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RAPD Analysis to Evaluate the Genetic Variation and Relationships in Japanese *Hemerocallis* spp.

By

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Summary：Random amplified polymorphic DNA (RAPD) was employed to estimate the genetic variation and relationship among species, varieties and individuals within varieties in *Hemerocallis* native to Japan. Twenty RAPD primers scored 248 bands, and almost all bands (96.7%) were polymorphic. Based on the polymorphic data, AMOVA showed the inter-variety variation was smaller than the inter-species variation, and a distribution reflecting the known classification about each variety by principal coordinates (PCO) and unweighted pair-group method arithmetic average (UPGMA) analysis were obtained. In addition, these analyses were able to genetically differentiate a lowland-type population within *H. dumortieri* var. *esculenta*. Furthermore, *H. aurantiaca* (Hama-kanzou) and *H. fulva* (No-kanzou and Yabu-kanzou) formed one cluster, suggesting that both species are genetically closely related. Our results indicate, for the first time, the genetic variation and relationships within Japanese *Hemerocallis* following the application of a DNA marker.

Key words：*Hemerocallis* spp., RAPD, AMOVA, PCO analysis, Cluster analysis

Introduction

The genus *Hemerocallis* L. (*Liliaceae*) is originally distributed centering on the Temperate zones in East Asia and includes about 30 species of flowering herbaceous perennials from Japan, Korea and China[1,2]. Many species and about 25,000 modern cultivars are widely grown in Asia, Europe and North America for their attractive flowers[1,3,4]. Varieties and populations, such as Nikko-kisuge, Musashino-kisuge, Tobishima-kanzou, Hama-kanzou, No-kanzou, Yabu-kanzou, among others, grow naturally in various areas throughout Japan. Chromosome composition of these varieties and their populations is similar to each other and the chromosome number is the same ($2n=22$: diploid), except for Yabu-kanzou ($2n=33$: triploid)[5]. Therefore, an inter-species hybrid can be easily produced by artificial pollination, and the progeny can also be fertile. However, each variety and population tends to be genetically differentiated by physiological, ecological and/or geographical isolation. Despite this, several features used to classify *Hemerocallis* showed continuous variation among varieties while intermediate types between themselves and the population of other natural habitats have also occasionally been found[2].

To date, the variability of *Hemerocallis* germplasm has been described in terms of morphology, growth, and the environmental adaptability of traits[1,6]. Several researchers have reported on variation and classification of *Hemerocallis* in Japan[7-10], although great inconsistencies in the classification of varieties or groups are evident. On the other hand, no biological or molecular analyses aimed at determining diversity and genetic relationships exist, except for the result that indicated mainly genetic variation among cultivars registered in the American *Hemerocallis* Society by AFLP markers[11].

Molecular markers are valuable in plant breeding, especially in studies on genetic diversity and on establishing genetic relationships. Random amplified polymorphic DNA (RAPD) markers have been used to generate vast quantities of data, leading to their rapid application addressing a diverse range of biological questions[12,13]. RAPD has the advantage that no prior knowledge of the genome under

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research is necessary, and can be employed across species using universal primers[14,15]. Due to its efficiency and convenience, RAPD has been used to make taxonomic comparisons of many plant species[13-20]. The analysis of multiple accessions individually within a variety or population is desired to estimate the genetic relationship among varieties or populations. In particular, to estimate the genetic structure of an allogamous species, the variation existing among individuals within a variety or population should be taken into account[21].

In this study, we have employed RAPD markers to estimate genetic variation within the Japanese *Hemerocallis* gene pool using 3–16 individuals per variety as material. These results provide the basis for determining how the present level of genetic variability might be best classified, managed and utilized by both breeders and geneticists.

Materials and Methods

Plant materials and DNA preparation

Three species, including five varieties and one cultivar of *Hemerocallis*, were used (Table 1). The plant materials were collected within each habitat reported by MATSUOKA and HOTTA (1966) and KITAMURA *et al.* (1967). Musashino-kisuge is indicated as a *forma* within var. *esculenta*[7]. The scientific name of each variety were followed KITAMURA *et al.* (1967) and HIYAMA (1965). Between three and 16 plants were randomly collected for each variety and cultivar, and then DNA of individual samples was extracted from the leaf of the plants by ISOPLANT II (Nippongene) according to the manufacturer's instructions.

