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Intraspecific Sequence Variation of Chloroplast DNA Reflecting Variety and Geographical Distribution of *Polygonum cuspidatum* (Polygonaceae) in Japan

Atsushi Inamura[1,*], Yayoi Ohashi[1], Etsuko Sato[1], Yoshika Yoda[1], Takehiro Masuzawa[1], Motomi Ito[2] and Koichi Yoshinaga[1,**]

[1] *Faculty of Science, Shizuoka University, Oya, Shizuoka, 422-8529 Japan*
[2] *Department of Systems Sciences (Biology), The University of Tokyo, Komaba, Meguro-ku, Tokyo, 153-8902 Japan*

To investigate the relationship between the evolution and the distribution of *Polygonum cuspidatum* in Japan, we analyzed the chloroplast DNA sequences of a region from the *rbcL* to the *accD* gene (ca. 1,420 bp), and found nucleotide variations at 22 sites in 68 samples. The phylogenetic relationship deduced from the sequence variations revealed the existence of at least five groups. The first group consisted of *P. cuspidatum* var. *cuspidatum* in the central part of Honshu; in Nagano, Yamanashi, and Shizuoka. The second, a sister of the first, consisted of those plants in Shizuoka-Itoigawa Line. The third group consisted of plants in the northern part of Japan including *P. sachalinense* in Hokkaido, *P. cuspidatum* var. *cuspidatum* in Aomori and var. *uzensis* in Akita. The fourth consisted of var. *uzensis* in the Tohoku District. The fifth consisted of var. *terminalis* in the Izu Islands. *P. cuspidatum* are differentiated according to their distribution, and two varieties, var. *terminalis* and var. *uzensis*, are differentiated genetically. *Polygonum sachalinensis*, a distinct species morphologically, fell into the accessions of *P. cuspidatum* on the phylogenetic tree obtained in the present study.

Key words: Chloroplast DNA — Intraspecific variation — Phylogeography — *Polygonum cuspidatum* — *Polygonum sachalinense*

Polygonum cuspidatum Siebold et Zucc. is a perennial herb widely distributed in the temperate zone of Asia. It grows at a wide range of altitudes, from lowlands to the alpine zone, as a pioneer plant. *Polygonum cuspidatum* is so variable in external morphology that several intraspecific classifications for the species have been proposed (Makino 1961, Kitamura and Murata 1963, Kitagawa 1982). *Polygonum cuspidatum* var. *terminalis* Honda is distributed in the Izu Islands. *Polygonum cuspidatum* var. *compacta* Hiyama located in the alpine zone is a small plant, and var. *uzensis* (Honda) Kitam., distributed in the Hokuriku and Tohoku Districts, has many hair-like protuberances on its leaves. Because extensive ecological and morphological variation is found, this species could be useful for studying speciation and intraspecific variation.

Chloroplast DNA (cpDNA) sequence divergence has been used extensively at the interspecific level and higher (Palmer *et al.* 1988). It is less commonly used for intraspecific studies, however, because of the slow evolutionary rate of sequence in the chloroplast genome (Wolfe *et al.* 1987). However, in some cases, the extent of intraspecific cpDNA diversity is high enough for population-level studies (Harris and Ingram 1991). In fact, cpDNA variation within species has provided insights into evolutionary processes of population differentiation (Terauchi *et al.* 1991, Soltis *et al.* 1991, Hong *et al.* 1993, Fujii *et al.* 1997, 1999).

In this study, we estimated the genetic differentiation and phylogenetic relationship among the populations of *Polygonum cuspidatum* in Japan based on the nucleotide sequence variation of a region from the *rbcL* to the *accD* gene of cpDNA. *accD* is a gene encoding a subunit of acetyl-CoA carboxylase and is rather variable; e.g., it is divided into ORF133 and ORF106 in the chloroplast genome of the monocot *Oryza sativa* (Hiratsuka *et al.* 1989) and is a mutational hotspot in the grass family (Morton and Clegg 1993). The intergenic spacer is also more variable than coding regions and provides phylogenetic information (Ogihara *et al.* 1991). We used the nucleotide sequences of those variable regions and discussed the genetic differentiation of *P. cuspidatum* in Japan.

