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# Elucidating the taxonomic rank of *Cladonia subulata* versus *C. rei* (*Cladoniaceae*)

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**Abstract** — *Cladonia subulata* and *C. rei* are two lichen species apparently closely related from a morphological viewpoint. Since both species also show a high morphological variability, it has been difficult to establish the limit between them, and their taxonomic classification has often been questioned. Nevertheless, they have different lichen substance contents. The present paper aims to clarify the taxonomy of *C. subulata* and *C. rei*. Their morphological, chemical, and anatomical variation is examined and correlated with the molecular data of three gene regions (ITS rDNA, *rpb2* and *ef1α*). The results of the analyses reveal two strongly supported monophyletic clades, correlated with the two taxa. We conclude that *C. subulata* and *C. rei* should be maintained as two different species.

**Key Words** — *Ascomycota,* secondary chemistry, sibling species, species delimitation

## Introduction

The lichens *Cladonia subulata* (L.) F.H. Wigg. and *Cladonia rei* Schaer. can be difficult to distinguish and therefore their taxonomic distinction has recently been questioned, particularly by Spier & Aptroot (2007). Traditionally, they have been regarded as two distinct species in spite of their great morphological similarity. *Cladonia subulata* is even the nomenclatural type species of the large genus *Cladonia* (Ahti 2000). The secondary metabolites, the presence of corticated areas at the base of podetia and the farinose or granular soredia are the main characters used to distinguish those species (Suominen & Ahti 1966, Wirth 1995, Brodo et al. 2001, James 2009).

Paus et al. (1993), who conducted an exhaustive revision of the morphological characters used to differentiate these species, concluded that none of them were sufficient to distinguish the two taxa. Nevertheless, they were attributed

Table 1. Specimens included in molecular study and GenBank accession numbers.

| Taxon | Code | Chemical | UV | $FeCl_3$ | Collection | ITS | *rpb2* | *ef1α* |
|---|---|---|---|---|---|---|---|---|
| *C. rei* | 2REI | HSEK | + | + | Canada, Ontario, S L58841 | FN868580 | HM243200 | HM243185 |
| *C. rei* | 3REI | HSEK | + | + | Sweden, Gästrikland, S F52894 | FN868581 | HM243201 | HM243186 |
| *C. rei* | 4REI | HSEK | + | + | Norway, Oslo, BG L86605 | FN868582 | HM243202 | HM243187 |
| *C. rei* | 5REI | HSEK | + | + | Canada, Newfoundland, BG L86394 | FN868583 | HM243203 | HM243188 |
| *C. rei* | 6REI | HSEK | + | + | USA, Minnesota, S F53070 | FN868584 | HM243204 | HM243189 |
| *C. rei* | 7REI | FUM, HSEK | + | + | Spain, Gerona, MACB 92216 | FN868585 | HM243205 | HM243190 |
| *C. rei* | 8REI | FUM, HSEK | + | + | Spain, Barcelona, MACB 100473 | FN868586 | HM243206 | HM243191 |
| *C. rei* | 11REI | HSEK | + | + | Slovakia, Trenčin, BRA 10005 | FN868591 | - | HM243192 |
| *C. rei* | 12REI | HSEK | - | + | Czech Republic, Central Bohemia, BRA 10044 | FN868592 | - | - |
| *C. rei* | 15REI | FUM, HSEK | + | + | Netherlands, Utrecht, Aptroot 68588 | FN868590 | HM243207 | HM243193 |
| *C. rei* | 16REI | HSEK | + | + | Japan, Akita, UPS L170710 | FN868593 | - | - |
| *C. rei* | 17REI | FUM, HSEK | + | + | Czech Republic, Karlovy Vary, J. Vondrák 7024 | FN868587 | HM243208 | HM243194 |
| *C. rei* | 18REI | FUM, HSEK | + | + | Czech Republic, South Bohemia, J. Vondrák 7006 | FN868588 | HM243209 | HM243195 |
| *C. rei* | 19REI | FUM, HSEK | + | + | Czech Republic, Karlovy Vary, J. Vondrák 7026 | FN868589 | - | HM243196 |
| *C. subulata* | 1SUBU | FUM | - | - | Spain, Asturias, MACB 93151 | FN868566 | HM243210 | HM243174 |
| *C. subulata* | 2SUBU | FUM | - | - | Spain, Ávila, MACB 93837 | FN868567 | HM243211 | HM243175 |
| *C. subulata* | 3SUBU | FUM | - | - | Sweden, Gästrikland, S F52879 | FN868568 | HM243212 | HM243176 |
| *C. subulata* | 4SUBU | FUM | - | - | Sweden, Halland, S F90966 | FN868569 | HM243213 | HM243177 |
| *C. subulata* | 5SUBU | FUM | - | - | Spain, Burgos, MACB 97275 | FN868570 | HM243214 | HM243178 |
| *C. subulata* | 6SUBU | FUM | - | - | Spain, Palencia, MACB 95159 | FN868577 | - | - |
| *C. subulata* | 7SUBU | FUM | - | - | Spain, La Rioja, MACB 96350 | FN868571 | HM243215 | HM243179 |
| *C. subulata* | 8SUBU | FUM | - | - | Portugal, Trás-os-Montes, MACB 93692 | FN868572 | HM243216 | HM243180 |
| *C. subulata* | 9SUBU | FUM | - | - | Chile, Navarino Island, MACB 92216 | FN868578 | - | - |
| *C. subulata* | 12SUBU | FUM | - | - | Slovakia, Moravia, BRA 10048 | - | - | HM243181 |
| *C. subulata* | 13SUBU | FUM | - | - | Netherlands, Utrecht, L Spier | FN868573 | HM243217 | - |
| *C. subulata* | 15SUBU | FUM | - | - | France, Midi-Pyrénées, L 75293 | FN868579 | - | - |
| *C. subulata* | 16SUBU | FUM | - | - | Czech Republic, Central Bohemia, J. Vondrák 6983 | FN868574 | - | HM243182 |
| *C. subulata* | 18SUBU | FUM | - | - | Denmark, Zealand, J. Vondrák 6967 | FN868575 | - | HM243183 |
| *C. subulata* | 19SUBU | FUM | - | - | Austria, Upper Austria, FB | FN868576 | HM243218 | HM243184 |
| *C. glauca* | 1GLAU | SQUA | | | Spain, Segovia, MACB 96751 | FN868594 | HM243219 | HM243197 |
| *C. glauca* | 3GLAU | BAR, THAM | | | Spain, Alava, MACB 96090 | FN868595 | HM243220 | HM243198 |
| *C. cenotea* | 1CENO | SQUA | | | Denmark, Hovedstaden, J. Vondrák 6965 | FN868596 | HM243221 | HM243199 |

