

Phylogeography of *Asparagus schoberioides* Kunth (Asparagaceae) in Japan

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ABSTRACT

To describe the phylogeographic structures of *Asparagus schoberioides* Kunth (Asparagaceae) in Japan, we investigated its nucleotide sequence variations with respect to its geographic distribution pattern. Sequencing of the internal transcribed spacer (ITS) 1 region in 29 samples of *A. schoberioides* revealed 20 polymorphic nucleotide sites. As a result, the 29 samples of *A. schoberioides* fell into 15 distinct haplotypes and phylogenetic analyses revealed these haplotypes fell into two major clades, Clade 1 and Clade 2. The haplotypes of Clade 1 were distributed chiefly along the Pacific Ocean side of Japan, while those of Clade 2 occurred mainly along the Japan Sea side. This result suggests that *A. schoberioides* has migrated via two routes in Japan.

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

1. Introduction

The genetic and geographic structures of natural plant populations are a consequence of ecological factors and historical events. Consequently, the amount of genetic variation within a species depends on its history and life strategy. Comparison of nucleotide sequences are the best way to analyze the history of plant populations, and phylogeographic studies employing nuclear DNA data have been used to test ever more sophisticated historical models [1-3]. In particular, nucleotide divergences of the nuclear DNA (nrDNA) showed relative higher values than those of chloroplast DNA (cpDNA) [4], although recombination of nrDNA is biased the concepts of homology [5].

Asparagus schoberioides Kunth is a perennial herb bearing small flowers with yellowish green perianth in a raceme-like inflorescence and reddish colored berries. This species ranged from Far East Russia, northern China, Korea, Sakhalin, Japan and Taiwan [6,7]. In Japan, its distribution extends from the northern-most part of Hokkaido down to Shikoku and Kyushu [6]. *A. schoberioides* is one of the most closely related species to the garden

asparagus, *A. officinalis* L. [8], which is the most economically important species in this genus. While the garden asparagus is not native to Japan, it is cultivated there and has escaped from the crop fields [6]. Recently, Ochiai *et al.* [9] and Ito *et al.* [10] reported successfully generating of interspecific hybrids between *A. schoberioides* and the garden asparagus. A natural hybrid between *A. schoberioides* and the garden asparagus has not been found to date, but the possibility of genetic introgression from crop to wild relatives suggests the escape of the garden asparagus from the crop fields may pose a substantial ecological risk for the environment and its biodiversity [2,11-16].

In Japan, molecular approaches have been used to analyze the intraspecific genetic variation of a number of different plant species (e.g., *Abies mariesii* [17]; *Aucuba chinensis* and *A. japonica* [18]; *Fagus crenata* [19-21]; *Purimula cunefolia* [22]; *Quercus serrata* and its allied species [23]; *Stachyurus praecox* [24], *Alpinia japonica*, *Arachnioides sporadosora*, *A. aristata*, *Daphne kiusiana*, *Elaeocarpus sylvestris* var. *ellipticus*, *Prunus zippeliana* [25]). Most of these studies were conducted based on the chloroplast DNA (cpDNA) sequence. Similarly, in our

previous study, we used primers designed by Taberlet *et al.* [26] and Nishizawa and Watano [27] to sequence approximately 3000 bp of 16 regions in the cpDNA of some *Asparagus* species [8]. However, we detected little variation in these regions and concluded that the cpDNA is not suitable for phylogenetic analyses of this species. The internal transcribed spacer (ITS) region is a powerful tool for resolving historical relationships among populations of widespread plants [28]. In fact, many researches with various species using ITS region had been reported in recent years [29-32]. For example, Yokoyama *et al.* [33] analyzed the ITS region of *Mitchella undulata* Siebold et Zucc. in Yakushima Island, they detected intraspecific variations that suggested this population had undergone rapid morphological modification. Thus, analysis of this region may permit plant relationships to be evaluated down to the intraspecific level.

