ISSN 1346-7565 Acta Phytotax. Geobot. 67 (1): 17–27 (2016)

Mitella amamiana sp. nov., the First Discovery of the Genus *Mitella* (Saxifragaceae) in the Central Ryukyus

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In this report, I describe the morphological characteristics, evolutionary relationships, and taxonomy of a newly discovered perennial *Mitella amamiana* sp. nov. (section *Asimitellaria*; Saxifragaceae) on Amami-Oshima Island in the central Ryukyus. The discovery is noteworthy because the genus *Mitella* has been considered absent from the central and southern Ryukyus. Using phylogenetic analyses based on nuclear ribosomal DNA (ETS and ITS) and the two copies of nuclear Granule-bound starch synthase genes (*GBSSI-A1* and *GBSSI-A2*), *M. amamiana* appears to have a sister relationship with *M. doiana* Ohwi, which is endemic to Yakushima island in the northern Ryukyus. *Mitella amamiana* is morphologically similar to *M. doiana* but clearly differs in the occasional presence of petals and the size of the leaves. Considering that plants of *Mitella* have low dispersability, the present discovery provides a valuable opportunity to characterize biogeographic associations across the Tokara gap, an ancient and continuous land break between the central and northern Ryukyus.

Key words: Amami-Oshima, biogeography, *Mitella amamiana*, new species, phylogeny, Tokara Gap

The Ryukyu islands (Ryukyus) are the southwest portion of the Japanese archipelago. They have long been the focus of various biological surveys because they represent one of the most species-rich and biogeographically unique areas in Japan (Kato & Ebihara 2010, Kubota *et al*. 2014), while Japan as a whole is a global biodiversity hotspot (Boufford *et al*. 2005, Mittermeier *et al*. 2011). Within the Ryukyus, three floristically distinct areas have been recognized; the northern, central and southern Ryukyus (Fig. 1), each of which is subdivided by >1000-m-deep sea channels known as the Tokara Gap (between the northern and central Ryukyus) and Kerama Gap (between the central and southern Ryukyus) (Nakamura *et al*. 2013).

The central and southern Ryukyus are characterized by subtropical climates and the absence of mountains above 700 m in height. These characteristics result in a uniqueness in the flora and fauna, with the absence of several species-rich groups of plants and animals typical of cool, temparate environments found in mainland Japan and Taiwan. These groups include cherries (*Cerasus*; Rosaceae), monkshoods (*Aconitum*; Ranunculaceae), primroses (*Primula*; Primulaceae), Asian salamanders (*Hynobius*; Hynobiidae), and bumblebees (*Bombus*; Apidae) (Yamazaki 1993, Kato 2000, Ohba 2001, Kadota 2006, Li *et al*. 2011).

Mitella L. (Saxifragaceae) is one such group of plants recorded from both mainland Japan and Taiwan, but not from the central and southern Ryukyus (Wakabayashi 2001), presumably because it prefers temperate moist riparian habitats along streams. *Mitella* consists of 20 perennial

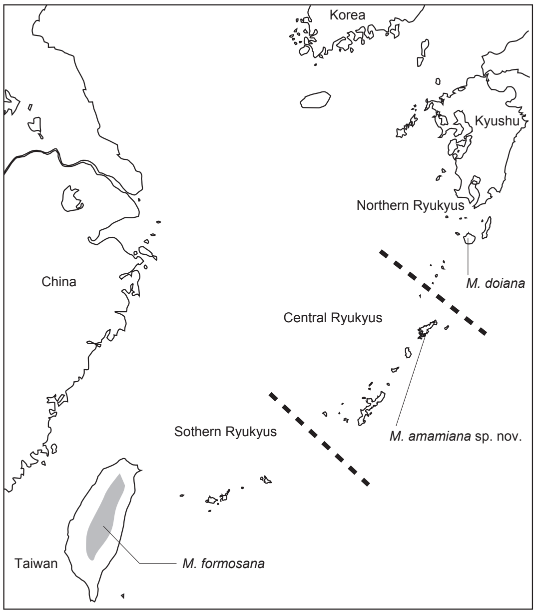

FIG. 1. Distribution of *Mitella,* including *M. amamiana* in the Ryukyus and Taiwan. Dashed lines indicate biogeographic boundaries, known as the Tokara and Kerama gaps, of the northern, central and southern Ryukyus.