FUM= fumarprotocetraric acid, HSEK= homosekikaic acid, SQUA= squamatic acid, BAR= barbatic acid, THAM = thamnolic acid.

a species rank based on their different habitat preferences. Spier & Aptroot (2007), on the contrary, concluded that as there are not enough characters to maintain the two taxa as independent they represent chemotypes of a single species. Syrek & Kukwa (2008) and James (2009), who have not accepted this viewpoint, retain *C. subulata* and *C. rei* as independent species.

The aim of this study is to resolve the complex *C. subulata*-*C. rei* and attempt to elucidate whether the complex represents two species or chemotypes of the one and the same species. To this end, three gene regions ITS rDNA, *rpb2* and *ef1*α have been analyzed in combination with morphological and anatomical characters. Recent studies using DNA sequence data have clarified relationships in several lichen species with high morphological similarities (Argüello et al. 2007, Ohmura & Kanda 2004, Amtoft et al. 2008).

## Material & methods

### Lichen material

A total of 241 specimens of *Cladonia subulata* and 60 of *C. rei* were studied. The samples selected for molecular and morphological study were chosen from several places within the geographical range of these species and are listed in Table 1. Some morphologically similar species, such as *C. glauca* Flörke and *C. cenotea* (Ach.) Schaer., were included (Suominen & Ahti 1966, Nourish 1977, Paus 1997, James 2009). *Cladonia cariosa* (Ach.) Spreng. was used as an outgroup because it was basal in the clade where *C. subulata* and *C. rei* were included by Stenroos et al. (2002) in their phylogenetic trees.

