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Lectotypification of *Usnea confusa* (Parmeliaceae, Ascomycota)

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Abstract The original material of *Usnea confusa* Asahina consists of several thalli glued on a cardboard. In order to avoid any future taxonomic confusion especially presence or absence of "isidiate soredia", a single specimen with numerous isidiofibrils developing on the soralia was chosen as lectotype. The lectotype of *U. confusa* contains usnic, salazinic, constictic acids and trace amount of protocetraric acid as the secondary substances. ITS rDNA sequences of Japanese and Taiwanese specimens that have the same morphology and chemistry with the lectotype form two distinct clades nested within the strongly supported clade representing the core group of *Usnea cornuta* (containing *U. cornuta* Körber s.str.). Our molecular phylogenetic result based only on ITS rDNA sequences doesn't allow to confirm or contradict the conspecificity of *U. confusa* with *U. cornuta*.

Key words: isidiofibrils, ITS rDNA, lectotype, lichenized fungi, phylogeny, soralia, taxonomy.

Introduction

In lichenology, the genus *Usnea* Adans. is known as one of the most difficult genera due to the high morphological variability within species (Clerc, 1998).

Among the more than 350 species of this genus widely distributed in the world (Lücking *et al.*, 2017), *Usnea cornuta* Körb. *s. lat.* is a good example showing such taxonomical difficulty to delimit the species. The diagnostic features of *U. cornuta s. lat.* defined by Gerlach *et al.* (2019) [as "*U. cornuta* aggregate" including morphospecies of *U. brasiliensis* (Zahlbr.) Motyka, *U. cornuta* and *U. dasaea* Stirt.] are the following characters: thallus erect-shrubby to subpendulous with more or less inflated branches constricted at their point of attachment; soralia minute with various shapes and ontogeny, ± covered with isidiomorphs; the ratio of cortex, medulla and axis (CMA) of main branches is of the *cornuta*-type (Truong *et al.*, 2011) or of the *brasiliensis*-type (Gerlach *et al.*, 2017), the density of medullary hyphae varies from dense to lax; the many chemotypes designated by main substances such as: salazinic, constictic, stictic, norstictic, protocetraric, psoromic, galbinic, thamnolic or lobaric acids; and the worldwide distribution occurring in Europe, Asia, North and South America, Australia, and Africa. After the finding that this group is polyphyletic by Truong *et al.* (2013), Gerlach *et al.* (2019) investigated species boundaries in this group and found at least nine strongly supported distinct lineages. Although *U. cornuta s. lat.* should be carefully revised based on morphological and chemical features in further researches, *U. cornuta s. str.* can be characterized by the minute and numerous soralia that are even with the cortex and confluent, a *cornuta*-type CMA, constictic, stictic, salazinic or norstictic acid chemotype. This group corresponds to "lineage 5" in the phylogenetic tree (Gerlach *et al.*, 2019).

To understand such complicated and cosmopo-

lite species, examinations of the related type specimens that were collected from various localities in the world and the molecular data of the local populations are prerequisite. However, in many cases, the original material of *Usnea* species is heterogeneous due to the high morphological similarity of closely related species. If we want to progress towards a sound taxonomy of the genus, it is thus very important to lectotypify such species represented by such heterogenous type material.

Usnea confusa Asahina was originally described from Japan (Asahina, 1956) and it is currently considered as a synonym of *U. cornuta* (Clerc, 2004). The original material of this species consists of 10 specimens glued on a cardboard (Fig. 1A). To avoid future taxonomic confusion, a single thallus is here lectotypified.

This paper further aims at providing ITS rDNA sequences of Japanese and Taiwanese collections corresponding morphologically, anatomically and chemically to the lectotype of *U. confusa* and integrating them in the general phylogeny of the group published by Gerlach *et al.* (2019).

Materials and Methods

This study is based on the examinations of herbarium specimens housed in the Department of Botany, National Museum of Nature and Science (TNS), Tsukuba, Japan.

Morphological observations for identification were made using a dissecting microscope and a bright field microscope. The ratios of thickness of the cortex, medulla, and axis for the branch were measured following the method of Clerc (1984, 1987). Cross sections of thallus were cut by hand with a razor blade, and observed after mounting in GAW (glycerin : ethanol : water, 1 : 1 : 1).

