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Phylogenetic Relationship of *Hydrangea macrophylla* (Thunb.) Ser. and *H. serrata* (Thunb.) Ser. Evaluated Using RAPD Markers and Plastid DNA Sequences

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In Japan, there are many genetic resources for breeding hydrangea cultivars, but it is difficult to utilize them effectively for breeding because of a lack of phylogenetic information. In this study, the phylogenetic relationship of *H. macrophylla* (Thunb.) Ser. f. *normalis* (E.H.Wilson) H.Hara and *H. serrata* (Thunb.) Ser. was evaluated by using RAPD markers and sequences of the plastid genes *rbc*L and *mat*K. The materials were collected from their wild populations throughout Japan. Both RAPD analysis and chloroplast DNA analysis indicated that the genetic diversity of *H. serrata* var. *serrata* was higher than that of *H. macrophylla* f. *normalis* or that of *H. serrata* (Thunb.) Ser. var. *yesoensis* (Koidz.) H.Ohba. These analyses revealed that *H. serrata* var. *serrata* of Japan was separated into two groups; i.e., eastern *serrata* group and western *serrata* group. The western *serrata* group was divided into two or three subgroups by single base substitutions in the *mat*K or *rbc*L fragment sequences. The results of chloroplast DNA analysis indicated that *H. serrata* of Shikoku, which was one of the western *serrata* subgroups, was evolutionarily differentiated from other western *serrata* subgroups. *Mat*K and *rbc*L sequences of the eastern *serrata* group were identical to those of *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis*. The *mat*K sequences of the eastern *serrata* group, *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis*, contained a duplication of 6 bp (GGTTAT), which was not found in the western *serrata* group or other *Hydrangea* species. Analysis of the *mat*K and *rbc*L sequences revealed that *H. serrata* var. *serrata* is paraphyletic and that the eastern *serrata* group, *H. macrophylla* f. normalis and *H. serrata* var. *yesoensis*, form a monophyletic group. The present study provided useful information for breeding hydrangea cultivars and for the taxonomic treatment of *H. macrophylla* and *H. serrata* including the varieties.

Key Words: *Hydrangea macrophylla*, *Hydrangea serrata*, *mat*K, RAPD, *rbc*L.

Introduction

Many hydrangea cultivars are descended from *Hydrangea macrophylla* (Thunb.) Ser. and *H. serrata* (Thunb.) Ser. (synonym: *H. macrophylla* subsp. *serrata* (Thunb.) Makino) introduced from Japan and China to Europe since the eighteenth century. The genetic diversity of cultivated hydrangeas is very low, because most cultivars are derived from the limited numbers of plants that were introduced into Europe. Recently, new attempts using wild populations of *H. serrata* or *H. macrophylla* for breeding hydrangea cultivars have been conducted. Wild plants of *H. serrata* as well as *H. macrophylla* distributed in Japan are expected to be genetic resources for breeding a wide variety of hydrangea cultivars; however, information on their genetic diversity and phylogenetic relationship has not been accumulated.

H. serrata (Thunb.) Ser. taxonomically consists of two varieties, var. *serrata* and var. *yesoensis* (Koidz.) H.Ohba. (*H. macrophylla* (Thunb.) Ser. var. *megacarpa* Ohwi is regarded as the synonym). *H. serrata* var. *serrata* is distributed widely in mountainous regions of Honshu (southward from Fukushima Pref.), Shikoku, Kyusyu, and the Korean Peninsula. On the other hand, wild plants of *H. macrophylla* (*H. macrophylla* (Thunb.) Ser. f. *normalis* (E.H.Wilson) H.Hara), endemic to Japan, are localized in warm coastal areas including Boso

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Peninsula, Izu Peninsula, Miura Peninsula, the Izu Islands, and a part of the Bonin Islands (Kita- and Minami-Iwo-To). There are several differences in morphological characteristics between *H. macrophylla* and *H. serrata* var. *serrata*. Leaves of *H. macrophylla* are larger, thicker, and glossier than those of *H. serrata* var. *serrata*. There are many trichomes on the surface of the leaves of *H. serrata* var. *serrata* (Sato and Tanaka, 1989). However, in *H. macrophylla* there are few trichomes on the surface of the leaves. Flower clusters of *H. macrophylla* are larger than those of *H. serrata* var. *serrata*. In *H. macrophylla*, the inflorescence setting position is generally at the top of primary shoots. In *H. serrata*, however, there are several inflorescence setting positions, which are the primary shoot type, lateral shoot type, and primary and lateral shoot type (Matsuno et al., 2008).

H. serrata (Thunb.) var. *yesoensis* (Koidz.) H.Ohba is distributed in northern areas of Japan, including Hokkaido and the Tohoku and Hokuriku districts. In general, *H. serrata* var. *yesoensis* grows in mountains with heavy snow in winter. Morphological characteristics of *H. serrata* var. *yesoensis* are between *H. macrophylla* and *H. serrata* var. *serrata*. In *H. serrata* var. *yesoensis*, the leaves are not glossy but have many trichomes on their surface similar to *H. serrata* var. *serrata* (Sato and Tanaka, 1989). The sizes of leaves and flower clusters of *H. serrata* var. *yesoensis* are large and very similar to those of *H. macrophylla*.

The relationship between *H. macrophylla* and *H. serrata* is still vague. Wilson (1923), Haworth-Booth (1984), and Ohba (2001) separated these taxa at the species level, and differences in nuclear DNA contents between these taxa have supported this taxonomic treatment (Zonneveld, 2004). On the other hand, Makino (1929) and McClintock (1957) united these taxa into the same species, i.e., *H. macrophylla* ssp. *serrata* and *H. macrophylla* ssp. *macrophylla*. The results of SSR analyses supported their treatment as a single species, as did Makino and McClintock (Reed and Rinehart, 2007; Rinehart et al., 2006).