RAPD amplification and data analysis

RAPD analysis was performed using 20 random 10-mer primers (Operon Co.) (Table 1). RAPD-PCR was carried out in a 20 µl volume containing 50 ng genomic DNA, 1X PCR mix (2 mM Tris-HCl pH 8.0, 10 mM KCl, 2 mM $MgCl_2$, 10 µM EDTA, 0.1 mM DTT, 0.05% Tween 20 and 0.05% Nonidet P-40), 200 µM dNTPs, 0.5 µM primer and 0.25 U *Taq* polymerase HS (Takara Bio Inc.). Amplification was performed with a PTC-100 thermal cycler (MJ Research Inc., USA) programmed for 3 min at 94℃, followed by 40 cycles of 1 min at 94℃, 2 min at 40℃ and 2 min at 72℃. The reaction was terminated with a final step of 5 min at 72℃, followed by a soak at 4℃ until recovery. Amplification products were separated by electrophoresis through a 1.5% (w/v) agarose gel in 1X TAE. DNA bands were stained with ethidium bromide, visualized under UV light and photographed. RAPD bands were scored manually as present (1) or absent (0). Only distinct major bands were chosen for this study. To estimate variation within populations, analysis of molecular variance (AMOVA)[22] was performed by using GenAlEx6[23]. The significance of the resulting variance components was tested through random permutations. To visualize the relationship among varieties, the genetic identity matrix and similarity measure matrix were subjected to principal coordinate (PCO) analysis[24] by using GenAlEx6[23]. Genetic relationships among varieties were investigated by cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA) based on genetic distances[25] by using the program MEGA ver. 5.1[26]. Bootstrap analyses were performed with 1000 replicates.

Results

RAPD analysis in this study was used to study 3–16 individuals per variety as choice material to detect genetic variation among and/or within varieties and species. Twenty primers used in this study showed 240 polymorphic bands out of 248 amplified bands. Total genetic variation was estimated as the total variance component in AMOVA (Table 2).

Table 2 shows the inter-species and inter-/intra-varietal genetic variance. The inter-species variance

Table 1 Plant materials used in the present study.

Hemerocallis spp.	Japanese or cultivar name	Cite of collection City / Prefecture	Assigned number
H. dumortieri			
var. *esculenta* f. *musashiensis*	Musashino-kisuge	Fuchu / Tokyo	Mu-1～5
var. *esculenta*	Nikkou-kisuge	Kumagaya / Saitama	Ni-1～12
〃	〃	Takayama / Gifu	Ni-13
〃	〃	Soma / Fukushima	Ni-14
〃	〃	Shakotan / Hokkaido	Ni-15
var. *exaltata*	Tobishima-kanzou	Sado / Niigata	To-1～3
〃	〃	Tobishima / Yamagata	To-4, 5
H. aurantiaca			
var. *littorea*	Hama-kanzou	Izu / Shizuoka	Ha-1～16
H. fulva			
var. *longituba*	No-kanzou	Ogawa / Saitama	No-1～3
var. *kwanzo*	Yabu-kanzou	unknown / Yamagata	Ya-1～3
H. sp1 x *H. sp2*	Stella de Oro	commercial	St-1～7

component was higher than the inter-varietal value, which indicates that genetic differentiation clearly occurs at the hierarchical level of both species and varieties. On the other hand, much variation was responsible for the intra-varietal genetic variance (38.35% of the total variation). Genetic differentiation at all hierarchical levels was highly significant ($p<0.001$).

