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Distinction of *Cladonia rei* and *C. subulata* based on molecular, chemical and morphological characteristics

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Abstract: *Cladonia rei* and *Cladonia subulata* are morphologically similar, but chemically different cup lichens of dry grasslands and nutrient-poor ruderal habitats. Recently, *C. rei* has been synonymized with *C. subulata* on the basis of combined morphological and chemical investigations. However, doubts remained due to a molecular divergent North American sample of *C. rei* compared to European *C. subulata*. To clarify the situation, using molecular methods, we analysed chemically different European samples of *C. rei* and *C. subulata*, as well as other morphologically or chemically similar *Cladonia* species. Molecular data show that European and North American samples of *C. rei* belong to the same clade, which is closely related to *C. fimbriata* and followed by a subclade with *C. coniocraea* and *C. ochrochlora*. The subclade of *C. subulata* appears to be distinct from *C. rei*. In concordance with molecular data, the presence of homosekikaic acid is the determining chemical feature for *C. rei*. In addition, *C. humilis* and *C. innominata* proved to be molecularly distinct species.

Key words: *Cladonia conista*, *Cladonia fimbriata*, *Cladonia humilis*, *Cladonia innominata*, homosekikaic acid, ITS, chemotype

Introduction

The genus *Cladonia* belongs to one of the most morphologically diverse and species-rich genera of lichens (Stenroos *et al*. 2002). Based on morphological and chemical characters, Sandstede (1931) accepted 69 species of *Cladonia* in central Europe. Furthermore, these were divided into a vast range of morphotypes. Improvements in the analysis of lichen chemistry increased the number of species recognized in subsequent years and made it increasingly difficult to distinguish taxa on the basis of morphological features or simple spot tests. In addition, the variability observed in chemical features raised doubts about the taxonomic status of several chemically defined species. These may, in fact, represent chemical strains (Lamb 1951; Leuckert *et al*. 1971). The idea was then widely accepted in central Europe and applied to the so-called "*Cladonia pyxidata* group". Within this group, Wirth (1994) included under the name *C. pyxidata* ssp. *grayi* (G. Merr. ex Sandst.) V. Wirth the following chemically different taxa: *C. cryptochlorophaea* Asahina, *C. grayi* G. Merr. ex Sandst., *C. merochlorophaea* Asahina and *C. novochlorophaea* (Sipman) Brodo & Ahti. For other species, Goward (1999) and Ahti & Hammer (2002) included under the name *C. humilis* the chemically different *C. humilis* (With.) J. R. Laundon and *C. innominata* Lendemer [syn. *C. conista* (Nyl.) Robbins]. Spier & Aptroot (2007) recently united *C. subulata* (L.) F. H. Wigg. and *C. rei* Schaer. under the name *C. subulata*.

On the other hand, other studies indicated that the biogenetic pathway of secondary lichen products could reflect phylogenetic relationships (Culberson 1986), and that the range of morphologically and chemically distinguished taxa could be extended by a range of molecular sibling species (Stenroos *et al*. 2002; Vondrák *et al*. 2009).

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The concepts of *Cladonia subulata* and *C. rei* changed over time. Vainio (1887) classified species known currently as *C. coniocraea* (Flörke) Spreng., *C. humilis, C. ochrochlora* Flörke, *C. subulata* and *C. rei* as varieties or formae of *C. fimbriata* (L.) Fr. Later, Sandstede (1931) accepted *C. coniocraea, C. fimbriata, C. ochrochlora, C. rei* and *C. subulata* (syn. *C. cornutoradiata*) at the species level, as well as *C. major* (K.G. Hagen) Sandst., which is currently included in *C. fimbriata*. Sandstede understood *C. cornutoradiata* Coem. (including f. *subulata*) to be a morphologically variable taxon reacting P+ red (fumarprotocetraric acid) and UV− (or + bluish) in contrast to *C. nemoxyna* (Ach.) Arnold (= *C. rei*), reacting P− (no fumarprotocetraric acid) and UV+ white (homosekikaic acid). This means that fumarprotocetraric acid-containing individuals of *C. rei* were regarded as *C. cornutoradiata*, a practice followed also by Ozenda & Clauzade (1970). Nevertheless, Zopf (1908) had already found in specimens of *Cladonia fimbriata* var. *cornuto-radiata* f. *nemoxyna* (Ach.) Vainio (= *C. rei*) an acid he named nemoxynic acid, found to be identical to homosekikaic acid by Asahina (1938). Asahina noticed that *C. nemoxyna* frequently contains fumarprotocetraric acid as an accessory component. The name "*Cladonia rei* Schaer. 1823" came into common use rather late. Østhagen (1976) showed that *C. nemoxyna* is a synonym of *C. rei* and that the type material of *C. rei* contains both homosekikaic and fumarprotocetraic acids. Although the type material of *C. nemoxyna* contains only homosekikaic acid, Østhagen (1976) pointed out that he found no differences in morphology between the fumarprotocetraric acid-containing and fumarprotocetraric acid-lacking species. These were therefore regarded as chemotypes. Detailed studies on the chemical differentiation of *C. rei* were carried out by Suominen & Ahti (1966), Paus *et al.* (1993), Paus (1997), Günzl & Fischer (2004) and Syrek & Kukwa (2008). They discovered that the fumarprotocetraric acid-containing chemotype of *C. rei* is the most common chemotype in Central Europe. They also found that all content-gradients of fumarprotocetraric acid are represented, and, in most cases, small amounts of sekikaic acid as well that are not correlated with any morphological trends. Paus *et al.* (1993) revealed that *C. rei* prefers bare soil and is quite frequently found in disturbed sites often associated with *C. chlorophaea* (Flörke ex Sommerf.) Spreng., whereas *C. subulata* has a wider ecological amplitude, preferring more stable, humus-rich soils and acidic sand. In dry grassland communities, both species can occur together (Dolnik 2005) but they can also show preferences for certain other communities (Biermann 1999; Fischer 2003). A high tolerance of *C. rei* on sites heavily contaminated with metals was found by Coppins & van den Boom (1995) and Cuny *et al.* (2004) near zinc works, by Ernst (1995) under galvanized crash barriers along roads, and by Hadjúk & Lisická (1999) in the vicinities of copper smelters.

