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Congruence between Pollination Morphs and Genotypes Based on Internal Transcribed Spacer (ITS) Sequences of Nuclear Ribosomal DNA in *Cimicifuga simplex* (*Ranunculaceae*)

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Cimicifuga simplex (*Ranunculaceae*) has been classified into several morphs or races based on external morphology, pollinator guild, or genotype. However, no attempt has been made to elucidate whether the subdivisions based on the different characters are congruent or not. Here, we genotyped the three pollination morphs reported previously, and found that they were genetically differentiated and each morph consistently corresponds to one of the seven ITS genotypes described in a different study. The three morphs were distributed parapatrically or allopatrically along altitudinal gradients. In accordance with the genetic differentiation of the pollination morphs, the degree of pubescence of leaf margin was found to be a diagnostic external character to discriminate pollination morph II from morphs I and III.

Key words: Altitudinal distribution, *Cimicifuga*, ecological differentiation, ecotype, genetic differentiation, pubescence.

Cimicifuga simplex Wormsk. ex Turcz. (*Ranunculaceae*) is a perennial herb widely distributed in East and Northeast Asia (Nakai 1916, Emura 1970, Pellmyr 1986). It occurs from sea level to over 3000 m in Japan except for the Okinawa island chain (Pellmyr 1986, Shimizu 1997). It is 0.4–2.5 m tall, 50–100 small, white flowers are arranged in a simple raceme, and some shorter lateral racemes may occur (Tamura 1982, Pellmyr 1986, Shimizu 1997).

Cimicifuga simplex has been classified

into several morphs or races based on external morphology, pollinator guild, karyotype or genotype (Nakai 1916, Emura 1970, Pellmyr 1986, Shimizu 1997, Compton et al. 1998, Yamaji et al. 2005). However, there is little consensus on how the subdivisions should be circumscribed and no attempt has been made to elucidate whether the subdivisions based on the different characters are congruent or not.

Pellmyr (1986) grouped *C. simplex* into three pollination morphs based on their pollinator guilds around Nikko, central Honshu, Japan.

Morph I occurs at altitudes over 1300 m and typically reaches 1.5–2.5 m in height. Morph II occurs at or below 950 m and is as tall as morph I. This morph more often has more lateral racemes than the others. Morph III grows in very dark, moist habitats within and below the altitudinal range of morph II. This morph is less than 1.0 m tall. Morphs I and III are pollinated by bumblebees (Hymenoptera: Apidae) while morph II is frequently visited by fritillaries (Lepidoptera: Nymphalidae) which are attracted to a two-component fragrance of the flowers (Pellmyr 1986, Groth et al. 1987). As they are difficult to distinguish only by external morphology, Pellmyr (1986) delimited the pollination morphs comprehensively based on plant height, floral fragrance, flowering season, and so on. We regard this grouping based on pollination morph important because pollination morph may be related to reproductive isolation caused by differential pollinator fauna.

Yamaji et al. (2005) divided *C. simplex* into seven genotypes based on polymorphism of ITS sequences including four homozygote genotypes (types 1–4) and three heterozygote genotypes (types 1+2, 1+3, and 3+4). They further elucidated the distribution pattern of the genotypes as follows; genotype 1: central Honshu, genotype 2: from northern Honshu (Pacific Ocean side) to central Honshu and areas at high altitude in Shikoku and Kyushu, genotype 3: widely distributed throughout Japan, and genotype 4: in areas at low altitude in Shikoku, Kyushu and western Honshu. However, the concordance of the genotypes with the pollination morphs proposed by Pellmyr (1986) was not investigated.

Reproductive isolation could result from the formation of ecotypes adapted to different pollinator environment via the processes of geographical, temporal and ethological isolation (Grant 1949, Stebbins 1970, Campbell and Aldridge 2006). Indeed, Pellmyr (1986) discussed that *C. simplex* radiated along two different lines, in pollinator guilds and in

flowering season, and suggested the possibility of incipient speciation. However, he did not show whether the pollination morphs of *C. simplex* are genetically differentiated or not. Furthermore, Yamaji et al. (2005) did not relate their genotypes to Pellmyr's (1986) pollination morphs. In this study, we examine the concordance of the pollination morphs with the nuclear ribosomal DNA types. Additionally, we suggest that the degree of pubescence of leaf margin is a useful external character to identify one of the three pollination morphs.

