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# Ribosomal DNA and β-tubulin data do not support the separation of the lichens *Usnea florida* and *U. subfloridana* as distinct species

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The lichens *Usnea florida* and *U. subfloridana* have since long been recognised as distinct species. They show many similarities in morphology, but have different reproductive strategies. *Usnea florida* is always provided with many apothecia and produces no specialised asexual propagules. *Usnea subfloridana* has soralia, isidiomorphs and occasionally apothecia. Phylogenetic analyses based on continuous sequences of the ITS and LSU regions of the nuclear ribosomal DNA and the gene coding for β-tubulin, show that specimens of the two species form one monophyletic group of intermixed specimens, and not two groups corresponding to morphology, which would have been expected if two species were at hand. The 'species pair' concept in lichenology is discussed. Other *Usnea* species included in the study are: *U. articulata*, *U. barbata*, *U. ceratina*, *U. filipendula*, *U. hirta*, *U. rigida* and *U. wasmuthii*.

## INTRODUCTION

Recently, the progress in our understanding of the taxonomy of the lichenized genus *Usnea* (*Parmeliaceae*, *Lecanorales*, *Ascomycota*) has increased considerably due to the work of, particularly Clerc (1984, 1987), Clerc & Herrera-Campos (1997), Halonen *et al.* (1998, 1999), and Ohmura (2001). Despite these efforts, *Usnea* still includes some poorly understood and morphologically variable species. These species are sometimes recognised by a few cardinal characters only, such as chemical constituents or the presence/absence of morphological features (e.g. apothecia, soralia). With the rapid recent development and application of molecular techniques, lichenologists have gained new tools to test hypotheses based on morphology and to investigate whether named morphotypes constitute phylogenetic species or not.

A well-known example of pragmatically distinguished species are the two sympatric species *Usnea florida* and *U. subfloridana*, which have different dispersal strategies. Both species are short and shrubby, have a black base and more or less papillate branches. *U. florida* is fertile and usually produces many apothecia but no asexual propagules. *U. subfloridana* has isidiate soralia, but apothecia are only rarely formed. In Europe *U. florida* often grows in environments with high conservation value. The number of such areas is rapidly decreasing, and *U. florida* is often considered a threatened or vulnerable species (Thor & Arvidsson 1999, Arup *et al.* 1997, Türk & Hafellner 1999). *U. subfloridana* is a rather common species, found in a wide variety of environments.

Several authors have assumed that *Usnea florida* and *U. subfloridana* form a species pair (Seaward & Hitch 1982, Clerc 1984, James *et al.* 1992), with *U. florida* as the fertile, primary species and *U. subfloridana* as the derived sterile, secondary species. The species pair concept, commonly used in lichenology, has its origin in a paper by Du Rietz (1924) in which he discussed the taxonomic significance of different types of vegetative propagules in relation to geographic distribution and differences in ecology between morphologically similar taxa with different dispersal strategies. Later, Poelt (1963) presented a hypothesis to explain the relatively low number of fertile lichenized taxa in Europe. According to this, the rapid change of environmental conditions due to the glaciations, in combination with the topography of Europe, made asexual dispersal

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strategies advantageous. Poelt (1970) also regarded the sterile taxa to be descendants of fertile taxa (as apomictic species, Poelt 1994). The species pair concept in lichenology has been extensively debated. Mattsson & Lumbsch (1989) reviewed the historical background to the development of the concept, and Tehler (1982) provided an early critique focussing on the treatment of clone-forming lichens. The species pair concept has been applied to different degrees in different groups of lichens. Poelt (1970) considered *Usnea florida* a primary species without mentioning any connected secondary species.

*Usnea florida* and *U. subfloridana* are morphologically indistinguishable, with the exception of the characteristics associated with their different dispersal strategies (Clerc 1984). Here, we investigate how the two taxa are related to each other. We do this by analysing the phylogeny of European populations of *U. florida* and *U. subfloridana*, to test if the two taxa represent distinct species. If the predefined morphological groups correspond to monophyletic groups resulting from the parsimony analyses, we would interpret them as phylogenetic species (Grube & Kroken 2000). Secondly, we would like to contribute to the discussion of lichen species pairs, by analysing this case.