Most analyses of the phylogenetic relationship between *H. macrophylla* and *H. serrata* have been conducted using hydrangea cultivars as representative samples of *H. macrophylla*. However, it is unclear whether these cultivars are pure *H. macrophylla* or not because *H. serrata* was also included in the breeding materials introduced from Japan into Europe. Yamamoto (1979) considered that 'Rosea', which was one of the most important breeding materials introduced from Japan, corresponded to a Japanese local variety 'Himeajisai'. He pointed out that 'Himeajisai' was likely to be a natural hybrid between *H. macrophylla* and *H. serrata* (var. *yesoensis*, or var. *serrata*) having intermediate features of morphology and growth habits between these two.

It is important for the elucidation of the phylogenetic relationship between *H. macrophylla* and *H. serrata* that the materials used for analyses are free from crossing between these species. Moreover, samples of *H. serrata* var. *yesoensis* should be treated as a distinct entity from both *H. macrophylla* and *H. serrata* var. *serrata* in having intermediate morphological characteristics between *H. macrophylla* and *H. serrata* var. *serrata*.

Random amplified polymorphic DNA (RAPD) has been widely used to study genetic relationships in many plant species because this technique is simple, requires a small amount of DNA, does not require information on the DNA sequence and is economical (Williams et al., 1990). While RAPD markers are frequently used for phylogenetic analysis of samples within the same species, plastid gene sequences are used for phylogenetic analysis between species, genera or families. Sequence data of the plastid genes *rbc*L and *mat*K has contributed to elucidating the phylogenetic relationships of Hydrangeaceae species (Hufford et al., 2001).

The purpose of this research was to evaluate the phylogenetic relationships among *H. macrophylla* f. *normalis*, *H. serrata* var. *serrata* and var. *yesoensis*, and to provide useful information for breeding hydrangeas. In the present study, we analyzed the genetic diversity and relationships of *H. macrophylla* f. *normalis*, *H. serrata* var. *serrata* and var. *yesoensis* collected from wild populations throughout Japan by using RAPD markers and sequences of the plastid genes *rbc*L and *mat*K.

Materials and Methods

Plant materials

The taxon of each *Hydrangea* individual used in this study was assigned based on Ohba's classification system of the genus *Hydrangea* (Ohba, 2001). Seven *H. macrophylla* f. *normalis*, fifteen *H. serrata* var. *serrata*, four *H. serrata* var. *yesoensis*, and one *H. serrata* (Thunb.) Ser. var. *angustata* (Franch. et Sav.) H.Ohba were used for RAPD analysis (Table 1). Figure 1 shows the place where each individual grew as a wild plant. Only individuals whose geographic origins were clear were chosen for this study. One *H. hirta* (Thunb.) Siebold et Zucc. was used as the outgroup. For analyses of plastid sequences, three *H. macrophylla* f. *normalis*, three *H. serrata* var. *serrata*, five *H. serrata* var. *yesoensis*, one *H. luteovenosa* Koidz., and one *H. petiolaris* Siebold et Zucc. were added to the individuals used for RAPD analysis (Table 1; Fig. 1). The geographic origin of the three *H. serrata* var. *serrata* was the Republic of Korea.

RAPD analysis

Total DNA was extracted using the CTAB method (Doyle and Doyle, 1987). Thirteen random 10-mer primers (A01, A02, A04, A05, A07, A08, A09, A10, A11, A13, A17, A18, A20; Operon Technologies, Inc., Alameda, CA, USA) were used for PCR amplification (Table 2). PCR amplification reactions with primers A01, A02, A04, A05, A07, A08, A10, A20 were car-

Table 1. List of *Hydrangea* plants evaluated by RAPD analysis and plastid sequence analyses.

