Reconsideration for Occurrence of *Mazus goodenifolius* (Phrymaceae) in Miyazaki Prefecture, Japan using Molecular and Morphological Data

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Abstract   Molecular analysis using ITS data and morphological observation were applied for reconsidering an occurrence of *Mazus goodenifolius* in Miyazaki Prefecture, Kyushu District of Japan. The analyses and observation indicated that the herbarium specimen collected from Miyazaki, and identified as *M. goodenifolius* by Yamazaki (1950, 1993) should be newly treated as *M. miquelii*. The distribution of *M. goodenifolius* in Japan is limited to the Ryukyus.

Key words : ITS, Japan, *Mazus*, Miyazaki, Ryukyus.

Introduction

The genus *Mazus* Lour. (Phrymaceae) consists of about 25 species with the centre of species diversity in China (Li, 1954; Walker, 1976; Hsieh, 2000). This genus has been traditionally included in the family Scrophulariaceae, but recent molecular phylogenetic studies have reclassified it in Phrymaceae (APGIII, 2009). In Japan, three species are reported as naturally occurring: *M. goodenifolius* (Hornem.) Pennell (Fig. 1A) studied herein, *M. miquelii* Makino (Fig. 1B) and *M. pumilus* (Burm.f.) Steenis (Yamazaki, 1993).

Mazus goodenifolius (Fig. 1A) is a biennial herb, distributed in Japan, Taiwan and New Guinea (Yamazaki, 1993). Following Yamazaki (1993), within Japan, this species is thought to occur in Miyazaki Prefecture, a southeastern part of Kyushu District, and Yaku, Amami and Okinawa Islands of the Ryukyus Archipelago (the Ryukyus) (Fig. 2). This species rarely occurs in the Ryukyus: only one and four localities were

respectively known in Amami Island (Kagoshima Prefecture, 2003) and Okinawa Island (Yokota and Hiraiwa, 2006); and its locality information for Yaku Island is thought to be insufficient (Kagoshima Prefecture, 2003). Therefore, this species is treated as a critically threatened species of Japan (Japanese Ministry of Environment, 2012).

Yamazaki (1950, 1993) reported the occurrence of this species in Miyazaki based on a single herbarium specimen (*N.Maruyama s. n.* [TNS701013]; Fig. 1C). This species has never been collected again from Miyazaki, and thus it is treated as a data deficient species by Miyazaki Prefecture (2000). The herbarium specimen of *N.Maruyama s. n.* (Fig. 1C) was originally determined as *Mazus englerianus* Bontati by T. Nakai; this name is now treated as a synonym of *M. miquelii* (Korea Forest Service, since 2010). Thereafter, Yamazaki (1950) determined the herbarium specimen as *M. fauriei* Bontati thought to be endemic to Taiwan (e.g. Hsieh, 2000). Subsequently, Yamazaki (1969) redetermined

Fig. 1.   Plants of two *Mazus* species and the voucher specimen for the putative occurrence of *M. goodenifolius* in Miyazaki Prefecture. A. *M. goodenifolius* (*HU451*; photographed on August 24, 2014 in Amami Island, Kagoshima). B. *M. miquelii* (*HU423*; photographed on August 16, 2014 in Tsukuba, Ibaraki). C. *M. goodenifolius* as identified by Yamazaki (1950, 1993) (*N.Maruyama s. n.* [TNS701013]; collected on April 4, 1948 in Nichinan, Miyazaki). Bars indicate 1 cm for A and B.

the herbarium specimen as *M. yakushimensis* Sugim. ex T.Yamaz., a species considered as endemic to Japan. Finally, Yamazaki (1993) treated *M. yakushimensis* as a synonym of *M. goodenifolius* and considered the species to be distributed in Japan and Taiwan. The occurrence of *M. goodenifolius* proposed by Yamazaki (1950, 1993) was supported by most major references of Japanese-national and domestic flora (Yamazaki, 1981, 1993; Shimabuku, 1997; Hatusima, 2004).

