Phytotaxa 599 (3): 139–149
https://www.mapress.com/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.599.3.1

Spiranthes minamitaniana (Orchidaceae), a new orchid species from Japan based on morphological and phylogenetic data

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Abstract

The delimitation of species within the genus *Spiranthes* (Orchidaceae) is problematic due to the intricate interplay of phenotypic plasticity, ecological variation, and hybridization, making identification that relies solely on morphology inadequate. In light of morphological and phylogenetic data, we have described a self-pollinating species, *S. minamitaniana* within the *Spiranthes sinensis* species complex. *Spiranthes minamitaniana* shares several morphological features with *S. hachijoensis*, such as glabrous inflorescence rachis, ovaries, and sepals, a degenerated rostellum, pollinia without a viscidium, and papillate lip basal callosities. However, it can be distinguished by several morphological traits, including semicircular, weakly- or non-3-lobed stigma, and more conspicuous anther cap. Although *S. minamitaniana* is morphologically most similar to *S. hachijoensis*, phylogenetic analysis strongly suggested that *S. minamitaniana* has independently acquired a selfing reproductive mode and constitutes a separate genetic cluster from it. As such, this taxon is morphologically and phylogenetically distinct from other closely related species. Consequently, this taxon is morphologically and phylogenetically distinct from other closely related species.

Key words: Orchidaceae, integrative taxonomy, reproductive isolation, SNP data, species delimitation, *Spiranthes sinensis* species complex

Introduction

The genus *Spiranthes* (Richard 1817) (Orchidaceae) encompasses ca. 50 species that are widely distributed throughout tropical and temperate regions of the Americas, Eurasia, and Australia. (Dueck *et al.* 2014; Pace *et al.* 2019; Surveswaran *et al.* 2017, 2018). Nonetheless, the identification of closely related species within the genus based solely on morphological characteristics is challenging due to the presence of phenotypic plasticity, convergent morphological features, and instances of hybridization (Dueck *et al.* 2014; Pace *et al.* 2019; Pace & Cameron 2017; Suetsugu *et al.* 2020, 2023b; Surveswaran *et al.* 2017, 2018; Tao *et al.* 2018).

The Old World *Spiranthes sinensis* (Persoon 1807: 511) Ames (1908: 53) species complex has particularly been challenging, due to its wide distribution and morphological variation (Hayakawa *et al.* 2013; Hsu & Chung 2014; Hu & Barretto 1976; Lin & Lin 2011). We note that there is ongoing debate as to which scientific name is the earliest for each component of *S. sinensis* species complex, owing to their morphological complexity (Pace *et al.* 2019; Surveswaran *et al.* 2018). Here, we considered the following eight species (*S. australis* R. Brown (1810: 219), *S. flexuosa* Lindley (1824: 823), *S. hachijoensis* Suetsugu (2023a: 345), *S. hongkongensis* S. Y. Hu & Barretto (1976:2), *S. maokensis* M.C. Pace (2019: 56), *S. nivea* T.P. Lin & W.M. Lin (2011: 320), *S. sinensis*, and *S. sunii* Boufford & Zhang (2008: 261) as the accept species (Pace *et al.* 2019; Suetsugu *et al.* 2023b).

Although it was previously believed that only one species of *Spiranthes*, *S. australis*, existed on the Japanese mainland, recent research by Suetsugu *et al.* (2023b) provides strong morphological, phylogenetic, and ecological

Accepted by Pankaj Kumar: 1 Jun. 2023; published: 7 Jun. 2023

evidence for the separation of *S. hachijoensis*. Given that some taxa within the *S. sinensis* complex have recently been recognized (Pace *et al.* 2019; Surveswaran *et al.* 2017) and many orchid species have recently been described on the Japanese mainland (Oh *et al.* 2022; Suetsugu 2021; Suetsugu *et al.* 2021), more *Spiranthes* species may remain unrecognized on the Japanese mainland. As partially anticipated, an additional unknown taxon of the genus *Spiranthes* was discovered in the Miyazaki Prefecture, located in the Kyushu region on the Japanese mainland. Although this taxon exhibits morphological similarity to *S. hachijoensis*, displaying characteristics such as a glabrous inflorescence rachis, ovaries and sepals, a degenerated rostellum, pollinia without a viscidium, and distinct papillate lip basal callosities, it can be distinguishable by several morphological traits, such as weakly- or non-3-lobed stigma and more conspicuous anther cap.

However, additional evidence will be required to fully elucidate its identity, given that species delimitation within the genus *Spiranthes* is problematic due to the intricate interplay of phenotypic plasticity, convergent morphological features, and hybridization (Lindley 1857). A potential solution to distinguish closely related species would be to implement a high-throughput sequencing technology that enables simultaneous sequencing of numerous loci (Suetsugu *et al.* 2022, 2023b; c; Suyama & Matsuki 2015). Ecological data based on breeding systems can further clarify whether morphologically distinct populations should be considered separate, reproductively isolated species (Suetsugu *et al.* 2022, 2023b; c). In this study, by integrating results from molecular phylogenetics, morphology, field observations, and reproductive biology, we reveal that it represents a cryptic species that shows a high level of molecular divergence from its closely related species. Therefore, we have described it as *S. minamitaniana*, named after the collector (Tadashi Minamitani) of the type specimens.

