American Journal of Plant Sciences, 2011, 2, 765-775
doi:10.4236/ajps.2011.26091 Published Online December 2011 (http://www.SciRP.org/journal/ajps)

Nucleotide Sequence Variations in a Medicinal Relative of Asparagus, *Asparagus cochinchinensis* (Lour.) Merrill (Asparagaceae)

Tatsuya Fukuda[1*], In-Ja Song[2], Takuro Ito[3], Hiroshi Hayakawa[4], Yukio Minamiya[4], Akira Kanno[5], Hokuto Nakayama[6], Jun Yokoyama[7]

[1]Faculty of Agriculture, Kochi University, Nankoku, Japan; [2]Subtropical Horticulture Research Institute, Jeju National University, Jeju, Korea; [3]Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan; [4]United Graduate School of Agricultural Sciences, Ehime University, Monobe, Japan; [5]Graduate School of Life Sciences, Tohoku University, Sendai, Japan; [6]Graduate School of Science, University of Tokyo, Tokyo, Japan; [7]Depertment of Biology, Faculity of Science, Yamagata University, Yamagata, Japan.
Email: [*]tfukuda@kochi-u.ac.jp

Received February 20th, 2011; revised March 15th, 2011; accepted April 6th, 2011.

ABSTRACT

To determine the evolutionary history of Asparagus cochinchinensis (*Asparagaceae*), *we investigated the geographic pattern of its nucleotide sequence variations in Japan, Taiwan, South Korea and China. We found* 21 *polymorphic nucleotide sites by sequencing the internal transcribed spacer* (*ITS*) *region, which gave rise to a total of* 15 *haplotypes, labeled A to O. The A-type was found only in inland China* (*Guizhou and Sichuan*), *and the other haplotypes in China extended to several lineages*; *therefore, A. cochinchinensis may have its origin in the interior of China. The I-type has large distribution area and it also experienced a quick expansion in relative recent years. Haplotype differentiation was observed between the eastern and western side of the Central Mountain Ridge in Taiwan. Two lineages were found in Japan, one in the Yaeyama Islands and the other in remaining areas of Japan, implying that A. cochinchinensis independently colonized in Japan at least twice.*

Keywords: *Asparagus cochinchinensis*, *Internal Transcribed Spacer* (*ITS*), *Phylogeny*, *Phylogeography*

1. Introduction

Genetic variation within species is one of the fundamental underpinnings of biological diversity [1]. The distribution of genetic variation is shaped by processes that are extrinsic to the species, such as ecological events or selective regimes, as well as intrinsic factors, such as the type of mating system. When considered in a geographical context, the structure of this variation is a product of current genetic exchange within a species as well as historical relationships between populations. By taking into account the genealogical relationships between haplotypes as well as their geographical distribution, phylogeographical methods can potentially determine the historical and recurrent population-level processes that shape current patterns of variation. Moreover, genetic lineages are often localized geographically and may share a common history, such as episodes of vicariance, routes of migration, colonization, and recent population expansions [2-4]. Such historical insights can be obtained by identifying the haplotype variations, which are then overlaid upon a geographical frame, reflecting the spatiotemporal dynamics of the organism studied.

The number of phylogenetic studies based on molecular data has grown enormously in recent years, and most of the recent studies are concerned with closely related species or variation within species. In particular, the use of molecular markers has considerably improved our knowledge about how past events shape the genetic diversity within a species [2-4]. In angiosperms, for example, the application of phylogenetic analysis is emerging as an important and practical tool for the study of economically important species such as rice, maize, sunflower and cassava, and their relatives [5-13]. These analyses also helped to unveil the domestication processes of these crops, and the ancestors and close relatives of a given species have repeatedly provided sources of useful traits to genetically impoverished crops. Moreover, con-

scious efforts to search for desirable traits in plants have been underway for the past century, and in recent decades species with desirable traits have come to be regarded as important biological resources in need of conservation [14]. The population structure and history of a given species is thus an important research focus, because this knowledge is needed to design concerted efforts for conservation of the species and to understand the evolutionary processes leading to genetic diversity. Recently, various intergenic spacers have been widely analyzed to assess the genetic variability of wild plants [15-22]. These markers are known to be mostly neutral with relative high mutation rates, and, in association with the geological history, provide information to estimate the extent of seed dispersal and track migration routes [23-27].

The genus *Asparagus* (Asparagaceae) is a large group distributed in arid and sub-arid regions of the Old World [28-35]. This genus is well known by virtue of the fact that it includes commercially important species of vegetables, most notably *A. officinalis*, as well as some ornamentally or medicinally important species such as *A. asparagoides*, *A. scandens*, *A. plumosus*, and *A. falcatus* [36]. *A. cochinchinensis* (Lour.) Merrill is one of the medicinally important species in the genus, and has small flowers in raceme-like inflorescences, whitish-colored berries, and swollen roots [37]. It is mainly distributed along seashores in temperate regions from China to Japan [31,37]. Here, to enhance our understanding of the evolutionary history of Asparagaceae, we describe the DNA polymorphisms of the internal transcribed spacer (ITS) region in *A. cochinchinensis* and discuss how these genetic variations spread within the present distribution with morphological differentiation.

2. Materials and Methods

2.1. Plant Materials

Thirty-seven samples of *Asparagus cochinchinensis* were examined in this study (**Table 1**). *A. lycopodineus* Wall. ex Baker, *A. schoberioides* Kunth and *A. officinalis* L. were selected as outgroups on the basis of phylogenetic analyses of the genus *Asparagus* [38]. Vouchers for all species sampled in this study have been deposited in the Herbarium, Graduate School of Science, Tohoku University (TUS), and the Herbarium of Tsumura Laboratory (THS).

2.2. Morphological Analysis

Length and width of a phylloclade vary extensively in *Asparagus cochinchinensis* [37]. Therefore, a preliminary morphological analysis of *A. cochinchinensis* and its allied species was conducted by measuring the continuous macromorphological variables of length and width of 5 phylloclades in each individual. Measurements were taken with a digital caliper. Phylloclade measurements were taken from flowering individuals.

2.3. DNA Extraction, Amplification, and Sequencing

Total DNA was isolated from 200 - 300 mg of phylloclades with a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA), according to the manufacturer's protocol. Isolated DNA was resuspended in Tris-EDTA (TE) buffer and stored at –20˚C until use.

Of *Asparagus* species, only *A. cochinchinensis* has two relatively large deletions, which are located between the *ndhC* and *trnV* genes in chloroplast DNA (cpDNA) and are 95 bp and 347 bp in length [39]. To identify *A. cochinchinensis*, therefore, two regions of cpDNA including indels were amplified using two pairs of primers (**Table 2**) [40].

