The Genetic Characteristics of an Endangered Population of *Mitella furusei* var. *subramosa* (Saxifragaceae) from Shikoku, Japan

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Abstract   To study the genetic characteristics of an isolated population of *M. furusei* var. *subramosa* from Uwajima City, Ehime Prefecture ("Tsushima-cho" population), we conducted nucleotide sequencing of two plastid DNA regions (*trnH-psbA* intergenic spacer and *matK* gene) and three nuclear DNA regions (nuclear ribosomal external transcribed spacer and internal transcribed spacer [ETS and ITS], *GBSSI-A0*, and *GBSSI-A1* genes) from the plants collected in the population. We revealed that the population has a unique genetic profile, which rules out the possibility of the origin of the population by an artificial introduction. Moreover, the present phylogenetic analyses suggested its close affinity to the populations from Kii Peninsula, Honshu. This finding may be attributable to the ancient distribution of the plants along with the ancient Kitan River system. Moreover, no genetic variation or heterozygosity was detected for all the sequenced DNA regions in the plant population. The genetic uniqueness, the poor genetic diversity, and the very scarce number of the surviving plants all indicate the urgent need for the conservation of the population.

Kew words : *Asimitellaria*, Ehime Prefecture, endangered species, endemic species, Kitan River.

Introduction

Japanese Archipelago hosts a rich and unique flora and is recognized as one of the biodiversity hotspot of the world. The number of endemic angiosperm species of Japan accounts for 1586 (Kato and Ebihara, 2011), and some of them are the members of the plant groups that have the center of diversity in Japan, shaping the floristic characteristics of the region (Okuyama, 2010). One of such plant groups is the genus *Mitella* section *Asimitellaria*, a perennial lineage with 12 endemic species and varieties dominating in characteristic riparian habitat of Japan. By making use of their endemic diversity, *Asimitellaria* has served as a useful model for studying a variety of issues to elucidate the evolutionary origin of the floristic diversity of the archipelago. These issues include pollinator-driven flower evolution (Okuyama *et al.*, 2004, 2008), introgressive hybridization (Okuyama *et al.*, 2005), the evolution of reproductive isolation (Okuyama and Kato, 2009), and the origin of polyploidy and the floristic disjunction between East Asia and North America (Okuyama *et al.*, 2012). Further accumulation of information on the natural history of *Asimitellaria* will provide a comprehensive view on the origin of the floristic diversity of the archipelago through the window of this model plant group.

One of the *Asimitellaria* species with wider distribution is *M. furusei*, in which two taxonomic varieties distinguished by the petal morphology, var. *furusei* and var. *subramosa*, are known. The distribution of the former ranges from west of Honshu Island to north of Kyushu,
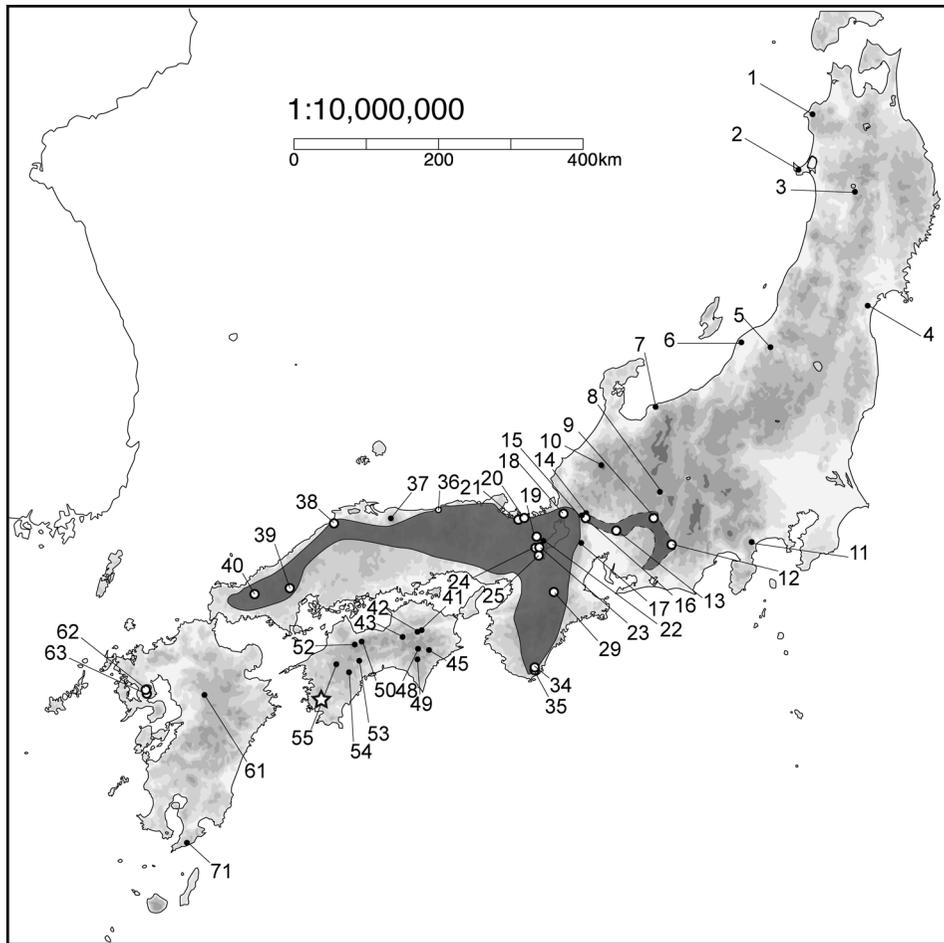
Fig. 1. The distribution map of *M. furusei* and the collection locality of the plants analyzed in the present study. The area in gray enclosed with solid line represents the distribution of *M. furusei* var. *subramosa*, whereas the area in white enclosed with broken line represents that of *M. furusei* var. *furusei*. The star indicates the "Tsushima-cho" population. Open and closed circles each represent the localities where *M. furusei* and the other species were collected, respectively. The population ID numbers correspond to those of Okuyama and Kato (2009).