Materials and methods

Morphological and ecological observations

We compared the morphological characteristics of *S. australis*, *S. flexuosa*, *S. hachijoensis*, *S. hongkongensis*, *S. minamitaniana*, *S. nivea* (including *S. nivea* var. *papillata*), and *S. sinensis* using herbarium specimens deposited in KYO, KPM, TAIF, TI, and TNS, as well as by observing living plants collected in Laos, Japan, and Taiwan during fieldwork between 2011 and 2021. These morphological characteristics were evaluated using a stereomicroscope and measured with a digital caliper. The morphological differences between the species in the *S. sinensis* complex were also examined by reviewing the relevant literature and by consulting online digitized plant collections, such as JSTOR Global Plants (http://plants.jstor.org/). The breeding system of *S. minamitaniana* was also examined through field observations (n = 10) and greenhouse pollinator-exclusion experiments (n = 4). At least one voucher specimen for samples collected during our field survey from each population were deposited in KYO, TAIF, TNS, and SPMN (Table S1). The herbarium acronyms follow Index Herbariorum (Thiers 2022).

MIG-seq-based high-throughput genomic analysis

For the multiplexed inter-simple sequence repeat (MIG)-seq analysis, 12 *S. australis* individuals, 14 *S. hachijoensis* individuals, 12 *S. minamitaniana* individuals, 3 *S. nivea* individuals (including 2 *S. nivea* var. *papillata* individuals) and 11 *S. sinensis* individuals from Japan and Taiwan were used. It should be noted that *S. minamitaniana* and *S. australis* grew in sympatry within a few dozen meters of each other in one sampling locality (Takanabe, Miyazaki Pref. [TKN population]). Genomic DNA was extracted from silica-dried leaves using the CTAB method (Doyle & Doyle 1990). A MIG-seq library was prepared as described by Suyama *et al.* (2022) and sequenced using the MiSeq system (Illumina, San Diego, CA, USA) and MiSeq Reagent Kit v3 (150 cycle). The raw MIG-seq data from 8 *S. australis* individuals, 14 *S. hachijoensis* individuals, 3 *S. nivea* individuals (including 2 *S. nivea* var. *papillata* individuals) and 11 *S. sinensis* individuals had previously been deposited at the NCBI Sequence Read Archive (SRA accession number PRJNA907989) for Suetsugu *et al.* (2023a). The raw MIG-seq data from the remaining 4 *S. australis* individuals and 12 *S. minamitaniana* individuals was deposited at the NCBI Sequence Read Archive (SRA accession number PRJNA978262).

After removing primer sequences and low-quality reads (Suetsugu *et al.*, 2021), 14747358 reads (283603 ± 7787 reads per sample) were obtained from 16629700 raw reads (319802 ± 8933 reads per sample). Stacks 2.62 pipeline was used for *de novo* single nucleotide polymorphism (SNP) discovery (Rochette *et al.* 2019), with the following parameters: minimum depth of coverage required to create a stack (m) = 3, maximum distance allowed between stacks (M) = 2, and number of mismatches allowed between sample loci while building the catalog (n) = 2. SNP sites with high heterozygosity (Ho ≥ 0.6) were removed, SNP sites with fewer than three minor alleles were filtered out, and only SNPs retained by six or more samples were extracted. Finally, 8300 loci 17656 SNPs (average missing rate = 0.676) were provided for the subsequent analyses. SNP-based maximum-likelihood (ML) phylogeny was inferred using RAxML v. 8.2.10 (Stamatakis 2014), with a GTR substitution model with Lewis' ascertainment bias correction and 1000 bootstrap replicates. Additionally, the Neighbor-Net network was constructed using the uncorrected p distance matrix and ignoring ambiguous sites, with the use of SplitsTree4 4.14 (Huson & Bryant 2006).

Results and discussion

Morphological characteristics of *Spiranthes minamitaniana*

Spiranthes minamitaniana can be easily distinguishable from *S. australis* with an overlapping distribution by glabrous (vs. densely pubescent) inflorescence rachis, ovaries, and sepals as well as papillate (vs. glabrous) lip basal callosities. Additionally, *S. minamitaniana* differs from the allogamous taxa, including *S. australis, S. flexuosa, S. maokensis, S. sinensis, and S. sunii*, by its modified rostellum and stigma morphology, which is associated with its self-fertilizing reproductive mode. In contrast to the allogamous taxa, which possess a well-developed rostellum separating the stigma from the pollinaria and pollinia with a viscidium, *S. minamitaniana* has a degenerated, hardly visible rostellum, and its pollinia lack a viscidium (Fig. 1–3). Based on morphological similarities, such as the glabrous inflorescence rachis and papillate lip callosities, as well as the subsequent discussion on phylogenetic and ecological characteristics, it is likely that *S. minamitaniana* is more closely related to *S. sinensis* than *S. australis.* However, *S. minamitaniana* can be distinguished from *S. sinensis* not only by its column structure but also by its smaller flower size and labellum that only weakly bends, typically less than 270°, in contrast to *S. sinensis*, which has a strongly recurved labellum that bends nearly 360°.

FIGURE 1. *Spiranthes minamitaniana* in its type locality. (A) Habit. (B) Inflorescence with whitish cleistogamous flower. (C–D) Flower. Scale bars: 30 mm (A) and 5 mm (B–D).

FIGURE 2. *Spiranthes minamitaniana* with reddish pink normal flower (*Tadashi Minamitani Ss146*, KYO). (A) Inflorescence. (B) Flower, lateral view. (C) Flower, dorsal view. (D) Dorsal sepal. (E) Petal. (F) Lateral sepal. (G) Labellum. (H) Close-up of basal callosities of labellum. (I) Ovary and column. (J) Column, ventral view. (K) Column, dorsal view. (L) Pollinarium. Scale bars: 20 mm (A), 3 mm (B–G), 0.5 mm (H) and 1 mm (I–M).

