and one primer did not reveal any polymorphisms. Our phylogeny indicated four separate groups, with dissimilarity values ranging from 0.2 to 0.3 according to the phylogeny based on RAPD results. Some variation was observed between northern, central, and southern *H. puniceum* populations. Extensive variation in flower color, shape, and size was detected among accessions of the hybrid group. While *H. puniceum* is the most widely distributed species from the northern to the southern regions, *H. x ‘Johnsonii’* has been observed only in the northern region, but not in the central or southern regions, so the distribution of *Hippeastrum* within Vietnam might depend on adaptation to environmental conditions. Our results have distinguished the *đỏ dai* and the *cam dai* groups, although a single scientific name has been applied to both extant groups in Vietnam. The present study is the first report on the presence and distribution of, and genetic variation among *Hippeastrum* accessions grown in Vietnam, which will be valuable information for *Hippeastrum* breeding programs.

**Key words**: genetic variation, *Hippeastrum*, RAPD analysis, Vietnam

INTRODUCTION

*Hippeastrum* is an ornamental flowering bulb that belongs to a genus *Hippeastrum* which comprised of more than 60 species (Dole and Wilkins, 2004; Banerji et al., 2011) in the Amaryllidaceae family (Traub, 1949; Rees, 1992, Meerow, 1988; Banerji et al., 2011). Because they originated in the subtropical Americas, from eastern Brazil to the southern central Andes of Peru, Argentina, and Bolivia (Traub, 1949; Meerow, 1988; Okubo, 1993), members of this genus are adapted to growth in tropical and subtropical regions. Under natural conditions, *Hippeastrum* grows in the summer, goes dormant in the winter, and bears beautiful flowers from the end of spring to summer. *Hippeastrum* is not only well adapted to growth in pot culture, but also for use as a cut flower due to the longevity of its blooms (about 14 d at 20–22°C), which enables them to compete comercially with other popular cut flowers (Read, 2004). Many species in this genus and their hybrids have large, colorful flowers that are prized as Christmas and New Year ornaments (Silberbush et al., 2003).

In Vietnam, *Hippeastrum* (commonly referred to as *hippeastrum* or *amaryllis* in English, or as *loa kèn* đở, *mắc chu lan*, *tứ diện*, or *làn hường* in local languages) has been introduced and grown as an ornamental plant in home gardens, as a landscape plant, or as a potted plant in many regions. With advancements in the diversity of many characters such as flower color, longevity, and ease of planting and care, *Hippeastrum* is becoming one of the most valuable commercial flowers. There is a huge diversity of different hybrids of *Hippeastrum* plants on the market today (Robert et al., 2006); however, there is little documentation of *Hippeastrum* growing in Vietnam. Recently, there have been many attempts to breed new *Hippeastrum* varieties, but a lack of important information regarding the distribution of and genetic variation within the genus can increase the time and costs of the breeding process.

To date, several molecular techniques have been developed to complement traditional methods for evaluating the diversity of plant populations. RAPD is such a technique that has been widely applied for genetic analysis of many genera of flowering bulb. Lee et al. (1996) successfully applied RAPD markers to establish a classification system for lilies. In another study, Hamada and Hagimori (1996) used 60 RAPD primers to identify variation among 12 lily varieties while Chakrabarty et al. (2007) traced the relatedness among *Hippeastrum* varieties in the germplasm collection at the National Botanical Research Institute in Lucknow, India using RAPD analysis. RAPD has also been used for cultivar identification (fingerprinting) (Swoboda and Bhalla, 1997). The efficiency RAPD as a technique for identifying polymorphisms tends to increase with increasing primer length (Yamagishi et al., 2002). Recently, many authors reported that the combination of RAPD analysis and morphological observations can be very effective for studying plant genetic variation (Geraldine et al., 1995; Helena et al.,...
Plant materials

Bulbs of 25 Hippeastrum accessions were collected from 17 provinces in Vietnam including Caobang, Haiphong, HaGiang, Langson, Sonla, Laichau, Hungyen, Ninhbinh, Hanam, Hanoi, Hatinh, Thuathienhue, Daklak, Hochimin, Bentre, Soctrang and Lamdong. The sites from which the bulbs were collected are shown in Fig. 1. At the collection sites, the plants were not identified by a species or varietal name. The growers had classified them only according to major flower characteristics such as flower shape, size, color, and flower throat color were recorded at each collection site. Subsequent DNA analysis was carried out in the laboratory at the Applied Biological Sciences, Faculty of Agriculture, Saga University, Japan.