For phylogenetic analysis in this species, we amplified the ITS1 region with primers designed by White *et al*. [41]. DNA was amplified by PCR in a 50 µl reaction volume containing approximately 50 ng total DNA, 10 mmol/l Tris-HCl buffer (pH 8.3) with 50 mmol/L KCl and 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 1.25 units *Taq* DNA polymerase (TAKARA) and 0.5 µmol/L of each primer. We used the following thermal cycle profile for amplification: 1.5 min at 94˚C, 2 min at 48˚C, and 3 min at 72˚C for 40 cycles, followed by 15 min of final extension at 72˚C. After amplification, reaction mixtures were subjected to electrophoresis in 1% - 2% low-melting-temperature agarose gels for 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.

2.4. Data Analysis

Sequences for the ITS region were prealigned with the CLUSTAL X program [42]. Alignment for this region required the inclusion of an indel. This indel was coded as binary (0 or 1) and treated as the fifth character.

Phylogenetic analysis and a test of clade support were conducted using the PAUP* program (version 4.0b10) [43]. Maximum parsimony analyses were carried out via a heuristic search with TBR branch swapping and the MULPERS option. Multiple islands of the most parsimonious trees [44] were identified using the heuristic option with 100 random sequence additions. To estimate confidence levels of monophyletic groups, the bootstrap

Table 1. List of taxa, sources and haplotypes of plant materials.

Taxon	OTU name	Locality	Haplotype
Asparagus cochinchinensis (Lour.) Merrill			
var. *cochinchinensis*	ACChinaSC1	CHINA: Sichuan, Gulin	A
	ACChinaSC2	CHINA: Sichuan, Gulin	A
	ACChinaGZ	CHINA: Guizhou, Anshun	A
	ACChinaHN	CHINA: Hainan, Sanya	B
	ACChinaAH	CHINA: Anhui, Shitai	J
	ACChinaHB	CHINA: Hubei, Xianning	K
	ACChinaHK	CHINA: HongKong, Mt.Victoria	I
	ACTaiwanHL	TAIWAN: Hualien, Chosi	C
	ACTaiwanTC	TAIWAN: Taichung, Hoping	H
	ACTaiwanNT	TAIWAN: Nantou, Mt. Nankao-shan	F
	ACTaiwanTP	TAIWAN: Taipei, Kungliao	C
	ACTaiwanKS	TAIWAN: Kaohsiung, Mt. Tsuyun-shan	G
	ACTaiwanKL	TAIWAN: Keelung, Hepingdao	I
	ACSouthKorea	SOUTH KOREA: Jeollanam-do, Yochon	I
	ACIriomote	JAPAN: Okinawa, Iriomote Isl.	D
	ACYonaguni	JAPAN: Okinawa, Yonaguni Isl.	E
	ACMiyako	JAPAN: Okinawa, Miyako Isl.	I
	ACYakushima	JAPAN: Kagoshima, Yakushima Isl.	I
	ACKagoshima1	JAPAN: Kagoshima, Kimotsuki, Uchinoura	I
	ACKagoshima2	JAPAN: Kagoshima, Kimotsuki, Nejime	I
	ACMiyazaki	JAPAN: Miyazaki, Koyu, Kawaminami	L
	ACFukuoka1	JAPAN: Fukuoka, Munakata, Oshima	I
	ACFukuoka2	JAPAN: Fukuoka, Munakata, Genkai	I
	ACKochi	JAPAN: Kochi, Tosa, Kagami	N
	ACKagawa	JAPAN: Kagawa, Takamatsu	M
	ACEhime	JAPAN: Ehime, Hojo	I
	ACTokushima1	JAPAN: Tokushima, Kaifu, Mugi	N
	ACTokushima2	JAPAN: Tokushima, Tokushima	M
	ACOkayama	JAPAN: Okayama, Okayama	N
	ACAwaji1	JAPAN: Hyogo, Awaji Isl. (Tsuna, Ichinomiya)	N
	ACAwaji2	JAPAN: Hyogo, Awaji Isl. (Mihara, Nandan)	N
	ACWakayama1	JAPAN: Wakayama, Higashimuro, Taiji	I
	ACWakayama2	JAPAN: Wakayama, Nishimuro, Shirahama	O
	ACShizuoka1	JAPAN: Shizuoka, Numazu	I
	ACShizuoka2	JAPAN: Shizuoka, Ito	I
	ACKanagawa	JAPAN: Kanagawa, Kamakura	I
A. cochinchinensis (Lour.) Merrill			
var. *pygmaeus* Makino	ACpygmarus	JAPAN: cultivated in Tohoku Univ.	I
Outgroup			
A. officinalis L.		JAPAN: cultivated in Tohoku Univ.	*****
A. schoberioides Knuth		JAPAN: Shimane, Yatsuka, Shimane	*****
A. lycopodineus Wall. Ex Baker		CHINA: Sichuan, Tianquan	*****

Table 2. Sequences of primer pairs in amplification of the two deletion regions of cpDNA (Kanno *et al.* 1997).

Region	Primer	Sequence (5'-3')
Deletion A	DelA-Fw	GGGTAGAGAATCGTTGCCTC
	DelA-Rv	CCAATCGCGGTCTTTCCCTA
Deletion B	DelB-Fw	GGCGAACAATTCGACAGACC
	DelB-Rv	CGAGTCCGTATAGCCCTAAC

method was employed with 1000 replications [45].

3. Results

To identify *Asparagus cochinchinensis*, we first amplified two regions that have been reported to include relatively large deletions in cpDNA [39]. All samples of *A. cochinchinensis* had two large deletions, but other *Asparagus* species including in the sister groups of *A. cochinchinensis* [38], did not have the deletions (**Figure 1**). Thus, the presence of deletions is a definite characteristic that can be used to identify *A. cochinchinensis* even if samples have insufficient morphological features. We then compared the phylloclade morphology of *A. cochinchinensis* with that of *A. lycopodineus* (**Figure 2**), and found that *A. cochinchinensis* has narrow or linear phylloclades, *A. lycopodineus* has broader phylloclades than those of *A. cochinchinensis*. *A. cochinchinensis*, individuals from China had greater diversity in phylloclade sizes and forms than those from Taiwan, South Korea and Japan. Among samples from China, those from Hainan had the largest size of phylloclades (**Figure 2**). Samples from Guizhou and Sichuan also had broader phylloclades than those from other areas and came close to the size range of *A. lycopodineus* (**Figure 2**). Other samples from China were within the range of variance of samples from Taiwan, South Korea, and Japan.