After extended morphological and chemical investigations of European material, Spier & Aptroot (2007) synonymized *C. rei* with *C. subulata*. They demonstrated an overlapping morphology of distinguishing features such as a corticated base of podetia, presence of squamules, and development of cups. They also argued that secondary lichen substances, such as fumarprotocetraric and homosekikaic acid are taxonomically of minor importance. We tested this hypothesis by analysing molecular, chemical, and morphological differences in *C. subulata* and *C. rei* from several European countries. We used the sequence from the internal transcribed spacer (ITS) of the nuclear rDNA gene cluster that was also used by Stenroos *et al.* (2002) for their extended phylogenetic analysis of the genus *Cladonia*. The hypotheses we tested were that: 1) *C. subulata* and *C. rei* are distinct species; 2) *C. rei* includes two chemical races, which are neither morphologically nor molecularly distinct; and 3) *C. subulata* is more closely related to *C. ochrochlora*, *C. coniocraea* and *C. fimbriata* containing only the fumarprotocetraric chemosyndrome than it is to *C. rei* and *C. novochlorophaea* containing the homosekikaic chemosyndrome.

TABLE 1. *Origin of chemically analysed specimens of* Cladonia subulata *(n = 75) and* Cladonia rei *(n = 90)*

	C. rei	*C. subulata*
Austria	0	1
Czech Republic	2	9
Denmark	1	1
France	1	0
Germany	31	41
Italy	3	0
New Zealand	2	1
Poland	4	4
Russia	41	12
Slovakia	2	1
St. Pierre & Miquelon	1	0
Sweden	1	3
Switzerland	1	2

Material and Methods

Material

The morphological investigations were based on 90 samples of *Cladonia rei*, chemically defined by the presence of homosekikaic acid and traces of sekikaic acid (regardless of whether fumarprotocetraric acid is present or not), and 75 samples of *C. subulata*, chemically defined by the presence of fumarprotocetraric acid and absence of homosekikaic acid. All samples were analysed by thin-layer chromatography. Taking into account their large morphological variability and geographical distribution range in Europe, seven samples of *C. rei* and eight samples of *C. subulata* were chosen for molecular analysis. The origin of the material used in the chemical analysis is summarized in Table 1. Samples of *C. nemoxyna* (n = 12) and *C. cornutoradiata* (n = 52), determined by Johann Heinrich Sandstede (1859–1951) and deposited in Munich (M), were analysed in addition to the herbarium material collected by the authors. Specimens of *C. cornutoradiata* with the chemosyndrome of *C. rei* were revised and regarded as *C. rei* (n = 4). Specimens are deposited in Munich (M), Kiel (KIEL), Poznań (POZ) and in the private herbarium of C. Dolnik. All molecularly analysed samples are stored in M. A complete list of all specimens analysed is available from the corresponding author.

To test the relationship between *C. rei* and *C. subulata*, other members of the section *Cladonia* were also analysed. These included *C. coniocraea* (6 samples), *C. fimbriata* (3), and *C. ochrochlora* (1), all of which are sorediose and have slender podetia. The wide scyphose and chemically divergent *C. merochlorophaea* (2), *C. novochlorophaea* (2), *C. humilis* (2) and *C. innominata* (1) were also included.

Cladonia humilis was chosen as an out-group species, as it belongs to a sister clade in *Cladonia* section *Cladonia* (Stenroos *et al.* 2002). Stenroos *et al.* (2002) follow a broad species concept of *C. humilis* including *C. humilis* s. str. (containing fumarprotocetraric acid and atranorin) and *C. innominata* (fumarprotocetraric and bourgeanic acids); both chemotypes were included in our analysis.

Morphological and chemical analyses

Paus *et al.* (1993) and Spier & Aptroot (2007) demonstrated that features currently used to distinguish *Cladonia rei* from *C. subulata* are unreliable. Therefore, we compared other features such as cup proliferation and form of apothecia. As young podetia of *C. rei* and *C. subulata* are morphologically indistinguishable, we used only well-developed podetia to describe the characteristics of each sample.

According to Spier & Aptroot (2007), more than half of the samples of *C. rei* and *C. subulata* bear cups. We distinguished between: 1) cups with and without short-stalked proliferations (Fig. 1A, e–g); 2) cups with long-stalked proliferations (Fig. 1B, c–f). According to Sandstede (1931), prominent apothecia are typical for *C. rei* so we distinguished between: 1) prominent apothecia wider than the scyphus occurring at the end of a slender scyphus or short-stalked cup proliferations (Fig. 1A, j) or at the blunt end of stout podetia, where they are as wide as the podetium tip (Fig 1A, c & d); and 2) without apothecia or minute ones (Fig. 1A, a & b, e–i, Fig. 1B).

Chemical analysis (thin-layer chromatography, TLC) was carried out with solvent system A according to Culberson & Ammann (1979), using standard Merck silica gel 60F254 plates and *C. symphycarpia* (Flörke) Fr. (Öland population/Sweden, containing atranorin, norstictic and bourgeanic acid) as control. Since the amount of homosekikaic acid can vary within one scyphus, we used the upper part of large podetia or an entire podetium for our analysis.

DNA extraction, PCR and sequencing

For the molecular analysis, we selected species tested by TLC analysis, with wide distribution ranges and morphological variability. The upper part of a single podetium was used for DNA isolation following the CTAB method (Rogers & Bendich 1985), as modified by Cubero *et al.* (1999). PCR was used for the amplification of the ITS from the isolated DNAs using the primers, cycling conditions and instruments described in Beck *et al.* (2002). Purification of the products with Macherey-Nagel columns (Macherey-Nagel, Düren) was followed by 30 cycles of sequencing reaction (95 °C for 10 s, 50 °C for 15 s, 60 °C for 3 min) using the primers ITS1F and ITS4 (White *et al.* 1990), and the Big Dye Terminator Reaction Kit 3.1 (Perkin-Elmer Inc., Wellesley, MA, USA). Sequences were obtained using an ABI 3730 48 capillary automatic sequencer. Fragments were assembled with the aid of the Staden package (http://staden.sourceforge.net/). GenBank accession numbers for all newly obtained ITS sequences, including voucher specimen details, are listed in Table 2.

