

RAPD marker assisted evaluation of chloroplast DNA variation in twelve hosta taxa

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Abstract

Hostas are major ornamental plants for perennial shady nurseries. They have striking foliage with attractive flowers, and their leaves are sometimes used as vegetables in Japan and Korea. The objective of this study was to estimate the genetic identity and genetic distance of twelve hosta taxa and to build a UPGMA cluster for them using Random Amplified Polymorphic DNA (RAPD). Rhizomes of hostas were collected from Shikoku Island, Japan. Newly emerged unfurled hosta leaves were disrupted with Nuclei Lysis solution and then purified the genomic cpDNA. The cpDNA was dehydrated as a final point for extraction. We amplified the cpDNA of twelve taxa by means of 20 RAPD 10mer markers (Kit A: OPA-01 to OPA-20). The amplified DNA was run in 0.8% agarose gel using 1000 kb DNA ladder. Out of 20 RAPD markers, the genomic cpDNA was successfully amplified for fourteen. Fourteen markers generated a total of 102 fragments. We found maximum genetic distance (0.655) and minimum genetic identity (0.520) between *H. sieboldiana* - *H. longissima* taxa and between *H. sieboldii* - *H. longissima*. Estimated minimum genetic distance (0.071) and maximum genetic identity (0.931) were found between *H. kikutii* var. *polyneuron* - *H. longipes* var. *caduca*. The UPGMA dendrogram revealed the relationship among the 12 hostas. The results confirmed our hypothesis of large genetic variability among the taxa, which distinguishes the genotypes clearly. The RAPD 10mer markers were found useful in application to the evaluation of the genetic variability and diversity within taxa.

Keywords: Genetic distance, Genetic identity, Giboshi, Plantain lily, UPGMA.

Abbreviation: AFLP_Amplified fragment length polymorphism, cpDNA_Chloroplast DNA; ISSR_Inter simple sequence repeat; G_D _Nei's genetic distance; G_L _Nei's genetic identities; RAPD_Random amplified polymorphic DNA; SSR_Simple sequence repeats; TAE_Tris-Acetate-EDTA; UPGMA_Unweighted pair group method with arithmetic mean.

Introduction

Hosta is the genus of a popular ornamental perennial plant found in USA, Canada, Britain, Japan and China. It is generally used for its remarkable foliage and sometimes used as a vegetable in Japan. Genus *Hosta* includes approximately 70 species; so far Asia is the native region of 43 species which originated in Japan, Korea and China (Schmid, 1991). *Hosta* species are highly variable morphologically (Chung et al., 1991), and are differentiated by their floral character (Schmid, 1991). Meanwhile, cultivars are differentiated by leaf and overall plant characteristics (Grenfell, 1996). Maekawa (1940) first distinguished 31 Japanese and 7 Chinese and Korean species morphologically; and later, he reduced the number to 24 (Maekawa, 1969). Fujita (1976) also gave a report on the morphological characteristics of the Japanese species. Phenotypic evaluations of any living organism have limitations reflecting understanding at the gene level. The phenotypic characteristic[s] is [are] inherited genetically and expressions are controlled by environmental conditions. The classification of the *Hosta* genus is mainly based on morphological characteristics and therefore, systematic study is still incomplete. Estimation of genetic distance is important for a better understanding at the gene level. Genetic diversity of crop plants can be studied in

inbred lines, pure lines or clones, in the form of germplasm accessions, species and populations. Diversity can occur at the genetic (variation in genes and genotypes), species (species richness) and ecosystem (species communities and their environment) levels. Molecular markers are the key tool for determining the relationship between genes (Ye et al., 2008). DNA polymorphism profiling is a useful technique for facilitating direct and reliable measurement of genetic variability and divergence. These techniques support the characterization of genotypes and the examination of tissues to distinguish physiological stages of plant; the outcome of these characterizations and examinations is autonomous to environmental conditions (Anita et al., 2004). Since the 1990s, the multi-locus marker system has been used to estimate plant genetic variation due to its ability to produce numerous amplicons and since sequence information is not required for molecular characterization (Koopman et al., 2008). DNA markers are generally used for the identification of genotypes and the determination of genetic similarity or dissimilarity. Molecular markers (RAPD, AFLP, SSR and ISSR) contribute to genetic divergence in terms of distinct morphological characteristics (Rezende et al., 2009). RAPD is used for the study of genetic relationships (Anita et al., 2004) and genetic variation (Salhi et al., 2005) because of its simplicity and low cost (Costa et al., 2011, Silva et al., 2012).

