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Genetic Differentiations among the Populations of *Salvia japonica* (Lamiaceae) and Its Related Species

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Morphological and genetic variations within *Salvia japonica* (Lamiaceae) and its related species in Japan were analyzed for clarifying their taxonomic significance. The genetic variations were explored through chloroplast and nuclear ribosomal DNA sequences and allozyme polymorphisms. Since chromosome numbers characterized the genus of *Salvia*, we also examined whether the karyotypes were different. We examined 58 populations of *S. japonica* and 14 populations of others species of *Salvia*. Among the populations of *S. japonica* represented four forms (f. *japonica*, f. *longipes*, f. *lanuginosa* and f. *albiflora*). The size of chromosomes were various among *Salvia* spp. Based on the allozyme as well as the DNA sequence, the populations of *S. japonica* separated from the others *Salvia* species. The populations of *S. japonica* exhibited four combinations of the morphological characters. However, these combinations did not correlate to the four forms of *S. japonica*. In addition, the morphological variations did not correlate to the allozyme and DNA sequences. It is suggested that the four morphological variations as well as the four form of *S. japonica* should not considered to be a taxonomic unit; accordingly, *S. japonica* were considered to be still at the early stage of speciation process.

Key words: allozyme, DNA, morphological variations, *Salvia japonica*

INTRODUCTION

Much debate still exists as to which mechanism is responsible for the majority of speciation events. The process of speciation in plants at minimum must involve: (i) divergence in some feature(s), usually morphological, such that plants are distinguishable; (ii) development of reproductive isolation sufficient to maintain these distinguishing features (Crawford 1985). The various ways by which divergence and reproductive isolation develop represent the modes of speciation. From these considerations, it follows that any study of speciation must consider the factors isolating species and the amount of genetic divergence between species. Various classifications of the modes of speciation have been proposed recently (Grant 1981; Gottlieb 2003); all have merit and they differ primarily in emphasis on different aspects of the process. The outline of Grant (1981) referred modes of plant speciation to two basic categories; i.e. evolutionary divergence (or primary speciation) and hybrid speciation. Primary speciation may involve either quantum or geographical speciation. The former is a rapid process whereas the latter involves gradual divergence. Hybrid speciation may occur at the diploid or polyploidy levels (Takano & Okada 2002).

Salvia japonica is a widespread species in Japan and vicinity areas, such as Korea, China, Taiwan and so on, while the other 8 species of *Salvia* are endemic species to Japan. Murata and Yamazaki (1993) treated that *S. japonica* Thunb., *S. isensis* Nakai ex Hara, *S. lutescens* (Koidz.) Koidz., *S. ranzaniana* belongs to the series *Japonicae* C. Y. Wu, where as *S. pygmaea* Matsum. belongs to series *Appendiculatae*. *Salvia japonica* contains morphological variations, and is divided into two varieties, namely, var. *japonica* Thunb. and var. *formosana* Murata. Further *S. japonica* var. *japonica* is composed of four forms, namely, f. *albiflora* Hiyama, f. *japonica*, f. *lanuginosa* (Franch) Stib., and f. *longipes* (Nakai) Sugimoto (Murata 1952). The information about wide-range morphological variations in *S. japonica* suggests that the taxonomic treatment within the species remains questionable. Murata and Yamazaki (1993) regarded *S. japonica* as the most variable in form of leaves. The question is whether they belong to the same species, or they can be divided into several taxa that are distinguishable based on genetic characteristics.

We analyzed variations of morphological characters in *S. japonica* and compared those variations with genetic variations detected from chloroplast DNA, nuclear ribosomal DNA and allozymes within *S. japonica* and its related species in Japan.

MATERIALS AND METHODS

Sample Collection. A total sum of 2,138 individuals of *Salvia* were used. They were include in *S. japonica* (58 populations), *S. lutescens* (two populations), *S. nipponica* (three populations), *S. glabrescens* (three populations), *S. pygmaea* (one population), *S. isensis* (one population), *S. plebeia* (one population), *S. hayatana* (one population), *S. arisanensis* (one population), and *S. ranzaniana* (one population). All of 72 species were examined their allozyme polymorphism to know genetic differentiation among them (Table 1). Among populations of *S. japonica*, the individual

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Table 1. Species, form, and name of populations, localities, numbers of individuals for allozyme analysis and chromosomes observation of *Salvia* spp. and morphological characters of *S. japonica* populations