## MATERIAL AND METHODS

### *Specimens studied*

Recently collected specimens of *Usnea florida* and *U. subfloridana* in the traditional sense were selected for molecular analysis (Table 1). However, two *U. subfloridana* specimens had both soralia and apothecia. The *U. florida* and *U. subfloridana* specimens originated from different geographical areas in Europe. In addition, several other *Usnea* species were sampled.

### *Extractions and PCR amplifications*

Total DNA from lichen specimens was extracted using the Qiagen DNeasy Plant Mini Kit. For the *Usnea* specimens the central axis only was used for extractions, to minimise the risk of contamination by photobionts and lichenicolous fungi. We chose to investigate two unlinked gene loci: β-tubulin and the most commonly utilised part of the genome for lichen studies at the species level, the ITS region of the nuclear ribosomal DNA, together with the more conservative LSU region.

Polymerase chain reaction (PCR) amplification and automated sequencing of the β-tubulin, ITS and LSU genes was conducted using the settings utilised by Döring *et al.* (2000). The following PCR primer pairs were used (Glass & Donaldson 1995): ITS1F – LR3, nu-LSU-155-5′ – LR5, nu-LSU-155-5′ – LR6 (Döring *et al.* 2000, Gardes & Bruns 1993, Vilgalys http://www.botany.duke.edu/fungi/mycolab/primers.htmweb-site). Sequencing primers used were: AL1R, ITS1F, ITS4, nu-LSU-155-5′, nu-LSU-362-5′, LR1, LR3, LR5, LR0R, LR3R (Döring *et al.* 2000, Gardes & Bruns 1993, Vilgalys (see above), White *et al.* 1990).

### *Sequence alignment and parsimony analysis*

The sequences were automatically aligned using the Clustal algorithm as implemented in BioEdit (http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html) and then adjusted manually,

**Table 1.** Specimens included in the study.

| Specimens | Collection | Labcode | GenBank accession no. | | Origin |
|---|---|---|---|---|---|
| | | | ITS-LSU | β-tubulin | |
| *Platismatia glauca* | *Articus, 673*, UPS | KPG 52 | | AF502271 | Sweden, Uppland |
| *P. glauca* | *Mattsson, 4007*, UPS | KPG 52 | AF058035 | | Sweden, Uppland |
| *Usnea articulata* | *Articus, 617*, UPS | KAR 29 | AJ457139 | AF502258 | England, Devon |
| *U. articulata* | *Articus, 615*, UPS | KAR 30 | AJ457140 | AF502259 | England, Somerset |
| *U. barbata* | *Ulitska, L-9261*, UPS | KA 7 | AJ457138 | AF502257 | Sweden, Västmanland |
| *U. ceratina* | *Articus, 606*, UPS | KC 20 | AJ457141 | AF502260 | England, Devon |
| *U. ceratina* | *Articus, 607*, UPS | KC21 | AJ457142 | AF502261 | England, Somerset |
| *U. filipendula* | *Articus, 502*, UPS | KFP 13 | AJ457149 | AF502268 | Sweden, Uppland |
| *U. filipendula* | *Coppins, 519*, UPS | KFP 18 | AJ457150 | AF502269 | Scotland, East Lothian |
| *U. florida* | *Articus, 428*, UPS | KF 1 | AJ457143 | AF502262 | Sweden, Östergötland |
| *U. florida* | *Articus, 500*, UPS | KF 2 | AJ457145 | AF502264 | Sweden, Östergötland |
| *U. florida* | *Articus, 450*, UPS | KF 10 | AJ457144 | AF502263 | Finland, Karelia |
| *U. florida* | *Articus, 522*, UPS | KF 26 | AJ457146 | AF502265 | England, Devon |
| *U. florida* | *Articus, 57*, UPS | KF 43 | AJ457147 | AF502266 | Sweden, Västergötland |
| *U. florida* | *Mattsson, 4001*, UPS | KF 44 | AJ457148 | AF502267 | Sweden, Uppland |
| *U. hirta* | *Coppins, 521*, UPS | KH 24 | AJ457151 | AF502270 | Scotland, East Lothian |
| *U. rigida* | *de los Rios & Grube*, GZU | KRI 47 | AJ457152 | AF502272 | Austria, Steiermark |
| *U. subfloridana* | *Articus, 511*, UPS | KS 3 | AJ457154 | AF502274 | Sweden, Östergötland |
| *U. subfloridana* | *Articus, 512*, UPS | KS 6 | AJ457156 | AF502275 | Sweden, Östergötland |
| *U. subfloridana* | *Articus, 514*, UPS | KS 7 | AJ457157 | AF502276 | Sweden, Uppland |
| *U. subfloridana* | *Articus, 423*, UPS | KS 12 | AJ457153 | AF502273 | Sweden, Östergötland |
| *U. subfloridana* | *Articus, 674*, UPS | KS 45 | AJ457155 | AF502278 | Sweden, Dalsland |
| *U. wasmuthii* | *Articus, 652*, UPS | KW 40 | AJ457158 | AF502277 | England, Somerset |