Numerous studies have used RAPD marker to identify genetic diversity and divergence for hosta (Sauve et al., 2005; Ling et al., 1995), flowering and ornamental plants (Pinheiro et al., 2012; Rodrigues et al., 2012; Rezende et al., 2009; Ye et al., 2008). Genetic variation of *Hosta* species was studied by isozyme analysis (Chung, 1994). RAPD is useful for identifying variations in species with low genetic variability when other techniques fail to reveal differences among the individuals (Bowditch et al., 1994). Hundreds of hosta cultivars with variegated, golden, blue or green leaves are available in the nursery trade. These new cultivars often sell at high prices in Europe, America and Japan. All hybridization of this plant, which has been done by non-specialists, has produced all of these cultivars. It is important to engage specialist breeders to develop new cultivars, but there is a lack of information on the molecular characterization of the hostas. The degree of gene difference between species may reflect their potentiality to develop breeding strategy. This study seeks to identify the extent of genetic differentiation or unity among hostas. It was hypothesized that RAPD markers will afford a better understanding of the variability and relationships among the studied taxa at the gene level. To test this hypothesis, our study estimated the genetic identity, genetic distance and UPGMA clustering of twelve taxa of hosta using RAPD markers.

Results

Banding pattern

A total of twenty RAPD markers were amplified for twelve hosta taxa. No band was found for six of the markers (OPA-05, OPA-06, OPA-13, OPA-14, OPA-16, and OPA-17). We selected fourteen of the twenty markers, which had generated 102 fragments in total. All taxa showed polymorphic bands for OPA-01, OPA-02 and OPA-3 markers. The fingerprints of fourteen RAPD markers over twelve taxa are shown in Fig 1. The maximum number of bands was found for *H. longissima* (40) and the second highest was found for *H. sieboldiana* and *H. sieboldii* (35). The minimum number of bands was observed for *H. nakaiana* (14) (Fig 2).

Genetic distance and identities

Pairwise G_D and G_I for comparison between twelve hostas were shown in Table 1. Maximum G_D (0.655) and minimum G_I (0.520) values were found for *H. sieboldiana* - *H. longissima*, also between *H. sieboldii* - *H. longissima* (Table 1). On the other hand, minimum G_D (0.071) and maximum G_I (0.931) values were found for *H. kikutii* var. *polyneuron* and *H. longipes* var. *caduca* (Table 1).

Cluster analysis

A dendrogram comprising twelve hosta taxa and UPGMA cluster had three clades for these taxa (Fig 3). Clade I comprised *H. sieboldiana*, *H. alismifolia*, and *H. longissima* while Clade III comprised *H. kiyosumiensis* and *H. montana*. Rest of the taxa belonged to Clade II. In the UPGMA cluster, very close average-linkage relationships were observed between *H. kiyosumiensis* and *H. montana*; between *H. alismifolia* and *H. longissima*; and between *H. sieboldiana* and the *H. alismifolia* - *H. longissima* cluster. Similarly, average linkage was found between *H. kikutii* var. *caput-avis* and *H. longipes* var. *caduca*; between *H. tardiva* and *H. longipes* var. *gracillima*; and between *H. nakaiana* and *H.*

kikutii var. *polyneuron*. *H. sieboldii* had a very close average-linkage to *H. kikutii* var. *caput-avis* and the *H. longipes* var. *caduca* cluster.