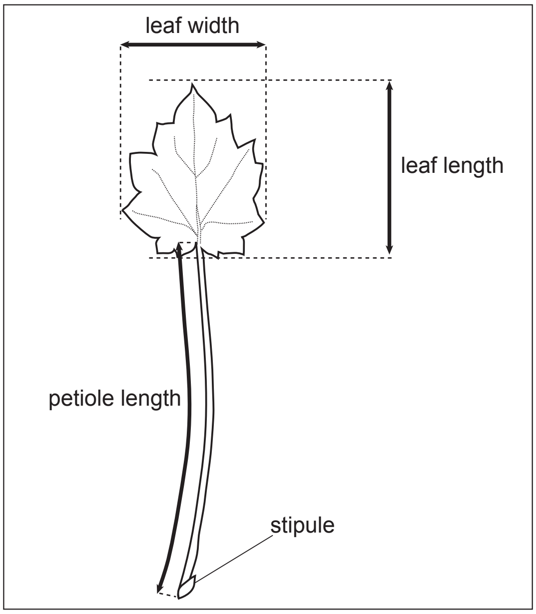

FIG. 2. Illustration of traits measured for leaf morphology.

species with a typical eastern Asia-North America distribution. Species of *Mitella* are characterized by 5-merous flowers with linear, often pinnately cleft petals and the capsular fruits oriented upward at maturity as an adaptation to splash-cup dispersal. Recent molecular phylogenetic analyses revealed that the genus is not a monophyletic group and consists of at least six distinct lineages (Okuyama *et al*. 2008). Among them, section *Asimitellaria* Wakab., with 10 species and two varieties described to date, is the largest lineage endemic to Japan and Taiwan (Wakabayashi 2001). In Japan, 9 out of 11 native species of *Mitella*, including the southernmost species, *M. doiana* from Yakushima in the northern Ryukyus, belong to section *Asimitellaria*.

Recently, however, Hidekazu Morita, a local naturalist, discovered *Mitella* on Amami-Oshima in the central Ryukyus. Because those plants are morphologically unique, I here describe them as a novel species, discuss its phylogenetic placement within the genus and characterize its morphological, evolutionary, and biogeographic relationships with other species.

Materials and Methods

Plants from Amami-Oshima Island used in this study were collected from one of the native populations, and cultivated in Tsukuba Botanical Garden. A subset of the collections was deposited in the herbarium of the National Museum of Nature and Science (TNS) as voucher specimens. Morphological measurements were conducted for each of these newly collected specimens as well as for specimens of *M. doiana*. Measurements of leaf traits are shown in Fig. 2. The specimens and number of individuals examined are listed in Table 1. For each individual, leaf blade length and width and petiole length of 2–4 leaves were measured. To determine the statistical significance of the morphological differences, two-way analysis of variance (ANOVA) was conducted using individuals and species as the sources of variation.

For nucleotide sequencing, total genomic DNA was extracted from fresh leaves of three living individuals (MAM-01, MAM-02, and MAM-12) of the newly discovered plants accord-

TABLE 1. Specimens used for morphological measurements.

Populations	Specimen	Individual ID in Fig.4
Mitella amamiana		
Kagoshima Pref., Amami city, Sumiyo-cho	TNS01024777	A1
	TNS01024774	A2
	TNS01240158	A3
	TNS01240158	A4
	TNS01240157	A5
Mitella doiana		
Kagoshima Pref., Yakushima-cho, From Kosugidani to Mt. Nagata-dake 1	TNS00795715	D1-6
Kagoshima Pref., Yakushima-cho, Kosugidani	TNS00045397	D7
Kagoshima Pref., Yakushima-cho, Northwest slope of Mt. Nagata-dake 1	TNS00763191	D8
Kagoshima Pref., Yakushima-cho, From Kosugidani to Mt. Nagata-dake 2	TNS00651893	D9-13
Kagoshima Pref., Yakushima-cho, Northwest slope of Mt. Nagata-dake 2	TNS00763139	D14

ing to the method described in Okuyama *et al*. (2005). Nuclear ribosomal ETS and ITS (nrDNA), and the two copies of granule-bound starch synthase A (*GBSSI-A*) were amplified and sequenced according to methods described previously (Okuyama & Kato 2009, Okuyama & Uno 2012, Okuyama *et al*. 2012). Accordingly, two datasets, nrDNA and *GBSSI-A*, were generated for phylogenetic analyses. The datasets included most of the available DNA sequences of *Mitella* (number of operational taxonomic units was 171 and 44 for nrDNA and *GBSSI-A* datasets, respectively), especially from section *Asimitellaria* (Okuyama & Kato 2009, Okuyama & Uno 2009, Okuyama *et al*. 2012, and present study). Alignment was conducted manually following the aligned datasets used in previous studies (Okuyama & Kato 2009, Okuyama & Uno 2009, Okuyama *et al*. 2012). Gaps were coded as separate characters using the complex indel coding methods of Simmons & Ochoterena (2000). Phylogenetic tree searches were conducted using PAUP*4.0a140 (Swofford 2003) under the maximum parsimony (MP) criterion with exactly the same settings as in Okuyama & Kato (2009). To examine the robustness of the phylogenetic inference based on MP, maximum likelihood tree searches with 100 replicate bootstrapping were also conducted using RaxML (Stamatakis 2014) with the GTRGAMMAI model. All nucleotide sequences newly generated in this study were deposited in DDBJ under accession Nos. LC77264–LC77267; the aligned data matrices used in this study were deposited in Treebase under submission ID18492.

