



Standard Paper

Usnea dasopoga (Ach.) Nyl. and *U. barbata* (L.) F. H. Wigg. (Ascomycetes, Parmeliaceae) are two different species: a plea for reliable identifications in molecular studies

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Abstract

Using molecular data to delimit species or reconstruct their evolutionary history is now widely used across all organisms. However, such analyses can suffer from poor or false specimen identifications leading to incorrect conclusions. Here we show that the use of misidentified specimens in a phylogenetic framework resulted in questionable conclusions in a previously published study (Mark *et al.* 2016). Using morphological, chemical and statistical analyses on the specimens used in that study, we found support for *Usnea barbata* and *U. dasopoga* being morphologically and anatomically distinct species with separate clusters in the molecular phylogeny. Furthermore, our revision of specimen identifications refutes the synonymization of *U. substerilis* with *U. lapponica*. In conclusion, we discuss the issue of correct identification of voucher specimens in DNA databases and conclude with some general suggestions to avoid false specimen identifications in phylogenetic studies.

Keywords: GenBank, lichens, misidentification, taxonomy, *Usnea lapponica*, *Usnea perplexans*, *Usnea substerilis*

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Introduction

The hyper diverse *Usnea* Adans. is a fruticose genus of lichenized Ascomycetes with the second highest number of species in the Parmeliaceae after the genus *Xanthoparmelia* (Thell *et al.* 2012). However, although various accounts have been proposed (Wirtz *et al.* 2006; Kirk *et al.* 2008; Thell *et al.* 2012; Clerc 2016), the exact number of species worldwide is still unknown. This is due to the fact that several species have been synonymized over the last decades (Swinscow & Krog 1978; Stevens 1990; Clerc 1997, 2004, 2011a, 2016; Clerc & Herrera-Campos 1997; Ohmura 2001; Mark *et al.* 2016), or newly described (Zhao *et al.* 1975; Swinscow & Krog 1979; Clerc & Herrera-Campos 1997; Herrera-Campos & Clerc 1998; Stevens 1999; Halonen 2000; Clerc 2006, 2007, 2011a, b; Wirtz *et al.* 2008; Truong *et al.* 2011; Truong & Clerc 2012, 2013, 2016; van den Boom *et al.* 2015; Mark *et al.* 2016; Gerlach *et al.* 2017; Bungartz *et al.* 2018; Clerc & Otte 2018). Moreover, the use of molecular data seems to reveal a much higher species diversity than previously thought (Wirtz *et al.* 2006; Gerlach *et al.* 2018). In the context of the tremendous impact of molecular data on systematics, one of the main remaining questions concerns the congruence between morphological characterization of species and phylogenetic delimitation based on DNA sequence data.

Furthermore, it is well known that there is a huge problem of taxonomic misidentifications in public DNA databases today (Bridge *et al.* 2003; Vilgalys 2003; Nilsson *et al.* 2006; Bidartondo *et al.* 2008; Kang *et al.* 2010; Ko *et al.* 2011; Hofstetter *et al.* 2019). As a consequence, we sometimes have to deal with questionable interpretations of molecular clades, as shown for instance in the genus *Usnea* by Clerc & Otte (2018).

In *Usnea*, almost all studies so far have shown that the traditional species concept based on morphology, anatomy and chemistry (Clerc 1998) is generally confirmed (Ohmura 2001; Kelly *et al.* 2011; Saag *et al.* 2011; Truong *et al.* 2013; Clerc & Otte 2018; Gerlach *et al.* 2018). In their important and innovative molecular study based on a six-locus dataset, Mark *et al.* (2016) evaluated the congruence of morphological and molecular data in delimiting species, using a set of 19 phenotypically delimited *Usnea* species (144 specimens) collected in Europe and North America, which resulted in a Bayesian 50% consensus tree inferred by BEAST (Mark *et al.* 2016: fig. 1). They found contrasting results: on the one hand, the morphology of eight species was congruent with molecular data; on the other, the delimitation of 11 species based on the traditional species concept was not confirmed by the phylogenetic tree. However, among these 11 hypothesized non-monophyletic species, three had already been synonymized with other taxa in previous studies: *Usnea subfloridana* Stirt. with *U. florida* (L.) F. H. Wigg. (Articus *et al.* 2002), and *Usnea diplotypus* Vain. and *U. chaetophora* Stirt. with *U. dasopoga* (Ach.) Nyl. (Clerc 2011b; see ‘Systematics of the Taxa Involved in This Paper’ below). The first part of the tree (Mark *et al.* 2016: fig. 1a) contains species that are most of the time

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well characterized by their secondary chemistry, and molecular data therefore agree quite well with the current traditional species concept, secondary chemistry being a good specific marker. With one exception (Clerc & Otte 2018), the second part of the tree (Mark *et al.* 2016: fig. 1b) contains species that cannot be separated on the basis of their chemistry. In this tree, two features deserve attention: 1) the separation of *Usnea barbata* (L.) F. H. Wigg. and *U. dasopoga* seems not to be supported by molecular data; 2) the synonymization of *U. substerilis* with *U. perplexans* (syn. *U. lapponica*) seems to be supported by molecular data. Clerc (2011b) discussed the taxonomy of the last four species and considered *U. barbata*, *U. dasopoga*, *U. perplexans* and *U. substerilis* as forming distinct species based mainly on morphological and anatomical characters. For this reason, the first author requested the loan of the voucher specimens used in the Mark *et al.* (2016) study in order to re-evaluate the identification of these specimens. The present study is the result of this re-evaluation. To avoid identifications based solely on expert opinion, we conducted a discriminant analysis using previous measurements of the thickness of the cortex, the medulla and the central axis in *U. barbata* and *U. dasopoga* since these characters are known to be diagnostic and can be used to distinguish the two species (Clerc 2011b).

We conclude with a plea for more accurate specimen identifications in molecular phylogenetic studies and finish with four suggestions which, if complied with, should help achieve this goal in lichens in general, and in *Usnea* in particular.