To construct a phylogenetic tree, we determined the sequences of the ITS1 region from 37 samples of *Asparagus cochinchinensis* and three outgroups, *A. lycopodineus*, *A. officinalis* and *A. schoberioides*. Two types for the length of the ITS1 region of *A. cochinchinensis* were found. Six of 37 samples in *A. cochinchinensis* were 249 bp and the remainder was 250 bp. We analyzed a data set of ITS1 sequences including 21 parsimony-informative characters with one indel and obtained three parsimonious trees of 44 steps with a consistency index (CI) of 0.886 and a retention index (RI) of 0.911. A strict consensus tree is shown in **Figure 3**.

In the phylogenetic tree, all samples of *Asparagus cochinchinensis* composed a monophyletic group with 15 haplotypes, denoted A to O-type (**Figures 3**, **4**). The relationship of the haplotypes to the location of each individual is indicated in **Table 1**. The sequences of each

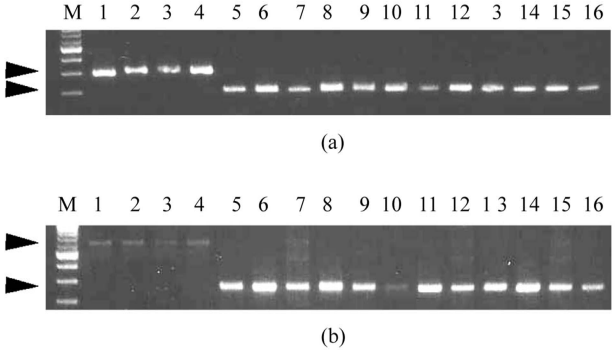

Figure 1. The length variation of cpDNA among *Asparagus cochinchinensis* and its allied species. Panel (a) shows DelA and panel (b) shows DelB (Kanno *et al.* 1997). M, 100-bp ladder; 1. *Asparagus virgatus*; 2. *A. officinalis*; 3. *A. schoberioides*; 4. *A. lycopodineus*; 16. *A. cochinchinensis* var. *pygmaeus*. The remaining lanes contain *A. cochinchinensis* var. *cochinchinensis* from 5. Sichuan in China; 6, Hainan in China; 7. Anhui in China; 8. Hubei in China; 9. Taipei in Taiwan; 10, Kaohsiung in Taiwan; 11, Nantou in Taiwan; 12. Chonranam in South Korea; 13, Okinawa (Iriomote Isl.) in Japan; 14. Okayama in Japan; 15. Kanagawa in Japan. Arrowheads indicate expected length of PCR products.

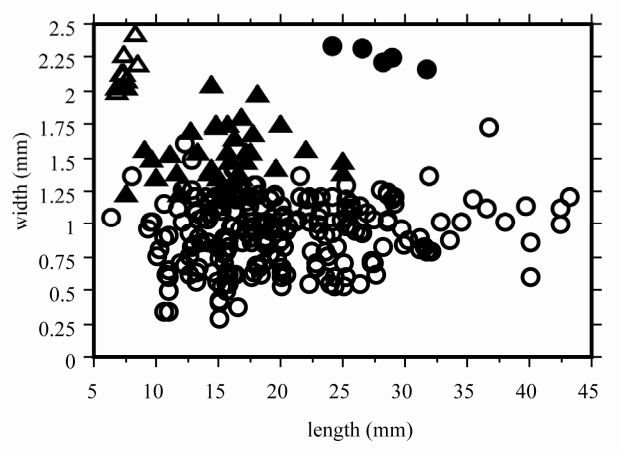

Figure 2. Relationships between the length and the width of the phylloclades in *Asparagus cochinchinensis*. ▲, A-type; ●, B-type; ○, remaining haplotypes; △, *Asparagus lycopodineus*.