Individual number	Taxon	Cultivar name	Original habitat[z]	Color of the petaloid sepal	Voucher	Accession number[y] *mat*K/*rbc*L	RAPD analysis	Plastid sequence analyses
M1	*H. macrophylla* f. *normalis*	—	Kita-Iwo-To, Bonin Islands	white	TI-Uemachi-M1	AB754549/AB755637	○	○
M2	*H. macrophylla* f. *normalis*	—	Nii-jima, Izu Islands	white	TI-Uemachi-M2	AB754550/AB755638	○	○
M3	*H. macrophylla* f. *normalis*	Hatsushimo	Oshima, Izu Islands	anthocyan color	TI-Uemachi-M3	AB754551/AB755639	○	○
M4	*H. macrophylla* f. *normalis*	Miyaketokiwa	Miyake-jima, Izu Islands	white	TI-Uemachi-M4	AB754552/AB755640	○	○
M5	*H. macrophylla* f. *normalis*	—	Izu Peninsula, Shimoda, Shizuoka	anthocyan color	TI-Uemachi-M5	AB754553/AB755641	○	○
M6	*H. macrophylla* f. *normalis*	Fuirijogasaki	Izu Peninsula, Ito, Shizuoka	anthocyan color	TI-Uemachi-M6	AB754554/AB755642	○	○
M7	*H. macrophylla* f. *normalis*	—	Boso Peninsula, Chiba	anthocyan color	TI-Uemachi-M7	AB754555/AB755643	○	○
M8	*H. macrophylla* f. *normalis*	—	Miura Peninsula, Miura, Kanagawa	anthocyan color	TI-Uemachi-M8	AB754556/AB755644	—	○
M9	*H. macrophylla* f. *normalis*	—	Boso Peninsula, Futtsu, Chiba	anthocyan color	TI-Uemachi-M9	AB754557/AB755645	—	○
M10	*H. macrophylla* f. *normalis*	—	Boso Peninsula, Tateyama, Chiba	anthocyan color	TI-Uemachi-M10	AB754558/AB755646	—	○
Y1	*H. serrata* var. *yesoensis*	Temariezo	Showa, Fukushima	anthocyan color	TI-Uemachi-Y1	AB754559/AB755647	○	○
Y2	*H. serrata* var. *yesoensis*	Sahashinosyo	Tokamachi, Niigata	anthocyan color	TI-Uemachi-Y2	AB754560/AB755648	○	○
Y3	*H. serrata* var. *yesoensis*	—	Otsuchi, Iwate	anthocyan color	TI-Uemachi-Y3	AB754561/AB755649	○	○
Y4	*H. serrata* var. *yesoensis*	—	Yuwa, Akita	anthocyan color	TI-Uemachi-Y4	AB754562/AB755650	○	○
Y5	*H. serrata* var. *yesoensis*	—	Sendai, Miyagi	anthocyan color	TI-Uemachi-Y5	AB754563/AB755651	—	○
Y6	*H. serrata* var. *yesoensis*	—	Nishikawa, Yamagata	anthocyan color	TI-Uemachi-Y6	AB754564/AB755652	—	○
Y7	*H. serrata* var. *yesoensis*	—	Kitakami, Iwate	anthocyan color	TI-Uemachi-Y7	AB754565/AB755653	—	○
Y8	*H. serrata* var. *yesoensis*	—	Ugo, Akita	anthocyan color	TI-Uemachi-Y8	AB754566/AB755654	—	○
Y9	*H. serrata* var. *yesoensis*	—	Towada, Aomori	anthocyan color	TI-Uemachi-Y9	AB754567/AB755655	—	○
S1	*H. serrata* var. *serrata*	kuju	Mt. Kuju, Oita	anthocyan color	TI-Uemachi-S1	AB754568/AB755656	○	○
S2	*H. serrata* var. *serrata*	hyugakonjo	Mt. Shiba, Miyazaki	anthocyan color	TI-Uemachi-S2	AB754569/AB755657	○	○
S3	*H. serrata* var. *serrata*	Higoshibori	Kumamoto	anthocyan color	TI-Uemachi-S3	AB754570/AB755658	○	○
S4	*H. serrata* var. *serrata*	Iyatemari	Miyoshi, Tokushima	anthocyan color	TI-Uemachi-S4	AB754571/AB755659	○	○
S5	*H. serrata* var. *serrata*	Tsuruginomai	Kami, Kochi	anthocyan color	TI-Uemachi-S5	AB754572/AB755660	○	○
S6	*H. serrata* var. *serrata*	Oniji	Saijo, Ehime	anthocyan color	TI-Uemachi-S6	AB754573/AB755661	○	○
S7	*H. serrata* var. *serrata*	Mihaginishiki	Hagi, Yamaguchi	anthocyan color	TI-Uemachi-S7	AB754574/AB755662	○	○
S8	*H. serrata* var. *serrata*	Komochishichidanka	Masuda, Shimane	anthocyan color	TI-Uemachi-S8	AB754575/AB755663	○	○
S9	*H. serrata* var. *serrata*	Shichidanka	Mt. Rokko, Hyogo	anthocyan color	TI-Uemachi-S9	AB754576/AB755664	○	○
S10	*H. serrata* var. *serrata*	—	Higashiomi, Shiga	anthocyan color	TI-Uemachi-S10	AB754577/AB755665	○	○
S11	*H. serrata* var. *serrata*	—	Minamiechizen, Fukui	anthocyan color	TI-Uemachi-S11	AB754578/AB755666	○	○
S12	*H. serrata* var. *serrata*	—	Shirakawa, Gifu	white	TI-Uemachi-S12	AB754579/AB755667	○	○
S13	*H. serrata* var. *serrata*	Kurenai	Iida, Nagano	anthocyan color[x]	TI-Uemachi-S13	AB754580/AB755668	○	○
S14	*H. serrata* var. *angustata*	Amagiamacha	Izu Peninsula, Shizuoka	white	TI-Uemachi-S14	AB754581/AB755669	○	○
S15	*H. serrata* var. *serrata*	Fujinoshirayuki	Mt. Fuji	white	TI-Uemachi-S15	AB754582/AB755670	○	○
S16	*H. serrata* var. *serrata*	Kiyosumisawa	Boso Peninsula, Kamogawa, Chiba	white[w]	TI-Uemachi-S16	AB754583/AB755671	○	○
K1	*H. serrata* var. *serrata*	Kaikyo	Jeju Island, Republic of Korea	anthocyan color	TI-Uemachi-K1	AB754584/AB755672	—	○
K2	*H. serrata* var. *serrata*	KankokusanYamaajisai	Republic of Korea	anthocyan color	TI-Uemachi-K2	AB754585/AB755673	—	○
K3	*H. serrata* var. *serrata*	—	Seoul, Republic of Korea	anthocyan color	TI-Uemachi-K3	AB754586/AB755674	—	○
H1	*H. hirta*	—	Otsu, Shiga	—	TI-Uemachi-H1	AB754587/AB755675	○	○
L1	*H. luteovenosa*	Mangetsu	Ehime	white	TI-Uemachi-L1	AB754588/AB755676	—	○
P1	*H. petiolaris*	—	Kyoto, Kyoto	white	TI-Uemachi-P1	AB754589/AB755677	—	○

[z] The place where the sample was growing as a wild plant.
[y] Accession number for sequence data at DDBJ.
[x] The color chages from white to red with age.
[w] White sepals with red margins.

ried out in a total reaction volume of 25 μL containing 1 × Taq buffer, 15 ng genomic DNA, 0.2 mM dNTPs, 12.5 pmol primers, and 1.25 units AmpliTaq DNA polymerase Stoffel fragment (PerkinElmer, Waltham, MA, USA). DNA fragments were amplified by repeating 45 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min using a GeneAmp PCR system 2400 thermal cycler (PerkinElmer). PCR amplification reactions with primers A09, A11, A13, A17, A18 were carried out in a total reaction volume of 10 μL containing 1 × Taq buffer, 10 ng genomic DNA, 0.25 mM dNTPs, 5 pmol primers, and 0.5 units Blend Taq-Plus DNA polymerase (Toyobo, Osaka, Japan). DNA fragments were amplified by repeating 45 cycles of 94°C for 30 sec, 36°C for 30 sec, and 72°C for 1.5 min using a MJ Mini thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was conducted for 2.5 h at 100 V, using a 2% (w/v) agarose gel in Tris-borate-EDTA (TBE) buffer with 10 μL PCR product from each sample. Staining was performed with ethidium bromide.