The aims of this study were to describe the phylogeographic structures of *A. schoberioides* especially in Japan on the basis of its ITS region and to analyze the distribution of these genetic variations relative to the distribution of *A. schoberioides*.

2. Materials and Methods

2.1. Plant Materials

Twenty-nine samples of *Asparagus schoberioides* were examined in this study (Table 1). *A. kiusianus* Makino and *A. officinalis* were selected as outgroups on the basis of phylogenetic analyses of the genus *Asparagus* [8]. 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. DNA Extraction, Amplification, and Sequencing

Total DNAs were isolated from 200 - 300 mg of phylloclades with a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA) used according to the manufacturers' protocols. The isolated DNA was resuspended in TE and stored at -20°C until use.

For phylogenetic analysis, we amplified the ITS1 region with primers designed by White *et al.* [34]. Double-stranded DNA was amplified by incubation at 94°C for 2 min followed by 40 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. After the amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gels to purify of the amplified products. We sequenced the purified PCR products using a DYEnamic ET-terminator Cycle Sequencing Kit (Amersham Pharmacia) and a Model 373A auto-

mated sequencer (Applied BioSystems) according to the manufacturers' instructions. For sequencing, we used the same primers as those used for amplification.

2.3. Data Analysis

ITS region sequences were aligned with the CLUSTAL X program [35]. Phylogenetic analysis and a test of clade support were conducted using the PAUP* program (version 4.0b10; [36]). Maximum parsimony analyses were carried out via a heuristic search with TBR branch swapping and MULPERS option. Multiple islands of the most parsimonious trees [37] were identified using the heuristic option with 100 random sequence additions. To estimate confidence levels of monophyletic groups, the bootstrap method with 1000 replications were employed [38].

3. Results

We determined the ITS1 region sequence in 29 samples of *Asparagus schoberioides* and two outgroup species, namely *A. kiusianus* and *A. officinalis*. The ITS1 region of all *A. schoberioides* plants was 249 bp in length. Variable sites in this region are shown in Table 2. Fifteen ITS1 haplotypes were obtained from *A. schoberioides*, and the sequence of each haplotype has been deposited in the DDBJ/EMBL/GenBank international DNA data bank (Table 1). There are no indels among the ITS sequences of *A. schoberioides* and its allied species.

When we used the ITS1 sequence data set in phylogenetic analyses, we obtained 78 most parsimonious trees of the 28 steps with a consistency index (CI) of 0.71 and a retention index (RI) of 0.92. One of the most parsimonious trees is shown in Figure 1.

In our phylogenetic tree, all *A. schoberioides* samples formed the monophyletic group and two clades (Clade 1 and Clade 2 hereafter) in *A. schoberioides* were recognized. The relationship between the clades and the localities of the individuals is indicated in Table 1. The correspondence between haplotypes and samples is shown in Figure 2. The geographic distribution of the samples is shown in Figure 3. Clade 1 consists of the following fourteen samples: China, South Korea, Hokkaido1, Hokkaido3, Hokkaido4, Hokkaido5, Hokkaido6, Miyagi1, Miyagi2, Saitama, Yamanashi, Nagano1, Nagano2 and Shizuoka. Clade 2 contains the following fifteen samples: Russia, Hokkaido2, Iwate, Niigata1, Niigata2, Niigata3, Nagano3, Kyoto1, Kyoto2, Kyoto3, Hyogo, Shimane, Yamaguchi, Fukuoka and Nagasaki. Thus, in our phylogenetic analysis, the samples from Hokkaido and Nagano extend into the two lineages that form Clades 1 and 2 (Figures 1, 3). Apart from these samples, Clade 1 consists of samples from the Pacific