Lichen substances were examined using thin layer chromatography (TLC) (Culberson and Johnson, 1982). Solvent B system (hexane : methyl tert-butyl ether : formic acid, 140 : 72 : 18) was used for all TLC analyses.

DNA extraction followed a modified CTAB protocol (Hosaka, 2009).

For DNA amplification, 10 µL of PCR mix contained 1 µL genomic DNA extraction, 0.25 µL of each primer (10 pmol/µL) and 5 µL Emerald-Amp PCR Master Mix (TaKaRa Bio Inc.). PCR amplification of the ITS rDNA region (including ITS1, 5.8S rDNA and ITS2) was performed using the primer set of ITS1F (Gardes and Bruns, 1993) as the 5′ primer and LR1 (Vilgalys and Hester, 1990) as the 3′ primer. In some cases, when no PCR product was appeared by ITS1F/LR1 primer pair, USITS1-F as the 5′ primer and USITS2-R as the 3′ primer (Ohmura, 2008) were used for the PCR. PCR cycling conditions were 94℃ (3 min), followed by 11 cycles of 95℃ (30 sec), 62℃ to 52℃ (30 sec) with annealing temperatures lowered by 1℃ between cycles, and 72℃ (1 min), followed by 30 cycles at 52℃ annealing temperature and a final extension at 72℃ (7 min). Sequencing was done on an ABI Prism 3130x genetic analyzer (Applied Biosystems) using the BigDye Terminator ver. 3.1 Cycle Sequencing Kit according to the manufacturer's instructions.

The sequences were aligned in MAFFT ver. 7 (Katoh *et al.*, 2017) using the default settings. After removing sites with gaps, missing data and ambiguous data, the resulting alignment of 483 sites was used for the molecular phylogenetic analyses.

The maximum likelihood (ML) (Felsenstein, 1981) and neighbor-joining (NJ) (Saitou and Nei, 1987) analyses with the best nucleotide substitution model were performed. Kimura 2-parameter (Kimura, 1980) plus gamma distribution (K2P+G) was selected for the model. The bootstrap values (Felsenstein, 1985) with 1,000 replicates for ML and NJ were shown on the branches only when both are ≥ 50% simultaneously. All calculations were conducteed in MEGA 7.0.18 (Kumar *et al.*, 2016).

The sample data for molecular analyses and their DDBJ/EMBL/GenBank accession numbers for the obtained ITS rDNA sequences are shown in Table 1.