while that of the latter is restricted in central Honshu Island (Fig. 1). Although it is one of the commonest species in western Honshu Island, the populations out of Honshu Island are scarce and are recognized as endangered.

An interesting example of the isolated populations of *M. furusei* var. *subramosa* has been known from Uwajima City, Ehime Prefecture (hereafter referred to as "Tsushima-cho" population) since 1993, when a local botanist Masaharu Hyodo had discovered it (Wakabayashi, 1995). Because that is the only population of *Mitella*

furusei known from Shikoku Island, and is fairly isolated from other populations, its biogeographic relationships to other populations as well as its genetic characteristic remain enigmatic. Such information is important not only to examine the biogeographic link between the populations, or to know the ancient migration/dispersal history, but also to build the conservation policy of this population, which is ranked as "critically endangered (CR)" in the Red Data Book of Ehime Prefecture (Ehime Prefecture, 2003).

Thus, to elucidate the genetic characteristics of

this "Tsushima-cho" population of *M. furusei* var. *subramosa*, we conducted nucleotide sequencing of two plastid DNA regions and three nuclear DNA regions (*trnH-psbA* intergenic spacer, *matK* gene, nuclear ribosomal ETS and ITS, *GBSSI-A0*, and *GBSSI-A1* genes) of six individual plants collected in the population. These DNA regions have been extensively surveyed already throughout the distribution ranges of *M. furusei* and its sister species *M. pauciflora* and *M. koshiensis* (Okuyama and Kato, 2009; Okuyama *et al.*, 2012), making them possible to examine the phylogeographic relationships among the populations and species. Through this genetic survey of the population, we illustrate the genetic uniqueness of the population and propose that the urgent action for the conservation of the population is needed.

Materials and Methods

Data collection

One of the authors (YO) visited the population on Sep. 26, 2011, and found only six individuals of the plants. Judging from the condition of the population on the time of discovery (M. Hyodo, personal communication), the population is apparently in decline and the size of the plants were all very small (Fig. 2), likely because of closure of the forest canopy. To obtain total genomic DNA, a leaf was collected from each of the six plant individuals. The entire plants were also collected from three of them to keep and regenerate them in the nursery of Tsukuba Botanical Garden, as we considered that the population is no longer self-sustainable.