Materials and Methods

The plant materials used in this study are listed in Table 1. The accessions of *Polygonum cuspidatum* Siebold et Zucc. were collected at various sites from Aomori to Kagoshima Prefecture in Japan. Those of *Polygonum sachalinense* Fr. Schm., a closely related species to *P. cuspidatum*, were collected in two localities in Hokkaido and one locality in Toyama Prefecture. Our aim was to elucidate the natural distribution of the plants. Therefore, plants growing at

* Present address: Forensic Science Laboratory, Shizuoka Prefectural Police Headquarters, Shizuoka
** Corresponding author. e-mail: sckyosi@ icp.shizuoka. ac.jp

Table 1. The materials of *Polygonum* and their sources analyzed for cpDNA variation

Taxon and population	Sample No.[d]
Polygonum cuspidatum Siebold et Zucc.[a]	
1. Higashitsugaru, Aomori, Honshu	502 (Bt)
2. Kitashiga Highlands, Shimotakai, Nagano, Honshu	I04 (Et), I05 (Et)
3. Chiisagata, Nagano, Honshu	401 (Qt)
4. Alt. 2,700 m, Mt. Yatsugatake, Nagano, Honshu	035 (It), 036 (It)
5. Nagiso, Nagano, Honshu	015 (Oa), 019 (Oa)
6. Mt. Fuji[b], Yamanashi, Honshu	C01 (Ja), C02 (Ja), C04 (Ja), C05 (Ja), C06 (Ja), C07 (Ja), C09 (Ja), C11 (Jt)
7. Minamitsuru, Yamanashi, Honshu	2 (Ja), 4 (Ja)
8. Fujinomiya, Shizuoka, Honshu	10 (Ja)
9. Alt. 2,640 m, Mt. Fuji[c], Shizuoka, Honshu	23 (Ja)
10. Itoh, Shizuoka, Honshu	B01 (Kt)
11. Shizuoka Univ., Shizuoka, Honshu	28 (Jt)
12. Mountain area of Shizuoka, Shizuoka, Honshu	026 (Nt), 027 (Jt), 029 (Mt)
13. Haibara, Shizuoka, Honshu	033(Jt)
14. Minamimuro, Mie, Honshu	K01 (Ja)
15. Amada, Kyoto, Honshu	202 (Qt)
16. Kinosaki, Hyogo, Honshu	204 (Qa), 208 (Ra)
17. Yazu, Tottori, honshu	211 (Qa)
18. Tomata, Okayama, Honshu	214 (Qa), 217 (Sa), 218 (Qa)
19. Okayama, Okayama, Honshu	106 (Qt)
20. Teshima Island, Kagawa, Shikoku	301 (Sa)
21. Okawa, Kagawa, Shikoku	006 (Qa)
22. Usuki, Oita, Kyushu	022 (Tt)
23. Miyakonojo, Miyazaki, Kyushu	021 (Tt)
24. Sakurajima, Kagoshima, Kyushu	034 (Tt)
Polygonum cuspidatum var. *compacta* Hiyama.	
25. Alt. 2,400 m, Mt. Fuji[b], Yamanashi, Honshu	C03 (Jt)
Polygonum cuspidatum var. *uzensis* (Honda) Kitam.	
26. Shenboku, Akita, Honshu	005 (Bt)
27. Shiwa, Iwate, Honshu	505 (Ct)
28. Nishiiwai, Iwate, Honshu	507 (Ca)
29. Tagajo, Miyagi, Honshu	509 (Ct)
30. Watari, Miyagi, Honshu	510 (Ct)
31. Haramachi, Fukusima, Honshu	511 (Ct)
32. Kitashiga Highlands, Shimotakai, Nagano, Honshu	I01-I03 (Ca), I06 (Ct)
33. Jyoganji River, Toyama, Honshu	F01 (Ct)
34. Sho River, Higashitonami, Toyama, Honshu	F04 (Ct)
Polygonum cuspidatum var. *terminalis* Honda	
35. Oshima Island, Tokyo	A01-A04 (Ut), A06 (Ut), A07 (Ut)
36. Miyakejima Island, Tokyo	G01 (Vt), G04 (Vt), G07 (Vt)
37. Mikurajima Island, Tokyo	J01 (Wt)
38. Hachijyojima Island, Tokyo	H03 (Wt), H04 (Wt)
Poygonum sachalinense Fr. Schm.	
39. Sapporo, Hokkaido	604 (At)
40. Yoichi, Hokkaido	605 (At)
41. Joganji River, Toyama, Honshu	F02 (Ht)
Polygonum weyrichii Gross var. *alpinum* Maxim	
Alt. 2,400 m, Mt. Fuji[b], Yamanashi, Honshu	D01-D03 (Out)

The number on the top of each collection site is shown in Fig. 4.

