Phylogenetic Background of the Glabrous and Early Blooming *Spiranthes sinensis* (Orchidaceae) Collected in Kumamoto Prefecture, Japanese Mainland

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(Accepted on June 28, 2016)

The orchid *Spiranthes sinensis* has been classified into separate varieties based on the presence or absence of hairs on their inflorescence stems and ovaries: *S. sinensis* var. *amoena*, which is considered puberulous, and *S. sinensis* var. *sinensis*, which is glabrous. The current study analyzed the internal transcribed spacer region of the nuclear DNA and the *trnL–F* intergenic spacer region of the chloroplast DNA to characterize the glabrous population of *S. sinensis* found in Kumamoto Pref., Japan. The results indicated that the DNA sequences of the Kumamoto specimens were identical to those of *S. sinensis* var. *amoena*, which infers that these glabrous specimens should be considered a hairless variant of *S. sinensis* var. *amoena* rather than a new distribution record for *S. sinensis* var. *sinensis*. The current study also found that the flowering season of the *S. sinensis* population in Kumamoto Pref. was somewhat earlier than usual for *S. sinensis* var. *amoena* (late March to late April vs. June for *S. sinensis* in Kumamoto Pref. and usual *S. sinensis* var. *amoena*, respectively), indicating that the Kumamoto population is ecologically distinct from typical *S. sinensis* var. *amoena*.

**Key words:** Flowering phenology, hair, ITS, *Spiranthes sinensis, trnL–F.*

The genus *Spiranthes* L. C. Rich. (Orchidaceae) contains approximately 50 species widely distributed across tropical and temperate regions of the Americas, Eurasia, and Australia (Satomi 1982, Chen et al. 2009). However, the classification of species within the genus is complicated by their polymorphism. For example, *Spiranthes sinensis* (Pers.) Ames var. *amoena* (M. Bieb.) H. Hara has been studied taxonomically, morphologically, geographically, ecologically, and cytologically (e.g., Tanaka 1965, Hara 1969, Honda 1976, Natsume and Natsume 2002, Tatarenko et al. 2010, Iwata et al. 2012), and separated into several varieties and forms based on their morphological and ecological variations, including size (*S. sinensis* var. *amoena* f. *gracilis* F. Maek., nom. nud), flowering season (*S. sinensis* var. *amoena* f. *autumnus* H. Tsukaya), floral color (pink, white: *S. sinensis* var. *amoena* f. *albescens* Honda, green: *S. sinensis* var. *amoena* f. *viridiflora* (Makino) Ohwi), or floral size and shape (Kitamura 1964, Maekawa 1971, Sawa 1980, Tsukaya 2005a).

The presence or absence of hairs on the inflorescence stems and ovaries of *Spiranthes*
Spiranthes sinensis has also been used as a diagnostic trait to distinguish two related varieties, *S. sinensis* var. *amoena* and *S. sinensis* var. *sinensis*, respectively (Hatusima 1968). The two varieties also vary greatly in their geographical distribution with *S. sinensis* var. *amoena* found on the Japanese mainland (northern Ryukyus and northward) as well as in Korea, Taiwan, China, the Far East of Russia, the Himalayas and as far south as Malaysia, Indonesia, Australia, New Zealand, and the southwest Pacific (Tsukaya 2005b). *Spiranthes sinensis* var. *sinensis* is limited to Japan (central and southern Ryukyus), Taiwan, and South China (Maekawa 1971, Satomi 1982). In contrast, glabrous specimens of *S. sinensis* have rarely been documented in Kanto (Odakura 1982, Tsukaya 2005a), Shikoku (Sawa 1980, Hayakawa et al. 2013), and Kyushu districts (Nakajima and Ohba 2012) of the Japanese mainland. These glabrous specimens could be examples of *S. sinensis* var. *amoena* that have lost their hairs or new distribution records for *S. sinensis* var. *sinensis* (Sawa 1980, Kobayashi et al. 2009, Hayakawa et al. 2013).