Total genomic DNA was extracted using the modified CTAB method as follows. First, the leaf materials were frozen in liquid nitrogen, and were ground in 1.5 ml microtube using zirconia beads and TissueLyser (Qiagen, Hilden, Germany). To remove polysaccharides and polyphenols, the granulated leaf materials were quickly washed using the wash buffer, which contains 0.1M HEPES (pH8.0), 2% (v/v) 2-mercaptoethanol, 9 mg/ml ascorbic acid, and 10.2 mg/ml polyvinylpyrrolidone. After washing, the materials were subjected to the modified CTAB procedures, in which DNA was first collected by isopropanol precipitation and eluted with an excessive amount (300 μl) of TE buffer, followed by an additional ethanol/sodium acetate precipitation and a rinse with 70% ethanol.

Polymerase chain reactions (PCRs) were conducted using Ex Taq polymerase (TaKaRa, Tokyo, Japan) aided with Ampdirect plus (Shimadzu, Kyoto, Japan) with 30 cycles of denaturation at 94℃ for 10 sec, primer annealing at 55–60℃ (depending on the melting temperatures of the primer oligonucleotides) for 10 sec, and

Fig. 2.　The condition of the surviving plants of *M. furusei* var. *subramosa* in "Tsushima-cho" population. All of these plants were so small that probably they are incapable of flowering and fruiting.
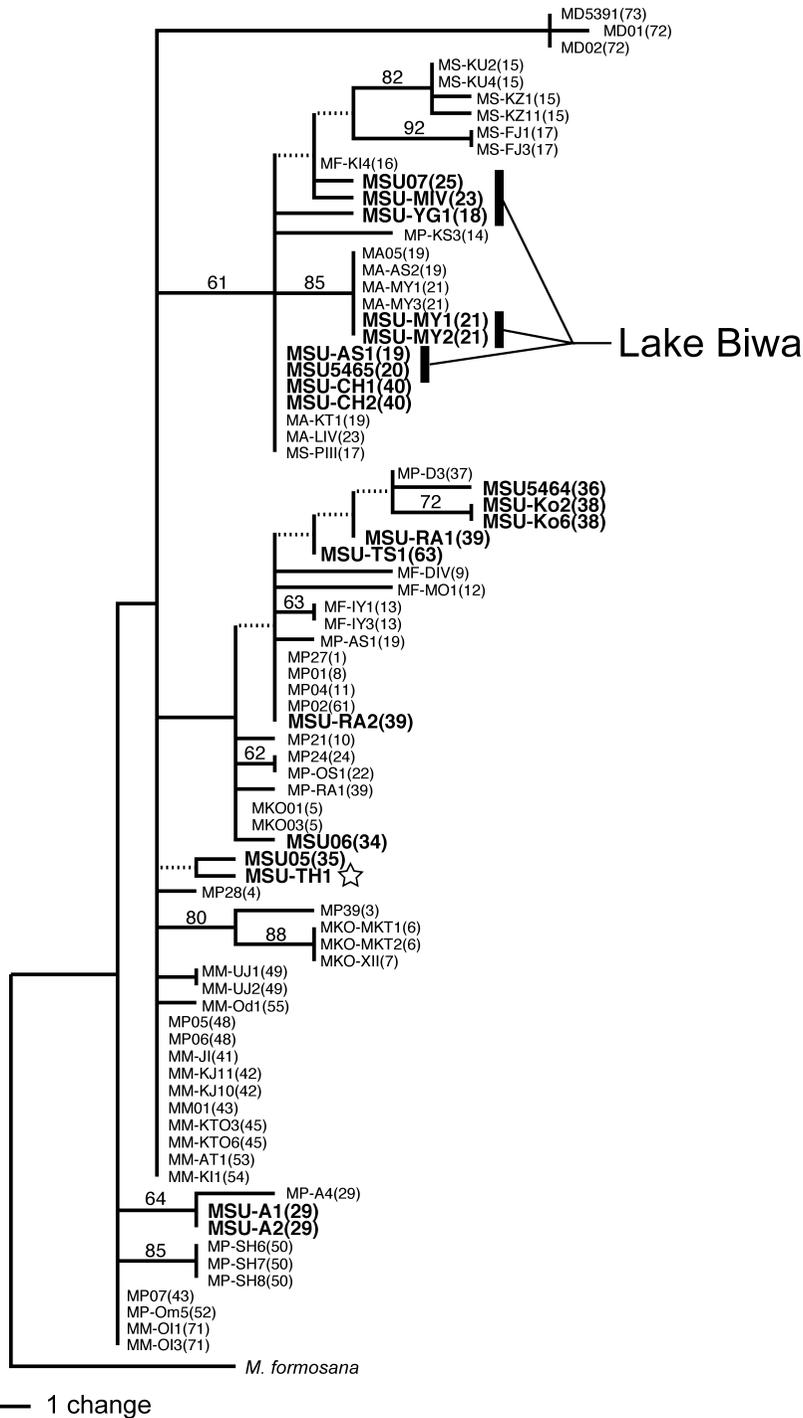
Fig. 3. One of the maximum parsimony (MP) trees of the combined plastid *trnH-psbA* and *matK* dataset (Tree Length for ingroup = 73). The number above branches are bootstrap supports. Branches that collapse in the strict consensus tree are shown by dashed lines. The taxon names are abbreviated as following; *M. acerina*: MA, *M. doiana*: MD, *M. furusei* var. *furusei*: MF, *M. furusei* var. *subramosa*: MSU, *M. koshiensis*: MKO, *M. pauciflora*: MP, *M. stylosa* var. *stylosa*: MS, *M. stylosa* var. *makinoi*: MM