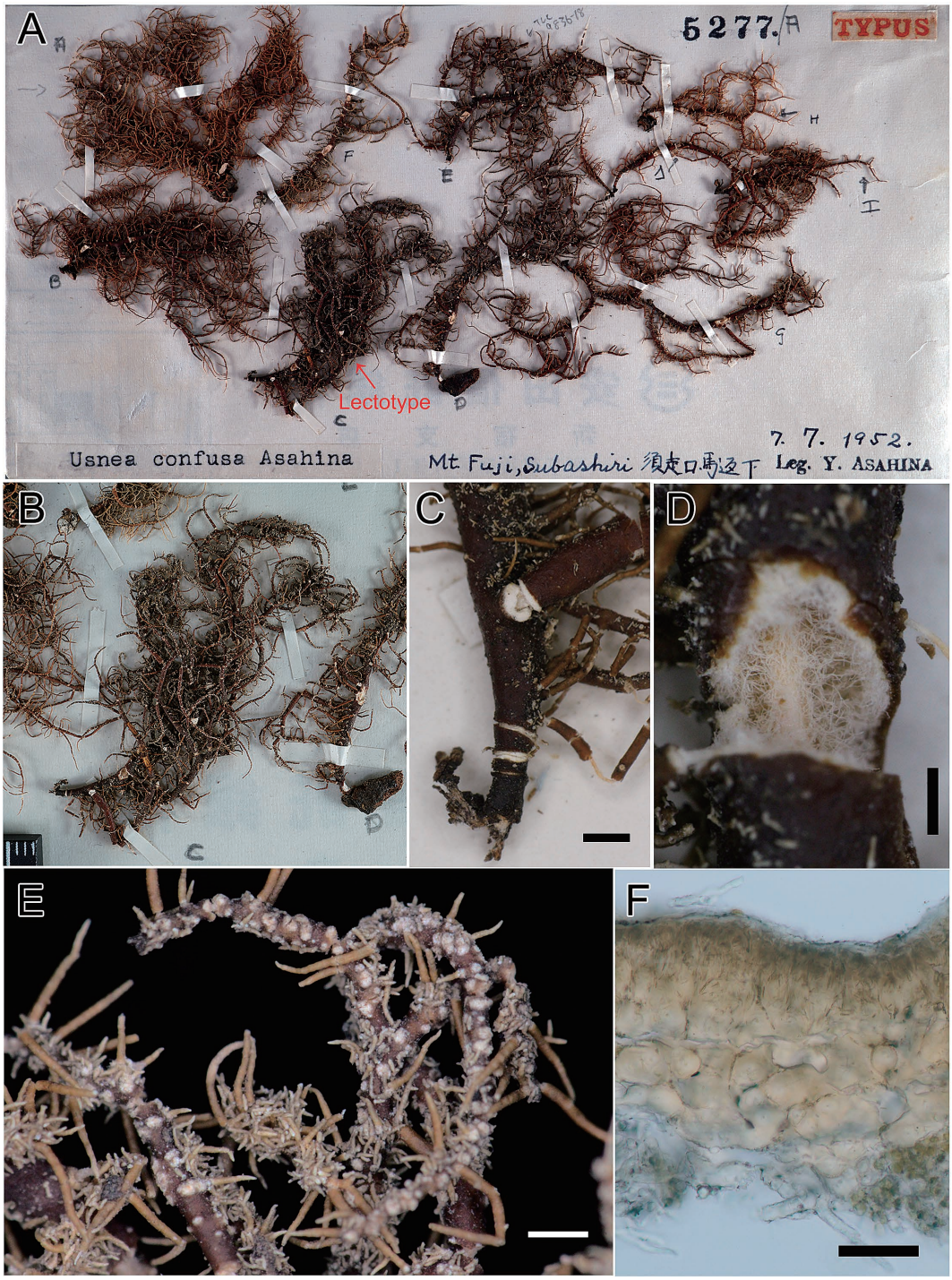

Fig. 1. Lectotype of *Usnea confusa* Asahina. A. Thallus "C" on the sheet was designated as the lectotype. B. Thallus. C. Base of the thallus. D. Cortex, medulla and axis in a slightly inflated branch. E. Isidiofibrils on soralia. F. *Merrillii*-type cortex. Scales: C=1 mm, D, E=0.5 mm, F=50 µm.

Table 1. DDBJ/EMBL/Genbank accession numbers of samples used for molecular analyses in this study

Taxon	Voucher of Y. Ohmura (YO)* or OTU name	Accession no. for ITS rDNA sequence	Specimens (YO)* which have the identical sequence	Reference
Usnea brasiliensis	bras-13BR	MF669812	—	Gerlach *et al.* (2019)
Usnea cornuta s.str.	corn-19BR	MF669861	—	Gerlach *et al.* (2019)
	corn-32US	JQ837300	—	Truong *et al.* (2013)
	corn-113BR	MF669835	—	Gerlach *et al.* (2019)
	corn-171BR	MF669869	—	Gerlach *et al.* (2019)
	corn-176BR	MF669847	—	Gerlach *et al.* (2019)
	corn-198BR	MF669872	—	Gerlach *et al.* (2019)
	YO7376	LC479119		This study
	YO8494A	LC479120	YO8494B, YO8494C	This study
	YO8506B	LC479121	—	This study
	YO10392B	LC479122	YO6218, YO6977, YO7270, YO7276, YO7333, YO8509, YO8850, YO8855B, YO8688B	This study
	YO10417	LC479123	YO8855A	This study
	YO10991	LC479124	—	This study
Usnea rubrotincta	YO10300	LC479125	—	This study
Usnea sp. [=*U. cornuta s. lat. sense* Gerlach *et al.* (2019)]	corn6-28BR	MF669826	—	Gerlach *et al.* (2019)
	corn6-83BR	MF669865	—	Gerlach *et al.* (2019)
	corn6-90BR	MF669866	—	Gerlach *et al.* (2019)
	corn6-170BR	MF669868	—	Gerlach *et al.* (2019)

*The detail collection data for voucher specimens of YO are shown in the paragraph of specimens used in the phylogenetic analyses in the main text except YO10300 (*Usnea rubrotincta* Stirt.) which was collected in Japan, Shikoku, Prov. Iyo (Pref. Ehime): Furuiwaya, Kumakogen-cho, Kamiukena-gun (N33°40′, E132°58′), on trunk of dead tree, elevation about 500 m, 11 November 2013 (TNS).