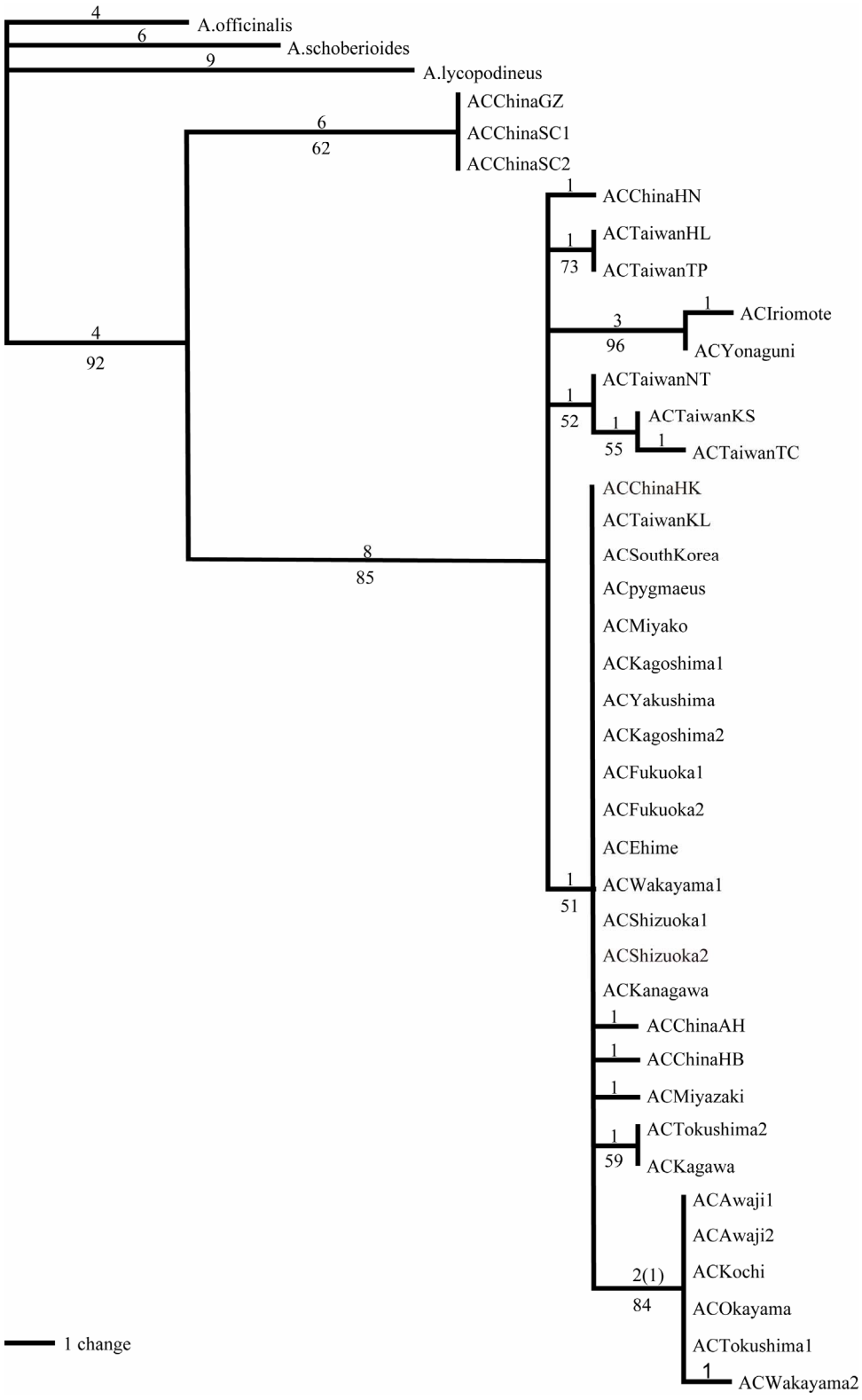

Figure 3. Phylogenetic relationships of haplotypes of *Asparagus cochinchinensis* and outgroups. The numbers above the branches indicate the values of synapomorphic characters and the number of parenthesis indicates an indel. The numbers below the branches indicate the bootstrap value. AC, *Asparagus cochinchinensis*. For other abbreviations, see Table 1.

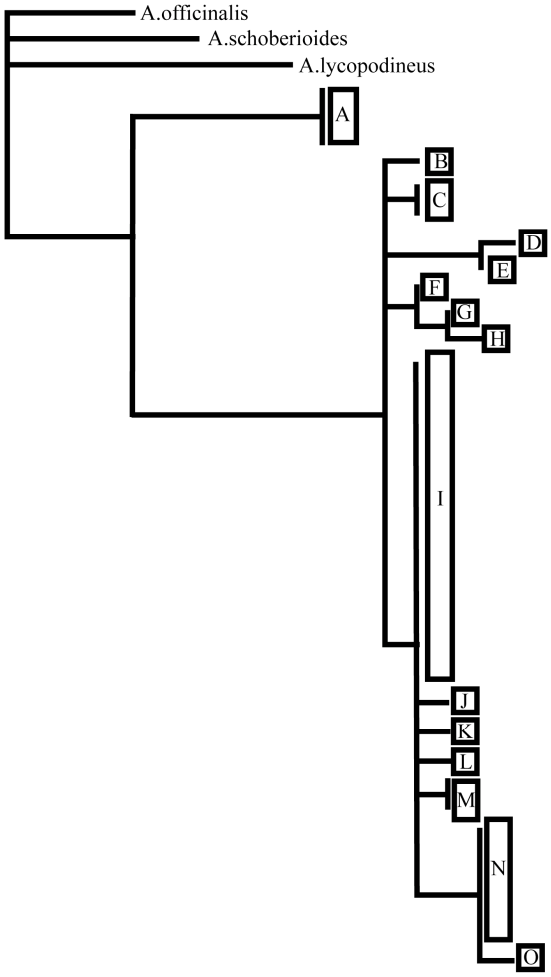

Figure 4. Schematic of the correspondence between haplotypes and OTUs in Figure 3.

haplotype have been deposited in the DDBJ/EMBL/GenBank international DNA data bank (**Table 3**). The geographic distribution of each haplotype is shown in **Figure 5**. Among this monophyletic group, individuals from Guizhou and Sichuan in China were same haplotype (A-type) and were distinguished from other haplotypes in *A. cochinchinensis* by six apomorphic nucleotide substitutions. The remaining fourteen haplotypes, from B to O, were monophyletic, as supported by a relatively high bootstrap value (85%). Of these haplotypes, the I-type was the most widespread haplotype and occurred in China, South Korea, and Japan. The other types had more restricted distributions than the I-type.

The geographical distributions of the haplotypes are shown in **Figure 5** and **Table 1**. Five haplotypes (A, B, I, J and K) were found in China; 5 haplotypes (C, F, G, H and I) occurred in Taiwan; and 1 haplotype (I) was found in South Korea. In Japan, seven haplotypes (D, E, I, L, M, N and O) were found from the Yaeyama Islands in the south to Kanto Distinct in central Honshu (Kanagawa). The D- and E-types formed a monophyletic group, as did the N- and O-types. The monophyly of the N- and O-types was supported by a 1-bp indel as well as one synapomorphic substitution. Among the 7 haplotypes in Japan, the I-, L-, N-, M-, and O-types, which occur from Yakushima Island to Kanagawa, were monophyletic with the J- and K-types in China. These results suggested that individuals in this clade have a different origin from those in the Yaeyama Islands (Iriomote and Yonaguni Islands), and at least two lineages of *Asparagus cochinchinensis* exist in Japan.

Table 3. List of GenBank accession numbers of nucleotide sequences for the taxa examined.

Taxon	Accession Number
Asparagus officinalis	AB195716
A. schoberioides	AB195562
A. lycopodineus	AB195563
A. cochinchinensis	
var. *cochinchinensis*	
(A-type)	AB195564
(B-type)	AB195565
(C-type)	AB195566
(D-type)	AB195567
(E-type)	AB195568
(F-type)	AB195569
(G-type)	AB195570
(H-type)	AB195571
(I-type)	AB195572
(J-type)	AB195573
(K-type)	AB195574
(L-type)	AB195575
(M-type)	AB195576
(N-type)	AB195577
(O-type)	AB195578
A. cochinchinensis	
var. *pygmaeus*	AB195579

The dwarf-type of *Asparagus cochinchinensis* (less than 20 cm high) is known as *A. cochinchinensis* (Lour.) Merrill var. *pygmaeus* Makino and is cultivated as ornamental purposes [31]. Our phylogenetic result in- dicated that this variety is of the I haplotype (**Figures 2**, **3**), that is, of the most widespread haplotype in Japan.