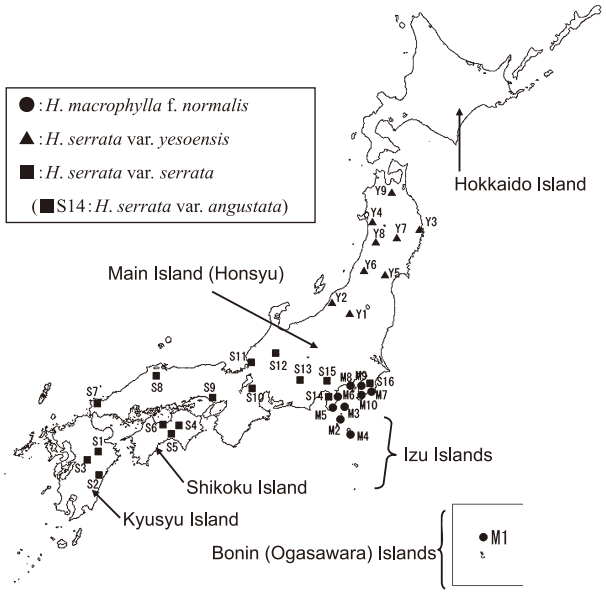

Fig. 1. The place where the sample grew as a wild plant.

The presence or absence of bands in the stained gels was used to calculate genetic similarities. Genetic similarity was calculated as $Sij = 2\ Nij/(Ni + Nj)$, where Nij is the number of markers shared by individuals i and j, Ni is the number of markers found in individual i, and Nj is the number of markers found in individual j (Dice, 1945). The similarity matrix was subjected to clustering analysis using UPGMA in the program SPSS for Windows, version 15.0 (SPSS, Chicago, IL, USA). Estimates of statistical support for the resulting clusters were obtained from UPGMA bootstrap analysis with 1000 replicates using the program PHYLIP 3.67 (Felsenstein, 2007).

Analysis of rbcL and matK sequences

Total DNA of additional individuals for analysis of plastid gene sequences was extracted using a MagExtractor TM-Plant Genome-Kit (Toyobo, Osaka, Japan). The primers used to amplify the fragments of *rbc*L and *mat*K were designed according to highly conserved regions within *H. macrophylla* (GenBank accession no. AB236030 for *mat*K, L11187 for *rbc*L),

Table 2. Sequences of primers for amplification of RAPD markers and cpDNA fragments.

Application	Primer	Sequence
RAPD analysis	OPA-01	5′-CAGGCCCTTC-3′
	OPA-02	5′-TGCCGAGCTG-3′
	OPA-04	5′-AATCGGGCTG-3′
	OPA-05	5′-AGGGGTCTTG-3′
	OPA-07	5′-GAAACGGGTG-3′
	OPA-08	5′-GTGACGTAGG-3′
	OPA-09	5′-GGGTAACGCC-3′
	OPA-10	5′-GTGATCGCAG-3′
	OPA-11	5′-CAATCGCCGT-3′
	OPA-13	5′-CAGCACCCAC-3′
	OPA-17	5′-GACCGCTTGT-3′
	OPA-18	5′-AGGTGACCGT-3′
	OPA-20	5′-GTTGCGATCC-3′
Amplification of *rbc*L	HmrbcL-F1	5′-AAGCTGGTGTTAAAGATTACAAATTGACT-3′
	HmrbcL-R1	5′-AAATTTGATTTCCTTCCATACCTCAC-3′
Amplification of *mat*K	HmmatK-F1	5′-TCGAACTAGATAGATATCATCAACACGAC-3′
	HpmatK-R1	5′-TCGGGATAATTTACATTTACACGGTCTC-3′
Sequencing reaction	rbcL-F-seq1	5′-AAGCTGGTGTTAAAGATT-3′
	rbcL-R-seq1	5′-AAATTTGATTTCCTTCC-3′
	matK-F-seq1	5′-TCGAACTAGATAGATATCATCA-3′
	pmatK-R-seq1	5′-TCGGGATAATTTACATTT-3′

H. anomala (GenBank accession no. AF323202 for *rbc*L), *H. quercifolia* (GenBank accession no. AF323203 for *rbc*L), *H. aspera* (GenBank accession no. AJ429277 for *mat*K), and *H. paniculata* (GenBank accession no. AB236029 for *mat*K). The primer sequences are presented in Table 2. PCR amplification reactions were carried out in a total reaction volume 50 μL containing 1 × KOD plus buffer, 20 ng genomic DNA, 0.2 mM dNTPs, 20 pmol primers, and 1.0 unit KOD plus polymerase (Toyobo). DNA fragments were amplified by repeating 35 cycles of 94°C for 15 sec, 58°C for 30 sec, and 68°C for 2.0 min using a MJ Mini thermal cycler (Bio-Rad Laboratories). PCR products were purified using a PCR-M Clean Up System (Viogene, Taipei, Taiwan) in order to remove excess primers and dNTPs after amplification. Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Sequencing reactions were carried out with the primers shown in Table 2 by repeating 25 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. The sequencing reaction products were purified through spin columns and then applied to a 3130xl Genetic Analyzer (Applied Biosystems).

Multiple alignments of the DNA sequences were obtained using the CLUSTAL W program implemented in MEGA5 (Tamura et al., 2011). Two indels were found in *mat*K alignments and they were excluded from the data for constructing the phylogenetic tree. The multiple alignments were used to construct maximum parsimony trees with 500 bootstraps in MEGA5.

