



Morphological and molecular evaluation of Japanese *Plicanthus* R.M.Schust. (Anastrophyllaceae, Marchantiophyta)

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ABSTRACT: *Plicanthus birmensis* and *P. hirtellus* have been recognized in Japan. These species could be distinguished by features of ciliate leaf lobes and underleaves, and anatomy of stems. However, intermediate forms between these species can be found in Japan, and the circumscriptions of these species are unclear among Japanese populations. We evaluate morphological and molecular variations in Japanese *Plicanthus* to address the taxonomic identity of Japanese populations. The Japanese samples identified as *P. birmensis*, *P. hirtellus* and intermediate forms between them were nested within a clade. Among Japanese populations no significant correlation between morphological characters and phylogenetic relationships is confirmed, and the diagnostic characters to delimit these two species in Japan should be recognized as infraspecific variation. Thus, we recognize only one species, *Plicanthus hirtellus* in Japan.

KEY WORDS: Anastrophyllaceae, *Plicanthus hirtellus*, *Plicanthus birmensis*, phylogenetic relationships, infraspecific variation.

INTRODUCTION

Plicanthus R.M.Schust. is a liverwort genus belonging to the family Anastrophyllaceae. Four species are recognized within the genus worldwide (Söderström *et al.*, 2016): *P. birmensis* (Steph.) R.M.Schust., *P. difficilis* (Steph.) L.Söderstr. & Váňa, *P. giganteus* (Steph.) R.M.Schust., *P. hirtellus* (Web.) R.M.Schust. Among them, *P. birmensis* and *P. hirtellus* have been recognized in Japan (Kitagawa, 1965 as *Chandonanthus birmensis* and *C. hirtellus*; Katagiri and Furuki, 2018). These species could be distinguished by the following characters: 1–2 ciliate underleaves with cilia confined to basal part, nearly entire leaf lobes, and thin-walled, hyaline, non-trigonus medulla cells of stems for *P. birmensis* vs. numerous ciliate underleaves with cilia throughout margin from base to apex, numerous ciliate leaf lobes, and thick-walled, yellow, trigonus medulla cells of stems for *P. hirtellus* (Kitagawa, 1965; Udar and Kumar, 1982 as *C. birmensis* and *C. hirtellus*). For the reason mentioned above, Udar and Kumar (1982) said that the distinguishing features between them are sufficiently stable and both taxa deserve independent status. However, several authors have considered these taxa to be conspecific (Hattori, 1966; Grolle, 1995; Kis and Pócs, 1997 as *C. birmensis* and *C. hirtellus*; Schuster, 2002a, b). Grolle (1995) said that *P. birmensis* should be regarded as merely a weak or depauperate form of *P. hirtellus*. Kis and Pócs (1997) pointed out the similarity of their oil bodies and considered them possibly conspecific. Hattori

(1966) also said that *P. birmensis* may possibly be a not well-developed form of *P. hirtellus* because of the existence of various intergrading forms between them.

These intergrading forms between *P. birmensis* and *P. hirtellus* can be found in Japan (Kitagawa, 1965; Kodama, 1971; Ando *et al.*, 1975 as *C. birmensis* and *C. hirtellus*). Actually, Kitagawa (1965) argued that it was often almost impossible to find any reliable characters to distinguish these two species and it might be better to regard *P. birmensis* as a variety of *P. hirtellus*. However, he refrained from proposing conspecific treatment and retained these taxa at species rank since he did not examine the type specimen of *P. hirtellus* nor sufficient specimens from tropical regions. Schuster (2002b) described *P. hirtellus* and *P. birmensis* as a complex that needs further study.

In the present study, we evaluate morphological and molecular variation in Japanese *Plicanthus* to address the taxonomic identity of Japanese populations.

MATERIALS AND METHODS

Morphological comparison

To evaluate morphological variation in Japanese populations, leaves and underleaves from the middle part of the stem were selected. Based on the leaf characters we provisionally identified the materials under the following three names.