### Morphological and chemical data

The samples were identified on the basis of morphology and secondary metabolites. The presence/absence of cortex at the base of podetia, presence/absence of squamules, branching type of podetia (type I: branched antler-like; type II: unbranched or forked at the apex), and cup shape of the podetia were studied macroscopically with a stereomicroscope, and the soredial size was measured under the light microscope. Microscopic measurements of the podetial wall thickness were carried out on sections cut with a freezing microtome. Iodine reactions were tested using Lugol's solution after pre-treatment with 10% KOH. In addition, transverse and lengthwise sections at the base of the podetia were made and stained with lactophenol blue solution. The stereome surface was observed by Scanning Electron Microscopy (SEM) in longitudinal sections of the podetia. Statistical analyses were done by Statgraphics 5.1 computer program. The continuous characters normality and homogeneous variance were subject to analysis of variance (ANOVA) in association with the resulting clades of the phylogenetical analyses. Continuous characters that did not fulfill the normality and homogeneous variance were analyzed by Kruskal-Wallis test. The Kolmogorov-Simirnov test was used to check normality and Levence statistic to check the homogeneous variance. Binary characters were subjected to a test of contingency tables based on $\chi^2$ –statistic test.

Chemical composition was checked by thin layer chromatography (TLC) according to the standardized procedures of White & James (1985), with solvent systems A and

B. Moreover, 60 samples were visualized under UV light (Table 1), and $FeCl_3$ reaction (alcoholic dissolution to 10%) was checked on 188 specimens (Table 1).

### DNA extraction and PCR

Total DNA was extracted using DNeasy Plant Mini Kit (Quiagen) following the manufacturer's instructions. The DNA was dissolved in 200 µl of buffer included in the kit. Three genetic regions were selected: ITS rDNA, *rpb2* partial gene, and *ef1α* partial gene. The primers used to amplify the nuclear ITS rDNA were ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990), alternatively 1780-5'F/LSU0012 (Piercey-Normore & DePriest 2001) or ITSCld /ITSClr (Pino-Bodas unpubl. data). The *rpb2* partial gene was amplified using nested PCR. The first PCR was performed with the primer pair RPB2-5F/ RPB2-7cR (Liu et al. 1999); 1 µl of the first amplification served as DNA template for a second reaction using the primers RPB2dRaq (5' GCTGCTAAGTCTACCAT 3') /RPB2rRaq (5' ATCATGCTTGGAATCTC 3') newly designed in this study. The primers used to amplify *ef1α* partial gene were CLEF-3F/CLEF-3R (Yahr et al. 2006). The amplification program for ITS rDNA was: initial denaturation at 94 ºC for 5 min; 5 cycles of 94 ºC for 30 s, 54 ºC for 30 s and 72 ºC for 1 min; and 33 cycles of 94 ºC for 30 s, 48 ºC for 30 s and 72 ºC for 1 min; with a final extension at 72 ºC for 10 min. The amplification program for *rpb2* was: initial denaturation at 94 ºC for 5 min; 40 cycles of 95 ºC for 30 s, 52 ºC for 30 s and 72 ºC for 2 min; with a final extension at 72 ºC for 10 min. The amplification program for *ef1*α was: initial denaturation at 94 ºC for 5 min; 35 cycles of 95 ºC for 30s, 55 ºC for 30s and 72 ºC for 1 min; with a final extension at 72 ºC for 10 min. PCRs were carried out with Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, UK). Amplifications were prepared for a 25 µl final volume. PCR was performed using the MJ Reseach-PTC-200 termocycler (Massachusetts). The PCR products were purified using the QIAquick Kit (QIAGEN, Valencia, California, USA).

### DNA sequencing

The primers for sequencing reactions were those used in PCR amplification. The sequencing reactions were done at the Secugen S. L. (CIB, Madrid, Spain) or Macrogen (Korea) sequencing service (www.macrogen.com). Sequencher™ program (Gene Codes Corporation, Inc, Ann Arbor, Michigan, USA) was used to assemble the consensus sequence from the two strands of each isolate.

### Sequence alignments and data analysis

The sequences were manually aligned with SE-AL v2.0a11 Carbon (Rambaut 1996) with each region aligned separately. The transitions and transversions were considered for aligning the sequences. The ambiguous positions were removed.