Results and Discussion

Lectotypification of Usnea confusa

Usnea confusa Asahina, Lich. Jpn. 3: 97, 1956. Lectotype (designated here): JAPAN, Honshu, Prov. Suruga, Subashiri-guchi, Mt. Fuji, 7 July 1952, Y. Asahina 5277, thallus "C" as shown in Fig. 1A, (TNS!). % C/M/A = 4.6/38.2/14.5. Chemistry: usnic, salazinic, constictic and protocetraric (trace) acids.

Although Ohmura (2001) already selected the lectotype of *U. confusa* as being the material collected by Y. Asahina with collection number 5277 (TNS), further studies showed that this material was in fact heterogeneous. Among the ten thalli glued on the cardboard marked as "TYPUS" and prepared by Y. Asahina (Fig. 1A), two of them correspond to *U. hondoensis* Asahina that has uninflated branches with punctiform soralia and the chemistry of usnic, norstictic and salazinic acids (thalli "F" and "H" in Fig. 1A, annotated by Y. Ohmura as "*U. pangiana*" in 1997). The eight remaining thalli belong to the same species corresponding to the description of *U. confusa*, and they have the same chemistry, i.e. usnic, salazinic, constictic and protocetraric (trace) acids. However, the amount of isidiofibrils on soralia in each thallus varies very much. To avoid any confusion in future taxonomic treatment, we selected one thallus bearing abundant isidiofibrils (thallus "C" in Fig. 1) according to the mention "soredia isidiosa" in the protologue (Asahina, 1956).

Isidiofibrils

The term of isidiofibril was defined by Truong *et al.* (2011), as being the secondary development of an axis as soon as a certain size is reached during the growth of an isidiomorph. This was already reported in *U. hakonensis* Asa-

hina, an Asian taxon (Ohmura, 2001), and it is here further observed in *U. confusa* (or *U. cornuta* s.str.). Morphologically, isidiofibrils may be confused with isidiomorphs but they usually are taller and slender, with sometimes a sinuose irregular form. Isidiofibrils are thus an older stage of isidiomorphs and more studies are necessary to access whether this character might be used to delimit species in the taxonomy of the genus *Usnea*. The amount of isidiofibrils varies depending on the developmental stage of thallus and on some environmental parameters of the habitat. Then the presence of isidiofibrils would be more the result of the older age of a thallus or of environmental changes than of the genetic makeup of the species.

Molecular analyses

A total of 18 sequences of ITS rDNA (491 bp) for the Japanese and Taiwanese specimens of *U. cornuta* morph with usnic, salazinic, constictic and protocetraric (trace) acids was obtained in this study, and six haplotypes were recognized among them. Based on the molecular phylogenetic analyses, two distinct clades ("confusa 1" and "confusa 2") (bootstrap values for ML/NJ = 85/85 and 88/91 respectively) were formed within the strongly supported clade of *U. cornuta s. str.* (bootstrap values: 96/98) (Fig. 2). The topology of the tree is not in conflict with the one shown in Gerlach *et al.* (2019). Thus, the tree in Fig. 2 clearly shows that *U. confusa* belongs to the core group of *U. cornuta* (Gerlach *et al.* 2019: lineage 5). On the base of morphological, anatomical and chemical studies, Clerc (2004) considered *U. confusa* being a synonym of *U. cornuta*. At first glance, our results might support this synonymy. However, our study neither really confirms or contradicts this affirmation (Fig. 2). Internal relationships of this group are not well supported and Gerlach *et al.* (2019) emphasized the need of fine-scale studies with a larger sampling over the entire distribution range of the lineage. Within each clade of "confusa 1" and "confusa 2", the differences among the sequences of haplotypes are only one residue (homology is 99.8%). In contrast, the difference between the sequences of the clades "confusa 1" and "confusa 2" is 7 to 11 residues (homology is 97.8 to 98.6%). No morphological and chemical difference were found between the specimens representing these two clades. So far, other haplotypes of *U. cornuta s. str.* were not detected from Japan and Taiwan. It is then possible that the eastern Asian population of *U. cornuta* are on the way of speciation. However probably due to the use for the phylogenetic analyses of ITS rDNA sequences only, the relationships among the *U. cornuta* core group are not well resolved. The use of multigene analyses or of high-throughput sequencing technology will be necessary to improve the deep relationships inside the *U. cornuta* group to settle this case.