[a] We could not identify forma elats Hiyama, because there were no flowers.

[b] Shouji-guchi Mountain Trail, alt. 1,000 m to 2,400 m.

[c] Fujinomiya-guchi Mountain Trail.

[d] Letters in parentheses represent the haplotype of *Polygonum* (see Fig. 2).

roadside, in parks constructed artificially, around graves, *etc.* were not analyzed, except sample No. 28, which has been kept in a plant garden of Shizuoka University as a standard strain. About five young leaves were collected from each individual, wrapped with wet tissue paper, and kept in a plastic bag. They were carried back to the laboratory, washed with distilled water and stored at −20 C until the extraction of DNA. In total, 65 individuals of *P. cuspidatum* and three individuals of *P. sachalinense* from 68 populations (patches) were collected. In addition, we used *P. weyrichii* Fr. Schm. var. *alpinum* Maxim. as an outgroup for rooting trees.

Total DNA was extracted from the leaves following the CTAB method (Dellaporta *et al.* 1983) with a slight modification. The extraction buffer containing 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 1% CTAB and 0.1% 2-mercaptoethanol was supplemented with 1.25% SDS and polyvinylpolypyrolidone (1 g/3 g leaves). The DNA sample was treated with RNase A and purified by CsCl-EtBr centrifugation (Sambrook *et al.* 1989).

In our previous study (unpublished), a gene library of *Polygonum cuspidatum* (sample No. 28 in Table 1) was constructed and a plasmid pLOW containing *rbcL* was selected by Southern hybridization. Based on the nucleotide sequence (acc. no. AB019030), the primer pair rbcL-4: 5′-GATCTTGCTCGCGAGGGTAACGA-3′ and accD A-1: 5′-TTTGAACAGCATCGGTTAAACCTGT-3′ was prepared. In the present study, this primer pair was used to amplify the DNA between *rbcL* and *accD* in the chloroplast genome of each sample. The PCR products electro-eluted from agarose gels were either cloned into pUC18 or directly used for sequencing. DNA sequencing was carried out using an ABI PRISM 310 system (PERKIN ELMER) with Dye Primer Cycle Sequencing Kits. The primer RA-1: 5′-CTTCTACCCATC-CTGTATATTGTC-3′ was used to read the internal sequences. Every mutation was determined at least twice by re-amplification and sequencing for confirmation.

The sequences were aligned using Clustal W (Thompson *et al.* 1996) and then revised manually. The alignment is available from K. Yoshinaga on request. We applied the Maximum Parsimony method using PAUP* 4.0b3 (Swofford 2000) for phylogenetic analysis. The gaps were treated as missing values. We did not use the presence or absence of gaps as characters because the gaps found in the present dataset were merely differences of numbers of mono-nucleotide repeats. To test the confidence levels, the bootstrap method (Felsenstein 1985) was employed with 1,000 replicates.

Table 2. Samples of *Polygonum*

Clade	Haplotype	Sample	Acc. No.
I	Kt	B01 (Shizuoka)	AB045190
	It	035, 036 (Mt. Yatsugatake, Nagano)	AB045203
	Jt	C03[c], C11 (Mt. Fuji, Yamanashi); 28, 027, 033 (Shizuoka)	AB045189
	Et	I04, I05 (Nagano)	AB045186
	Ja	2, 4 (Yamanashi); 10 (Shizuoka); 23 (Mt. Fuji, Shizuoka); C01, C02, C04-C07, C09 (Mt. Fuji, Yamanashi); K01 (Mie)	AB045188
II	Ht	F02[d] (Toyama)	AB045187
	Mt	029 (Shizuoka)	AB045191
	Nt	026 (Shizuoka)	AB045192
	Oa	015, 019 (Nagano)	AB045193
III	Bt	502 (Aomori); 005[a] (Akita)	AB045183
	At	604[d], 605[d] (Hokkaido)	AB045182
IV	Ca	I01[a]-I03[a] (Nagano), 507[a] (Iwate)	AB045184
	Ct	I06[a] (Nagano), 505[a] (Iwate); 509[a], 510[a] (Miyagi); 511[a] (Fukushima); F01[a], F04[a] (Toyama)	AB045185
V	Ut	A01[b]-A04[b], A06[b], A07[b] (Oshima Island)	AB045199
	Vt	G01[b], G04[b], G07[b] (Miyakejima Island)	AB045200
	Wt	H03[b], H04[b] (Hachijyojima Island); J01[b] (Mikurajima Island)	AB045201
	Ra	208 (Hyogo)	AB045196
	Sa	217 (Okayama), 301 (Kagawa)	AB045197
	Qa	204 (Hyogo); 211(Tottori); 214, 218 (Okayama); 006 (Kagawa)	AB045194
	Qt	401 (Nagano); 202 (Kyoto); 106 (Okayama)	AB045195
	Tt	021 (Miyazaki), 022 (Oita), 034 (Kagoshima)	AB045198
Out		D01[e]-D03[e] (Mt. Fuji. Yamanashi)	AB054202