Spiranthes minamitaniana is somewhat similar to two autogamous taxa *S. hongkongensis* and *S. nivea* in having a degenerated rostellum and pollinia without a viscidium (Lin & Lin 2011; Pace *et al.* 2019; Suetsugu & Hayakawa 2019; Surveswaran *et al.* 2017). However, *S. minamitaniana* can be distinguished from *S. hongkongensis* by its glabrous (vs. densely pubescent) rachis, ovaries, and sepals. *Spiranthes minamitaniana* also differs from *S. nivea* in terms of its papillate (vs. nearly glabrous) labellum disc, papillate (vs. glabrous) basal labellum callosities and glabrous (vs. sparsely pubescent) rachis, ovaries, and sepals. Consequently, *S. minamitaniana* is most similar to *S. hachijoensis* in having glabrous inflorescence rachis, ovaries and sepals and papillate lip basal callosities (Fig. 4). However, it is distinguishable from *S. hachijoensis* by the semicircular, weakly- or non-3-lobed stigma (vs. distinctly trilobed stigma), and the more conspicuous anther cap. In addition, *S. minamitaniana* tends to have a sublaxly arranged with an average distance of 1.7–3.0 mm between flowers (vs. densely arranged flowers with an average distance of 1.2–2.3 mm in *S. hachijoensis*), a shorter length ratio of the rachis to the inflorescence (1/4–1/3 vs. 1/3–1/2 in *S. hachijoensis*), and more strongly bent flowers against the ovary (in contrast to weakly bent flowers against the ovary in *S. hachijoensis*; Fig. 4).

FIGURE 3. *Spiranthes minamitaniana* with whitish cleistogamous flower (*Tadashi Minamitani Ss147*, KYO). (A) Inflorescence. (B) Flower and bract. (C) Flower. (D) Ovary and column. (E) Column, ventral view. (F) Column, dorsal view. Scale bars: 1 mm. Photographed by Masayuki Ishibashi.

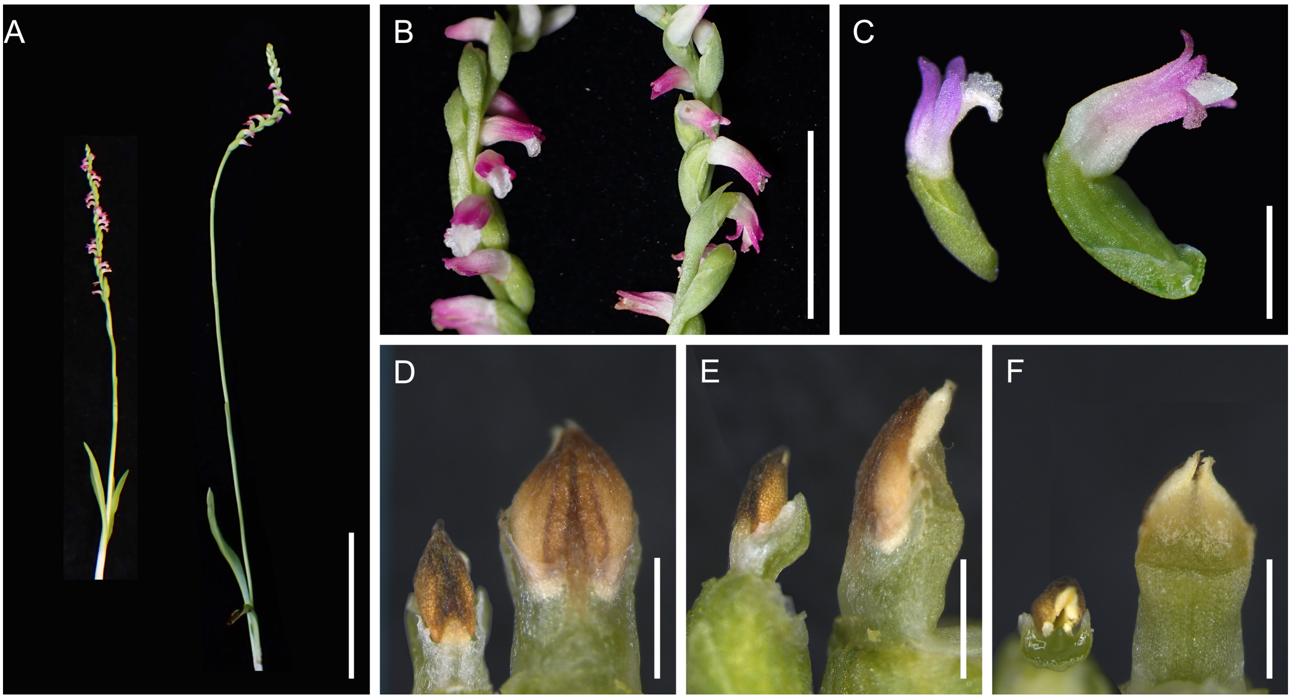

FIGURE 4. Comparison between *Spiranthes hachijoensis* (*Nahoko Fukudome Ss203*, KYO: left) and *S. minamitaniana* (*Tadashi Minamitani Ss204*, KYO: right). (A) Habit. (B) Inflorescence. (C) Flower. (D) Column, dorsal view. (E) Column, lateral view. (F) Column, ventral view. Scale bars: 50 mm (A), 10 mm (B), 3 mm (C) and 1 mm (D–F). Photographed by Kenji Suetsugu.

Ecological characteristics of *Spiranthes minamitaniana*

Spiranthes minamitaniana flowers do not face pollinator limitations in their natural habitat. A nearly 100% rate of fruit set was observed in all the natural plants examined, as well as in all the greenhouse-grown plants that lacked pollinators. Given that all seeds examined were either monoembryonic or devoid of embryos in *S. minamitaniana*, and such seeds are indicative of sexual reproduction (Catling 1982; Sun 1996), it is likely that autogamy, rather than agamospermy, is the mechanism for the high rate of fruit set. This is due to the presence of a degenerated rostellum which facilitates contact between the pollinia and the upper portion of the stigmatic surface. Possibly as a result of its dominant autogamous strategy, *S. minamitaniana* even produces sporadically cleistogamous flowers (Fig. 3), which may conserve energy and resources that might otherwise be invested in attracting pollinators.