The ITS rDNA region is considered as a good DNA barcoding marker to recognize fungal taxa including lichenized fungi (Begerow *et al.* 2010; Orock *et al.* 2012). However, our study agree with Badotti *et al.* (2018) who stated that the use of this marker only might in some cases not be sufficient to separate fungal species, especially when the speciation is a recent event and processes like incomplete lineage sorting and reticulation are at work, obscuring species boundaries and relationships (Naciri and Linder, 2015).

Specimens used in the phylogenetic analyses (all vouchers in TNS). **JAPAN**. Hokkaido. Prov. Kushiro: Aikappu, Akkeshi-cho, Akkeshi-gun (N43°01′09″, E144°50′20″), on bark of *Cerasus sargentii*, elevation about 80 m, 28 May 2012, Y. Ohmura *et al.* 8850; Tobai, Akkeshi-cho, Akkeshi-gun (N42°59′54″, E144°53′31″), on twig of *Abies sachalinensis*, elevation about 80 m, 28 May 2012, Y. Ohmura *et al.* 8855A, 8855B. Honshu. Prov. Musashi (Tokyo Metropolis): Inamura-iwa, Nippara, Okutama-machi, Nishitama-gun (N35°50′28″, E139°02′00″), on trunk of *Juniperus rigida*, elevation about 800 m, 24 September 2015, Y. Ohmura 10991. Prov. Kii (Pref. Wakayama): Fukuchiin Temple, Mt. Koya, Koya-cho, Ito-gun (N34°12′, E135°35′), on bark of pine tree, elevation 820 m, 2 March 2012, Y. Ohmura 8494 & A. Frisch; Kiyotaka-Inari

