

# 高知県産無毛型ネジバナ(ラン科) の系統的背景について

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Katsura Ito<sup>3</sup>, Shin-ichi Tebayashi<sup>3</sup>, Hiroaki Ikeda<sup>1</sup>, Tatsuya Fukuda<sup>3</sup>:  
**Phylogenetic background of a glabrous individual of  
*Spiranthes sinensis* var. *amoena* (Orchidaceae) collected in  
Kochi Prefecture, Japan**

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**Abstract**

*Spiranthes sinensis*, a terrestrial orchid, has morphological and ecological variations such as plant size, flowering season, floral colour, and hair density on the inflorescence stems and ovaries. *Spiranthes sinensis* var. *amoena* has been described as having puberulous inflorescence stems and ovaries, while these in *S. sinensis* var. *sinensis* are considered to be glabrous. In Japan, *S. sinensis* var. *amoena* grows on a wide area of the mainland (the Northern Ryukyus and northward). By contrast, the distribution of *S. sinensis* var. *sinensis* is limited to the Central and Southern Ryukyus. We found a glabrous individual of *S. sinensis* in Kochi Prefecture, Japan, which has identical DNA sequences of internal transcribed spacer (ITS) region of nuclear DNA and *trnL-F* intergenic spacer region of chloroplast DNA to *S. sinensis* var. *amoena*. Thus, this glabrous individual should be included in *S. sinensis* var. *amoena*.

**Key words** : hair, internal transcribed spacer (ITS), *Spiranthes sinensis* var. *amoena*, *trnL-F*

**Introduction**

The genus *Spiranthes* L.C.Rich. (Orchidaceae) is distributed in tropical to temperate regions and contains approximately 50 species (Satomi 1982; Xinqi et al. 2009). *Spiranthes sinensis* (Pers.) Ames var. *amoena* (M.Bieb.) H.Hara, a terrestrial orchid, has morphological and ecological variations such as plant size (*S. sinensis* var. *amoena* f. *gracilis* F.Maek., nom. nud), flowering season (*S. sinensis* var. *australis* (R.Br.) H.Hara et Kitam. ex. Kitam. f. *autumnus* H.Tsukaya), floral colour (pink, white: *S. sinensis* var. *amoena* f. *albescens* Honda, green: *S. sinensis* var. *amoena* f. *viridiflora* (Makino) Ohwi), floral size and shape, and density of hairs on the inflorescence stems and ovaries (Kitamura 1964; Maekawa 1971; Sawa 1980; Tsukaya 2005a). The varieties of *S. sinensis* have been studied taxonomically (Kitamura 1964; Hara 1969; Tsukaya 2005b), ecologically (Tsukaya 1994; Iwata et al. 2012), morphologi-

cally (Honda 1976; Sawa 1980; Natsume and Natsume 2002), geographically (Kitamura 1964; Hatusima 1968), and cytologically (Tanaka 1965; Tatarenko et al. 2010).

The presence or absence of hairs on the inflorescence stems and ovaries constitutes a diagnostic trait to distinguish 2 related varieties, *Spiranthes sinensis* var. *amoena* and *S. sinensis* var. *sinensis*, respectively (Hatusima 1968). *S. sinensis* var. *amoena* grows on a wide area of the Japanese mainland (the Northern Ryukyus and northward), and also in Korea, Taiwan, China, the far east of Russia, the Himalayas, southward to Malaysia, Indonesia, Australia, New Zealand, and the southwest Pacific (Tsukaya 2005b). By contrast, the distribution of *S. sinensis* var. *sinensis* is limited to Japan (the Central and Southern Ryukyus), Taiwan, and South China (Maekawa 1971; Satomi 1982). It is widely believed that the distributions of the 2 varieties are separated by the Tokara strait

in Japan (Hatusima 1968), but glabrous individuals of *S. sinensis* have been observed in the Japanese mainland (Sawa 1980; Odakura 1982; Tsukaya 2005a).

These glabrous individuals could be *S. sinensis* var. *amoena* which lost hairs (Tsukaya 2005a) and DNA sequences would be helpful to test this possibility. No variations are found in *trnL-F* intergenic spacer of the chloroplast DNA (cpDNA) of *S. sinensis* var. *amoena* (including *S. sinensis* var. *amoena* f. *gracilis* and *S. sinensis* var. *australis* f. *autumnus*) collected from a wide area of the Japanese mainland, but their *trnL-F* intergenic spacer sequences significantly differ from those of *S. sinensis* var. *sinensis* collected in Okinawa (Tsukaya 2005b).