Material and Methods

Morphological, anatomical and chemical analyses

The voucher specimens of the *U. barbata-dasopoga* group analyzed by Mark *et al.* (2016) and deposited in TU were requested on loan and studied morphologically, anatomically and chemically. Morphology and anatomy were studied with a Leitz stereomicroscope. According to Clerc (2011b), anatomical characters are important in distinguishing the two species. Anatomical measurements of the cortex, medulla and central axis were carried out in longitudinal sections of branches at $\times 40$ magnification. The percentage thickness of cortex/medulla/axis of the total branch diameter (CMA), as well as the ratio axis/medulla (A/M) of all the cited specimens were calculated according to Clerc (1987). A new indicator, the ratio medulla/cortex (M/C), is introduced and used here.

Chemical analyses were performed by thin-layer chromatography (TLC) following Culberson & Ammann (1979), with solvent B modified according to Culberson & Johnson (1982).

Identification of the voucher specimens used in Mark *et al.* (2016)

Identification of the 31 loaned voucher specimens was conducted blindly without knowing the specimen's position on the phylogenetic tree of Mark *et al.* (2016: fig. 1b). Our identifications are reported in Fig. 1 and Table 1. The voucher specimens were labelled reporting the code of the specimens analyzed by Mark *et al.* (2016: fig. 1b).

Statistical analyses

All former anatomical measurements were added to a database compiled over the years by the first author on the *U. barbata-dasopoga*

group. This database initially contained measurements of 112 *U. barbata* and 104 *U. dasopoga* specimens and is available on Github (<https://Github.com/YNaciri-cjbg/Usnea>). The specimens of Mark *et al.* (2016), as identified by the first author (PC), were added and classified as *barbata*-PC and *dasopoga*-PC. Means and variance were computed for the four groups in R (R Development Core Team 2016) as well as boxplots. For mean comparisons, a Kruskal-Wallis rank test was preferred as non-normality and/or heteroscedasticity were confirmed for nearly all variables using Bartlett tests and Shapiro tests, respectively. Since Kruskal-Wallis tests give only a general statement that differences exist among groups but do not identify which groups are different from others, pairwise tests were also carried out between groups. In the latter case, the significance level was adjusted using a false discovery rate correction with $n = 6$ (Benjamini & Yekutieli 2001). We therefore used adjusted levels of 0.0204, 0.0041 and 0.0004 for nominal levels of 5%, 1% and 0.1%, respectively.

In a second step, a linear discriminant analysis was conducted on the same dataset. The initial 216 individuals were used to find the discriminant function and the newly identified specimens from Mark *et al.* (2016) were added *a posteriori* for assignment to the two species. The analysis was conducted in R using the MASS package version 7.3–51.4 (Venables & Ripley 2002) and the function *lda*. The function *stepclass* of the package *klaR* (Weihs *et al.* 2005) was additionally used to identify variables that better discriminate the two species in a linear discriminant analysis framework (*lda*). The R program is also available on Github (<https://Github.com/YNaciri-cjbg/Usnea>).

Systematics of the Taxa Involved in This Paper

Usnea barbata is a pendulous species characterized by the distinctly irregular branches with slightly to strongly inflated segments (the thickest branch diameter is, most of the time, distant from the basal part), the thin cortex, and the rather large and dense to lax medulla (Clerc 2011b). Chemistry: usnic, \pm salazinic acids.

Usnea chaetophora Stirt. is a pendulous species characterized by main branches formed by numerous, short segments separated by distinct annulations. These characters were found to be present in specimens corresponding to *U. barbata*, as well as in specimens of *U. dasopoga*, and are therefore considered to be phenotypic modifications due to environmental conditions. The type specimen of *U. chaetophora* shows the typical characters of *U. dasopoga* and is therefore considered as a synonym of the latter species (Clerc 2011b). Chemistry: usnic, \pm salazinic acids.

Usnea cylindrica P. Clerc is closely related to *U. dasopoga*. It differs from the latter species mainly by the filamentose branching type with almost no primary thicker branches (all branches are nearly of the same diameter) (Clerc 2011b). Molecular studies are needed to test the validity of this species. Chemistry: usnic, salazinic acids.

Usnea dasopoga is a pendulous species characterized by the cylindrical to tapering or slightly irregular branches (the thickest branch diameter is most of the time close to the basal part), the thick cortex and the rather thin and dense medulla (Clerc 2011b). Chemistry: usnic, \pm salazinic acids.

Usnea diplotypus Vain. is a species based on specimens collected on rocks in Scandinavia (Vainio 1925). Except for *U. sphacelata* R. Br. and *U. pyrenaica* Motyka, there are no primarily saxicolous species in Europe. However, many of the European *Usnea* species might grow saxicolous under optimal environmental conditions (Clerc 2011b). The type specimen of *U. diplotypus* corresponds in