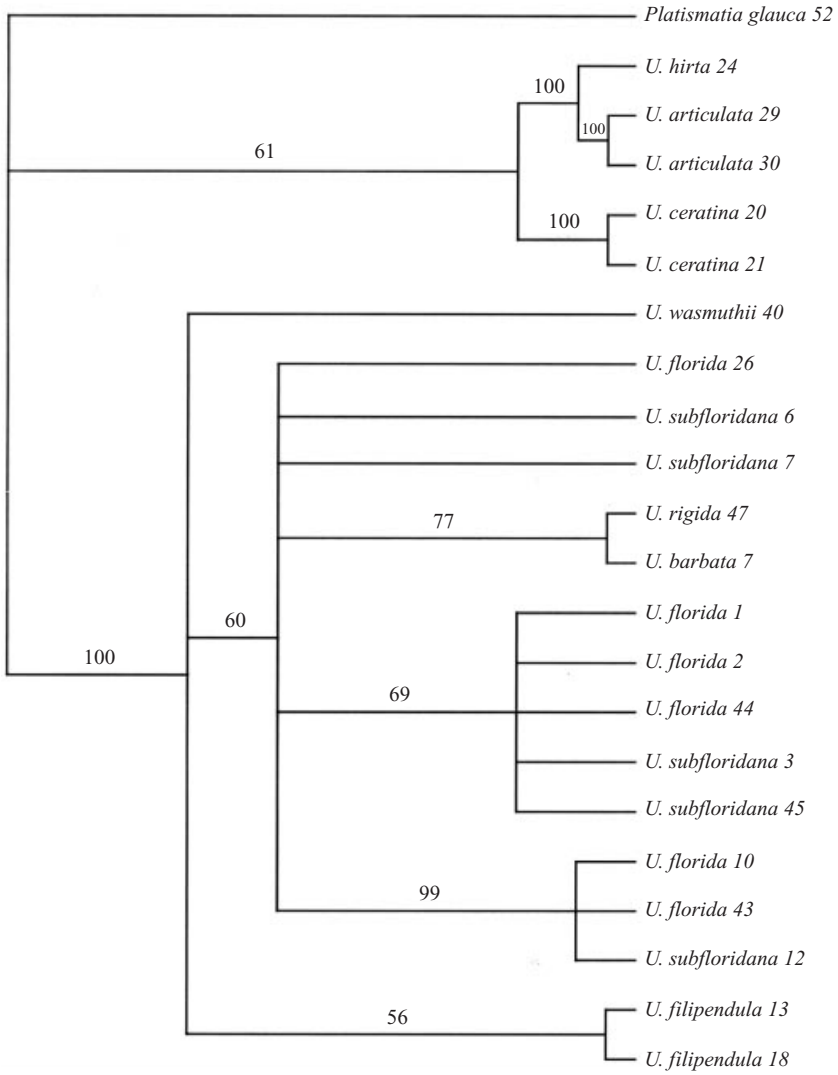


**Fig. 1.** Strict consensus tree of the β-tubulin matrix (jack-knife values above the branches).

particularly regarding areas including insertion sites. The alignment is available from the corresponding author.