Discussion

In this study variation among the twelve hostas was assessed using fourteen successfully amplified RAPD markers (Fig 1). RAPD markers provided valuable genetic similarity and dissimilarity information for these *hostas*. The RAPD markers showed polymorphisms and large variability that distinguished the genotypes clearly. The high level of genetic dissimilarity among hostas (ranging from 0.071 to 0.655) shows a considerable level of genetic variation in the *hostas* and indicates a quite extensive genetic base. *H. kikutii* var. *caput-avis* and *H. kikutii* var. *polyneuron* had the same flowering characteristics and very little difference in morphological characteristics; a similar condition has been observed for *H. longipes* var. *gracillima* and *H. longipes* var. *caduca* (Fujita, 1976; Schmid, 1991). In the current study, we found very high genetic identity and low genetic distance (G_I : 0.833 and G_D : 0.182) between *H. kikutii* var. *caput-avis* and *H. kikutii* var. *polyneuron*; and between *H. longipes* var. *gracillima* and *H. longipes* var. *caduca* (G_I : 0.804 and G_D : 0.218) (Table 1). These results revealed that *H. kikutii* var. *caput-avis* and *H. kikutii* var. *polyneuron* were very similar genetically, as were *H. longipes* var. *gracillima* and *H. longipes* var. *caduca*. Interestingly, genetic study using RAPD markers revealed that *H. kikutii* var. *polyneuron* differed little genetically (i.e., maximum percentage of identity) from *H. longipes* var. *caduca* (G_D : 0.071 and G_I : 0.931) (Table 1), although the two are quite different phenotypically. We found maximum genetic distance and minimum identity between *H. sieboldiana* - *H. longissima* and between *H. sieboldii* - *H. longissima*. Phenotypic variability is often confined because plant developmental stages and appearance influenced by environment do not afford an understanding of characteristics. RAPD markers generated banding patterns irrespective of plant developmental stage or growing environment. We identified different hostas from the phenotypic analysis by Fujita (1976) and Schmid (1991). However, the results of our study demonstrated the complex nature of the genotypic and phenotypic variations. Sauve et al. (2005) also found complexity in phenotypic and genotypic variations of hosta taxa. It has been found that phenotypically different genotypes may show variations in a few loci, while phenotypically similar genotypes may be completely different (Rodrigues et al., 2012). Similar to our study, genetic variation among the taxa through RAPD markers have been investigated in several plants (Arif et al., 2010; Da-Mata et al., 2009; Lin et al., 2009). RAPD primers are a useful tool for differentiation at the species level (Choo et al., 2009), which can reflect both coding and non-coding regions of the genome (Vanijajiva et al., 2005). In the current study, we found the RAPD marker assisted genetic variation and genetic relationship among taxa. The genetic variations within hosta taxa were directly related to the RAPD marker technique. The results of the RAPD marker assisted genetic variability analysis for hostas should be useful to hosta breeders in their hybridization work.

Table 1. Pairwise Nei genetic distance matrix (G_D) (below diagonal) and Nei genetic identity matrix (G_I) (above diagonal) of twelve taxa of hosta from the binary data of fourteen RAPD markers.

	A	B	C	D	E	F	G	H	I	J	K	L
A	-	0.647	0.608	0.520	0.539	0.627	0.657	0.608	0.676	0.647	0.657	0.667
B	0.435	-	0.647	0.657	0.598	0.588	0.735	0.686	0.676	0.706	0.716	0.725
C	0.498	0.435	-	0.520	0.618	0.667	0.696	0.686	0.814	0.784	0.696	0.627
D	0.655	0.420	0.655	-	0.569	0.637	0.588	0.598	0.569	0.598	0.608	0.657
E	0.618	0.514	0.482	0.565	-	0.794	0.765	0.735	0.706	0.735	0.725	0.696
F	0.466	0.531	0.405	0.451	0.231	-	0.775	0.725	0.794	0.804	0.775	0.725
G	0.420	0.307	0.362	0.531	0.268	0.256	-	0.892	0.843	0.833	0.824	0.833
H	0.498	0.376	0.376	0.514	0.307	0.321	0.114	-	0.833	0.843	0.814	0.784
I	0.391	0.391	0.206	0.565	0.348	0.231	0.171	0.182	-	0.931	0.843	0.775
J	0.435	0.348	0.243	0.514	0.307	0.218	0.182	0.171	0.071	-	0.853	0.745
K	0.420	0.335	0.362	0.498	0.321	0.256	0.194	0.206	0.171	0.159	-	0.853
L	0.405	0.321	0.466	0.420	0.362	0.321	0.182	0.243	0.256	0.294	0.159	-

Here, A = *H. sieboldiana*, B = *H. alismifolia*, C = *H. sieboldii*, D = *H. longissima*, E = *H. tardiva*, F = *H. longipes* var. *gracillima*, G = *H. nakaiana*, H = *H. kikutii* var. *caput-avis*, I = *H. kikutii* var. *polyneuron*, J = *H. longipes* var. *caduca*, K = *H. kiyosumiensis*, and L = *H. montana*.

