Comparing with Phylogenetic Trees Inferred from cpDNA, ITS Sequences and RAPD Analysis in the Genus *Asparagus* (Asparagaceae)

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This genus *Asparagus* (Asparagaceae) includes commercially important species of vegetable as well as some ornamentally or medicinally important species, and it needs molecular markers to conduct precious identification. Therefore, we compared results of phylogenetic analyses based on different molecular approaches for this genus. To reconstruct phylogenetic trees, we used three data sets, sequences of some chloroplast DNA (cpDNA) regions and the internal transcribed spacer (ITS) 1 region, and random amplified polymorphic DNA (RAPD) for seven samples selected from all subgenera in this genus. Both trees of the ITS region and the RAPD analysis have higher resolutions than those of cpDNA sequences. However, the tree of RAPD analysis has low a value of consistency index (CI) due to parallelism of many characters. The tree of ITS1 sequences provided appropriate resolution and a high CI value. Therefore, ITS sequencing is overall the most efficient approach for clarifying the phylogenetic relationship among the species of *Asparagus*.

Keywords : *Asparagus*, nrDNA, *pet*, phylogeny

INTRODUCTION

Asparagus L. (Asparagaceae) is the large genus containing approximately 300 species of three subgenera, *Asparagus*, *Protasparagus* and *Myrsiphyllum*, distributed from subarid to arid regions in the Old World (Dahlgren et al., 1985; Clifford and Conran, 1987; Chen and Tamanian, 2000). This genus is well known by virtue of the fact that it includes commercially important species of vegetable, most notably *A. officinalis*, as well as some ornamentally or medicinally important species such as *A. asparagoides*, *A. scandens*, *A. plumosus* and *A. falcatus* (Chen and Tamanian, 2000). Moreover, *A. cochinchinensis* is one of the medicinally important species in the genus *Asparagus*, which distributed in temperate regions from China to Japan, and the swollen and dried root of this species is a crude drug called as Tiandong in China and as Tenmondo in Japan (Namba, 1993).

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In general, the morphological characters in the genus *Asparagus* are quite simple and most key characters to identify the species in this genus exist in the vegetative parts above ground such as phylloclades, flowers, and fruits, so that it is impossible to identify each species by its dried roots. Therefore, it needs to make genetic markers to identify each species of this genus, but there is little genetic information. Recently, Fukuda et al. (2005) conducted a phylogenetic analysis of *Asparagus* based on chloroplast DNA (cpDNA) sequences, but could not obtain a phylogenetic tree with enough resolution for the species relationship of this genus. Comparing with substitutions of cpDNA sequences between species of *Asparagus* and those of other plant groups, the analysis attributed little variation of cpDNA detected in *Asparagus* to the rapid radiation events of the genus (Fukuda et al., 2005). Rigorous phylogenetic methodology has provided more precious resolution of the closest relationships of distinctive plant lineages than was achieved before the use of such approaches in systematics (Soltis and Soltis, 1998). Understanding of phylogenetic relationships among plant groups gained through molecular phylogenetic studies has offered insights into the site of origin and morphological characteristics of those species. For this purpose, the most appropriate approach and generic region for studying a given taxonomic level or type of evolutionary question needs to be selected carefully.

In this case, other approaches by the sequencing and/or the PCR-mediated technique are needed to reconstruct a more precise phylogenetic tree or to make a genetic marker of this genus. In this study, we compared two analyses, a sequencing analysis of internal transcribed spacer (ITS) and a random amplified polymorphic DNA (RAPD) analysis, and discussed whether these approaches are appropriate tools for resolving phylogenetic relationships among species of this genus.

MATERIALS AND METHODS

Seven *Asparagus* species namely, *A. officinalis* L., *A. schoberioides* Kunth, *A. cochinchinensis* (Lour.) Merrill, *A. asparagoides* L., *A. scandens* Thunb., *A. virgatus* Baker and *A. plumosus* Baker, were examined in this study (Table 1). They belong to three different subgenera in the traditional classification of the genus *Asparagus* (Clifford and Conran, 1987). Vouchers for all species used in this study are also listed in Table 1 and have been deposited in the Herbarium, Graduate School of Science, Tohoku University (TUS), and the Herbarium of Tsumura Laboratory (THS).

Total DNA was isolated from fresh leaf tissues based on the method described by Doyle and Doyle (1987). Isolated DNA was resuspended in TE and stored at -20°C until use. Double-stranded DNA was amplified after incubation at 94°C for 2 min, by 30 cycles of incubation at 94°C for 1.5 min, 48°C for 2 min, and 72°C for 3 min, with a final extension at 72°C for 15 min. For

Table 1 List of taxa and GenBank accession numbers of nucleotide sequences in this study.