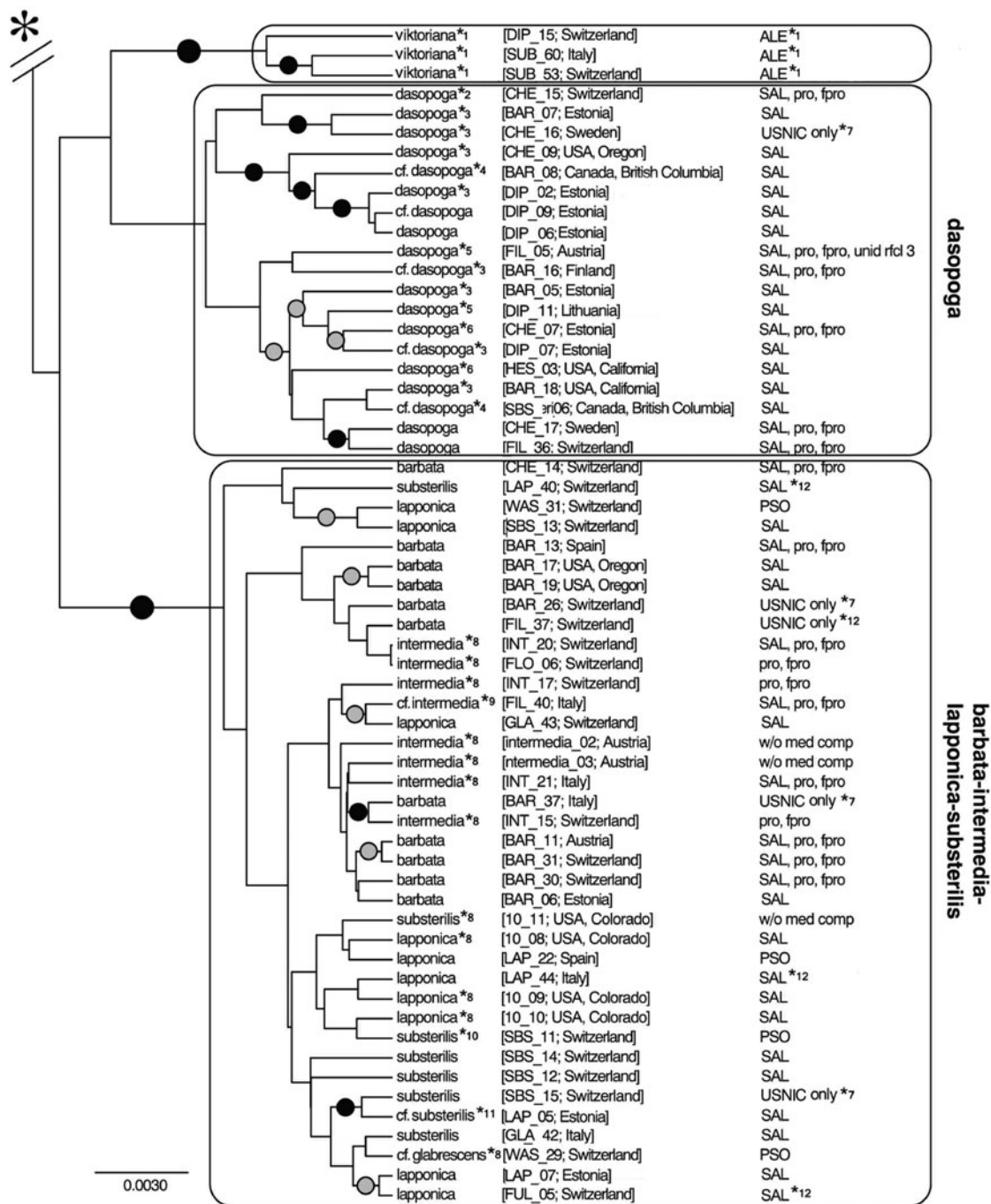


Fig. 1. The phylogenetic tree of the *Usnea barbata-dasopoga* group from Mark *et al.* (2016: fig. 1b) with the new identifications provided in this study, with permission of Springer Nature (License no. 4634240338179). Identifications in Mark *et al.* (2016): *1, *Usnea praetervisa* (Asahina) P. Clerc, see Clerc & Otte (2018); *2, *U. cf. cylindrica*; *3, *U. barbata*; *4, *U. diplotypus*; *5, *U. barbata/dasopoga*; *6, *U. chaetophora*; *8, identification not checked by the authors; *9, *U. dasopoga*; *10 & *11, *U. lapponica*. TLC results in Mark *et al.* (2016): *7, fumar- and protocetraric acids as main substances; *12, pannaric and pannaric acid-6-methylester as main substances. Main substances are given in capitals and accessory substances are in lower case. For full details see Mark *et al.* (2016).

all its characters to a short saxicolous morphotype of *U. dasopoga*. This short morphotype is consistent with the fact that, due to harsher saxicolous conditions, individuals growing on this substratum might not have the opportunity to become pendant. *Usnea diplotypus* was therefore considered as a synonym of *U. dasopoga* (Clerc 2011b). Chemistry: usnic, \pm salazinic acids.

Usnea perplexans Stirt. (syn. *U. lapponica* Vain.) is a short sorediate species with irregular branches and large excavate soralia

without isidiomorphs. It seems to be closely related to *U. substerilis*, differing from this species by the strongly excavate soralia surrounded by a disintegrating cortex. Chemistry: usnic, \pm salazinic, \pm barbatic, \pm psoromic, \pm caperatic acids.

Usnea substerilis Motyka is a short sorediate species with irregular branches, large soralia remaining more or less superficial on branches, with no disintegration of the cortex, and with (few) isidiomorphs. It seems to be closely related to *U. perplexans* (see

Table 1. Specimen identifications (ID) according to Mark *et al.* (2016) and to this study, together with details of the main morphological characters: comments on ID, on voucher material and on TLC results, with CMA, A/M and M/C measurements and the results of the linear discriminant analysis (LDA). The order of specimens follows that of the phylogenetic tree of Mark *et al.* (2016: fig. 1b) from top to bottom.