Data analysis

In addition to the 32 newly produced sequences, further sequences were downloaded from GenBank for

FIG. 1. Morphology of *Cladonia* species. A, *Cladonia rei*, morphological variability characterized by simple podetia (a), by stout podetia with short branching patterns (b, c, h, i, j) or narrow cups (e, f, g, h, j), by no (g) or only short proliferations (e, f, h, j) and often prominent apothecia (c, d, j); B, *Cladonia subulata*, morphological variability characterized by simple subulate podetia (a), by podetia with narrow cups with long and subulate proliferations (c, d, e, f), or by antler-like branching patterns without cups (b, g, h, i, j, k) and mostly minute apothecia. Scales: A & B = 1 cm.

comparison. We included 7 sequences produced by Stenroos *et al.* (2002): *C. fimbriata* (AF455224), *C. humilis* (AF455209), *C. merochlorophaea* (AF455227), *C. ochrochlora* (AF455192), *C. rei* (AF455191) and *C.*

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TABLE 2. *Voucher data and GenBank accession numbers for newly sequenced* Cladonia *species. All vouchers are deposited in* M

Taxon	Lichen acids (major)	Morphotype	Voucher	Barcode Number	GenBank No. ITS
C. subulata	fumarprotocetraric acid	antlers, farinose	Poland. Wielkopolska, Nowe Jastrzębsko, *Zarabska* 27 ix 2007	M-0155392	GU188418
C. subulata	fumarprotocetraric acid	antlers, farinose	Germany, SH, PI, Liether Kalkgrube, 2008, *Dolnik* 1049	M-0155390	GU188422
C. subulata	fumarprotocetraric acid	narrow cups, no proliferation, farinose	Germany, SH, PI, Liether Kalkgrube, 2008, *Dolnik* 1051	M-0155387	GU188421
C. subulata	fumarprotocetraric acid	antlers, farinose to granulose-sorediate	Germany, SH, RD, Warderfeld, 2008, *Dolnik* 1062	M-0155391	GU188419
C. subulata	fumarprotocetraric acid	antlers, granulose-sorediate	Germany, SH, NF, Löwenstedter Sandberge, 2007, *Dolnik* 953	M-0155388	GU188423
C. subulata	fumarprotocetraric acid	antlers, farinose	Poland, Wielkopolska, Chrośnica, *Zarabska* 27 ix 2007	M-0155389	GU188420
C. subulata	fumarprotocetraric acid	subulate, farinose	Poland, Wielkopolska, Jastrzębsko Stare, *Zarabska* 20 ix 2007	M-0155383	GU188425
C. subulata	fumarprotocetraric acid	cups with long proliferaton, farinose	Sweden, Sk, Falsterbo, *Dolnik* 20 iii 2008	M-0155386	GU188424
C. rei	homosekikaic, tr. sekikaic, fumarprotocetraric acid	minute cups without or with short proliferation, farinose, small apothecia	Germany, SH, RD, Warderfeld, 2008, *Dolnik* 1059	M-0155382	GU188402
C. rei	homosekikaic, tr. sekikaic, fumarprotocetraric acid	unbranched, farinose, prominent apothecia	Denmark, SJy, Kliplev, *Dolnik* 28 iii 2008	M-0155380	GU188400
C. rei	homosekikaic, tr. sekikaic, fumarprotocetraric acid	narrow cups with short stalked proliferation, farinose, apothecia prominent	Germany, SH, NF, Beltringharder Koog, *Dolnik* 20 vii 2005	M-0155381	GU188403
C. rei	homosekikaic, tr. sekikaic, fumarprotocetraric acid	trumpets, farinose to granulose-sorediate, small apothecia	Germany, SH, RD, Schachtholm, 2006, *Dolnik* 602	M-0155377	GU188401
C. rei	homosekikaic, tr. sekikaic, fumarprotocetraric acid	furcate, farinose, prominent apothecia	Poland, Wielkopolska, Kuźnica Zbąska, *Zarabska* 18 ix 2007	M-0155384	GU188399
C. rei	homosekikaic, tr. sekikaic acid	narrow cups, long proliferation, farinose, small apothecia	Poland, Wielkopolska, Jastrzębsko Stare, *Zarabska* 20 ix 2007	M-0155385	GU188398
C. rei	homosekikaic, tr. sekikaic acid	antlers, farinose, small apothecia	Poland, Wielkopolska, Chrośnica, *Zarabska*, 27 ix 2007	M-0155378	GU188397
C. novochlorophaea	homosekikaic, sekikaic, fumarprotocetraric acid	broad granulose-sorediate cups	Germany, SH, RD, Owschlager Moor, 2008, *Dolnik* 977	M-0155373	GU188414

TABLE 2. *Continued*

Taxon	Lichen acids (major)	Morphotype	Voucher	Barcode Number	GenBank No. ITS
C. novochlorophaea	homosekikaic, sekikaic acid	broad granulose-sorediate cups	Sweden, Sk, Falsterbo, *Dolnik* 20 iii 2008	M-0155372	GU188415
C. merochlorophaea	merochlorophaeaic, 4-O-methylcryptochlorophaeic, fumarprotocetraric acid	broad granulose-sorediate cups	Germany, SH, RD, Fockbeker Moor, 2008, *Dolnik* 972	M-0155370	GU188417
C. merochlorophaea	merochlorophaeaic, 4-O-methylcryptochlorophaeic, fumarprotocetraric acid	broad granulose-sorediate cups	Germany, SH, RD, Felmer Moor, 2008, *Dolnik* 980	M-0155371	GU188416
C. coniocraea	fumarprotocetraric acid	subulate, farinose	Denmark, SJy, Frøslev Mose, *Dolnik* 28 iii 2008	M-0155361	GU188407
C. coniocraea	fumarprotocetraric acid	subulate, farinose	Germany, SH,SL, Jardelunder Moor, 2008, *Dolnik* 1064	M-0155362	GU188409
C. coniocraea	fumarprotocetraric acid	subulate, farinose	Poland, Wielkopolska, Łomnica, *Zarabska* 21 ix 2007	M-0155364	GU188410
C. coniocraea	fumarprotocetraric acid	subulate, farinose	Poland, Wielkopolska, Glinno, *Zarabska* 18 ix 2007	M-0155363	GU188411
C. coniocraea	fumarprotocetraric acid	trumpet, farinose, no corticated inner cup	Germany, NI, OHZ, Franzhorn, 2008, *Dolnik* 1028	M-0155375	GU188408
C. coniocraea	fumarprotocetraric acid	subulate, farinose	Poland, Wielkopolska, Bolewicko, *Zarabska* 07.10.2005	M-0155374	GU188412
C. ochrochlora	fumarprotocetraric acid	trumpet, farinose, corticated inner cup	Germany, SH, RD, Dosenmoor, 2007, *Dolnik* 733	M-0155376	GU188413
C. fimbriata	fumarprotocetraric acid	farinose cups	Germany, SH, SL, Jardelunder Moor, 2008, *Dolnik* 1065	M-0155367	GU188404
C. fimbriata	fumarprotocetraric acid	farinose cups	Germany, SH, SL, Warderfeld, 2008, *Dolnik* 1009a	M-0155366	GU188405
C. fimbriata	fumarprotocetraric acid	granulose-sorediate cups	Germany, SH, RD, Warderfeld, 2008, *Dolnik* 1060	M-0155360	GU188406
C. humilis	atranorin, fumarprotocetraric acid	broad farinose cups	Germany, SH, RD, Warderfeld, 2008, *Dolnik* 1057	M-0155369	GU188395
C. humilis	atranorin, fumarprotocetraric acid	broad farinose cups	Germany, SH, PI, Liether Kalkgrube, 2008, *Dolnik* 1053	M-0155368	GU188396
C. innominata	bourgeanic, fumarprotocetraric acid	broad farinose cups	Germany, SH, OD, Höltigbaum, 2006, *Dolnik* 389	M-0155365	GU188394