The analyses of the data matrices were made by PAUP* 4.0 beta 8 (Swofford 1998). The heuristic search was performed with 1000 random addition sequence replicates, the TBR branch swapping option and MulTrees option were ON. Gaps were treated as missing data; uninformative characters were excluded from the analysis.

Jack-knifing for rapid identification of well-supported monophyletic groups (Farris *et al*. 1997) was performed by using PAUP*. The jack-knife settings were 1000 jack-knife replicates with JAC-emulation, and nominal deletion of characters 36.79% and retaining groups with frequency $> 50\%$ and 10 random replicates and MulTrees OFF. The tree was rooted by using *Platismatia glauca* (*Parmeliaceae*) as outgroup.

### *Chemistry*

HPTLC was performed according to the methods of Arup *et al*. (1993).

## RESULTS

### *Morphology*

All *Usnea florida* specimens included here have apothecia and no soralia, whereas all *U. subfloridana* specimens studied have soralia, with two specimens also having apothecia.

### *Chemistry*

The following substances were identified: usnic, thamnolic, squamatic, and alectorialic acids. *Usnea subfloridana* specimens contain usnic, thamnolic and in one case alectorialic acid. The chemistry of the *U. florida* specimens varies; in addition to the chemotype containing usnic and thamnolic acids, chemotypes containing usnic, thamnolic and alectorialic acids, or usnic and squamatic acids, occur in the material studied. The chemotypes do not form monophyletic groups.

### *Data matrix*

The data matrices contain 22 taxa. All sequences are new and were produced by the authors. The matrix of