Results

RAPD analysis

A total of 426 RAPD markers were generated with 13 primers in all individuals and 413 markers were polymorphic. Based on the UPGMA dendrogram, the individuals except for *H. hirta* were separated into two major groups with a similarity value of 0.56 (Fig. 2). One group consisted of *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis* individuals with high bootstrap support (95%). The other group consisted of *H. serrata* var. *serrata* individuals and *H. serrata* var. *angustata*. *H. macrophylla* f. *normalis* was separated into a group of Honshu individuals (M5, M6, M7) and a group of Izu and Bonin Islands individuals (M1, M2, M3, M4) with a similarity value of 0.68. On the other hand, *H. serrata* var. *serrata* was divided into groups from the eastern (S12, S13, S15, S16) and western (S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11) areas of Japan with a similarity value of 0.57. The *H. serrata* var. *angustata* individual (S14) was assigned as a member of the eastern group of *H. serrata* var. *serrata* (tentatively called the eastern *serrata* group). Furthermore, the western group of *H. serrata* var. *serrata* (western *serrata* group) was divided into a subgroup of Honshu individuals (S7, S8, S9, S10, S11) and the subgroup of Shikoku and Kyushu individuals (S1, S2, S3, S4, S5, S6) with a similarity value of 0.59. The Shikoku individuals (S4, S5, S6) were separated from Kyusyu individuals (S1, S2, S3) within the subgroup. UPGMA dendrogram suggested that *H. macrophylla* and *H. serrata* var. *yesoensis* were genetically similar, and that the genetic diversity of *H. serrata* var. *serrata* was greater than that of *H. macrophylla* f. *normalis* or *H. serrata* var. *yesoensis*.

Analysis of matK sequences

The *mat*K gene in *H. macrophylla* is 1521 bp long (AB236030, Setoguchi et al., 2006). Sequences of partial *mat*K fragments (1421–1427 bp) used for alignments in this study covered about 93–94% of the *mat*K gene. The aligned *mat*K matrix using all individuals, including *H. hirta*, *H. luteovenosa*, and *H. petiolaris*, consisted of 1431 bp, of which 32 positions were variable. Two indels

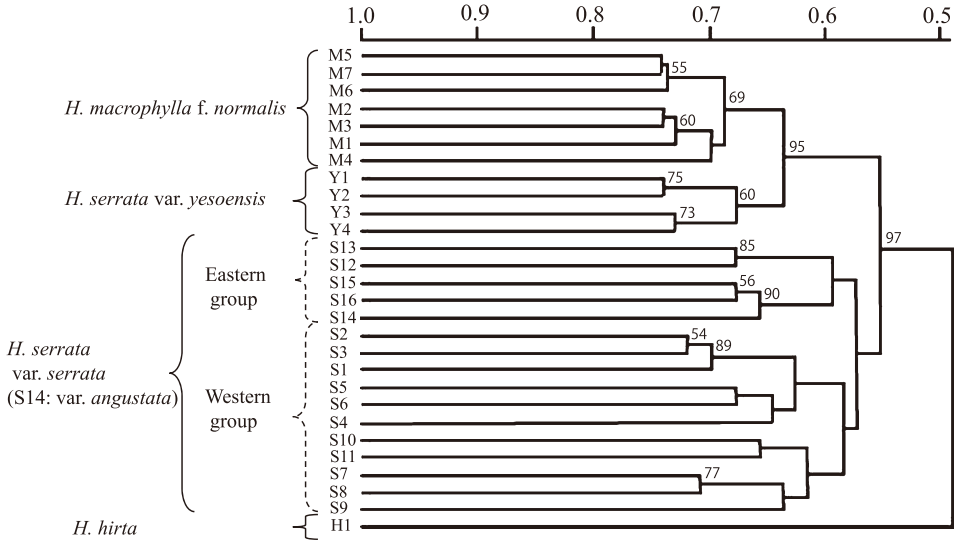

Fig. 2. UPGMA dendrogram based on Dice similarities calculated from RAPD data on 27 individuals of *H. macrophylla* and *H. serrata*. One individual of *H. hirta* was used as an outgroup. Bootstrap values (%) from 1000 replicates are indicated above the branches when over 50%.

Table 3. Substitution and duplication events observed in the aligned *mat*K fragment sequences (1431 bp) of *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis, H. serrata* var. *serrata*, and *H. serrata* var. *angustata*.

Informative position	Sequence region	Individual
336	AGAGGA**A**AAATTC	All individuals of *H. macrophylla* f. *normalis* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1–Y9), 5 individuals of *H. serrata* var. *serrata* (S10, S12–S13, S15–S16, The group of eastern area of Japan), *H. serrata* var. *angustata* (S14)
	AGAGGA**C**AAATTC	10 individuals of *H. serrata* var. *serrata* (S1–S9, S11, The group of western area of Japan), 3 Korean individuals of *H. serrata* var. *serrata* (K1–K3)
764	ATCAAG**G**AAAATC	All individuals of *H. macrophylla* f. *normalis* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1-Y9), 13 individuals of *H. serrata* var. *serrata* (S1–S3, S7–S13, S15–S16, Japanase group except for Shikoku Island), *H. serrata* var. *angustata* (S14), 3 Korean individuals of *H. serrata* var. *serrata* (K1–K3)
	ATCAAG**T**AAAATC	3 individuals of *H. serrata* var. *serrata* (S4–S6, Shikoku Island group)
1379–1384	GGTTAT**GGTTAT** (6 bp replication)	All individuals of *H. macrophylla* f. *normalis* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1–Y9), 5 individuals of *H. serrata* var. *serrata* (S10, S12–S13, S15–S16, The group of eastern area of Japan), *H. serrata* var. *angustata* (S14),
	GGTTAT------ (no replication)	10 individuals of *H. serrata* var. *serrata* (S1–S9, S11, The group of western area of Japan) 3 Korean individuals of *H. serrata* var. *serrata* (K1–K3)