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

Taxon	Sample name	Locality	Collector	Haplotype	Acc. no.
<i>Asparagus schoberioides</i> Kunth	Russia	RUSSIA: Sakhalin, Dovie-alexandrowsk	Kudo 25406	K	AB196766
	China	CHINA: Liaoning, Dalian, Lushun	Suzuki <i>s.n.</i>	D	AB196739
	SouthKorea	SOUTH KOREA: Junranam, Gwangyang, Okryong	Im 21864	E	AB196767
	Hokkaido1	JAPAN: Hokkaido, Kamikawa, Toma	Deguchi 8326	A	AB196744
	Hokkaido2	JAPAN: Hokkaido, Oshima, Matsumae	Kudo 25410	F	AB196741
	Hokkaido3	JAPAN: Hokkaido, Iburi, Tomakomai	Kudo 25407	A	AB196745
	Hokkaido4	JAPAN: Hokkaido, Okushiri Isl. Mt. Kamui	Kudo 25411	B	AB196743
	Hokkaido5	JAPAN: Hokkaido, Abashiri, Abashiri	Kudo 25409	A	AB196742
	Hokkaido6	JAPAN: Hokkaido, Hidaka, Samani	Yamaji <i>s.n.</i>	A	AB196760
	Iwate	JAPAN: Iwate, Mt. Hayachine	Endo <i>s.n.</i>	M	AB196747
	Miyagi1	JAPAN: Miyagi, Tome, Towa	Sugaya & Soma 4203	A	AB196752
	Miyagi2	JAPAN: Miyagi, Shiroishi, Mt. Ohagi	Suzuki 366	A	AB196751
	Saitama	JAPAN: Saitama, Chichibu	Yamaji <i>s.n.</i>	A	AB196759
	Yamanashi	JAPAN: Yamanashi, Hokuto	Yamaji <i>s.n.</i>	A	AB196765
	Niigata1	JAPAN: Niigata, Nakakubiki, Kasuga	Iwano 1177	H	AB196756
	Niigata2	JAPAN: Niigata, Nishikubiki, Nou	Iwano 5257	N	AB196758
	Niigata3	JAPAN: Niigata, Nishikanbara, Maze	Takeuchi <i>s.n.</i>	I	AB196757
	Nagano1	JAPAN: Nagano, Minamisaku, Minamimaki	Takahashi 21353	A	AB196755
	Nagano2	JAPAN: Nagano, Chiisagata, Aoki	Hisauchi <i>s.n.</i>	C	AB196754
	Nagano3	JAPAN: Nagano, Kitasaku, Karuizawa	Kimura <i>s.n.</i>	L	AB196753
	Shizuoka	JAPAN: Shizuoka, Fujinomiya, Nehara	Konta <i>et al.</i> 771	A	AB196762
	Kyoto1	JAPAN: Kyoto, Takeno, Amino	Tsugaru & Miyahara 26674	I	AB196748
	Kyoto2	JAPAN: Kyoto, Kumano, Kumihama	Tsugaru & Takahashi 27970	H	AB196749
	Kyoto3	JAPAN: Kyoto, Maizuru, Kanmuri Isl.	Tsugaru & Takahashi 30054	I	AB196750
	Hyogo	JAPAN: Hyogo, Kobe, Fukiai	Miyake 3869	G	AB196746
	Shimane	JAPAN: Shimane, Yatsuka, Shimane	Miki <i>s.n.</i>	I	AB196761
	Yamaguchi	JAPAN: Yamaguchi, Toyoura, Toyoura	Imada 4414	I	AB196764
	Fukuoka	JAPAN: Fukuoka, Munakata	Watanabe <i>s.n.</i>	J	AB196740
	Nagasaki	JAPAN: Nagasaki, Tsushima Isl., Shimoagata	Ohashi & Soma 7454	I	AB196763
	Outgroup				
<i>A. kiusianus</i> Makino		JAPAN: Fukuoka, Munakata	Watanabe 93		AB196738
<i>A. officinalis</i> L.		JAPAN: Miyagi, Furukawa (cult.)	Komatsu 041118		AB195716

Table 2. Apomorphic characters of the ITS1 sequences obtained from *Asparagus schoberioides* and two outgroups.