Taxa	Locality	Collectors	Subgenus	<i>petB</i> intron	<i>petD-rpoA</i>	ITS
<i>Asparagus asparagoides</i> L.	cultivated in Tohoku University	Fukuda 041401	<i>Myrsiphyllum</i>	AB177709	AB177733	AB245517
<i>A. cochinchinensis</i> (Lour.) Merrill	TAIWAN: Keelung, Hepingdao	Yokoyama & Tsukaya <i>s.n.</i>	<i>Asparagus</i>	AB177711	AB177735	AB195572
<i>A. officinalis</i> L.	JAPAN: Miyagi, Furukawa (cult.)	Komatsu 041118	<i>Asparagus</i>	AB177722	AB177746	AB195716
<i>A. plumosus</i> Baker	cultivated in Tohoku University	Fukuda 041404	<i>Protasparagus</i>	AB177724	AB177748	AB246319
<i>A. scandens</i> Thunb.	cultivated in Tohoku University	Fukuda 041406	<i>Myrsiphyllum</i>	AB177727	AB177751	AB245518
<i>A. schoberioides</i> Kunth	JAPAN: Shimane, Yatsuka	Miki <i>s.n.</i>	<i>Asparagus</i>	AB177728	AB177752	AB195562
<i>A. virgatus</i> Baker	cultivated in Tohoku University	Fukuda 041417	<i>Protasparagus</i>	AB177732	AB177756	AB245519

the ITS1 region, we amplified using the primers, namely, ITS2 and ITS5 designed by White et al. (1990). DNA was amplified by PCR in a 50 μ l reaction volume containing approximately 50 ng total DNA, 10 mM Tris-HCl buffer (pH 8.3) with 50 mM KCl and 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (TaKaRa) and 0.5 μ M of each primer. After amplification, reaction mixtures were subjected to electrophoresis in 1.0% low-melting-temperature agarose gels for a purification of amplified products. We sequenced the purified PCR products using a DYEnamic ET-terminator Cycle Sequencing Kit (Amersham Pharmacia) and a Model 373A automated sequencer (Applied BioSystems) according to the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification. Sequences for each region were prealigned with the CLUSTAL X program (Thompson et al., 1997), and then ambiguously aligned regions were manually corrected to minimize indels. Alignment for all DNA regions required the inclusion of several indels. These indels were coded as binary (0 or 1) and treated as the fifth characteristic. The two cpDNA regions sequenced were combined into a single data set because the chloroplast genome is not subject to recombination. Phylogenetic analysis and a test of clade support were conducted using a PAUP* (version 4.0b10; Swofford, 2002). Maximum parsimony analyses were carried out via a heuristic search with TBR branch swapping and MULPERS option. Multiple islands of the most parsimonious trees (Maddison, 1991) were identified using the heuristic option with 100 random sequence additions. To estimate confidence levels of monophyletic groups, the bootstrap method with 1000 replications was employed (Felsenstein, 1985). All sequences were deposited in the DDBJ/EMBL/GenBank International DNA databases (Table 1).

In the RAPD study, forty random primers (10 mer) (OPI01 through 20 and OPJ01 through 20, Operon Technologies) and twelve base random primers (twelve mer) (DNA Oligomer Set A-2; Wako Pure Chemical Industries, Ltd.) were used in this study. The sequences of these primers are available from the listed companies. PCR was performed in 25 μ l of a reaction mixture that contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 8 mM MgCl₂, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.32 μ M of a single random primer, 50 ng of total DNA and 1–2 units of *Taq* DNA polymerase (Gibco BRL). The enzyme was added after the heating of samples for 4 min at 94°C.

PCR was conducted for 40 cycles, with each cycle consisting of 1 min at 94°C, 1 min at 42°C and 2 min at 72°C. The reaction mixture was finally incubated for 5 min at 72°C. PCR was performed with a GeneAmp PCR System 9600 (Perkin Elmer Cetus). The products of PCR were analyzed using gel electrophoresis in 1.0% agarose gels in 1x TBE buffer. Gels were stained with ethidium

Fig. 1 An example of the RAPD analysis (A) and the southern blot analysis (B) of *Asparagus* species. Arrows indicate the bands using southern blot analysis. Above and below bands in the southern blot analysis (B) indicate the bands which is showing arrow 1 and 2, respectively. M: marker (digested by *Hind* III), 1: *A. cochinchinensis*, 2: *A. plumosus*, 3: *A. officinalis*, 4: *A. scandens*, 5: *A. schoberioides*, 6: *A. asparagoides*, 7: *A. virgatus*.

bromide and bands of DNA were visualized on an UV transilluminator (Fig. 1A). Moreover, we performed Southern Hybridization to clarify the homology in some of the polymorphic DNA in the RAPD experiment (Fig. 1B). PCR products were electrophoresed in 1.0% agarose gels in 1x TBE buffer. Hybridization was performed at 42°C in a solution containing 50% formamide, 5x Saline-Sodium Citrate (SSC), 0.1% N-lauroylsarcosine, 0.02% Sodium Dodecyl Sulfate (SDS) and 2% blocking reagent (Roche Co.). A single band was excised from the gel, used as a hybridization probe and labeled with the PCR DIG Probe Synthesis Kit (Roche Co.). Washes were performed twice in 2xSSC, 0.1% SDS at room temperature for 5 min and twice in 0.5xSSC, 0.1% SDS at 65 °C for 15 min. Results were scored for computer analysis on the basis of the presence or absence of each amplified product ("1" for the presence and "0" for the absence of a homologous band). The genetic relationships among seven species of *Asparagus* were evaluated using the same methods as the phylogenetic analysis of cpDNA and ITS sequences, because RAPD analysis is one of the appropriate tools for resolving phylogenetic relationships in interspecific comparisons (Rieseberg, 1996).