were found in the matK alignments. One indel was a 4 bp insertion specific to *H. petiolaris*. The other indel was a 6 bp (GGTTAT) insertion. Two single base substitutions and a duplication of 6 bp (GGTTAT) were found among individuals of *H. macrophylla* f. *normalis* and *H. serrata* (Table 3). There were no differences in the *mat*K fragment sequences between *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis*. *H. serrata* var. *serrata* and *H. serrata* var. *angustata* were divided into two groups according to a single base substitution and a duplication of 6 bp (GGTTAT). One *serrata* group consisted of individuals (S10, S12, S13, S14, S15, S16) from the eastern area of Japan. The *mat*K fragment sequence of this group was identical to those of *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis* and contained a duplication of 6 bp (GGTTAT). The other *serrata* group consisted of individuals (S1, S2, S3, S4, S5, S6, S7, S8, S9, S11, K1, K2, K3) from the western area of Japan and Korea. Neither this *serrata* group nor the outgroup including *H. hirta*, *H. luteovenosa*, and *H. petiolaris* contained the 6 bp duplication in their *mat*K sequences. In the maximum parsimony tree based on sequences of *mat*K, *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis*, and the eastern *serrata* group were clustered together (Fig. 3). The tree indicated that *H. serrata* var. *serrata* was paraphyletic. The S10 individual was assigned as a member of the eastern *serrata* group according to analysis of *mat*K sequences, despite the fact that it belonged to the western *serrata* group according to RAPD analysis. The *H. serrata* var. *angustata* individual (S14) was assigned as a member of the eastern *serrata* group. The Shikoku group (S4, S5, S6) was discriminated from the western *serrata* group by the substitution of a nucleotide. The maximum parsimony tree indicated that the genetic diversity of *H. serrata* var. *serrata* was greater than that of *H. macrophylla* f. *normalis* or *H. serrata* var. *yesoensis*.

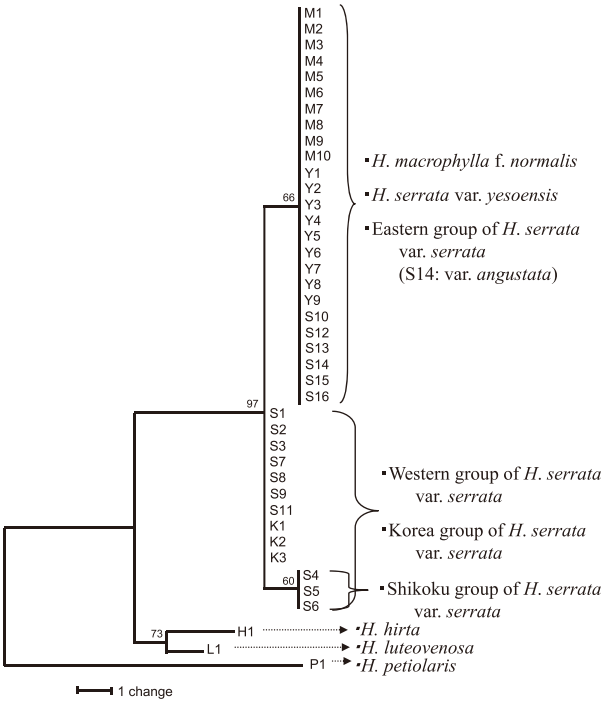

Fig. 3. The maximum parsimony tree based on *mat*K fragment sequences of 38 individuals of *H. macrophylla* and *H. serrata*. *H. hirta*, *H. luteovenosa*, and *H. petiolaris* individuals were used as the outgroup. A tree out of the 10 most parsimonious trees is shown. Tree length = 23, CI = 1.00, RI = 1.00. Bootstrap values (%) from 500 replicates are indicated above the branches when over 50%.

Analysis of rbcL sequences

The aligned *rbc*L matrix consisted of 1257 bp, of which 24 positions were variable. Four events of single base substitutions were found in the *rbc*L fragment sequences among individuals of *H. serrata* var. *serrata* (Table 4). On the other hand, there were no differences in the *rbc*L fragment sequences among individuals of *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis*.

Table 4. Substitution events observed in the aligned *rbc*L fragment sequences (1257 bp) of *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis*, *H. serrata* var. *serrata*, and *H. serrata* var. *angustata*.

Informative position	Sequence region	Individual
45	TCAACC**T**GGAGTT	All individuals of *H. macrophylla* f. *normalis* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1–Y9), 13 individuals of *H. serrata* var. *serrata* (S1–S7, S10–S13, S15–S16), *H. serrata* var. *angustata* (S14), 2 Korean individuals of *H. serrata* var. *serrata* (K1, K2)
	TCAACC**C**GGAGTT	2 individuals of *H. serrata* var. *serrata* (S8, S9), 1 Korean individual of *H. serrata* var. *serrata* (K3)
670	AGGGCT**G**TATGTG	All individuals of *H. macrophylla* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1–Y9), 12 individuals of *H. serrata* var. *serrata* (S1–S3, S7–S13, S15–16, Japanese group except for Shikoku Island), *H. serrata* var. *angustata* (S14), 3 Korean individuals of *H. serrata* var. *serrata* (K1–K3)
	AGGGCT**A**TATTTG	3 individuals of *H. serrata* var. *serrata* (S4–S6, Shikoku Island group)
674	CTGTAT**G**TGCCAG	All individuals of *H. macrophylla* f. *normalis* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1–Y9), 12 individuals of *H. serrata* var. *serrata* (S1–S3, S7–S13, S15–S16, Japanese group except for Shikoku Island), *H. serrata* var. *angustata* (S14), 3 Korean individuals of *H. serrata* var. *serrata* (K1–K3)
	CTATAT**T**TGCCAG	3 individuals of *H. serrata* var. *serrata* (S4–S6, Shikoku Island group)
966	CGATTT**G**ATTGAA	All individuals of *H. macrophylla* f. *normalis* (M1–M10), All individuals of *H. serrata* var. *yesoensis* (Y1–Y9), 5 individuals of *H. serrata* var. *serrata* (S10, S12–S13, S15–S16, The group of eastern area of Japan), *H. serrata* var. *angustata* (S14)
	CGATTT**T**ATTGAA	10 individuals of *H. serrata* var. *serrata* (S1–S9, S11, The group of western area of Japan), 3 Korean individuals of *H. serrata* var. *serrata* (K1–K3)

