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Genetic Diversity in Multilocus Nuclear Genes of *Phyllodoce nipponica* (Ericaceae)

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Abstract To find useful loci for phylogenetic as well as phylogeographic studies of *Phyllodoce nipponica* (Ericaceae), I sequenced 16 loci of previously reported nuclear genes from 12 individuals covering the entire range of this species. Twelve of 16 loci were polymorphic, in which nine loci harbored informative for unraveling intra-specific genetic structure. In addition, the genetic relationship among 12 individuals apparently contradicted the expectation from previous phylogeographic study based on cpDNA.

Key words: alpine plants, endemic, nuclear genes, phylogeography.

Introduction

Phylogeography, where revealed a current geographic distribution of genetic variations mostly within a species, is one of approaches for understanding a biogeographic history (Avise, 2000). The current geographic structure could lead to inferring a history of range shifts following the Pleistocene climatic oscillations; i.e., location of refugia and colonization routes (e.g., Taberlet *et al.*, 1998). Furthermore, more detailed history such as historical introgression and population admixtures could be also demonstrated by the phylogeographic investigation (Petit *et al.*, 2003).

Traditionally, phylogeographic studies relied exclusively on genetic variations of chloroplast DNA (cpDNA; e.g., Comes and Kadereit, 1998) because the maternal inheritance of cpDNA is appropriate for unraveling a biogeographic history via seed dispersal. However, cpDNA haplotypes have been sometimes shared with closely related or distantly related species due to introgression (e.g., Kikuchi *et al.*, 2010; Pelser *et al.*, 2012). In the case of cpDNA capture followed by introgression, a genetic structure such as phylogenetic trees was inconsistent between genes in cpDNA and nuclear DNA (nDNA; Rieseberg and Soltis, 1991). In addition, the geographic structure of genes would be shaped by somewhat stochastic processes by genetic drifts (Rosenberg and Nordborg, 2002), and therefore, geographic structure elucidated by a single locus such as haplotype of cpDNA may bias inferring a biogeographic history (Hickerson *et al.*, 2010 and references therein). Consequently, multilocus investigations are desirable for understanding population divergence and geographic structure (Schaal *et al.*, 1998; Hewitt, 2001), and obtaining genetic variations from nDNA was a common approach (Schönswetter *et al.*, 2005; Eidesen *et al.*, 2007; Bai *et al.*, 2010). Notably, sequencing multilocus nDNA could more precisely elucidate a number of independent genealogies and be appropriate for phylogeographic studies based on demographic parameters and including hypotheses testing (Knowles, 2009).

In the phylogeography in the Japanese alpine

plants, unambiguous genetic differentiations in cpDNA between northern and southern Japan were found from many species (Fujii and Senni, 2006; Ikeda *et al.*, 2006, 2008, 2009), by which a distinct history of Pleistocene range shifts were inferred for each region. In contrast, two studies did not find such an unambiguous geographic differentiation, where a few haplotype dominantly distributed throughout the Japanese archipelago (*Arcterica nana*: Ikeda and Setoguchi, 2006; *Phyllodoce nipponica*: Ikeda and Setoguchi, 2007). According to the previous study, a single migration event in the latter two species may result in the current range in the Japanese archipelago. Nevertheless, these minor genetic structures in cpDNA may not precisely represent biogeographic histories, but be shaped by stochastic effects for haplotype distribution. Previous AFLP investigation confirmed a homogenous genetic structure in haplotypes of *Arcterica nana* by using genetic variations throughout genome (Ikeda and Setoguchi, 2009). In contrast, any studies have never been conducted to evaluate the unique genetic structure of *Phyllodoce nipponica*. According to the previous study (Ikeda and Setoguchi, 2007), this species harbored a widespread haplotype along with high genetic diversity in central Japan as well as unique haplotypes in western Japan. Thereby, persisting populations throughout Pleistocene range shifts were inferred in central and western Japan, while populations in northern Japan would be originated by colonization from central Japan. To understand the biogeographic history of alpine flora throughout the Pleistocene range shifts, the genetic structure and subsequent biogeographic history of *P. nipponica* was further clarified by multilocus investigation.