Fig. 2 The phylogenetic trees by the RAPD analysis (A), by the ITS sequences (B), and by the cpDNA sequences (C). The number of the branches indicates the synapomorphic characters; the bootstrap values are in bold (only those more than 50 % are indicated on the tree).

Table 2 Genetic distances among species of *Asparagus* based on results of RAPD analysis.

	<i>A.cochinchinensis</i>	<i>A.plumosus</i>	<i>A.officinalis</i>	<i>A.scandens</i>	<i>A.schoberioides</i>	<i>A.asparagoides</i>
<i>A.plumosus</i>	0.0367					
<i>A.officinalis</i>	0.0189	0.0341				
<i>A.scandens</i>	0.0267	0.0356	0.0456			
<i>A.schoberioides</i>	0.0207	0.0419	0.0156	0.0591		
<i>A.asparagoides</i>	0.0485	0.0261	0.0464	0.0404	0.0481	
<i>A.virgatus</i>	0.0277	0.0303	0.0298	0.0274	0.0315	0.0529

RESULTS AND DISCUSSION

In cpDNA analysis, published sequences of the *petB* intron and the intergenic region of *petD-rpoA* could gain some characters to reconstruct the phylogenetic tree in *Asparagus*. A data set comprising 660 characters from a combination of the *petB* intron and the *petD-rpoA*, including 15 indels, was analyzed. We obtained the 11 most parsimonious trees of the 27 steps with a consistency index (CI) of 0.85 including autapomorphies, or 0.60 excluding autapomorphies, and a retention index (RI) of 0.56. One of the most parsimonious trees is shown in Fig. 2C. When we used the ITS1 sequence data set in phylogenetic analyses, we obtained the two most parsimonious trees of the 54 steps with a consistency index (CI) of 0.94 including autapomorphies, or 0.86 excluding autapomorphies and a retention index (RI) of 0.84. One of the most parsimonious trees is shown in Fig. 2B. In the RAPD analysis, out of the 243 amplification products scored in total, 219 (90%) were polymorphic. An example of the RAPD analysis and the southern blot analysis of *Asparagus* species are shown in Fig. 1. In this example, the profile generated by RAPD analysis was composed of two to five major bands. In the southern blot analysis, *A. scandens* and *A. asparagoides* had the same amplified fragments. Genetic distances are shown in Table 2. We obtained a single most parsimonious tree of the 322 steps with a consistency index (CI) of 0.68 including autapomorphies, or 0.56 excluding autapomorphies, with a retention index (RI) of 0.38 (Fig. 2A).

The foregoing applications illustrate the potential for applying efficiently generated polymorphic DNA markers to a wide array of systematics in various plant groups (Soltis and Soltis, 1998). In our phylogenetic analysis, *A. plumosus* was a monophyletic group with *A. asparagoides* in RAPD analysis (Fig. 2A), however, *A. plumosus* was a monophyletic group with *A. virgatus* in ITS and cpDNA sequences (Fig. 2B and C). The phylogenetic trees using ITS and cpDNA sequences showed the same branching patterns. Both phylogenetic trees using ITS sequences and RAPD analysis could have higher resolution than those of cpDNA sequences (Fig. 2). The phylogenetic tree of RAPD has many syn- and aut-apomorphic characters in all lineages of *Asparagus* (Fig. 2A). However, RAPD fragments often vary in band intensity and the differences could result from following reasons such as primer mismatch, relative sequence abundance and so on (Williams et al., 1993). Fainter bands are generally less robust in RAPD experiments and this fact suggests that varying degrees of primer mismatch may account for many band intensity differences (Heun and Helentjaris, 1993). These problems may mislead results. Although our analysis of RAPD could detect many variations, and the homologous bands were confirmed by the southern blot analysis, the low value of CI (0.68) indicates that many of the polymorphic characters are still the results of parallelisms. This is probably based on the fact that the *Asparagus* species used in our study had relatively large phylogenetic distances among them to compare by RAPD analyses. Therefore, RAPD analysis in this genus may be useful for the comparison among populations of the same species or among closely related species in the genus.

The application of the ITS sequences comparison has been most widely used in the estimation of phylogenetic relationships among species, and we also could obtain the phylogenetic tree with the appropriate resolution (Fig. 2B). In our study, although the number of phylogenetic informative

characters in the ITS tree is smaller than those of the RAPD analysis, the value of CI in the ITS tree (CI=0.94) is higher than those of the RAPD. Therefore, it is considered that the ITS tree is the most reliable of other approaches in the phylogenetic analysis of *Asparagus*. The ITS sequencing is the most appropriate approach for clarifying the phylogenetic relationship among species of *Asparagus*, which had experienced the rapid radiation events.

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