Species/forms	Population name	Locality of populations	N	Specimen/voucher	Morphological variations			
					Internode: long (L)/ short (S)	Stem: erect (E)/ decumbent (D)	Leave margin*	Leaflet base**
S. japonica								
f. *japonica*	J1	Katano, Osaka Pref.	57	Sudarmono *et al.* Jap01/01 BO	L	E	D	S
f. *japonica*	J2	Hoshida, Osaka Pref.	46	Sudarmono *et al.* Jap01/02 BO	S	E	C	T
f. *japonica*	J3	Himuro-dai, Hirakata, Osaka Pref.	39	Sudarmono *et al.* Jap03/03 BO	S	E	C	T
f. *japonica*	J4	Tsuburo, Nara Pref.	42	Sudarmono *et al.* Jap03/04 BO	L	D	C	T
f. *japonica*	J5	Sakura, Ouda-cho, Nara Pref.	27	Sudarmono *et al.* Jap03/05 BO	S	E	C	T
f. *japonica*	J6	Hatano, Oyodo-cho, Yoshino-gun, Nara Pref.	38	Sudarmono *et al.* Jap03/06 BO	L	D	C	T
f. *japonica*	J7	Shimagahara, Mie Pref.	45	Sudarmono *et al.* Jap03/07 BO	L	E	D	S
f. *japonica*	J8	Ueno, Mie Pref.	25	Sudarmono *et al.* Jap03/08 BO	S	E	C	T
f. *japonica*	J9	Oyamada-mura, Mie Pref.	22	Sudarmono *et al.* Jap03/09 BO	S	E	C	T
f. *japonica*	J10	Iwagami, Aoyama-cho, Mie Pref.	26	Sudarmono *et al.* Jap03/10 BO	L	D	C	T
f. *japonica*	J11	Funa-cho, Toba , Mie Pref.	30	Sudarmono *et al.* Jap03/11 BO	L	D	C	T
f. *japonica*	J12	Ise, Mie Pref.	41	Sudarmono *et al.* Jap03/12 BO	L	D	C	T
f. *japonica*	J13	Asakuma river, Kinshinshi, Mie Pref.	19	Sudarmono *et al.* Jap03/13 BO	S	E	C	T
f. *japonica*	J14	Mt. Asakuma, Ise, Mie Pref.	22	Sudarmono *et al.* Jap03/14 BO	L	E	D	S
f. *longipes*	J15	Isobe-cho, Mie Pref.	37	Sudarmono *et al.* Jap03/15 BO	L	D	C	T
f. *japonica*	J16	Nachi Katsuura-cho, Wakayama Pref.	22	Sudarmono *et al.* Jap01/16 BO	S	E	C	T
f. *japonica*	J17	Ooshima island, Wakayama Pref.	15	Sudarmono *et al.* Jap01/17 BO	L	E	D	S
f. *japonica*	J18	Kasagi-cho, Kyoto Pref.	28	Sudarmono *et al.* Jap03/18 BO	S	E	C	T
f. *japonica*	J19	Wazuka-cho, Kyoto Pref.	23	Sudarmono *et al.* Jap03/19 BO	S	E	C	T
f. *japonica*	J20	Hiyoshi, Kyoto Pref.	45	Sudarmono *et al.* Jap03/20 BO	S	E	C	T
f. *japonica*	J21	Ukyoku, Umegahata-cho, Kyoto Pref.	37	Sudarmono *et al.* Jap03/21 BO	L	D	C	T
f. *japonica*	J22	Nougami, Keihoku, Kyoto Pref.	36	Sudarmono *et al.* Jap03/22 BO	L	D	C	T
f. *japonica*	J23	Kyoutanabe, Kyoto Pref.	49	Sudarmono *et al.* Jap03/23 BO	L	E	D	S
f. *japonica*	J24	Ritto, Shiga Pref.	45	Sudarmono *et al.* Jap03/24 BO	S	E	C	T
f. *japonica*	J25	Koonan-cho, Shiga Pref.	37	Sudarmono *et al.* Jap03/25 BO	L	E	D	S
f. *japonica*	J26	Tsuchiyama-cho, Shiga Pref.	34	Sudarmono *et al.* Jap03/26 BO	L	E	D	S
f. *japonica*	J27	Mt. Hiezan, Shiga Pref.	29	Sudarmono *et al.* Jap03/27 BO	L	E	S	D
f. *japonica*	J28	Sakauchi-mura, Kawakami, Gifu Pref.	42	Sudarmono *et al.* Jap03/28 BO	L	D	C	T
f. *japonica*	J29	Oodaira, Hamakita , Shizuoka Pref.	19	Sudarmono *et al.* Jap03/29 BO	L	E	D	S
f. *longipes*	J30	Mt. Hooraiji, Hoorai-cho, Aichi Pref.	25	Sudarmono *et al.* Jap03/30 BO	L	E	D	S
f. *japonica*	J31	Shinshiro, Aichi Pref.	14	Sudarmono *et al.* Jap03/31 BO	L	E	D	S
f. *lanuginosa*	J32	Mt. Asayama, Hakone-cho, Kanagawa Pref.	19	Tsukaya *et al.* Jap03/32	L	E	D	S
f. *japonica*	J33	Yamazaki-cho, Hyogo Pref.	27	Sudarmono *et al.* Jap02/33 BO	L	D	C	T
f. *japonica*	J34	Kaibara-cho, Hyogo Pref.	25	Sudarmono *et al.* Jap03/34 BO	L	E	D	S
f. *japonica*	J35	Chikusa-cho, Hyogo Pref.	42	Sudarmono *et al.* Jap03/35 BO	L	E	D	S
f. *japonica*	J36	Oohara-cho, Okayama Pref.	19	Sudarmono *et al.* Jap03/36 BO	L	E	S	D
f. *japonica*	J37	Kanba, Maniwa-gun, Okayama Pref.	33	Sudarmono *et al.* Jap03/37 BO	L	E	S	D
f. *japonica*	J38	Katsuyama-gun, Okayama Pref.	29	Sudarmono *et al.* Jap03/38 BO	L	E	S	D
f. *japonica*	J39	Yatsuka-mura, Maniwa-gun, Okayama Pref.	35	Sudarmono *et al.* Jap03/39 BO	L	E	S	D
f. *japonica*	J40	Chizu-cho, Tottori Pref.	42	Sudarmono *et al.* Jap03/40 BO	S	E	C	T
f. *longipes*	J41	Shiraichi, Higashi Hiroshima, Hiroshima Pref.	14	Sudarmono *et al.* Jap03/45 BO	L	E	D	S
f. *japonica*	J42	Meise-gun, Kamiyama-cho, Tokushima Pref.	24	Sudarmono *et al.* Jap03/42 BO	L	E	D	S
f. *japonica*	J43	Tokushima, Tokushima Pref.	18	Sudarmono *et al.* Jap03/43 BO	L	E	D	S
f. *japonica*	J44	Yamashiro-cho, Tokushima Pref.	40	Sudarmono *et al.* Jap03/44 BO	L	D	C	T
f. *japonica*	J45	Oono, Yamashiro-cho, Tokushima Pref.	31	Sudarmono *et al.* Jap03/41 BO	L	D	C	T
f. *longipes*	J46	Uma-gun, Ehime Pref.	23	Sudarmono *et al.* Jap03/46 BO	L	D	C	T
f. *japonica*	J47	Uwa-cho, Ehime Pref.	23	Sudarmono *et al.* Jap03/47 BO	L	D	C	C
f. *japonica*	J48	Oozu, Ehime Pref.	21	Sudarmono *et al.* Jap03/48 BO	L	D	C	C
f. *longipes*	J49	Kochi, Kochi Pref.	15	Sudarmono *et al.* Jap03/49 BO	L	D	C	T
f. *japonica*	J50	Susaki, Kochi Pref.	27	Sudarmono *et al.* Jap03/50 BO	L	E	S	D
f. *japonica*	J51	Kadogawa-cho, Miyazaki Pref.	36	Sudarmono *et al.* Jap02/51 BO	L	E	D	S
f. *longipes*	J52	Hyuuga, Miyazaki Pref.	14	Sudarmono *et al.* Jap02/52 BO	L	E	S	D
f. *japonica*	J53	Aoidake, Miyazaki Pref.	36	Sudarmono *et al.* Jap02/53 BO	L	E	S	D
f. *japonica*	J54	Mimata-cho, Miyazaki Pref.	25	Sudarmono *et al.* Jap02/54 BO	L	E	D	S
f. *japonica*	J55	Nishi Myakonojou-cho, Miyazaki Pref.	16	Sudarmono *et al.* Jap02/55 BO	L	E	D	S
f. *japonica*	J56	Kitamata-mura, Kagoshima Pref.	69	Sudarmono *et al.* Jap02/56 BO	L	E	D	S
f. *japonica*	J57	Takarabe-cho, Kagoshima Pref.	40	Sudarmono *et al.* Jap02/57 BO	L	E	D	S
f. *japonica*	J58	Amami isl. (Amami oshima), Kagoshima Pref.	21	Sudarmono *et al.* Jap03/58 BO	L	D	C	T
S. nipponica	N1	Kinkasan island, Oshika-cho, Miyagi Pref.	23	Okada *et al.*				
	N2	Chizu-cho, Tottori Pref.	26	Sudarmono *et al.* Jap03/66 BO				
	N3	Miyagi Pref.	13	Sudarmono *et al.* Jap05/67 BO				
S. glabrescens	G1	Miyazu, Kyoto Pref.	24	Sudarmono *et al.* Jap03/68 BO				
	G2	Tsuge-mura, Tenri, Nara Pref.	25	Sudarmono *et al.* Jap05/69 BO				
	G3	Kamiawa, Ueno, mie Pref.	25	Sudarmono *et al.* Jap05/70 BO				
S. pygmaea	Py	Sumiyoh river, Amami isl., Kagoshima Pref.	35	Okada *et al.* Jap03/71				
S. isensis	I	Mt. Asakuma, Mie Pref.	37	Okada *et al.* Jap03/72				
S. lutescens								
var. *crenata*	L1	Shinshiro, Aichi Pref.	7	Tsukaya *et al.* Jap04/73				
var. *lutescens*	L2	Mt. Yazu, Hisai, Mie Pref.	40	Sudarmono *et al.* Jap05/74 BO				