(1) *Plicanthus birmensis* (Fig. 1A–E): plants with entire leaf lobes, underleaves with 1–2 cilia confined to

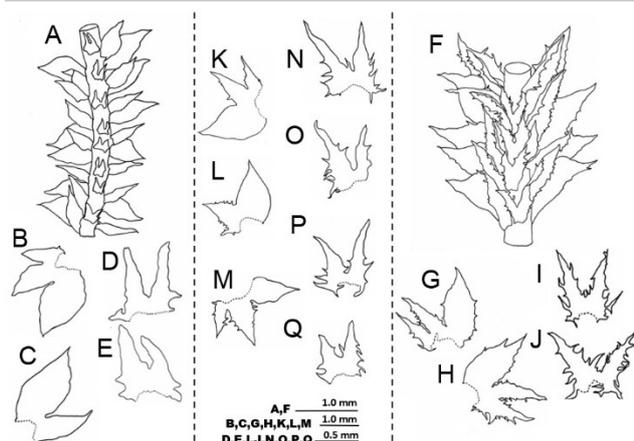


Fig. 1. Morphological variation of Japanese *Plicanthus*. **A–E**: entire form (identified as *P. birmensis*). **F–J**: ciliate form (identified as *P. hirtellus*). **K–Q**: intermediate form. **A, F**: part of shoot. **B, C, G, H, K, L, M**: leaves. **D, E, I, J, N, O, P, Q**: underleaves. **A–E** drawn from K. Amamoto 12, **F–J** from K. Amamoto 10, **K, L, N, O** from K. Amamoto 24, **M, P, Q** from K. Amamoto 16. All specimens are deposited in HIRO.

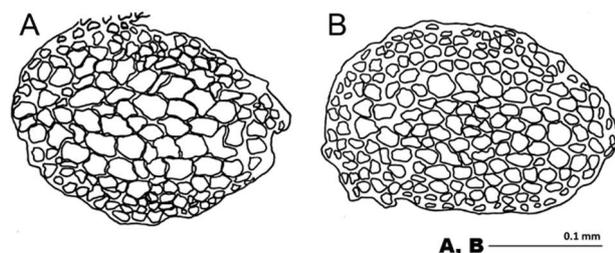


Fig. 2. Variation of medullary cells in stem cross section. **A**. Thin-walled, hyaline, non-trigonal medullary cells form. **B**. Thick-walled, yellowish, trigonal medullary cells form. **A** drawn from K. Amamoto 11, **B** from K. Amamoto 27. All specimens are deposited in HIRO.

basal margin (in leaves and underleaves).

(2) *Plicanthus hirtellus* (Fig. 1F–J): plants with numerous ciliate leaf lobes, underleaves with cilia throughout margin from base to apex (in leaves and underleaves).

(3) *Plicanthus* intermediate form (Fig. 1K–Q): plants that do not fall into the above two phenotypes. For instance, adaxial lobe ciliate but the other lobes entire, or adaxial lobe and middle lobe ciliate but abaxial lobe entire.

In addition to leaf characters, we also observed medullary cells in cross section of the middle part of the stem, which Udari and Kumar (1982) used for distinguishing *P. birmensis* and *P. hirtellus* (Fig. 2). Voucher information of each specimen is listed in Table S1.

Molecular phylogenetic analyses

We selected the following four loci for phylogenetic analysis: chloroplast ribulose 1, 5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*), ribosomal protein S4 (*rps4*), *trnL_{UAA}-trnF_{GAA}* region including the *trnL* intron and the *trnL-trnF* intergenic spacer (*trnL-F*)

and nuclear internal transcribed spacer (ITS). The protocol for DNA extraction and PCR amplification followed earlier publications (Suzuki *et al.*, 2013; Inoue and Tsubota 2014). PCR products were purified by using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Duren) following the manufacturer's protocols. Purified PCR products were sequenced by MacroGen Japan Corp. (Tokyo, Japan). Primers for PCR and DNA sequencing are listed in Table S2. Sequences obtained in the present study have been submitted to the DNA Data Bank of Japan (DDBJ), a member of International Nucleotide Sequence Database Collaboration (INSDC).

Based on the preliminary phylogenetic analyses and the results of Katagiri and Inoue (2018), and Bakalin *et al.* (2020), we selected 26 OTUs of *Plicanthus* as ingroup, and four OTUs of *Hattoria*, *Tetralophozia* and *Vietnamiella* as outgroup. Accession numbers for newly obtained and already published sequences are provided in Table 1.