Specimen names	Mark <i>et al.</i> (2016) ID	This study ID	Comments on ID	Comments on material	Comments on TLC	Cortex %	Medulla %	Axis %	A/M	M/C	LDA ¹
CHE15	<i>cf. cylindrica</i>	<i>dasopoga</i>	*1	–	–	9	22.5	36	1.6	2.5	+
BAR07	<i>barbata</i>	<i>cf. dasopoga</i>	*2	*6	–	11	23.5	31	1.3	2.1	+
CHE16	<i>barbata</i>	<i>dasopoga</i>	*1	–	*9	12	16	44	2.8	1.3	+
CHE09	<i>barbata</i>	<i>dasopoga</i>	*1	–	–	8	20	44	2.2	2.5	+
BAR08	<i>diplotypus</i>	<i>cf. dasopoga</i>	*3	*6/*7	–	8.5	26.5	30	1.1	3.1	–
DIP02	<i>barbata</i>	<i>dasopoga</i>	*1	–	–	12	13	50	3.8	1.1	+
DIP09	<i>dasopoga</i>	<i>cf. dasopoga</i>	*1	–	–	9	19	44	2.4	2.1	+
DIP06	<i>dasopoga</i>	<i>dasopoga</i>	*1	*6	–	10	15	50	3.3	1.5	+
FIL05	<i>barbata/dasopoga</i>	<i>dasopoga</i>	*1	*8	–	9.5	18	45	2.6	1.9	+
BAR16	<i>barbata</i>	<i>cf. dasopoga</i>	*3	*6/*8	–	9.5	28.5	24	0.8	3	–
BAR05	<i>barbata</i>	<i>dasopoga</i>	*1	–	–	7.5	17	51	3	2.3	+
DIP11	<i>barbata/dasopoga</i>	<i>dasopoga</i>	*1	*7	–	8.5	13.5	55	4	1.6	+
CHE07	<i>chaetophora</i>	<i>dasopoga</i>	*1	*8	–	9	21	39	1.9	2.3	+
DIP07	<i>barbata</i>	<i>cf. dasopoga</i>	*1	*6/*7	–	9.5	19	43	2.3	2	+
HESO3	<i>chaetophora</i>	<i>dasopoga</i>	*1	–	–	9	22.5	36	1.6	2.5	+
BAR18	<i>barbata</i>	<i>dasopoga</i>	*1	*7	–	10.5	21.5	36	1.7	2	+
SBS06	<i>diplotypus</i>	<i>cf. dasopoga</i>	*1	*6/*7	–	10.5	16.5	46	2.8	1.6	+
CHE17	<i>dasopoga</i>	<i>dasopoga</i>	*1	–	–	10.5	19.5	39	2	1.9	+
FIL36	<i>barbata</i>	<i>dasopoga</i>	*1	–	–	9.5	23.5	34	1.4	2.5	+
CHE14	<i>barbata</i>	<i>barbata</i>	*4	*8	–	5.5	23.5	42	1.8	4.3	+
BAR13	<i>barbata</i>	<i>barbata</i>	*4	–	–	5	23.5	43	1.9	4.7	+
BAR17	<i>barbata</i>	<i>barbata</i>	*4	–	–	5	21.5	47	2.2	4.3	+
BAR19	<i>barbata</i>	<i>barbata</i>	*4	–	–	3	28.5	37	1.3	9.5	+
BAR26	<i>barbata</i>	<i>barbata</i>	*4	*8	*9	5.5	30.5	27	0.9	5.5	+
FIL37	<i>barbata</i>	<i>barbata</i>	*4	–	*10	5	24	41	1.7	4.8	+
FIL40	<i>dasopoga</i>	<i>cf. intermedia</i>	*5	–	–	7.5	21	42	2	2.8	NA
BAR37	<i>barbata</i>	<i>barbata</i>	*4	–	*9	5	30.5	29	0.9	6.1	+
BAR11	<i>barbata</i>	<i>barbata</i>	*4	–	–	6	30.5	27	0.9	5.1	+
BAR31	<i>barbata</i>	<i>barbata</i>	*4	–	–	7.5	19	47	2.4	2.5	–
BAR30	<i>barbata</i>	<i>barbata</i>	*4	*6/*7	–	5.5	22.5	45	2	4.1	+
BAR06	<i>barbata</i>	<i>barbata</i>	*4	–	–	6.5	29.5	30	1	4.5	+

LAP44	<i>lapponica</i>	<i>perplexans</i>	-	-	NA	NA	NA	NA	NA	NA	NA	NA
SBS15	<i>substerilis</i>	<i>substerilis</i>	-	-	NA	NA	NA	NA	NA	NA	NA	NA
LAP05	<i>lapponica</i>	<i>cf. substerilis</i>	-	*6/*7	NA	NA	NA	NA	NA	NA	NA	NA
FUL05	<i>lapponica</i>	<i>perplexans</i>	-	-	NA	NA	NA	NA	NA	NA	NA	NA

1. + = *a priori* morphological identification confirmed *a posteriori* by the discriminant analysis; - = *a priori* morphological identification not confirmed *a posteriori* by the discriminant analysis; NA = not available.
 *1 = CMA, M/C values and morphology in accordance with *U. dasopoga*.
 *2 = The packet contains a mixture of both species, *U. barbata* and *U. dasopoga*. Mark *et al.* (2016) performed the TLC on the *U. dasopoga* specimen and the DNA was probably extracted from the same specimen.
 *3 = CMA, M/C values not in accordance with *U. dasopoga*; morphology in accordance with *U. dasopoga*.
 *4 = CMA, M/C values and morphology in accordance with *U. barbata*.
 *5 = This specimen has no soralia or apothecia. It displays cylindrical main branches without swellings and an M/C close to *U. dasopoga* values. However, the pale basal part, the absence of soralia and the cortex thickness would indicate another taxon. It is tentatively interpreted here as a thin morphotype of the *U. intermedia* group.
 *6 = Poor material, not well developed, or damaged or stressed by insects or lichenicolous fungi.
 *7 = Short or juvenile specimens.
 *8 = Basal part partly or totally missing.
 *9 = The TLC analysis of Mark *et al.* (2016) could not be confirmed. Protocetraric and fumarprotocetraric acids were never found alone as main medullary substances in this group, at least in Eurasian specimens (Ohmura & Onimaru 2010; Clerc 2011b; Ohmura *et al.* 2017). In this group, protocetraric acid always occurs as an accessory substance with salazinic acid. Their presence alone as main substances in the TLC analysis by Mark *et al.* (2016) is most probably due to a contamination with small *Boyoria fuscescens* (Gyeln.) Brodo & D. Hawksw. branches that are present in some of these collections.
 *10 = The TLC analysis of Mark *et al.* (2016) could not be confirmed. Panmaric acid has thus far never been found in the genus *Usnea*. Our TLC analysis confirms the absence of this compound in this specimen. The result from Mark *et al.* (2016) is probably due to a contamination.

under this species above) and some individuals are extremely difficult to identify with certainty. Chemistry: usnic, ±salazinic, ±barbatic, ±psoromic, ±caperatic acids.