Results

Characteristics of Mitella amamiana

Mitella amamiana grows on a wet rocky wall in the spray of a small waterfall in an evergreen forest on Amami-Oshima (Fig. 3A, B). Only four populations, each less than 500 m apart, have been found (Hidekazu Morita, personal communication). The total number of individuals in these populations is roughly around 1,000, yet the habitat area is extremely small. The plants are therefore vulnerable to human impact and thus special care is required to conserve them. According to the IUCN Red List Categories and Criteria. Version 3.1 (IUCN, 2001), the status of *M. amamiana* is equivalent to endangered (EN) based on its occurrence in only four locations and the area in which it grows is declining or fluctuating (criterion B2).

Mitella amamiana is morphologically similar

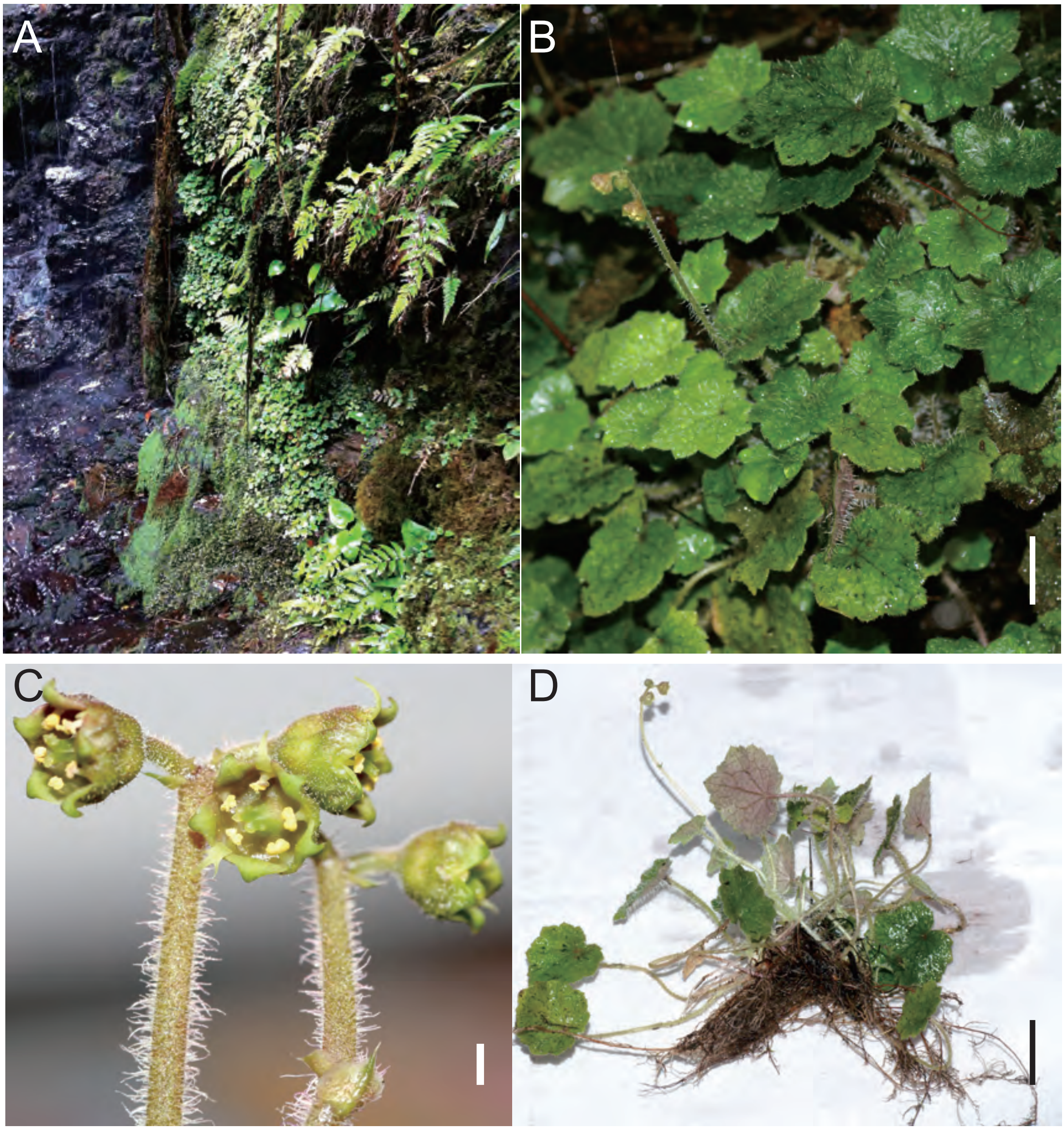

FIG. 3. *Mitella amamiana* Y. Okuyama. A: Wild population on wet rock wall on Amami-Oshima Island. B: Flowering individual in the wild. Scale = 1 cm. C: Close-up of flowers. Note two petals in flower in center. Scale = 1 mm. D: Fresh whole plant (specimen *TNS1240158*).