Given that autogamy is known to be an isolating mechanism among sympatric species (Suetsugu 2022; Sun 1996), the predominantly autogamous breeding system of *S. minamitaniana* probably aids in maintaining its reproductive isolation from its closely related and sympatric species *S. australis*. Furthermore, phenological isolation can be detected between *S. minamitaniana* and *S. austlalis*. Around the type locality, *S. austlalis* blooms from late-June to early-July, more than 1 month before *S. minamitaniana*, which flowers from early-May to mid-May. Thus, a combination of contrasting breeding systems and phenological isolation likely results in premating isolation from *S. australis*. *Spiranthes minamitaniana* and its putative ancestral species *S. sinensis* are geographically and reproductively isolated, with distinct flowering phenology. While *S. minamitaniana* is found on the restricted area of Kyushu Island, *S. sinensis* distributes more southern regions (with a northern limit of Amami-Ohshima Island, Ryukyu Islands) with a blooming period at least one month earlier than *S. minamitaniana* (Maekawa 1971; Suetsugu & Hayakawa 2019). Therefore, according to the biological species concept, which defines a species as populations that interbreed in nature, *S. minamitaniana* is distinct from *S. sinensis* due to geographical, flowering phenological, and reproductive differences.

Although *S. minamitaniana* and *S. hachijoensis* have never been observed growing sympatrically, they are both found in Miyazaki Prefecture and have concurrent flowering periods, thus there is a possibility of potential hybridization. However, as both species are adapted for self-fertilization and the likelihood of outcrossing is extremely low, the formation of hybrids is unlikely. Notably, the recognized habitats of *S. minamitaniana* are restricted to wetlands or marshes, while *S. hachijoensis* and *S. australis* occur primarily in drier grasslands. Therefore, niche partitioning based on habitat preference at the local scale may also contribute to reproductive isolation.

Phylogenetic characteristics of *Spiranthes minamitaniana*

Our MIG-seq-based ML phylogenetic analysis revealed that the seven *Spiranthes* taxa form two strongly supported clades [*S. australis* + *S. nivea* and *S. sinensis* + *S. hachijoensis* + *S. minamitaniana*] with 100% bootstrap support (Fig. 5). This finding also indicated that *S. minamitaniana* is not closely related to the sympatric species *S. australis*. Additionally, even in the location where *S. minamitaniana* and *S. australis* grow sympatrically within a few dozen meters in TKN population, no individuals of both species possessing genetic components of the other species were detected, indicating the absence of natural hybrids in this case, even in sympatry (Fig. 6).

Our phylogenetic analyses also indicated that *S. minamitaniana* constitutes a separate genetic cluster from its morphologically most similar taxon *S. hachijoensis* (Fig. 5–6). These analyses also refute the possibility of their simultaneous origin from outcrossing taxa. Based solely on morphological and ecological data, we could not dismiss the likelihood of *S. minamitaniana* and *S. hachijoensis* concurrently acquiring a selfing reproductive mode, with *S. hachijoensis* differentiating into a lineage characterized by greater column reduction. In the shared origin scenario, *S. minamitaniana* and *S. hachijoensis* would be clustered together within a single branch in Splitstree. However, our results demonstrated that both taxa were positioned on distinct, elongated branches at the base of the network. This suggests independent divergence from their ancestral outcrossing species (most likely *S. sinensis*). Furthermore, *S. hachijoensis* plants gathered from the nearest locality to *S. minamitaniana* (NAN population) displayed no genetic components of the latter, suggesting few hybridization opportunities due to their self-fertilization ability (Fig. 6). Taken together, these findings indicate that *S. minamitaniana* is phylogenetically distinct from *S. hachijoensis*.

Conclusion

The transition from cross-fertilization to self-fertilization plays a significant role in promoting reproductive isolation, making it a driving force behind speciation (Wright *et al.* 2013; Suetsugu *et al.* 2023a). However, given that the shift from outcrossing to selfing is a common evolutionary transition in flowering plants (Wright *et al.* 2013), description of all the self-fertilizing ecotypes as separate species based on the biological species concept could potentially lead to confusion.

