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Chloroplast DNA phylogeography of *Pedicularis* ser. *Gloriosae* (Orobanchaceae) in Japan

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Abstract Phylogeographic analyses using chloroplast DNA (cpDNA) variation were performed for *Pedicularis* ser. *Gloriosae* (Orobanchaceae). Eighty-one plants of eighteen populations of six species (*P. gloriosa, P. iwatensis, P. nipponica, P. ochiaiana, P. sceptrum-carolinum, P. grandiflora*) were analyzed. Fifteen distinct haplotypes were identified based on six cpDNA regions: the intergenic spacer between the *trnT* and *trnL* 3'exon, *trnL* 3'exon–*trnF*, *atpB*–*rbcL*, *accD*–*psaI*, the *rpl16* intron, and the *trnK* region (including the *matK* gene). Via phylogenetic analyses of the haplotypes, two continental species, *P. sceptrum-carolinum* and *P. grandiflora*, were placed at the most ancestral position in the trees. The former species is widely distributed in the Eurasian continent, and the latter is distributed in Far East Asia. Two robust major cpDNA clades (Clades I and II) were revealed in the Japanese Archipelago, although the statistical values of monophyly of these clades were weak. Clade I included the haplotypes (A-1, A-2, B-1, B-2, and J) of three species (*P. gloriosa, P. iwatensis,* and *P. ochiaiana*), and Clade II included seven haplotypes (C-D, E-1, E-2, and F-H) of *P. nipponica.* These results suggest that this series originated on the Eurasian continent, and, subsequently, populations at the eastern edge of the continent differentiated into the two Japanese lineages.

Key words chloroplast DNA・endemic taxa・Japanese Archipelago・Orobanchaceae ・

1 *Pedicularis* ser. *Gloriosae*・phylogeography

Introduction

It is generally thought that the Japanese flora is derived from invasions from the Eurasian or Asian continents via Sakhalin, the Kuril Islands, the Korean peninsula, and the Ryukyu Islands during previous geological epochs (Hotta 1974; Maekawa 1998). Evidence for this hypothesis is provided by the fact that many widespread Japanese species also exist on the Asian continent (Hara 1959; Ohwi 1953, 1965). However, we should also consider the possibility that not only dispersal events, but also vicariance events have occurred, given that the Japanese Archipelago was a part of the Asian continent before the Neogene (at least ~24 million years ago) (Iijima and Tada 1990). It has also been reported that approximately 40% of the plant species in Japan are endemic (Hotta 1974; Murata 1977, 2000). Furthermore, more than 20 endemic genera in various plant groups (*Glaucidium, Ranzania, Sciadopitys, Wasabia* etc.) are known to exist in Japan (Hotta 1974). These features of the Japanese flora suggest that a large proportion of the plants in Japan originated or differentiated within the Japanese Archipelago. Therefore, in order to understand the evolutionary history of the Japanese flora, it is important to clarify the origins of Japanese endemic plant groups. However, there have been few studies of the phylogenetic divergence patterns of endemic plant groups in the Japanese flora based on objective data, except for studies of plants of the Ryukyu Islands (Hiramatsu *et al*. 2001; Setoguchi 2001; Setoguchi et al. 2006) and the Bonin

Islands (Ito and Pak 1996; Ito 1998; Setoguchi and Watanabe 2000; Soejima and Nagamasu 2004; Takayama et al. 2005).

Pedicularis ser. *Gloriosae* Prain (Orobanchaceae) are perennial herbs, which are distributed from Europe to northern Asia, including the Japanese Archipelago (Prain 1890; Li 1948; Tsoong 1955; Yang et al. 1998). They are often found in wet, sunny meadows. This series is characterized by the presence of large flowers with edentate corollas that are frequently densely villose on the lower margin of the galea. According to Li (1948), seven species are included in the series: *P. grandiflora* Fischer, *P. sceptrum-carolinum* L., *P. odontochila* Diels, *P. gloriosa* Bisset et S. Moore, *P. nipponica* Makino, *P. iwatensis* Ohwi, and *P. ochiaiana* Makino. Although *P. grandiflora* was divided into another independent series by some authors (ser. *Grandiflorae* Prain) (Prain 1890; Tsoong 1955; Yang et al. 1998), the system presented by Li (1948), taken in the broad sense, was adopted in the present study; that is, *P. grandiflora*, *P. sceptrum-carolinum*, and *P. odontochila* are considered to be continental species, and the remaining four species are considered to be endemic Japanese species. Of the continental species, *P. sceptrum-carolinum* is widely distributed from Europe to northern Asia, whereas *P. grandiflora* is distributed only in Far East Asia, and *P. odontochila* is found only in Shaanxi (Qinling) in north-central China (Yang et al. 1998). Distribution of the Japanese species is shown in Fig. 1; *P. iwatensis* is distributed in

northern Honshu, *P. nipponica* is found on the Sea of Japan side from central to northern Honshu, whereas *P. gloriosa* occurs on the Pacific Ocean side of central Honshu (mainly in the Kanto district), and *P. ochiaiana* is distributed only near the summits of the mountains on Yakushima Island, Kyushu. Thus, approximately half of the species in this group are endemic to Japan, and it is thought a suitable material for clarifying the differentiation process of an endemic plant in Japan.

In this study, phylogeographic investigation of *Pedicularis* ser. *Gloriosae* including two continental species, *P. grandiflora* and *P. sceptrum-carolinum* was conducted based on cpDNA sequence variation. The efficiency and merits of cpDNA for plant phylogeography have been discussed in many studies (e.g. Clegg and Zurawski 1992; Soltis et al. 1992; Soltis and Soltis 1998, Avise 2000, 2004). In the present study, six cpDNA regions were used (total sequence length, approximately 7,000 bp) in order to obtain enough cpDNA variation. According to these analyses I inferred the phylogeographic history and origin of the Japanese endemic species of *Pedicularis* ser. *Gloriosae*.