to *M. doiana*, with a relatively small plant size, having only 1–3 flowers on an inflorescence, and a papillose seed surface (Fig. 1), but differs in the overall plant size and the occasional presence of one to three petals per flower. The length of the leaf blade and petiole on herbarium specimens of *M. amamiana* are consistently greater in *M. doiana* (Fig. 4; see largest leaf within each individual). The length and width of the leaf blade and the length of the petiole are all significantly greater in *M. amamiana* than in *M. doiana* (two-way ANOVA, Table 2). The linear petals (when present) sometimes have one or two lobes on one or both sides (Fig. 3C). Based on these distinct characteristics, I describe the plant as a new species.

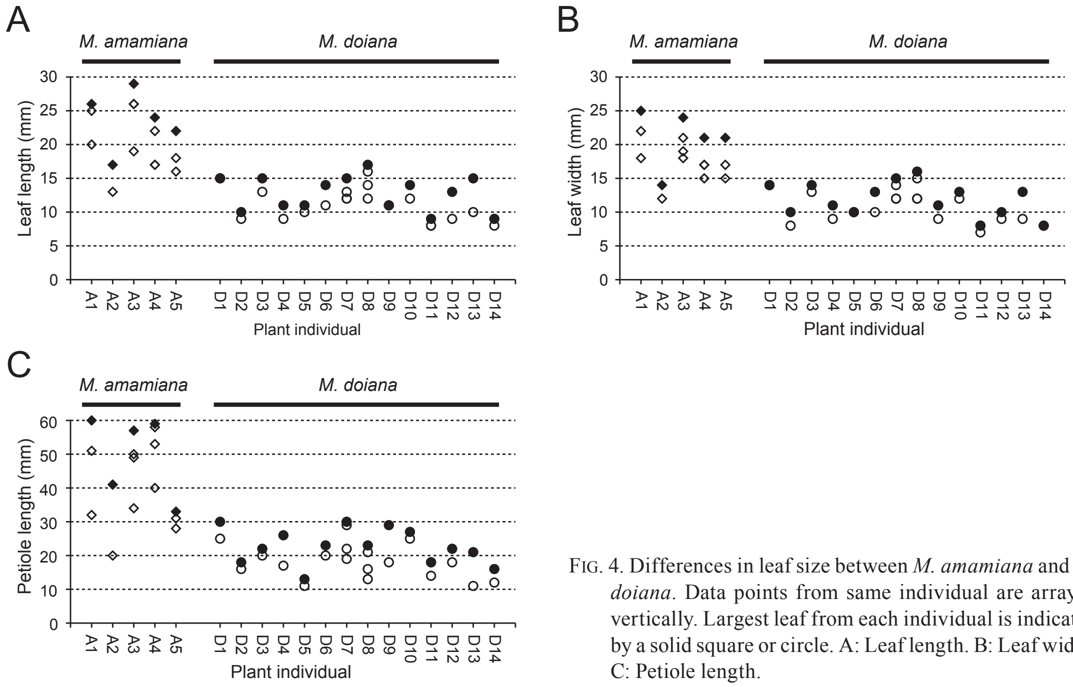

FIG. 4. Differences in leaf size between *M. amamiana* and *M. doiana*. Data points from same individual are arrayed vertically. Largest leaf from each individual is indicated by a solid square or circle. A: Leaf length. B: Leaf width. C: Petiole length.

TABLE 2. Results of two-way ANOVA for morphological variables between and within species.

Source of variation	Leaf length	Leaf width	Petiole length
Species	*Df* = 1	*Df* = 1	*Df* = 1
	F = 142	*F* = 138	*F* = 118
	$P = 6.63\times10^{-13}$	$P = 9.52\times10^{-13}$	$P = 6.54\times10^{-12}$
Individual	*Df* = 17	*Df* = 17	*Df* = 17
	F = 3.05	*F* = 3.76	*F* = 2.22
	$P = 3.71\times10^{-3}$	$P = 7.58\times10^{-4}$	$P = 2.76\times10^{-2}$

Mitella amamiana Y. Okuyama, **sp. nov.**—Figs. 3 & 7

Diagnosis: Similar to *Mitella doiana* Ohwi but larger and occasionally having petals (vs. completely lacking petals), with leaf blades 1.7–2.9 cm long and 1.4–2.4 cm wide (vs. 0.9–1.7 cm long and 1–1.6 cm wide), and petioles 3.3–6 cm (vs.1.3–3 cm) long.

Typus. JAPAN, Kagoshima Pref., Amami City, Sumiyo-cho, 24 February 2012, *Okuyama, Y., Goto N., Tabata, M., OK120225* (holotype: *TNS [1240156]*; isotypes: *TNS [1240158]*).