Table 1. Continued

Species/forms	Population name	Locality of populations	N	Specimen/voucher	Morphological variations			
					Internode: long (L)/ short (S)	Stem: erect (E)/ decumbent (D)	Leave margin*	Leaflet base**
S. plebeia	P1	Kizu River, Kyotanabe, Kyoto Pref.	10	Sudarmono *et al*. Jap04/75 BO				
S. hayatana	H	Wu shinpi, Wu-yen Chiao, Taiwan	36	Okada *et al.*				
S. arisanensis	A	Mt. Ho-huan Shan, Taiwan	24	Okada *et al.*				
S. renzaniana	R	Shinogo, Kitayama vill., Wakayama Pref.	33	Okada *et al*. Okada5698				
Total individuals			2,138					

*Leaf margin: crenate (C); dentate (D); serrate (S); **Leaflet base: truncate (T); shallowly cuneate (S); deeply cuneate (D); N = number of individuals

from four different populations that have peculiar karyotype and morphological characters, i.e. population Yamazaki, Hyogo Pref.(J33), population Nachi-katsuura, Wakayama Pref. (J16), population Kanba, Okayama Pref. (J37), and population Kaibara, Okayama (J34) were used for DNA analysis. In addition, individuals of *S. nipponica* as well as *S. glabrescens* from three different localities were also used for DNA analyses. Template DNA of twenty-three taxa of *Salvia* and three taxa of outgroup (Table 2) were analyzed for estimating the relationships among species. These taxa included *S. japonica*, which was analyzed from four individuals of different populations, *S. nipponica* and *S. glabrescens*, which were analyzed from three individuals of different localities, and others *Salvia* species, which their DNA sequences were obtained from DNA Data Bank of Japan. Living materials transplanted in the screen house of Botanical Gardens, Faculty of Science, Osaka City University. Voucher specimens are kept in BO (Herbarium Bogoriense) and Botanical Gardens, Osaka City University.

Variations of Morphological Characters. In order to examine the morphological variations of leaves, all individuals of 58 populations of *S. japonica* were observed (Table 1). Four morphological characters were examined, those are, (i) the internode length, i.e., Long (L): more than 5 cm or Short (S): less than 1.5 cm; (ii) the stem habit, i.e., erect (E) or decumbent (D); (iii) the leaf margin, i.e., crenate (C): tooth rounded, dentate (D): tooth obtuse angled, or serrate (S): tooth acute angled; (iv) the leaflets base, i.e., truncate (T), shallowly cuneate (S), or deeply cuneate (D) (Table 1).

Chromosome Observation. Chromosome number and the photomicrographs obtained during our examinations revealed that chromosome lengths of the best cell of all taxa referred Table 1. Growing root tips were incubated in 0.05% colchicine aqueous solution for 2 hours at 18 °C. They were fixed with the fixative fluid (ethanol: chloroform:glacial acetic acid = 2:1:1) for more than 45 minutes at 5 °C. The root tips were then macerated with 1N HCl at 60 °C for 18 seconds. The meristematic tissues were stained with 2% aceto-orcein for 5-10 minutes on a slide glass. After then, one drop of 45% acetic acid was added and the slide glass was covered by cover slip and gently squashed.