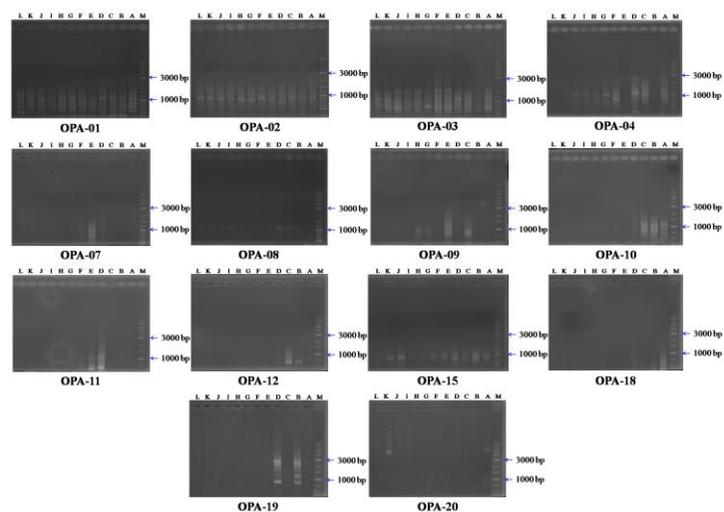


Fig 1. The fingerprints for fourteen RAPD markers for studied taxa of hosta . Here, A = *H. sieboldiana*, B = *H. alismifolia*, C = *H. sieboldii*, D = *H. longissima*, E = *H. tardiva*, F = *H. longipes* var. *gracillima*, G = *H. nakaiana*, H = *H. kikutii* var. *caput-avis*, I = *H. kikutii* var. *polyneuron*, J = *H. longipes* var. *caduca*, K = *H. kiyosumiensis*, L = *H. montana* and M = DNA ladder.

Table 2. A list of the twenty RAPD primers used for the amplification of twelve *Hosta* taxa in the present study.

Primer Name	Sequences (5' - 3')	Primer Name	Sequences (5' - 3')
OPA-01	CAGGCCCTTC	OPA-11	CAATCGCCGT
OPA-02	TGCCGAGCTG	OPA-12	TCGGCGATAG
OPA-03	AGTCAGCCAC	OPA-13	CAGCACCCAC
OPA-04	AATCGGGCTG	OPA-14	TCTGTGCTGG
OPA-05	AGGGGTCTTG	OPA-15	TTCCGAACCC
OPA-06	GGTCCCTGAC	OPA-16	AGCCAGCGAA
OPA-07	GAAACGGGTG	OPA-17	GACCGCTTGT
OPA-08	GTGACGTAGG	OPA-18	AGGTGACCGT
OPA-09	GGGTAACGCC	OPA-19	CAAACGTCGG
OPA-10	GTGATCGCAG	OPA-20	GTTGCGATCC

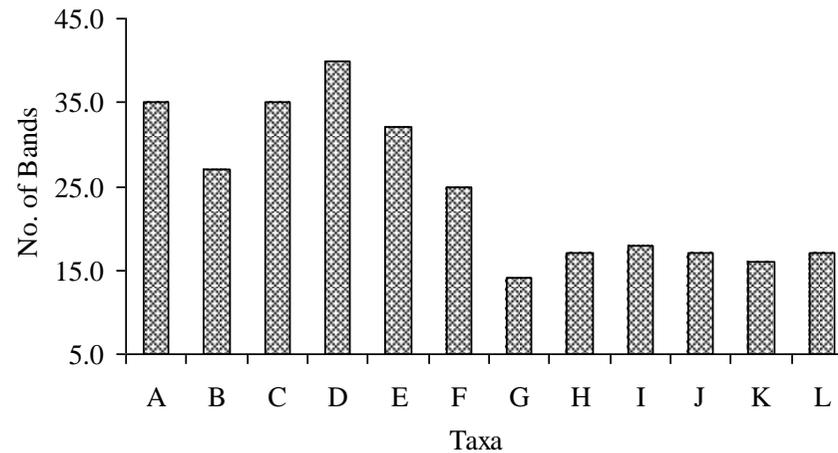


Fig 2. Total number of bands from binary data for twelve *Hosta* taxa.

Here, A = *H. sieboldiana*, B = *H. alismifolia*, C = *H. sieboldii*, D = *H. longissima*, E = *H. tardiva*, F = *H. longipes* var. *gracillima*, G = *H. nakaiana*, H = *H. kikutii* var. *caput-avis*, I = *H. kikutii* var. *polyneuron*, J = *H. longipes* var. *caduca*, K = *H. kiyosumiensis*, and L = *H. montana*

