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Usnea nipparensis and *U. sinensis* form a 'species pair' presuming morphological, chemical and molecular phylogenetic data

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Abstract. Phylogenetic relationships between *Usnea nipparensis* and *U. sinensis*, caperatic acid containing *Usnea* species, were examined based on ITS rDNA, and the phylogenetic position of *U. nipparensis* was inferred based on multi-locus gene analysis using ITS rDNA, nuLSU, and *MCM7*. Although *U. nipparensis* and *U. sinensis* have a sorediate and an esorediate shrubby thallus, respectively, and in general look quite different, other detailed morphological and chemical features are similar. Analysis of the ITS rDNA sequences suggests their close relationship, but also confirms the independence of both species, and that they most likely form a 'species pair' based on morphological, chemical and molecular phylogenetic data. Phylogenetic trees based on both multi-locus gene and ITS rDNA alone strongly support that *U. nipparensis* and *U. angulata* belong to the same clade.

Key words: Asia, caperatic acid, ITS rDNA, lichenized fungi, nuLSU, *MCM7*, phylogeny, taxonomy

Introduction

The genus *Usnea* (*Parmeliaceae*) is known as one of the most difficult genera to identify due to the high morphological variability within species (Clerc 1998), while recent studies with molecular data using correctly identified specimens made big progress to understand the species concept and phylogeny of this difficult group (Ohmura 2002, 2008; Ohmura & Kanda 2004; Wirtz et al. 2008; Kelly et al. 2011; Lumbsch & Wirtz 2011; Saag et al. 2011; Truong et al. 2013; Truong & Clerc 2016; Clerc & Otte 2018; Gerlach et al. 2017, 2019; Ohmura & Clerc 2019).

Among c. 350 species of *Usnea* worldwide (Lücking et al. 2017), clarifying 'species pairs' would be an interesting matter to discuss, considering their distribution, dispersal strategy and evolution. The 'species pair' concept (Poelt 1970, 1972; Tehler 1982; Mattsson & Lumbsch 1989) is generally applied to a pair of taxa morphologically, anatomically and chemically similar, but that can be distinguished by their sexual vs. asexual reproductive strategies. The 'primary species' produces fruiting bodies and sexual spores, while its counterpart, the 'secondary species' is vegetatively dispersed by soredia, isidia, or fragmentation.

Regarding the genus *Usnea*, *U. florida* (L.) F.H. Wigg. and *U. subfloridana* Stirt. are a good example of 'species pair', being the primary and secondary species respectively (Clerc 1984). Several other species pairs were also proposed in the genus *Usnea* by Walker (1985) (i.e., *U. aurantiacoatra – U. antarctica*; *U. perpusilla – U. sphacelata*; *U. trachycarpa – U. subantarctica*) and by Shen et al. (2012) (i.e., *U. orientalis* Motyka – *U. pygmoidea*). However, molecular phylogenetic analyses using single- or multi-locus genetic data have not supported most of these relationships and they considered that they are conspecific (Articus et al. 2002; Seymour et al. 2007; Saag et al. 2011; Wirtz et al. 2012; Mark et al. 2016) except *U. aurantiacoatra – U. antarctica* that were revealed as independent species by using microsatellite analysis (Lagostina et al. 2018) and RADseq (Grewe et al. 2018). Since the relationship for *U. orientalis – U. pygmoidea* was not tested by phylogenetic analysis in Shen et al. (2012), these ITS rDNA sequences were also incorporated into the analysis in this present study.

The main aim of this study is to examine the relationship between *U. nipparensis* Asahina and *U. sinensis* Motyka based on nuclear ITS rDNA (including partial 18S rDNA, ITS1, 5.8S rDNA, ITS2, and partial 28S rDNA). *Usnea nipparensis* is a sorediate taxon with rounded soralia which are distinctly stipitate, and produces usnic and caperatic acids, and atranorin (±) or usnic, caperatic, and stictic acid group (Ohmura 2001, 2012). In contrast,