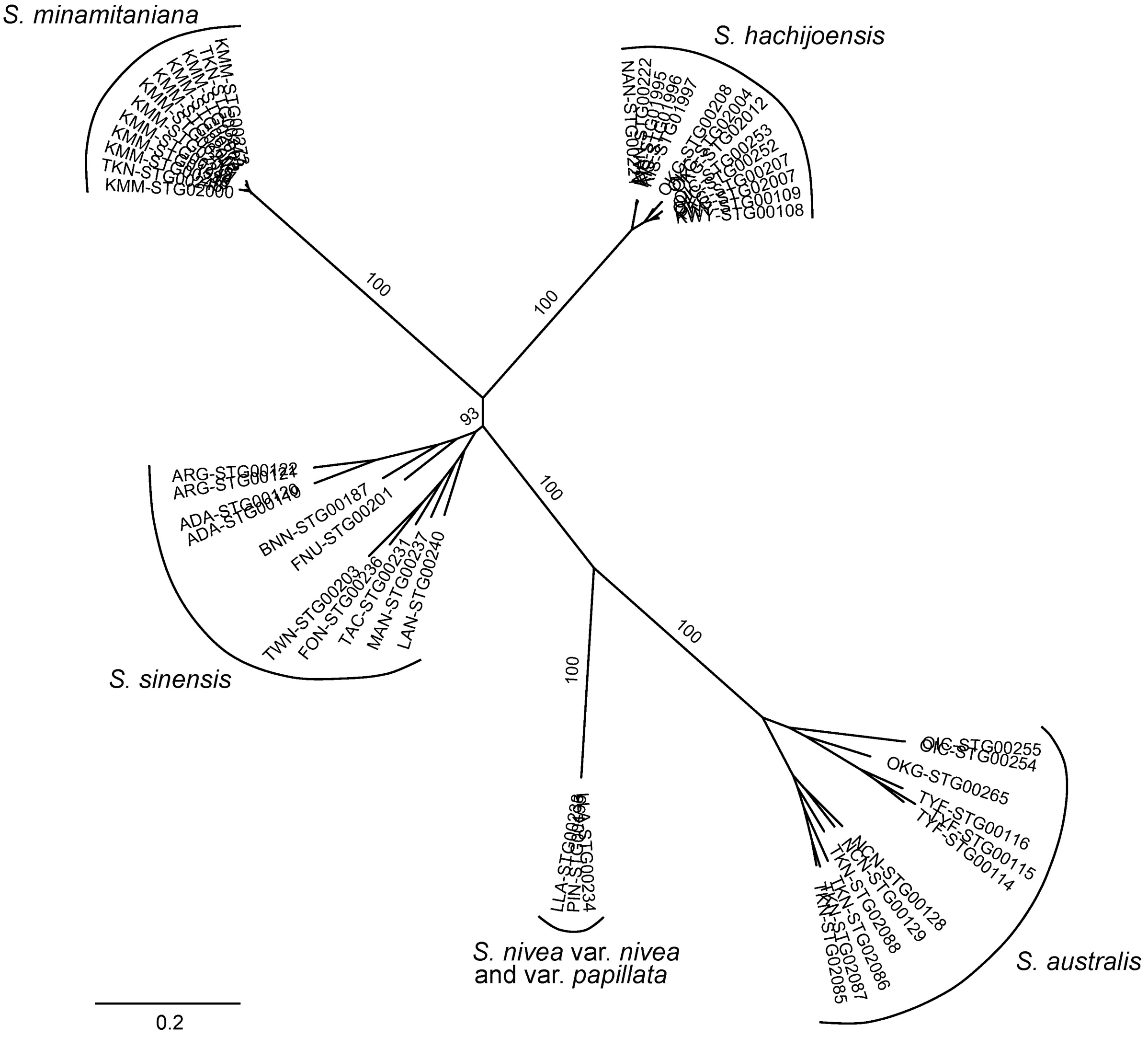

FIGURE 5. Phylogenetic tree of *Spiranthes minamitaniana* and its closely related taxa reconstructed from MIG-seq data. Bootstrap values for intraspecific clades are not shown. Branch length represents the average number of substitutions per site.

Specifically, within the *S. sinensis* species complex, some self-fertilizing variants coexist with their cross-fertilizing counterparts in the same geographical areas. These self-fertilizing variants are also genetically intermixed with the cross-fertilizing ones, leading to their identification as synonymous with their cross-fertilizing ancestors (Pace *et al.* 2019). Contrastingly, *S. minamitaniana*, found in Miyazaki, Kyushu, presents a different distribution range and flowering time compared to *S. sinensis* located south of the Ryukyu Islands. Additionally, the genetic distinction between *S. minamitaniana* and *S. sinensis* is equivalent to those found between other pairs of species within the complex, although we cannot rule out the possibility that *S. minamitaniana* might be phylogenetically nested within *S. sinensis* when considering specimens from broader geographic areas. In summary, we have gathered multifaceted evidence supporting the recognition of *S. minamitaniana* as a distinct species, which includes evidence from the morphological species concept, the biological species concept, and (at least) partial evidence of genetic isolation.

Taxonomic treatment

Spiranthes minamitaniana Suetsugu, *sp. nov.* (Figs. 1–3)

Type. JAPAN. Miyazaki Pref.: Koyu County, Kawaminami Town, Kawaminami Marsh, 13 May 2018, *Tadashi Minamitani Ss146-1* (holotype: KYO!, herbarium sheet and spirit collection labeled as the same specimen).

Diagnosis. *Spiranthes minamitaniana* is morphologically most similar to *S. hachijoensis* but differs from *S. hachijoensis* by semicircular, weakly- or non-3-lobed stigma, and more conspicuous anther cap.