Results

Statistical analyses

Once re-identified, the specimens from Mark *et al.* (2016) were labelled as *dasopoga*-PC or *barbata*-PC. The statistical analyses (Fig. 2) show that for all variables, significant differences among groups exist ($P < 0.05$). In the first dataset (216 specimens), *U. barbata* and *U. dasopoga* are found to be significantly different in all variables ($P < 0.001$, Table 2) except for branch diameter, which is similar for both species ($P = 0.171$). The newly identified *dasopoga*-PC individuals are not statistically different from *U. dasopoga* except in branch diameter ($P = 0.0004$), whereas the newly identified *barbata*-PC individuals are not statistically different from *U. barbata* except in central axis ($P = 0.0058$), medulla ($P = 0.0128$) and axis/medulla ($P = 0.0096$).

As branch diameter was not significantly different between the two species, this variable was discarded from subsequent analysis. Using *stepclass*, the best discriminant variable between *U. dasopoga* and *U. barbata* was found to be the ratio medulla/cortex (M/C) with a correctness rate of 0.94. The 216 *U. dasopoga* and *U. barbata* individuals were used to compute the linear discriminant function. Only nine samples were classified as the ‘wrong’ species, which corresponds to an error rate of 4.2%. The 30 *dasopoga*-PC and *barbata*-PC specimens were subsequently assigned to one of the two species using the previous linear function. Only three of them (10%) were misclassified according to the discriminant function (Table 1): BAR31 identified as *U. barbata* and *a posteriori* classified as *U. dasopoga*, and both BAR08 and BAR16 identified as *U. dasopoga* and *a posteriori* recognized as *U. barbata* in the discriminant analysis.

Specimen identification

Table 1 provides the results of our morphological, anatomical and chemical studies on 35 specimens identified by Mark *et al.* (2016) as *U. barbata*, *U. cylindrica* P. Clerc (*U. dasopoga* aggr.), *U. dasopoga* (syn.: *U. chaetophora*, *U. diplotypus*), *U. perplexans* (syn. *U. lapponica*) and *U. substerilis*, as well as the new identification for some specimens. The means and standard deviations for *U. barbata* and *U. dasopoga* based on new identifications are shown in Table 2.

Discussion

Usnea barbata and U. dasopoga

In this study, based on anatomical and morphological data (Figs 2–6, Tables 1 & 2), all specimens of the *barbata-chaetophora-dasopoga-diplotypus* clade of Mark *et al.* (2016: fig. 1b) were found to correspond to *U. dasopoga* (Fig. 1): nine specimens identified as *U. barbata* by Mark *et al.* (2016) were newly identified as *U. dasopoga* (we did not take into consideration here the two specimens FIL05 and DIP11 identified as *U. barbata/dasopoga* by Mark *et al.* (2016)). Five of these nine specimens (CHE16, CHE09, DIP02, BAR05 and FIL36) represented well-developed material for which the identification was straightforward, whereas the four remaining specimens (BAR07, BAR16,