[a] *Polygonum cuspidatum* var. *uzensis*. [b] *Polygonum cuspidatum* var. *terminalis*. [c] *Polygonum cuspidatum* var. *compacta*. [d] *Polygonum sachalinense*. [e] *Polygonum weyrichii*.

Results and Discussion

Sixty-eight DNA samples were prepared from each individual and a region of about 1,420 bp between *rbcL* and *accD* was amplified. Nucleotide sequences were then determined. All sequences were deposited in DDBJ/EMBL/Genbank DNA database (for accession numbers, see Table 2). Sequence variations were found at 22 sites, as shown in Fig. 1 and summarized in Fig. 2. Among the 68 accessions of *Polygunum cuspidatum* and *P. sachalinense*, we found 20 variable sites and two indels. Based on these sequence variations, we recognized 19 haplotypes in *P. cuspidatum* and two haplotypes in *P. sachalinense* (Table 2).

Genetic distances measured by Kimura's 2-parameter method ranged 0-0.008 among plants of *Polygonum cuspidatum* (including *P. sachalinense*), and 0.075-0.801 between *P. weyrichii* var. *alpinum* and *P. cuspidatum* (including *P. sachalinense*). The intraspecific genetic distances observed here were almost equal or slightly lower than those in previous studies in which intraspecific phylogeny had been well resolved using cpDNA data in Japanese plants (0.001-0.017 in *Pedicularis chamissonis*, Fujii *et al.* 1997; 0.001-0.009 in *Primula cuneifolia*, Fujii *et al.* 1999).

Among the variable sites, site 3 was of interest because the change here always involved three continuous bases, AAA or TTT, which we assumed to be caused by a single