Materials and Methods

Sampling of taxa and cpDNA makers

1
2 A total of 81 plants from eighteen populations of six species of *Pedicularis* ser.
3 *Gloriosae* were sampled (Table 1). For *P. gloriosa* and *P. nipponica,* samples from
4 several populations were collected from throughout the range of these species.
5 Furthermore, in order to check intra-populational variation, 10-16 individuals were
6 analyzed as a representative population of each Japanese species (see, Table 1). In the
7 continental species, the samples of *P. sceptrum-carolinum* were collected from Germany
8 and China, and the sample of *P. grandiflora* was collected from Russia. I was unable to
9 include the Chinese species, *P. odontochila,* because it is an almost unknown species
10 whose record consists of only a single specimen (Yang et al. 1998). Samples of *P.*
11 *yezoensis* Maxim., *P. resupinata* L. (ser. *Resupinatae* Maxim.), and *P. schistostegia*
12 Vved. (ser. *Comosae* Maxim.) were also collected to use as outgroups for phylogenetic
13 analyses (Yamazaki 1993). The *Pedicularis* species close relatives to ser. *Gloriosae*
14 have not been clarified at present, therefore, the above three outgroups were selected
15 randomly from same subgroup *Allophyllum* (Li 1948). Voucher specimens of the
16 samples collected were deposited in the Makino Herbarium (MAK), Tokyo Metropolitan
17 University and the Herbarium, Faculty of Science, Kanazawa University (KANA).
18 Leaves of the sample plants were dried and preserved in silica gel. After
19 powdering the leaves in a mixer mill (Vibration Mill type MM300, Retsch, Haan,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1 Germany), total genomic DNA was extracted from 0.01 g of the powder using a slightly
2 modified version of the CTAB method described by Doyle and Dickson (1987). For all
3 plants, the non-coding region between the *trnT* (UGU) and *trnL* (UAA) 3'exons of
4 cpDNA (Taberlet et al. 1991) was sequenced for recognition of cpDNA haplotypes (ca.
5 1,200bp). This region was chosen for exhaustive analysis, because that showed
6 relatively high cpDNA polymorphisms among the several accessions in the preliminary
7 analysis. This region consists of the three parts (intergenic spacer between *trnT–L*
8 5'exon, 35 bp of *trnL* 5'exon and *trnL* intron). The haplotypes were inferred from the
9 site changes and insertions/deletions (indels) of the region. Next, to infer the
10 phylogenetic relationships among the populations of ser. *Gloriosae*, five regions were
11 further sequenced: the intergenic spacers between *trnL* (UAA) 3'exon and *trnF* (GAA)
12 (Taberlet et al. 1991), *atpB* and *rbcL* (Terachi 1993; Fujii et al. 1997), and *accD* and
13 *psaI* (Small et al. 1998), *trnK* region (including coding region of *matK*) (Johnson and
14 Soltis 1994), and *rpl16* intron (Jordan et al. 1996; Klechner and Clark 1997). In this
15 analysis, a single representative sample for each population of the six species of ser.
16 *Gloriosae* was analyzed. The intra-populational cpDNA variation was hardly detected in
17 the present study (for a detailed description, see below), therefore it is considered that
18 the relationships among the populations are able to estimate only using a single
19 representative sample per population.

PCR amplification and sequencing

All six cpDNA regions mentioned above were amplified by polymerase chain reaction (PCR). The PCR reaction mixtures contained 50-100 ng template DNA, 4 μL of 10X PCR buffer, 0.2 mM of each deoxyribonucleotide, 2.0 mM of $MgCl_2$, 0.4 μM of each of the primer pairs, and 1.0 U of ExTaq DNA polymerase (Takara Bio Inc., Tokyo, Japan) in a total volume of 40 μL. The PCR program ran for 3 min at 94℃ for initial denaturation, followed by 30 cycles of denaturation at 94℃ for 1 min, primer annealing at 55℃ for 1 min and extension at 72℃ for 2 min. The reactions were then extended by 7 min at 72℃.

The PCR products for direct sequencing were excised from 1% agarose gels and purified using a GENECLEAN III Kit (Qbiogene, Inc., Carlsbad, CA, USA) to remove the non-incorporated primers and nucleotides. Sequencing reactions were carried out using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc., Foster City, CA, USA). The sequencing reaction products were purified, concentrated by EtOH precipitation, and then applied to an ABI Prism 377 automated DNA sequencer (Applied Biosystems) with Long Ranger gels (Cambrex Corp., East Rutherford, NJ, USA).

Sequence alignment and phylogenetic analyses

Alignment of the sequences was performed manually using the DNASIS-Mac program version 3.0 (Hitachi Software Engineering, Tokyo, Japan). The boundaries of the coding and non-coding regions were determined by comparing the sequences to the corresponding sequences in *Nicotiana tabacum* L. (Shinozaki et al. 1986). Indels were generally placed so as to maximize the number of matching nucleotides in corresponding sequences.