extension at 72℃ for 1–2.5 min (depending on the length of the target DNA regions). The amplified DNA were treated with ExoSAP-IT (GE Healthcare, USA) and directly sequenced using an ABI 3130 DNA sequencer and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA). The oligonucleotide primers used for PCR and nucleotide sequencing are those described elsewhere (Okuyama and Kato, 2009; Okuyama *et al.*, 2012).

For phylogenetic analysis, the nucleotide sequences of two plastid DNA regions, *trnH-psbA* and *matK*, and three nuclear DNA regions, nuclear ribosomal ETS and ITS, and the two duplicated copies of granule-bound starch synthase I-A (*GBSSI-A0* and *GBSSI-A1*) were newly generated from a individual (MSU-TH1) of *M. furusei* var. *subramosa* from the "Tsushima-cho" population. To evaluate genetic variation of the population, additional 1, 2, and 5 individuals were also sequenced for plastid DNA, ETS and ITS, and the latter 1.5-kbp regions of *GBSSI-A0,* and *GBSSI-A1*, respectively. In addition, the nucleotide sequences of *GBSSI-A0* and *GBSSI-A1* were also sequenced from an individual each of *M. furusei* var. *furusei* from population No. 16 (MF-KI4; Fig. 1, Table 1) and *M. furusei* var. *subramosa* from population No. 29 (MSU-A1; Fig. 1, Table 1). The nucleotide sequences newly generated in this study were deposited in DDBJ/Genbank under accession numbers AB690312-AB690321.

Four datasets, plastid DNA, nuclear ribosomal DNA, *GBSSI-A0*, and *GBSSI-A1*, which include all the available DNA sequences of *Asimitellaria* species (Okuyama and Kato, 2009; Okuyama *et al.*, 2012; and present study), were prepared for phylogenetic analyses. As the aim of this study is to understand the phylogenetic position of the "Tsushima-cho" population of *M. furusei* var. *subramosa*, only the *Asimitellaria* taxa closely related to *M. furusei* var. *subramosa* were included. That is, for plastid DNA dataset, *M. furusei*, *M. pauciflora*, *M. koshiensis*, *M. acerina*, *M. stylosa*, and *M. doiana* were included as ingroup, and for nuclear DNA dataset, *M. furusei*, *M. pauciflora*, *M. koshiensis* were included as ingroup. Others were used as outgroups. Alignment gaps were coded as separate characters by using the methods of Simmons and Ochoterena (2000). Phylogenetic tree searches was conducted using PAUP*4.0b10 (Swofford, 2003) under the maximum parsimony criterion with exactly the same settings as in Okuyama and Kato (2009).

Results

Plastid DNA phylogeny

A phylogenetic analysis of plastid DNA dataset with 1679 aligned characters resulted in the maximum parsimony (MP) trees with almost identical topology to that of Okuyama and Kato (2009), except for the inclusion of MSU-TH1. Figure 3 illustrates one of the MP trees with tree length of 73 steps. Although the resolution of the tree was low, *M. furusei* var. *subramosa* collected from around Lake Biwa were only grouped in a clade with 61% bootstrap support, although MSU-TH1 was not grouped in the clade. Moreover, MSU-TH1 was found to have a unique nucleotide sequence.