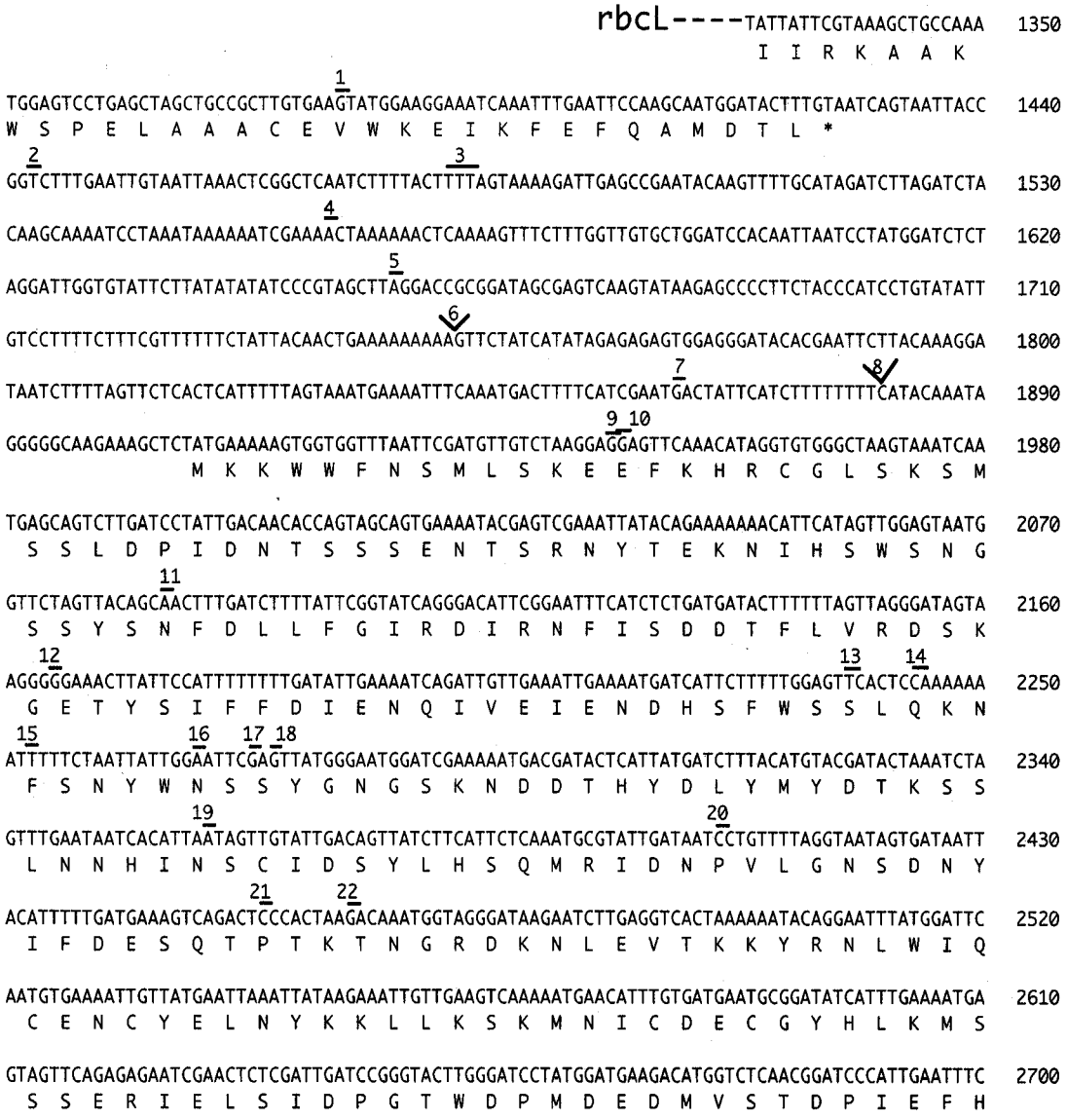

Fig. 1. Nucleotide sequence of a part of the chloroplast DNA from *Polygonum cuspidatum* grown in a plant garden of Shizuoka University. The numbering of the nucleotides from the translation start site of *rbcL* is shown on the right. Deduced amino acid sequences of the 3′ portion of *rbcL* and 5′ portion of *accD* are shown in the lower part of the nucleotide sequence with the one-letter code. Numbers in the upper part of the nucleotide sequence indicate mutation sites.

Clade	Haplotype	Mutation site																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
I	Kt	G	T	TTT	A	A	A--	G	--	G	G	A	A	TC	C	T	A	G	G	A	C	C	G
	It	G	T	TTT	A	A	---	G	--	G	G	A	G	TC	C	T	A	G	G	A	C	C	G
	Jt	G	T	TTT	A	A	A--	G	--	G	G	A	G	TC	C	T	A	G	G	A	C	C	G
	Et	G	T	TTT	A	A	AA-	G	--	G	G	A	G	TC	C	T	A	G	G	A	C	C	G
	Ja	G	T	AAA	A	A	A--	G	--	G	G	A	G	TC	C	T	A	G	G	A	C	C	G
II	Ht	G	T	TTT	A	A	---	G	--	G	A	A	G	TC	C	T	G	T	G	C	C	A	G
	Mt	G	T	TTT	A	A	AAA	G	-T	G	G	A	G	TC	C	T	G	T	G	C	C	A	G
	Nt	G	T	TTT	A	A	A--	G	--	G	G	A	G	TC	C	T	G	T	G	C	C	A	G
	Oa	G	T	AAA	A	A	A--	G	--	G	G	A	G	TC	C	T	G	T	G	C	C	A	G
III	Bt	G	C	TTT	A	A	A--	G	--	G	G	A	G	TC	C	T	A	T	G	A	C	A	G
	At	G	C	TTT	A	A	A--	G	--	G	G	A	G	GA	C	T	A	T	G	A	C	A	G
IV	Ca	G	T	AAA	A	C	A--	G	TT	G	G	A	G	TC	A	T	A	T	C	A	T	A	G
	Ct	G	T	TTT	A	C	A--	G	TT	G	G	A	G	TC	A	T	A	T	C	A	T	A	G
V	Ut	A	T	TTT	A	A	A--	A	TT	G	G	A	G	TC	C	T	A	T	G	A	T	A	A
	Vt	A	T	TTT	C	A	A--	G	TT	G	G	A	G	TC	C	T	A	T	G	A	T	A	G
	Wt	A	T	TTT	A	A	A--	G	TT	G	G	A	G	TC	C	T	A	T	G	A	T	A	G
	Ra	G	T	AAA	A	A	A--	G	-T	G	G	G	G	TC	C	T	A	T	G	A	T	A	G
	Sa	G	T	AAA	A	A	AAA	G	-T	G	G	A	G	TC	C	T	A	T	G	A	T	A	G
	Qa	G	T	AAA	A	A	A--	G	--	G	G	A	G	TC	C	T	A	T	G	A	T	A	G
	Qt	G	T	TTT	A	A	A--	G	-T	G	G	A	G	TC	C	T	A	T	G	A	T	A	G
	Tt	G	T	TTT	A	A	A--	G	TT	A	G	A	G	TC	C	G	A	T	G	A	T	A	G