It is necessary to clarify the occurrence of this species in Miyazaki for estimating its extinction risk in Japan. In the present study, we conducted molecular analyses using ITS data and morphological observations on the herbarium specimen collected from Miyazaki (*N.Maruyama s. n.*)

together with plants of the three *Mazus* species in Japan, to enable resolution of the purported occurrence of *M. goodenifolius* in Miyazaki.

Materials and Methods

Plant materials

Taxonomic treatment and identification for *Mazus* plants follows Yamazaki (1993). In the present study, a single herbarium specimen (*N.Maruyama s. n.*; Fig. 1C), has been collected from Miyazaki, (Yamazaki, 1950, 1993) and is deposited in the herbarium of National Museum of Nature and Science (TNS). Total genomic DNA was extracted from the herbarium specimen for the present molecular phylogenetic analyses. We also obtained four plants of *M. goodenifolius* from

Amami Island (1 plant from 1 locality) and Okinawa Island (3 from 3); three plants of *M. miquelii* from Tsukuba, Hyogo and Miyazaki, Japan; two plants of *M. pumilus* from Kagoshima and Okinawa Island of the Ryukyus, Japan (Table 1 and Fig. 2).

Eleven operational taxonomic units (OTUs) were derived, comprising an outgroup member and ten ingroup members consisting of the herbarium specimen *N.Maruyama s. n.* from Miyazaki and the recently collected nine plants of three *Mazus* species. Voucher specimens for the present collections were deposited in the TNS herbarium.

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from leaves taken from one of four plants present on the herbarium specimen sheet of *N.Maruyama s. n*, and the collections from each locality. Leaves were dried rapidly in silica gel followed by DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The ITS region was amplified using polymerase chain reaction (PCR) with an iCycler (Bio-Rad, Hercules, CA, USA). Forward primer AB101 (5′-ACG AAT TCA AGG TCC GGT GAA TGT TTC G-3′) and reverse primer AB102 (5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′) were used for PCR (Douzery *et al.*, 1999). Amplifications were performed using the TaKaRa Sapphire Amp Fast Master Mix (TaKaRa, Otsu, Shiga, Japan) with Sapphire Amp (TaKaRa). The PCR profile was 35 cycles of 5 s at 94℃, 5 s at 50℃, and 5 s at 72℃ after an initial denaturing for 3 min at 94℃. PCR products were checked by electrophoresis before purification using illustra ExoProStar (GE Healthcare, Tokyo, Japan). Cycle sequencing reaction was performed with the BigDye™ Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA). For sequencing, the PCR primers above and additional-internal primer of ITS2N (5′-TCGCTGC-GTTCTTCATC-3′) and ITS3N (5′-GATGAA-GAACGCAGCGA-3′; T. Yukawa, personal communication). The Sanger sequencing prod-

ucts were then purified using ethanol precipitation. Automated sequencing was performed on a 3130xl Genetic Analyzer (Applied Biosystems). The electropherograms were analyzed using ATGC ver. 4.01 (Genetyx Co., Tokyo, Japan). Sequence data from this study were deposited in the DDBJ database (http://www.ddbj.nig.ac.jp/; Table 1).

Phylogenetic analyses

DNA sequences were aligned using the program ClustalW 1.8 (Thompson *et al.*, 1994), after which they were manually adjusted. Phylogenetic analyses were conducted based on a Bayesian approach using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford, 2002) using as an outgroup *Lancea tibetica* Hook. f. & Thoms. (Tank *et al.*, 2006; Xia *et al.*, 2009). Based on the Bayesian phylogenetic analysis, the hierarchical likelihood ratio test (hLRT) implemented in MrModeltest 2.2 (Nylander, 2004) was used to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed, each with a random starting tree and four chains (one cold and three heated). The MCMCMC length was 2 million generations, and the chain was sampled every 100th generation from the cold chain. The first 5000 sample trees (25% of the total 20,000 sample trees) were discarded as burn-in after checking that the average standard deviation of split frequencies (ASDSF) reached a stationary state at ＜0.01 thereafter. As a guide to convergence, the potential scale reduction factors (PSRFs) were close to 1.0 for all parameters in an output table. A 50% majority consensus tree of the output tree file from MrBayes was generated using TreeView (Page, 1996).