RAPD analysis

Total DNA was extracted from 1–2 g of fresh young leaves of each Hippeastrum accession using the CTAB method, as described by Murray and Thompson (1980), with minor modifications, as described by Yamashita et al. (2000). The PCR was programmed as: initial denaturation for 30 s at 94°C, followed by 45 cycles of 30 s at 94°C, 2 min at 40°C, and 3 min at 72°C; the final extension for 7 min at 72°C. The final reaction mixture was cooled down to 4°C.

One hundred RAPD primers were tested for the amplification of genomic sequences; 60 random arbitrary 10-bp primers were obtained from Operon Technologies, Inc., (Alameda, California) (designated as primers OPB through OPF), and 40 arbitrary 12-bp primers were included in the A primer set from BEX (Tokyo, Japan) (designated as primers A00 through A40). Amplification of RAPD markers from DNA samples from each of six accessions was carried out in triplicate for each PCR run. Only primers that consistently produced clear bands were selected for further experiments.

DNA amplification products were separated by agarose gel electrophoresis. Agarose (1% w/v) gel with 1X TAE (40 mM Tris–acetate, 1 mM EDTA (pH 8.5) buffer, and electrophoresis was conducted at 70 V/cm for 1.30 h. Gels were stained with ethidium bromide (EtBr), visualized under UV light, and images were captured using a digital camera FAS–III (Toyobo Co. Ltd., Osaka, Japan). All reactions were repeated at least three times and only those bands that were reproducible on all runs were used for further analysis. Molecular Weight Marker II (HindIII/EcoRI digested Lambda DNA) was used as a reference to estimate the size of the DNA fragments.

RAPD bands on gels were scored as present (+) or absent (−) for each accession studied. To estimate the genetic distance among the strains, the genetic similarity measure (F) for each of the RAPD fragments was calculated using the formula of Nei and Li (1979), F=2 Mxy/Mx+My, where Mxy is the number of shared fragments between the strains ‘x’ and ‘y’; Mx is the number of fragments scored as strain ‘x’; and My is the number of fragments scored as strain ‘y’. Distance values were calculated as 1 – F. The UPGMA clustering method in MEGA version 5 (Tamura et al., 2011) was used to generate a phylogeny.

RESULTS

Floral observation

Considerable variation in the floral characters was detected among the accessions we examined (Table 1, Fig. 2). Based on floral characteristics, four different groups of Hippeastrum were distinguished, and were designated cam dài (orange), dò dài (scarlet red), dò cam dài (red orange), and hybrids, respectively.

Seven cam dài accessions (H37, H53–2, H5, and H79) had small orange trumpet-shaped flowers with a yellowish green throat and a large white star at the base. No variation in the floral characters was observed among cam dài accessions collected from different geographical locations. Some of the floral characteristics of the dò cam dài accessions (H6 and H72) were similar to those of the cam dài accessions, except for the flower and throat color. The flowers of these accessions were red-orange with a yellowish green throat, and a large white star and a red ring at the base. The dò dài accessions (H4, H22, H53–1, H5, and H79) had trumpet-shaped, scarlet red flowers with a yellow-
Extensive variation was observed in all the floral characters of the hybrid group. Hybrid flower size was either medium or large. Among hybrid accessions, two (H31–2 and H104) had triangular–shaped flowers, whereas the others had flowers that were star–shaped. Hybrid flower colors varied from dark red (accession H67), to pure white (accession H27), or bi–color (accessions H101, H84, H104, H31–2, H77, and H85). Differences were also observed in the coloration of the flower throats of hybrid accessions. Six of nine accessions had a red ring at the base of the flower throat while accession H67 had a large dark red star throat. Accessions H28 and H88 were easy to distinguish from other accessions due to their pale pink flowers with netted venation.

**RAPD analysis**

From the 100 RAPD primers tested, 25 that consistently produced clear bands were chosen for genotyping the Hippeastrum accessions. RAPD analysis revealed a high degree of genetic diversity among these accessions. The number of bands generated by each primer varied from 3 (for primers A06 and A21) to 14 (for primers OPB–05 and A15), with an average of 9.2 bands per primer. In this study, 24 out of 25 primers produced polymorphic amplification products. A total of 230 distinct bands were produced, of which 167 were polymorphic (about 72.6%).
detected per primer ranged from 1 (primer A06) to 13 (primer A15), while primer A22 revealed no polymorphism (Table 2). Some primers produced identical RAPD banding patterns for the Hippeastrum accessions with similar physical floral characters, especially flower color. One RAPD marker (about 2000 bp in size) was detected in all the đỏ cam đạ and đỏ đạ accessions using primer OPF–03. The primer OPA–04 yielded a RAPD marker (about 830 bp in size) only in the đỏ đạ accessions (Fig. 3). The RAPD marker detected using primer OPF–13 (about 400 bp in size) could distinguish the đỏ đạ accessions from the other accessions. Moreover, one RAPD marker (about 1375 bp in size) was revealed only in the cam đạ accessions collected from the southern and central regions, but was not detected in accessions collected from the northern region by using primer OPF–16.

