Random Amplified Polymorphic DNA (RAPD) Analysis of Three Japanese *Aster* Species

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Summary RAPD analysis was carried out using 60 random primers to investigate molecular genetic characterization of the amphidiploid *Aster microcephalus* var. *ovatus* and its presumable diploid parents. One of the presumable parents, *A. iinumae*, possesses S-type karyotype consisting of shorter chromosomes, which is considered to be derived from L-type karyotype consisting of longer chromosomes, characteristic to another presumable parent, *A. ageratoides*. In all, 611 reproducible fragments were amplified, and the fragment-band size range was from 50 to 12000 bp in the three species. Cluster analysis of RAPD data showed that the three species had quite similar genetic distances from each other with high values. The karyotypes of both *Aster ageratoides* and *A. microcephalus* var. *ovatus* include a set of L-type chromosomes, and the two species possess about 100 specific RAPD bands in common. The common band number between *A. ageratoides* and *A. microcephalus* var. *ovatus* was quite higher than those between *A. ageratoides* and *A. iinumae*, and between *A. iinumae* and *A. microcephalus* var. *ovatus*. This indicates that many fragments found in the L-type chromosomes do not exist in the S-type chromosomes, which could have caused chromosome size reduction from L-type to S-type.

Key words Molecular characterization, RAPD, *Aster*, Karyotype, Cluster analysis.

Aster L. (Asteraceae) comprises about 400 species distributed widely in the northern hemisphere (Noyes and Rieseberg 1999). Due to the existence of many closely related genera (Bremer 1994), the generic circumscription of this genus has been controversial. There has also been a generic controversy over East Asian *Aster*. Based on morphological information of the length of the pappus, Kitamura (1937) segregated three small genera, *Kalimeris* Cass., *Heteropappus* Less., and *Miyamayomena* Kitamura (= *Gymnaster* Kitamura), from *Aster* s.l. However, molecular phylogenetic trees drawn by subsequent works suggested that these three genera should be merged together with *Aster* s.l. (Ito *et al.* 1995, 1998). The molecular works showed multiple origins of *Kalimeris* and *Miyamayomena* derived from different ancestors of *Aster*, and *Heteropappus* also derived from *Aster* independently.

Previous cytogenetic studies using the Japanese genera have revealed that most species of *Kalimeris* had karyotypes consisting of significantly smaller chromosomes (S-type karyotype) than those of other genera (L-type karyotype) (Huziwaru 1957a, b, 1958, 1967, Shindo 1967, Gu 1989). Consequently, these works indicated that the lengths of the pappus and chromosomes could have been shortened in parallel evolution.

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Fig. 1. Somatic chromosome complements of *Aster ageratoides* var. *ageratoides* (a), *Aster iinumae* (b), and *Aster microcephalus* var. *ovatus* (c). Bar= $\times\mu\text{m}$.

Recent cytogenetic studies of *Aster* deal mainly with chromosome size changes and polyploidization in three species, *Aster ageratoides* Turcz. var. *ageratoides* (designated as *A. leiophyllus* Franch. & Sav.), *Aster iinumae* Kitam. ex Hara (designated as *K. pinnatifida* (Matsum.) Kitam.), and *A. microcephalus* var. *ovatus* (Franch. & Sav.) Soejima et Mot. Ito (designated as *A. ovatus*) (Matoba *et al.* 2005, 2007). *Aster ageratoides* has karyotypes consisting of significantly larger chromosomes (L-type karyotype) than those of *A. iinumae* (S-type karyotype) (Fig. 1). Both species are diploids. Interestingly, despite the large size difference between L- and S-type chromosome sets, their chromosome formulas are quite similar; they consist of 2 satsubmedian-, 14 median-, and 2 submedian-centromeric chromosomes (Matoba *et al.* 2005). Additionally, the tetraploidal LS-type karyotype, considered to be an amphidiploid between them, is found in *A. microcephalus* var. *ovatus* (Huziwaru 1957b, Tara 1977, Matoba *et al.* 2005, 2007) (Fig. 1).

It is known that the genome size of a plant species is greatly affected by the amount of repetitive sequences and ploidy levels. The difference in the number of repetitive sequences contributes significantly to genome size, organization of chromosomes, and evolution of plant species (Bennetzen 2000). Although previous studies have indicated that *Kalimeris* was derived from *Aster* s.str. (Ito *et al.* 1995, 1998), there has been no attempt to search caused-DNA sequences directly connected to genome size reduction in this lineage.

The random amplified polymorphic DNA (RAPD) method of DNA amplification (Williams *et al.* 1990) has been widely used as a means of generating genetic markers in many organisms. The RAPD technique could identify genotypes directly and help to mitigate complications arising from earlier cytological and morphological studies (Das 2008). However, in recent molecular work on angiosperms, RAPD generated-DNA sequences were more repetitive in the genome (Koo *et al.* 2005), and were obtained preferentially from a genome with karyotypes consisting of large chromosomes (Hoshi *et al.* 2010).