The phylogenetic relationships among the populations of ser. *Gloriosae* were inferred by maximum parsimony (MP) and Bayesian methods, using six cpDNA regions. In the MP analysis, the trees were constructed based solely on substitution data using PAUP* version 4.0b10 (Swofford 2003). In the analysis, we used the branch and bound search option, with MulTrees on, and addition sequence option set at "furthest". The clade support was estimated by bootstrap analysis (Felsenstein 1985) based on 10,000 replicates using PAUP, and decay analysis (Bremer 1988, Donoghue et al. 1992) using Autodecay version 4.0 programs (Eriksson 1998; Eriksson et al. 1998). For Bayesian analysis, the best-fit substitution models were determined using MrModeltest ver. 2.2 (Nylander 2004), with Akaike's information criterion (AIC, Akaike 1974). The best-fit

model for the present cpDNA data was GTR+G with gamma shape = 0.1968 (Tavaré 1986). The Bayesian analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). In the analyses, Metropolis-coupled Markov Chain Monte Carlo (MC3) sampling was performed with four chains that were run for 4,000,000 generations, saving the current tree to a file every 100 generations. Default cold and heated chain parameters were used. At the end of each run, we considered the sampling of the posterior distribution to be adequate if the average standard deviation of split frequencies was <0.01. MC3 runs were summarized and further investigated for convergence of all parameters, using the "sump" and "sumt" commands in MrBayes. Trees prior to log likelihood stabilization and convergence (burn in = 10,000) were discarded before a majority rule consensus tree was generated. Posterior probabilities were estimated on 30,000 trees.

Estimation of divergence time

To evaluate possible rate heterogeneity of molecular evolution among the cpDNA haplotypes of species of ser. *Gloriosae*, relative rate tests were performed using the 1D tests of Tajima (1993) with MEGA version 2.1 (Kumar et al. 2001). This method involves the comparison of two sequences relative to a third (outgroup) sequence. In

this study, the corresponding sequence was obtained from *P. yezoensis*.

In order to establish a preliminary estimate of the divergence time for the major cpDNA clades within ser. *Gloriosae*, molecular evolutionary rates obtained from other species (Richardson et al. 2001; Mummenhoff et al. 2004) were used for the calculation. For the *Pedicularis* species, because, we do not have the estimated molecular evolution rates of the non-coding regions of cpDNA, and not have the information of age of fixed nodes. The calibrated rates of a *trnL–F* non-coding region were estimated as follows: 1.30×10^{-9} substitutions per site per year (s/s/y), *Inga* (Leguminosae); 4.87×10^{-9} s/s/y, *Phylica* (Rhamnaceae); 8.24×10^{-9} s/s/y, *Aichryson* (Crassulaceae) (Richardson et al. 2001); $1.3–2.8 \times 10^{-9}$ s/s/y, *Lepidium* (Brassicaceae) (Mummenhoff et al. 2004). Despite the wide range of variance, I used $1.30–8.24 \times 10^{-9}$ s/s/y as the rate of a *trnL–F* non-coding region. In the present calculation, only nucleotide sequences of *trnL–F* were cut out from other nucleotide sequences in order to evaluate it as accurately as possible, and the pairwise genetic distances among the haplotypes were calculated by GTR+G model obtained from MrModeltest as mentioned above. The divergence time (t) between the cpDNA clades were calculated by following formula: $t = D / 2K$, where D = the mean genetic distance and K = the estimated molecular evolution rates mentioned above (Sanderson 1998).

Results

Detected cpDNA haplotypes

The length of the non-coding region between the *trnT* (UGU) and *trnL* (UAA) 3'exon of cpDNA varied from 1234-1307 bp. The polymorphic characters in the region included 95 nucleotide substitutions and 42 indels among all accessions, as well as 40 site changes and 17 gaps among the populations of ser. *Gloriosae* (Table 2). Based on the polymorphic characters, twelve distinct cpDNA haplotypes (Types A-L; Table 1) were recognized in ser. *Gloriosae*. In the Japanese species, six cpDNA haplotypes were found in *P. nipponica* (Types C-H), and most haplotypes were endemic to population except for haplotype E (Mt. Sumon-dake and Mt. Naeba-san). On the other hand, all other species showed single cpDNA haplotypes (*P. gloriosa*, Type A; *P. iwatensis*, Type B; *P. ochiaiana*, Type I). In the continental species, two populations (Germany and China) of *P. sceptrum-carolinum* were distinguished by seven nucleotide substitutions and one indel (Types J and K), and *P. grandiflora* in Russia had an endemic haplotype (Type L).

Intra-populational cpDNA variation was detected in one population of *P. nipponica* (the Asahi-dake Mountains, northern Honshu, Japan) (Fig. 1). In 16 analyzed

individuals of the population, two variable indels were detected that were located at sequence positions 62-63 and 868, which were poly-AT and poly-T variation. Three haplotypes (D-1, 2 and 3) were recognized based on the observed variation, and all private haplotypes at the population of Asahi-dake Mountains. In 16 analyzed individuals, D-1 haplotype was detected 10 individuals, D-2 was 4 individuals, and D-3 was 2 individuals. In this study, the sample possessed major haplotype (D-1) was used for further sequencing analyses, as represented by the population. The nucleotide sequence data for minor haplotypes (D-2 and 3) were deposited in DDBJ/EMBL/GenBank (accession numbers AB280533 and AB280534).

Further sequencing for phylogenetic analysis

To clarify the phylogenetic relationships among populations of ser. *Gloriosae*, five cpDNA regions representative of each population of the six species and three outgroup species were further sequenced. The polymorphic characters within the samples of ser. *Gloriosae* of each region are summarized in Table 2. The total length of the combined data set after multiple alignments of the six regions was 6,920 bp; 466 site changes and 88 gaps were inferred among all accessions including outgroups, and 206 site changes and 58 indels were detected within the series. The calculated values of uncorrected

p-distance (Nei and Kumar 2000) within the series ranged from 0–0.0132, and the genetic distances based on GTR+G model (Tavaré 1986) within the series ranged from 0–0.0140 (S1). Three cpDNA haplotypes were further distinguished by the five non-coding regions: Type A-2 in *P. gloriosa*, Type B-2 in *P. iwatensis*, and Type E-2 in *P. nipponica* (Table 1). The three haplotypes were distinguished from Type A-1, B-1, E-1 by 1 bp nucleotide, 1 indel (7 bp), and, 1 bp site change and 1 indel (14 bp), respectively. Therefore, a total of 15 cpDNA haplotypes were recognized in the accessions of ser. *Gloriosae*. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank DNA databases under accession numbers AB280429 to AB280532, and, AB280945 and AB280946 .