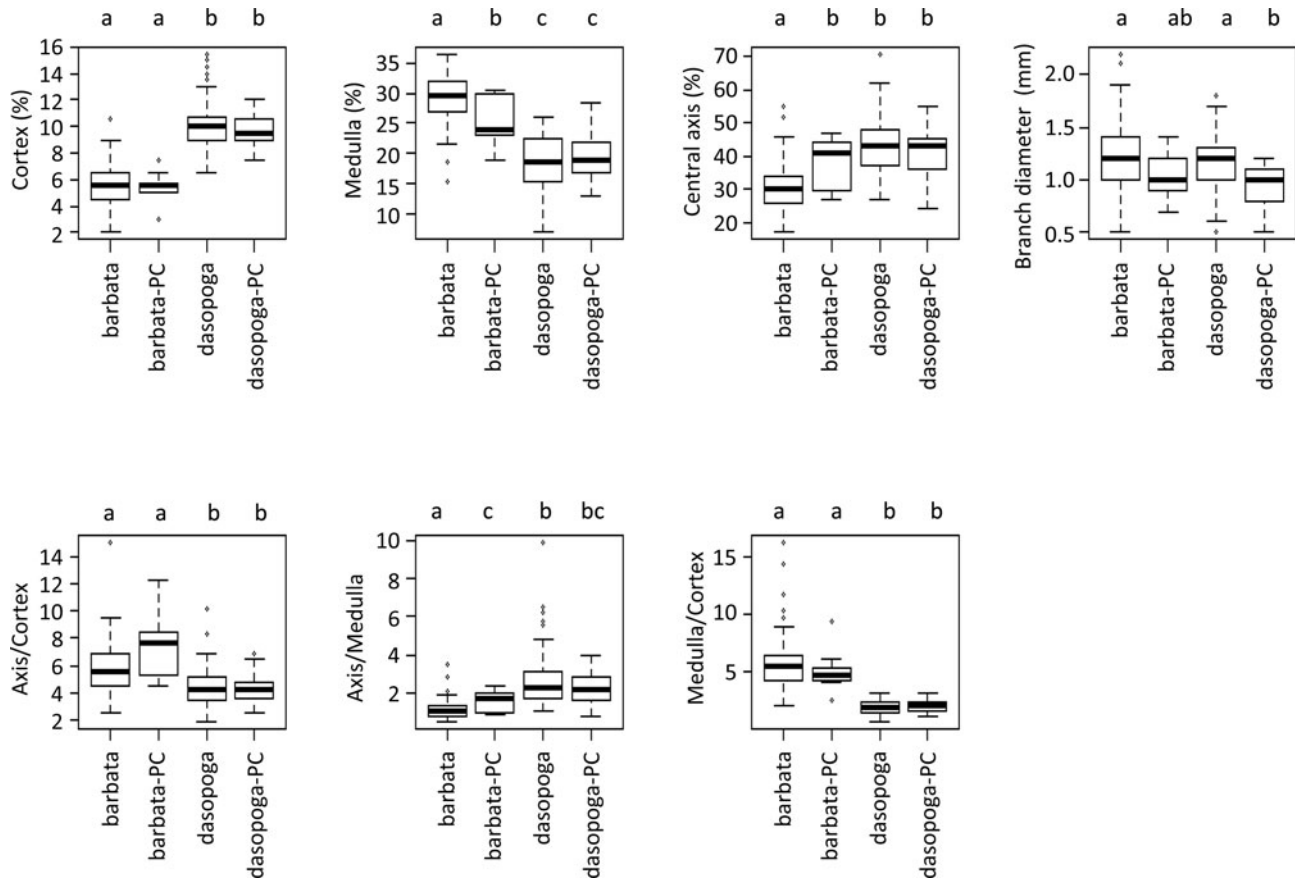


Fig. 2. Boxplots for *Usnea barbata* ($n = 112$) and *U. dasopoga* ($n = 104$). The two groups *dasopoga-PC* ($n = 19$) and *barbata-PC* ($n = 11$) are the samples used by Mark *et al.* (2016) that were re-identified in this study. The upper letters refer to Wilcoxon pairwise tests: groups with different letters are significantly different at the adjusted level of 5% ($P < 0.0204$).

BAR18 and DIP07) consisted of poorly developed, juvenile or damaged material, for which the morphological identification was difficult or uncertain and needed to be backed up by the discriminant analysis based on anatomical characters, and more specifically the new character M/C. Out of the 19 specimens now identified as *U. dasopoga*, two were considered as being misclassified according to the discriminant function (Table 1) based on anatomical characters (CMA, A/M and M/C values). BAR08 and BAR16 were *a priori* identified as *U. cf. dasopoga* and *a posteriori* recognized as *U. barbata* in the discriminant analysis. BAR08 is a damaged specimen (Table 1) and the intermediate CMA and M/C values might well be a consequence of this damage. However, the morphological characters speak distinctly for *U. dasopoga*. The thallus of BAR16 is incomplete, with the basal part lacking (Table 1). Since in *U. dasopoga* the thickest branch diameter is close to the basal part, the CMA and M/C measurements were probably not taken at the correct place, which could explain their atypical values for *U. dasopoga*. Following our new identifications, the *barbata-chaetophora-dasopoga-diplotypus* clade of Mark *et al.* (2016: fig. 1b) now corresponds well to *U. dasopoga* and we suggest renaming it the *U. dasopoga* clade (Fig. 1), although it is not strongly supported in the phylogenetic tree (Mark *et al.* 2016: fig. 1b).

The *barbata-intermedia-laponica-substerilis* clade of Mark *et al.* (2016: fig. 1b) consists of several morphologically well-defined species, such as *U. barbata*, *U. perplexans* (syn.: *U. laponica*) and *U. substerilis* (see Clerc 2011b). These taxa are closely

related and probably diverged recently, showing various degrees of incomplete lineage sorting (Naciri & Linder 2015; Mark *et al.* 2016). This could be the reason why they are all polyphyletic in the phylogenetic tree of Mark *et al.* (2016).

All the specimens of this well-supported *barbata-intermedia-laponica-substerilis* clade (Fig. 1) indeed belong to the *U. barbata* group, characterized by the irregular branches with slightly to strongly inflated segments (Fig. 5), presence or absence of soralia, a rather large and dense to lax medulla (Fig. 6), and a basal part that is often concolorous with main branches or brownish to brownish black.

If we consider only *U. barbata* s. str., all specimens of this clade were correctly identified by Mark *et al.* (2016) (see Fig. 1 of this study). In our study, only BAR31 was considered misidentified according to the discriminant function and *a posteriori* classified as *U. dasopoga* (Table 1). However, the general morphology of this specimen leaves no doubt about its identification as *U. barbata* (see ‘Specimen identification’ above). Under extreme environmental conditions, some specimens might display extreme CMA values, as is certainly the case here. *Usnea intermedia*, usually with many apothecia but without asexual propagules, forms a so-called ‘species pair’ (Poelt 1970) with *U. barbata* (Halonen *et al.* 1998). It is thus not surprising that they sometimes constitute a strongly supported group (BAR37 and INT15) in the phylogeny of Mark *et al.* (2016: fig. 1b). The only disturbing element here is the presence of the specimen FIL40, identified as *U. dasopoga* by Mark *et al.* (2016). However, a critical

Table 2. Mean and standard deviation on the 216 individuals assigned to the two species *Usnea barbata* and *Usnea dasopoga* for seven quantitative variables and associated tests.

Characters	<i>U. barbata</i> (n = 112)	<i>U. dasopoga</i> (n = 104)	Bartlett K-squared statistics (df = 1)	Shapiro W statistics (df = 111; 103)	Kruskal-Wallis Chi-squared statistics (df = 1)
Cortex	5.58 ± 1.33	10.10 ± 1.78	8.77**	** ; ***	150.9 ***
Medulla	29.24 ± 3.75	18.38 ± 4.51	3.57 ^{ns}	** ; ^{ns}	141.9***
Central axis	30.43 ± 6.57	42.98 ± 8.47	6.88**	*** ; ^{ns}	95.4***
Branch diam.	1.24 ± 0.33	1.16 ± 0.25	8.41**	* ; ^{ns}	1.9 ^{ns}
Central axis/Cortex	5.74 ± 1.77	4.40 ± 1.24	12.68***	*** ; ***	40.9***
Central axis/Medulla	1.10 ± 0.44	2.65 ± 1.42	123.54***	*** ; ***	120.3***
Medulla/Cortex	5.67 ± 2.12	1.90 ± 0.61	130.13***	*** ; ^{ns}	155.7***

n = number of individuals included in the analysis; df = degree of freedom; ^{ns} = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.



Fig. 3. *Usnea dasopoga* (*U. dasopoga* FIL_05/Austria in Mark *et al.* (2016)). Cylindrical, not swollen primary and secondary branches. Scale = 1 mm. In colour online.