In this study, RAPD analysis was carried out using 60 random primers to investigate the molecular genetic characterization of the amphidiploid *A. microcephalus* var. *ovatus* and its parental diploid-species with different karyotypes in chromosome size.

Table 1. Source of the materials of three species of *Aster*

Species	Chromosome number (2n)	Karyotype formula	Ploidy level	Strains (source and cultivar)	Accession code
<i>Aster ageratoides</i> var. <i>ageratoides</i>	18	18L	Diploid	Miyazaki Prefecture, Japan	20080819 #1–2
<i>Aster iinumae</i>	18	18S	Diploid	Niigata Prefecture, Japan	20081104 #02–3
<i>Aster microcephalus</i> var. <i>ovatus</i>	36	18L+18S	Tetraploid	Osaka Prefecture, Japan	20090604 1–1

^a L: large size chromosome, S: small size chromosome (see Matoba *et al.* 2007)

Materials and methods

Plant materials

The plant materials of three species of *Aster* used are listed in Table 1. To extract total genomic DNA, leaf materials of the plants were obtained from cultivated plants. All of the plants used were cultivated in the Laboratory of Plant Environment Science, Department of Plant Science, School of Agriculture, Tokai University.

DNA extraction

The present procedure of total genomic DNA extraction is a modification of the CTAB method of Doyle and Doyle (1990). The DNAs were extracted from fresh leaves of the living plant individuals. The fresh leaves were frozen in liquid nitrogen and homogenized to a fine powder. The powdered samples were gently dispersed in an extraction buffer containing 1 M Tris–HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, and 0.5% mercaptoethanol, and were treated with the buffer for 1 h at 55°C. After the treatment, they were extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1) for 15 min each, and the DNAs were precipitated with an equal volume of isopropyl alcohol.

RAPD amplification

RAPD amplification was conducted following the instructions of the RAPD decamer Kit (Operon Technologies, Alameda, CA, USA). To optimize the polymerase chain reaction (PCR) amplification conditions, experiments were carried out with varying concentrations of DNA template, primers, and *Taq* DNA polymerase. Sixty primers of OPA, OPB, and OPC series were selected from the RAPD primer set of Operon Technologies (Alameda, CA, USA).

Amplification was performed on a PCR thermocycler, Program Temp Control System (Astec, PC-708), with 20 µl reaction mixtures containing 20 ng of template DNA, 0.2 mM of each dNTP (dATP, dTTP, dCTP and dGTP), 0.5 units of *Taq* DNA polymerase (Toyobo, TAP-211), 10 pmol of primer, 10 mM Tris–HCl, 50 mM KCl, and 1.5 mM MgCl₂. The amplification regime was performed using the following program: 92°C for 3 min followed by 45 cycles at 92°C for 1 min, 40°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Amplified PCR products were performed onto an agarose gel (1.0% w/v) (Nacalai Tesque, Kyoto, Japan) electrophoresis (iMyrun IMR-201) in 0.5×TAE buffer at 100 V for 50 min. The gel was visualized by ethidium bromide staining and photographed under a UV illuminator (Funakoshi, NTM-20).

Data analysis

All RAPD bands were repeated twice using the same reaction mixture in order to confirm the reproducible amplification of scored fragments. The bands exhibited in both repeated experiments were counted as reproducibly-amplified DNA fragments, and were scored for the construction of the data matrix. The bands that appeared at the same position as the control RAPD samples (with-

Fig. 2. RAPD products from total DNAs. *Aster microcephalus* var. *ovatus* (1), *Aster iinumae* (2) and *Aster ageratoides* var. *ageratoides* (3) was amplified by OPA-01 (a) and OPA-12 (b) RAPD primers. 1 kb Plus DNA Ladder was used for molecular marker (M).

out any template DNA) were excluded. Each band selected was scored for presence (1) or absence (0) of a band. The pooled data matrix was entered into the StatPartner version 2.0 package. A dendrogram was constructed by employing the single linkage method (Sneath, 1957) and UPGMA (unweighted pair group method with arithmetic average) (Sokal and Sneath 1963) to group individuals into discrete clusters.

Results and discussion

RAPD profiling

The stability of RAPD bands for characterizing the molecular difference within the three *Aster* species was well demonstrated with the purified genomic DNA (Fig. 2). A total of 60 decamer random sequences of three primer series (OPA, OPB, and OPC) were initially screened to select proper primers for RAPD analysis. All primers were examined in each of the three *Aster* species accessions for testing RAPD-generated banding patterns. To obtain solid data on band scoring, smear PCR products or obscure bands were removed from the pooled matrix. After the initial electrophoretic-test, 44 primers showed stable bands across all the species, and all other primers showed stable bands in either one or two species. Each primer amplified less than ten varying numbers of DNA fragments. Thus, the obtained bands with all 60 primers were used for further analysis.