Sequences of each locus were aligned separately using the program MAFFT ver. 7.515 (Katoh and Standley, 2013) with some manual adjustment on the sequence editor of MEGA ver. 7.0.21 (Kumar *et al.*, 2016). Gaps were treated as missing data. Two data matrices were produced: a concatenated matrix of *rbcL*, *rps4* and *trnL-F*, and ITS. Phylogenetic analyses were performed based on maximum likelihood and Bayesian inferences (ML/BI). The optimal nucleotide substitution model was selected based on the corrected Akaike information criterion (AICc; Sugiura, 1978) for ML and the Bayesian information criterion (BIC; Schwarz, 1978) for BI using Kakusan4 (ver. 4.0.2016.11.07; Tanabe, 2011). RAxML ver. 8.2.12 (Stamatakis, 2014) was used for ML using GTR + Gamma model, with a rapid bootstrap analysis of 10,000 replicates. MrBayes ver. 3.2.7a (Ronquist *et al.*, 2012) was used for BI using F81 Gamma model for first codon position of *rbcL*, JC69 + Gamma for second codon position of *rbcL*, HKY85 + Gamma for third codon position of *rbcL*, JC69 + Homogeneous for first codon position of *rps4*, HKY85 + Homogeneous for second and third codon positions of *rps4*, HKY85 + Gamma for *trnL-F*, and HKY85 + Gamma for ITS, with 10,000,000 generations, sampling tree every 1,000 generations. Convergence was assessed using Tracer ver. 1.7.1 (Rambaut *et al.*, 2018). A 50 % majority-rule consensus tree was calculated after the convergence of the chains and discarding 25 % of the sampled trees as burn-in.

RESULTS AND DISCUSSION

The chloroplast data matrix had a total length of 2,822 bp, of which 157 sites (6 %) were variable, and 63 sites (40 % of the variable sites) were parsimony informative. The nuclear data matrix had a total length of 829 bp, of which 136 sites (16 %) were variable, and 50 sites (37 % of the variable sites) were parsimony informative. No topological conflict was detected between ML and BI trees which

**Table 1.** List of taxa used for the phylogenetic analyses with voucher information and accession numbers. Newly obtained accession number is shown in bold.

Taxon	ID	Voucher information	Accession number			
			<i>rbcL</i>	<i>rps4</i>	<i>trnL-F</i>	ITS
Ingroup						
<i>Plicanthus birmensis</i>	JP01	Japan: Gifu, Amamoto 11 (HIRO)	LC648966	LC648988	LC649010	LC649037
<i>Plicanthus birmensis</i>	JP02	Japan: Hiroshima, Amamoto 12 (HIRO)	LC648967	LC648989	LC649011	LC649038
<i>Plicanthus birmensis</i>	JP03	Japan: Aichi, Amamoto 30 (HIRO)	LC648973	LC648995	LC649017	LC649044
<i>Plicanthus birmensis</i>	JP04	Japan: Mie, Amamoto 29 (HIRO)	LC648980	LC649002	LC649024	LC649051
<i>Plicanthus birmensis</i>	JP05	Japan: Fukuoka, Amamoto 35 (HIRO)	LC648981	LC649003	LC649025	LC649032
<i>Plicanthus birmensis</i>	JP06	Japan: Miyazaki, Amamoto 1113 (HIRO)	LC648985	LC649007	LC649029	LC649035