The results from RAPD analysis were used to calculate genetic dissimilarity coefficients in the pair–wise comparisons among the accessions. Genetic distances ranged from a minimum value of 0.008 between accession H142 and accession H147, to a maximum value of 0.357 between accessions H53 and H5 (Fig. 4). The dendrogram developed from the genetic dissimilarity values revealed by RAPD analysis is shown in Fig. 5. At genetic dissimilarity values greater than 0.3, two distinct clusters of accessions were observed. The first cluster included 16 Hippeastrum accessions of the đỏ cam đạ and the hybrid groups, while the second cluster included nine accessions from the cam đạ and the đỏ cam đạ groups. Within the first cluster, two subgroups were clearly identified with a dissimilarity value greater than 0.2. Accessions with similar flower color, shape, and size were always in

<table>
<thead>
<tr>
<th>Order</th>
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<th>Sequence</th>
<th>Company</th>
<th>Total no. of bands</th>
<th>No. of polymorphic bands</th>
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<td>5’–CGGCCCTTC–3’</td>
<td>Operon</td>
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<td>9</td>
</tr>
<tr>
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<td>AATGCGGCTG</td>
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<td>10</td>
<td>7</td>
</tr>
<tr>
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<td>OPA–17</td>
<td>GACGCGTTGT</td>
<td>Operon</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>OPA–12</td>
<td>TGCGCCATAG</td>
<td>Operon</td>
<td>9</td>
<td>6</td>
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<tr>
<td>5</td>
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<td>GTTTCGCTCC</td>
<td>Operon</td>
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</tr>
<tr>
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<td>TGCCGCTTTC</td>
<td>Operon</td>
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<td>11</td>
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<td>TTCAAGCTACCA</td>
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</tr>
<tr>
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<td>A26</td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>230</td>
<td>167</td>
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Fig. 4. Dissimilarity values based on RAPD analysis among the *Hippeastrum* accessions in Vietnam.

Fig. 5. Dendrogram of the *Hippeastrum* accessions in Vietnam developed from RAPD data.
close proximity in the dendrogram (e.g., accessions H52 and H84, or H28 and H88, or H77 and H85). These accessions clustered into three small branches of the dendrogram, which indicates their relatively close relationship. In a second cluster, two different subgroups could be distinguished. One included the cam dãi accessions, and the other included two dã ca m dãi accessions. Although all of the cam dãi accessions were relatively close, they were separated into two small branches on the dendrogram. The accessions H53, H59, and H37 collected from the northern region (Haichong, Hagiang, and Caobang provinces, respectively) were grouped into one branch. However, the accessions H96, H56, H147, and H142 collected from the central and southern regions (Thuathienhue, Hochiminh, Bentre, and Soctrang provinces, respectively) were clustered on another short branch, indicating their close relationship.

**DISCUSSION**

Based on floral observation and results of the RAPD analysis in the present study, four different groups of *Hippeastrum* cultivated in Vietnam were revealed. Those groups could be identified as cam dãi, or *Hippeastrum puniceum* (Lamk.) Kuntze (syn. *Hippeastrum equestre* Herb.); dã dãi, or *Hippeastrum* x ‘Johnsonii’; dã ca m dãi, or *Hippeastrum striatum* Lamareck ; and the lai or hybrid group. These findings are not in agreement with those of other authors as to the number of extant *Hippeastrum* species and cultivars currently in Vietnam. Previous reports had indicated only two *Hippeastrum* species cultivated in Vietnam as *H. reticulatum* Herb. (a subspecies *H. reticulatum* Herb. var. striatifolia Herb.), and *H. puniceum* (Lamk.) Kuntze (syn. *H. equestre* Herb.) (Ho, 1999; Do et al., 2007).

There is also an apparent contradiction in classification based on the flower color of *H. puniceum* (syn. *H. equestre* Herb.) in Vietnam. According to Do et al. (2007), the flowers of *H. puniceum* are red or orange; however, Ho (1999) reported flowers of this species to be red. In our study, accessions that were identified as *H. puniceum* had orange flowers with yellowish green throats and a large white star at the base, the same as described for *H. puniceum* by Traub (1949), Okubo (1993), and Read (2004).