DNA Extraction and Amplification. Total DNA was isolated from 0.7 to 1.5 grams of fresh or silica gel-dried leaves, using a modification of the 2x cetyltrimethylammonium bromide (CTAB) extraction protocol of Doyle and Doyle (1987). The chloroplast DNA (hereafter cpDNA) sequences were amplified with primer pairs *rbcL* 1-1 as the forward primer and *rbcL* NN3-2 as the reverse primer (Hasebe *et al*. 1994), and for the intergenic spacer region of *trnL-F* FRF as the forward primer and 5FR as the reverse primer (Sudarmono & Okada 2007). The highest yields of polymerase chain reaction (PCR) products of *rbcL* and *trnL-F* were achieved using the following conditions. The PCR reaction mixture consisted of 5 µl of 5% rTaq-polymerase (TAKARA, Japan) reaction buffer, 4 µl of 0.2 mM each dNTP, 2.5 µl of 20 pM each primer, 0.25 µl of 0.5 units Taq DNA polymerase, 10-50 ng of 5 µl template total DNA and mess up by sterilized water in a total volume of 50 µl. The PCR samples were heated to 94 °C for 3 min, followed by 37 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min 30 sec, and a final extension at 72 °C for 5 min.

Additional pairs of forward and reverse sequence primers used for the amplification of nuclear ribosomal DNA (hereafter nrDNA) in this study were ITS A and ITS B (Blattner 1999). The PCR reaction with the ITS primers included 5 µl dimethyl sulfoxide (DMSO) with a reduced amount of sterilized water to compensate. The PCR conditions were denaturation at 94 °C for

Table 2. Accession number (DNA Data Bank of Japan) of DNA sequences of the taxa studied

Haplotype	Accession number		
	rbcL	*trnL-F*	*ITS*
Salvia arisanensis	AB295063	AB295074	AB295085
*S. glabrescens*MIY	AB295064	AB295075	AB295086
*S. glabrescens*SUG	AB295065	AB295076	AB295087
*S. glabrescens*KAM	AB295066	AB295077	AB295088
S. hayatana	AB295067	AB295078	AB295089
S. isensis	AB266221	AB266231	AB266241
S. japonica			
f. *albiflora*	AB266220	AB266230	AB266240
f. *japonica*	AB266219	AB266229	AB266239
f. *lanuginosa*	AB266217	AB266227	AB266237
f. *longipes*	AB266218	AB266228	AB266238
*S. japonica*YAZ/33	AB295068	AB295079	AB295090
*S. japonica*16	AB295096	AB295100	AB295104
*S. japonica*34	AB295097	AB295101	AB295105
*S. japonica*37	AB295098	AB295102	AB295106
S. lutescens			
var. *crenata*	AB266223	AB266233	AB266243
var. *lutescens*	AB266222	AB266232	AB266242
var. *intermedia*	AB295099	AB295103	AB295107
*S. nipponica*KIN	AB295069	AB295080	AB295091
*S. nipponica*CHZ	AB295070	AB295081	AB295092
*S. nipponica*OSK	AB295071	AB295082	AB295093
S. pygmaea	AB295072	AB295083	AB295094
S. plebeia	AB295073	AB295084	AB295095
S. ranzaniana	AB287373	AB287374	AB287375
Lamium amplexicaule	AB266225	AB266235	AB266245
L. purpureum	AB266224	AB266234	AB266244
Glechoma hederacea	AB266226	AB266236	AB266246

5 min, followed by 35 cycles of 94 °C for 1 min 30 sec, 60 °C for 30 sec, 72 °C for 40 sec, and a final extension at 72 °C for 5 min.

Amplified fragments were subjected to electrophoresis in a 1.5 % agarose gel and purified using Microspin S-300 HR Columns following the manufacturer's protocol (GE Healthcare Biosciences, USA). DNA cycle sequencing with BigDye Terminators v1.1 (Applied Biosystems, USA) and PCR primers were performed in 10 µl volumes on the cleaned PCR products (25 cycles, 10 sec denaturation at 96 °C, 5 sec annealing at 50 °C, and 4 min extension at 60 °C for *rbcL* and *trnL-F*, or 25 cycles, 10 sec denaturation at 96 °C, and 4 min annealing/ extension at 60 °C for ITS). Cycle sequencing reactions were purified by ethanol precipitation, and then denatured in HiDi Formamide 25 µl, 95 °C for 2 min. The denatured samples were cooled on ice and run on an ABI PRISM 310 genetic analyzer (Perkin Elmers Co., Applied Biosystems, USA).

Sequence Alignment and Phylogenetic Analysis. Alignments were obtained using the program BioEdit 5.0.9 (Hall 2005), and adjusted visually. Alignments of *rbcL* and the intergenic spacer region of *trnL-F* of cpDNA were combined. The alignment of the nrDNA region included the ITS1-5.8S rDNA-ITS2 region. Gaps were treated as missing data. Phylogenetic relationships were analyzed using maximum parsimony (MP) approaches with a strict consensus, implemented with the computer program, PAUP*, Phylogenetic Analysis Using Parsimony, version 4.0 Beta10 (Swofford 2002). Heuristic searches were conducted with SIMPLE addition, tree-bisection-reconnection (TBR) branch swapping, and MULPARS options. Bootstrap analysis (Felsenstein 1985) was performed with PAUP* v4.0 using 1,000 bootstrap replications to assess the amount of support for monophyletic groups. Branch lengths were used in preference to cladogram, in which nucleotide substitutions occurring between taxa and character-state changes were detected by distance-based methods.

Allozymic Analysis. Young, fresh leaves of about 0.5 cm^2 from every individual were used for allozymic analyses. They were homogenized with 0.1 M TRIS-HCl grinding buffer, pH 7.5. The extract was absorbed by filter paper (Whatmann No. 3) and run on a 12% starch gel (horizontal electrophoresis system) and on a 7.5-10% polyacrylamide gel (vertical electrophoresis system) (Sudarmono & Okada 2007). A total of eight enzyme systems were analyzed. Six of the eight enzyme systems, Phosphoglucoisomerase (PGI), Phosphoglucomutase (PGM), Menadione reductase (MNR), Isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6-PGD), and Malate dehydrogenase (MDH), were analyzed using the horizontal system. The remaining two systems, Aspartate aminotransferase (AAT), and Shikimate dehydrogenase (SKDH), were resolved using vertical gel electrophoresis as described by Shiraishi (1988). Staining was followed with the procedure of Soltis *et al.* (1983), with some modification in buffer pH from pH 8.0-8.5 to pH 7.5 for PGM. Genetic interpretation of the present isozyme gel banding pattern was based on the evaluation of allozymic polymorphisms in other well documented investigations (Shield *et al.* 1983; Kephart 1990; Syamsuardi & Okada 2002).