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U. sinensis is an esorediate taxon usually with abundant apothecia, and produces usnic, norstictic, caperatic, and salazinic acids (±) as chemical compounds (Ohmura 2001, 2012). Their overall morphology looks different, but they have similar growth size (up to c. 30 cm), anisotomic-dichotomous branching, ratio of cortex/medulla/axis ('%C/%M/%A', see Clerc 1987) [(5.9–)7.1–12(–14)/18–28(–32)/(20–)26–44(–47) (0.9–1.7 mm in diam.) for *U. nipparensis* vs. (5.8–)8.1–13(–17)/(14–)16–25(–31)/(28–)32–45(–50) (0.8–1.9 mm in diam.) for *U. sinensis*], cortex structure (*ceratina*-type plectenchymatous cortex), and chemistry (caperatic acid and ±*β-orcinol* depsidones) (Ohmura 2001). Phylogenetic position of *U. nipparensis* was also inferred based on multi-locus gene analysis using ITS rDNA, nuLSU, and *MCM7* in the light of general phylogeny of the subgenus *Usnea* published by Truong and Clerc (2016).

Materials and methods

This study is based on the examinations of herbarium specimens housed in the National Museum of Nature and Science (TNS), Tsukuba, Japan (File S1).

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 & 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 EmeraldAmp PCR Master Mix (TaKaRa Bio Inc.). PCR amplification of ITS rDNA was performed using the primer set of ITS1F (Gardes & Bruns 1993) as the 5' primer and LR1 (Vilgalys & Hester 1990) as the 3' primer; for nuLSU, LR0R (Vilgalys, unpubl.) as the 5' primer and LR5 (Vilgalys & Hester 1990) as the 3' primer were used; and for *MCM7*, X-Mcm7-F (Leavitt et al. 2011) as the 5' primer and X-Mcm7-R (Leavitt et al. 2011) as the 3' primer were used. PCR cycling conditions were 94°C (3 min), followed by 11 cycles of 95°C (30 sec), 62°C to 52°C (30 sec) with annealing temperatures lowered by 1°C between cycles, and 72°C (1 min), followed by 30 cycles at 52°C annealing temperature and a final extension at 72°C (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 Version 7 (Katoh et al. 2019) using the default settings. Each data set (ITS rDNA, nuLSU, and *MCM7*) was separately aligned. After removing sites with gaps, missing data and ambiguous data, the data were concatenated. The resulting alignment of 1,512 sites for the multi-locus data set (File S2) or 457 sites for ITS rDNA (File S3) was used for the molecular phylogenetic analyses.

The maximum likelihood (ML) (Felsenstein 1981) and neighbor-joining (NJ) (Saitou & Nei 1987) analyses were performed with the best nucleotide substitution model [TN93+G model (Tamura-Nei 1993) for multi-locus analysis and K2+G model (Kimura 1980) for ITS rDNA analysis]. The bootstrap values (Felsenstein 1985) with 1,000 replicates for ML and NJ were shown on the branches only when both were ≥ 50% simultaneously. All calculations were conducteed in MEGA 10.1.8 (Kumar et al. 2018).

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

Results and discussion

Phylogenetic position of *Usnea nipparensis* in the subgenus *Usnea*

The topology of the molecular phylogenetic tree based on the multi-locus dataset of ITS rDNA, nu LSU and *MCM7* obtained in this study (Fig. 1) is not in conflict with the one shown in Truong & Clerc (2016). The clades or nodes of NEUROPOGON and USNEA-1 to USNEA-4 were formed in the same order as in Truong & Clerc (2016) but USNEA-3 clade was not formed in this tree even with weak support value. Within the USNEA-4 clade, some branches in the tree were insufficiently supported by the bootstrap values. This is, because unlike Truong & Clerc (2016), the tree was calculated with less alignment data, removing the sites with gaps and missing data. Such treatment for alignment is generally desirable for phylogenetic analysis, because different regions of DNA or amino acid sequences evolve under different evolutionary forces (Kumar et al. 2018).

Usnea nipparensis formed a monophyletic clade with *U. angulata* Ach. with high support values (ML/NJ = 95/90). The phylogenetic position of *U. nipparensis – U. angulata* clade within the USNEA-4 clade could not be inferred from the current data.

Ohmura (2002) showed a weak relationship (< 50% support value) in the NJ tree based only on ITS rDNA between *U. nipparensis* and *U. mutabilis* Stirt., which contains murolic acid complex (fatty acids). However, the tree in this study based on multi-locus gene analyses with ML and NJ methods was also unable to improve the weak support value for the relationship.