Phylogenetic relationships among the accessions of ser. *Gloriosae*

The MP analysis of 15 cpDNA haplotypes recognized from the six regions in ser. *Gloriosae* resulted in three parsimonious trees using the branch and bound search option. The trees required 542 steps; Consistency Index (CI) excluding uninformative characters = 0.8640, Retention Index (RI) = 0.9213. The strict consensus tree (MP tree) of the three trees is shown in Fig. 2. The MP tree displayed the following phylogenetic relationships. First, the cpDNA haplotypes of ser. *Gloriosae* formed a clade with 100%

bootstrap value (decay index (DI) = 91) against the outgroups. Second, the two continental species (*P. sceptrum-carolinum* and *P. grandiflora*) were placed at the ancestral position in the series. Third, the Japanese accessions were positioned derivatively, and constituted a single clade with 51% value (DI = 1). Two major clades were revealed within the Japanese species clade: Clade I, which included the haplotypes of three species (A-1, 2, B-1, 2, I) with a 90% bootstrap value (DI = 5), of which *P. gloriosa* and *P. iwatensis* formed a monophyletic group with a 71% value (DI = 1); and Clade II, which consisted of seven haplotypes (C-D, E-1, 2, F-H) of *P. nipponica* with a bootstrap value of 94% (DI = 6). Within Clade II, the haplotype of Mt. Iouzen (H) was placed at the most ancestral position, and the remaining six haplotypes formed a clade with 100% bootstrap value (DI = 10). In Clade II excluding Type H, two subclades were identified: one including the haplotypes of the populations of Mt. Yakeishi-dake (C), Asahi-dake Mountains (D), and the Tsugaike Highland (G) with a bootstrap value of 82% (DI = 2), and the other including the haplotypes of three populations: Mt. Sumon-dake (E-1), Mt. Naeba-san (E-2), and Yomogi Pass (F)) with a bootstrap value of 100% (DI = 7). In the former subclade, the haplotypes of Mt. Yakeishi-dake (C) and Asahi-dake Mountains (D) formed a monophyletic group with a 91% value (DI = 3).

Majority-rule (50%) consensus tree of Bayesian analysis based on a GTR+G model is presented in Fig. 3. The tree was also the same topology as that of the MP tree.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1 The posterior probabilities of most clades were high (> 95%), excluding the clade of
2 Japanese *Gloriosae* (90%) and that of *P. gloriosa* and *P. iwatensis* (86%). The
3 discordance between bootstrap values in MP analysis and posterior probabilities in
4 Bayesian analysis was notified by several studies (Huelsenbeck et al. 2002, Alfaro et al.
5 2003, Douady et al. 2003), therefore, nodes with high posterior probabilities and low
6 bootstrap support are considered with caution in this study.
7

8 Estimation of divergence time among major lineages

9

10 To evaluate possible rate heterogeneity among *Gloriosae* lineages, relative rate
11 tests were performed using the 1D tests of Tajima (1993), but found no significant rate
12 heterogeneity in any pairwise comparisons of the cpDNA sequences (data not shown).
13 For the estimation of divergence time among major cpDNA clade, the pairwise genetic
14 distance based on GTR+G model among the haplotypes were calculated using *trnL–F*
15 region (ca. 1,000 bp). The ranges of genetic distances among all accessions were
16 0–0.039, and those among the accessions of ser. *Gloriosae* ranged from 0–0.015. The
17 average genetic distance among the haplotypes of continental species and Japanese
18 species was 0.010±0.0020, which implies a range of diversification time of 0.61–3.8
19 million years ago (Myr). The average genetic distance between the haplotypes of Clade

I and Clade II was 0.0064±0.0019, which implies a range of diversification time of 0.39–2.5 Myr.

Discussion

In the present study, most cpDNA haplotypes detected in *Pedicularis* ser. *Gloriosae* were endemic to each population except for haplotype A-1 of *P. gloriosa* (Table 1). In other words, there were little species and a population that shared the same haplotype. Furthermore, although the intra-populational variation of each species was examined only by single representative population in this study, those were detected only in the Asahi-dake Mountains in *P. nipponica.* The variation in the population was poly-AT and poly-T variation, and the three haplotypes detected were endemic to the population (Types D-1, 2, and 3). These facts suggest that the cpDNA variation detected in this study characterize the each species or population of ser. *Gloriosae.*

MP and Bayesian trees showed that the accessions of ser. *Gloriosae* constituted a single clade with high bootstrap value, and it was inferred that the continental species *P. grandiflora* and *P. sceptrum-carolinum* had ancestral position in the clade (Figs. 2 and 3). As mentioned above, *P. grandiflora* is distributed in Far East Asia, and *P. sceptrum-carolinum* is distributed from Europe to northern Asia. These

facts suggest that this series originated in Eurasian Continent, and, subsequently, the populations of eastern edge of the continent differentiated into Japanese lineages. In the present analysis, the monophyly of the Japanese accessions including four species was supported by weak bootstrap values in MP tree and by posterior probabilities (< 95%) in Bayesian tree (Figs. 2 and 3). Therefore, it is unclear whether the four Japanese species originated through a single event. However, it is evident that at least two cpDNA clades were differentiated from a part of the continental lineages around the Japanese Islands (Clades I and II, for a detailed description, see below).