Nuclear ribosomal ETS and ITS phylogeny

A phylogenetic analysis of nuclear ribosomal ETS and ITS DNA dataset with 1173 aligned characters resulted in MP trees with almost identical topology to that of Okuyama and Kato (2009), again except for the inclusion of MSU-TH1. Figure 4 illustrates one of the MP trees with tree length of 55 steps. The tree separates *M. furusei* var. *subramosa*into the four distinct groups, i.e., Lake Biwa, Japan Sea-1, Japan Sea-2, and Kii Peninsula, although the "Kii Peninsula" group was not supported as a monophyletic group. Obviously, MSU-TH1 had a unique sequence similar to those of the "Kii Peninsula" group, suggesting the affinity of the population to "Kii Peninsula" group.

MP01(8)
MP21(10)
MP-KS3(14)
64 ┌ MP04(11)
└ MF-KI4(16)
MSU5464(36)
57 ┌ **MSU-CH1(40)**
├ **MSU-CH2(40)**
├ **MSU-TRN1(62)**
└ **MSU-TS1(63)**
84 ┌ **MSU-RA1(39)**
└ **MSU-RA2(39)**

Japan Sea-1

MKO01(5)
MKO03(5)
63 MKO-M5(5)
MKO-MKT1(6)
MKO-MKT2(6)
MKO-XII(7)
MP-A4(29)
MP-Om5(52)
60 MP-SH6(50)
MP-SH8(50)
MP-RA1(39)
MF-IY1(13)
MF-IY3(13)
MSU05(35)
MSU06(34)
57 **MSU-A1(29)**   Kii Peninsula
MSU-A2(29)
MSU-TH1☆
89 ┌ MF01(12)
├ MF-MO1(12)
└ MF-DIV(9)
MP-SH7(50)

MP02(61)
MP03(19)
MP24(24)
MP-AS1(19)
MP-OS1(22)
63 ┌ MP05(48)
└ MP06(48)
MP-D3(37)
MP27(1)
51 MP31(2)
MP28(4)
MP39(3)

MSU01(19)
MSU02(24)
MSU07(25)
MSU-YG1(18)
MSU04(19)
MSU-MIV(23)
MSU-AS1(19)
63 **MSU-MY1(21)**
MSU-MY2(21)
MSU5465(20)
MSU03(19)

Lake Biwa

72

74

MP07(43)
MP22(49)
MSU023N(38)
89 ┼ **MSU-Ko2(38)**   Japan Sea-2
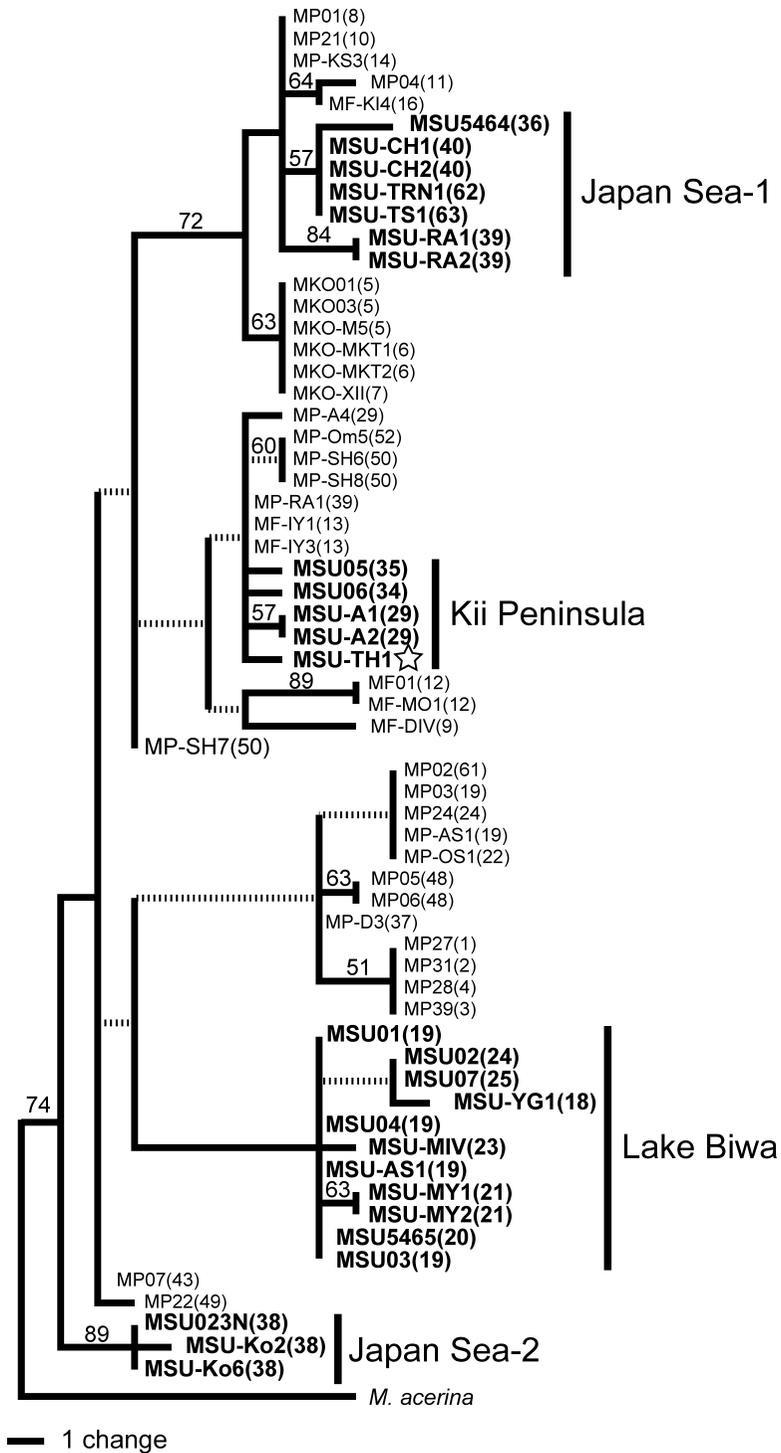**MSU-Ko6(38)**
M. acerina