Phylogenetic relationship of *Usnea nipparensis*, *U. sinensis*, and the related species

Six sequences of ITS rDNA for *U. nipparensis* and ten sequences for *U. sinensis* were analyzed within the subgenus *Usnea* using the same dataset of ITS rDNA sequences used in the multi-locus analysis and sequences of *U. orientalis*, *U. pygmoidea*, and the related taxa in order to test the hypotheses of species pair relationships. The samples of *U. nipparensis* consist of two chemotypes:

Table 1. Vouchers and their GenBank accession numbers. New sequences are in bold.

Species	Voucher	Chemistry*	ITS rDNA	nuLSU	*MCM7*	Reference
Usnea angulata	Peru; 85 (G)	NOR	JQ837291	JQ837376	JQ837336	Truong et al. (2013)
U. aff. *brasiliensis*	Madeira; 44 (G)	PRO	JQ837294	JQ837379	JQ837338	Truong et al. (2013)
U. clerciana	Galapagos; 125 (G)	SAL	JQ837311	JQ837395	JQ837354	Truong et al. (2013)
U. cornuta	Madeira; 43 (G)	SAL	JQ837302	JQ837387	JQ837345	Truong et al. (2013)
U. crocata	Peru; 35 (G)	PRO	JQ837303	JQ837388	JQ837346	Truong et al. (2013)
U. croceorubescens	Japan; Y. Ohmura 3144D (TNS)	SAL	AB051654	–	–	Ohmura (2002) (as '*U. pangiana*')
U. dasaea	Peru; 41 (G)	STI	JQ837305	JQ837390	JQ837348	Truong et al. (2013)
U. dasaea	Ecuador; 81 (G)	GAL	JQ837306	JQ837391	JQ837349	Truong et al. (2013)
U. glabrata	Switzerland; 113 (G)	STI	JQ837313	JQ837397	JQ837356	Truong et al. (2013)
U. intumescens	Japan; Y. Ohmura 3112 (TNS)	ATR (tr), CPS, PSO	AB051641	–	–	Ohmura (2002)
U. mutablis	Japan; Y. Ohmura 4407 (TNS)	ATR, EA2, MUR	AB051650	KR995436	KR995691	Ohmura (2002); Divakar et al. (2015)
U. nipparensis	Japan; Y. Ohmura 3825 (TNS)	CAP	AB051652	**LC576903**	**LC576905**	Ohmura (2002); **this study**
U. nipparensis	Japan; Y. Ohmura 6274 (TNS)	CAP	**LC576907**	–	–	**This study**
U. nipparensis	Japan; Y. Ohmura 6282 (TNS)	CAP	AB623075	–	–	Ohmura (2002); **this study**
U. nipparensis	Japan; Y. Ohmura 9054 (TNS)	CAP, NOR, STI	**LC576908**	–	–	**This study**
U. nipparensis	Japan; Y. Ohmura 12248 (TNS)	CAP	**LC576909**	–	–	**This study**
U. nipparensis	Japan; Y. Ohmura 12249 (TNS)	CAP	**LC576910**	–	–	**This study**
U. orientalis	Taiwan; L4625 (TNM)	SAL**	FJ494942	–	–	Shen et al. (2012)
U. orientalis	Taiwan; L4653 (TNM)	SAL**	FJ494943	–	–	Shen et al. (2012)
U. orientalis	Taiwan; L4669 (TNM)	SAL**	FJ494944	–	–	Shen et al. (2012)
U. orientalis	Taiwan; L4673 (TNM)	SAL**	FJ494945	–	–	Shen et al. (2012)
U. perhispidella	Peru; 137 (G)	STI	JQ837290	JQ837375	JQ837335	Truong et al. (2013)
U. pygmoidea	Japan; Y. Ohmura 2736	SAL	AB051657	–	–	Ohmura (2002)
U. pygmoidea	Japan; Y. Ohmura 3144C	NOR, STI	AB051658	–	–	Ohmura (2002)
U. rubicunda	Bolivia; 38 (G)	SAL	JQ837316	JQ837399	JQ837358	Truong et al. (2013)
U. rubicunda	Madeira; 75 (G)	STI	JQ837319	JQ837402	JQ837361	Truong et al. (2013)
U. silesiaca	Ecuador; 88 (G)	SAL	JQ837331	JQ837412	JQ837370	Truong et al. (2013)
U. sinensis	Taiwan; Y. Ohmura 7313 (TNS)	CAP, NOR	**LC576911**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 7314 (TNS)	CAP, NOR	**LC576912**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 7369 (TNS)	CAP, NOR	**LC576913**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 7390 (TNS)	CAP, NOR	**LC576914**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 7408 (TNS)	CAP, NOR (tr)	**LC576915**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 7611 (TNS)	CAP, NOR	**LC576916**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 10375 (TNS)	CAP, NOR	**LC576917**	–	–	**This study**
U. sinensis	Taiwan; Y. Ohmura 10439 (TNS)	CAP, NOR	**LC576918**	–	–	**This study**
U. sinensis	Taiwan; G. Kokubugata 10895C (TNS)	CAP, NOR	**LC576919**	–	–	**This study**
U. sinensis	Taiwan; L4766 (TNM)	NOR**	FJ494953	–	–	Shen et al. (2012)
U. sphacelata	Antarctica; F564 (NIPR)	–	AB103542	**LC576904**	**LC576906**	Ohmura & Kanda (2004); **this study**
U. subaranea	Ecuador; 123 (G)	–	JQ837292	JQ837377	JQ837337	Truong et al. (2013)
U. subdasaea	Galapagos; 22 (G)	GAL	JQ837329	JQ837410	JQ837368	Truong et al. (2013)
U. subglabrata	Bolivia; 25 (G)	STI	JQ837312	JQ837396	JQ837355	Truong et al. (2013)
U. subrubicunda	USA; 76 (G)	PRO	JQ837332	JQ837413	JQ837371	Truong et al. (2013)