The genetic identities and genetic distances for each pair-wise combination of populations were also estimated following Nei (1978). In this study the unbiased genetic identity was used to accommodate bias, because of small sample size (< 50 individuals). Allele frequencies were analyzed using POPGENE ver. 1.31 (Yeh *et al.* 1999).

For the analysis of genetic relationships between 72 populations of *Salvia* based on allozymic polymorphisms, We used the Unweighted Pair Group Method using Arithmetic averages (UPGMA) phylogram and employed NTSYS-pc 2.0 (Rohlf 2000).

RESULTS

Morphological Variations. Among the morphological characters of *S. japonica*, i.e., the internode length: long (L) or short (S), the stem habit: erect (E) or decumbent (D), the leaf margin: crenate (C), dentate (D) or serrate (S), and the leaflet base: truncate (T), shallowly cuneate (S) or deeply cuneate (D), could be combined into 36 combinations. However, only four combinations were found in this study (Table 1). Those are; 12 populations showed SECT: the combination with short internode (S), erect stem (E), crenate leaf margin type (C) and truncate leaflet shape (T), 17 populations had LDCT: long internode (L), decumbent stem (D), crenate leaf margin (C) and truncate leaflet (T), eight populations displayed LESD: long internode (L), erect stem (E), serrate leaf margin (S) and deeply cuneate leaflet (D), and 21 populations exhibited LEDS: long internode (L), erect stem (E), dentate leaf margin (D), shallowly cuneate leaflet (S). These four combinations might be considered as taxonomic units. However, the geographic distribution of the majority populations which have LEDS combination of morphological characters tend to be separated each other (Table 1).

Chromosome Analysis. Chromosome numbers of the *S. japonica* (Figure 1), *S. lutescens, S. isensis, S. pygmaea, S. hayatana, S. arisanensis, S. nipponica, S. glabrescens* and *S. plebeia* were 2n = 16. This is the same result as the report by Funamoto *et al.* (2000), but different from that of Wu and Huang (1975) who reported 2n = 16-18 for species of *S. japonica*. Mitotic metaphase chromosomes varied ranging from 0.8 to 3.8 µm in length (Figure 1). The smallest chromosome of each species karyotipe ranged from 0.8 µm (*S. japonica* f. *longipes*) to 2.0 µm (*S. arisanensis*). The longest chromosome of those karyotype was 2.0 up to 3.8 µm. However, the longest chromosome of the karyotypes of *S. japonica* Yamazaki populations (Hyogo Pref.) was various (2.4 – 3.8 µm). Chromosome complements of *S. nipponica*, *S. glabrescens, S. pygmaea*, and *S. isensis* were similar, they consisted of two subtelocentrics, eight submetacentrics and six metacentrics. Satellite chromosomes were observed only in *S. japonica*, *S. glabrescens*, and *S. pygmaea*. Chromosomes of individuals in Yamazaki population had one set chromosome having longer satellite than the short arm. This finding was the first time so far.

The Phylogeny Constructed from DNA. Monophyly of *Salvia* studied was shown clearly with maximum bootstrap

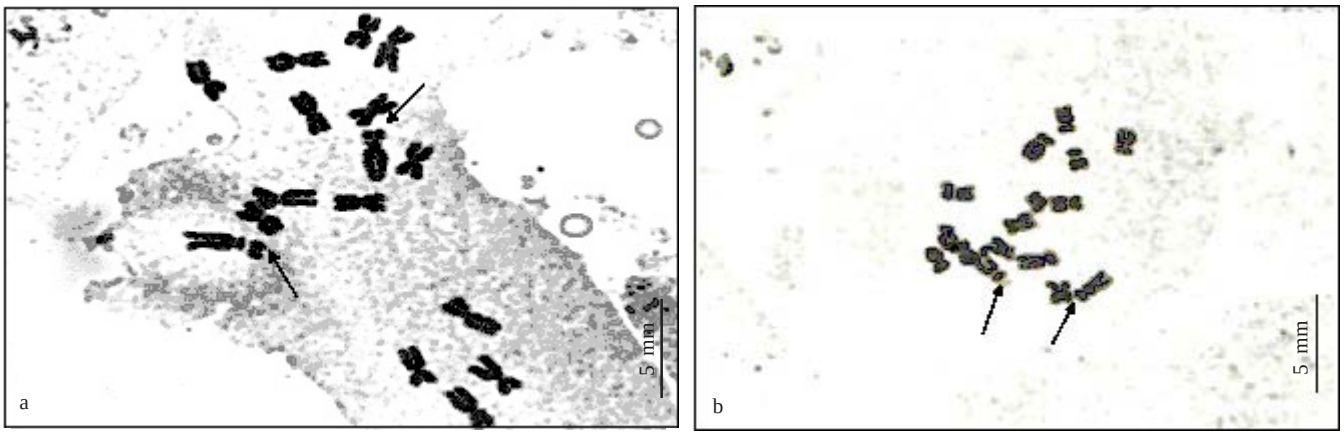

Figure 1. Mitotic metaphase chromosomes of *S. japonica* from Ichinomiya, Kaibara-cho, Hyogo Pref (J34) 2n = 16 (a), and *S. japonica* from Yamazaki, Hyogo Pref (J33) 2n = 16 (b). Arrows indicate satellite chromosome.

value (100%) in the phylogenetic tree, which constructed based on combined data of the cpDNA (*rbcL* and intergenic spacer *trnL-trnF*) and ITS region of nrDNA (Figure 2). There were two clades in *Salvia* in this study, one of them was the clade of *Salvia* of subg. *Allagospadonopsis* with high bootstrap values (100%), while the other consisted the clade of subg. *Salvia* and subg. *Sclarea* with moderate bootstrap values (79%). In this clade, *S. glabrescens* and *S. nipponica* formed a subclade supported high bootstrap values (100%). Clade of subg. *Allagospadonopsis* consisted of all taxa of *S. japonica* (60% bootstrap value), clade of *S. isensis*, *S. lutescens*, and *S. ranzaniana* (69% bootstrap value), and clade of *S. hayatana*, *S. arisanensis* (77% bootstrap value) and *S. pygmaea*. Branch lengths of *Salvia* species from the node of the *Salvia* clade varied from 77 changes per site in f. *longipes*, f. *japonica* to 101 changes per site in *S. plebeia* (Figure 2).