In this study, I sequenced multiple nuclear genes of *Phyllodoce nipponica* to find polymorphic markers that were appropriate for further phylogeographic and phylogenetic studies. The previously published EST of *Rhododendron* (Wei *et al.*, 2005) and phytochrome genes in Ericaceae (Ikeda and Setoguchi, 2010) were used for designing primers. Furthermore, geographic structure of nuclear genes was tentatively depicted using the polymorphic loci to provide insights into further investigations.

Materials and Methods

DNA experiments

Twelve locations were selected, which can represent the entire range of *Phyllodoce nipponica* (Fig. 1). DNA samples for PCR were extracted from dried leaves that were collected in previous study (Dai, Iid, Shi, Kis, Yat, Hak, Ohm, and Dsn; Ikeda and Setoguchi, 2007) or for the present screening (Por, Aki, Nyo, Asa) using DNeasy Kit (Qiagen, Hilden, Germany). One individuals of each population were used.

Genetic variations were evaluated for 16 loci that were originated from EST for *Rhododendron* (B1, B2, B3, B4, B6, B7, C11, C12, C16, C18, C20, C26, and C35: Wei *et al.,* 2005) and phytochrome genes (*PHYB* and *PHYE*: Ikeda and Setoguchi, 2010; *PHYA*: originally designed by degenerate PCR). These loci were selected because electropherograms of sequences were unambiguously detected, when sequencing one or two individuals for most of the ESTs (Wei *et al.,* 2005). PCR amplification was conducted using fast reaction polymerase (SapphireAmp® Fast PCR Master Mix; TAKARA Bio, Shiga, Japan) following the manufacturer's protocol, with the exception of the final reaction volume (4 μL in this study). Amplification was performed with an initial denaturation for 2 min at 98℃ followed by 40 cycles of denaturation for 10 s at 98℃, annealing for 5 s at 56℃, and extension for 15 s at 72℃. After purification of PCR product using ExoSAP-IT (USB corporation, Ohio, USA), PCR products were directly sequenced using an ABI 3130 Genetic Analyzer (POP-7 polymer and 50-cm capillary; Applied Biosystems, Foster City, CA). The entire sequences were determined using both directions. For the efficiency of further multilocus investigation, original primers were designed for most loci (Table 1), where whole sequencings between forward and reverse primers can be completely

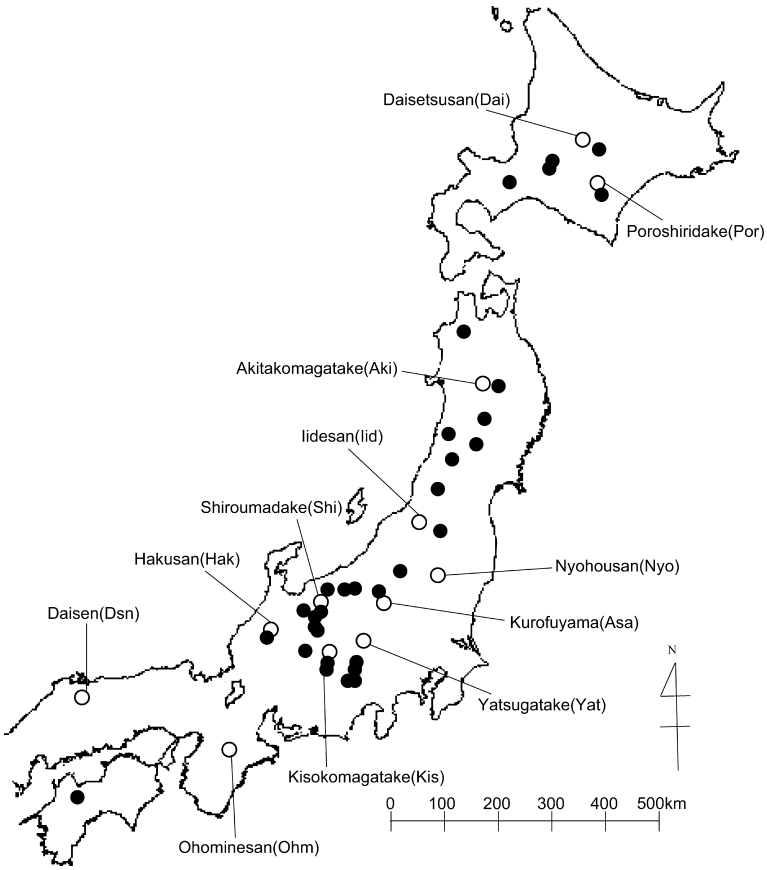

Fig. 1. A map of distribution of *Phyllodoce nipponica*. The black circles indicated the distribution of this species. White with names of location followed by abbreviation in parentheses represented populations in the present screening.

determined from each direction. Assembly and alignment were performed using CLC DNA Workbench (CLC bio, Aarhus, Denmark). In total, approximately 5,619 bp from 16 loci (ca. 160–521 bp per locus) were analyzed. Sequences with singleton or heterozygous sites were confirmed by multiple rounds of PCR and sequencing.