TABLE 3. *Presence of cup proliferations and apothecia as morphological features to distinguish between* Cladonia rei *and* Cladonia subulata

	n	cups present % (*n*)	cup margin with long proliferations % (*n*)	prominent apothecia present % (*n*)
Cladonia rei	90	64 (58)	16 (9)	36 (32)
Cladonia subulata	75	64 (48)	69 (33)	10 (7)

subulata (AF455180, AF455181). The 39 sequences were aligned using the program BioEdit (Hall 1999). For the phylogenetic analysis, all alignment positions with less than 75% gaps were used, resulting in a data matrix with 409 characters. *Cladonia humilis* and *C. innominata* were used as out-group taxa. Three different analyses were performed: Maximum Parsimony (MP) and Maximum Likelihood (ML) calculations using PAUP* 4.0b10 (Swofford 2003), and Maximum Likelihood estimations using RAxML version 7.0.3 (Stamatakis 2006). For the MP analysis, gaps were treated as missing data, and multistate character interpreted as uncertainty. A heuristic search with 10 000 random-addition sequence replicates was performed using tree bisection-reconnection (TBR) branch-swapping, with MulTrees on and steepest-descent option not in effect. Bootstrap analysis with 5000 replicates, with 25 random-addition sequence replicates each, and with search parameters as above, has been used to estimate branch support. For the ML analysis, the optimal substitution model was tested with hierarchical likelihood ratio tests and by successive evaluation under the Akaike Information Criterion using the program Modeltest v.3.06 (Posada & Crandall 1998). The symmetrical model (Zharkikh 1994) including rate variation among sites was suggested as the best fitting model for the data. A heuristic search with 500 random-addition sequence replicates was performed using the parameters Nst = 6, Rmat = (1·1233 4·3293 1·9908 0·6999 9·4337), Rates = gamma, Shape = 0·5470 and Pinvar = 0. Bootstrap analysis with 500 replicates, with 10 random-addition sequence replicates each, and search parameters as above has been used to estimate branch support. An alternative assessment of the data has been carried out using RAxML and a GAMMA-GTR model using standard settings and the empirical base frequencies pi(A): 0·200025, pi(C): 0·271850, pi(G): 0·275323 and pi(T): 0·252802. Branch support has been calculated using 500 bootstrap repetitions and the settings detailed above.

Results

Morphological and chemical data

Our observations confirm the high morphological variability with overlapping morphological characters in *C. rei* and *C. subulata* found by Paus *et al.* (1993) and Spier & Aptroot (2007). Apart from the positive UV+ white reaction in *C. rei* resulting from the presence of homosekikaic acid, the long and subulate proliferations occurring on the cup margin are much more pronounced in *C. subulata* than in *C. rei*. Prominent apothecia are much more common in *C. rei* (Table 3). These two morphological characters found in both species are not absolute, but in combination, they give both species a specific appearance, which is visible in well-developed samples (Fig. 1). The two chemotypes of *C. rei* (chemotype I: fumarprotocetraic and homosekikaic; chemotype II: homosekikaic with traces of sekikaic, but no fumarprotocetraic acid) show a weak geographical pattern, since chemotype I was found to be more common in the German material (87%), whereas chemotype II was more common in the Kaliningrad Oblast (66%).

Molecular data

The MP analysis of the ITS nrDNA indicated that out of the 409 characters 285 were constant, 31 variable, but parsimony uninformative, and 93 parsimony informative. The search resulted in 12 equally parsimonious trees of 180 steps length (CI = 0·756, RI = 0·932, RC = 0·704). The ML analysis produced a single best-scoring tree with a likelihood score of 1550·96588, and the RAxML search resulted in one tree with a final ML optimization likelihood of −1584·557787. All search methods resulted in similar phylogenetic trees, with only minor differences in poorly supported branches. Figure 2 shows the ML tree with the bootstrap support values obtained with various methods.