━ 1 change

Fig. 4.   One of the maximum parsimony (MP) trees of the nuclear ribosomal ETS and ITS dataset (Tree Length = 55 for ingroup). The populations of *M. furusei* var. *subramosa* are separated into four phylogenetic groups, i.e., Lake Biwa, Kii Peninsula, Japan Sea-1, and Japan Sea-2, respectively. Other descriptions are as in Fig. 3.
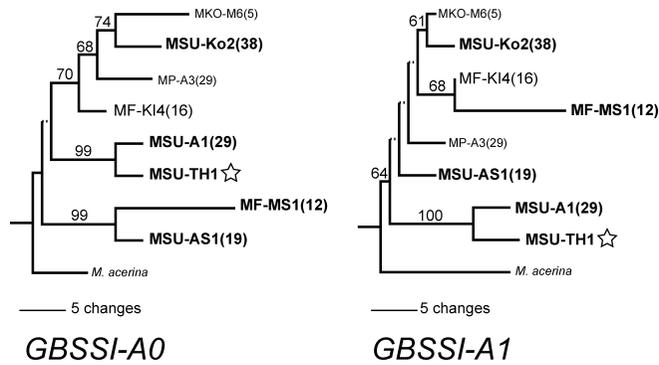
Fig. 5.   One of the maximum parsimony (MP) trees of the *GBSSI-A0* dataset (Tree Length = 67 for ingroup) and the *GBSSI-A1* dataset (Tree Length = 50 for ingroup). Other descriptions are as in Fig. 3.

Nuclear GBSSI-A0 and GBSSI-A1 phylogeny

Phylogenetic analyses of *GBSSI-A0* and *GBSSI-A1* DNA datasets with 2405 aligned characters resulted in MP trees as shown in Fig. 5. In these datasets, the *M. furusei* var. *subramosa* plants from the three (Lake Biwa, Japan Sea-2, and Kii Peninsula) out of the four distinct groups of Fig. 4 were represented with MSU-AS1, MSU-Ko2, and MSU-A1, respectively. MSU-TH1 again had unique sequences, and formed sister relationships to MSU-A1 in both of the MP trees with high bootstrap supports (99% and 100%, respectively), again suggesting the affinity of the population to "Kii Peninsula" group.

Genetic variation

Although we sequenced >1.5-kbp of plastid DNA for 2 individuals, >1.1-kbp of nuclear ribosomal ETS and ITS DNA for 3 individuals, and >1.5-kbp of *GBSSI-A0* and *GBSSI-A1* for 6 individuals of *M. furusei* var. *subramosa* from "Tsushima-cho" population, no genetic variation or heterozygosity was detected. This was contrasting especially with the relatively large genetic diversity often observed in *GBSSI-A0* and *GBSSI-A1* as in MF-KI4, in which each of these loci was heterozygous with 10 and 1 SNPs, respectively.