Based on the MP phylogenetic analysis, characters were treated as unordered, and character transformations were equally weighted. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony

Table 1. Plant materials of *Mazus* investigated in the present study and their ITS accession numbers

Species	Collection locality (altitude)	Abbreviation*	Voucher no.**	ITS Accession no.***	Type
INGROUP					
M. goodenifolius	Japan: Ryukyu, Amami Island, Amami (118 m)	R-AMM	HU451 (TNS)	LC027727[a]	*a*
	Japan: Ryukyu, Okinawa Island, Ogimi (158 m)	R-OKN1	HU218 (TNS)	LC027728[a]	*b*
	Japan: Ryukyu, Okinawa Island, Ogimi (107 m)	R-OKN2	HU486 (TNS)	LC027729[a]	*b*
	Japan: Ryukyu, Okinawa Island, Motobu (291 m)	R-OKN3	HU196 (TNS)	LC027730[a]	*b*
	Japan: Kyushu, Miyazaki, Nichinan (formerly Agata)	K-MYZ2	*N.Maruyama s. n.* (TNS)	LC027734[a]	*c*
M. miquelii	Japan: Honshu, Ibaraki, Tsukuba (313 m)	H-IBR	HU423 (TNS)	LC027731[a]	*d*
	Japan: Honshu, Hyogo, Yabu (150 m)	H-HYG	GK10636 (TNS)	LC027732[a]	*e*
	Japan: Kyushu, Miyazaki, Tano (80 m)	K-MYZ1	GK9438 (TNS)	LC027733[a]	*e*
M. pumilus	Japan: Ryukyu, Nakano-shima (15 m)	R-NKN	GK11311 (TNS)	LC027735[a]	*f*
	Japan: Ryukyu, Okinawa Island, Ogimi (158 m)	R-OKN1	HU224 (TNS)	LC027736[a]	*f*
OUTGROUP					
Lancea tibetica	China: Sichuan		XZ-2007–0525 (PE)	FJ172736[b]	*g*

* Referring to Fig. 1.
** HU: H. Umemoto; GK: G. Kokubugata.
*** [a] The present study; [b] Xia *et al.* (2009). These ITS sequence data were deposited in the DDBJ database.
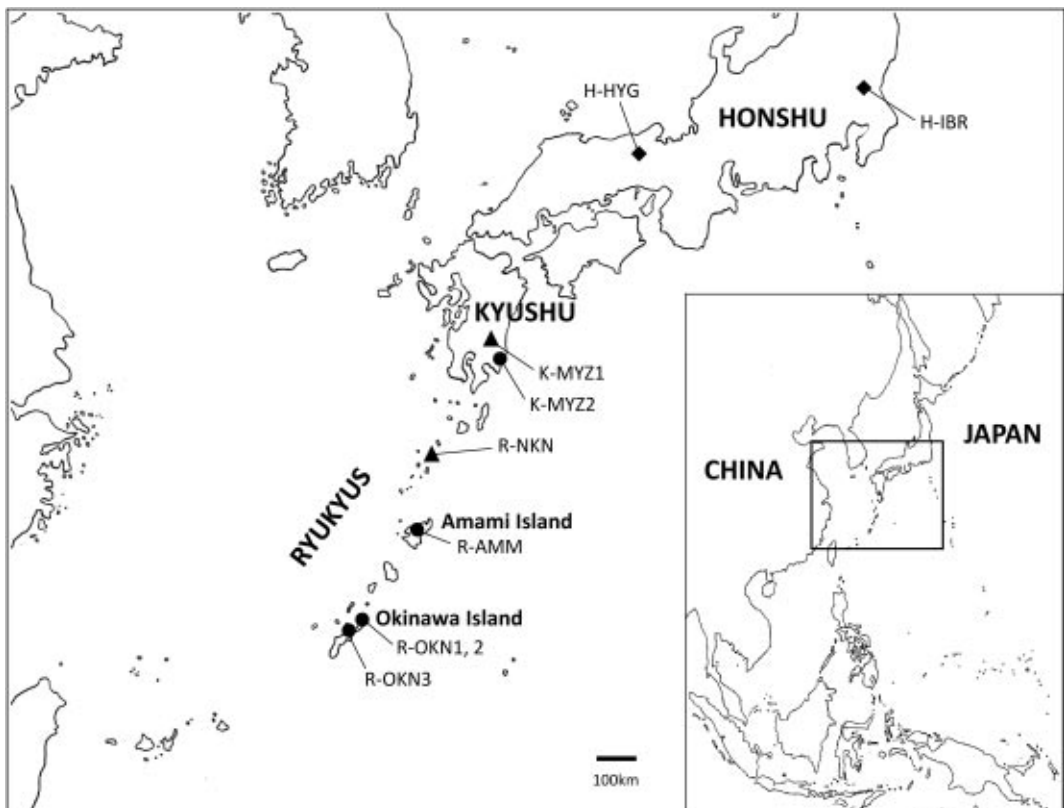
Fig. 2. Map showing collection localities of nine *Mazus* plants in the present study. Abbreviations refer to localities listed in Table 1. ●: *M. goodenifolius*. ◆: *M. miquelii*. ▲: *M. pumilus*.