4. Discussion

The usefulness of the ITS region as a molecular marker for the indirect estimation of infraspecies relationships depends on the level of phylogenetic resolution [46]. The

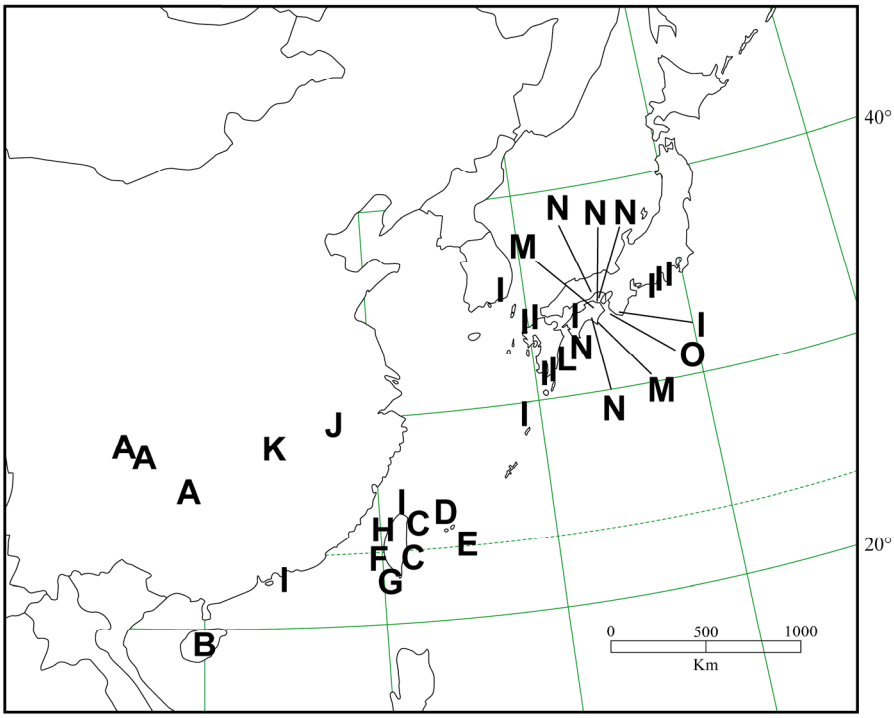

Figure 5. Geographical distribution of haplotypes in *Asparagus cochinchinensis*. Letters in this figure indicate the locations of the haplotypes.

ITS region had faster divergence rates and subsequently higher phylogenetic resolution than regions of cpDNA [47]. In our previous study, we used primers designed primers by Taberlet *et al.* [48] and Nishizawa and Watano [49] to sequence approximately 3000 bp of 16 regions in cpDNA including frequently analyzed regions for some *Asparagus* species, but found little variation in these regions [38]. As a result, we concluded that cpDNA is powerless for phylogenetic studies within this species. In contrast, we found in current study that the ITS1 region of *A. cochinchinensis* possesses a relatively high level of haplotype diversity (15 haplotypes) and nucleotide variation (up to 4.2%). This region, therefore, provides sufficient phylogenetic resolution at a population level within the species. Our results on the usefulness of the ITS1 region are accordance with the results of Beckler and Holtsford [5] who used the ITS phylogenetic tree to reveal the recent derivation of maize from teosinte, and of Dick *et al.* [50], who concluded on the basis of various ITS phylogenetic trees that the ITS region is a powerful tool for resolving historical relationships among populations of widespread plants. Here, we revealed that the ITS region of *A. cochinchinesis* have 21 polymorphic nucleotide sites leading 15 haplotypes and one of haplotyps (A-type in China) diverged deeply compared with other haplotypes. These results show the evolutionary origin and phylogeographic pattern of *A. cochinchinensis* in eastern Asia.

4.1. Phylogenetic and Morphological Analyses of *Asparagus cochinchinensis* and Its Allied Species

Our results indicate that some mutations have accumulated in the ITS1 region of *Asparagus cochinchinensis* between the A-type and the other haplotypes at the basal position of this phylogenetic tree (**Figures 3**, **4**), suggesting the relatively ancient divergence of the A-type. The A-type comprised individuals from inland China (Guizhou and Sichuan). Our morphological analysis indicated that *A. cochinchinensis* has narrow or linear phylloclades, and *A. lycopodineus* has broader phylloclades than those of *A. cochinchinensis*, a result that agreed well with the description of Chen and Tamanian [37]. The phylloclades of the A-type individuals tended to be broader than those of other haplotypes, except for the B-type from Hainan (**Figure 4**). These results suggest that the A-type *A. cochinchinensis* has differentiated genetically and morphologically from those of other areas. Although the A-type is highly differentiated, two specific deletions on cpDNA (Kanno *et al.* [39]) were presented in the type (**Figure 1**). Therefore, *A. cochinchinensis* has two main lineages of nuclear genomes, the A-type and all remain-

ing haplotypes.

What is the history of the evolution of the A-haplotype? The morphological characteristics of the A-type are intermediate between other *Asparagus cochinchinensis* haplotypes and its most closely related species, *A. lycopodineus* on the basis of the ITS phylogeny of *Asparagus* (Fukuda *et al*. unpublished data). Two possible explanations would be consistent with the establishment of the A-type. One is that the A-type of *A. cochinchinensis* appeared as an intermediate form in the course of gradual differentiation from *A. lycopodineus* to *A. cochinchinensis*. The other is that A-type of this species may have experienced ancient hybridization events with related species and subsequent allelic recombination and concerted evolution. A previous phylogenetic study using cpDNA sequences suggested that genetic differentiation among species of the genus *Asparagus* is poor [38]. Therefore, it is possible that interspecific hybrids are generated in various combinations of species. In fact, successful production of interspecific hybrids between *A. officinalis* and *A. schoberioides*, and *A. officinalis* and *A. kiusianus* are known [51,52]. Moreover, interlocus-concerted evolution of the ITS region is well known [53-55]. Interlocus-concerted evolution has affected the heterogeneity of nucleotide substitution and is generally regarded as a mechanism that homogenizes copies of a gene in a genome. Once an interspecific hybrid occurs and its proportion by chance increases interlocus-concerted evolution may operate to fix a new haplotype in this species. From the view of the hybridization explanation, however, the ITS1 sequences of A-type must have experienced quite complicated recombination events with repeated crossing over throughout its range. Thus, the simpler explanation of the former view that *A. cochinchinensis* differentiated gradually from *A. lycopodineus* may be more plausible based on the present data. Extensive comparisons of genetic data between *A. lycopodineus* and *A. cochinchinensis* should be made to confirm these explanations for the origin of the A-type.

4.2. Phylogeographic Pattern of *Asparagus cochinchinensis*

In this study, we investigated the geographical structure of haplotype distribution in *Asparagus cochinchinensis*. Although the A-type varied from the other haplotypes, a relatively low level of genetic variation was detected in the ITS1 region among the haplotypes from B to O. This may reflect geographical patterns of haplotype distribution associated with more recent events. China had the greatest diversity of haplotypes. Our results indicated that haplotypes in China (A, B, I, J, and K) were spread throughout the phylogenetic tree and included the most deeply divergent lineage (the A-type). This suggests that *A. cochinchinensis* had its origin in China. A recent phylogenetic analysis of the genus *Asparagus* indicated that *A. cochinchinensis* is most closely related to *A. lycopodineus* (Fukuda *et al*. unpublished data), which occurs from inland China to India [37]. Taken together with the fact, *A. cochinchinensis* appears to have originated in the interior of China. Then, how did the haplotypes expand their distribution beyond inland China?