Discussion

Biogeographic perspectives

The "Tsushima-cho" population of *M. furusei* var. *subramosa* is remarkably isolated from other populations, being about 200 km distant from the nearest population (Fig. 1). Because of the low dispersal ability (hydrochory; Savile, 1953) and strict riparian habitat requirement of the *Asimitellaria* plants, the genetic profile of the isolated population likely reflects an intriguing biogeographic history, and thus motivates the present study. By a fairly intensive genetic characterization of the population, we revealed that the population has a unique genetic profile, which rules out the possibility of the origin of the population by an artificial introduction. Despite an extensive sampling of the *M. furusei* populations, no nucleotide sequence was found to be identical to that of the "Tsushima-cho" population in total of the five DNA regions sequenced, suggesting "Tsushima-cho" population has experienced a distinct genealogical history which could be at least traced back to 0.2 Mya, if we simply apply a base substitution rate of 3.0×10^{-9} s/s/y (Wolfe *et al.*, 1987) for the entire sequenced plastid DNA. Moreover, the present phylogenetic analyses suggested its close affinity to the populations from Kii Peninsula, Honshu ("Kii Peninsula" group; Fig. 4 and 5). This result suggests the biogeographic link of the plants along with the Pacific Ocean side.

Various studies have suggested that the gene flow of the plant species with hydrochory is restricted within a river system (Liao and Hsiao, 1998; Keller, 2000; Pollux *et al.*, 2007). This view is contrasting with the present observation, where obviously no river system interconnects between the populations of Kii Peninsula and "Tsushima-cho." Apart from the long-range dispersal hypothesis, a plausible explanation of the genetic link between the population is to attribute it to an ancient river system during the late Pleistocene (from 60 to 10 kyr ago), when a land bridge connected Honshu, Shikoku, and Kyushu islands. The ancient Kitan River system, which ranges across Shikoku and western Honshu to near Kii Peninsula, is the candidate that interconnected the populations. In a study of hydrochorous riparian shrub species, *Rhododendron ripense* (Ericaceae), a strong association between the genetic structure among populations and the ancient river system was observed (Kondo *et al.*, 2009). Therefore, the plants with the similar habitat requirement, *M. furusei* var. *subramosa*, could have the similar biogeographic history to that of *R. ripense*. More extensive and comprehensive survey of the population genetic structures of *M. furusei* var. *subramosa* would be needed to examine this hypothesis.

Urgent conservation needs

In Red Data Book Ehime (Ehime Prefecture, 2003), *M. furusei* var. *subramosa* is ranked as CR, and need for the conservation of the habitat environment is emphasized, yet it is also noted as "there is no immediate threat of extinction". However, as eight years have passed since the publication of the book, the situation has changed. Now, no flowering individual can be found in the population (M. Hyodo, personal communication), and the population number is obviously in decline, leaving only ＜10 individuals. This is probably because of the closure of the forest canopy after the move of a hotspring lodge (Haraigawa-onsen) nearby the population on the year 1998. Deer browsing might be also the cause of the population decline, as is observed in

many forest floor vegetations in Japan (Takatsuki, 2009).

The immediate threat of the population is also confirmed in the present study by the low genetic diversity of the population, where no apparent genetic variation was observed among the ＞4.1 kbp DNA region. Although at present there is no quantitative measure of the genetic diversity of the sequenced regions in the other populations, it would be reasonable to mention that the population is poor in genetic diversity, as we often see the genetic variation of these sequences in a population or even in an individual (heterozygosity) of the *Asimitellaria* plants (Okuyama and Kato, 2009; Y. Okuyama unpublished data). Apparently the remaining plants in the population are no longer self-sustainable, thus we have decided to conserve them *ex situ*. One of the author (YO) collected three plants from the population and introduced them in the nursery of Tsukuba Botanical Garden. A month has passed since we transplanted them to pots with well-fertilized soil. The plants have grown well and now they are large enough to bear fruits in the next fruiting season, in which we are expecting a successful propagation of the plants for the possible future re-introduction. However, this effort would be useless unless the remediation of the original environment is made, for example by thinning of the *Cryptomeria* forest. Urgent actions are needed to conserve this unique and biogeographically important but isolated population of the species.

Acknowledgments

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