PCO analysis based on the genetic similarity matrix clustered five varieties and one modern cultivar into four groups reflecting genome composition and species taxa (Fig. 1). The first and second PCO axis accounted for 40.3 and 22.8% of total variation, respectively. *H. dumortieri* and a group encompassing *H. fulva* and *H. aurantiaca* separated along the first axis. Only 'Stella de Oro' was distant from the three species, along the second axis. Musashino-kisuge, Nikkou-kisuge and Tobishima-kanzou (all *H. dumortieri*) were intermingled and plotted without a clear division among varieties or *forma*. Only two individuals of Nikkou-kisuge (Ni-6 and -8) were distributed far from the *H. dumortieri* group, and approached the *H. fulva* group. No-kanzou and Yabu-kanzou (both *H. fulva*) were clearly separated, and each individual within each variety was positioned close to the other. The individuals of Hama-kanzou (*H. aurantiaca*) were very close to each other relative to the intra-varietal distribution of the other two species. Cluster analysis (UPGMA) based on the same data indicated more clearly the relation among varieties shown by PCO analysis. That is, *H. dumortieri* formed a cluster consisting of two sub-clusters, except for two Nikkou-kisuge individuals (Ni-6 and Ni-8). Musashino-kisuge and a population of Nikkou-kisuge at Kumagaya in Saitama formed one subcluster. On the other hand, Nikkou-kisuge individuals in Gifu, Fukushima and Hokkaido and Tobishima-kanzou were clustered together. In *H. fulva* and *H. aurantiaca*, three varieties formed one large cluster without a sub-cluster consisting of No-kanzou and Yabu-kanzou, although a small cluster was formed for each variety. Two individuals of Nikkou-kisuge (Ni-6 and Ni-8) formed part of the same cluster as Hama-kanzou and No-kanzou. 'Stella de Oro' was connected to the large *H. dumortieri* cluster with a long clade.

Discussion

To assess genetic variation and relationships among

Table 2 Analysis of molecular variance (AMOVA) for RAPD data sets showing the variation apportioned among species, varieties and individuals.

Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation	Probability
Among species	3	924.917	16.331	36.89	<0.001
Among varieties	3	237.327	10.964	24.76	<0.001
Within varieties	47	798.071	16.980	38.35	
Total	53	1960.315	44.275		

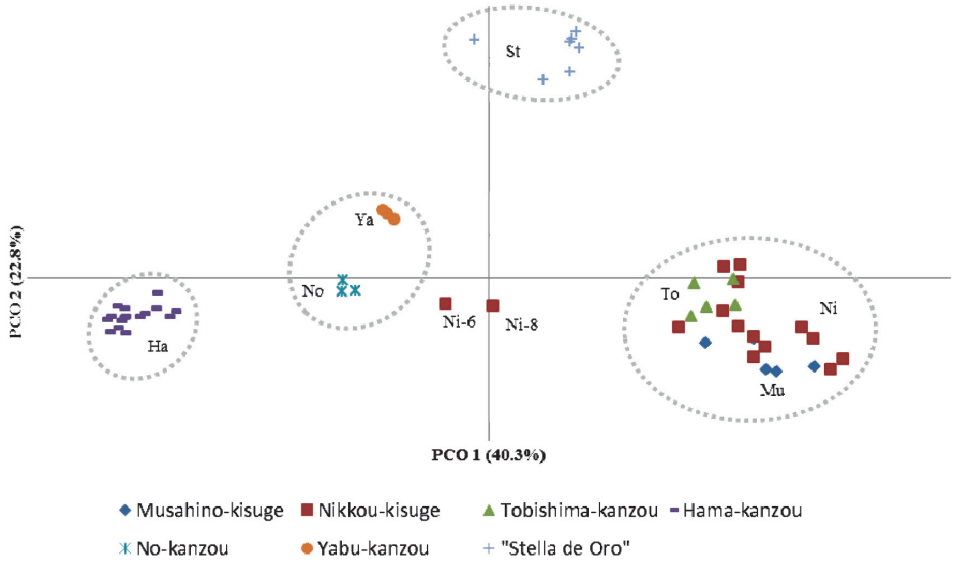

Fig. 1 Principal coordinate plots on the genetic similarity matrix of five varieties and one cultivar of *Hemerocallis* by using RAPD. The number beside the symbols corresponds to those shown in Table 1.