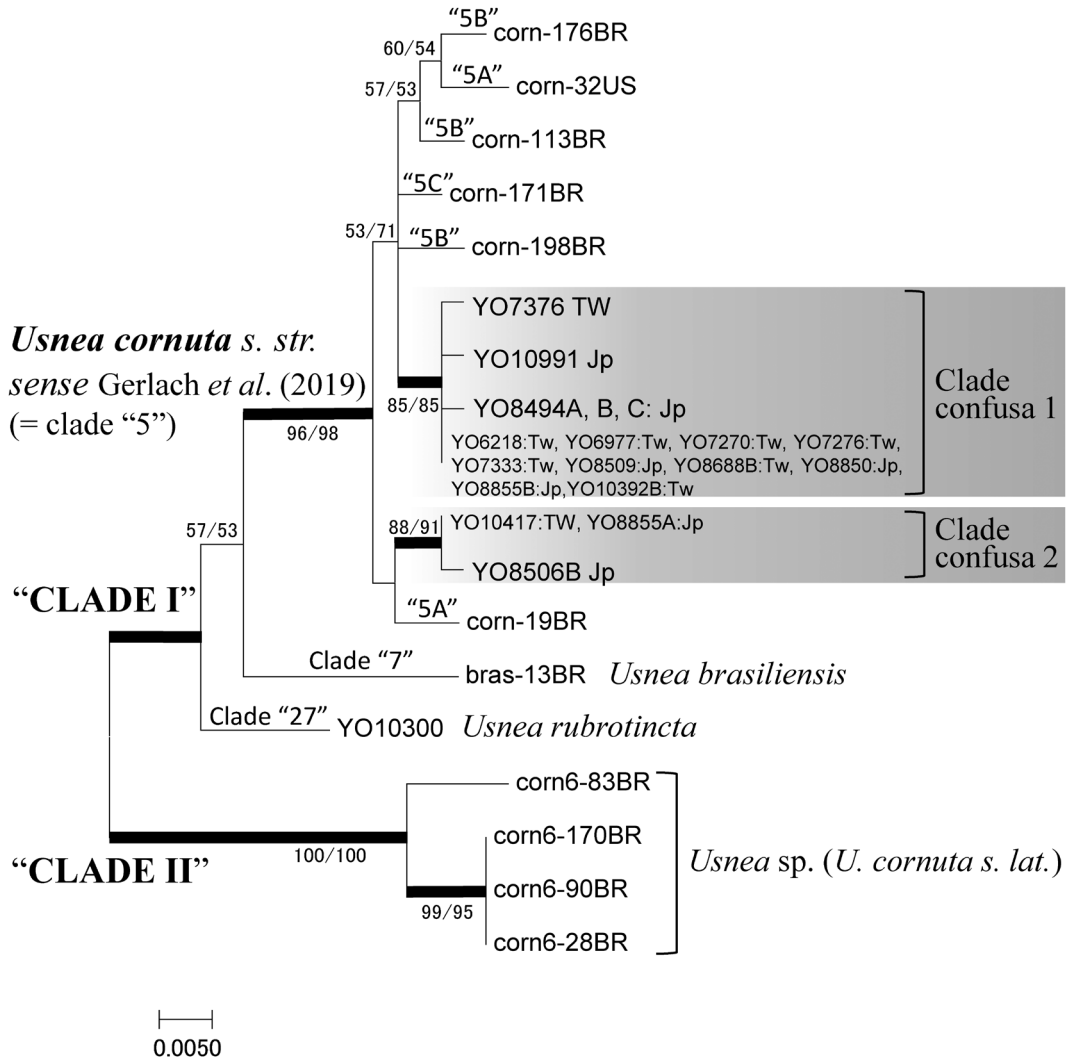

Fig. 2. Molecular phylogenetic tree of *Usnea cornuta* and the related taxa based on ITS rDNA sequences. The tree was constructed by ML method, and the reliability of each branch was tested by ML and NJ methods. The bootstrap values for ML/NJ analyses are shown on the branches only when both are ≥ 50% simultaneously. Bold branches indicate they are ≥ 80% simultaneously. The OTU names indicated the vouchers of Y. Ohmura (YO) and those used in Gerlach *et al.* (2019) (see Table 1). Jp and Tw indicating after ":" of YO vouchers are Japan and Taiwan as the collection locality. Clade numbers or names in the double quotations are identical with those in Gerlach *et al.* (2019). The topology of this tree with statistically supported branches is not conflicted with that in Gerlach *et al.* (2019).

Shrine, Mt. Koya, Koya-cho, Ito-gun (N34°13′, E135°36′), on wooden pole, elevation 820 m, 3 March 2012, Y. Ohmura 8509 & A. Frisch; Okunoin, Mt. Koya, Koya-cho, Ito-gun (N34°13′, E135°36′), on bark of *Cryptomeria japonica*, elevation 820 m, 3 March 2012, YO8506B. **TAIWAN**. Miaoli Co.: Xuejian Recreation Area, Taian Township (N24°25′36″, E121°00′53″), on trunk of *Prunus* sp., elevation 1886 m, 8 October 2013, Y. Ohmura 10392B; the same locality (N24°26′44″, E121°01′32″), on bark of *Pinus taiwanensis*, elevation 2150 m, 8 October 2013, Y. Ohmura 10417. Taichung Co.: between 0 km and 6.8 km point of mountain trail, en route from

Shiyuan Yakou to Mt. Nanhu (N24°23′14″, E121°21′29″), on bark of broad-leaf deciduous tree, elevation 1977 m, 30 September 2010, Y. Ohmura 7333; the same locality, on bark of *Pinus* sp., Y. Ohmura 7276; the same locality (N24°23′29″, E121°21′09″), on bark of *Betula* sp., elevation 1948 m, 30 September 2010, Y. Ohmura 7270; the same locality (N24°22′30″, E121°21′08″), on bark of *Chamaecyparis obtusa*, elevation 2249 m, 30 September 2010, Y. Ohmura 7376. Nanto Co.: Keitau, 9 November 1968, U. Mizushima s.n. (TNS-L-22389); Renluen (N23°42′27″, E120°55′08″), on bark of ever green tree, elevation 1699 m, 30 August 2008, Y. Ohmura 6218; en route from Sunghsueh Hostel to Chengkung No. 2, 3 Cabin, Mt. Chilai, Taroko National Park (N24°07′01″, E121°18′58″), on bark of *Tsuga* sp., elevation about 2900 m, 29 September 2009, Y. Ohmura 6977. Chiayi Co.: Mt. Alishan, Alishan Township (N23°31′, E120°48′), on bark of *Chamaecyparis formosensis*, elevation 2340 m, 4 October 2011, Y. Ohmura 8688B.

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