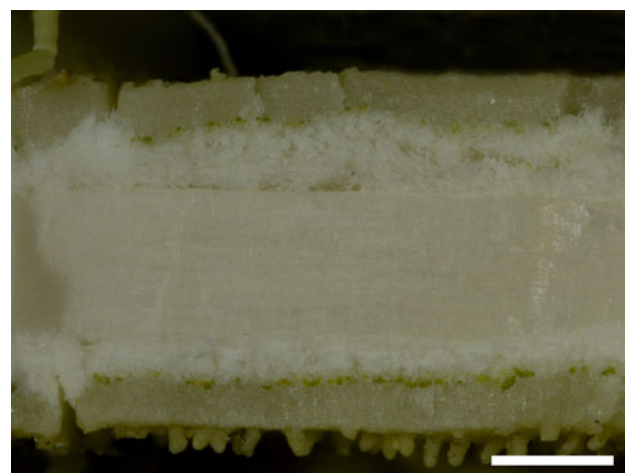


Fig. 4. *Usnea dasopoga* (*U. dasopoga* CHE_17/Sweden in Mark *et al.* (2016)). Transversal section of main branch with thick cortex, thin and compact medulla and large central axis. Scale = 200 μm. In colour online.

analysis of its morphology (soralia absent) reveals that it does not correspond to *U. dasopoga* but probably to a thin morphotype without soralia of the *Usnea barbata-intermedia* group (see 'Specimen identification'). A final worrying element is the presence of *Usnea* cf. *glabrescens* (Vain.) Vain. (WAS29) in this *barbata-intermedia-lapponica-substerilis* clade (Fig. 1). *Usnea glabrescens* belongs to the *U. fulvoraegens-glabrescens* clade in fig. 1 of Mark *et al.* (2016). Unfortunately we were not able to check the identity of WAS29, however, it probably corresponds either to *U. perplexans* or to *U. substerilis*. Chemistry is a good indicator here since, although occurring in specimens of *U. glabrescens* s. str. collected in the United Kingdom, psoromic acid was never found in Swiss specimens (WAS29 was collected in Switzerland) while it is a relatively frequent substance in *U. perplexans* or *U. substerilis*.

Finally, after a re-evaluation of the identification of specimens by Mark *et al.* (2016), and on the basis of a discriminant analysis, we conclude that the existing molecular data do not support the conspecificity of *U. barbata* and *U. dasopoga*. On the contrary, although the *U. dasopoga* clade is not strongly supported, the data of Mark *et al.* (2016) seem to be congruent with the morphological and anatomical data, at least for two well delimited taxa, *U. barbata* and *U. dasopoga*.

Usnea perplexans (syn.: *U. lapponica*) and *U. substerilis*

Optimally developed specimens of *U. perplexans* and *U. substerilis* can be easily separated on the basis of soralia morphology and the presence/absence of isidiomorphs (Clerc 2011b). SBS15 and LAP05, respectively identified by Mark *et al.* (2016) as *U. substerilis* and *U. perplexans*, have a well-supported sister relationship (Mark *et al.* 2016: fig. 1b) and for this reason these authors proposed to synonymize *U. substerilis* with *U. perplexans*. However, LAP05 is such a poor specimen, with only one small branch (2 cm long) with few thin lateral branches and few soralia, that a confident identification is difficult. The presence of small isidiomorphs on two soralia (Fig. 7) would, however, speak for *U. substerilis*. We therefore suggest that the synonymization of *U. substerilis* under *U. perplexans* proposed by Mark *et al.* (2016) should not be accepted as it is based on a poor specimen whose identification is not established with certainty.

A Plea for Reliable Identifications in Molecular Studies

We acknowledge the importance and the quality of the molecular work carried out by Mark *et al.* (2016). They used a high number of genes compared to other studies in this group and up-to-date

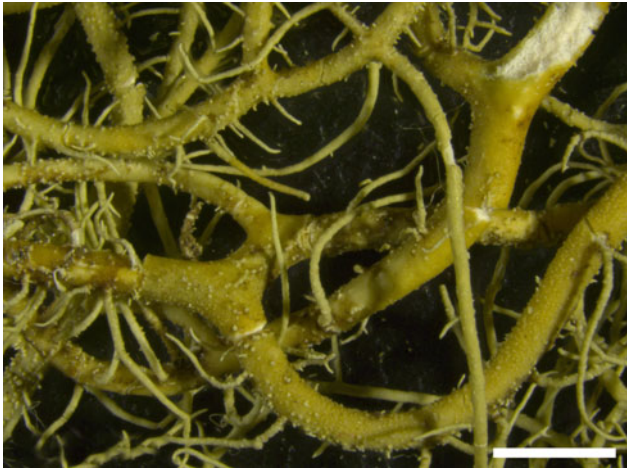


Fig. 5. *Usnea barbata* (*U. barbata* BAR_06/Estonia in Mark *et al.* (2016)). Irregular and more or less swollen main branches. Scale = 1 mm. In colour online.

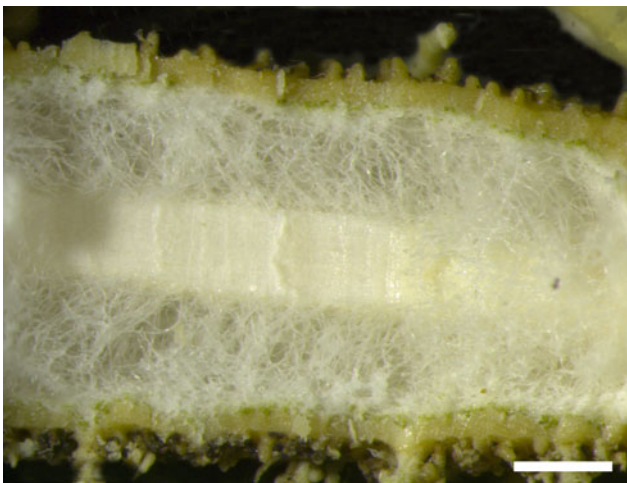


Fig. 6. *Usnea barbata* (*U. barbata* BAR_11/Sweden in Mark *et al.* (2016)). Transversal section of main branch with thin cortex, large and dense to lax medulla, and thin central axis. Scale = 200 μ m. In colour online.