search was performed with 200 replicates of random additions of sequences with ACCTRAN character optimization, tree bisection–reconnection (TBR) branch swapping, and MULTREES and STEEPEST DESCENT options on. Statistical support for each clade was assessed using bootstrap analysis (Felsenstein, 1985). A thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap values (BS).

Morphological observation of the herbarium specimen (N.Maruyama s. n. [TNS])

We checked corolla and stolon morphologies as these are considered valuable characters to identify *Mazus* species (Yamazaki, 1993; Hsieh, 2000).

Results

Phylogenetic analyses based on ITS

After alignment of the sequences from the eleven OTUs, a matrix of 654 bp was obtained for the ITS sequence. Five ITS types (*a–f*) were recognized in ten ingroup members: type *a*, *b*

and *c* for *M. goodenifolius* including the Miyazaki plant (*N.Maruyama s. n.*); type *d* and *e* for *M. miquelii*; type *f* for *M. pumilus* (Table 1, Fig. 2).

The model of GTR + I was selected for the Bayesian analysis. The 50% majority rule consensus tree of all post-burn-in trees is depicted with mean branch lengths and posterior probabilities (PPs; Fig. 3). In the MP analysis, 32 of 46 variable characters were parsimony-informative, and two equally parsimonious trees of 87 steps were obtained with CI = 0.989; RI = 0.980; RC = 0.969. Because the topology of the MP strict consensus tree (not shown) was compatible with that of the Bayesian 50% majority rule consensus tree, the bootstrap percentages (BSs; 1,000 replicates) were plotted on the Bayesian tree (Fig. 3).