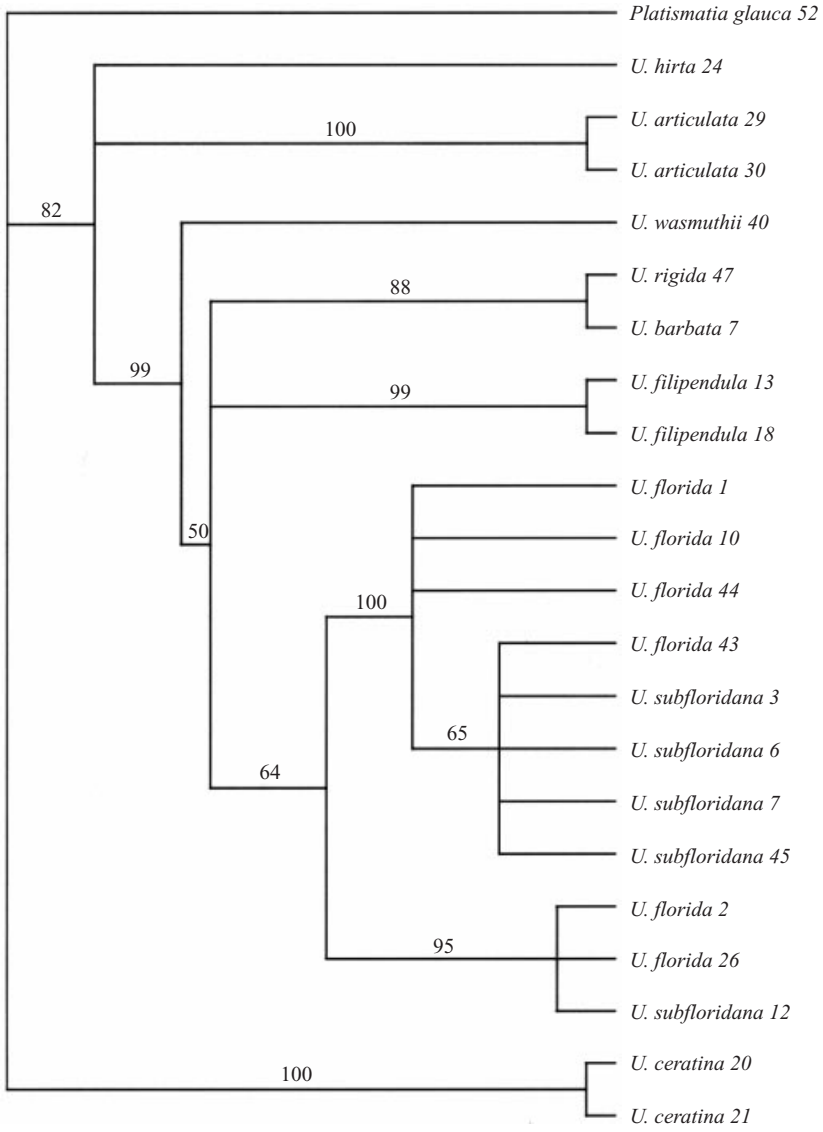


**Fig. 2.** Strict consensus tree of the combined ITS–LSU matrix (jack-knife values above the branches).

β-tubulin has 351 aligned sites of which 51 are parsimony informative. The ITS–LSU region contains 1405 aligned sites of which 58 are parsimony informative (45 of these are located in the ITS and 13 in the LSU region). The ITS region consists of 559 sites and the sequenced part of the LSU rDNA of 846 sites. There is a 57 bases long insertion present in the LSU region at position 1023 relative *Saccharomyces cerevisiae*. This insertion is found in all *Usnea florida* and *U. subfloridana* specimens and in one *U. articulata* specimen, but does not occur in the other *Usnea* specimens studied. The LSU region of *U. hirta*, *U. ceratina* 21 and *U. florida* 43 was only partially sequenced (546, 533 and 592 bases respectively).

### *Parsimony analysis*

The strict consensus trees of the separate and the combined analyses are presented and the jack-knife values are written above the branches (Figs 1–3).

The β-tubulin analysis resulted in two most parsimonious trees. The tree length is 80 steps (CI = 81, RI = 89). The analysis of the ITS–LSU matrix resulted in three most parsimonious trees with a tree length of 90 steps (CI = 77, RI = 87). The data sets were also combined and this analysis resulted in twelve most parsimonious trees of 179 steps (CI = 75 and RI = 85).

*Usnea florida* and *U. subfloridana* in the traditional, morphologically based sense, did not form monophyletic groups in any of the analyses. In each of the analyses there are highly supported groups with specimens of both species being mixed. The β-tubulin analysis (Fig. 1) shows two groups of intermixed specimens (j = 69, 99) and some specimens with unresolved relationships. In the ITS–LSU analysis (Fig. 2), *U. florida* and *U. subfloridana* form one monophyletic group (j = 64), within this group are two strongly supported groups with intermixed specimens (j = 95 and 100). Also in the combined analysis (Fig. 3), *U. florida* and *U. subfloridana* form one monophyletic