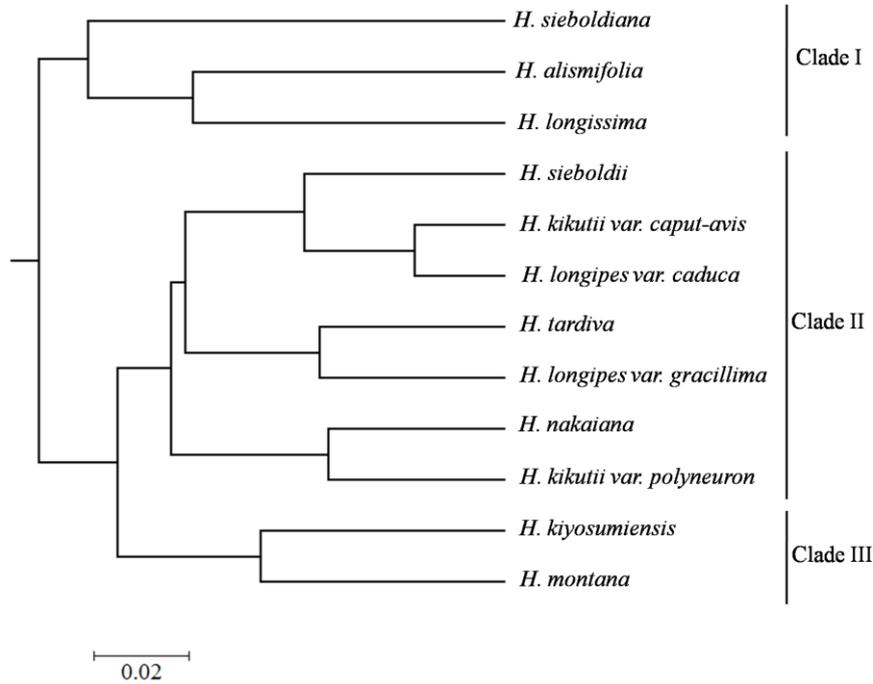


Fig 3. UPGMA cluster for twelve hosta taxa based on fourteen successfully generated fragments of RAPD markers. The dendrogram was constructed by successive (agglomerative) clustering using an average-linkage method.

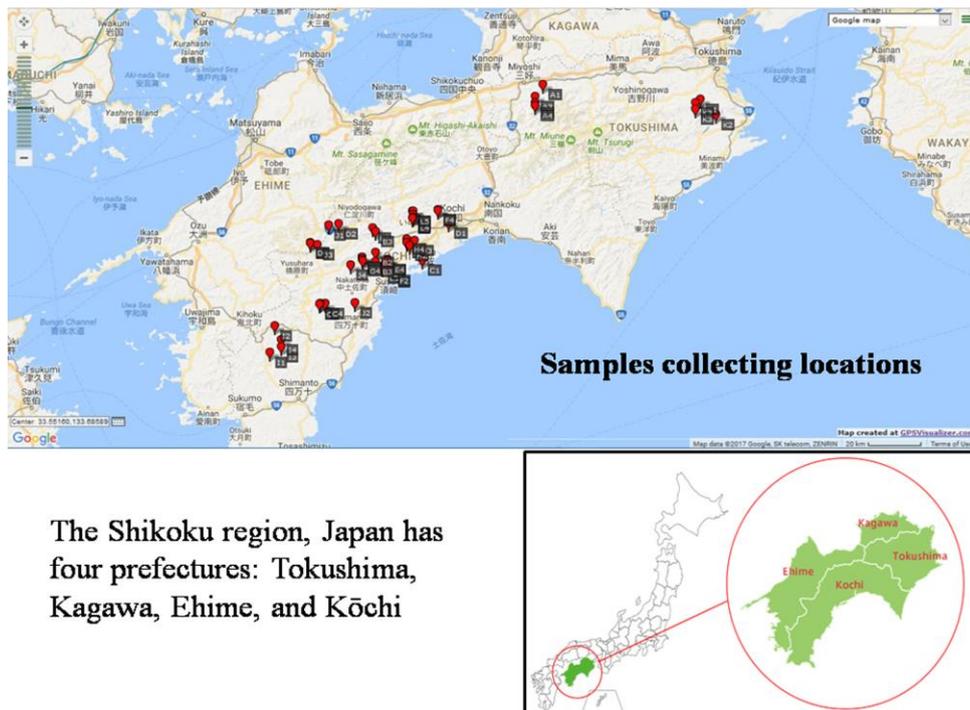


Fig. 4. Sample collection localities of hostas, collected from Shikoku Island, Japan. GPS data were plotted in GPS Visualizer for visualization of the sample collection site. Source: Map of Shikoku Region, Japan was Japanese Encyclopedia. Here, A1-A4 = *H. sieboldiana*, B1-B4 = *H. alismifolia*, C1-C4 = *H. sieboldii*, D1-D4 = *H. longissima*, E1-E4 = *H. tardiva*, F1-F4 = *H. longipes* var. *gracillima*, G1-G4 = *H. nakaiana*, H1-H4 = *H. kikutii* var. *caput-avis*, I1-I4 = *H. kikutii* var. *polyneuron*, J1-J4 = *H. longipes* var. *caduca*, K1-K4 = *H. kiyosumiensis*, L1-L5 = *H. montana*