Fig. 2. Nucleotide sequence variation of chloroplast DNA in *Polygonum*. An amino acid replacement substitution is indicated by a gray box and a synonymous substitution by an open box. Spaces (—) were introduced to adjust sequence length. Shading indicates mutation site within the coding region.

mutation event. Parsimony analysis using PAUP 4.0b3 produced ten minimum length trees (Fig. 3). These trees required 24 steps, and had consistency (CI) and retention (RI) indexes of 0.875 and 0.880, respectively. We constructed a strict consensus tree of these trees and performed 1,000 bootstrap resamplings. The resulting bootstrap support is shown on the branches of the tree (Fig. 3). Of all the variable sites, only site 3 showed homoplasy when the positions of each mutation on the shortest tree were restored with ACCTRAN optimization. In some cases, haplotypes that differ only at site 3 were found in the same locality, so this site might be a hot spot in *Polygonum cuspidatum*. We also calculated the bootstrap value of each branch with the dataset omitting site 3 (values are shown in parentheses in Fig. 3). In the strict consensus tree obtained, five major clades were recognized. Samples belonging to each clade are shown in Table 2. The first, second and third groups shared an amino acid replacement at site 20 in *accD*. The first group had synonymous substitutions at site 17 and an amino acid replacement at site 21 in *accD*, and was supported with a bootstrap value of 76%. This group consisted of *P. cuspidatum* var. *cuspidatum* in the central part of Honshu; ten accessions on Mt. Fuji in Shizuoka and Yamanashi, two on Mt. Yatsugatake in Nagano, two in Kitashiga, Nagano, five in Shizuoka, two in Yamanashi and one in Minamimuro, Mie (Fig. 4). The second commonly had amino acid replacements at sites 16 and 19 in *accD* and was supported with a 76% bootstrap value. This group consisted of *P. cuspidatum* var. *cuspidatum* in Nagiso, Nagano, a mountain area in Shizuoka City, and *P. sachalinense* in Toyama. The third group consisted of *P. sachalinense* in Hokkaido, *P. cuspidatum* var. *cuspidatum* in Aomori and var. *uzensis* in Akita, all located in Northern Japan. They had a synapomorphic substitution at site 2, supported with a 63% bootstrap value. In addition, *P. sachalinense* in Hokkaido had an amino acid replacement at site 13 in *accD*. The fourth group shared two amino acid replacements at sites 14 and 18 in *accD* and one substitution in the spacer region, and was supported by a bootstrap value of 84%. They were distributed widely in the Tohoku and Hokuriku Districts; two in Iwate, two in Miyagi, one in Fukushima, two in Toyama, and four in Kitashiga, Nagano. All of them were identified as var. *uzensis* from their morphological characteristics. The fifth group consisted of var. *terminalis* in the Izu Islands, which had an amino acid replacement at site 1 in *rbcL*, supported with a 67% bootstrap value. All the samples from