Within the Japanese species, two major clades were identified (Figs. 2 and 3), one of which consisted of the populations of *P. gloriosa, P. iwatensis*, and *P. ochiaiana* (Clade I), and the other of which included those of *P. nipponica* (Clade II). The two clades were geographically structured; the species of the former clade are distributed primarily on the Pacific Ocean side of the Japanese Archipelago, while those of the latter clade are found primarily on the Sea of Japan side (Fig. 1). The environmental differences between the Pacific Ocean and Japan Sea sides is conspicuous, especially in the amount of snowfall in winter, between the regions on either side of the axis of the Japanese Archipelago. It is known that there are many plant species or intraspecific variation corresponding to the environmental difference between the two regions, for example, *Camellia japonica* L. (Theaceae), *Cephalotaxus harringtonia* (Knight) K.

Koch (Cephalotaxaceae), *Torreya nucifera* (L.) Sieb. et Zucc. (Taxaceae), and *Hamamelis japonica* Sieb. et Zucc. (Hamamelidaceae) (Hara 1959; Fukuoka 1966; Hotta 1974; Yamazaki 1983). Therefore, the diversification of the two major cpDNA clade of Japanese *Gloriosae* might have caused by regional environmental differences in Japanese Islands.

The Clade I consisted of three species and inter-populational variation in *P. gloriosa* and *P. iwatensis* were nearly zero (Fig. 3). The average genetic distance based on GTR+G model between haplotype J (*P. ochiaiana*) and other haplotypes of Clade I was 0.0051±0.00027 (S1). On the other hand, Clade II consists of only the accessions of *P. nipponica*, and inter-populational variation in the species was relatively high (Fig. 3). The average genetic distance between haplotype H (Mt. Iouzen) and other haplotypes of Clade II was 0.0067±0.00055 (S1). In short, the intra-specific genetic variation within *P. nipponica* was higher than the inter-specific genetic variation within Clade I species. However, according to Tajima (1993)'s test, no significant evolutionary rate heterogeneity was found in any pairwise comparisons of the cpDNA sequences. The three species of the Pacific Ocean clade are clearly distinguished by the form of the corolla or calyx (Yamazaki 1993), however there has been no report to date of geographic variation or intraspecific taxa within *P. nipponica*. These results suggest that *P. nipponica* has retained a constant external morphology at time that was relatively

longer than that of the species of Clade I.

In the Clade I, the haplotype J of *P. ochiaiana* was positioned most ancestrally, and those of *P. gloriosa* and *P. iwatensis* formed a sister group (Figs. 2 and 3), suggesting that the Clade I of the Pacific Ocean side first diverged into the population of Yakushima Island, and then separated into the populations of the Kanto district and the Tohoku district. After that, each population further might evolve to species level seen today (i.e., *P. ochiaiana*, *P. gloriosa* and *P. iwatensis*). On the other hand, in the Clade II of Japan Sea side, the accession of the Mt. Iouzen population (type H), which is the southern limit of the distribution of *P. nipponica* (Fig. 1), had the most ancestral position within the clade of the species (Figs. 2 and 3). This might mean that the population diverged earlier than other populations. In Clade II excluding haplotype H, the distribution of haplotypes exhibited curious geographic structure. One subclade including the populations Mt. Sumon-dake, Yomogi Pass, and Mt. Naeba-san, made a single clade in the central part of distribution area of the species. On the contrary, another subclade showed a disjunct distribution in populations of the Mt. Yakeishi-dake and Asahi-dake Mountains of northern Honshu and Tsugaike Highland of central Honshu (Fig. 1). This phylogeographic structure in Japan Sea side area might mean the complicate evolutionary history or diversification process of *P. nipponica.*

The preliminary estimates of divergence times between the continental (*P.*

sceptrum-carolinum) and Japanese accessions were 0.61—3.8 Myr. These divergence times correspond to the middle of the Pliocene (Tertiary) to the middle of the Pleistocene (Quaternary). The prototype of the present Japanese Archipelago had almost been formed by the Pliocene (Iijima & Tada, 1990). Furthermore, the evidence of climatic cooling in Japan from the Pliocene to the Pleistocene was suggested according to fossil data (Tanai, 1972; Momohara, 1994) and pollen analysis (Tsukada, 1983). In the Pleistocene, it has been suggested that the cycle of glacial advances and retreats occurred around the world (Bradley, 1999), and it is believed that the last glacial period (ca. 10,000—70,000 years ago) greatly influenced the present plant distribution, including the most Japanese plants. My divergence estimates suggest that Japanese accessions were derived from common ancestors with continental lineages for at least 0.61 Myr. Therefore, the Japanese clades might have diversified until the middle Pleistocene, and differentiated into several species or geographically structured within a species due to the several climatic changes (cooling and warming) that preceded the last glacial period.

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Figure Legends

Figure 1. Location of sampling sites of *Pedicularis* ser. *Gloriosae* for cpDNA analysis in Japan. Gray shading represents the distribution area of each species.

Figure 2. Strict consensus of three equally most parsimonious trees among the cpDNA haplotypes of species of *Pedicularis* ser. *Gloriosae*. Numbers above the branches are bootstrap values in percentage based on 10,000 replicates, and those under the branches are decay index values. The letter after each population name indicates the cpDNA haplotype.

Figure 3. Majority-rule (50%) consensus tree based on Bayesian phylogenetic analysis of 15 cpDNA haplotypes of species of *Pedicularis* ser. *Gloriosae*. Values above lines are posterior probabilities (%).The best-fit substitution models (GTR+G model) were determined by MrModeltest ver. 2.2 (Nylander 2004). The letter after each population name indicates the cpDNA haplotype.

Table 1 Materials, and their sources, analyzed for cpDNA variation of *Pedicularis* ser. *Gloriosae*.