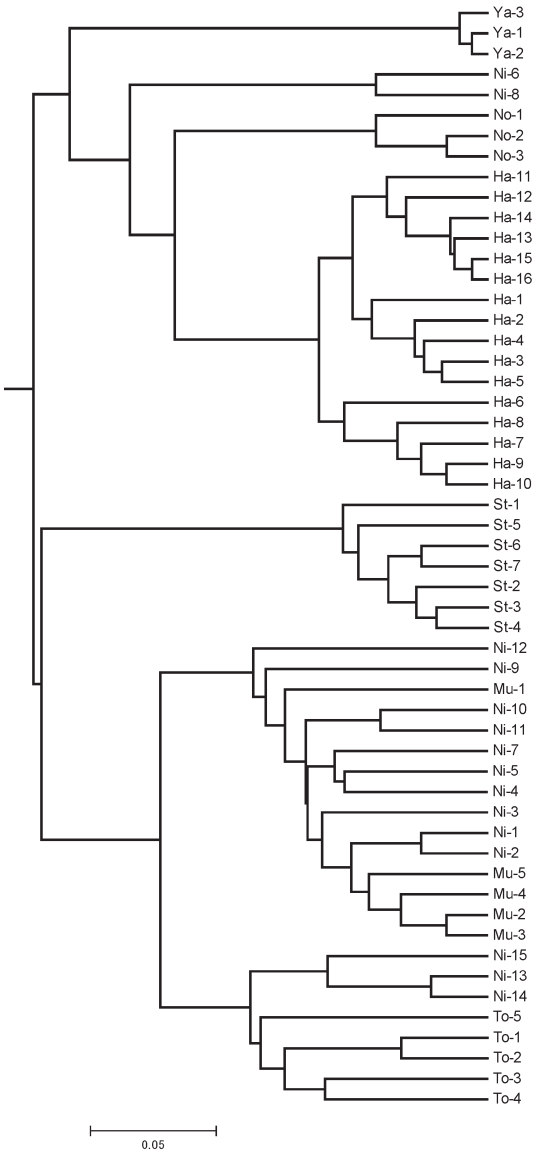

Fig. 2 UPGMA dendrogram based on genetic distance estimated by using RAPD among five *Hemerocallis* varieties and one cultivar. The assigned numbers next to lines correspond to those shown in Table 1.

species, varieties and individuals within varieties of Japanese *Hemerocallis* in this study, RAPD analysis was conducted with 3-16 individuals per variety. In theory, the use of multiple individuals per variety will allow for the determination and suitable evaluation of genetic variability between varieties, including the variability within a variety. Moreover, analysis of multiple individual genotypes per variety provides highly informative data about a population's genetic structure[21,27].

AMOVA indicated that genetic differentiation occurs at both the species' and varieties' hierarchical levels. On the other hand, the genetic variance within varieties indicated broad genetic variation within varieties. The plots of individuals of *H. dumortieri* varieties were more dispersed than those of *H. fulva* and *H. aurantiaca* individuals in PCO analysis. Despite the overlap between individuals of *H. dumortieri* varieties, this variation among or within varieties was divided into two subclusters in cluster analysis. Nikkou-kisuge, of Japanese origin, is distributed in grass fields on mountains and alps in the north of Japan from central Honshu through to Hokkaido while Tobishima-kanzou populations grow near the coast at Tobishima in Yamagata and at Sado in Niigata[2,9,28]. SATAKE *et al.* (1982) also reported that Nikkou-kisuge sometimes grows gregariously on seashore slopes while Tobishima-kanzou is often considered to be Nikkou-kisuge of Tobishima-type, suggesting that some seashore types of Nikkou-kisuge were introduced to Tobishima and Sado. Tobishima-kanzou is classified as a different variety from Nikkou-kisuge, namely var. *exaltata* of a variant of Nikkou-kisuge based on the difference in the flowering period. On the other hand, Musashino-kisuge and individuals of Nikkou-kisuge in Saitama formed another subcluster, both growing wild on particular low-altitude mountains in Kanto district. More specifically, Musashino-kisuge and individuals of Nikkou-kisuge in Saitama grow naturally only in an area termed Sengen-yama (Mt. Sengen) and Kannon-yama (Mt. Kannon). The latter population is called locally Kannonyama-kisuge. Both populations are accounted for as lowland Nikkou-kisuge type, which is a type of Nikkou-kisuge that has adapted to warm areas. In addition, Musashino-kisuge has been classified as *forma musashiensis* within var. *esculenta*[5,7]. However, lowland type Nikkou-kisuge and Nikkou-kisuge from cold districts have not been genetically differentiated by previous reports although our results suggest that they can be genetically differentiated.