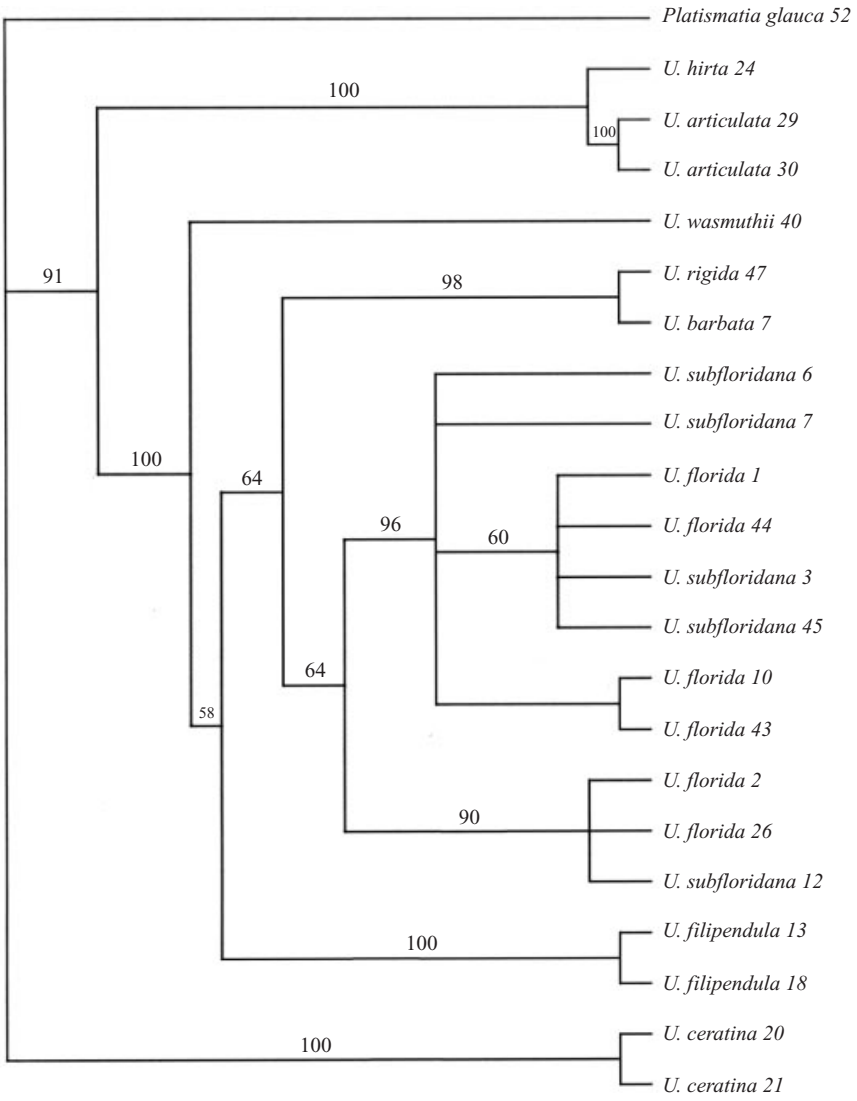


**Fig. 3.** Strict consensus tree of the combined β-tubulin and ITS–LSU matrix (jack-knife values above the branches).

group (j = 64) and within this group two groups with high support (j = 90, 96) are found. The β-tubulin tree is not as resolved as the ITS–LSU tree and the grouping of the *U. florida* and *U. subfloridana* specimens differ between the analyses.

There is a well-supported monophyletic group in all analyses (j = 99–100), containing *Usnea florida*, *U. subfloridana*, *U. barbata*, *U. rigida*, *U. filipendula* and *U. wasmuthii*. Also, *U. barbata* and *U. rigida* form a group with high jack-knife values (77–98, Figs 1–3) which is present in all three analyses. The specimens of *U. ceratina* and *U. articulata*, respectively, form well-supported, monophyletic groups (j = 100). *U. hirta* is the sister group to *U. articulata*; this, however, has no support in one of the analyses.

## DISCUSSION

It is clear from our results that neither *Usnea florida* nor *U. subfloridana* form monophyletic groups in any of the analyses. Hence, we conclude that the β-tubulin and nuclear rDNA data investigated suggest that there are no natural groups corresponding to the reproductive strategies. Together, the two taxa form one monophyletic group of intermixed specimens. The jack-knife support value for this group in the combined analysis (Fig. 3) is comparatively low (j = 64), however, but the group contains two strongly supported groups (j = 90, 96) which includes specimens of both taxa. It is thus better to treat *Usnea florida* and *U. subfloridana* as one polymorphic species. Our investigation, including two unlinked loci, is in accordance with the suggestions by Grube & Kroken (2000) to use more than one locus to exclude the possibility that we may deal with two separate species.

The results also show that the sexual and asexual modes of reproduction and dispersal may be optional within one species. This results in the occurrence of specimens with sexual, vegetative, or with combined sexual and vegetative reproduction, as have been observed in *U. florida–U. subfloridana*. We do not know

what factors regulate the kind of reproduction that a lichen individual may show. The observation that fertile '*U. subfloridana*' specimens (i.e. specimens producing both vegetative propagules and apothecia) usually occur at *U. florida* sites, may indicate that unknown environmental conditions induce the production of apothecia and that the production of vegetative propagules is sometimes repressed. This seems to be the case also in many other sorediate lichens, such as *Hypogymnia physodes*, *H. tubulosa* and *Parmelia sulcata*, to name just a few. This would explain why *Usnea florida* in the traditional sense is limited to certain areas, while *U. subfloridana* in the traditional sense has a wider distribution.