Taxa, locality[1], voucher[2], and collector	No. of plants	CpDNA haplotype[3]
Pedicularis sect. *Lasioglossa* ser. *Gloriosae*		
Japanese species		
P. gloriosa Bisset et S. Moore		
Mt. Mitsutouge-yama, Yamanashi, Honshu, Alt. 1400m, MAK322927, N. Fujii	3	A-1
Misaka-touge, Yamanashi, Honshu, Alt. 1300m, MAK322928, N. Fujii	3	A-1
Mt. Jyumai-yama, Shizuoka, Honshu, Alt. 1360m, MAK322929, N. Fujii	3	A-2
Mt. Odake-san, Tokyo, Honshu, Alt. 1200m, MAK323191, N. Fujii	10	A-1
The Akagi-san Mountains, Gunma, Honshu, Alt. 1460m, MAK336041, N. Fujii	1	A-1
P. iwatensis Ohwi		
The Akitakomagadake Mountains, Akita, Honshu, Alt. 1200m, MAK322849, N. Fujii	12	B-1
Mt. Yakushi-dake, Akita, Honshu, Alt. 770m, MAK322848, N. Fujii	3	B-2
P. nipponica Makino		
Mt. Yakeishi-dake, Iwate, Honshu, Alt. 1000-1200m, MAK322850, N. Fujii	3	C
The Asahi-dake Mountains, Yamagata, Honshu, Alt. 1300-1500m, MAK322851, N. Fujii	16	D
Mt. Sumon-dake, Niigata, Honshu, Alt. 1480m, MAK322852, N. Fujii	3	E-1
Mt. Naeba-san, Nagano, Honshu, Alt. 1300-1700m, MAK336042, N. Fujii	1	E-2
Yomogi pass, Mts. Tanigawa-dake, Gunma. Honshu, Alt. 1300m, MAK320225, N. Fujii	3	F
Tsugaike Highland, Mts. Shirouma-dake, Nagano, Honshu, Alt. 2000m, MAK320906, N. Fujii	3	G
Mt. Iouzen, Ishikawa, Honshu, Alt. 900m, MAK336044, N. Fujii	1	H
P. ochiaiana Makino		
Mt. Kuromi-dake, Yakushima Island, Kagoshima, Kyusyu, Alt. 1750m, MAK322931, N. Fujii	13	I
Continental species		
P. sceptrum-carolinum L.		
Federsee, Baden-Wuerttemberg, Germany, Alt. 570m, W. Licht	1	J
Tayuan forest, Great Hsingan Mountain, Heilungkiang province, China, Alt. 800m, MAK351811, Y.-C. Zhu	1	K
P. grandiflora Fisch.		
Maritime Province of Siberia, Russia (N44° 10’06.7”, E135° 39’08.0”, Alt. 1m), Coll. K.Ueda and C. Suyama, Voucher specimen (in KANA)	1	L
Total No. of samples	81	
Outgroups		
P. yezoensis Maxim. (sect. *Pedicularis*)		
Mt. Chogadake, Nagano, Honshu, 2500m, MAK310467, N. Fujii	1	
P. resupinata L. (sect. *Pedicularis*)		
Mt. Gassan, Yamagata ., Honshu, Alt. 1500m, MAK304317, N. Fujii	1	
P. schistostegia Vved. (sect. *Bicuspidatae* Maxim.)		
Rebun Island, Hokkaido, Alt. 20m, KANA198102, N. Fujii	1	

[1]For localities of Japanese samples, see Fig. 1

[2]KANA: Herbarium of Kanazawa University, MAK: Makino Herbarium, Tokyo Metropolitan University

[3]CpDNA haplotypes were inferred from the sequences between the *trnT* and *trnL* non-coding regions of cpDNA. The three haplotypes, A-2, B-2 and E-2, were differentiated from A-1, B-1 and E-1, respectively, by further sequencing of five non-coding regions (*trnL-trnF*, *atpB-rbcL*, *accD-psaI*, *trnK* region, and *rpl16* intron).

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Table 2 Comparison of the lengths, polymorphic characters and values of uncorrected sequence divergences among the analyzed regions of cpDNA within *Pedicularis* ser. *Gloriosae*.

Region	Length (bp)	NS[a]	Indel[b]	P[c] (%)
The region for cpDNA haplotypes recognition				
The non-coding region between *trnT* (UGU) and *trnL* (UAA) 3'exon	1234-1307	40	17	0-1.59
trnT – *trnL* 5'exon (IGS)	670-734	24	14	
trnL 5'exon	35	1	0	
trnL intron	496-510	14	3	
trnL 3'exon	32	1	0	
Additional sequencing regions for phylogenetic analysis				
The intergenic spacer between *trnL* 3'exon and *trnF* (GAA)	368-379	9	6	0-1.64
trnL 3'exon – *trnF* (IGS)	354-365	9	6	
A part of *trnF*	14	0	0	
The intergenic spacer between *atpB* and *rbcL*	710-736	16	8	0-0.99
The intergenic spacer between *accD* and *psaI*	942-975	31	7	0-1.70
trnK region	2426-2447	72	11	0-1.20
trnK 5'exon – *matK* (intron)	710-718	18	4	
matK gene	1523-1533	50	5	
matK – *trnK* 3'exon (intron)	186-206	4	2	
rpl16 intron	875-890	38	9	0-2.28
Total	6595-6702	206	58	0-1.32

[a] Numbers of nucleotide substitutions.
[b] Numbers of insertion/deletion.
[c] Uncorrected p-distances (Kumar *et al*. 1993).