The large monophyletic group of haplotypes except A-type is divided into five lineages: one in Hainan as mentioned above, two in Taiwan, one in the Yaeyama Islands, and one involved mixed locality from China, Taiwan, South Korea, and Japan. Except haplotypes found in inland China (J- and K-type), all of these haplotypes are distributed in the archipelago along eastern coast of the Eurasian continent. These facts indicate that *Asparagus cochinchinensis* experienced relative rapid expansion of its distribution range to the east and islands in the archipelago. The monophyletic group consisting of I- to O-type includes samples from wide range of locality: Japan, South Korea, Taiwan and China. Especially, I-type is found in the coastal area from Japan to south China. These facts suggest that the I-type of *A. cochinchinensis* might have expanded its range to these areas in more recent years than the expansion event with haplotype diversification mentioned above. Therefore, from our analysis, *A. cochinchinensis* experienced relative quick expansions of distribution range at least twice.

In Taiwan, a lofty backbone range, the Central Mountain Ridge, basically runs the axis of the island and has numerous peaks above 3000 m in elevation. Therefore, some local topographical barriers in the central zone may have blocked or limited the migration to the opposite coasts, with considerable effects on the genetic structure of plant species. In our study, significant genetic differences were detected between *Asparagus cochinchinensis* individuals on the island of Taiwan. Except for the presence of haplotype I in Keelung, which is the northern most area in Taiwan, the samples from the eastern region in Taiwan (Huelien and Taipei) had the same haplotype (C-type), and the haplotypes from Nantou, Kaohsiung and Taichung in the region of coastal to western Taiwan formed a single monophyletic group (F, G, and H). A further analysis using more samples from Taiwan is needed to confirm hypotheses of the geographical structure of haplotype distribution in this area.

The D- and E-types are only found in the Yaeyama Islands (Iriomote and Yonaguni Islands), may have been involved in a different colonization event from the latter group. The islands are geographically located in the southwestern-most part of Japan and close to Taiwan. This

area is the transition zone from subtropical to temperate climate, and vascular plants from both temperate and tropical floras are observed [56]. Although our phylogenetic analysis could not resolve whether *Asparagus cochinchinensis* in the Yaeyama Islands is phylogenetically grouped with Japan or Taiwan. The second lineage in Japan (the I- to O-types) formed a monophyletic group with haplotypes from Anhui and Hubei in China, Keelung in Taiwan and Jeollanam-do in South Korea (**Figures 2**, **3**). Moreover, few apomorphic characters have accumulated within this monophyletic group. These facts suggest that *A. cochinchinensis* might have expanded its range to these areas in recent years. Considering these results, the ITS variation we see today may have been generated after Japan became separated from the Eurasian continent.

The interest in trying to link geographic distribution and evolution of the ITS region with organism evolution rests on the wide use of such markers for phylogeny as well as on the increasing number of cases documenting complicated evolution in plants. The ITS region analyzed in this work provides a robust phylogeographic hypothesis for the evolution of *Asparagus cochinchinensis.* This hypothesis, based on molecular information, is a timely contribution that allows unbiased interpretation of evolutionary history. Additional molecular markers may lend support to this scenario, and multiple markers should be surveyed within this species to find any variation. This work will help to reinterpret existing ITS data sets and facilitate the interpretation of new ones.

5. Acknowledgements

We wish to thank Drs. T. Nakamura, H. Tsukaya, H. Yamaji, M. Kawata, M. Maki, T. Yamashiro, T. Ochiai, P.-Y. Yun, H. Ashizawa, Y. Mashiko, M. Nakada, R. Shinohara, M. Komatsu, S.-Y. Kim, M. Hirai, M. Shiba, K. Kondo, E. Miki, and H. Tokairin for providing much help and advice. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, by the Sasakawa Scientific Research Grant from The Japan Science Society, and Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education Science and Technology (2010-0029630).

REFERENCES

[1] M. Nei and S. Kumar, "Molecular EVolution and Phylogenetics," Oxford University Press, New York, 2000, pp. 3-16.

[2] J. C. Avise, J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb and N. C. Saunders, "Intraspecific Phylogeography: The Mitochondrial DNA Bridge between Population Genetics and Systematics," *Annual Review of Ecology, Evolution, and Systematics*, Vol. 18, 1987, pp. 489-552.

[3] J. C. Avise, "Molecular Markers, Natural History, and Evolution," Chapman & Hall, New York, 1994. doi:10.1007/978-1-4615-2381-9

[4] J. C. Avise, "Phylogeography: The History and Formation of Species," Harvard University Press, Cambridge, 2000.

[5] E. Beckler and T. Holtsford, "*Zea* Systematics: Ribosomal ITS Evidence," *Molecular Biology and Evolution*, Vol. 13, No. 4, 1996, pp. 612-622.

[6] B. A. Schaal and K. M. Olsen, "Gene Genealogies and Population Variation in Plants," *Proceedings of the National Academic of Sciences of the United States of America*, Vol. 97, No. 13, 1999, pp. 7024-7029.

[7] K. M. Olsen, "Population History of *Manihot esculenta* (Euphorbiaceae) Inferred from Nuclear DNA Sequences," *Molecular Ecology*, Vol. 11, No. 5, 2002, pp. 901-911. doi:10.1046/j.1365-294X.2002.01493.x

[8] K. M. Olsen and M. D. Purugganan, "Molecular Evidence on the Origin and Evolution of Glutinous Rice," *Genetics*, Vol. 162, No. 2, 2002, pp. 941-950.

[9] K. Liu, M. M. Goodman, S. Muse, J. S. C. Smith, E. S. Buckler and J. Doebley, "Genetic Structure and Diversity among Maize Inbred Lines as Inferred from DNA Microsatellites," *Genetics*, Vol. 165, No. 4, 2003, pp. 2117-2128.