In our analyses, the resolution of the *U. florida–U. subfloridana* specimens in the separate β-tubulin and the ITS–LSU analyses differ somewhat. This is not surprising when different phenotypes belong to a single species. A similar pattern can be seen in a recently published study by Myllys *et al.* (2001) where the ITS and β-tubulin data result in slightly different trees.

Other recent molecular studies (e.g. Lohtander *et al.* 1998a, b, Myllys *et al.* 1999) focussing on putative lichen species pairs, have also concluded that in most investigated cases, specimens do not form monophyletic groups corresponding to their reproductive strategy, which would have been the expected result if they represent distinct natural entities. Clearly, *U. florida–U. subfloridana* does not represent a species pair in the sense of Poelt. Many *Usnea* species show a wide morphological variation and it is hard to know which characters are reliable for their identification. Some taxa, though, show striking features, making them easily recognised. Fertile shrubby *Usnea* specimens lacking vegetative reproductive structures (*U. florida*), are easily identified (most *Usnea* species in Europe do not produce apothecia) and such a concept of *U. florida* is of course attractive in its simplicity. It does not, however, represent a natural, monophyletic, group. Some lichen species may be able to switch between sexual and vegetative reproduction depending on environmental conditions, as is possibly the case in *U. florida–U. subfloridana*. This may be common in lichens, and further 'species pairs' may prove to be non-monophyletic assemblages.

Although our analyses only contain a limited selection of other *Usnea* species, we can identify some additional groupings within the genus. In the β-tubulin, ITS–LSU and the combined analyses *U. florida–U. subfloridana*, *U. barbata*, *U. rigida*, *U. filipendula* and *U. wasmuthii* form a strongly supported group of species. Clerc (1992) has already pointed out the close similarity between *U. subfloridana* and *U. wasmuthii*. However, in our study these two species do not form a monophyletic group, but are included in a well-supported group together with other species. A more unexpected result is that *U. barbata*, *U. rigida* and *U. filipendula* also seem to be closely related to *U. florida–U. subfloridana*. This indicates that thallus shape (shrubby *vs* pendent) does not necessarily reflect phylogenetic relations within *Usnea*.

The oldest name in the *U. florida–U. subfloridana* complex is *Lichen floridus* L. 1753, which is also the type species of the genus *Usnea* (Jørgensen, James & Jarvis 1994). Even if a full nomenclatural survey is beyond the scope for this investigation, we may conclude that *U. florida–U. subfloridana* should be treated as one species and this species should be called *U. florida*. In this connection, we would like to point out that *U. florida–U. subfloridana* in the fertile stage still requires strong conservation attention. The fertile specimens of *U. florida–U. subfloridana* usually only occur in areas with high species diversity and still function well as bioindicators for old, species-rich forests.

## CONCLUSIONS

The traditional way of delimiting *Usnea florida* and *U. subfloridana* is not consistent with the results from parsimony analyses of molecular data from the β-tubulin, and nuclear ribosomal (ITS and LSU) DNA. Specimens of *U. florida* and *U. subfloridana* form one monophyletic group of intermixed specimens. Thus, we should treat the fertile and sorediate specimens of these taxa as belonging to one, polymorphic species, with the name *U. florida*. Our results also contribute to the discussion on the species concepts in lichenized fungi-morphologically easily recognised groups of individuals may not represent monophyletic taxa, and some species may be able to switch between sexual and vegetative reproduction depending on environmental conditions. Molecular analyses, combined with careful morphological investigations, will hopefully enable us to resolve many of the remaining problems in *Usnea*, one of the most widely known but at the same time most poorly understood groups of lichenised ascomycetes.

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