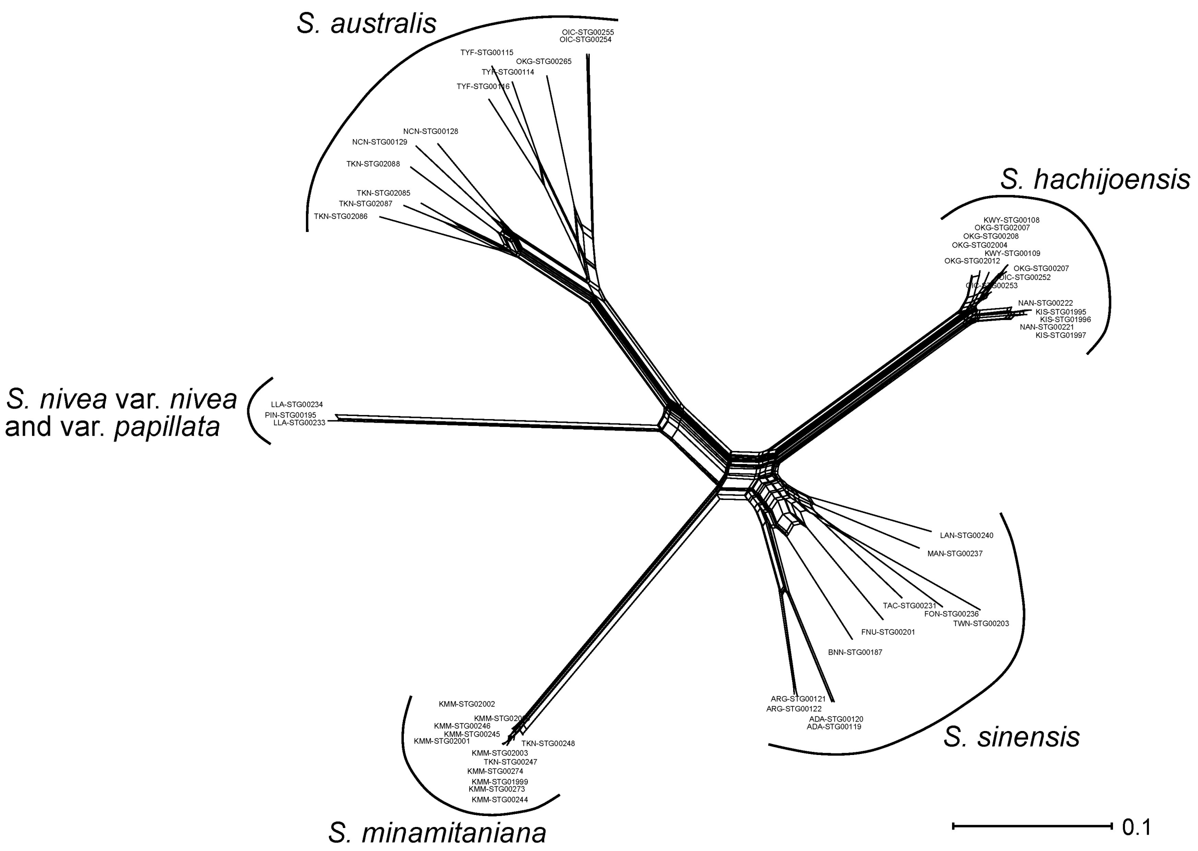

FIGURE 6. Neighbor-Net network of *Spiranthes minamitaniana* and its closely related taxa reconstructed based on uncorrected *p* distance calculated from 17656 SNPs.

Description. Plants 15–35 cm tall. Roots fleshy, fasciculate, slender to tuberous, up to ca. 5 mm in diameter. Leaves 2–6, basal, forming a rosette, erect and spreading, narrowly lanceolate, 21–76 × 3–7 mm, apex acute to acuminate, with an indistinct petiole-like base. Inflorescence terminal, racemose erect, 15–35 cm, glabrous, with 1–3 sterile bracts sheathing the peduncle; rachis 4.1–14.3 cm, with 24–49 spirally arranged flowers; floral bracts shorter or longer than pedicellate ovary, ovate-lanceolate, 5.4–8.6 mm long, 2.0–3.6 mm wide, glabrous, apex acuminate. Ovary sessile or with inconspicuous pedicel, pale green, ellipsoid-obovoid, 3.5–6.9 mm long, 1.5–2.7 mm wide, glabrous. Flowers resupinate, horizontal or nodding, barely to weakly opening. Dorsal sepal entirely white or pinkish purple with a basal white part, narrowly triangular, 3.3–4.2 mm long, 1.2–1.5 mm wide, glabrous, apex obtuse or rarely acute, connivent with petals and forming a hood over column. Lateral sepals entirely white or pinkish purple with a basal white part, narrowly triangular to lanceolate, slightly oblique at approximately halfway of the length, 3.3–4.2 mm long, 0.8–1.0 mm wide, glabrous, apex obtuse or acute. Petals entirely white or pinkish purple with a basal white part, linear to oblanceolate, occasionally slightly oblique at approximately halfway of the length, 2.7–3.3 mm long, 0.8–1.0 mm wide, glabrous, apex rounded. Labellum white, weakly recurved downward at about two-thirds the distance from the claw to labellum apex, oblong to slightly constricted near the reflection and then dilating below, centrally papillate near the apex, 3.2–4.1 mm long, 1.4–1.6 mm wide below the callosities, 1.4–1.6 mm wide at widest point below recurvature; margin entire to slightly undulating from the base until the area of recurvature, below point of recurvature margin becoming ruffled and lacerate; two basal callosities transparent, obovate to subglobose, papillate, 0.5–0.7 mm long, 0.5–0.6 mm wide. Column translucently white to pale green dorsally, pale green ventrally, clavate, 1.6–1.9 mm long; anther cap yellow-brown, ovate, partly embedded on the upper part of column, 1.0–1.6 mm long; pollinia 2, each 2-partite, yellow, granular-farinaceous, without viscidium at the narrower end, 1.0–1.1 mm long; rostellum narrow, typically lacking; stigma semicircular, weakly- or non-3-lobed, filled with viscid liquid, 0.4–0.9 mm long. Fruit ellipsoid-obovoid, 4.9–9.4 mm long, 2.2–4.0 mm wide, glabrous. Seed fusiform, ca. 0.6 mm long; embryo 1, ellipsoid, ca. 0.15 mm long.

Japanese name. Osuzu-neji-bana

Distribution. Japan (Kawaminami Marsh and Takanabe Marsh, Miyazaki Pref.). Despite the long-term searches by the authors during the flowering season around the populations, the species has yet to be discovered elsewhere.

The scarcity of additional sightings during surveys specifically targeting *S. minamitaniana* suggests that the species is quite rare and has a highly fragmented distribution. However, *S. minamitaniana* may have been mistaken for more widespread species, such as *S. hachijoensis*, which share a similar morphology. Further surveys and detailed morphological examination of *Spiranthes* in wetlands may reveal a broader distribution, given that *S. minamitaniana* has only been observed in wetlands or marshes.

Conservation status. *Spiranthes minamitaniana* is currently recognized in only two populations located ca. 10 km apart, where approximately 30 individuals have been observed in total. Therefore, it can be considered critically endangered, according to IUCN criteria (IUCN 2019) under D1 criteria, in which the number of mature individuals is less than 50.