After each gene region was separately analyzed, a matrix combining the three studied gene regions was constructed in which we included only taxa for which sequences of all three gene regions were available. Both individual regions and the combined matrix were analyzed using Maximum Parsimony (MP) and Bayesian Analysis. MP analyses were conducted with PAUP* version 4.0b10 (Swofford 2002) using heuristic search with 500 replicates and TBR Branch-swapping option. Bootstrap analyses were performed with 10.000 replicates, using the fast-step option. MrModeltest (Nylander 2004) was used for selecting the best evolution model (Table 2) for each region. Bayesian analyses were

carried out by MrBayes 3.1 (Huelsenbeck & Ronquist 2001). The posterior probabilities were approximated by sampling trees using Markov Chain Monte Carlo (MCMC). The posterior probabilities of each branch were calculated by counting the frequency of trees visited during MCMC analysis. Model parameters were estimated in each analysis for 2.000.000 generations sampled in 12 simultaneous chains and every 100$^{th}$ was saved into a file. Plots of likelihood were examined for each run to determine the number of generations required to reach stationarity (burn-in) by Tracer v.1.0. (http://tree.bio.ed.ac.uk/software/tracer/). Then, the MCMC convergence was evaluated by performing cumulative and sliding window analyses of posterior probability and among-run variability of cumulate and split frequencies using the online application AWTY (Nylander et al. 2008). The initial 2000 trees were discarded. Using the "sumt" command of MrBayes, the 50% majority-rule consensus tree was calculated from 36,000 trees sampled after reaching likelihood convergence to calculate the posterior probabilities of the tree nodes. The statistical congruence among the different regions was tested using ILD test (Farris et al. 1994; Huelsenbeck et al. 1996) carried out with PAUP. A conflict between ITS and *rpb2* and ITS and *ef1α* was found. The incongruities detected among the different data sets appeared in the *C. rei* clade. When incongruities appear among the different data sets, these sets can be analyzed as a whole or separately. This work followed the methodology proposed by Wiens (1998), who advises to separately analyze each data set and to assess the support of each clade; then to carry out a combined analysis of all the data sets, finally deeming as questionable those parts of the tree where incongruities are found.

## Results

### Phylogenetic analyses

In this work, 80 new sequences have been generated, of which 32 are of ITS rDNA, 22 of *rpb2,* and 26 of *ef1α*. The alignment of the ITS rDNA region contained 582 positions while the *rpb2* and *ef1α* alignments contained 891 and 612, respectively.

The MP analyses based on ITS rDNA region generated 500 equally parsimonious trees of 127 steps. The likelihood parameters of Bayesian analyses are shown in Table 2. Both analyses generated topologically similar trees. The majority Bayesian consensus tree (Fig. 1A) shows three strongly supported monophyletic clades. One clade groups all the specimens delimited as *C. subulata*; another clade includes all the samples identified as *C. rei*; and the third clade comprises the samples of *C. glauca* and *C. cenotea*. Within the *C. rei* clade, two strongly supported subclades appear. In both subclades, the specimens come from different geographical origins (Table 1).

The MP and Bayesian analyses based on *rpb2* partial gene display a similar topology (Fig. 1B). The MP analysis generated 500 equally parsimonious trees, 162 steps long. The rest of the parameters, together with the likelihood values of the Bayesian analysis are shown in Table 2. As in the ITS rDNA analyses, three strongly supported clades appear, one corresponding to *C. subulata*, another to *C. rei* and a third including *C. glauca* and *C. cenotea*. Only one strongly