In the present study, we report a glabrous in-

dividual of *Spiranthes sinensis* variety in Kochi Prefecture, Shikoku, Japan (Fig. 1 I–III). We show that this individual is the hair-loss type of *S. sinensis* var. *amoena* based on morphological and DNA sequence data.

#### Materials and methods

All individuals of *Spiranthes sinensis* varieties examined in this study were collected in the fields in late June to July 2012 (Table 1). We randomly collected a total of 1018 individuals from 7 localities (Nankoku, 265; Monobe River, 284; Kagami River, 148; Tsukuba, 122; Tateyama1, 98; Tateyama2, 82; and Iori, 19). Two populations (Nankoku and Monobe River in Nankoku City, Kochi Prefecture) were close to the locations where glabrous individuals of

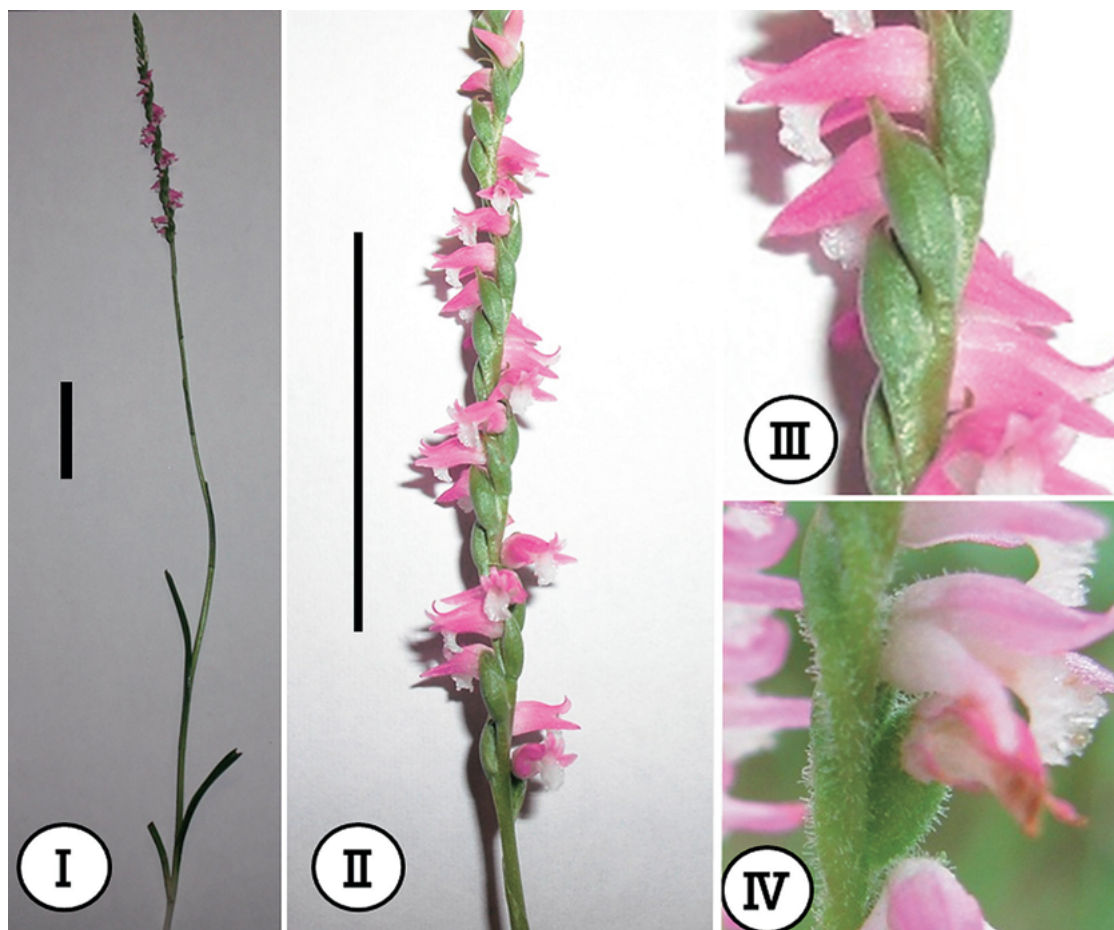


Fig. 1. Inflorescences of *Spiranthes sinensis* variety. I–III: plant with glabrous inflorescence stems and ovaries collected in Monobe River, Kochi Prefecture (Jun/28/2012; MBK0235286). Bar = 3 cm for I and II; IV: plant with a puberulous inflorescence stem and ovaries in Takayama city, Gifu Prefecture.