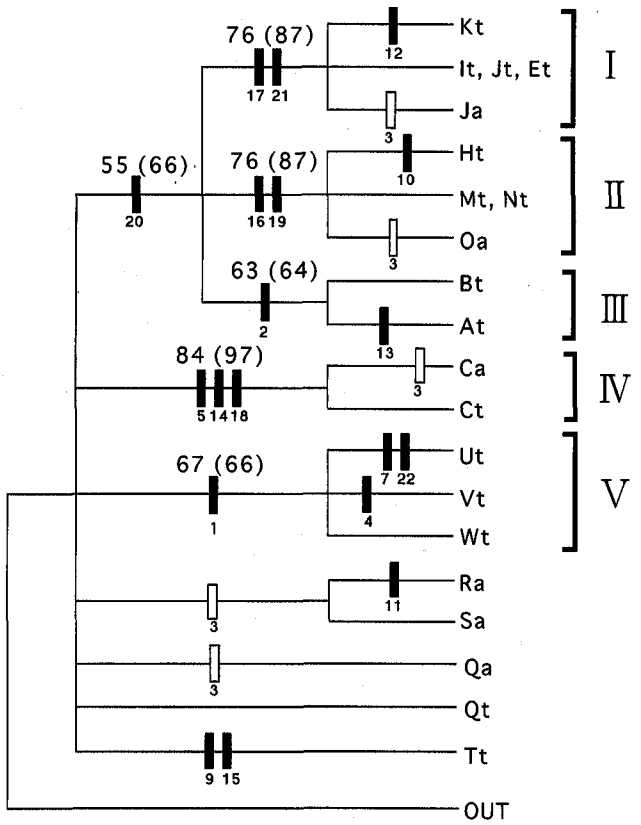

Fig. 3. The strict consensus tree of 10 equally parsimonious trees generated using PAUP* 4.0b3a based on the nucleotide sequence variation of chloroplast DNA. Bootstrap values with 1,000 replicates are indicated above each branch. The Bootstrap values calculated with the dataset omitting site 3 are given in parentheses. Substitutions are indicated by solid boxes or open boxes (at site 3). Indels were neglected. The tree was rooted using *Polygonum weyrichii* as an outgroup.

Oshima Island had a substitution at site 7 and a synonymous substitution at site 22, while those from Miyakejima Island had a substitution at site 4. These results suggest that the var. *terminalis* on each island were genetically isolated from one another.

The remaining haplotypes (Qa, Qt, Ra, Sa and Tt), their relationships unresolved, formed a polychotomy at the base of the tree (Fig. 3). All of them were identified as *Polygonum cuspidatum* var. *cuspidatum*. It has two synapomorphic substitutions and all accessions belonging to this haplotype are located in Kyushu. The accessions belonging to Qa, Qt, Ra and Sa were located in western Honshu and Shikoku, except one, found in Nagano.

In the present study, we analyzed four intraspecific taxa of *Polygonum cuspidatum* and a closely related species, *P. sachalinense*. *Polygonum cuspidatum* var. *terminalis* and var. *uzensis* (except one accession in Akita) were recognized as valid taxa, since their accessions formed a monophyletic group (Table 1, Fig. 3). They also have distinct distributions, var. *terminalis* in the Izu Islands and var. *uszensis* in the Tohoku and Hokuriku districts of Honshu. The present result suggests that they were derived from *P. cuspidatum*, and had differentiated genetically as well as morphologically. On the other hand, var. *compacta*, found in the alpine zone, had not differentiated genetically according to the present study, suggesting that this taxon may merely be an alpine ecotype. In this study, we examined only one sample belonging to this taxon and it is necessary to examine samples of other localities to confirm this result.

In the strict consensus tree, the groups I, II and III made a monophyletic group, although the bootstrap value was not high. This result suggests close phylogenetic relationships among them. The distributions of group I and II are similar, however, that of group III is disrupted by the distribution of group IV (var. *uzensis*). A similar pylogeographic pattern had been reported in *Primula cuneifolia* and *P. nipponica* (Fujii *et al.* 1999). The distribution of *Primula cuneifolia* is divided by the area of *P. nipponica* in Tohoku. It is possible that they have the same historical background, however, more examples are needed to understand this phylogeographic pattern in Japan.

In this study, we examined three accessions of *Polygonum sachalinense*. However, they did not group together; two accessions of Hokkaido belonged to group III and an accession of Toyama belonged to group II. In the beginning of this study, we intended to use *P. sachalinense* as an outgroup, but they were dispersed among the ingroup OTUs in the end. There are two possible explanations for this result. One is that *P. sachalinense* is a polyphyletic species derived from *P. cuspidatum*. The other is that cytoplasmic gene flow (Rieseberg *et al.* 1991, Whittemore and Schaal 1991) between *P. cuspidatum* and *P. sachalinense* had occurred. Because *P. sachalinense* has distinct morphological characteristics distinguishing it from *P. cuspidatum*, the latter explanation is preferable. This hypothesis is also supported by the fact that one accession of var. *uzensis* in Akita belonged to group III rather than group IV in which all the other accessions of var. *uzensis* belonged. To clarify the relationship between *P. cuspidatum* and *P. sachalinense*, a study of more accessions of P. *sachalinense* is needed.