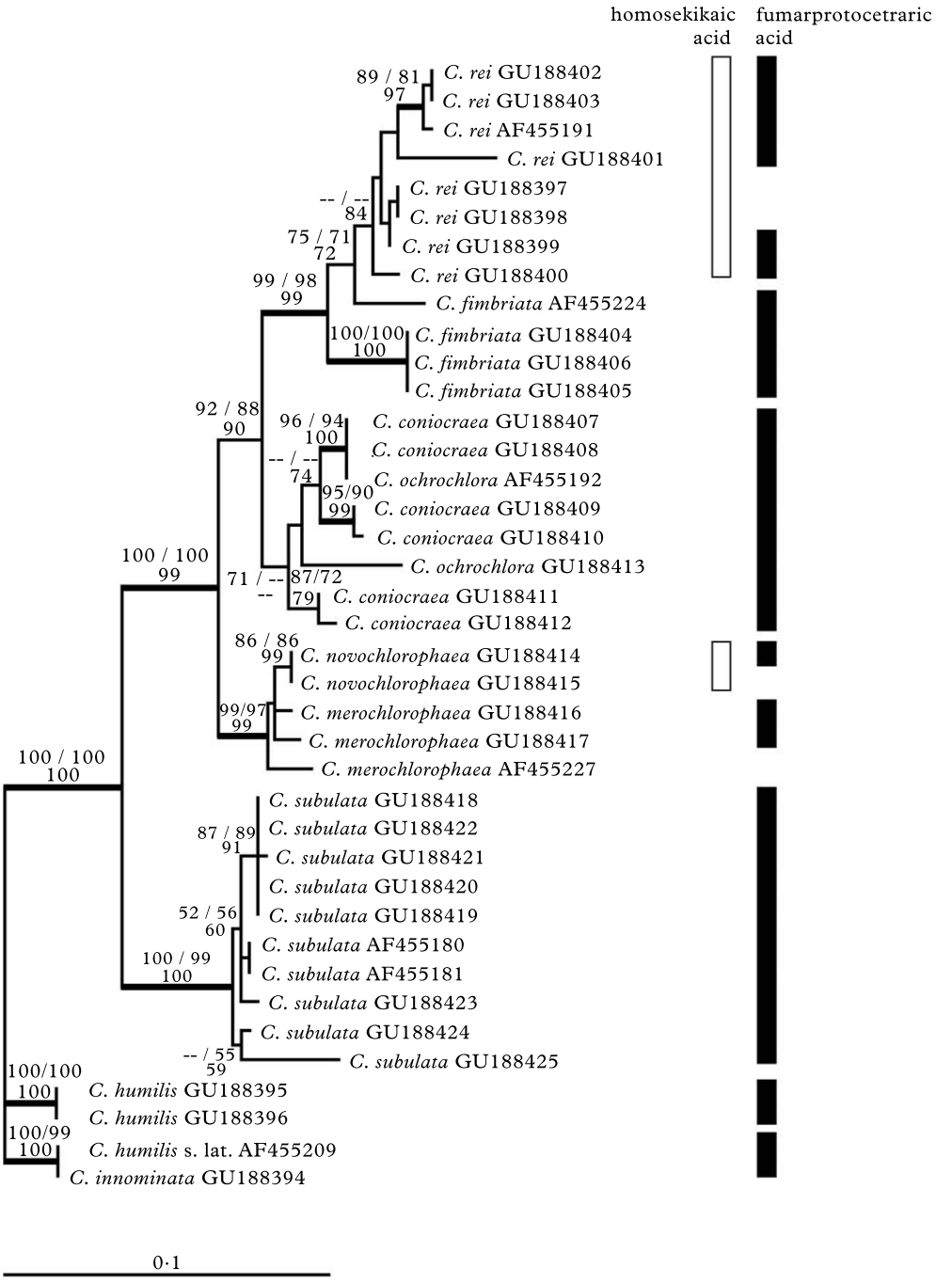

FIG. 2. Analysis of members of *Cladonia* sect. *Cladonia* inferred in a ML search from ITS nrDNA data. Shown is the best-scoring tree of the ML search (see section Material and Methods for details). Figures associated with branches indicate bootstrap support values inferred by three independent analyses: MP search using PAUP (upper row, left side), ML search using PAUP (upper row, right side) and search using RAxML (lower row). Branches with bootstrap support higher than 90 in all three methods are marked by bold lines.

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The phylogenetic analysis clearly separated *Cladonia subulata* and *C. rei*, which is in accordance with the results obtained by Stenroos *et al.* (2002). They are morphologically similar, but chemically and genetically distinct, confirming our first hypothesis of two distinct species. Furthermore, *C. rei* forms a clade together with *C. merochlorophaea*, *C. ochrochlora* and *C. fimbriata*.

Homosekikaic acid is present in all samples of *C. rei*, thus supporting the current use of homosekikaic acid as a diagnostic feature of this species. Samples of *C. rei* in which fumarprotocetraric acid was detected (chemotype I) are grouped with those without fumarprotocetraric acid (chemotype II), confirming our second hypothesis and the current practice to regard these chemotypes has no taxonomic value. The Canadian sample of *C. rei* (AF455191, Stenroos *et al.* 2002) groups with European material. *Cladonia fimbriata* from Chile (AF455224, Stenroos *et al.* 2002) is separated from the *C. rei* clade in all three analyses, but without bootstrap support. Furthermore, this specimen does not group with the European samples of *C. fimbriata*. A granulose sorediate sample of *C. fimbriata* (GU188406), originally determined as *C. chlorophaea*, is not separated from farinose forms of *C. fimbriata* (GU188404, GU188405). This suggests that the size of soredia may be of minor importance in separating *C. fimbriata* from other members of the *C. chlorophaea* group containing fumarprotocetraric acid as the major component. The current morphological concept of *C. chlorophaea* is discussed in more detail by Kowalewska *et al.* (2008: 65) and is not the focus of this study. In *C. rei* and *C. subulata*, the size of soredia also varies from farinose to granulose, indicating a low taxonomic value of this character in this group.

The next clade contains samples representing *C. coniocraea* and *C. ochrochlora*, however, they are intermingled. The *C. ochrochlora* type *sensu* Sandstede (1931) is characterized by slender podetia with narrow cups and is therefore similar to some morphotypes of *C. rei*. Nevertheless, the corticated inner cup and the lack of homosekikaic acid are reliable characteristics for distinguishing between *C. rei* and these species. More distant from *C. rei* is the clade including *C. novochlorophaea* and *C. merochlorophaea*. There are also two chemotypes in *C. novochlorophaea* (Fig. 2); one contains fumarprotocetraric acid (GU188414) and the other does not (GU188415). The only chemical difference between *C. rei* and *C. novochlorophaea* is the co-dominance of sekikaic and homosekikaic acids in *C. novochlorophaea*, whereas, in *C. rei*, sekikaic acid occurs only in trace amounts. Thus, our third hypothesis is refuted, since the presence of homosekikaic acid does not indicate a closer relationship of *C. rei* to *C. novochlorophaea*.

All samples of *Cladonia subulata* contain fumarprotocetraric acid as the major chemical component and they form a well-supported group. Regarding morphology, both anisodiametric antler-like branching patterns and cup-bearing patterns can be found. *Cladonia humilis* s. lat. was demonstrated to be non-monophyletic. Both chemotypes form well-supported groups, and the sample '*Cladonia humilis*' AF455209 from China (Stenroos *et al.* 2002) is grouped together with *C. innominata* from Germany; both samples contain bourgeanic and fumarprotocetraric acid.