*S. sinensis* variety had previously been found (Sawa 1980; Kobayashi et al. 2009). We microscopically observed the hair density on the inflorescence stems, and categorized individuals into 4 groups: (1) much ( $>41/\text{mm}^2$ ); (2) less ( $21\text{--}40/\text{mm}^2$ ); (3) a few ( $>0\text{--}20/\text{mm}^2$ ); and (4) zero ( $0/\text{mm}^2$ ). A voucher specimen of glabrous *S. sinensis* (*H.Miyata and K.Ohga MBK0235286*) was deposited in the Herbarium at the Makino Botanical Garden, Kochi (MBK).

For the molecular analyses, we used a total of 4 individuals—2 from Monobe River (1 with and 1 without hairs) and 2 from Tsukuba (both with hairs). Total DNA was isolated from fresh leaves using a Plant Genomic DNA Mini Kit (Viogene, Sunnyvale, CA, USA), according to the manufacturer's protocol. We amplified the internal transcribed spacer (ITS) regions (ITS1, 5.8S rRNA, and ITS2) of nuclear DNA (nrDNA) with ITS4 and ITS5 primers (White et al. 1990) and the cpDNA *trnL-F* intergenic spacer with e and f primers (Taberlet et al. 1991). The isolated DNA was amplified by PCR in a 50- $\mu\text{L}$  reaction solution containing approximately 50 ng of total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM of each dNTP, 1.25 units of *Taq* DNA polymerase (Takara Bio Inc.,

Shiga, Japan), and 0.5  $\mu\text{M}$  of each primer. We applied the following thermal cycle profile for amplification, using a PCR Thermal Cycler Dice system (Takara): 1 min at 94  $^\circ\text{C}$ , 2 min at 48  $^\circ\text{C}$ , and 2 min at 72  $^\circ\text{C}$  for 45 cycles, followed by 15 min of final extension at 72  $^\circ\text{C}$ . After amplification, the PCR products of the ITS and *trnL-F* intergenic spacer regions were subjected to electrophoresis in 1.0% low-melting-temperature agarose gels, to purify amplified products. We sequenced the purified PCR products using a BigDye Terminator ver. 3.1 kit (Applied Biosystems, Foster, CA, USA) and an ABI Prism 3100 genetic analyser (Applied Biosystems), according to the manufacturer's instructions. The sequences of the nrDNA ITS regions and cpDNA *trnL-F* intergenic spacer region have been registered in the DDBJ/EMBL/GenBank International DNA databases as AB740173-6 and AB823666-9 (Table 3).

## Results and Discussion

The data for hair density of the inflorescence stems in *Spiranthes sinensis* variety are summarized in Table 2. In Monobe River, 259 (91.2%), 19 (6.7%), 5 (1.8%), and 1 (0.4%) individuals were categorized as much, less, a few,

Table 1. Localities of *Spiranthes sinensis* variety used in this study.

Population name	Locality	Latitude	Longitude	Number of Samples
Nankoku	Border between Nankoku city and Konan city, Kochi Prefecture	N33°55'	E133°67'	265
Monobe River	Monobe, Nankoku city, Kochi Prefecture	N33°55'	E133°68'	284
Kagami River	Kagamigawa-cho, Kochi city, Kochi Prefecture	N33°55'	E133°50'	148
Tsukuba	Kohyadai, Tsukuba city, Ibaraki Prefecture	N36°01'	E140°06'	122
Tateyama1	Ashikuraji, Nakaniikawa-gun Tateyama-machi, Toyama Prefecture	N36°51'	E137°26'	98
Tateyama2	Ashikuraji, Nakaniikawa-gun Tateyama-machi, Toyama Prefecture	N36°35'	E137°29'	82
Iori	Iori, Nakaniikawa-gun Kamiichi-machi, Toyama Prefecture	N36°38'	E137°33'	19

Table 2. Hair density of the inflorescence stems in *Spiranthes sinensis* variety in 2012.