In summary, *Polygonum cuspidatum* are differentiated according to their distribution, and two varieties, var. *terminalis* and var. *uzensis*, are differentiated genetically. On the other hand, *P. sachalinensis*, a distinct species morphologically, fell among accessions of *P. cuspidatum* on the phylogenetic tree obtained in the present study. One cause of this result could be cytoplasmic gene flow between the two species. More detailed study including an examination of nuclear genome phylogeny will be needed to understand these relationships.

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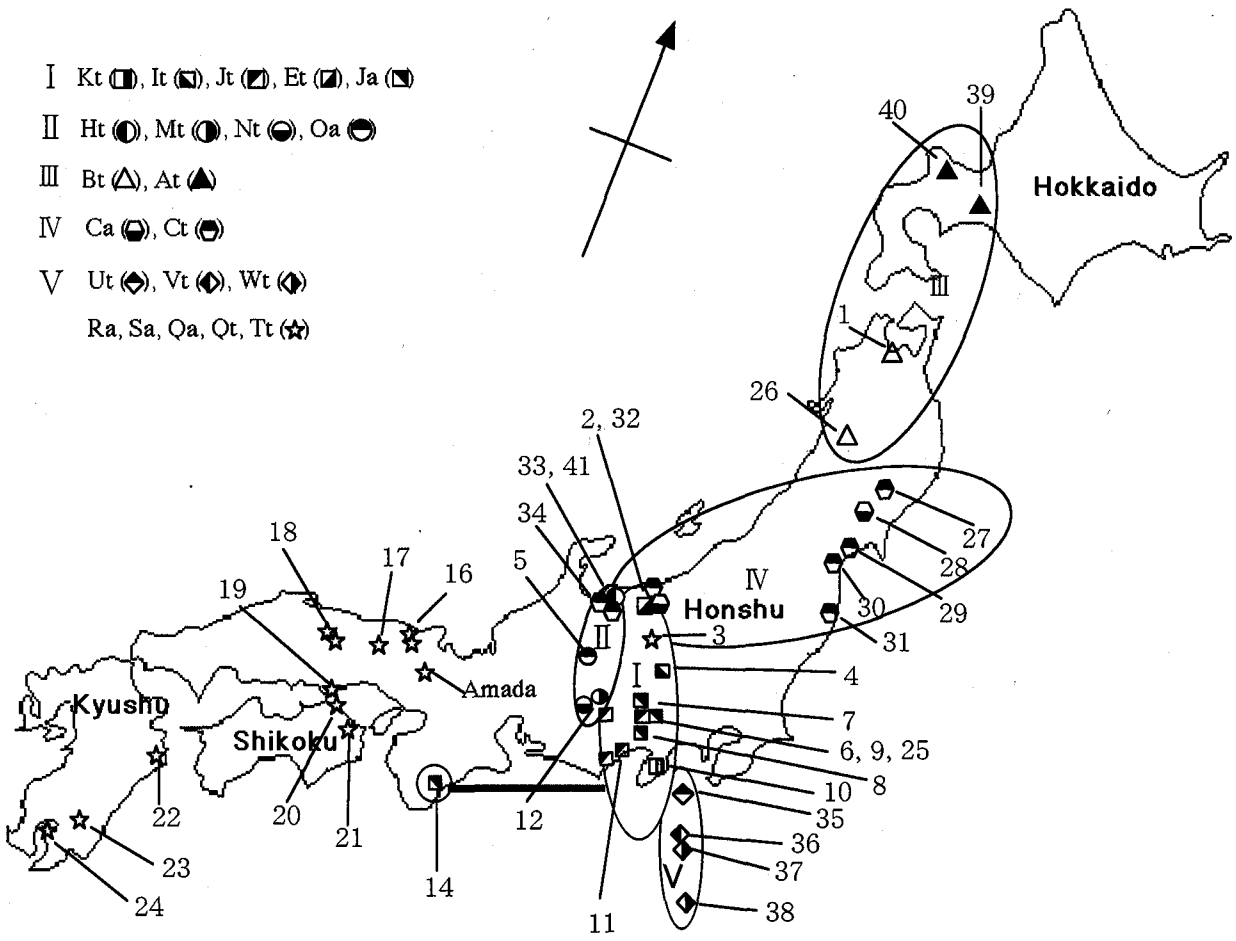

Fig. 4. Geographical distribution of clades and haplotypes of cpDNA in *Polygonum cuspidatum* and *P. sachalinense*. Accessions in clade I are indicated by squares, those in clade II by circles, those in clade III by triangles, those in clade IV by hexagonals, those in clade V by diamonds, and the rest by stars. The number of each collection site is shown in Table 1.

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