Discussion

As previously found by Stenroos *et al.* (2002), our results confirm that *Cladonia rei* and *Cladonia subulata* are not closely related. The ITS region differentiated sequences of *Cladonia* sect. *Cladonia* presented in Stenroos *et al.* (2002) quite well. The phylogenetic tree we have constructed in this study for several collections of European *C. rei* and *C. subulata* is consistent within itself and with the chemical data and the phylogeny found by Stenroos *et al.* (2002) for these species. We have refrained, therefore, from analysing other genes. Sequences of the ITS region are applied widely and have proved useful in many phylogenetic studies at the levels of genus and species in lichens (e.g., Myllys *et al.* 1999; Tretiach *et al.* 2009). We also

found that the North American specimen of *C. rei* (AF455191, Stenroos *et al.* 2002) does not differ from European material. Such differences were suggested by Spier & Aptroot (2007). The results presented are in perfect agreement with the current species concept of *C. rei*, in which the presence of homosekikaic acid as the main chemical component is considered characteristic of the species (Suominen & Ahti 1966; Paus *et al.* 1993; Hammer 1995), and fumarprotocetraric acid is treated as a common accessory component. Both chemotypes of *C. rei* have a wide distribution range in Europe. However, the fumarprotocetraric acid-containing chemotype is predominant in central Europe [The Netherlands, 73%, n = 59 (Spier & Aptroot 2007); Germany, 78%, n = 128 (Günzl & Fischer 2004); Poland, 70%, n = 228 (Syrek & Kukwa 2008)]. Chemotype II is more common in eastern parts of Europe [Kaliningrad Oblast (66%, n = 41, this study], and Finland [69%, n = 59 (Suominen & Ahti 1966)], where the climate is more continental. There is no obvious explanation for this geographical pattern. The combined molecular and chemical analysis allows a new assessment of morphological features characteristic of *C. rei* and *C. subulata*, a notoriously difficult group to handle, especially in biodiversity studies or vegetation surveys. According to our results, *C. fimbriata* is most closely related to *C. rei*. At first glance, this is surprising, since *C. fimbriata* possesses farinose and much shorter podetia with broader cups and does not contain homosekikaic acid. Nevertheless, *C. homosekikaica* Nuno, a rare lichen with the chemical pattern of *C. rei* (Culberson *et al.* 1985) and the morphology of *C. fimbriata*, could serve as the missing link between both species. The specimen collected by T. Feuerer from Chile containing only fumarprotocetraric acid and assigned to *C. fimbriata* (Stenroos *et al.* 2002) appears to be unrelated to European material of *C. fimbriata*, but requires further analysis on a broader scale that includes more material.

The homosekikaic acid-containing *C. novochlorophaea* forms a subclade with *C. merochlorophaea* and is not as closely related to *C. rei* as put forward in our third hypothesis. This underlines the fact that the metadepside homosekikaic acid can occur in independent subclades of *Cladonia* species (Stenroos *et al.* 2002) and thus has little or no relevance in establishing phylogenetic relationships above the species level, although distinct patterns of biogenetic relationships in biosynthetic pathways of secondary lichen products should be taken into account (Culberson 1986). According to our ITS sequences, the samples of *C. novochlorophaea* and *C. merochlorophaea* from central Europe are very closely related. This was already presumed by Sipman (1973), who treated both taxa as varieties of *C. merochlorophaea*. So far, the chemical pattern is the only discernible feature differentiating these taxa. The chemosyndrome of *C. merochlorophaea* with the depsides merochlorophaeic and 4-*O*-methylcryptochlorophaeic acids, and the chemosyndrome of *C. novochlorophaea* with homosekikaic and sekikaic acids are biosynthetically closely related (Culberson *et al.* 1985), thus supporting in this case a closer relationship. In North American populations, gene flow between chemotypes of the *C. chlorophaea* complex has been observed (Culberson *et al.* 1988; DePriest 1994), thus indicating the need for further studies. It is therefore beyond the scope of this article to focus on the phylogenetic relationship of *C. chlorophaea*, *C. merochlorophaea* and *C. novochlorophaea*. The molecular studies of Stenroos *et al.* (2002) show that further analyses are necessary in order to clarify which species contain several chemotypes, as was shown for the two chemotypes of *Cladonia rei*, and which chemical composition could be used as a diagnostic feature to distinguish between different species as, for example, in *C. arbuscula* (Wallr.) Flot. and *Cladonia mitis* Sandst. (Myllys *et al.* 2003).

Our samples of *C. subulata* from Europe form a homogenous group that includes the specimens used by Stenroos *et al.* (2002). In their phylogenetic study, *C. subulata* forms a subclade with morphologically very different corticated species such as *C. macrophyllodes* Nyl. and *C. turgida* Hoffm. This makes it difficult to find morphological homologies,

and provides further evidence that similar morphological patterns have developed independently, as did some chemical patterns.

The presence of phenolic acids can be important for distinguishing between morphologically similar species, such as *C. humilis* and *C. innominata*. The molecular differences between these species can be explained by the biogenetic relationship of lichen substances proposed by Culberson (1986), where fatty acids such as bourgeanic acid and paradepsidones such as atranorin follow different biosynthetic pathways. The well-supported differences in the sister subclades of both species underline genetic differences between these species and between gene loci other than those responsible for the biosynthesis of the mentioned secondary substances.

In central and western Europe, *C. humilis* is by far the most common chemotype and *C. innominata* is rare. *Cladonia innominata* predominates further to the east in Europe, i. e., in Poland (Kowalewska *et al.* 2008) and in the Kaliningrad Oblast (Dolnik & Petrenko 2003). Tønsberg (1985) suggests a more oceanic distribution for *C. humilis* in Europe, whereas *C. innominata* has more continental site preferences. Both species also occur in North and South America and Australia (Archer 1989), but, so far, no geographical differences are known. Laundon (1984) suggested reducing *C. conista* (= *C. innominata*) to the rank of a variety of *C. humilis*, and Archer (1989) described it under the name *C. humilis* var. *bourgeanica* A. W. Archer. For nomenclatorial reasons, Lendemer (2008) replaced the invalid name "*C. conista*" by the new "*C. innominata*." Ecological differences between the two sibling species are not known, but we can see once more, two molecularly and chemically separate species, which are morphologically difficult to distinguish. Our results support the view of Lumbsch (1998), that there is neither a simple scheme for accepting all chemotypes as sibling species, nor a scheme neglecting chemical variations on the species level. In the present study, molecular analysis proved to be helpful in detecting examples of both possibilities in *Cladonia* sect. *Cladonia*.