Data Analysis

Alleles of each locus were determined directly. If electropherograms of sequences showed two or more dual peaks, combinations of polymorphisms and subsequent haplotype phases of each locus were determined by Bayesian statistical methods (Stephens *et al.,* 2001; Stephens and Donelly, 2003).

Following summary statistics of each locus were calculated using DnaSP ver. 5.0 (Librado and Rozas, 2009); the number of polymorphisms and alleles, gene (haplotype) diversity, nucleotide diversity (Nei, 1987) and Tajima's *D*. The significant deviation from expected values under neutral equilibrium was evaluated by 10,000 coalescent simulations implemented by DnaSP.

Maximum-likelihood (ML) tree was estimated to reveal the genetic relationship among individuals. Sequences from 16 loci were concatenated with separate partitions for each locus. Substitution models for each partition were estimated using TREEFINDER (Jobb *et al.*, 2004). The significance of nodes was evaluated by 1,000

Table 1. Primers for the present screening

Locus	Primer Name	Primer sequences	Application*	Reference
B1	ErB1-F	AGCCTACGGAGAGGTCATCA	P and S	Wei *et al.*, 2005
	ErB1-R	CAATCTCCTGTGCTCTGCAA	P and S	Wei *et al.*, 2005
B2	ErB2-F	AACCCTCGTGGAAGTCCTTT	P and S	Wei *et al.*, 2005
	ErB2-R	TCGGCTATAGTTTGGGCTTC	P and S	Wei *et al.*, 2005
B3	ErB3-F	CTGATAACAGTGAGGATCGGA	P and S	Wei *et al.*, 2005
	ErB3-R	TTCCATGCTGGAATGAGAG	P and S	Wei *et al.*, 2005
B4	ErB4-F	ATTTCTGCCGCTTCCGTTAT	P and S	Wei *et al.*, 2005
	ErB4-R	TGCTACAACCAGCCCATAAA	P and S	Wei *et al.*, 2005
B6	ErB6-F	CCAGGTACTGTCGTGGTGTG	P and S	Wei *et al.*, 2005
	ErB6-R	AGCGCACGAAAGCATCTTAT	P and S	Wei *et al.*, 2005
B7	ErB7-F	GATGAACCTTTGGCTCTGGA	P and S	Wei *et al.*, 2005
	ErB7-R	CACGAACGAGGAACATGGTA	P and S	Wei *et al.*, 2005
C11	ErC11-F	AACCCGAGCATCTTACATGG	P and S	Wei *et al.*, 2005
	ErC11-R3	GATATACCTTGTGGCAGT	P and S	Original
C12	ErC12-F2	GGATAGTTGCTCAACTTCTATGT	P and S	Original
	ErC12-R	CAGTTCTTCAATCCCCATGC	P and S	Wei *et al.*, 2005
C16	ErC16-F	CATGAGACAAGCAGGGAGGT	P and S	Wei *et al.*, 2005
	ErC16-R	CCCCATCAGGCTTAAAAACTT	P and S	Wei *et al.*, 2005
C18	ErC18-F	CGAGGTGACACAACTGGATG	P and S	Wei *et al.*, 2005
	ErC18-R	CGCCATATTGAAGCGAACTC	P and S	Wei *et al.*, 2005
C20	ErC20-F2_2	CATCCCACTAATGCTCAA	P and S	Original
	ErC20-R	AAGTTGATGTTCCGGTTGTTG	P and S	Wei *et al.*, 2005
C26	ErC26-F	GAGAGCGGTCCTGAAGTTTG	P and S	Wei *et al.*, 2005
	ErC26-R	GACAGCCAATGCTGTGCTTA	P and S	Wei *et al.*, 2005
C35	ErC35-F	ATGCTGAGGATTCCAGTGCT	P and S	Wei *et al.*, 2005
	ErC35-R	GCACTAGGAAGGTGGAGACG	P	Wei *et al.*, 2005
	ErC35-R2	CCAAAGTATCACTAAGGCCT	S	Original
PHYA	ErPHYA-F	TGCTTATTGGCTCTCGAT	P and S	Original
	ErPHYA-R	AGACTGCAAGCGGGTA	P and S	Original
	degA-F1	CTSCATGCWGAYTTTGAGGAGTCAGG	dPCR	Original
	degA-R1	GTGAGYTCAAAAACYTCYTGAACCATW-GTRTCA	dPCR	Original
PHYB	ErPHYB-F	GTTTGCCATCACACTTCTGCT	P and S	Ikeda and Setoguchi, 2010
	ErPHYB-R	GAAGAACGTGGATGCATCCT	P and S	Ikeda and Setoguchi, 2010
PHYE	ErPHYE-F	GCAGTGCAATCACAGAAACT	P and S	Ikeda and Setoguchi, 2010
	ErPHYE-R	GACCCCATGTTGGCCATGTA	P and S	Ikeda and Setoguchi, 2010