Materials and Methods

Plant material

Field survey and sampling were conducted in the Norikura and Utsukushigahara highlands (Table 1, Fig. 1). In the Norikura highlands, Kuraigahara (KR) population was located at an elevation of 2330 m, Suzuran (SZ) population at 1450 m, and Inakoki (IN) population at 860 m. In Utsukushigahara highlands, Utsukushigahara Skyline (US) population was located at the elevation of 1480 m, Hinokisawa (HS) at 1350 m, Takabocchi (TB) at 1150 m, and Gakenoyu (GK) was at 980 m.

In a preliminary survey, we found the number of hairs on leaf margin was very different between morph II and the other morphs. Thus, we compared the degree of pubescence among the morphs. Three terminal leaflets from each of randomly selected plants in the seven populations were collected (33 plants at KR, 5 at US, 21 at SZ, 5 at HS, 22 at TB, 27 at IN, and 16 at GK), and the number of hairs per 5 mm length along leaf margin was counted for haphazardly selected three portions per leaflet under stereo microscope. The mean values were used as data for an individual plant.

Altitudinal distribution

The altitudinal distribution of the three morphs of *Cimicifuga simplex* was surveyed in August–October 2010–2012 along altitudinal gradient (800–2400 m) in Norikura highlands

Table 1. Pollination morphs, ITS genotypes and pubescence of leaf margin in *Cimicifuga simplex* collected from seven populations in central Honshu, Japan

Population	Number of genotyped plants	Pollination morph	Genotype of ITS	Floral fragrance	Flowering period	Major flower visitors
Norikura highlands						
KR	24	I	3	not sweet	August–September	bumblebees
SZ	10	II	2	sweet	September	butterflies
IN	6	III	1 + 3	not sweet	September–October	flies and syrphids
Utsukusigahara highlands						
US	5	I	3	not sweet	August	bumblebees
HS	9	II	2	sweet	September	butterflies
TB	15	II	2	sweet	September	butterflies
GK	13	III	1 + 3	not sweet	September–October	flies and syrphids
Population	Number of hairs per 5 mm length along leaf margin (mean ± SD)	Latitude (N)	Longitude (E)	Elevation (m)	DDBJ number	
Norikura highlands						
KR	1.1 ± 0.9	36°7'15"	137°34'18"	2330	AB777700–AB777723	
SZ	40.1 ± 7.2	36°7'18"	137°37'36"	1450	AB777724–AB777733	
IN	0.1 ± 0.3	36°9'48"	137°45'59"	860	AB777734–AB777739	
Utsukusigahara highlands						
US	1.5 ± 0.8	36°14'49"	138°3'16"	1480	AB777740–AB777744	
HS	54.5 ± 4.4	36°15'8"	138°2'27"	1350	AB777760–AB777768	
TB	47.0 ± 4.3	36°8'51"	138°1'19"	1150	AB777745–AB777759	
GK	0.1 ± 0.3	36°9'20"	138°0'40"	980	AB777769–AB777781	

and Utsukushigahara highlands in Nagano, central Honshu, Japan (Fig. 1). Among patchily distributed *C. simplex* populations, seven large populations located at elevations of 860 m, 1450 m and 2330 m in the Norikura highlands, and 980 m, 1150 m, 1350 m and 1480 m in the Utsukushigahara highlands were intensively surveyed (Table 1, Fig. 1). We discriminated pollination morphs by plant height, floral fragrance and flowering season (cf. Pellmyr 1986); morph I: reaches 1.5–2.5 m in height,

emits non-sweet floral scent, flowering occurs from mid August to early September, and is mainly visited by bumblebees, morph II: reaches 1.5–2.5 m in height, gives off strong sweet fragrance from flowers, flowering occurs throughout September, and is mainly visited by butterflies, morph III: is less than 1.0 m tall, emits non-sweet floral scent, flowering occurs from late September to early October, and is mainly visited by flies and syrphids. The determination of floral scent type was conducted