Materials and methods

Plant materials

First, rhizomes of *H. sieboldiana*, *H. alismifolia*, *H. sieboldii*, *H. longissima*, *H. tardiva*, *H. longipes* var. *gracillima*, *H. nakaiana*, *H. kikutii* var. *caput-avis*, *H. kikutii* var. *polyneuron*, *H. longipes* var. *caduca*, *H. kiyosumiensis*, and *H. montana* were collected from Shikoku area, Japan (Fig. 4) and planted in pots to emerge new leaves. Four to five plants of each taxon were grown. Newly emerged unfurled hosta leaves were collected [4 samples for each taxon for repetition (except *H. montana*; 5 samples)] for cpDNA extraction.

Extraction of cpDNA

40-60 mg of newly emerged hosta leaves were washed in 70% ethanol. The leaves were kept in a microcentrifuge tube with a Zirconia bead. Nuclei Lysis solution (600 µl) was added to the microcentrifuge tube and disrupted with a Micro Smash MS-100 bead beater (at 3500 rpm for 3 minutes) (Tomy Seiko, Tokyo, Japan). We followed the manufacturer's instructions (Wizard[®] Genomic DNA Purification Kit, Promega, Wisconsin, USA) for the purification of genomic cpDNA. We dehydrated the purified genomic cpDNA with DNA Rehydration solution (100 µl) as a final stage of extraction. The resultant dehydrated cpDNA was preserved at 4°C, was ready for use in the amplification.

Amplification of cpDNA

We used RAPD 10mer Kit A (OPA-01 to OPA-20) (eurofins, Tokyo, Japan). We amplified the extracted cpDNA using twenty RAPD primers (Table 2).

Components and conditions for RAPD-PCR

We used 13 µl of distilled water; 2 µl each of DNA, primer and 2 µl *Taq* polymerase reaction buffer; 1 µl dNTP; and 0.2 µl *Taq* DNA polymerase for RAPD-PCR. PCR conditions were as follows: (i) 94°C for 10 min; (ii) 94°C for 1 min; (iii) 36°C for 1 min; (iv) 72°C for 2 min; (v) 45 cycles of steps ii to iv; (vi) 72°C for 10 min; (viii) hold at 4°C.

Gel electrophoresis

5 µl amplified DNA solution (4 µl Amplified DNA + 1 µl loading dye) was run into 0.8% agarose gel for 45 minutes at 75 volt and 1×TAE buffer was used to dip gel in the electrophoresis chamber. We used 1000 kb DNA ladder for gel electrophoresis. We repeated the process (from amplification to gel electrophoresis) at least 2 times to avoid the error in the separation of different sized molecules.

Data analysis

PyElph was used for gel image analysis (Pavel and Vasile, 2012) to obtain binary data for the presence (1) or absence (0) of band. The total number of bands, pairwise Nei genetic distance and identity matrix (Nei, 1972) for twelve taxa were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012). We built the UPGMA dendrogram by implementing Neighbor from the PHYLIP-3.695 package (Felsenstein, 2005). The output file was subjected to TreeView (Page, 1996) to explore the dendrogram.

Conclusion

RAPD provided indiscriminately observed genetic variations of the entire hosta genome. Floral and leaf characteristics of ornamental and leafy vegetables, respectively, are characteristics essential to commercial value. Though sometimes the floral and leaf characters of hostas were highly similar, genetically they were not. Phenotypically dissimilar hosta genotypes can vary in a few loci but phenotypically alike genotypes can differ completely. We expect that the RAPD assisted genetic variability analysis and the association among the studied hosta taxa that will be of considerable practical value to hosta breeders.

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Conflict of interest

We would hereby like to declare that there is no conflict of interests that could possibly arise.

Authors Contribution

Hasan Mehraj conceived, designed, and performed the experiment, analyzed the data and wrote this manuscript. Kazuhiko Shimasaki contributed the plant materials and the chemicals required. Kouhei Ohnishi demonstrated the whole experimental process. Kazuhiko Shimasaki and Kouhei Ohnishi were responsible for supervising the experiment. Subarna Sharma assisted in the performance of the entire experimental process.

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