methodologies (the multispecies coalescent and species delimitation analyses). We also acknowledge the importance of molecular data that currently revolutionize and challenge systematics as well as species delimitations based on traditional morphological data. Taxonomy, however, remains central to sequence-mediated species identification. Today, limited taxonomic expertise, as well as time and funding constraints, make barcoding approaches an efficient solution for species identification and in-depth fungal inventories, especially in difficult fungal genera. It is thus critical that DNA sequences are annotated with correct identifications. Sadly taxonomists are on the Red List now and it becomes more and more difficult to find taxonomic experts who can correctly identify species in difficult groups of organisms (Buyck 1999). It is currently much easier and less time consuming to train a good molecular phylogeneticist than a good and experienced classical taxonomist, since it requires years of training and experience to produce accurate identifications. This situation significantly impacts the quality of data in taxonomic identifications in public DNA databases. For example, Bridge *et al.* (2003) suggested that up to 20% of the named fungal



Fig. 7. *Usnea substerilis* (*U. lapponica*_05/Estonia in Mark *et al.* (2016)). Soralia with isidiomorphs on thin lateral branches. Scale = 500 μ m. In colour online.

sequences in public databases may be misidentified. This is in line with Nilsson *et al.* (2006), who affirmed that more than 10% of all publicly available fungal ITS sequences have incorrect taxonomic annotations, and with Ko *et al.* (2011) who suggested that as many as 86% of fungal endophyte sequences from public databases are incorrectly named. This is confirmed in our study where we show that 28% of the specimens studied by us ($n = 35$) and belonging to the *barbata-chaetophora-dasy-poga-diplotypus* and *barbata-intermedia-lapponica-substerilis* clades of Mark *et al.* (2016) were misidentified in their evaluation of traditionally circumscribed species of the genus *Usnea* using molecular data. We would like to point out here that in calculating this number we did not consider *U. chaetophora*, *U. cylindrica* and *U. diplotypus* as being wrongly identified by Mark *et al.* (2016) since their synonymy with *U. dasopoga* can be a matter of opinion. This number of 28% can, however, be reduced to 17% if we consider only the six well-developed and typical specimens for which the identification was straightforward (BAR05, CHE09, CHE16, DIP02, FIL36 and FIL40). The questionable identification by Mark *et al.* (2016) of the four other specimens (BAR07, BAR16, BAR18 and DIP07) was based on poor or juvenile, not well-developed material or specimens damaged and stressed by insects or lichenicolous fungi. Therefore, it is clear that their identification was difficult and made easier here by using the new M/C character and the discriminant analysis.

With 17%, Mark *et al.* (2016) are in the lowest part of the range when considering the data presented by the existing literature on false fungal identifications (Bridge *et al.* 2003; Vilgalys 2003; Nilsson *et al.* 2006; Bidartondo *et al.* 2008; Kang *et al.* 2010; Ko *et al.* 2011; Hofstetter *et al.* 2019). However, even a low percentage of mistakes in specimen identifications might blur the best phylogenetic trees, as evidenced here.


When morphological and molecular data are not congruent in phylogenetic studies, it is of the highest importance that the specimen identification issue is addressed before the conclusion of non-congruence of the data. Consequently, we would like to suggest the following recommendations as a road map to avoid erroneous conclusions.

1. When evaluating traditionally circumscribed species of difficult groups using molecular data, molecular phylogeneticists should make sure that they collaborate with experts of these groups. This will ensure optimal species identification in their study.

- For each specimen it is of course important to make sure that the material studied is not a mixture of two or more species. Herbarium packets of *Usnea* species often and typically contain more than one species. Two similar species can be completely entangled and it might require an expert eye to separate them. For instance, BAR07 contained both *U. barbata* and *U. dasopoga*, and BAR26, BAR 37, CHE16 and SBS15 were most probably contaminated with small branches of *Bryoria* spp. (presence of protocetraric as main substance) or with other unknown species as shown by the presence of pannaric acid (FIL37, FUL05 and LAP44).
- The use of badly collected material, which makes identification much more difficult and uncertain, should be avoided. For example, in *Usnea*, the basal part constitutes a very important diagnostic character. In the study of Mark *et al.* (2016), the basal part was unfortunately lacking in BAR16, BAR26, CHE07, CHE14 and FIL05.
- Evaluating traditionally circumscribed species of difficult groups using molecular data is not the same as using a barcoding strategy to identify poorly developed specimens. Only optimally developed specimens, easy to identify and representative of the studied species, should be used whereas badly developed or poor quality material, or stressed specimens attacked by fungi or insects (BAR07, BAR08, BAR16, BAR30, DIP06, DIP07, LAP05 and SBS06), juvenile specimens (BAR08, BAR18, BAR30, DIP07, DIP11, LAP05 and SBS06), as well as atypical material difficult to identify (e.g. FIL40), should be avoided.

In conclusion, we would like to emphasize the fact that we recognize the important contribution of Mark *et al.* (2016) to the understanding of the systematics and phylogeny of the genus *Usnea*. Among other issues, the excellent molecular data acquired in this work question the polyphyly of both *U. glabrescens* var. *fulvovoreagens* Räsänen and *U. subfloridana* Stirt., and the placement of *U. pacificana* Halonen nested within *U. glabrescens*. Moreover, these data confirmed the monophyly of several species, such as *U. cavernosa* Tuck., *U. silesiaca* Motyka and *U. wasmuthii* Räsänen, and allowed a new species to be described (Clerc & Otte 2018). Furthermore, Mark *et al.* (2016) provided data showing a possible rapid diversification of the genus *Usnea*, especially in the *U. barbata-intermedia-laponica-substerilis* clade probably containing young species lacking monophyly due to incomplete lineage sorting. As a conclusion, we want to argue that our intention was not to lead a charge against their work, but firstly to show that *U. barbata* is different from *U. dasopoga*, a statement confirmed by their molecular data, and secondly to draw attention to the importance of correct taxonomic identification of DNA samples.

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