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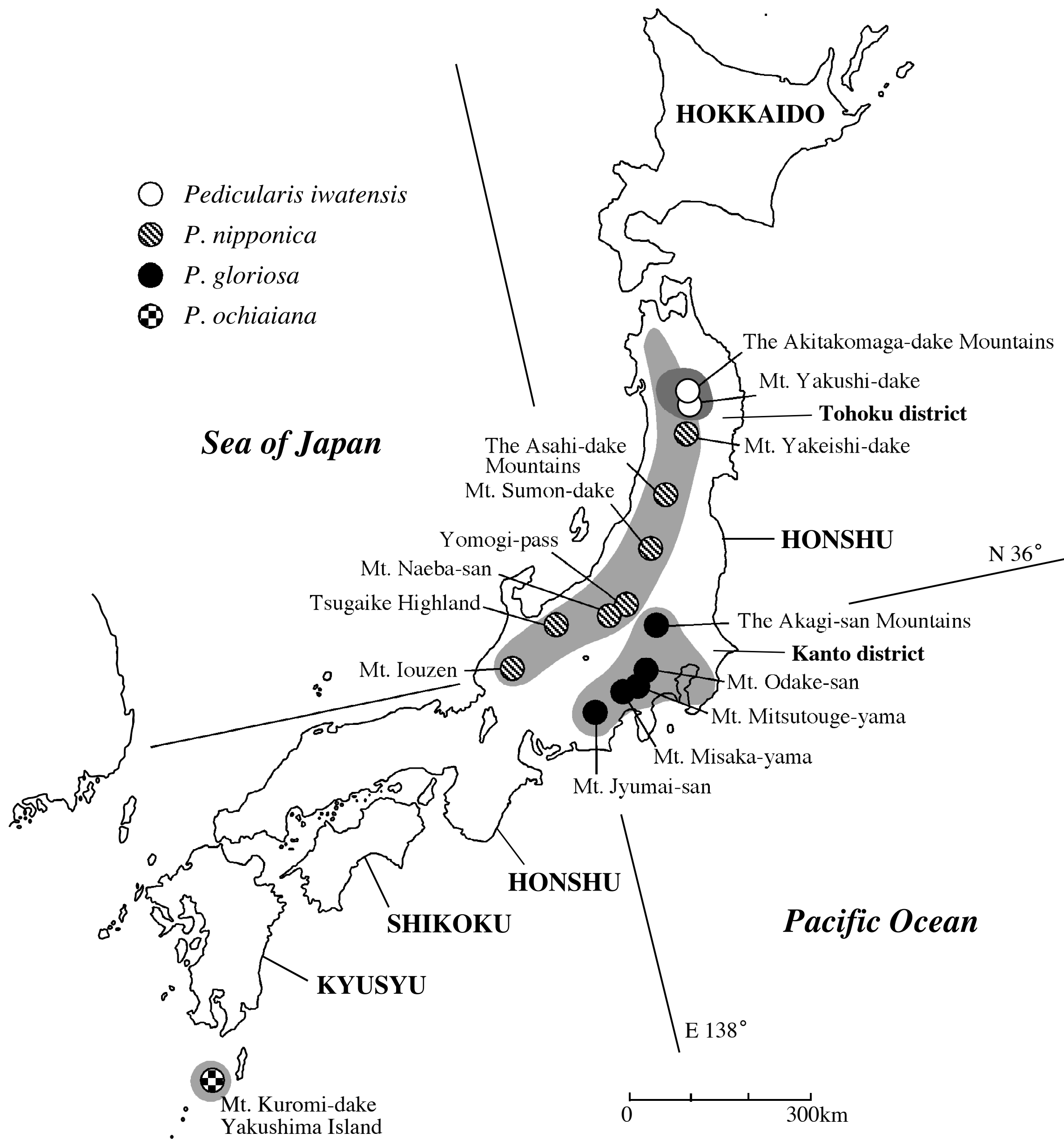

Fig. 1.

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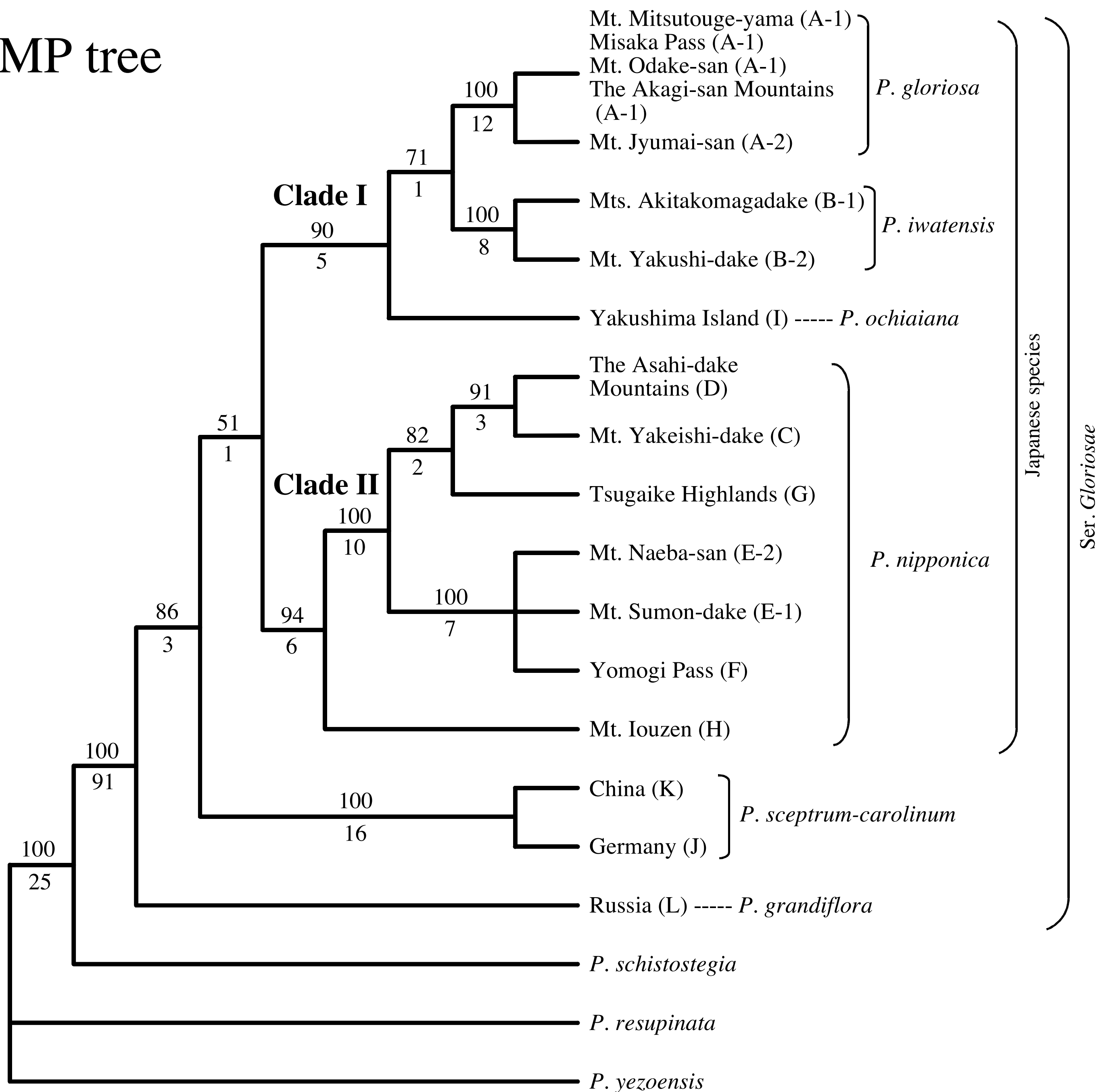