Herbs, perennial. Rhizome creeping. Radical leaves tufted; blade ovate, deeply cordate, acute, (1.3)–1.7–2.9 cm long, (1.2)–1.4–2.4 cm wide, shallowly lobulate, toothed, upper surface densely hirsute, dark green, lower surface hirsute mostly on nerves, pale green often with purplish tinge; petiole (2–)3.3–6 cm long, hirsute and slightly glandular pubescent, with fused axillary stipules at base; stipules scarious, ovate to oblong, entire, slightly hairy near apex. Flowering stems (3)–6–11 cm long, erect, hirsute and glandular pubescent. Inflorescences racemes, 1–3-flowered. Bracteole oblong, entire. Flowers March to April; pedicel 3–5 mm long, densely glandular pubescent. Calyx tube campanulate-obconic, sparsely glandular dotted, 2–3 mm long; calyx lobes 5, triangular-ovate, ca. 1 mm long and 1.3 mm wide, apex acute, abaxial surface glandular dotted, adaxial surface glabrous, erect, slightly recurved near tip, greenish. Petals occasionally present, green, subulate-linear or sometimes with one or two lobes on one or both sides, glabrous or with a few glandular dots. Stamens 5, alternate calyx lobes, ca. 1 mm long at anthesis;

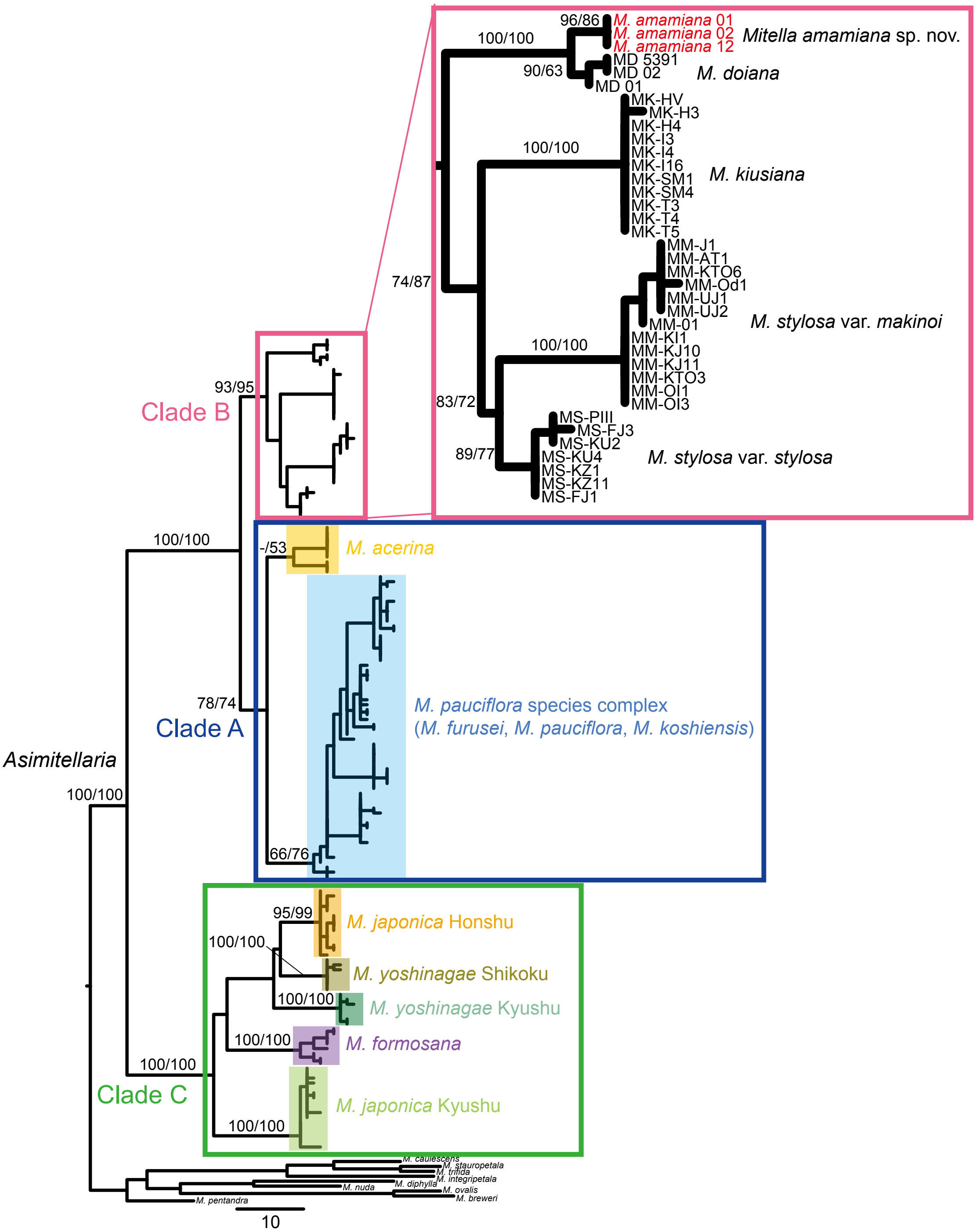

FIG. 5. One of the 7,600 most-parsimonious trees (L=475, CI=0.7516, RI=0.9723) obtained from the nrDNA dataset. Nodal support values by bootstrapping are indicated near major branches (MP/ML).