We reported an additional population of glabrous *Spiranthes sinensis* in Kumamoto Pref., Kyushu, on the Japanese mainland. In addition, the population is characterized by earlier blooming (late March and late April; Tadao Matsusaka pers. comm.) than the usual *S. sinensis* var. *amoena* (June). Since the flowering season of *S. sinensis* var. *sinensis* is March to April in central and southern Ryukyus, such early flowering may indicate that glabrous *S. sinensis* in Kumamoto Pref. is a disjunctive distribution of *S. sinensis* var. *sinensis*.

A previous study (Tsukaya 2005b) has shown that the *trnL–F* intergenic spacer sequence in the chloroplast DNA (cpDNA) and the internal transcribed spacer (ITS) sequence of the nuclear DNA (nrDNA) of *S. sinensis* can be used to distinguish *S. sinensis* var. *amoena* samples collected on the Japanese mainland from *S. sinensis* var. *sinensis* sample collected on Iriomote Island in Okinawa Pref., Japan.

Therefore, we investigated the DNA sequences in ITS and *trnL–F* regions of glabrous *S. sinensis* in Kumamoto Pref. to determine if the population is a disjunctive distribution of *S. sinensis* var. *sinensis* or hairless variants of *S. sinensis* var. *amoena*.

**Materials and Methods**

**Field sampling**

*Spiranthes sinensis* in the current study were collected in Kamiamakusa City, Kumamoto Pref. on 17 March 2016. A total of seven specimens were collected at the bud stage and transplanted under laboratory conditions (ca. 25°C on a 12:12 h light:dark cycle) so their floral traits, such as the shape of their floral structures and the presence/absence of hairs on the inflorescence stems and ovaries, could be investigated after they bloomed between late March and late April. Two voucher specimens of glabrous *Spiranthes sinensis* were deposited in the TNS herbarium (T. Matsusaka & A. Hashimoto HH108, HH109, 17 March 2016).

**Molecular analysis**

We used two glabrous *Spiranthes sinensis* specimens for the molecular analysis. Total DNA was isolated from leaves using a DNeasy® Plant Mini Kit (Qiagen KK, Tokyo, Japan), according to the manufacturer’s protocol. We amplified the ITS and *trnL–F* intergenic spacer regions by using primers of ITS4 and ITS5 (White et al. 1990) and e and f (Taberlet et al. 1991), respectively. The isolated DNA was amplified by PCR in 16 µL reaction solutions each containing 0.5 µM of each forward and reverse primer, 1.5 mM of MgCl₂, 0.2 mM of each dNTP, and 0.05 units of TaKaRa Ex Taq® DNA polymerase (Takara Bio Inc. Shiga, Japan) in a 1× concentration buffer supplied by the manufacturer. We used the following thermal cycle profile for amplification in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA): 1 min at 94°C, 2 min at 48°C, and 2 min at 72°C for
45 cycles, followed by 15 min of final extension at 72°C. Following amplification, 2 µL of each product was loaded onto 1.0% agarose gels for electrophoresis. The PCR products were purified with ExoSAP-IT® (USB Corporation, Cleveland, OH, USA) and directly sequenced in both directions using a BigDye® Terminator kit ver. 3.1 (Applied Biosystems, Foster, CA, USA) and an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems) following the manufacturer’s instructions. The primers used for amplification were also used for sequencing. The sequences analyzed in this study were deposited in the DDBJ/EMBL/GenBank International DNA databases (ITS: LC155423-4 and trnL–F: LC155425-6).

Results and Discussion
Frequency of glabrous individuals
Although glabrous specimens of *Spiranthes sinensis* have been previously documented on the Japanese mainland (Sawa 1980, Odakura 1982, Tsukaya 2005a), their frequency within the populations has been low. For example, even though six glabrous specimens were found in Nankoku City, Kochi Pref., they accounted for only 0.4% of the total Nankoku populations (Sawa 1980). A similar study at nearby Monobe, Nankoku City, Kochi Pref., found only one glabrous individual among the 284 flowering plants assessed (Hayakawa et al. 2013). In contrast, the results of the current study found that five of the seven *S. sinensis* samples collected in Kamiamakusa City, Kumamoto Pref. were glabrous. Furthermore, the two remaining specimens had markedly fewer hairs than normal puberulous *S. sinensis* var. *amoena* (Fig. 1).