Additional specimens examined (paratype). JAPAN. Miyazaki Pref.: Koyu County, Kawaminami Town, Kawaminami Marsh, 13 May 2018, *Tadashi Minamitani Ss146-2* (KYO); loc. cit., 6 May 2021, *Tadashi Minamitani Ss204-1* (KYO); loc. cit., 6 May 2021, *Tadashi Minamitani Ss204-2* (KYO); loc. cit., 6 May 2021, *Tadashi Minamitani Ss204-3* (KYO); loc. cit., 6 May 2021, *Tadashi Minamitani Ss204-4* (TNS); loc. cit., 6 May 2021, *Tadashi Minamitani Ss204-5* (TNS); Koyu County, Takanabe Town, Takanabe Marsh, 14 May 2018, *Tadashi Minamitani Ss147-1* (KYO, herbarium sheet and spirit collection labeled as the same specimen); loc. cit., 14 May 2018, *Tadashi Minamitani Ss147-2* (KYO).

Acknowledgments

We thank Tadashi Minamitani for providing specimens and photographs for this study. We also thank Masayuki Ishibashi for the skillful technical assistance with photography. We are also grateful to Takako Shizuka and Kazuma Takizawa for their technical assistance. Finally, we thank the curators of KYO, KPM, TAIF, TI, and TNS for providing access to herbaria or collection databases. This study was financially supported by PRESTO (JPMJPR21D6, KS) from the Japan Science and Technology Agency and by the Environment Research and Technology Development Fund (#4-2001, KS and YS) from the Ministry of Environment, Japan.

References

Ames, O. (1908) *Orchidaceae: Illustrations and studies of the family Orchidaceae*. Houghton, Mifflin. 53 pp.

Boufford, D.E. & Zhang, W. (2007) Spiranthes sunii Boufford & Wenheng Zhang, sp. nov.; a new rheophytic orchid from Gansu Province, China. *Harvard papers in botany* 13: 261–266.
https://doi.org/10.3100/1043-4534-13.2.261

Brown, R. (1810) *Prodromus florae novae Hollandae et insulae Van-Diemen*. 1st ed. Taylor, London. 592 pp.

Catling, P.M. (1982) Breeding systems of northeastern North American *Spiranthes* (Orchidaceae). *Canadian Journal of Botany* 60: 3017–3039.
https://doi.org/10.1139/b82-358

Doyle, J.J. & Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15.

Dueck, L.A., Aygoren, D. & Cameron, K.M. (2014) A molecular framework for understanding the phylogeny of *Spiranthes* (Orchidaceae), a cosmopolitan genus with a North American center of diversity. *American Journal of Botany* 101: 1551–1571.
https://doi.org/10.3732/ajb.1400225

Hayakawa, H., Ohga, K., Miyata, H., Arakawa, R., Ito, K., Tebayashi, S., Ikeda, H. & Fukuda, T. (2013) Phylogenetic background of a glabrous individual of *Spiranthes sinensis* var. *amoena* (Orchidaceae) collected in Kochi prefecture, Japan. *Journal of Phytogeography and Taxonomy* 61: 45–50.

Hsu, T.C. & Chung, S.W. (2014) *Spiranthes hongkongensis* (Orchidaceae): Newly recorded from Taiwan. *Taiwan Journal of Biodiversity* 16: 405–408.

Hu, S.Y. & Barretto, G. (1976) New species and varieties of Orchidaceae from Hong Kong. *Chung Chi Journal* 13: 1–34.

Huson, D.H. & Bryant, D. (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23: 254–267.
https://doi.org/10.1093/molbev/msj030

IUCN (2019) Guidelines for using the IUCN red list categories and criteria. Version 14. Available from: http://www.iucnredlist.org/documents/RedListGuidelines.pdf (accessed 6 October 2022)

Lin, T.P. & Lin, W.M. (2011) Newly discovered native orchids of Taiwan (IV). *Taiwania* 56: 315–322.

Lindley, J. (1824) *Spiranthes flexuosa*. *The Botanical Register* 10: t. 823.

Lindley, J. (1857) Contributions to the Orchidology of India.—No. 1. *Journal of the Proceedings of the Linnean Society of London.Botany* 1: 170–190.
https://doi.org/10.1111/j.1095-8339.1857.tb02444.x

Maekawa, F. (1971) *The wild orchids of Japan in color.* Seibundo-shinkosha, Tokyo. 495 pp.

Oh, S.-H., Suh, H.-J., Seo, S.-W., Chung, K.-S. & Yukawa, T. (2022) A new species of *Goodyera* (Orchidaceae: Orchidoideae) from Korea and Japan. *Journal of Plant Biology* 65: 357–363.
https://doi.org/10.1007/s12374-022-09358-1

Pace, M.C. & Cameron, K.M. (2017) The systematics of the *Spiranthes cernua* species complex (Orchidaceae): Untangling the Gordian Knot. *Systematic Botany* 42: 1–30.
https://doi.org/10.1600/036364417X696528

Pace, M.C., Giraldo, G., Frericks, J., Lehnebach, C.A. & Cameron, K.M. (2019) Illuminating the systematics of the *Spiranthes sinensis* species complex (Orchidaceae): ecological speciation with little morphological differentiation. *Botanical Journal of the Linnean Society* 189: 36–62.
https://doi.org/10.1093/botlinnean/boy072

Persoon, C.H. (1807) *Synopsis plantarum, seu Enchiridium botanicum, complectens enumerationem systematicam specierum hucusque cognitarum.* Vol. 2. Parisiis Lutetiorum, C.F. Cramerum. 511 pp.
https://doi.org/10.5962/bhl.title.638

Richard, L.-C. (1817) *De orchideis europaeis annotationes, praesertim ad genera dilucidanda spectantes*. Ex typ. A. Belin, Parisiis. 232 pp.
https://doi.org/10.5962/bhl.title.15465