*Application: P, S and dPCR indicate primers used for PCR amplification, sequencing, and degenerate PCR amplification, respectively.

bootstrap resamplings using TREEFINDER.

Results

Twelve of 16 loci harbored at least one polymorphism among the present samples (Table 2). Among these polymorphic loci, all polymorphisms in three loci were singleton that occurred exclusively in one sample (C26, *PHYA*, and *PHYB*; $S = S_S$ in Table 2). Genetic variations were variable among polymorphic loci ($\pi =$ 0.0005–0.0075). The Tajima's *D* was not significantly deviated from zero.

Genetic relationship among individuals was shown in un-rooted ML tree (Fig. 2). All individuals except for Asa and Nyo were distinguished from each other. Individuals in northern Japan (Dai, Por, Aki) were highly diverged from remaining ones (bootstrap 100%). In addition, individuals in Kanto region, Asa and Nyo, harbored unique genetic structure (bootstrap 100%).

Discussion

The present screening of multilocus sequencing of *Phyllodoce nipponica* found a number of

Table 2. Summary statistics of each locus

Locus	*n*	bp	*S*	S_s	n_h	*H*	π	*D*
B1	24	186	3	0	3	0.681	0.0071	1.614
B2	24	287	0	0	1	0.000	0.0000	n.a.
B3	24	160	3	0	3	0.594	0.0067	0.843
B4	24	161	0	0	1	0.000	0.0000	n.a.
B6	24	220	0	0	1	0.000	0.0000	n.a.
B7	24	235	0	0	1	0.000	0.0000	n.a.
C11	24	419	2	0	3	0.598	0.0016	0.583
C12	24	440	9	2	4	0.681	0.0075	1.196
C16	24	477	1	0	2	0.431	0.0009	1.027
C18	24	359	2	1	3	0.507	0.0015	0.062
C20	24	480	6	0	6	0.801	0.0049	1.443
C26	24	441	3	3	3	0.236	0.0009	−1.256
C35	24	521	7	3	6	0.786	0.0038	0.179
PHYA	24	353	1	1	2	0.159	0.0005	−0.681
PHYB	24	510	2	2	3	0.304	0.0006	−0.890
PHYE	24	370	4	2	4	0.569	0.0030	0.059

n, number of sequences for analysis; bp, length of sequences for analysis; *S*, number of segregating sites; S_s, number of singletons; n_h, number of alleles; *H*, gene (haplotype) diversity; π, average number of pairwise nucleotide differences per site calculated based on all sites; *D*, Tajima's *D*.