									1	1	1	1	1	1	1	1	1	1	1	1
			3	3	3	4	4	9	0	2	3	5	5	6	6	7	7	7	8	9
sample	5	9	2	5	8	6	8	1	1	9	5	4	5	4	6	1	2	3	3	0
Russia	C	A	A	T	T	G	A	C	C	C	C	A	T	C	T	C	T	G	A	G
China	.	G	C	C	C	.	.	G	T	.	T	A	.	G	.
South Korea	.	G	C	C	C	.	.	G	T	A	.	G	.
Hokkaido1	.	G	C	C	C	.	.	G	A	.	G	.
Hokkaido2	.	G	.	.	C	A	.	G	.	.	.	C	A	.	.	.
Hokkaido3	.	G	C	C	C	.	.	G	A	.	G	.
Hokkaido4	.	G	C	C	.	.	.	G	T	.	.	A	.	G	.
Hokkaido5	.	G	C	C	C	.	.	G	Y	.	.	A	.	G	.
Hokkaido6	.	G	C	C	C	.	.	G	A	.	G	.
Iwate	G	.	.	.	C	A	.	.	.
Miyagi1	.	G	C	C	C	.	.	G	A	.	G	.
Miyagi2	.	G	C	C	C	.	.	G	A	.	G	.
Saitama	.	G	C	C	C	.	.	G	A	.	G	.
Yamanashi	.	G	C	C	C	.	.	G	A	.	G	.
Niigata1	.	.	C	G	.	.	.	C
Niigata2	G	.	.	.	C
Niigata3	G	.	.	.	C	A	.	.	T
Nagano1	.	G	C	C	C	.	.	G	A	.	G	.
Nagano2	.	G	C	C	C	.	.	G	.	T	A	.	G	.
Nagano3	G
Shizuoka	.	G	C	C	C	.	.	G	A	.	G	.
Kyoto1	G	.	.	.	C
Kyoto2	.	.	C	G	.	.	.	C
Kyoto3	G	.	.	.	C
Hyogo	.	G	C	G
Shimane	G	.	.	.	C
Yamaguchi	G	.	.	.	C
Fukuoka	A	.	G	.	.	.	C
Nagasaki	G	.	.	.	C
<i>A. kiusianus</i>	.	G	C	.	C	A	C	G	.	.	A	C	C	.	C	.	A	T	G	.
<i>A. officinalis</i>	T	G	C	.	C	.	C	G	T	.	.	C	C	.	.	.	A	T	G	.

Numbers indicate the position from the first nucleotide of 5' region of ITS sequence removing the primer region.