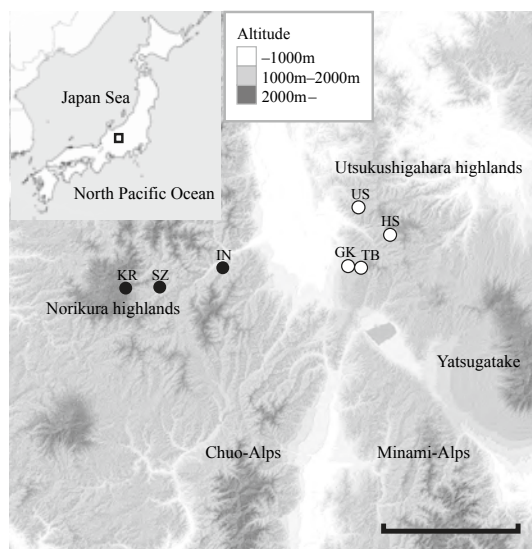


Fig. 1. Locality of the study sites in the Norikura highlands (black) and Utsukushigahara highlands (white), central Japan. Scale bar = 50 km. See text for details.

by more than one person.

Genotype determination

Five to twenty-four individuals were randomly sampled from each of the seven populations (Table 1), and total DNAs were isolated using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) from silica-gel dried leaf tissue, according to the manufacturer's protocol. The ITS region (215 bp of ITS1, 167 bp of 5.8S rDNA, and 186 bp of ITS2) were sequenced using primers Cs01 (TCG AAA CCT GCT TTG CAG AA) and Cs02 (AAC GGC TTC CAA GGG TTA TG) and Takara Ex Taq (Takara Bio, Shiga, Japan) for amplification by PCR. The following PCR temperature profile was used: (94°C, 3 min) × 1 cycle, (94°C, 1 min; 50°C, 1 min; 72°C, 2 min) × 25 cycles, and (72°C, 7 min) × 1 cycle. The PCR products were purified with ExoSap-IT (USB, Cleveland, Ohio, USA). We sequenced the purified PCR products using the BigDye Terminator Cycle Sequencing Kit ver. 1.1 and Model 3130 automated sequencer (ABI, Weiterstadt, Germany) following the

manufacturer's instructions. For sequencing, we used the same primers as those used for amplification. Sequences were aligned manually. All variant characters were compared in raw data on the automated sequencer, and the ITS genotype was determined as described in Yamaji et al. (2005) for each plant individual.

Results

In Norikura highlands, pollination morph I, II and III was found at elevations of 2330 m, 1450 m, and 860 m, respectively. In Utsukushigahara highlands, pollination morph I was found at the elevation of 1480 m, morph II at 1350 m and 1150 m, and morph III at 980 m. The number of hairs per 5 mm length along leaf margin was 1.1 ± 0.9 (mean \pm SD) in morph I, 47.4 ± 8.9 in morph II, and 0.1 ± 0.3 in morph III (Figs. 2 and 3, $P < 0.01$ for morph I vs. II, and for morph II vs. III, NS for morph I vs. III, Tukey's HSD test). Within each morph, the variation among populations were small (Table 1).

The congruence between pollination morphs and ITS genotypes was consistently detected. With no exceptions, morph I ($n = 29$) corresponded with genotype 3, morph II ($n = 34$) with genotype 2, and morph III ($n = 19$) with genotype 1+3 (Table 1). The other four genotypes (genotypes 1, 4, 1+2 and 3+4) described by Yamaji et al. (2005) were not detected in this study.