On the other hand, two individuals (Ni-6 and Ni-8) separated from Nikkou-kisuge in Saitama approached Hama-kanzou, No-kanzou and Yabu-kanzou group in PCO analysis. In particular, the cluster showing that Ni-6 and Ni-8 is genetically close to No-kanzou suggests that Ni-6 and Ni-8 are hybrids between Kannonyama-kisuge and No-kanzou.

In PCO analysis, Hama-kanzou (*H. aurantiaca*), No-kanzou and Yabu-kanzou (*H. fulva*), which were clearly differentiated varieties, formed one cluster. Furthermore, the fact that a subcluster formed by No-kanzou and Yabu-kanzou did not form in this cluster indicates that No-kanzou is genetically closer to Hama-kanzou than to Yabu-kanzou. Hama-kanzou grows near the seashore in Kanto district while No-kanzou grows wild around groves and forests in an inland area over an area west of Kanto. These two varieties resemble each other morphologically, in particular Hama-kanzou, which is considered to be a

variant of the seashore-type No-kanzou[2]. Matsuoka and Hotta (1966) reported that it is difficult to morphologically distinguish Hama-kanzou and No-kanzou, except for differences in their senescent character, i.e., deciduous or evergreen, respectively. Indeed, several reports have described Hama-kanzou to be classified as an *H. fluva* species, var. *littorea*[2,6]. Indeed, the cluster analysis, reflecting genetic distance, supports these reports that Hama-kanzou is in fact *H. fluva* var. *littorea*.

In this study, we showed that genetic differentiation is a progressive process taking place in each *Hemerocallis* variety and population. The results of genetic relationships among varieties obtained by RAPD analysis generally support the classifications made by previous reports. On the other hand, there is a need for two sets of more detailed investigations. The first is the assessment of genetic differentiation of a lowland-type population of Nikkou-kisuge grown in Saitama within var. *esculenta*. The second is the assessment of genetic distance between Hama-kanzou and No-kanzou. Since this is the first report showing the genetic relationships within the genus *Hemerocallis* growing in Japan, in the future, the application of more DNA markers may reveal more structural genetic diversity of Japanese *Hemerocallis* with a higher level of accuracy. Furthermore, if outcrossing between cultivars introduced from overseas and native individuals progresses, collapses of the genetic character of each species and variety is anticipated. In order to conserve Japanese *Hemerocallis*, establishment of a simple and rapid method such as RAPD for determining genetic classification is desired.

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日本在来 *Hemerocallis* spp. の RAPD 分析による遺伝的変異および類縁関係の評価

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要約：RAPD 法により，日本国内に自生の *Hemerocallis* 属内における種，変種および個体間の遺伝的変異を評価し，類縁関係を推定した。20 種のランダムプライマーを用いた PCR で，248 のバンドが検出され，そのうちの 240（96.7%）のバンドで多型がみられた。多型データに基づく AMOVA の結果，遺伝的変異は種間に比べて変種間で小さく，同データによる主座標分析および平均距離法によるクラスター解析により，各変種について既知の分類を反映した配置が得られた。さらに，これらの分析によって，*H. dumortieri* var. *esculenta* 内における低地タイプの個体群が遺伝的に区別可能であると共に，*H. aurantiaca*（ハマカンゾウ）および *H. fulva*（ノカンゾウ，ヤブカンゾウ）の両種が遺伝的に近縁であることが示唆された。本研究は，DNA マーカーを用いて日本在来の *Hemerocallis* 属内における遺伝変異および類縁関係を示した最初の報告である。

キーワード：*Hemerocallis* spp., RAPD, AMOVA, 主座標分析，クラスター解析

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