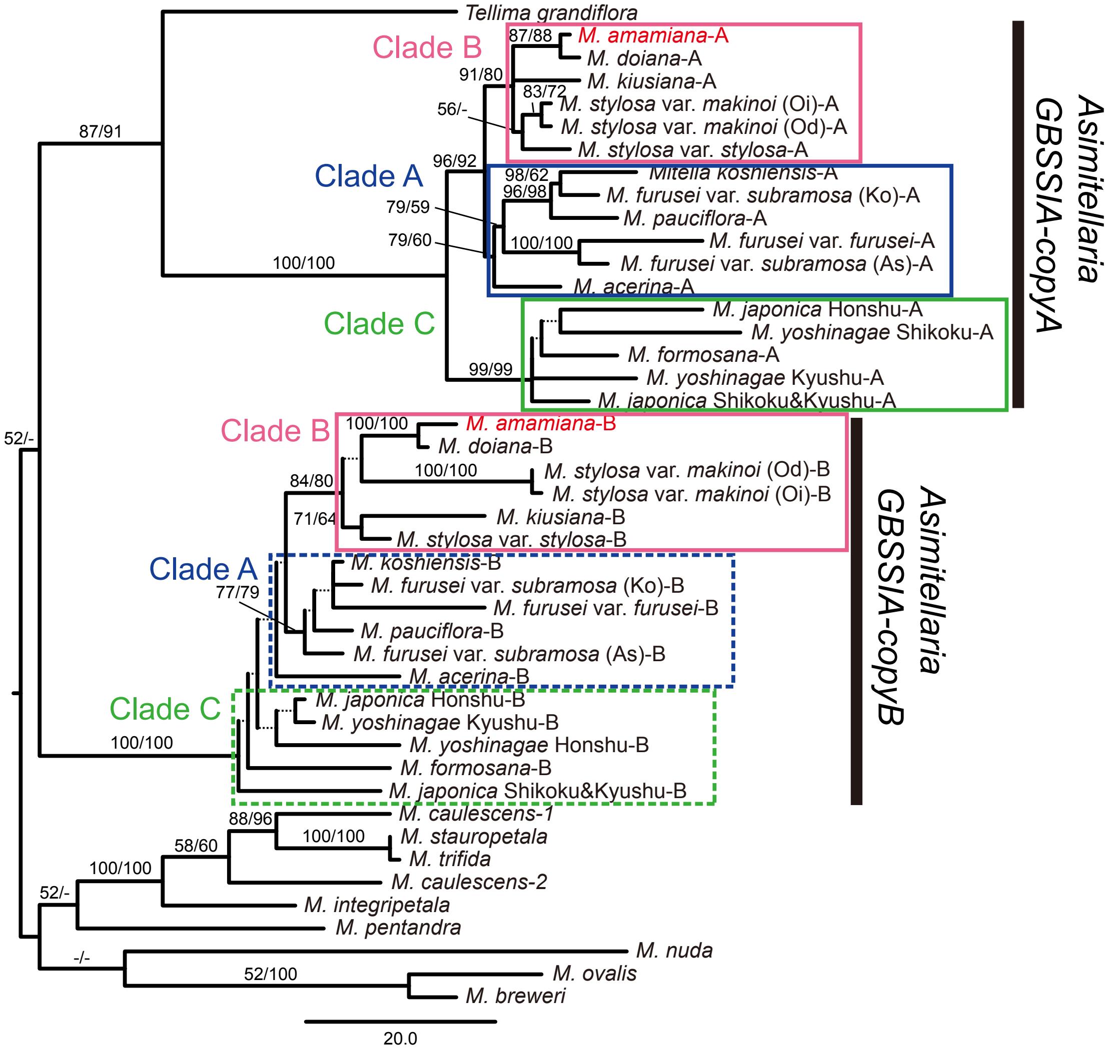

FIG. 6. One of the 2,435 most-parsimonious trees (L=634, CI=0.8344, RI=0.9200) obtained from the *GBSSI-A* dataset. Note that each individual of each species of *Asimitellaria* has two copies; namely, *GBSSIA-copyA* and *GBSSIA-copyB*; the copies form distinct phylogenies, each of which is comparable to the nrDNA tree. Branches not supported in the ML analysis are indicated by dashed lines; nodal support values by bootstrapping are indicated near major branches (MP/ML).

filaments ca. 0.6 mm long, green; anthers broadly orbicular, ca. 0.4 mm long, 0.7 mm wide, yellow. Disc inconspicuous. Ovary inferior. Styles 2, erect, 0.5–0.8 mm long, 1- or 2-lobed at tip, green, lobes spreading, stigma at apex of styles. Fruit a capsule, dehiscing between styles, 4–5 mm long, 5–6 mm wide; sparsely glandular dotted, ridged longitudinally. Seeds many, ovoid, ca. 1 mm long, 0.7 mm wide, scabrous and sparsely papillose, brownish.*Japanese name*. Amami-charumeru-so

Distribution. Japan, central Ryukyus, Kagoshima Pref., Amami-Oshima, Amami City, Sumiyo-cho, 300–430 m above sea level. Known only in this area.