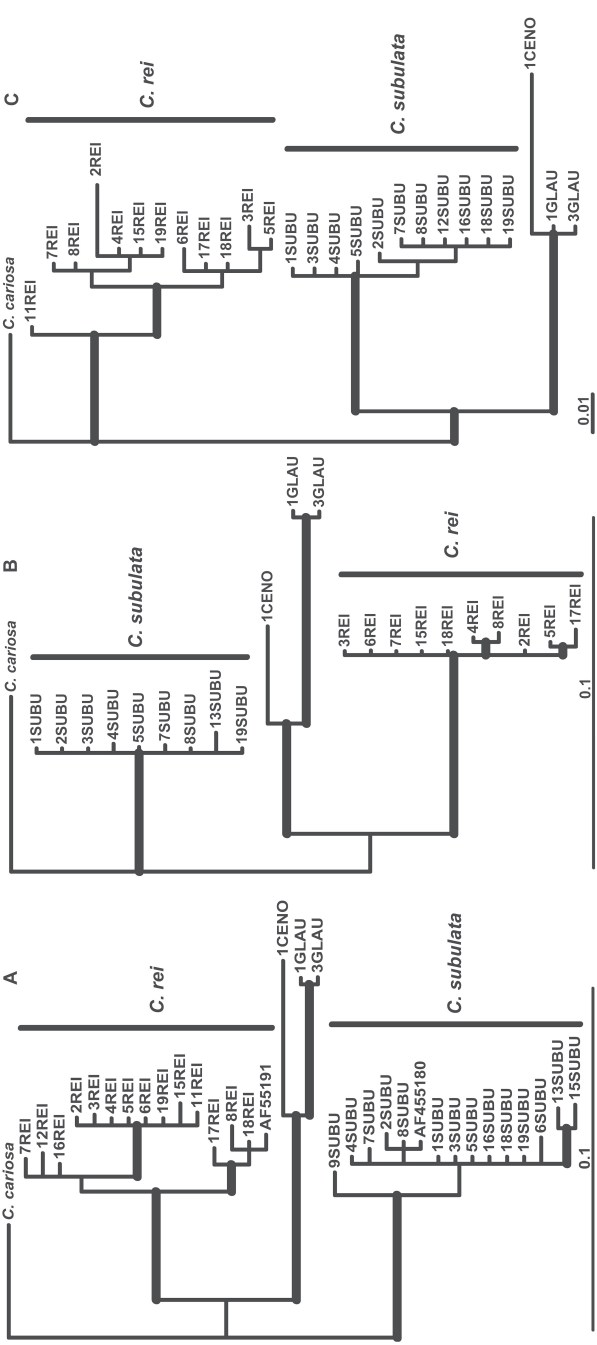


FIG. 1. Phylogeny of *Cladonia subulata* and *Cladonia rei*. The 50% consensus majority-rule tree from Bayesian/MCMC of three separate regions. Bold branches indicate a support of bootstrap ≥ al 70% and posterior probability ≥ 95%. A) ITS rnDNA B) *rpb2* C) *ef1α*.

supported subclade can be distinguished within the *C. rei* clade. However, it does not correspond to any of those appearing in the ITS rDNA analyses. The samples of this subclade have different geographical origins.

The MP analyses based on *ef1α* partial gene generated three equally parsimonious trees of 113 steps. The remaining MP parameters and Bayesian likelihood values are shown in Table 2. Analyses corresponding to this *ef1α* region also show three strongly supported monophyletic clades (Fig. 1C). In the *C. subulata* clade, one low-support subclade can be observed. The topologies of the MP and Bayesian consensus trees were not strictly identical. The MP tree shows *C. cenotea* apart from the *C. glauca* samples, while the Bayesian tree does not. The Bayesian analysis was repeated using GTR+I+G model and the result was the same.

Table 2. Information on MP analyses, evolutionary model and likelihood parameters of Bayesian analyses.

| | Parameter | ITS rDNA | rpb2 | ef1α | Combined |
|---|---|---|---|---|---|
| MP | CI | 0.8920 | 0.8377 | 0.9292 | 0.8667 |
| | RI | 0.9530 | 0.9448 | 0.9815 | 0.9518 |
| | RC | 0.8501 | 0.7915 | 0.9120 | 0.8249 |
| | informative characters | 73 | 98 | 66 | 232 |
| Bayesian analyses | Model | SYM+I | SYM+G | SYM+G | GTR+I+G |
| | -LnL | -1582.984 (0.07398) | -2128.058 (0.02592) | -1527.70 (0.00723) | -5172.63 (0.01232) |
| | π (A) | - | - | - | 0.2601 (0.00008) |
| | π (C) | - | - | - | 0.2519 (0.00008) |
| | π (G) | - | - | - | 0.2415 (0.00008) |
| | π (T) | - | - | - | 0.2465 (0.00007) |
| | r (A-C) | 0.4745 (0.00235) | 0.0468 (0.00031) | 0.0605 (0.00051) | 0.0591 (0.00016) |
| | r (A-G) | 0.2498 (0.00248) | 0.2375 (0.00165) | 0.2485 (0.00246) | 0.2472 (0.00067) |
| | r (A-T) | 0.1474 (0.00130) | 0.0873 (0.00055) | 0.0808 (0.00073) | 0.1150 (0.00028) |
| | r (C-G) | 0.0666 (0.00050) | 0.0326 (0.00022) | 0.0312 (0.00036) | 0.3326 (0.00009) |
| | r (C-T) | 0.4095 (0.00311) | 0.5272 (0.00246) | 0.5026 (0.00349) | 0.4944 (0.00093) |
| | r (G-T) | 0.0566 (0.00046) | 0.0684 (0.00044) | 0.0760 (0.00080) | 0.0608 (0.00017) |
| | α | - | 0.2748 (0.01024) | 0.3535 (0.04190) | 73.254 (0.00007) |
| | Pinvar | 0.6021 (0.00198) | - | - | 0.6233 (0.00795) |

Bayesian parameters: mean value (variance)
Models selected by AIC criterion using MrModeltest

The MP analyses based on the combined dataset generated 500 equally parsimonious trees of 405 steps long. The remaining parameters of the MP analyses, together with the likelihood values of the Bayesian analyses are shown in Table 2. Both analyses generated topologically similar trees (Fig. 2). Three strongly supported monophyletic clades appear, one corresponding to *C. subulata*, another to *C. rei,* and the third to *C. glauca* and *C. cenotea*.