Discussion

Our results suggest that the three pollination morphs of *Cimicifuga simplex* detected by Pellmyr (1986) are genetically differentiated. The altitudinal distribution of the pollination morphs in the two highlands (ca. 50 km away from each other) were identical; morphs I, II and III distributed at high, medium and low elevations, respectively (Table 1). The same altitudinal distribution pattern of the morphs was observed by Pellmyr (1986) in Nikko (ca. 250 km away from Norikura highlands in this study). According to Yamaji et al.'s (2005) description

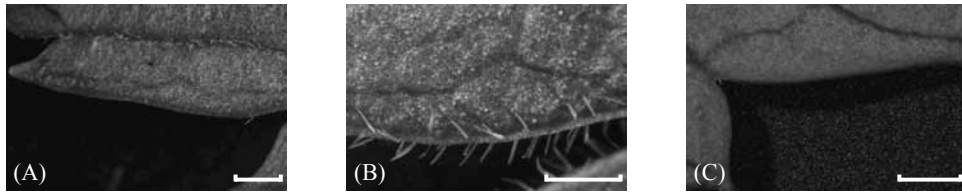


Fig. 2. Pubescence of leaf margin in pollination morphs of *Cimicifuga simplex*. Morph I (A) and morph III (C) are glabrous, and morph II (B) is pubescent. Scale bars = 1 mm.

of genotype distribution, it was suggested that there was a climatic correlation between horizontal distribution and vertical distribution of the three pollination morphs; morph I was found at high altitude in this study and is distributed in northern Japan as reported in Yamaji et al. (2005), morph III was seen at low altitude in this study and is distributed in southern Japan, and morph II occurred at comparatively high altitude in this study and is distributed in northern Honshu and disjunctively distributed at high altitude in southern Japan. The results of this study suggest that such allopatric distribution and differential pollinator fauna may have promoted the reproductive isolation between the types. Indeed, Yamaji et al. (2005) suggested reproductive isolation between genotype 2 (pollination morph II) and genotype 3 (morph I) because they lack heterozygote types. In accordance with the genetic differentiation of the pollination morphs, the degree of pubescence of leaf margin was found to be a diagnostic external character to discriminate pollination morph II from morphs I and III (Fig. 3).

In summary, the results suggest that *C. simplex* in central Japan is composed of at least three genetically differentiated pollination morphs and these morphs distribute parapatrically or allopatrically along altitudinal gradient. Further studies using more sensitive DNA markers will elucidate the history of divergence and hybridization among the three pollination morphs in Japan. Additionally, there is a need to confirm whether the pollination morphs of *C. simplex* occurring in other regions

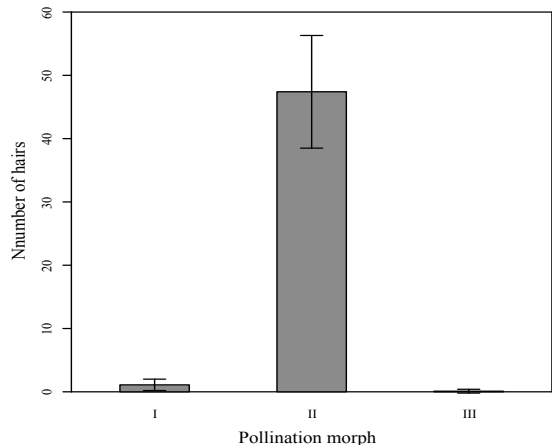


Fig. 3. Number of hairs per 5 mm length along leaf margin of the three pollination morphs (mean \pm SD). See text for details.

are similarly pollinated by those pollinators as described by Pellmyr (1986) and as found in this study.

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楠目晴花^a, 市野隆雄^{b,c}: サラシナショウマ (キンボウゲ科) における送粉型と核リボソーム DNA の ITS 領域に基づく遺伝子型の一致性

サラシナショウマは外部形態や送粉者ギルド、遺伝子型に基づいていくつかの型やレースに分類されてきた。しかしこれらの異なる形質に基づく分類が互いに一致しているかどうかはこれまで調べられていない。本研究では先行研究で報告された3つの送粉型について核DNAのITS領域の遺伝子型を特定し、それらが別の先行研究で見出された7タイプの遺伝子型とどう対応するかを調べた。その結果、3送粉型は3タイプの遺伝子

型にそれぞれ1対1で対応することが明らかになった。3送粉型は、標高傾度に沿って側所的または異所的な分布を示した。さらに、送粉型が遺伝的に分化していることに対応して、小葉周縁の毛の密度が送粉型I・IIIと送粉型IIを区別する形質であることが見出された。

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