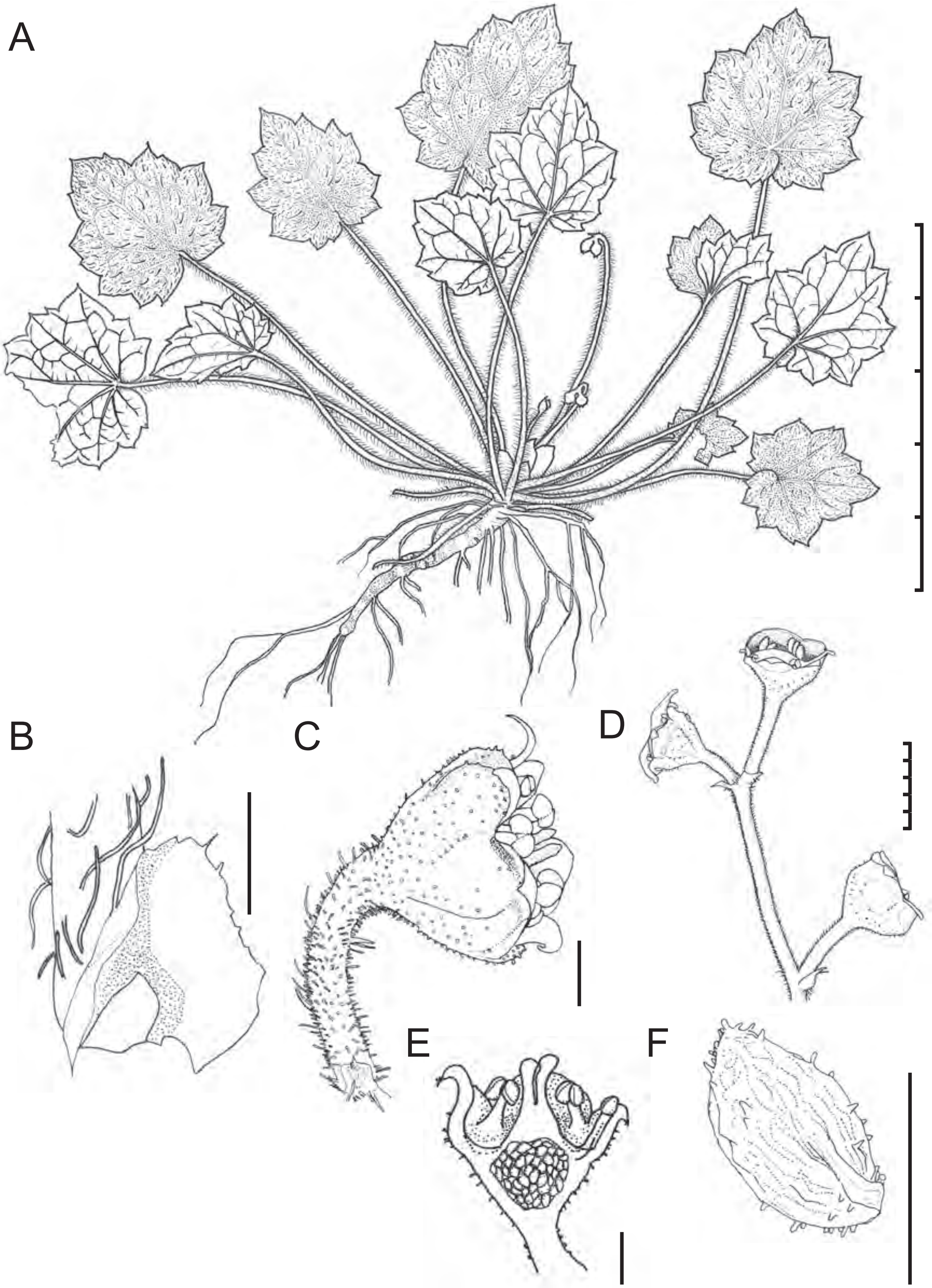

FIG. 7. *Mitella amamiana* Y. Okuyama. A: Whole plant. B: Stipule at the leaf base. C: Flower. D: Fruit. E: Vertical section of flower. F: Seed. Scale, A: 1 cm. B-F: 1 mm.

Other specimens examined. JAPAN, Kagoshima Pref., Amami City, Sumiyo-cho, 29 March 2011, *M. Tabata s.n.* (*TNS[01024774], TNS[01024777]*).