[10] Y. Vigouroux, Y. Matsuoka and J. Doebley, "Directional Evolution for Microsatellite Size in Maize," *Molecular Biology and Evolution*, Vol. 20, No. 9, 2003, pp. 1480-1483. doi:10.1093/molbev/msg156

[11] A. V. Harter, K. A. Gardner, D. Falush, D. L. Lentz, R. A. Bye and L. H. Rieseberg, "Origin of Extant Domesticated Sunflowers in Eastern North America," *Nature*, Vol. 430, 2004, pp. 201-205. doi:10.1038/nature02710

[12] I. M. Ehrenreich and M. D. Purugganan, "The Molecular Genetic Basis of Plant Adaptation," *American Journal of Botany*, Vol. 93, No. 7, 2008, pp. 953-962. doi:10.3732/ajb.93.7.953

[13] B. L. Gross and K. M. Olsen, "Genetic Perspectives on Crop Domestication," *Trends in Plant Science*, Vol. 15, No. 9, 2010, pp. 529-537. doi:10.1016/j.tplants.2010.05.008

[14] S. D. Tanksley and S. R. McCouch, "Seed Banks and Molecular Maps: Unlocking Genetic Potential from the Wild," *Science*, Vol. 277, No. 5329, 1997, pp. 1063-1066. doi:10.1126/science.277.5329.1063

[15] K. M. Olsen and B. A. Schaal, "Evidence on the Origin of Cassava: Phylogeography of *Manihot esculenta*," *Proceedings of the National Academic of Sciences of the United States of America*, Vol. 96, No. 10, 1999, pp. 5586-5591. doi:10.1073/pnas.96.10.5586

[16] J. Yokoyama, T. Fukuda, A. Yokoyama and M. Maki, "The Intersectional Hybrid between *Weigela hortensis* and *W. maximowiczii* (Caprifoliaceae)," *Botanical Journal of the Linnean Society*, Vol. 138, No. 3, 2002, pp. 369-380. doi:10.1046/j.1095-8339.2002.00033.x

[17] T. Fukuda, J. Yokoyama and H. Tsukaya, "The Evolutionary Origin of Indeterminate Leaves in Meliaceae: Phylogenetic Relationships among Species in the Genera *Chisocheton* and *Guarea*, as Inferred from Sequences of Chloroplast DNA," *International Journal of Plant Sciences*, Vol. 164, 2003, pp. 13-24. doi:10.1086/344741

[18] H. Tsukaya, T. Fukuda and J. Yokoyama, "Hybridization and Introgression between *Callicarpa japonica* and *C. mollis* (Verbenaceae) in Central Japan, as Inferred from Nuclear and Chloroplast DNA Sequences," *Molecular Ecology*, Vol. 12, No. 11, 2003, pp. 3003-3011. doi:10.1046/j.1365-294X.2003.01961.x

[19] J. Yokoyama, T. Fukuda and H. Tsukaya, "Morphological and Molecular Variation of *Mitchella undulata* Sieblod et Zucc., with Special Reference to Systematic Treatment of the Dwarf Form from Yakushima Island," *Journal of Plant Research*, Vol. 116, No. 4, 2003, pp. 309-316. doi:10.1007/s10265-003-0105-7

[20] T. Yamashiro, T. Fukuda, J. Yokoyama and M. Maki, "Molecular Phylogeny of *Vincetoxicum* (Apocynaceae-Asclepiadoideae) Based on the Nucleotide Sequences of cpDNA and nrDNA," *Molecular Phylogenetics and Evolution*, Vol. 31, No. 2, 2004, pp. 689-700. doi:10.1016/j.ympev.2003.08.016

[21] H. Tsukaya, "Gene Flow between *Impatiens radicaus* and *I. javensis* (Balsaminaceae) in Gunung Pangorango, Central Java, Indonesia," *American Journal of Botany*, Vol. 91, No. 12, 2005, pp. 2119-2123.

[22] T. Feng, S. R. Downie, Y. Yu, X. Zhang, W. Chen, X. He and S. Liu, "Molecular Systematics of *Angelica* and Allied Genera (Apiaceae) from the Hengduan Mountains of China Based on nrDNA ITS Sequences: Phylogenetic Affinities and Biogeographic Implications," *Journal of Plant Research*, Vol. 122, No. 4, 2009, pp. 403-414. doi:10.1007/s10265-009-0238-4

[23] C. Ferris, R. P. Oliver, A. J. Davy and G. M. Hewitt, "Native Oak Chloroplasts Reveal an Ancient Divide across Europe," *Molecular Ecology*, Vol. 2, No. 6, 1993, pp. 337-344. doi:10.1111/j.1365-294X.1993.tb00026.x

[24] S. Dumolin-Lapegue, M. H. Pemonge, L. Gielly, P. Taberlet and R. J. Petit, "Amplification of Oak DNA from Ancient and Modern Wood," *Molecular Ecology*, Vol. 8, No. 12, 1997, pp. 2137-2140. doi:10.1046/j.1365-294x.1999.00788.x

[25] S. Dumolin-Lapegue, B. Demesure, S. Fineschi, V. L. Corre and R. J. Petit, "Phylogeographic Structure of White Oaks Throughout the European Continent," *Genetics*, Vol. 146, No. 4, 1999, pp. 1475-1487.

[26] T. Fukuda, J. Yokoyama and H. Ohashi, "Phylogeny and Biogeography in *Lycium* (Solanaceae): Inferences from Chroloplast DNA Sequences," *Molecular Phylogenetics and Evolution*, Vol. 19, No. 2, 2001, pp. 246-258. doi:10.1006/mpev.2001.0921

[27] N. Fujii, N. Tomaru, K. Okuyama, K. Koike, T. Mikami and K. Ueda, "Chloroplast DNA Phylogeography of *Fagus crenata* (Fagaceae) in Japan," *Plant Systematics and Evolution*, Vol. 232, No. 1-2, 2002, pp. 21-33. doi:10.1007/s006060200024

[28] L. H. Bailey, "*Asparagus* L.," In: L. H. Bailey, Ed., *The Standard Cyclopedia of Horticulture*, The Macmillan Company, New York, 1944, pp. 406-411.

[29] F. J. Chittenden, "*Asparagus* L.," In; F. J. Chittenden, Ed., *Dictionary of Gardening*, Clarendon Press, Oxford, 1956, pp. 193-196.

[30] B. Valdes, "*Asparagus* L.," In: T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters and D. A. Webb, Eds., *Flora Europaeana*, Cambridge University Press, Cambridge, 1964, pp. 71-73.

[31] J. Ohwi, "*Asparagus* L.," In: F. G. Meyer and E. H. Walker, Eds., *Flora of Japan*, Smithonian Institution, Washington DC, 1965.

[32] V. L. Komarov, "*Asparagus* L.," In: V. L. Komarov, Ed., *Flora of the USSR*, Vol. IV., Israel Program for Scientific Translations, Jerusalem, 1968, pp. 325-339.