<i>Plicanthus birmensis</i>	MM01	Myammer: Kayah, Y. Inoue 7232 (HIRO)	LC648987	LC649009	LC649031	LC649033
<i>Plicanthus birmensis</i>	RU01	Russia: Primorskiy Kray, P-76-5-05 (KPABG)	-	-	EU791668	EU791791
<i>Plicanthus hirtellus</i>	JP07	Japan: Gifu, Amamoto 10 (HIRO)	LC648968	LC648990	LC649012	LC649039
<i>Plicanthus hirtellus</i>	JP08	Japan: Hiroshima, Amamoto 13 (HIRO)	LC648969	LC648991	LC649013	LC649040
<i>Plicanthus hirtellus</i>	JP09	Japan: Mie, Amamoto 25 (HIRO)	LC648977	LC648999	LC649021	LC649048
<i>Plicanthus hirtellus</i>	JP10	Japan: Mie, Amamoto 27 (HIRO)	LC648979	LC649001	LC649023	LC649050
<i>Plicanthus hirtellus</i>	JP11	Japan: Kochi, Amamoto 150 (HIRO)	LC648983	LC649005	LC649027	LC649053
<i>Plicanthus hirtellus</i>	JP12	Japan: Miyazaki, Amamoto 1112 (HIRO)	LC648984	LC649006	LC649028	LC649034
<i>Plicanthus hirtellus</i>	BT01	Bhutan: Bumthang, Long 28602 E	-	-	AY453785	-
<i>Plicanthus hirtellus</i>	CN01	China: Yunnan, Long 34407 E	KF852278	KF851383	-	-
<i>Plicanthus hirtellus</i>	NP01	Nepal, Long 30335 (E)	-	AM398304	-	-
<i>Plicanthus</i> intermediate form	JP13	Japan: Gifu, Amamoto 14 (HIRO)	LC648970	LC648992	LC649014	LC649041
<i>Plicanthus</i> intermediate form	JP14	Japan: Yamaguchi, Amamoto 16 (HIRO)	LC648971	LC648993	LC649015	LC649042
<i>Plicanthus</i> intermediate form	JP15	Japan: Yamaguchi, Amamoto 17 (HIRO)	LC648972	LC648994	LC649016	LC649043
<i>Plicanthus</i> intermediate form	JP16	Japan: Aichi, Amamoto 21 (HIRO)	LC648974	LC648996	LC649018	LC649045
<i>Plicanthus</i> intermediate form	JP17	Japan: Kyoto, Amamoto 22 (HIRO)	LC648975	LC648997	LC649019	LC649046
<i>Plicanthus</i> intermediate form	JP18	Japan: Nara, Amamoto 24 (HIRO)	LC648976	LC648998	LC649020	LC649047
<i>Plicanthus</i> intermediate form	JP19	Japan: Mie, Amamoto 26 (HIRO)	LC648978	LC649000	LC649022	LC649049
<i>Plicanthus</i> intermediate form	JP20	Japan: Fushushima, Nemoto 996 (private herbarium)	LC648982	LC649004	LC649026	LC649052
<i>Plicanthus</i> intermediate form	JP21	Japan: Miyazaki, Amamoto 1114 (HIRO)	LC648986	LC649008	LC649030	LC649036
Outgroup						
<i>Hattoria</i> <i>yakushimensis</i>	JP22	Japan: Yakushima Isl., Katagiri 4281 (NICH-488239)	LC376047	LC376048	LC376049	LC744594
<i>Tetralophozia</i> <i>filiformis</i>	CN02	<i>rbcL</i> , <i>rps4</i> : China: Yunnan, Shaw 5790 (DUKE) <i>trnL-F</i> , ITS: China: Yunnan, C-73-44-18 (VBGI) <i>rbcL</i> : Canada: Faubert 268.3 (GOET)	KF852352	KF851442	MZ229434	MZ231276
<i>Tetralophozia</i> <i>setiformis</i>	CASERU	<i>rps4</i> : Sweden: Soederstroem 2003/056 (BOL) <i>trnL-F</i> , ITS: Russia: Kamchatka, 99-06, 112052 (KPABG)	KC184764	AM398370	MZ229441	MZ231281
<i>Vietnamiella</i> <i>epiphytica</i>	VN01	Viet Nam: Lao Cai, V-9-7-17 (VBGI, KPABG)	MK290986	-	MK290984	MK277316