* Main chemistry except usnic acid for the specimen is shown. Abbreviations for the chemistries: ATR, atranorin; CAP, caperatic; CPS, conpsoromic (=2′-O-demethylpsoromic); EA2, Eumitrin A_2; GAL, galbinic; MUR, murolic acid complex; NOR, norstictic; PRO, protocetraric; PSO, psoromic; SAL, salazinic; STI, stictic; –, only usnic acid contain; (tr), trace in TLC. **Chemistry was examined by High Performance Liquid Chromatography (HPLC).

chemotype 1 (usnic and caperatic acid) for Ohmura 3825, 6274, 6282, 12248, and 12249; and chemotype 2 (usnic, norstictic, caperatic, and stictic acids) for Ohmura 9054. They form a monophyletic clade with high support values (ML/NJ=100/100) (Fig. 2). Therefore, the chemical difference seen in the *U. nipparensis* morphotype is certainly confirmed as a variation within a single species. All samples of *U. sinensis* examined by the author contain usnic, norstictic, and caperatic acids as major substances except Y. Ohmura 7408 (TNS) in which norstictic acid appeared as a faint trace in TLC. The amount of norstictic acid in *U. sinensis* is variable and sometimes not detected by TLC (see Ohmura 2002). In contrast, caperatic acid was not reported from the voucher specimen of