The Bayesian and MP analysis (Fig. 3) showed two major clades in the ingroup (Clades I and II). Clade I comprised two plants of *M. pumilus* (type *f*) with high statistical support (PP = 1.00/BP = 100%); and Clade II (1.00/97) comprised the remaining ingroup members. In Clade II, two subclades were recognized (Clade IIA and IIB): Clade IIA (1.00/93) comprised
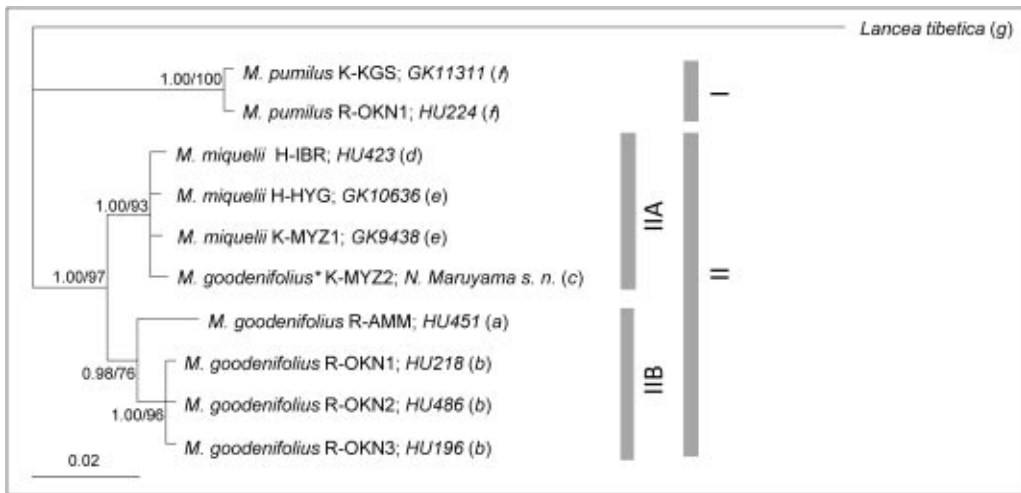

Fig. 3. The Bayesian 50% majority rule consensus tree of *Mazus* plants and an outgroup member based on nrITS sequences. The topology of the maximum parsimony strict consensus tree was compatible with the Bayesian tree. Alphabets in parentheses indicate nrITS types. Numerals above branches indicate Bayesian posterior probabilities (*upper*) and bootstrap percentages in the maximum parsimony analysis (*lower*; < − 50%).
* Identification according to Yamazaki (1950, 1993).

three plants of *M. miquelii* and the herbarium specimen of *N.Maruyama s. n.*; and Clade IIB (0.98/76) comprised four plants of *M. goodenifolius* from Amami and Okinawa Islands of the Ryukyus.

Morphological observation

In the four plants from the herbarium sheet (*N.Maruyama s. n.*), we could not observe corolla morphology, because corollas of the four plants were fragmentary, and size or shape could not be determined. With regard to stolon morphology, the four plants did not have developed stolons; however three of the four plants were well branched (Fig. 1C).

Discussion

The present molecular phylogenetic analyses revealed that the plant of *N.Maruyama s. n.* from Miyazaki, identified as *M. goodenifolius* by Yamazaki (1950, 1993) comprised a clade with three plants of *M. miquelii*, rather than the plants of *M. goodenifolius* from Amami and Okinawa Islands of the Ryuku, Japan (Fig. 3). Following Yamazaki (1993) and Hsieh (2000), *M. goodenifolius* and *M. miquelii* are distinguishable in corolla and stolon morphology: the former has a short corolla (0.7 to 1.0 cm long) and no stolons; while the latter has a longer corolla (1.5 to 2.0 cm) long and slender stolons. The plants on the herbarium specimen of *N.Maruyama s. n.* did not have developed stolons; however, it has been noted by Nemoto and Ohtsuka (1998) that it was not necessary that plants of *M. miquelii* always developed them, as this depended on seasonal and environmental factors. Furthermore, Nemoto and Ohtsuka (1998) stated that *M. miquelii* developed only short stolons until April, with most development during August to October. The plants on the herbarium sheet (*N.Maruyama s. n.*) were collected in early April and probably had not sufficient growing time to develop stolons. Therefore, the absence/presence of developed stolons is not a reliable key character to distinguish *M. miquelii* from *M. goodenifolius*.

In conclusion, based on molecular and morphological data, we identify the herbarium specimen collected from Miyazaki (*N.Maruyama s. n.*) as *M. miquelii* and not *M. goodenifolius*, thus disagreeing with Yamazaki (1950, 1993). As a consequence the distribution range of *M. goodenifolius* in Japan is limited in the Ryukyus.

References

APG III. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants. Botanical Journal of the Linnean Society 161: 105–121.