Rochette, N.C., Rivera-Colón, A.G. & Catchen, J.M. (2019) Stacks 2: Analytical methods for paired-end sequencing improve RADseq-based population genomics. *Molecular Ecology* 28: 4737–4754.
https://doi.org/10.1111/mec.15253

Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.
https://doi.org/10.1093/bioinformatics/btu033

Suetsugu, K. (2021) *Cremastra saprophytica* (Orchidaceae: Epidendroideae), a new leafless autonomously self-pollinating orchid species from Gifu Prefecture, Japan. *Phytotaxa* 527: 89–96.
https://doi.org/10.11646/phytotaxa.527.2.1

Suetsugu, K. (2022) Living in the shadows: *Gastrodia* orchids lack functional leaves and open flowers. *Plants, People, Planet* 4: 418–422.
https://doi.org/10.1002/ppp3.10281

Suetsugu, K., Fukushima, K., Makino, T., Ikematsu, S., Sakamoto, T. & Kimura, S. (2023a) Transcriptomic heterochrony and completely cleistogamous flower development in the mycoheterotrophic orchid *Gastrodia*. *New Phytologist* 237: 323–338.
https://doi.org/10.1111/nph.18495

Suetsugu, K. & Hayakawa, H. (2019) *Spiranthes hongkongensis* SY Hu & Barretto (Orchidaceae): Newly discovered orchid on Ishigaki Island, Ryukyu Islands, Japan. *Acta Phytotaxonomica et Geobotanica* 70: 205–208.

Suetsugu, K., Hirota, S.K., Hayakawa, H., Fujimori, S., Ishibashi, M., Hsu, T.-C. & Suyama, Y. (2023b) *Spiranthes hachijoensis* (Orchidaceae), a new species within the S. sinensis species complex in Japan, based on morphological, phylogenetic, and ecological evidence. *Journal of Plant Research* 136: 333–348.
https://doi.org/10.1007/s10265-023-01448-6

Suetsugu, K., Hirota, S.K., Hsu, T.-C., Kurogi, S., Imamura, A. & Suyama, Y. (2023c) *Monotropastrum kirishimense* (Ericaceae), a new mycoheterotrophic plant from Japan based on multifaceted evidence. *Journal of Plant Research* 136: 3–18.
https://doi.org/10.1007/s10265-022-01422-8

Suetsugu, K., Hirota, S.K., Nakato, N., Suyama, Y. & Serizawa, S. (2022) Morphological, ecological, and molecular phylogenetic approaches reveal species boundaries and evolutionary history of *Goodyera crassifolia* (Orchidaceae, Orchidoideae) and its closely related taxa. *PhytoKeys* 212: 111–134.
https://doi.org/10.3897/phytokeys.212.91536

Suetsugu, K., Hirota, S.K. & Suyama, Y. (2021) A new natural hybrid, *Goodyera ×tanakae* (Orchidaceae) from Japan with a discussion on the taxonomic identities of *G. foliosa*, *G. sonoharae*, *G. velutina*, *G. ×maximo-velutina* and *G. henryi*, based on morphological and molecular data. *Taiwania* 66: 277–286.

Suetsugu, K., Lin, J.-Z., Hsu, T.-C. & Hayakawa, H. (2020) Interspecific hybridization between *Spiranthes australis* and *S. sinensis*

(Orchidaceae) in southern Taiwan. *Acta Phytotaxonomica et Geobotanica* 71: 177–184. https://doi.org/10.18942/apg.201922

Sun, M. (1996) The allopolyploid origin of *Spiranthes hongkongensis* (Orchidaceae). *American Journal of Botany* 83: 252–260. https://doi.org/10.1002/j.1537-2197.1996.tb12702.x

Surveswaran, S., Gowda, V. & Sun, M. (2018) Using an integrated approach to identify cryptic species, divergence patterns and hybrid species in Asian ladies' tresses orchids (*Spiranthes*, Orchidaceae). *Molecular Phylogenetics and Evolution* 124: 106–121. https://doi.org/10.1016/j.ympev.2018.02.025

Surveswaran, S., Kumar, P. & Sun, M. (2017) *Spiranthes himalayensis* (Orchidaceae, Orchidoideae) a new species from Asia. *PhytoKeys* 89: 115–128. https://doi.org/10.3897/phytokeys.89.19978

Suyama, Y., Hirota, S.K., Matsuo, A., Tsunamoto, Y., Mitsuyuki, C., Shimura, A. & Okano, K. (2022) Complementary combination of multiplex high-throughput DNA sequencing for molecular phylogeny. *Ecological Research* 37: 171–181. https://doi.org/10.1111/1440-1703.12270

Suyama, Y. & Matsuki, Y. (2015) MIG-seq: an effective PCR-based method for genome-wide single-nucleotide polymorphism genotyping using the next-generation sequencing platform. *Scientific Reports* 5: 16963. https://doi.org/10.1038/srep16963

Tao, Z.-B., Ren, Z.-X., Bernhardt, P., Liang, H., Li, H.-D., Zhao, Y.-H., Wang, H. & Li, D.-Z. (2018) Does reproductive isolation reflect the segregation of color forms in *Spiranthes sinensis* (Pers.) Ames complex (Orchidaceae) in the Chinese Himalayas? *Ecology and Evolution* 8: 5455–5469. https://doi.org/10.1002/ece3.4067

Thiers, B. (2022) Index Herbariorum, New York Botanical Garden. Available from: http://sweetgum.nybg.org/science/ih/ (accessed 6 October 2022)

Wright, S.I., Kalisz, S. & Slotte, T. (2013) Evolutionary consequences of self-fertilization in plants. *Proceedings of the Royal Society B: Biological Sciences* 280: 20130133. https://doi.org/10.1098/rspb.2013.0133