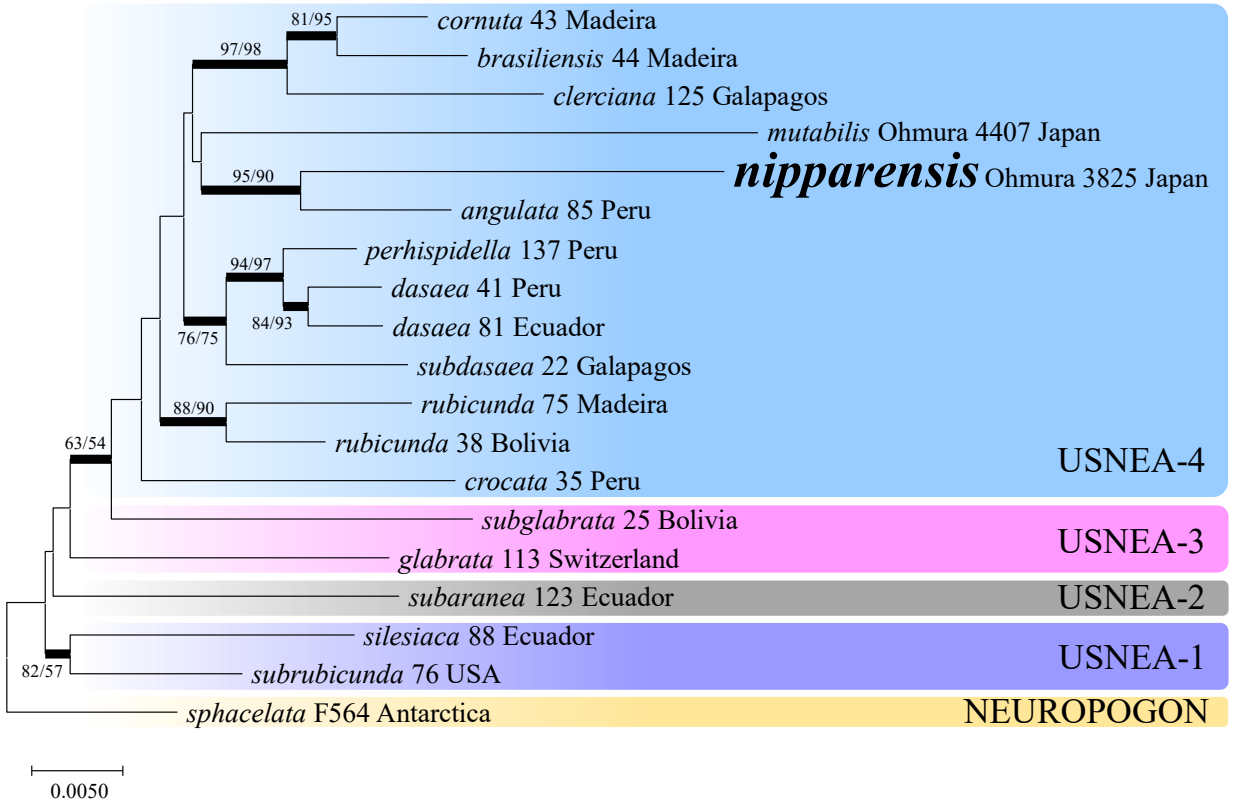

Figure 1. Phylogenetic position *of Usnea nipparensis* in the subgenus *Usnea* inferred from the multi-locus dataset of ITS rDNA, nu LSU, and *MCM7* genes. The tree was constructed using the NJ method, and the reliability of each branch was tested by ML and NJ methods. The bootstrap values for ML/NJ were generated from 1,000 replicates and shown on the thick branches only when both were ≥50% simultaneously. The OTU names indicated the taxon epithet, the voucher number, and location (see Table 1). The clade names are identical to those in Truong & Clerc (2016). All positions containing gaps and missing data were eliminated. There were a total of 1,512 positions in the final dataset.

the GenBank accession number FJ494953 (Shen et al. 2012). This is because it was examined by means of High Performance Liquid Chromatography (HPLC) that is generally difficult to detect fatty acids lacking benzene rings in the structure (Huneck et al. 1994). All samples of *U. sinensis* form a monophyletic clade with high support value (94/99) (Fig. 2).

The *U. nipparensis* and *U. sinensis* clades form a monophyletic clade together with support values (60/73). This *U. nipparensis* – *U. sinensis* clade forms a monophyletic clade with *U. angulata* with high support values (91/92). The chemistry of *U. angulata* is fundamentally the same as *U. sinensis*, e.g., containing usnic, norstictic and caperatic acids (Ohmura 2001), although the presence of caperatic acid in *U. angulata* was not confirmed in some studies (e.g., Awasthi 1986; Stevens 1999; Truong et al. 2013). Caperatic acid, a fatty acid, is usually detected on TLC with water, but it is sometimes ambiguous. It would be easily detected and identified by a microcrystal test in addition to TLC (Yoshimura & Kurokawa 1976). In fact, caperatic acid was detected from *U. angulata* collected in South America (specimens housed in TNS) (Fig. S1), although Truong et al. (2013) did not detect it from the South American materials. Morphology of *U. angulata* is distinctively different from *U. nipparensis* and *U. sinensis* in having a pendulous thallus with ridged to alate branches, the presence of punctiform soralia, and the %C/%M/%A [(5.6–)9.2–15(–17)/(6.7–)8.6–19(–28)/(25–)40–57(–61)] (Ohmura 2001, 2012). The tree suggests these three species might have evolved from a common ancestor, and the evolutionary order is supposed to be *U. angulata*, *U. sinensis*, and *U. nipparensis* from oldest to most recent (Fig. 2). Multi-locus analysis also supported the order for *U. angulata* and *U. nipparensis* (Fig. 1). Since the multi-locus genes, except ITS rDNA, were not available for *U. sinensis* in this study, the species was not included in the tree (Fig. 1).