Douzery, E. J. P., Pridgeon, A. M., Kores, P., Linder, H. P., Kurzweil H. and Chase, M. W. 1999. Molecular phylogenetics of *Diseae* (Orchidaceae): a contribution from nuclear ribosomal ITS sequences. American Journal of Botany 86: 887–899.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Hatusima, S. 2004. Flora of Kyushu. A List of Ferns and Flowering Plants from Kyushu including the Amami Islands. 343 pp. The Kagoshima University Museum, Kagoshima (in Japanese).

Hsieh, T. H. 2000. Revision of *Mazus* Lour. (Scrophulariaceae) in Taiwan. Taiwania 45: 131–146.

Japanese Ministry of the Environment. 2012. Red Data List (Plants). http://www.biodic.go.jp/rdb/rl2012/redList 2012_ikansoku.csv (in Japanese). [Accessed and verified on February 4, 2015]

Kagoshima Prefecture. 2003. Kagoshima Red Data Book. 657 pp. Kagoshima Environmental Research and Service, Kagoshima (in Japanese).

Korea Forest Service, since 2010. The Korean Plant Names Index. http://www.nature.go.kr/newkfsweb/kfs/idx/SubIndex.do?orgId＝ekpni&mn＝ENG_10 [Accessed and verified on February 4, 2015]

Miyazaki Prefecture. 2000. The Red Data Species of Plants and Animals in Miyazaki Prefecture. 384 pp. Miyazaki Kankyo-Kagaku Society, Miyazaki (in Japanese).

Li, H. L. 1954. The genus *Mazus* (Scrophulariaceae).

Brittonia 8: 29–38.

Nemoto, M. and Ohtsuka, T. 1998. Effect of short-growing indigenous plants growing in surroundings of crop fields on the suppression of weed colonization. Journal of Weed Science and Technology 43: 26–34 (in Japanese with English abstract).

Nylander, J. A. A. 2004. MrModeltest ver. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Page, R. D. M. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Computer Applications in Bioscience 12: 357–358.

Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Shimabuku, K. 1997. Check List of Vascular Flora of the Ryukyu Islands, Revised Edition. Kyushu University Press, Fukuoka (in Japanese).

Swofford, D. L. 2002. PAUP: phylogenetic analysis using parsimony, version 4.0b10. Sinauer Associates, Sunderland, MA.

Tank, D. C., Beardsley, P. M., Kelchner, S. A. and Olmstead, R. G. 2006. Review of the systematics of Scrophulariaceae s. l. and their current disposition. Australian Systematic Botany 19: 289–307.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Walker, E. H. 1976. Flora of Okinawa and the Southern Ryukyu Island. 1159 pp. Smithsonian Institution Press, Washington, DC.

Xia, Z., Wang Y.-Z. and Smith, J. F. 2009. Familial placement and relations of *Rehmannia* and *Triaenophora* (Scrophulariaceae s. l.) inferred from five gene regions. American Journal of Botany 96: 519–530.

Yamazaki, T. 1950. Notulae ad Scrophulariaceae Asiae Orientalis (1). Journal of Japanese Botany 25: 209–217 (in Japanese with English abstract).

Yamazaki, T. 1969. Supplement of the flora of Ryukyus and Formosana (7). Journal of Japanese Botany 44: 349–351 (in Japanese with English abstract).

Yamazaki, T. 1981. Scrophulariaceae. In: Satake, Y., Owhi, J., Kitamura, S., Watari, S. and Tominari, T. (eds.), Wild Flowers of Japan III, pp. 97–121. Heibonsha. Tokyo (in Japanese).

Yamazaki, T. 1993. Scrophulariaceae. In: Iwatsuki, K., Yamazaki, T., Boufford, D. E. and Ohba, H. (eds.), Flora of Japan IIIa, pp. 326–335. Kodansha, Tokyo.

Yokota, M. and Hiraiwa, A. 2006. *Mazus goodenifolius* (Hornem.) Pennell. In: Okinawa Prefecture (ed.), Red Data Okinawa, revised version, pp. 163. Okinawa Prefecture, Naha (in Japanese).