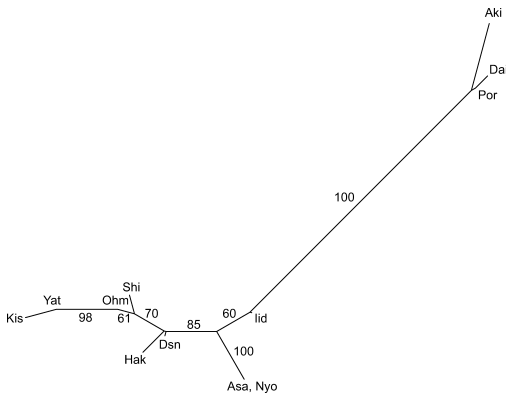

Fig. 2. An un-rooted genetic relationship among individuals that estimated by maximum-likelihood approach using sequences concatenating 16 loci. Numbers along branches indicate bootstrap values based on 1,000 resamplings.

useful loci for phylogenetic as well as phylogeographic studies. Because singleton polymorphisms harbored little information to elucidate a geographic structure, remaining nine loci would be good candidates for future investigation. Furthermore, the present screening of polymorphisms in nuclear genes exhibited an inconsistent geographic structure with a previous phylogeography based on cpDNA (Ikeda and Setoguchi, 2007). Reconciling the contradiction would elucidate a notable biogeographic history of this endemic species as well as of Japanese alpine flora. The present candidate loci could contribute to further studies.

Insights into geographic structure of nDNA

According to genetic structure shown by ML tree (Fig. 2), individuals in northern Japan (Dai, Por, Aki) were unambiguously distinguished from remaining ones in central and western Japan. The previous study revealed that this species harbored a dominant haplotype, haplotype A, throughout the entire range, while a number of haplotype was detected in central Japan (Ikeda and Setoguchi, 2007). Although the previous study detected significant differentiations among regions (12.1% of total variations), any unambiguous geographic structure was not noted in northern Japan. Thus, the present unique genetic structure of nDNA in northern Japan contradicted the previous structure of cpDNA.

Because all loci were following neutral expectation based on Tajima's *D*, natural selection was difficult to explain the unique genetic structure in northern Japan. A plausible explanation of the contradiction is introgression. The inconsistent genetic structure between cpDNA and nDNA was generally interpreted as introgression, where cpDNA was introgressed from other species and replaced by them (Rieseberg and Soltis, 1991). *P. nipponica* shares its distribution with *Phyllodoce aleutica* and *Phyllodoce caerulea* in both central and northern Japan and northern Japan, respectively. However, if introgression occurred from these relatives in either region, unique genetic structure would be exhibited in cpDNA. In contrast, *P. nipponica* harbored a homogenous geographic structure in cpDNA, while north-south differentiation in nDNA. Therefore, introgression from closely related species could unlikely explain the inconsistent genetic structure.

In contrast, introgression between two lineages

in *P. nipponica* would be more plausible; i.e., northern or southern lineages of *P. nipponica* have introgressed into another lineage, resulting in the cpDNA replacement as well as homogenous geographic structure. According to the northward migration inferred in the previous study (Ikeda and Setoguchi, 2007), introgression from the former inhabitant may have occurred in northern Japan following the postglacial colonization. If so, populations in northern Japan would be persisted throughout Pleistocene climatic oscillations. Although persistence of unique populations in northern Japan was consistent with biogeographic histories of most alpine plants (Fujii and Senni, 2006; Ikeda *et al.*, 2006, 2008, 2009), this history had never been postulated from a previous study. Accordingly, the present unique genetic structure in northern Japan may give a notable insight of biogeography of this species as well as Japanese alpine flora.

However, individuals in Aki and Por were not included in the previous study. This inconsistent sampling strategy may cause the presently distinct geographic structure. In addition, the previous study included only a small number of samples in this region, especially in Dai ($n = 3$) and Yuubari ($n = 1$), and therefore, may have not precisely elucidated the genetic structure in northern Japan. Consequently, further investigation including a number of samples and populations is necessary to evaluate the inconsistent genetic structure between cpDNA and nDNA.

In contrast to northern Japan, western Japan harbored unique haplotypes in the previous study (Ikeda and Setoguchi, 2007). Although the present study included a sample from Dsn, it was not specifically distinguished from others. Given that the western Japan did not harbor any closely related species, alternative hypothesis other than introgression would be needed for explaining the contradiction. The first hypothesis is that new mutations may have occurred in cpDNA after colonization. Alternatively, persisting populations were suffered from further colonization and introgression occurred from migrants into former inhabitant. These hypotheses are also examined by further study.

Accordingly, the present screening showed that geographic structure in nDNA was inconsistent with that of cpDNA. Polymorphic loci that determined in the present study could contribute to the future study for elucidating complete geographic structure of nDNA and re-examining the contradiction. An appropriate interpretation of both nDNA and cpDNA could give a novel insight into the biogeographic history of Japanese alpine flora.

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