Presuming 'species pair' for *Usnea nipparensis* and *U. sinensis*

Based on morphological, chemical, and molecular phylogenetic data, *U. nipparensis* and *U. sinensis* could be assumed as a 'species pair', and they are supposed to have evolved from the common ancestor of *U. angulata*. The 'species pair' concept is generally applied to two taxa having the same chemistry, but different reproductive strategies, one being esorediate and the other sorediate (Poelt 1972). Both *U. nipparensis* and *U. sinensis* have caperatic acid as the major compound. However, in addition to caperatic acid, *U. nipparensis* has ±stictic acid group and *U. sinensis* has norstictic and ±salazinic acids. In a strict sense, these species do not have exactly the same chemistry, but all of these additional substances are β-orcinol depsidones (Culberson 1969). The fact that the norstictic ±salazinic acids chemotype and the stictic acid group chemotype can occur within a single species, e.g., *U. glabrescens* var. *glabrescens* (Clerc & Otte 2018), suggests that the chemical differences in β-orcinol depsidones

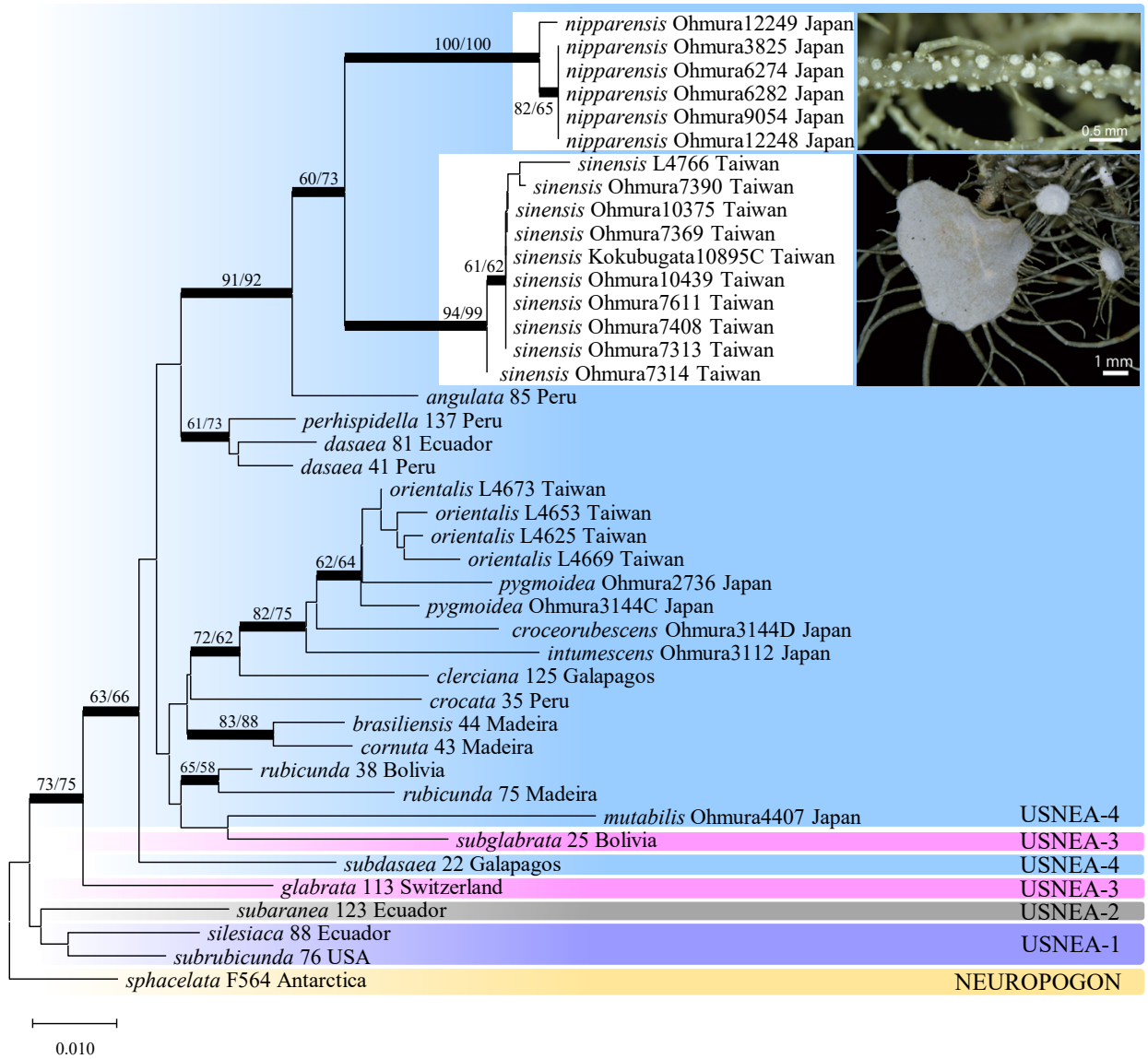

Figure 2. Species pair relationship for *Usnea nipparensis* and *U. sinensis* inferred from ITS rDNA. The tree was constructed using the NJ method, and the reliability of each branch was tested by ML and NJ methods. The bootstrap values for ML/NJ were generated from 1,000 replicates and shown on the thick branches only when both were ≥50% simultaneously. The OTU names indicated the taxon epithet, the voucher number, and location (see Table 1). The clade names are identical to those in Truong & Clerc (2016). All positions containing gaps and missing data were eliminated. There were a total of 457 positions in the final dataset.

of *U. nipparensis* and *U. sinensis* might be caused by a small evolutionary event or an unknown factor.

In the species pair concept, secondary species (vegetative lineage) are assumed to have arisen from primary species (sexual lineage) through a rare transition event, and the vegetative lineage is thought to be successful due to its superior ability to colonize and survive in marginal habitats (Buschbom & Mueller 2005). This idea could be also applied to the case of *U. sinensis* (primary species) and *U. nipparensis* (secondary species) from the phylogenetic result in this study. The known distribution of *U. sinensis* is narrower than that of *U. nipparensis*: i.e., *U. sinensis* is collected from Yunnan in the mainland of China and Taiwan, while *U. nipparensis* is recorded from Japan, Taiwan, Korea, India, and Nepal (Ohmura 2001). Although the distribution of *U. nipparensis* is currently restricted in South and East Asia, it