The topology of the maximum parsimony tree based on sequences of *rbc*L was almost identical to that of *mat*K, except for a few minor differences in the grouping of *H. serrata* var. *serrata* individuals (Fig. 4). The tree indicated that *H. serrata* var. *serrata* was paraphyletic. *H. serrata* var. *serrata* was divided into the eastern Japan group, Shikoku group, and western Japan and Korea group, which was subdivided into two subgroups. *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis* and the eastern *serrata* group were united into a single cluster. The S10 individual was assigned as a member of the eastern *serrata* group, which was in accordance with the results of *mat*K sequence analysis. The *H. serrata* var. *angustata* individual (S14) was assigned as a member of the eastern *serrata* group.

Discussion

H. serrata var. *serrata* is geographically distributed widely in Japan and also in the Korean Peninsula, whereas *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis* are endemic to Japan. In this study, we conducted phylogenetic analysis of *H. macrophylla* f. *normalis*, *H. serrata* var. *serrata*, and *H. serrata* var. *yesoensis* using individuals derived from wild populations in Japan for the purpose of providing useful information for breeding hydrangeas.

Both RAPD analysis and chloroplast DNA analysis indicated that the genetic diversity of *H. serrata* var. *serrata* was higher than that of *H. macrophylla* f. *normalis* or that of *H. serrata* var. *yesoensis*. The high genetic diversity of *H. serrata* var. *serrata* can be attributed to its wide-ranging geographical distribution, including Japan and the Korea Peninsula. Results of RAPD analysis indicated that *H. serrata* var. *serrata* of Japan was separated into two groups; i.e., eastern and western *serrata* groups (Fig. 2). This result was supported by phylogenetic analysis using chloroplast DNA

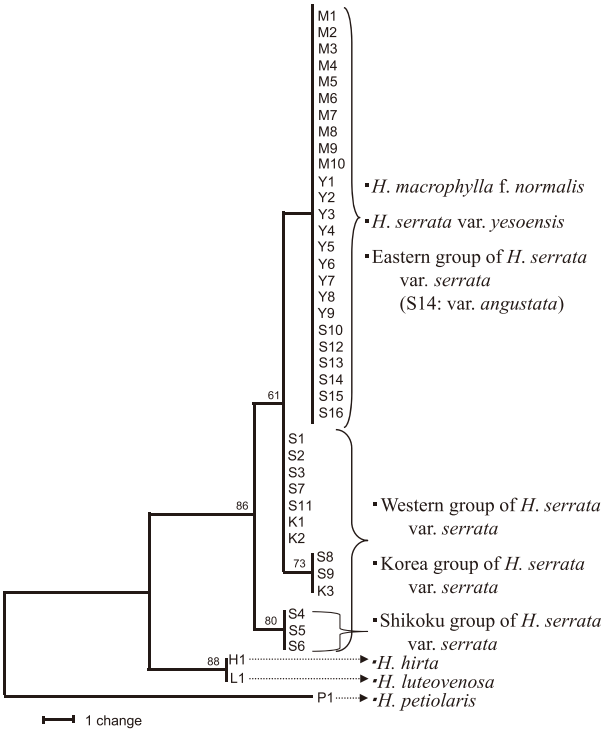

Fig. 4. The maximum parsimony tree based on *rbc*L fragment sequences of 38 individuals of *H. macrophylla* and *H. serrata*. *H. hirta*, *H. luteovenosa*, and *H. petiolaris* individuals were used as the outgroup. The most parsimonious was obtained and is shown. Tree length = 26, CI = 0.91, RI = 0.97. Bootstrap values (%) from 500 replicates are indicated above the branches when over 50%.

sequences (Figs. 3 and 4). The grouping of *H. serrata* var. *serrata* in this study was consistent with the geographical distribution of flower color. White flowers are predominant on the Pacific Ocean side of the eastern area of Japan, including Tokai district and Mt. Fuji. Plants S12, S14, S15, assigned as members of the eastern *serrata* group in this study, have white flowers. On the other hand, flowers with an anthocyan color are found in the area west of the Kinki district. All *serrata* individuals assigned as members of the western group have flowers with an anthocyan color.

In this study, the *serrata* individuals derived from the area west of the Suzuka Mountains were assigned as members of the western group, whereas *serrata* individuals derived from the area east of the Suzuka Mountains were assigned as members of the eastern group. Furthermore, individual S10, which was sampled from a wild population in Suzuka Mountains, was assigned as a member of the western *serrata* group by RAPD analysis, whereas it was included in the eastern *serrata* group in the analysis using chloroplast DNA sequences (Figs. 2, 3, and 4). These results indicate that the Suzuka Mountains are one of the borders between the eastern and western *serrata* groups.