Allozyme Variation. Seven enzyme systems (AAT, PGI, PGM, MNR, IDH, 6-PGD, and MDH) showed consistent banding patterns, and all of them were polymorphic. These enzyme systems contained 9 allozyme loci; AAT was composed of 2 loci (*Aat-1* and *Aat-2*), PGI 1 locus, PGM 1 locus (*Pgm-2*), MNR 1 locus, IDH 1 locus, 6-PGD 1 locus (*6-Pgd-2*) and MDH 2 loci (*Mdh-1* and *Mdh-2*). Three loci (*Pgm-2, Mdh-1,* and *Mdh-2*) were interpreted as a monomer, five loci (*Aat-1, Aat-2, Pgi, Idh,* and *6-Pgd-2*) as a dimer, and one locus (MNR) as tetramer according to the previous studies (Weeden & Wendel 1989; Kephart 1990).

Genetic Differentiation at the Species Level. Mean of total genetic diversity (H_T) of *S. japonica* was calculated as 0.408 (41%), most of which was partitioned as 26% within populations (H_S = 0.261) and 15% among populations (D_{ST} = 0.147) (Table 3). Almost all of loci showed high genetic diversity within populations except *Aat-1* and *Pgm-2*. Mean of genetic differentiation coefficient (G_{ST}) of *S. japonica* was 0.372, in the other words, 37% occurred among populations and 63% within populations. Genetic differentiation in *S. japonica* was similar to other conspecific species of *Salvia*, which their genetic diversity was majority occured among populations (Table 3). Although gene flow of *S. japonica* (*Nm* = 0.486) was lower than endemic species of *S. lutescens* (*Nm* = 2.38), it was comparable to the so-called "widespread species" (*Nm* = 0.149) or "out-crossing species by animal" (*Nm* = 0.634) (cf. Hamrick & Godt 1990).

Genetic Distance. Three main clusters were constructed within populations of *S. japonica*. The base cluster (cluster III) was composed of only population of J36, the second cluster (cluster II) consisted of populations J16, J52, J27, J53, J37, where as the remaining populations formed cluster I (Figure 3 & 4). Other *Salvia* species were separated from the cluster of *S. japonica*. However, *S. japonica* were closely related to the cluster of *S. lutescens*, *S. isensis*, *S. pycmaya*, and *S. ranzaniyana* (Figure 3).

Comparison Morphological Variations with Genetic Variations. Combinations of variations of morphological character of *S. japonica* did not correlate to allozymic variations (Figure 4). Cluster III consisted of only one population of f. *japonica* (Figure 4). Cluster II was composed of heterogeneous morphological variations, i.e. f. *japonica* with similar combination of morphological characters (LESD), population J16 of f. *japonica* with morphological combination SECT, and f. *longipes* (population J52). Cluster I contained highly heterogeneous morphological variations, i.e., populations with all of the combinations available (SECT, LDCT, LESD, and LEDS), f. *japonica*, f. *lanuginosa*, and f. *longipes* (see Table 1, Figure 4). Further, some subclusters seemingly existed, but no correlation was detected between subclusters and any morphological variations.

DISCUSSION

In general, it is accepted that in many cases the widespread species exhibit high level of genetic differentiation (Hamrick & Godt 1990). Beside highly genetic variations, *S. japonica* also has variability in morphological characters. Therefore, make confusion in describing infra-specific taxa. However, infra-specific taxa can be recognized and named if they exhibit clear delimitation from other taxa indicated by non overlapping discontinuity in one or more characters, and have a geographical basis (Brunell & Whitkus 1999). *Salvia japonica* had wide-range variations in morphological characters,