could be much wider because of the vegetative dispersal strategy. Indeed, *U. boomiana* P. Clerc, collected from the Canary Islands (van den Boom et al. 2015; G – holotype!), and the caperatic acid chemotype of *U. subeciliata* (Motyka) Swinscow & Krog, collected from Australia (Fig. 67 in Stevens 1999; specimens not seen), resemble to *U. nipparensis* both in morphology and chemistry. Further research using molecular phylogenetic analyses may solve the relationship between *U. nipparensis* and these species.

Although single- or multi-locus genetic data were usually not able to resolve species pair relationships in the genus *Usnea*, this study shows a presumable species pair relationship between *U. nipparensis* and *U. sinensis* based on ITS rDNA. In addition, these two species were suggested to have speciated from the common ancestor *U. angulata*. There should be many species pairs in the genus *Usnea* with different evolutionary histories. Species pairs having an old evolutionary history could be clarified by single- or multi-locus genetic data. However, recently

speciated taxa representing a species pair should be analyzed using fine scale markers, such as microsatellite and RADseq (Grewe et al. 2018; Lagostina et al. 2018).

This study also confirmed the close relationship between *U. orientalis* and *U. pygmoidea* [as a species pair hypothesized by Shen et al. (2012)], forming a monophyletic clade with support value (62/64) (Fig. 2). However, the independency of each species is needed to be examined with further data.

Insufficient resolution in molecular phylogenetic analysis using single- or multi-locus genetic data can cause incorrect interpretations, especially when it comes to test the conspecificity as pointed out by Grewe et al. (2018), but also while testing higher taxonomic groups in which results would vary depending on the analysis performed (Truong et al. 2013; Divakar et al. 2017). Future integrated studies with traditional careful α-taxonomy and fine-scale or genomic mega data for molecular analyses may solve difficult taxonomic problems that remain among the genus *Usnea*.

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Supplementary electronic material

Figure S1. Caperatic acid crystalized by microcrystal test with GE (glycerin: acetic acid, 1:3). Download file

File S1. Detailed information of specimens examined and housed in TNS and NIPR. Download file

File S2. Final alignment for the multi-locus data set removing sites with gaps, missing data and ambiguous data. Download file

File S3. Final alignment for the ITS rDNA data removing sites with gaps, missing data and ambiguous data. Download file

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