[33] R. M. T. Dahlgren, H. T. Clifford and P. F. Yeo, "*Asparagus* L.," In: R. M. T. Dahlgren, H. T. Clifford and P. F. Yeo, Eds., *The Families of the Monocotyledons*, Springer-Verlag Berlin, Heidelberg, 1985, pp. 140-142. doi:10.1007/978-3-642-61663-1

[34] H. T. Clifford and J. G. Conran, "*Asparagus* L.," In: A. S. George, Ed., *Flora of Australia*, Australian Government Publishing Service, Canberra, 1987, pp. 159-164.

[35] K. Kubituki and P. J. Rudall, "*Asparagus* L.," In: K. Kubituki, Ed., *The Families and Genera of Vascular Plants*, Vol. 3. Springer-Verlag Berlin, Heidenberg, 1998, pp. 125-128.

[36] D. K. Kar and S. Sen, "Chromosome Characteristics of *Asparagus*-Sapogenin Yielding Plant," *Cytologia*, Vol. 50, 1985, pp. 147-155. doi:10.1508/cytologia.50.147

[37] X. Chen and K. G. Tamanian, "*Asparagus* L.," In: Z. Wu and P. H. Raven, Eds., *Flora of China*, Vol. 24, Missouri Botanical Garden Press, St. Louis, 2000, pp. 139-146.

[38] T. Fukuda, H. Ashizawa, T. Nakamura, T. Ochiai, A. Kanno, T. Kameya and J. Yokoyama, "Molecular Phylogeny of the Genus *Asparagus* (Asparagaceae)," *Plant Species Biology*, Vol. 20, No. 2, 2005, pp. 121-132. doi:10.1111/j.1442-1984.2005.00131.x

[39] A. Kanno, Y.-O. Lee and T. Kameya, "The Structure of the Chloroplast Genome in Members of the Genus *Asparagus*," *Theoretical and Applied Genetics*, Vol. 95, No. 8, 1997, pp. 1196-1202. doi:10.1007/s001220050681

[40] T. Fukuda, I.-J. Song, T. Nakamura, M. Nakada, A. Kanno, T. Kameya, H. Yamaji, S. Terabayashi, S. Takeda, M. Aburada and J. Yokoyama, "Molecular Identification of Tiandong Derived from *Asparagus cochinchinensis* (Lour.) Merrill by Two Typical Deletions in cpDNA,"

Natural Medicines, Vol. 59, 2005, pp. 91-94.

[41] T. J. White, T. Bruns, S. Lee and J. Taylor, "Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics," In: M. Innis, D. Gelfand, J. Sninsky and T. J. White, Eds., *PCR Protocols*: *A Guide to Methods and Application*, Academic Press, San Diego, 1990, pp. 315-322.

[42] J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins, "The Clustal_X Windows Interface: Flexible Strategies for Multiple Sequence Alignment Aided by Quality Analysis Tools," *Nucleic Acids Research*, Vol. 25, No. 24, 1997, pp. 4876-4882. doi:10.1093/nar/25.24.4876

[43] D. L. Swofford, "PAUP*. Phylogenetic Analysis Using Parsimony (and Other Methods) Version 4.0b10," Sinauer Associates, Sunderland, 2002,

[44] W. P. Maddison, "The Discovery and Importance of Multiple Islands of Most-Parsimonious Trees," *Systematic Zoology*, Vol. 40, No. 3, 1991, pp. 315-328. doi:10.2307/2992325

[45] J. Felsenstein, "Confidence Limits on Phylogenies: An Approach Using the Bootstrap," *Evolution*, Vol. 39, 1985, pp. 783-791. doi:10.2307/2408678

[46] B. G. Baldwin, M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell and M. J. Donoghue, "The ITS Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny," *Annals of the Missouri Botanica Garden*, Vol. 82, No. 2, 1995, pp. 247-277. doi:10.2307/2399880

[47] T. Sang, D. J. Crawford and T. F. Stuessy, "ITS Sequences and the Phylogeny of the Genus *Robinsonia* (Asteraceae)," *Systematic Zoology*, Vol. 20, No. 1, 1995, pp. 55-64.

[48] P. Taberlet, L. Gielly, G. Pautou and J. Boubet, "Universal Primers for Amplication of Three Non-Coding Regions of Chloroplast DNA," *Plant Molecular Biology*, Vol. 17, No. 5, 1991, pp. 1105-1109. doi:10.1007/BF00037152

[49] T. Nishizawa and Y. Watano, "Primer Pairs Suitable for PCR-SSCP Analysis of Chloroplast DNA in Angiosperms," *Journal of Phytogeography and Taxonomy*, Vol. 48, 2000, pp. 67-70.

[50] C. W. Dick, K. Abdul-Salim and E. Bermingham, "Molecular Systematic Analysis Reveals Cryptic Tertiary Diversification of a Widespread Tropical Rain Forest Tree," *The American Naturalist*, Vol. 160, No. 12, 2003, pp. 691-703. doi:10.1086/379795

[51] T. Ochiai, T. Sonoda, A. Kanno and T. Kameya, "Interspecific Hybrids between *Asparagus schoberioides* Kunth and *A. officinalis* L.," *Acta Horticulturae*, Vol. 589, 2002, pp. 225-229.

[52] T. Ito, T. Ochiai, T. Fukuda, H. Ashizawa, T. Sonoda, T. Kameya and A. Kanno, "Potential of Interspecific Hybrids in Asparagaceae," *Acta Horticulturae*, Vol. 776, 2008, pp. 279-284.

[53] D. M. Hillis, C. Moritz, C. A. Porter and R. J. Baker, "Evidence for Biased Gene Conversion in Concerted Evolution of Ribosomal DNA," *Science*, Vol. 251, No. 4991, 1991, pp. 308-310. doi:10.1126/science.1987647

[54] T. Sang, D. J. Crawford and T. F. Stuessy, "Documentation of Reticulate Evolution in Peonies (*Paeonia*) Using Internal Transcribed Spacer Sequences of Nuclear Ribosomal DNA: Implications for Biogeography and Concerted Evolution," *Proceedings of the National Academic of Sciences of the United States of America*, Vol. 92, No. 15, 1995, pp. 6813-6817. doi:10.1073/pnas.92.15.6813

[55] J. Fuertes Aguilar, J. A. Rosselo and G. Nieto Feliner, "Nuclear Ribosomal DNA (nrDNA) Concerted Evolution in Natural and Artificial Hybrid of *Armeria* (Plumbaginaceae)," *Molecular Ecology*, Vol. 8, No. 8, 1999, pp. 1341-1346. doi:10.1046/j.1365-294X.1999.00690.x

[56] E. H. Walker, "Flora of Okinawa and the Southern Ryukyu Islands," Smithonian Institution Press, Washington DC, 1976.