Locality	Number of Plants	Haired			Non-haired
		much	less	a few	zero
Nankoku	265	256 (96.6%)	6 (2.3%)	3 (1.1%)	0 (0%)
Monobe River	284	259 (91.2%)	19 (6.7%)	5 (1.8%)	1 (0.4%)
Kagami River	148	139 (93.9%)	9 (6.1%)	0 (0%)	0 (0%)
Tsukuba	122	122 (100%) <sup>1)</sup>	—	0 (0%)	0 (0%)
Tateyama1	98	98 (100%) <sup>1)</sup>	—	0 (0%)	0 (0%)
Tateyama2	82	82 (100%) <sup>1)</sup>	—	0 (0%)	0 (0%)
Iori	19	19 (100%) <sup>1)</sup>	—	0 (0%)	0 (0%)
Total	1018 (697) <sup>2)</sup>	654 (93.8%)	34 (4.9%)	8 (1.1%)	1 (0.1%)

<sup>1)</sup> Not determined whether much or less.

<sup>2)</sup> Samples of Tsukuba, Tateyama 1 & 2, and Iori were excluded in the total number of individuals.

and zero, respectively. The hair density of *S. sinensis* variety varied according to location. However, more than 90% of individuals had much hair densities. We found a glabrous individual in Monobe River (Fig. 1 I–III), which could be identified as *S. sinensis* var. *sinensis* based on the presence of a glabrous inflorescence stem and ovaries. The glabrous individual had following traits: length and width of scape, 29.6 cm and 1.68 mm, respectively; 35 flowers; and 3 leaves of width  $2.82 \pm 1.00$  mm. Glabrous individuals of *S. sinensis* were previously found only in Nankoku City, Kochi Prefecture (Sawa 1980; Kobayashi et al. 2009) and these were assigned to *S. sinensis* var. *sinensis* (Kobayashi et al. 2009). Sawa (1980) found 6 glabrous individuals there which only account for 0.4% of the Nankoku population. Interestingly, this frequency was the same as that for our Monobe River population, indicating that glabrous *S. sinensis* grows sympatrically with *S. sinensis* var. *amoena* at low frequencies in these locations.

In the molecular analyses, we determined the sequences of the nrDNA ITS and cpDNA *trnL-F* intergenic spacer regions of *Spiranthes sinensis* individuals with or without glabrous inflorescence stems and ovaries, collected from Kochi and Ibaraki prefectures. These regions were previously shown to be effective for distinguishing *S. sinensis* var. *amoena* of the Japanese mainland from *S. sinensis* var. *sinensis*

of Okinawa (Tsukaya 2005b). In all of the *S. sinensis* individuals used in the present study, the lengths of the ITS and *trnL-F* intergenic spacer regions were 728 bp and 491 bp, respectively. The sequences of the ITS1 and *trnL-F* intergenic spacer regions in all of the *S. sinensis* individuals used in the present study were identical to those previously reported for *S. sinensis* var. *amoena* (AB187151, AB187153-6, and AB187158-9; and AB187135-40, AB187143-4, and AB187146-7, respectively) (Table 3). Thus, molecular data indicate that the glabrous *S. sinensis* variety found in Kochi Prefecture is closely related to *S. sinensis* var. *amoena* rather than to *S. sinensis* var. *sinensis* in spite of morphological similarity to the latter variety. These results are in accordance with those of Tsukaya (2005b), who demonstrated that *S. sinensis* varieties of the Japanese mainland and Okinawa could be clearly distinguished based on phylogeographic data, rather than on morphological and ecological variations.