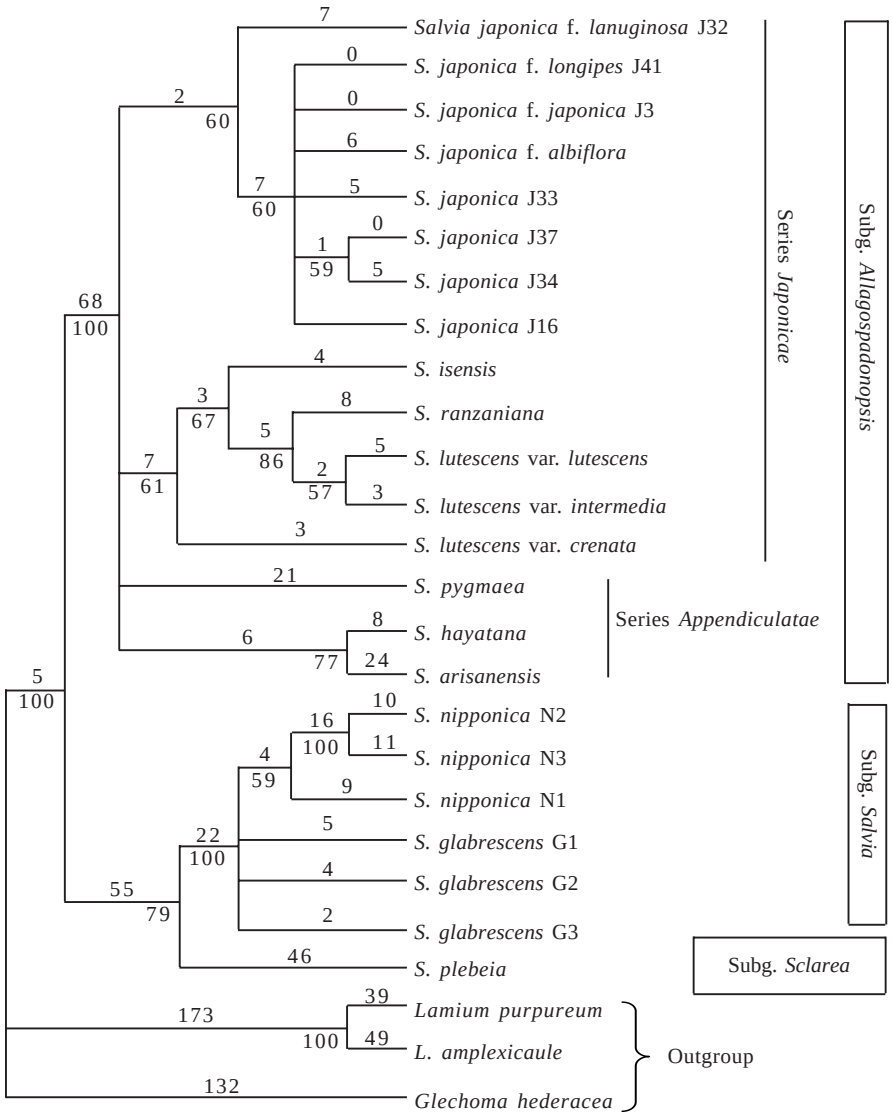

Figure 2. A strict consensus tree of 48 most parsimonious trees from combined analysis of *rbcL*, *trnL-F* and ITS DNA data set of 23 taxa of *Salvia*. Length 719, CI = 0.847, RI = 0.830. Bootstrap values are below nodes and branch lengths are above branches. Population numbers after the species names refer to Table 1.

Table 3. Genetic diversity indices and estimation gene flow for *S. japonica*, *S. lutescens*, and comparing with outcrossing and widespread species (Hamrick & Godt 1990). Standard error are in parentheses

Species	Genetic diversity indices				Gene flow *Nm*	References
	Hs	*Dst*	*Ht*	*Gst*		
S. japonica	0.261 (0.038)	0.147 (0.024)	0.408 (0.050)	0.372 (0.043)	0.486 (0.076)	Present study
S. lutescens	0.190 (0.059)	0.034 (0.017)	0.224 (0.062)	0.095 (0.036)	2.380 (1.890)	Present study
Outcrossing by animal	0.243 (0.010)		0.310 (0.010)	0.197 (0.017)	0.634	Hamrick and Godt (1990)
Widespread	0.267 (0.014)		0.347 (0.013)	0.210 (0.025)	0.149	Hamrick and Godt (1990)

Hs: the genetic diversity within populations; *Dst*: the genetic diversity among populations; *Ht*: the total genetic diversity; *Gst*: the coefficient of gene differentiation among populations; *Nm*: the gene flow estimate.

however, no one has attempted to examine its taxonomic significance from viewpoints of genetic relationships.

At the first, we tried to find the correlation between chromosomal variations and morphological ones. As the results, the peculiar satellite chromosome was found only in population Yamazaki, Hyogo Pref., it was not from any other populations. However, it had no relationships to any morphological variations. Then, we looked for the other points of view.

In general, it is accepted that ITS of nrDNA evidence is frequently useful for analysis of low taxonomic level (Mort & Crawford 2004), because the nuclear ribosomal DNA has topology of both parental species (e.g. Choi & Pak 1999). Therefore, we employed the ITS as well as cpDNA for analysing the relationships among *Salvia* species in Japan and understanding the situation of the variations observed in *S. japonica* (Sudarmono 2007).