Molecular analysis
Sequence data from the nrDNA ITS and cpDNA trnL–F intergenic spacer regions have previously been used to distinguish *S. sinensis* var. *amoena* specimens found on the Japanese mainland from *S. sinensis* var. *sinensis* found in Okinawa (Tsukaya 2005b). The lengths of the ITS and trnL–F intergenic spacer regions of *S. sinensis* in the current study were 728 bp and 454/412 bp, respectively, which are identical to the sequences previously reported for *S. sinensis* var. *amoena* (Table 1). The molecular data therefore provide strong evidence that the glabrous specimens in the current study were more closely related to *S. sinensis* var. *amoena*.
Table 1. The sequences obtained from *Spiranthes sinensis* samples in Japan

<table>
<thead>
<tr>
<th>Locus site (base pair)</th>
<th>trnL–F spacer</th>
<th>ITS</th>
<th>Accession</th>
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<td>1 1 2 2 4</td>
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<td>AB187151</td>
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<td>1 8 2 9 0 6 5</td>
<td>AB187136</td>
<td>AB187152</td>
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<td>7 0 4 1 2</td>
<td>6 8 3 6 6 6 5</td>
<td>AB187137</td>
<td>AB187153</td>
</tr>
</tbody>
</table>

*Spiranthes sinensis* var. *amoena*

Hokkaido, Hidaka: C G A A C A G T C G C A ? AB187135 AB187151 1
Hokkaido, Samai: C G A A C A G T C G C R ? AB187136 AB187152 1
Shizuoka, Sessokyo: C G A A C A G T C G C A ? AB187137 AB187153 1
Aichi, Okazaki: C G A A C A G T C G C A ? AB187138 AB187154 1
Hiroshima, Miyajima Isl.: C G A A C A G T C G C A ? AB187139 AB187155 1
Mie, Mt. Asama: C G A A C A G T C G C A ? AB187140 AB187156 1
Miyazaki, Takanabe: C G A A C A G T C G G A ? AB187141 AB187157 1
Ibaraki, Tsukuba (A): C G A A C A G T C G C A G AB823668 AB740173 2
Ibaraki, Tsukuba (B): C G A A C A G T C G C A R AB823669 AB740174 2
Kochi, Nankoku (puberulous): C G A A C A G T C G C A G AB823667 AB740176 2
Kochi, Nankoku (glabrous): C G A A C A G T C G C A G AB823666 AB740175 2

*S. sinensis* var. *sinensis*

Okinawa, Iriomote Isl.: A C C C A C T Y Y T G A ? AB187148 AB187165 1

*S. sinensis* variety

Kumamoto, Kamiamakusa (glabrous): C G A A C A G T C G C A G LC155425 LC155423 3
Kumamoto, Kamiamakusa (glabrous): C G A A C A G T C G C A G LC155426 LC155424 3

*a* Underline indicates difference from estival type of *S. sinensis* var. *amoena.*


than *S. sinensis* var. *sinensis.* These results are in accordance with those of a previous study that demonstrated *S. sinensis* populations from the Japanese mainland and Okinawa could be better distinguished based on molecular data, rather than according to their morphological and ecological variations (Tsukaya 2005b). Furthermore, the results of the current study are consistent with a previous molecular study (Hayakawa et al. 2013) which showed that a glabrous specimen of *S. sinensis* from Kochi Pref., Japan had identical ITS and *trnL–F* sequences compared to those of *S. sinensis* var. *amoena.*