Separation of *Cladonia rei* and *C. subulata* morphotypes in the field

This study has shown that *Cladonia rei* and *C. subulata* are quite distinct species. It is therefore worthwhile distinguishing them in vegetation surveys and biodiversity studies. Unfortunately, typical illustrations of *C. rei* have been rare in recent lichen handbooks, making it difficult to identify the habitus of this species. Good photographs of *C. rei* can be found in the North American lichen flora of Brodo *et al.* (2001), with prominent apothecia on short proliferations of cup margins, and in the Dutch lichen flora by van Herk & Aptroot (2004). Unfortunately, the photograph of *C. subulata* in van Herk & Aptroot (2004) shows young podetia with typical narrow cups and very short, blunt proliferations, which can occur not only in *C. subulata* but are also common in *C. rei*. We provide photographs of the morphological range of both species (Fig. 1) and recommend photographs in older literature such as Zopf (1908: *C. subulata*, Tab. 1, fig. 4; and *C. rei*, Tab. 2, fig. 1) and Sandstede (1931: *C. subulata*, Tab. 31, figs. 1, 2, 4 and 5; and *C. rei* Tab. 32, figs. 8 and 9). Paus *et al.* (1993) present morphological gradients of both species to underscore the difficulties of differentiation. As Paus *et al.* (1993) and Spier & Aptroot (2007) pointed out that several features, such as a corticated base of podetia (Syrek & Kukwa 2008), or presence of squamules and podetium surface (granulose to farinose), are unreliable features for differentiation, since they occur in both species. Nevertheless, we think that it is best to study herbarium material and note their morphologies before going out into the field. *Cladonia rei* is characterized by shallow scyphi, equal usually in diameter to the podetium base. Along the scyphus margin, there are numerous short, blunt proliferations often bearing prominent apothecia. *Cladonia subulata*, on the other hand, has deep scyphi, usually wider than the podetial base; the proliferations are mostly longer and acute (subulate) (*cf.* Hammer 1995). If the scyphus is not cup-bearing, older podetia of *C. subulata* may have an antler-like branching pattern with

few long and short acute branches (Fig. 1B, h–k). The morphological determination can be confirmed by a simple UV test (254 nm) with a philatelist's hand lamp; the homosekikaic acid in *C. rei* reflects whitish. Paus (1997) and Spier & Aptroot (2007) point out that, in a few cases, the concentration of homosekikaic acid may be very low so that the UV test results will be negative or poor, but, in general, the UV+ test is recommended by Paus *et al.* (1993) and by us to confirm the morphological determination of *C. rei*.

Although Sandstede (1931) described *C. rei* as having a strong white reflection under UV light, it should be stressed that the similar squamatic acid-containing *C. glauca* Flörke gives a much stronger white reflection under UV light, but is not cup bearing. Some morphs of *C. rei* resemble *C. cornuta* (L.) Hoffm., which is always UV negative (containing only fumarprotocetraric acid) and often has blackened podetium bases, or poorly developed morphs of the red-fruited *C. macilenta* Hoffm. complex, especially *C. bacillaris* (Ach.) Genth, which differs chemically in the presence of barbatic and didymic acid. Sometimes, in vegetation surveys, only poorly developed morphs of scyphus-bearing *Cladonia* species occur. In such a case, a TLC analysis provides reliable results in distinguishing *C. rei* from other species.

Conclusion

Although some individuals show overlapping morphology, our results clearly indicate that *Cladonia rei* and *C. subulata* are chemically and molecularly distinct species and should thus be accepted on the species level. Most morphotypes of both species have very specific characters and can be distinguished in the field without further auxiliary material, or with the help of a UV lamp. Nevertheless, for a definite determination, thin-layer chromatography is a prerequisite.

References

Ahti, T. & Hammer, S. (2002) *Cladonia*. In *Lichen Flora of the Greater Sonoran Desert Region*. Vol. 1 (T. H. Nash III, B. D. Ryan, C. Gries & F. Bungartz, eds): 131–158, Tempe: Lichens Unlimited, Arizona State University.

Archer, A. W. (1989) Two new lichens: *Cladonia paeminosa* and *C. humilis* var. *bourgeanica*. *Muelleria* **7:** 1–5.

Asahina, Y. (1938) Lichenologische Notizen (X). Über die Identität der Nemoxynsäure mit der Homosekikasäure. *Japanese Journal of Botany* **14:** 251–253.

Beck, A., Kasalicky, T. & Rambold, G. (2002) Mycophotobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. *New Phytologist* **153:** 317–326.

Biermann, R. (1999) Vegetationsökologische Untersuchungen der *Corynephorus canescens*-Vegetation der südlichen und östlichen Nordseeküste sowie der Kattegattinsel Læsø unter besonderer Berücksichtigung von *Campylopus introflexus*. *Mitteilungen der Arbeitsgemeinschaft Geobotanik in Schleswig-Holstein und Hamburg* **59:** 1–148.

Brodo, I. M., Sharnoff, S. D. & Sharnoff, S. (2001) *Lichens of North America*. New Haven and London: Yale University Press.

Coppins, B. J. & van den Boom, P. P. G. (1995) *Micarea confusa*: a new species from zinc- and cadmium-contaminated soils in Belgium and The Netherlands. *Lichenologist* **27:** 81–90.

Cubero, O. F., Crespo, A., Fatehi, J. & Bridge, P. D. (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Plant Systematics and Evolution* **216:** 243–249.

Culberson, C. F. (1986) Biogenetic relationships of the lichen substances in the framework of systematics. *Bryologist* **89:** 91–98.

Culberson, C. F. & Ammann, K. (1979) Standardmethode zur Dünnschichtchromathographie von Flechtensubstanzen. *Herzogia* **5:** 1–24.

Culberson, C. F., Culberson, W. L. & Johnson, A. (1985) Orcinol-type depsides and depsidones in the lichens of the *Cladonia chlorophaea* group (*Ascomycotina, Cladoniaceae*). *Bryologist* **88:** 380–387.

Culberson, C. F., Culberson, W. L. & Johnson, A. (1988) Gene flow in lichens. *American Journal of Botany* **75:** 1135–1139.

Cuny, D., Denayer, F.-O., de Foucault, B., Schumacker, R., Colein, P. & van Haluwyn, C. (2004): Patterns of metal soil contamination and changes in terrestrial cryptogamic communities. *Environmental Pollution* **129:** 289–297.

DePriest, P. T. (1994) Variation in the *Cladonia chlorophaea* Complex II: ribosomal DNA variation in the Southern Appalachian population. *Bryologist* **97:** 117–126.

Dolnik, C. (2005) Artenreichtum in Küsten-Sandtrockenrasen der Kurischen Nehrung und des Samlandes auf unterschiedlichen räumlichen Skalenebenen. In *Trockenrasen auf unterschiedlichen Betrachtungsebenen – Observation scales in dry grasslands* (H. Bültmann, T. Fartmann & T. Hasse, eds): 83–95. Havixbeck-Hohenholte: Wolf & Kreuels.

Dolnik, C. & Petrenko, D. E. (2003) Lichens of the Southern Curonian Spit in the Baltic Sea. *Botanicheskii Zhurnal* **88** (2): 41–59.

Ernst, G. (1995) *Vezdaea leprosa* – Spezialist am Straßenrand. *Herzogia* **11:** 175–188.