In all, 611 reproducible fragments were amplified, and the fragment-band size range was from 50 to 12000bp in three species. The generated bands were in the range of 50 to 12000bp in *A. ageratoides*, 50 to 12000bp in *A. iinumae*, and 200 to 12000bp in *A. microcephalus* var. *ovatus*. The maximum number of major amplicons generated by RAPD was 13, obtained with primers OPB-01 and OPC-01.

Each primer of OPB-16, OPB-19, and OPC-16 revealed a single band for each species, which is polymorphic among the three species. Ten bands were common to *A. ageratoides* and *A. iinumae*, and these bands were obtained from 10 primers. Seventy-seven common bands to *A. ageratoides* and *A. microcephalus* var. *ovatus* were obtained from 43 primers, while 27 common bands to *A. iinumae* and *A. microcephalus* var. *ovatus* were obtained from 22 primers. Ninety-seven and 51

Table 2. Genetic distances obtained from pairwise analysis of single linkage method among various *Aster* species based on RAPD analysis

Species	<i>A. ageratoides</i> var. <i>ageratoides</i>	<i>A. iinumae</i>	<i>A. microcephalus</i> var. <i>ovatus</i>
<i>A. ageratoides</i> var. <i>ageratoides</i>	0.00		
<i>A. iinumae</i>	15.87	0.00	
<i>A. microcephalus</i> var. <i>ovatus</i>	15.36	15.49	0.00

Fig. 3. Dendrogram of genetic relationships between three *Aster* species. The scale indicates the genetic distance derived from RAPD markers data. The cluster tree was determined with the single linkage method (Sneath 1957).

bands generated by 45 and 30 RAPD primers, respectively, were specific to *A. ageratoides* and *A. iinumae*. In contrast, 102 bands generated by 44 primers were specific to *A. microcephalus* var. *ovatus*.

Pairwise genetic distances among the three Japanese *Aster* species in the present study are shown in Table 2. The highest genetic distance was 15.875 with 252 character differences between *A. ageratoides* and *A. iinumae*, while the lowest genetic distance was 15.362 with 236 character differences between *A. ageratoides* and *A. microcephalus* var. *ovatus*. The genetic distance between *A. iinumae* and *A. microcephalus* var. *ovatus* showed an intermediate value of 15.492 with 240 character differences. Cluster analysis of the genetic distance values was performed to generate a dendrogram showing overall genetic relatedness among these three species (Fig. 3). The cluster tree showed that each species had almost the same branch length, although the first cluster comprised *A. ageratoides* and *A. microcephalus* var. *ovatus*, and *Aster iinumae* formed the next higher genetic distance cluster (Fig. 3).

The present RAPD result showed that the three species of Japanese *Aster* have quite similar genetic distances from each other with high values. This indicates that these species kept a high degree of genetic differentiation, which might be caused by a lot of fragments unique to each species. However, the common band number between *A. ageratoides* and *A. microcephalus* var. *ovatus* was higher than that between *A. ageratoides* and *A. iinumae*, and between *A. iinumae* and *A. microcephalus* var. *ovatus*. This indicates that the species with the L-type chromosomes shares many fragments that the S-type chromosomes lack. Current molecular work on *Drosera* suggested that the DNA fragments obtained by RAPD primers were preferentially amplified from the species containing a common genome consisting of large chromosomes (Hoshi *et al.* 2010). This amplifying preference led us to predict that RAPD-generated DNA fragments possessed many of the DNA sequences related to chromosome size changes.

For the three species in this study, *A. microcephalus* var. *ovatus* with the LS-type karyotype is considered to be an amphidiploid derived from hybridization between *A. ageratoides* with the L-type karyotype and *A. iinumae* with the S-type karyotype (Ito *et al.* 1995, 1998, Matoba *et al.* 2005, 2007). The S-type karyotype is suggested to be derived from the L-type karyotype by chromosome size changes (Ito *et al.* 1995, 1998). In our comparison of RAPD bands among the three species, the fragments shared by the species with the L-type and LS-type karyotypes, but not found in the S-type might be related to chromosome size reduction from L-type to S-type. The existence

of fragments specific to each species could be explained by their possible occurrence or disappearance after chromosome size changes or after establishing the LS-type karyotype by amphidiploidization events.

Further molecular analyses, including FISH, are necessary to find the causal sequences related to chromosome evolution with *Aster* species differentiation.

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