Among these *Hippeastrum* groups, *H. puniceum* is the most widely distributed in Vietnam. It is found in home gardens from the northern region to the southern region. It is also important to note that Vietnam has two distinct climate regions: a northern region that is characterized by four distinct seasons including a cold winter, and the southern and central regions that are characterized by two distinct seasons, hot and rainy or hot and dry. *H. puniceum* flowers at the beginning of the rainy season in the southern and central regions, but flowers at end of spring in the northern region, when the weather is still cold and dry. It seems that flower initiation in *H. puniceum* in Vietnam does not appear to depend on vernalization, but on exposure to dry conditions. Therefore, the study on the flower initiation of *H. puniceum* is essential. Moreover, RAPD analysis identified some genetic variation between the *H. puniceum* populations from the northern region compared to those from the southern and central regions. All of the accessions collected from the southern and central regions (H96, H56, H147, and H142) could be phylogenetically distinguished from those collected in the northern region (H53, H59, and H37) with this RAPD data. Thus, the variation observed between *H. puniceum* populations of the northern region and those of the southern and central regions could have resulted from the adaptation of each population to these different climactic conditions.

To date, the name *H. equestre* (syn. *H. puniceum*) has been applied to accessions belonging to both the dã dãi and the cam dãi groups in Vietnam. However, our study indicates that accessions of the dã dãi and cam dãi groups are likely separate, as depicted in the dendrogram developed from analysis of this RAPD data. There is significant genetic variation (genetic distance value >0.3) between these two groups. Also, based on observation of the floral characteristics of each accession, the dã dãi accessions could be redesignated as *H.* x ‘Johnsonii’, which is known to be a hybrid of *H. reginae* x *H. vittatum* (Traub, 1949; Meerow et al., 1990, and Read, 2004). *H.* x ‘Johnsonii’ is cultivated in the northern region for its scarlet red flowers, but is not grown in the southern or central regions. Its flowering season is at the end of spring, although it does flower rarely in summer or winter.

Plant classifications based on morphological characteristics can sometimes present problems for taxonomists, but RAPD analyses can help to resolve such ambiguities (Swoboda and Bhalla, 1997). It is not uncommon for incorrect scientific names to persist locally, as in the case of the classification of *Allium* species occurring in Vietnam. Pham et al. (2006) had previously distinguished the wakegi onion (*Allium wakegi* Araki) from the shallot (*Allium cepa* Agregatum group) using cytological, morphological, and RAPD analysis, although both had previously been classified as part of the *A. cepa* Agregatum group. Similarly, the present study is the first report distinguishing *H. puniceum* and *H.* x ‘Johnsonii’ in Vietnam.

*H. striatum* has not been officially reported to occur in Vietnam, but has only been informally observed in some northern provinces in Vietnam, including Hanoi, Thanhhoa, and Sonla provinces (personal observation). Although the flower size and shape of *H. striatum* are similar to those of *H. puniceum*, the genetic diversity between these two species is considerable (approximately 0.2). Due to small sample numbers, genetic variation among the *H. striatum* accessions has not yet been determined. Further studies of the morphological, cytological, and physiological characteristics of *H. striatum* are necessary to confirm whether those accessions are distinct at the species or lower level.

The demand for the new *Hippeastrum* cultivars is increasing in Vietnam and worldwide. Consequently, a number of new cultivars have been imported into the country for cultivation. The present study has revealed...
significant genetic variation among Hippeastrum accessions of the hybrid group. The available diversity of flower color, shape, and size of these new cultivars will be useful for Hippeastrum breeding in Vietnam. Among the hybrid accessions, hường sắc, or H. reticulatum Herb. var. striatifolia Herb., is particularly easy to identify due to its pale pink flowers with netted venation and a white stripe on the leaves (visual observation). This accession blooms in September, while others flower from March to May. According to Meerow (1988) the flower emergence of these groups may be affected by a photoperiodic response more than by low temperatures. In addition to providing valuable information on the relationships among and distribution of Hippeastrum species and varieties not only in Vietnam, our report also agrees with that of Chakrabarty et al. (2007) in that the RAPD method is useful for identifying new and existing hybrids, and for determining the relatedness of Hippeastrum accessions. The useful RAPD primers and the RAPD markers developed in the present study could be used in further research to analyse the relationships between Hippeastrum species, hybrids, and varieties not only in Vietnam, but also in other countries.

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