Fischer, P. (2003) Trockenrasen des Biosphärenreservates "Flusslandschaft Elbe". *Archiv Naturwissenschaftlicher Dissertationen* **15:** 1–287. Nümbrecht: Martina Galunder–Verlag.

Goward, T. (1999) *The Lichens of British Columbia, Illustrated Keys. Part 2 – Fruticose Species*. Victoria BC: Crown Publications.

Günzl, B. & Fischer, P. (2004) Ein Beitrag zur Verbreitung der Chemotypen ausgewählter *Cladonia*-Arten. *Aktuelle Lichenologische Mitteilungen N. F.* **13:** 20–25.

Hadjúk, J. & Lisická, E. (1999) *Cladonia rei* (lichenized *Ascomycotina*) on heavy metal-contaminated habitats near copper smelters at Krompachy (NE Slovakia). *Bulletin Slovenskej botanickej spoločnosti* **21:** 49–51.

Hall, T. A. (1999) BioEdit: a user-friendly biological sequence alignment 1 editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Hammer, S. (1995) A synopsis of the genus *Cladonia* in the Northeastern United States. *Bryologist* **98:** 1–28.

Kowalewska, A., Kukwa, M., Ostrowska, I., Jabłonska, A. Oset, M. & Szok, J. (2008) The lichens of the *Cladonia pyxidata-chlorophaea* group and allied species in Poland. *Herzogia* **21:** 61–78.

Lamb, I. M. (1951) On the morphology, phylogeny, and taxonomy of the lichen genus *Stereocaulon*. *Canadian Journal of Botany* **29:** 522–584.

Laundon, J. R. (1984) The typification of Withering's neglected lichens. *Lichenologist* **16:** 211–239.

Lendemer, J. C. (2008) Studies in lichens and lichenicolous fungi: notes on some taxa from eastern North America. *Mycotaxon* **104:** 325–329.

Leuckert, C., Ziegler, H. & Poelt, J. (1971) Zur Problematik der *Cladonia chlorophaea*-Gruppe in Mitteleuropa. *Nova Hedwigia* **22:** 505–534.

Lumbsch, H. T. (1998): The use of metabolic data in lichenology at the species and subspecific levels. *Lichenologist* **30:** 357–367.

Myllys, L., Lohtander, K., Källersjö, M. & Tehler, A. (1999) Applicability of ITS data in *Rocellaceae* (*Arthoniales, Euascomycetes*) phylogeny. *Lichenologist* **31:** 461–476.

Myllys, L., Stenroos, S., Thell, A. & Ahti, T. (2003) Phylogeny of bipolar *Cladonia arbuscula* and *Cladonia mitis* (*Lecanorales, Euascomycetes*). *Molecular Phylogenetics and Evolution* **27:** 58–69.

Østhagen, H. (1976) Nomenclatural note on *Cladonia nemoxyna*. *Bryologist* **79:** 242–246.

Ozenda, P. & Clauzade, G. (1970) *Les Lichens – Étude Biologique et Flore Illustrée*. Paris: Masson.

Paus, S. M. (1997) Die Erdflechtenvegetation Nordwestdeutschlands und einiger Randgebiete. *Bibliotheca Lichenologica* **66:** 1–222.

Paus, S., Daniels, F. & Lumbsch, T. (1993) Chemical and ecological studies in the *Cladonia subulata* complex in Northern Germany (Cladoniaceae, lichenised Ascomycotina). *Bibliotheca Lichenologica* **53:** 191–200.

Posada, D. & Crandall, K. A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Rogers, S. O. & Bendich, A. J. (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* **5:**69–76.

Sandstede, H. (1931) Die Gattung *Cladonia*. In *Rabenhost's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz* (A. Zahlbruckner, ed). **Vol. 9** (4,2). Leipzig: Akademische Verlagsgesellschaft.

Sipman, H. J. M. (1973) The *Cladonia pyxidata-fimbriata* complex in The Netherlands, with description of a new variety. *Acta Botanica Neerlandica* **22:** 490–502.

Spier, L. & Aptroot, A. (2007) *Cladonia rei* is a chemotype and synonym of *Cladonia subulata*. *Lichenologist* **39:** 57–60.

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22:** 2688–2690.

Stenroos, S., Hyvönen, J., Myllys, L., Thell, A. & Ahti, T. (2002) Phylogeny of the genus *Cladonia* s. lat. (*Cladoniaceae, Ascomycetes*) inferred from molecular, morphological, and chemical data. *Cladistics* **18:** 237–278.

Suominen, J. & Ahti, T. (1966) The occurrence of the lichens *Cladonia nemoxyna*, *C. glauca* and *C. polycarpoides* in Finland. *Annales Botanici Fennici* **3:** 418–423.

Swofford, D. L. (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sunderland, Massachusetts: Sinauer Associates.

Syrek, M. & Kukwa, M. (2008) Taxonomy of the lichen *Cladonia rei* and its status in Poland. *Biologia* **63:** 493–497.

Tretiach, M., Muggia, L. & Bauffo, L. (2009) Species delimination in the *Lepraria isidiata-L. santosii* group: a population study in the Mediterranean-Macronesian region. *Lichenologist* **41:** 1–15.

Tønsberg, T. (1985) Notes on *Cladonia asahinae, C. conista* and the *C. grayi*-group in Norway. *Gunneria* **51:** 1–26.

van Herk, K. & Aptroot, A. (2004) *Veldgids Korstmossen*. Utrecht: KNNV Uitgeverij.

Vainio, E. A. (1887) *Monographia Cladoniarum Universalis*. Helsingforsiae: J. Simelii.

Vondrák, J., Říha, P., Arup, U. & Søchting, U. (2009) The taxonomy of the *Caloplaca citrina* group (*Teloschistaceae*) in the Black Sea region; with contributions to the cryptic species concept in lichenology. *Lichenologist* **41:** 571–604.

White, T. J., Bruns, T. D., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, eds). San Diego: Academic Press.

Wirth, V. (1994) Checkliste der Flechten und flechtenbewohnenden Pilze Deutschlands – eine Arbeitshilfe. *Stuttgarter Beiträge zur Naturkunde, Serie A* (*Biologie*) **517:** 1–63.

Zharkikh, A. (1994) Estimation of evolutionary distances between nucleotide sequences. *Journal of Molecular Evolution* **39:** 315–329.

Zopf, W. (1908) Beiträge zu einer chemischen Monographie der Cladoniaceen. *Berichte der Deutschen Botanischen Gesellschaft* **36:** 51–113.

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