Fig. 2.

Bayesian tree

Mt. Mitsutouge-yama (A-1)
Misaka Pass (A-1)
Mt. Odakesan (A-1)
The Akagi-san Mountains (A-1)
Mt. Jyumai-san (A-2)
P. gloriosa
100
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Clade I
Mts. Akitakomagadake (B-1)
Mt. Yakushi-dake (B-2)
P. iwatensis
100
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Yakushima Island (J) ----- *P. ochiaiana*
The Asahi-dake Mountains (D)
Mt. Yakeishi-dake (C)
Tsugaike Highlands (G)
100
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Clade II
100
Mt. Naeba-san (E-2)
Yomogi Pass (F)
Mt. Sumon-dake (E-1)
100
P. nipponica
100
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Mt. Iouzen (H)
100
China (K)
Germany (J)
P. sceptrum-carolinum
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Russia (L) ----- *P. grandiflora*
P. schistostegia
P. resupinata
P. yezoensis
0.01 substitutions/site
Japanese species
Ser. *Gloriosae*

Fig. 3

Supplement 1 Genetic distance[a] matrix for the cpDNA haplotypes of *Pedicularis* ser. *Gloriosae*.

	A-1	A-2	B-1	B-2	C	D	E-1	E-2	F	G	H	I	J	K	L	out1	out2
Type A-1																	
Type A-2	0.00015																
Type B-1	0.00306	0.00321															
Type B-2	0.00305	0.00321	0[b]														
Type C	0.00801	0.00818	0.00705	0.00704													
Type D	0.00784	0.00800	0.00688	0.00687	0.00259												
Type E-1	0.00716	0.00733	0.00621	0.00620	0.00321	0.00335											
Type E-2	0.00700	0.00717	0.00605	0.00604	0.00305	0.00320	0.00015										
Type F	0.00749	0.00765	0.00654	0.00653	0.00352	0.00366	0.00060	0.00045									
Type G	0.00817	0.00833	0.00721	0.00720	0.00385	0.00368	0.00383	0.00352	0.00399								
Type H	0.00672	0.00689	0.00592	0.00591	0.00721	0.00705	0.00605	0.00590	0.00638	0.00737							
Type I	0.00526	0.00542	0.00479	0.00479	0.00984	0.00967	0.00832	0.00816	0.00865	0.01000	0.00787						
Type J	0.00812	0.00828	0.00748	0.00747	0.00999	0.01013	0.00944	0.00928	0.00977	0.01047	0.00915	0.00947					
Type K	0.00829	0.00845	0.00765	0.00764	0.01017	0.01031	0.00962	0.00946	0.00995	0.01065	0.00933	0.00948	0.00305				
Type L	0.01144	0.01161	0.01077	0.01076	0.01284	0.01333	0.01212	0.01196	0.01246	0.01404	0.01252	0.01234	0.01330	0.01365			
out1[c]	0.03935	0.03957	0.03796	0.03790	0.04080	0.04053	0.04068	0.03916	0.03979	0.04227	0.03980	0.03959	0.04110	0.04112	0.04252		
out2[c]	0.03136	0.03155	0.02986	0.02982	0.03285	0.03259	0.03151	0.03132	0.03191	0.03287	0.03178	0.03150	0.03253	0.03254	0.03470	0.01628	
out3[c]	0.03266	0.03285	0.03167	0.03162	0.03389	0.03407	0.03299	0.03279	0.03339	0.03425	0.03358	0.03258	0.03451	0.03476	0.03710	0.02710	0.01987

[a] Genetic distances were calculated based on GTR+G model obtained from MrModeltest (Nylander 2004), using six cpDNA regions.

[b] There was no evidence of nucleotide substitution between haplotype B-1 and B-2, both were distinguished only by a single insertion/deletion.

[c] out1, *P. schistostegia*; out2, *P. resupinata*; out3, *P. yezoensis*.