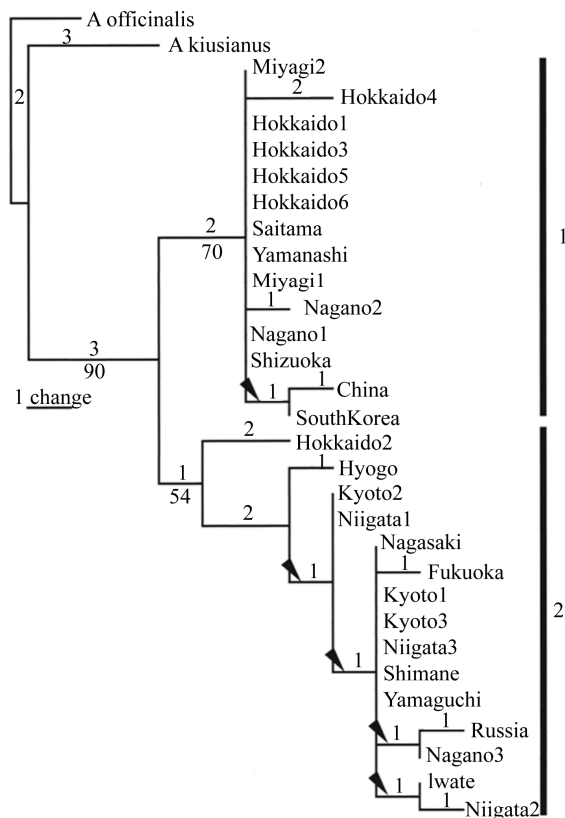


Figure 1. One of the 78 most parsimonious trees of 29 samples of *Asparagus schoberioides* and outgroups. The numbers above the branches indicate the synapomorphic characters; the bootstrap values are indicated below branches (only those more than 50% are indicated on the tree). Arrowheads indicate branches that do not appear in the strict consensus tree.

Oceanic side of northern and central Honshu in Japan, while Clade 2 is comprised of samples from the Japan Sea side of Honshu (**Figures 1, 3**). Moreover, some of the samples from the neighboring locations in Japan fall into different phylogenetic position within the clade (**Figures 1**).

4. Discussion

The phylogenetic analyses in this study indicate that Japanese *Asparagus schoberioides* could not be detected the haplotype of the garden asparagus, suggesting that there are no genetic introgressions from the crops or the escaped individuals of the crop fields into wild relatives. Moreover, our results suggest that the current distribution in *A. schoberioides* has employed two routes of expansion. One is along the Japan Sea side while the other is along the Pacific Oceanic side. This is consistent with the geographical features of Japan, which has a lofty backbone range that basically runs along the axis of Honshu and divides the island into two areas, namely, the

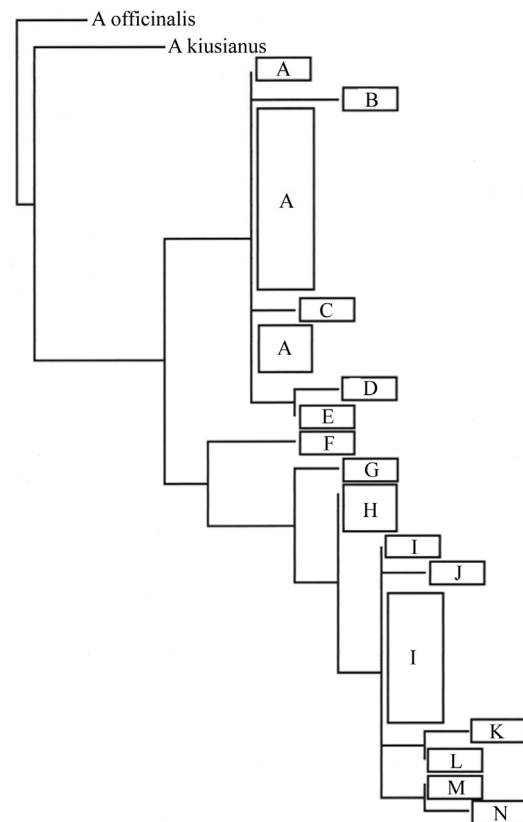


Figure 2. Schematic of the correspondence between haplotypes and samples in Figure 1.

Pacific Oceanic and the Japan Sea sides. The range affect the climate of both sides and may have blocked or limited the migration of plants to the opposite sides. This is supported by studies of plant intraspecies variation between the two sides. For example, the leaf of *Euptelea polyandra* Siebold et Zucc. growing on the Japan Sea side is broader than that of the same species growing on Pacific Oceanic side [39]. Yonekura and Ohashi [40] reported similar observations with regard to *Bistorta tenuicaulis* (Bisset et Moore) Nakai. Moreover, Fujii *et al.* [20] and Okura and Harada [21] found that a phylogenetic tree constructed on the basis of the cpDNA variability of *Fagus crenata* was roughly divided into two clades that were composed of populations growing on the Pacific Oceanic side and the Japan Sea side, respectively. Thus, the expansion of *A. schoberioides* may similarly have been limited by the mountain range, leading to two different lineages on the Pacific Oceanic and the Japan Sea sides.