differed only at poorly supported nodes. Figures 3 and 4 show the ML tree with supporting values from bootstrap and Bayesian posterior probabilities (BP/PP).

Monophyly of *Plicanthus* was supported with high supporting values in both chloroplast and nuclear trees. Although three clades were resolved within the *Plicanthus* (Clade I, II, III in Figs. 3, 4), the supporting values for each clade are low to moderate. All Japanese samples were resolved in clade III, except for one sample (JP05 in clade I). The Japanese samples identified as *P. birmensis*, *P. hirtellus* and intermediate forms between

them were nested within clade III, and the morphological traits of leaves and stem medullary cells did not reflect the phylogeny (Figs. 3 and 4). The single Japanese sample (JP05) was resolved in clade I with the samples from Bhutan, China and Russia, while no morphological differences were detected among the Japanese samples in clade I and III, and even the Myanmar sample in clade II.

These results support the views that *P. birmensis* and *P. hirtellus* are conspecific (Kitagawa, 1965; Hattori, 1966; Grolle, 1995; Kis and Pócs, 1997 as *C. birmensis* and *C. hirtellus*; Schuster, 2002a, b), and the diagnostic

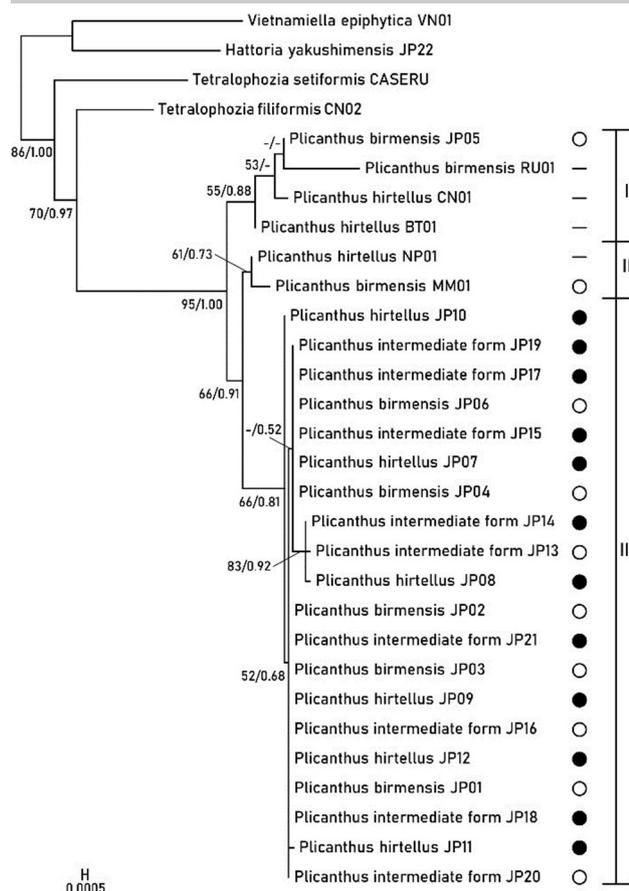


Fig. 3. Maximum likelihood tree of the genus *Plicanthus* based on the concatenated sequences of *rbcL*, *rps4* and *trnL-F*, depicted by RAxML. Supporting values more than 50 % obtained by RAxML for bootstrap probabilities (BP) and MrBayes for Bayesian posterior probabilities (PP) are shown on each branch (BP/PP). The root is arbitrarily placed on the branch leading to *Hattoria yakushimensis* and *Vietnamiella epiphytica*. ○. Thin walled, hyaline medullary cells. ●. thick walled, trigonous, yellowish medullary cells.

characters to delimit these two species in Japan should be recognized as infraspecific variation. Thus, we conclude that Japanese populations identified as *P. birmensis* or *P. hirtellus* are conspecific. Based on the nomenclatural priority, here we assign *Plicanthus hirtellus* (F. Weber) R.M. Schust to the Japanese populations. However, molecular data of the plants from Africa where *P. hirtellus* was originally described, have not been revealed. Further morpho-molecular evaluation will address the well-established circumscription of *P. hirtellus*.

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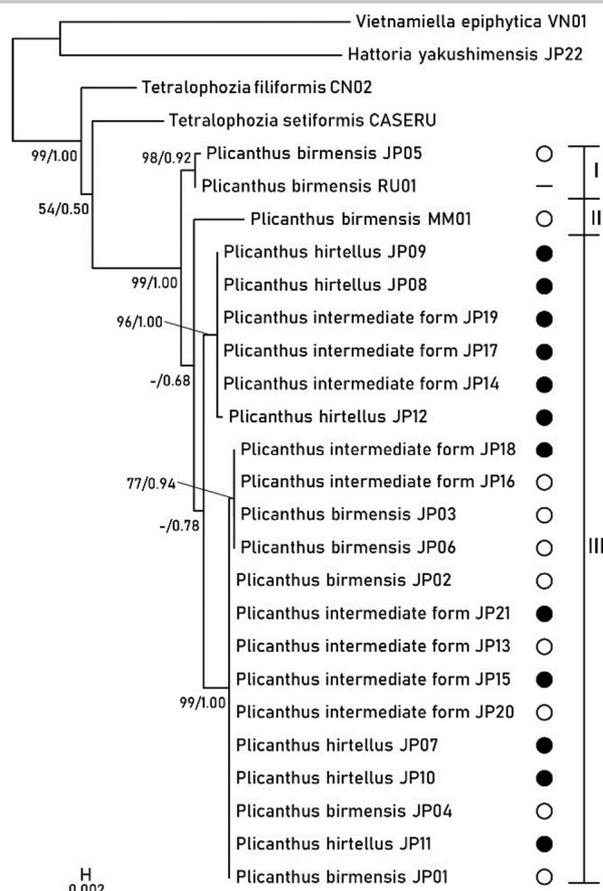


Fig. 4. Maximum likelihood tree of the genus *Plicanthus* based on ITS sequences, depicted by RAxML. Supporting values more than 50 % obtained by RAxML for bootstrap probabilities (BP) and MrBayes for Bayesian posterior probabilities (PP) are shown on each branch (BP/PP). The root is arbitrarily placed on the branch leading to *Hattoria yakushimensis* and *Vietnamiella epiphytica*. ○. Thin walled, hyaline medullary cells. ●. thick walled, trigonous, yellowish medullary cells.

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Supplementary materials are available from Journal Website