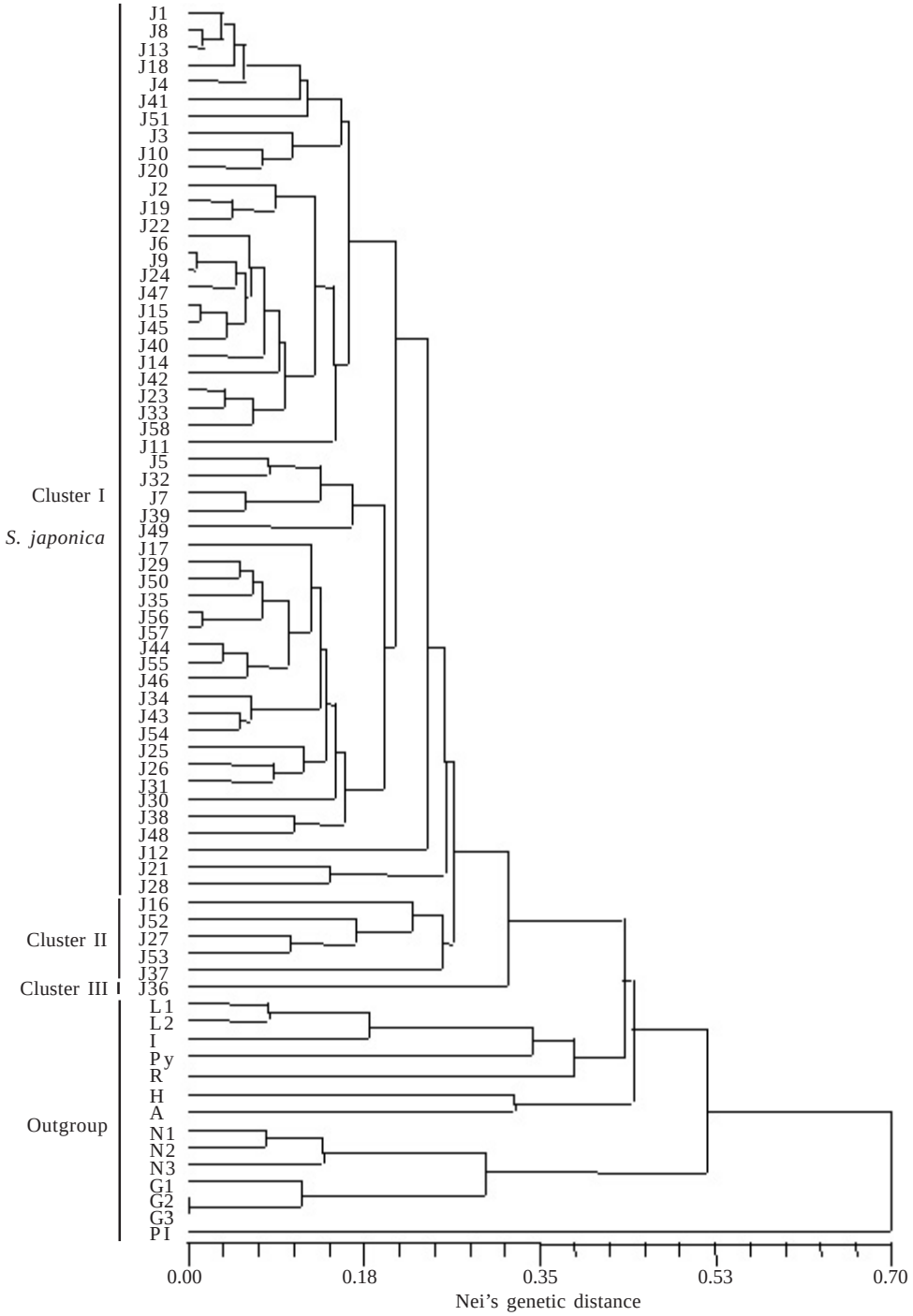

Figure 3. UPGMA dendrogram of allozyme divergence based on Nei's genetic distance among 72 populations of ten species of *Salvia*. Name of populations refer to Table 1. Cluster I of *S. japonica* are JI to J28 by column; cluster II are J16 to J37 by column, and cluster III is J36.

The results of cpDNA analysis as well as nrDNA suggested that at present, all of the *Salvia* species in Japan showed monophyly, although Walker *et al*. (2004) reported nonmonophyly of American species of *Salvia*. Further, the species belong to subg. *Allagospadonopsis*, namely, *S. japonica, S. isensis, S. lutescens, S. ranzaniana, S. pygmaea, S. hayatana*, and *S. arisanensis* were closely related to each other and formed one clade, and the other two subgenera, subg. *Salvia* and subg. *Sclarea*, formed the other clade. Relatively low bootstrap supports of DNA analyses in *S. japonica* were detected. *S. japonica* showed wide-range morphological variations in vegetative organs (Table 1) as well as characters of flower, which were recognized as intraspecific taxa, i.e., four forms (Murata 1952). We compared four combinations of morphological variations and four forms of *S. japonica* (Table 1) with genetic variations. As the results, these combinations and forms of *S. japonica* were not considered to be a taxonomic unit as indicated in the dendrogram (Figure 3), because they did not form any restricted clusters. Thus, various variations, i.e., morphological

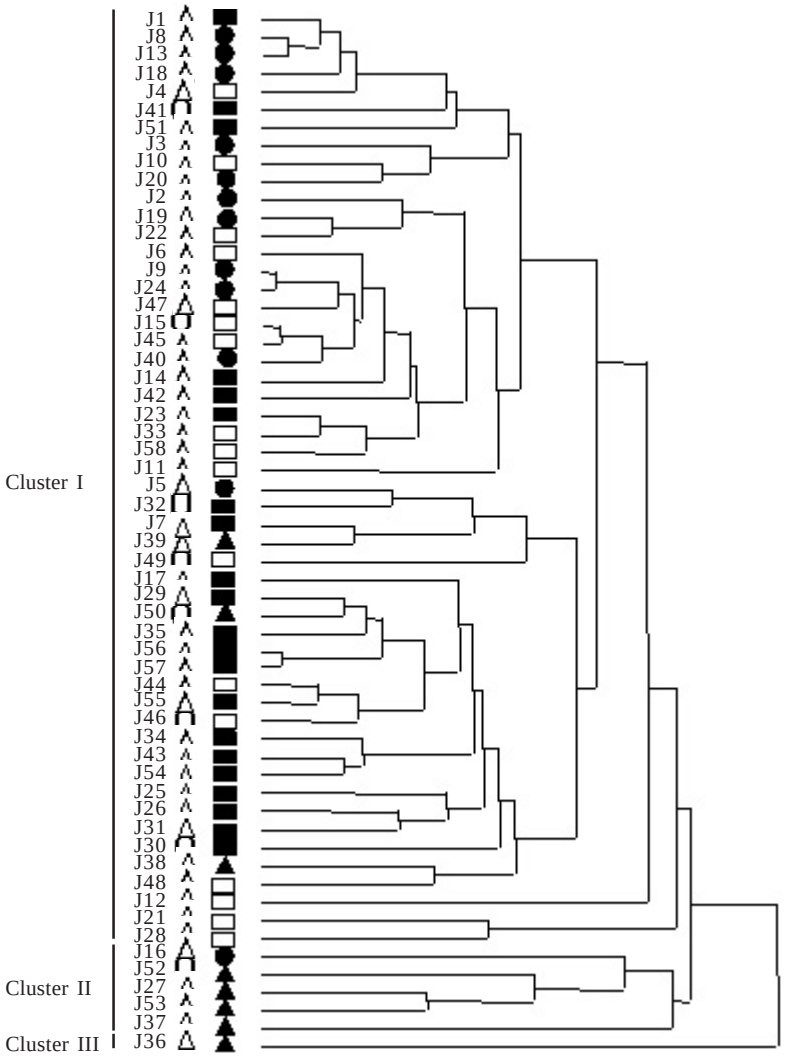

Figure 4. UPGMA dendrogram of allozyme divergence based on Nei's genetic distance between all investigated populations of *S. japonica*. Name of populations refer to Table 1. Populations of long internode, erect stem, dentate margin leaf, shallowly cuneate leaflet (LEDS) morphological characters are represented by filled box; populations of long internode, decumbent, crenate leaf, truncate leaflet (LDCT) morphological characters are represented by open box; populations of short internode, erect stem, crenate margin leaf, truncate leaflet (SECT) morphological characters are represented by filled circles; populations of long internode, erect stem, serrate margin leaf, deeply cuneate leaflet (LESD) morphological characters are represented by filled triangle; as well as *S. japonica* form *japonica* are represented by open triangle, form *longipes* are represented by O, and form *lanuginosa* are represented by U.

variations and genetic variations detected from DNA and allozymic analyses, observed in *S. japonica* were not evaluated as criteria to identified intraspecific taxonomic units. *S. japonica* populations might be still at the early stage of speciation process.

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