With regard to our phylogenetic analyses, Clade 1 showed relatively little mutation accumulation, whereas many more nucleotide substitutions were observed in Clade 2 (**Figure 1**). This discrepancy suggests that Clade 1 had different colonization or migration time from

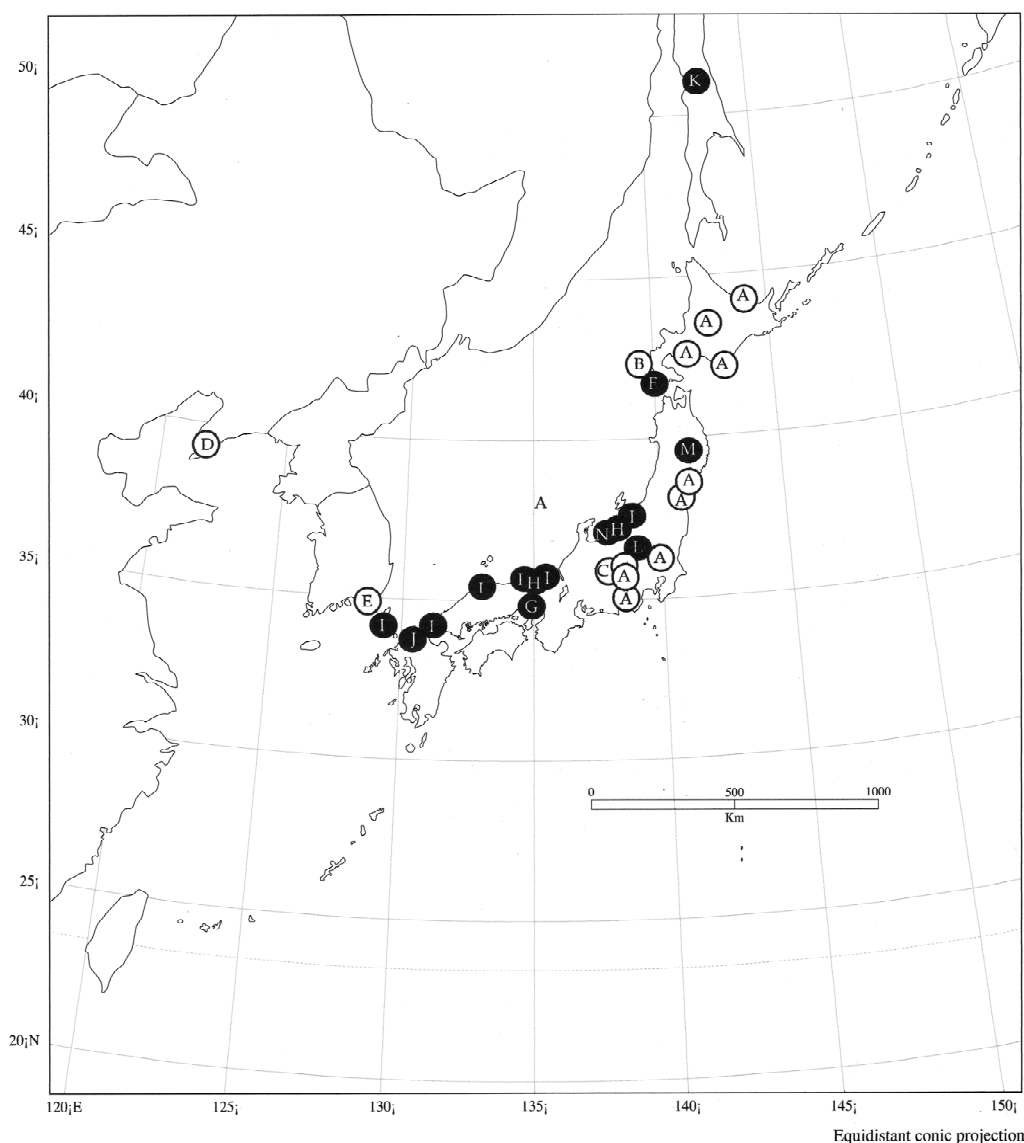


Figure 3. Geographical distribution of the *A. schoberioides* samples belonging to each clade. Alphabets indicate haplotypes of ITS1 (see Figure 2). ○: Clade 1, ●: Clade 2.