We conclude that the glabrous individual of *Spiranthes sinensis* variety found in Kochi Prefecture could be a hair-loss type of *S. sinensis* var. *amoena*. In the present study, we used only a single sample of the hair-loss type of *S. sinensis* var. *amoena*, and therefore investigation of additional samples is required. As suggested by Tsukaya (2005a, b), more comprehensive studies are necessary to settle this discrepancy between the morphological and

Table 3. The sequences obtained from *Spiranthes sinensis* samples in Japan

Locus site (base pair)	<i>trnL-F</i> spacer <sup>a</sup>					ITS <sup>a</sup>					Accession		References			
	1	1	2	2	4	1	1	2	2	4	<i>trnL-F</i>	ITS				
	7	2	7	4	8	1	1	8	2	9	0	6	5			
	7	0	4	4	1	2	6	8	3	6	6	6	5			
<i>Spiranthes sinensis</i> var. <i>amoena</i>																
Hokkaido, Hidaka	C	G	A	A	C	A	G	T	C	G	C	A	?	AB187135	AB187151	Tsukaya (2005b)
Hokkaido, Samai	C	G	A	A	C	A	G	T	C	G	C	<u>R</u>	?	AB187136	AB187152	Tsukaya (2005b)
Shizuoka, Sessokyo	C	G	A	A	C	A	G	T	C	G	C	A	?	AB187137	AB187153	Tsukaya (2005b)
Aichi, Okazaki	C	G	A	A	C	A	G	T	C	G	C	A	?	AB187138	AB187154	Tsukaya (2005b)
Hiroshima, Miyajima Isl.	C	G	A	A	C	A	G	T	C	G	C	A	?	AB187139	AB187155	Tsukaya (2005b)
Mie, Mt. Asama	C	G	A	A	C	A	G	T	C	G	C	A	?	AB187140	AB187156	Tsukaya (2005b)
Miyazaki, Takanabe	C	G	A	A	C	A	G	T	C	G	<u>Q</u>	A	?	AB187141	AB187157	Tsukaya (2005b)
Ibaraki, Tsukuba (A)	C	G	A	A	C	A	G	T	C	G	C	A	<u>G</u>	AB823668	AB740173	This study
Ibaraki, Tsukuba (B)	C	G	A	A	C	A	G	T	C	G	C	A	<u>R</u>	AB823669	AB740174	This study
Kochi, Nankoku (puberulous)	C	G	A	A	C	A	G	T	C	G	C	A	<u>G</u>	AB823667	AB740176	This study
<i>S. sinensis</i> var. <i>sinensis</i>																
Okinawa, Iriomote Isl.	<u>A</u>	<u>C</u>	<u>C</u>	<u>C</u>	<u>A</u>	<u>C</u>	T	Y	Y	T	<u>G</u>	A	?	AB187148	AB187165	Tsukaya (2005b)
<i>S. sinensis</i> variety																
Kochi, Nankoku (glabrous)	C	G	A	A	C	A	G	T	C	G	C	A	<u>G</u>	AB823666	AB740175	This study

<sup>a</sup> Underline indicates difference from estival type of *S. sinensis* var. *amoena*.



genetic traits of *S. sinensis* var. *sinensis* and *S. sinensis* var. *amoena*.

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ネジバナ (*Spiranthes sinensis* var. *amoena*) には、個体サイズ、花色、開花期、花の形態、花序の毛の多寡など多くの変異が報告されているが、日本本土 (トカラ海峡以北) において花序に毛のないネジバナが稀に発見されている。花序の毛の有無に関して、日本本土に産する有毛花序を持つものがネジバナ、奄美大島以南に産する無毛花序を持つものがナンゴクネジバナ (*S. sinensis* var. *sinensis*) として識別される。近年の遺伝的解析により、本土産のネジバナと沖縄産のナンゴクネジバナは遺伝的に異なることが示されているが、本土産の無毛型ネジバナ (ナンゴクネジバナ) の遺伝的解析は行われ

ておらず、系統的位置が不明なままである。そこで、高知県南国市で発見した無毛型ネジバナの遺伝的解析を行うことによって、本土産の無毛型ネジバナが、ナンゴクネジバナの隔離分布であるのか、ネジバナの形態変異であるのかを明らかにすることを目的とした。その結果、高知県産の無毛型ネジバナはネジバナと核遺伝子 ITS 領域および葉緑体遺伝子 *trnL-F* 領域において同一の塩基配列を持っていたため、本土に稀産する無毛型ネジバナはネジバナの形態変異である可能性が高いことがわかった。したがって、両変種の同定形質である毛の有無のみではネジバナとナンゴクネジバナを完全には識別できないため、形態的、生理的、生態的、遺伝的調査を改めて行う必要がある。

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