Results of chloroplast DNA analysis indicated that there was more genetic diversity within the western *serrata* group than within the eastern *serrata* group (Figs. 3 and 4). No differences were found in DNA sequences of either *mat*K fragments or *rbc*L fragments within the eastern *serrata* group, including the variety *Angustata* individual (S14). On the other hand, the western *serrata* group was divided into two or three subgroups by single base substitutions in the *mat*K or *rbc*L fragment sequences. Individuals S4, S5, S6, whose geographical origins were Shikoku Island, were distinguished from other members of the western *serrata* group on analysis of both *mat*K and *rbc*L. This result suggests that *H. serrata* var. *serrata* of Shikoku is evolutionarily differentiated from other western *serrata*. The *mat*K sequences of the western *serrata* group except for individuals of Shikoku were the same as those of Korean *serrata*. Furthermore, a single base substitution in *rbc*L sequences within the western *serrata* group, except for the Shikoku individuals, was found within the Korean *serrata* group (Table 4). These results indicate that the Korean *serrata* group and the western *serrata* group, except for the Shikoku samples, form a monophyletic group and that the single base substitution in the *rbc*L sequence occurred in a common ancestor.

Both phylogenetic trees based on RAPD markers and chloroplast DNA sequences suggest that *H. macrophylla* f. *normalis* as well as *H. serrata* var. *yesoensis* is monophyletic. On the other hand, phylogenetic trees based on the *mat*K and *rbc*L sequences indicate that *H. serrata* var. *serrata* is paraphyletic. Although RAPD analysis demonstrated that *H. serrata* var. *serrata* was monophyletic, the bootstrap value was low (27.5%). *Mat*K and *rbc*L sequences of the eastern *serrata* group were identical to those of *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis*. Both phylogenetic trees based on *mat*K and *rbc*L sequences indicated that *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis*, and the eastern *serrata* group form a monophyletic group, and that these taxa are descended from a common ancestor. This is supported by the geographic distribution of these taxa and the 6 bp duplication in the *mat*K sequence. *H. serrata* var. *serrata* is distributed more widely than *H. macrophylla* f. *normalis* and *H. serrata* var. *yesoensis*, and the distribution areas of *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis*, and the eastern *serrata* groups neighbor each other. This pattern of distribution supports the hypothesis that *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis*, and the eastern *serrata* group were derived from a common ancestor, which was *H. serrata* var. *serrata*. Moreover, this hypothesis is supported by the *mat*K sequences specific to these taxa. The *mat*K sequences of these taxa contained a duplication of 6 bp (GGTTAT) (Table 3). On the other hand, neither the *serrata* individuals, except for the eastern *serrata* group, nor the outgroup including *H. hirta*, *H. luteovenosa* and *H. petiolaris* contained the 6 bp duplication in their *mat*K sequences. In addition, other *Hydrangea* species in the databases (*H. heteromalla*, GU217275; *H. paniculata*, GU217276; *H. quercifolia*, GU217277; *H. arborescens*, GU217285; *H. involucrata*, GU217290; *H. sikokiana*, GU217291; *H. longipes*, GU217292; *H. sargentiana*, GU217293; *H. glabripes*, GU217294; *H. aspera*, GU217295; *H. serratifolia*, GU217300; *H. integrifolia*, GU217302; *H. seemannii*, GU217303; *H. anomala*, GU217304; *H. indochinensi*, GU217312; *H. scandens*, GU217328; *H. lobbii*, GU217330; *H. chungii*, GU217332; *H. angustipetala*, GU217336) did not contain the 6 bp duplication in their *mat*K sequences. The 6 bp duplication seems to be specific to *H. macrophylla* f. *normalis*, *H. serrata* var. *yesoensis*, and the eastern *serrata* group, so the duplication event probably occurred in their common ancestor.

There are two distinct interpretations of the taxonomic positions of *H. macrophylla* and *H. serrata*. One interpretation is that these taxa should be treated as different species (Haworth-Booth, 1984; Ohba, 2001; Wilson, 1923; Zonneveld, 2004). The other is that *H. serrata* var. *serrata* should be placed as a subspecies of *macrophylla* (Makino, 1929; McClintock, 1957; Reed and Rinehart, 2007; Rinehart et al., 2006). The results of this study revealed that *H. serrata* var. *serrata* consists of two groups; one group is a near relative of *H. macrophylla* and the other group is obviously different from *H. macrophylla*. Therefore, the interpretations of the taxonomic relationship between the *H. macrophylla* and *H. serrata* are possibly affected by the *serrata* group used for analysis if the *serrata* samples are restricted to only one *serrata* group. Furthermore, it is important for the elucidation of the phylogenetic relationships between

H. macrophylla and *H. serrata* that hydrangea cultivars are excluded from *macrophylla* samples, because it is unclear for most hydrangea cultivars whether they are absolutely free from crossing with *H. serrata*.

The present study provided valuable information for the taxonomic treatment of *H. macrophylla* and *H. serrata*, including the varieties. Analysis of the *mat*K and *rbc*L sequences revealed that *H. serrata* var. *serrata* was paraphyletic and that the eastern *serrata* group, *H. macrophylla* f. *normalis*, and *H. serrata* var. *yesoensis* formed a monophyletic group. However, these taxa have clearly distinct morphological and physiological characteristics and specific distribution areas, so it is reasonable that *H. macrophylla* and *H. serrata* should be treated as different species. Two concepts for the present *H. serrata* var. *yesoensis* are reliable; to treat it as a distinct species or as an infrataxa of *H. macrophylla* or *H. serrata*. Until further evidence for elucidation is obtained, its present treatment as a variety of *H. serrata* should be tentatively maintained because of their morphological similarities. This study indicates that *H. serrata* var. *serrata* is genetically polymorphic; however, we have not yet found morphological features except for flower color corresponding to a genetic separation. Further morphological and geographical studies as well as analysis of sequence data from more informative regions of the genome are needed for taxonomic evaluation of its infraspecific separation.

There are abundant genetic resources for hydrangea breeding in Japan. Effective utilization of these genetic resources for breeding hydrangeas could produce novel cultivars with excellent properties (e.g., ornamental value, resistance to environmental stress), and the results obtained in this study will provide useful information for breeding hydrangeas.

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