Flowering Phenology

*Spiranthes sinensis* var. *amoena* is known to have ecological variations, such as the timing of its flowering season (Tsukaya 1994). Most populations bloom in spring to early summer, while some bloom in late summer to autumn. Populations in the southern part of Japan tend to bloom earlier than those in the north, although the observed seasonal character is not thought to be caused by environmental factors such as the latitude or altitude of the habitat (Tsukaya 1994). Furthermore, it has also been noted that two sympatric populations of *S. sinensis* var. *amoena* within the same locality can have flowering periods that differ by as much as two months (Tsukaya 1994), even though their *trnL–F* and ITS1 sequences did not indicate any genetic variation between the two seasonally differentiated variants (Tsukaya 2005a, 2005b). Another change in flowering season was observed for the glabrous *S. sinensis* specimens investigated in the current study. Tsukaya (1994) showed that an assumed annual mean temperature can be used to predict the flowering time of spring flowering *S. sinensis* var. *amoena* populations. According to this correlation, the flowering of the population in the current study would be expected to occur in late June. Contrary to the expectation, *S. sinensis*
samples assessed in the current study were at the bud stage in mid-March and bloomed between late March and mid-April under cultivated conditions. Similarly, early blooming (late March and late April) was also observed in the natural condition of the investigated population (Tadao Matsusaka personal communication). Despite their earlier flowering, no morphological differences other than the presence or absence of hairs were observed between normal puberulous S. sinensis var. amoena and the glabrous Kumamoto specimens.

**Conclusion**

The glabrous samples collected from Kamiamakusa City had identical ITS and trnL–F sequences to those of *Spiranthes sinensis* var. *amoena* from the Japanese mainland. This fact indicates that the specimens collected in Kamiamakusa City, Kumamoto Pref. should be considered as the hairless variants of *S. sinensis* var. *amoena* rather than a new distribution record for *S. sinensis* var. *sinensis*. It is interesting to note that the glabrous phenotype within the population occurred at a much higher frequency than previously observed in *S. sinensis* var. *amoena* (Sawa 1980, Hayakawa et al. 2013), and that the Kamiamakusa specimens also flowered more than two months earlier than would usually be expected for *S. sinensis* var. *amoena*, both of which are characteristics more consentient with previous descriptions of *S. sinensis* var. *sinensis*. More comprehensive studies, as previously suggested (Tsukaya 2005a, 2005b), are therefore required to fully understand the biological and taxonomic complexities of *Spiranthes sinensis* var. *sinensis* and *S. sinensis* var. *amoena*.

We thank Dr. Tadao Matsusaka for his first discovery of glabrous and early blooming individuals of *Spiranthes sinensis* var. *amoena* in the investigated population; Drs. Tadao Matsusaka and Akihiko Hashimoto for their field study assistances; and Dr. Atsushi Ebihara, curator of the TNS herbarium, for permission to examine herbarium specimens. This work was supported by a Grant-in-Aid by the Japan Society for the Promotion of Science (15K18470).

**References**


末次健司a，早川宗志b：熊本県天草で採取された無毛かつ早咲きのネジバナ（ラン科）の系統的背景

日本産のネジバナSpiranthes sinensisは、花序の毛の有無に関して、日本本土（トカラ海峡以北）に産する有毛のものがネジバナS. sinensis var. amoena、奄美大島以南に産する無毛のものがナンゴクネジバナS. sinensis var. sinensisとして識別される。しかし日本本土においても花序に毛のないネジバナが稀に発見される。近年の遺伝的解析により、本土産のネジバナと沖縄県西表島産のナンゴクネジバナは遺伝的に異なったことが示されていた。また本土産の無毛型ネジバナの遺伝学的解析により、高知県産の個体群が異なっていた。ネジバナの形態変異の可能性が高いことが明らかになっている一方、他地域における系統的由来は不明なままである。そこで本研究では、熊本県上天草で発見した無毛型ネジバナの遺伝的解析を行い、こ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。本個体群のネジバナは、個体群のほとんどの個体が無毛型である点や、開花時期が通常のネジバナよりも2ヶ月程度早い点で、高知県産の無毛型ネジバナと、形態的に異なる特徴をもつ。しかし熊本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、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。本研究は、両変種の同定形質とされていた毛の有無のみならず開花期の違いにおいて、ネジバナとナンゴクネジバナを完全には識別できないことを示すものである。

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