Clade 2. Clade 1 consists of samples from the Pacific Oceanic side of Japan, South Korea and China. Since only one sample each from South Korea and China were employed, the genetic diversity of *A. schoberioides* in these regions could not be detected. There are two phylogeographic hypotheses of Japanese *A. schoberioides* belonging to Clade 1. One is that they had migrated from the mainland of China or the Korean Peninsula into Japan, at which point it expanded to Hokkaido and the Pacific Oceanic side. In this study, nucleotide variation of Clade 1 is very small, suggesting that there may have experienced extinct events in the western and the Japan Sea sides of Japan. The other is that they had migrated from the mainland of China into Hokkaido, and ex-

panded rapidly to the Pacific Oceanic side from Miyagi to Shizuoka of Honshu. In this case, they had migrated from the mainland of China into Korean Peninsula independently. However, it is not certain which hypothesis to support from our results.

In contrast to Clade 1, our phylogenetic analyses suggest that a common ancestor of Clade 2 originated from more ancient times than that of Clade 1. Clade 2 consists of samples from the Japan Sea side of Japan and Sakhalin, and does not include samples from the mainland of China or the Korean Peninsula, although not enough samples were collected from the Asian Continent to be certain of this. Therefore, the relationships between the Japanese and Asian Continent populations of *A. schober-*

rioides remain unclear at present.

Our phylogenetic analyses also indicate that within each clade, the phylogenetic relationship and geographic distribution do not correlate. For example, some neighboring locations in Japan belong to different phylogenetic position within the clade. Examples of this are the three samples from Niigata and the three Kyoto samples in Clade 2 (**Figure 1**). This suggests that *A. schoberioides* populations in Niigata and Kyoto consist of individuals that have experienced different histories. Furthermore, northern samples such as those from Sakhalin, Hokkaido and Iwate also appeared in various phylogenetic positions in Clade 2 (**Figure 1**). These results indicate that the migration of *A. schoberioides* along the Japan Sea side of Japan has occurred repeatedly.

Thus, our phylogeographic analysis has outlined the different histories of *A. schoberioides* between the Pacific Oceanic and the Japan Sea sides in Japan. What has aided the rapid migration of samples in Clade 1 and the frequent colonization of samples in Clade 2? One answer may lie in the fact that *A. schoberioides* bears reddish berries that may be bird-dispersed [41-44]. This may aid the rapid dispersal of seeds of *A. schoberioides* throughout the two routes of Japan.

In general, a morphologically recognized species may be regarded as a composite of subtly defined cryptic species each of which has equal status [45]. In fact, such a definition takes the systematic approach to an extreme which would appear to be unworkable in plants. For example, Yatabe *et al.* [46] concluded that there are some cryptic species of *Asplenium nidus* L. (Aspleniaceae) in West Java on the basis of sequence variations using *rbcL* of cpDNA. In this study, *A. schoberioides* had nucleotide differentiation between Clade 1 and Clade 2, suggesting that there are cryptic species with the geographical features of Japan.

5. Conclusions

There are no genetic introgression between *A. schoberioides* and *A. officinalis* (garden asparagus) and two migration routes of *A. schoberioides* exist in Japan. Such research, when applied to *A. schoberioides* and to other Japanese plant taxa, will provide new insights into the phylogeography of Japanese plants.

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