Etymology. The specific epithet refers to the name of the islands, Amami, to which Amami-Oshima belongs and where the type specimen was collected.

Phylogenetic position of Mitella amamiana

The number of sites in the aligned data matrices of nrDNA and *GBSSI-A* were 985 and 2,287, of which 233 and 204 were parsimony-informative, respectively. The phylogenetic tree based on nrDNA was almost identical with those reported in Okuyama & Kato (2009), where section *Asimitellaria* was subdivided into three clades A–C and most of the species (including some cryptic species to be described) were reconstructed as monophyletic groups (Fig. 5). Likewise, the phylogenetic tree based on *GBSSI-A* was concordant with that reported in Okuyama *et al.* (2012), where duplicated copies of the gene; namely, *GBSSI-A* copies A and B, each formed an independent phylogenetic tree that was comparable to the tree based on nrDNA (Fig. 6). Accordingly, in this study I obtained three independent phylogenetic trees to examine the evolutionary relationships of *M. amamiana*.

All three trees placed *M. amamiana* in Clade B as sister to *M. doiana*, with relatively strong branch support (87–100%). The results were consistent regardless of the phylogenetic criterion (MP or ML; Figs. 5 and 6). Moreover, in nrDNA, *M. amamiana* and *M. doiana* each formed a monophyletic clade with moderate to strong (63–96%) support (Fig. 5), indicating that the two groups of plants were closely related yet genetically distinct.

Discussion

Implication for phytogeography

Based on morphological and phylogenetic surveys, I found that the newly discovered *Mitella amamiana* is clearly distinct from other species of *Mitella*, including the most closely related species, *M. doiana*. The discovery of *M. amamiana* on Amami-Oshima in the central Ryukyus is surprising in two respects. First, the occurrence at 300–430 m elevation was unexpected, given that species of *Mitella* south of Kyushu Island (*M. doiana* on Yakushima and *M. formosana* in Taiwan, Fig. 1) are confined to elevations above 500 m elevations in habitats that are cool and moist for most of the year. The species of *Mitella* are highly susceptible to drought throughout their life cycle and show very low dispersability of the seeds, which are dispersed by a splash-cup mechanism mediated by raindrops (Savile 1975). For example, the populations of the closely related *M. kiusiana* on Kyushu Island are absent in the area associated with ignimbrite, which is a remnant of the last massive volcanic eruption of Mt. Aso 90,000 years ago (Kariyazaki, 1989). This suggests that *M. kiusiana* did not expand its range over the long period of time since the last volcanic activity. There may be unique environmental factors that have allowed the persistence of *M. amamiana* for at least thousands of years, which likely resulted from the unique terrain, climate, and vegetation in the area. Therefore, the entire area should be protected and special care taken to conserve *M. amamiana*.

Second, the sister relationship of *Mitella amamiana* to *M. doiana*, which is endemic to Yakushima Island in the northern Ryukyus, was also unexpected. It suggests the possibility that the two species are remnants of a continuous distribution of their ancestors across these regions. The major biogeographic gap between the central and northern Ryukyus (Fig. 1) has been attributed to the ancient breakup of the land connection between them (Tokara Gap). Although various hypotheses have been presented, the timing of the formation of the Tokara Gap dates back at least 1.3 million years (Mya) (Ota *et al.* 1998, Kimura, 2000). If the range of base substitution rates in the nrDNA ITS of herbaceous plants (1.72×10^{-9} to 8.34×10^{-9} substitutions/site/yr) as proposed in Kay *et al.* (2006) is applied to the whole nrDNA dataset (900 bp; when the less variable 5.8S region was excluded), the timing of the lineage split between *M. amamiana* and *M. doiana* can be roughly calculated as 0.3–1.3 Mya, which is marginally congruent with the age of the Tokara gap formation (>1.3 Mya). It is also possible that the species are the consequence of long distance dispersal between the two regions after formation of the Tokara gap. However, because the seeds and plant parts of *Asimitellaria* are extremely sensitive to desiccation and there is no evidence of long distance dispersal of any species of *Asimitellaria*, this scenario is a less likely explanation for the occurrence of *M. amamiana* and *M. doiana* across the Tokara Gap. Whichever the case, the present discovery is important in terms of illustrating the richness and uniqueness of the flora of the central Ryukyus, especially the Amami Islands.

I would like to thank Hidekazu Morita, Mitsutake Tabata, Seibun Higa, Masatsugu Yokota, and Goro Kokubugata who kindly informed me of the discovery and the current situation of the natural populations of *M. amamiana*. Mitsutake Tabata also kindly guided me to the natural population. I am also grateful to Kunihiko Uno who assisted with nucleotide sequencing of *M. amamiana* and Nana Goto who generated the helpful line drawing (Fig. 7A).

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Received June 16, 2015; accepted January 18, 2016