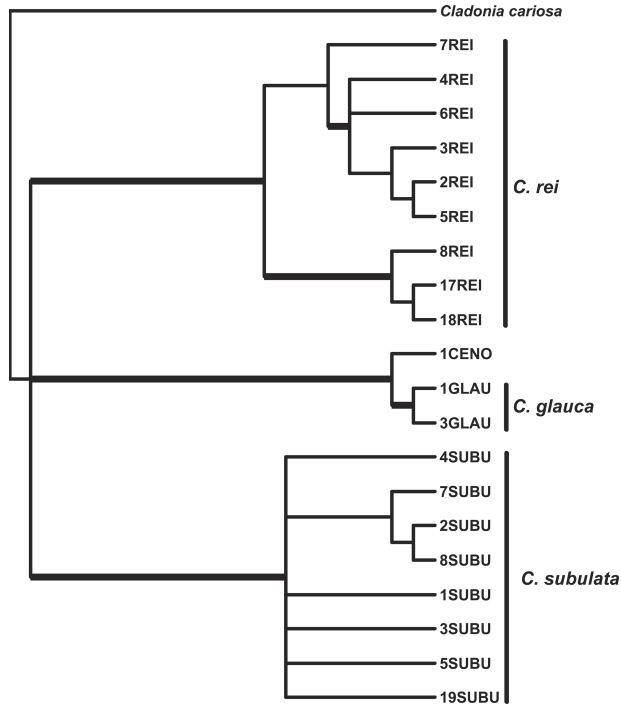


Fig. 2. The 50% consensus majority-rule tree based of combined data set (ITS rnDNA, *rpb2* partial gene and *ef1α* partial gene) from Bayesian/MCMC. The highly supported branches (bootstrap ≥ al 70% and posterior probability ≥ 95%) are indicated in bold.

The ILD-based congruence analysis revealed one conflict between the ITS rDNA + *rpb2* partial gene matrices and another conflict between the ITS rDNA + *ef1α* partial gene matrices. The cause of these incongruities lies in 4 samples of *Cladonia rei* (4REI, 8REI, 17REI and 18REI), which appear in different subclades in the analyses. The three data matrices were combined, however, in accordance with Wiens (1998).

### Morphological and chemical analysis

The SEM showed notable differences between the stereome surfaces of *Cladonia subulata* and *C. rei*. In *C. rei*, the internal face of the stereome lacks pores, while *C. subulata* samples display a reticulated stereome with pores (Fig. 3). Furthermore, under the light microscope the transverse and lengthwise podetial sections (Fig. 4) reveal stereome hyphae that are thinner in *C. subulata* (2-3 μm diam.) than in *C. rei* (3.75-5 μm diam.). In both cases, the stereome hyphae are arranged lengthwise along the podetia.

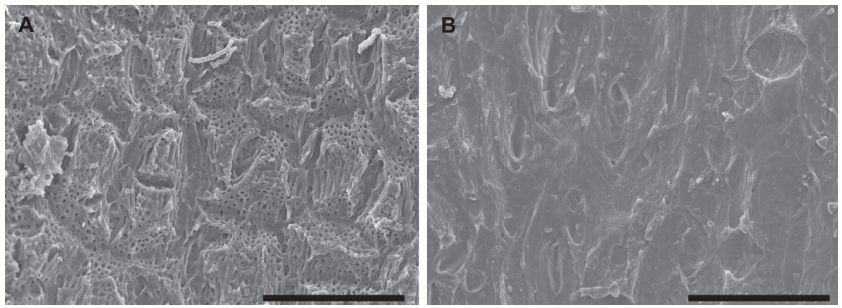


Fig. 3. SEM micrographs of the stereome surface.
A) *Cladonia subulata*. B) *C. rei*. Bar = 100 μm.

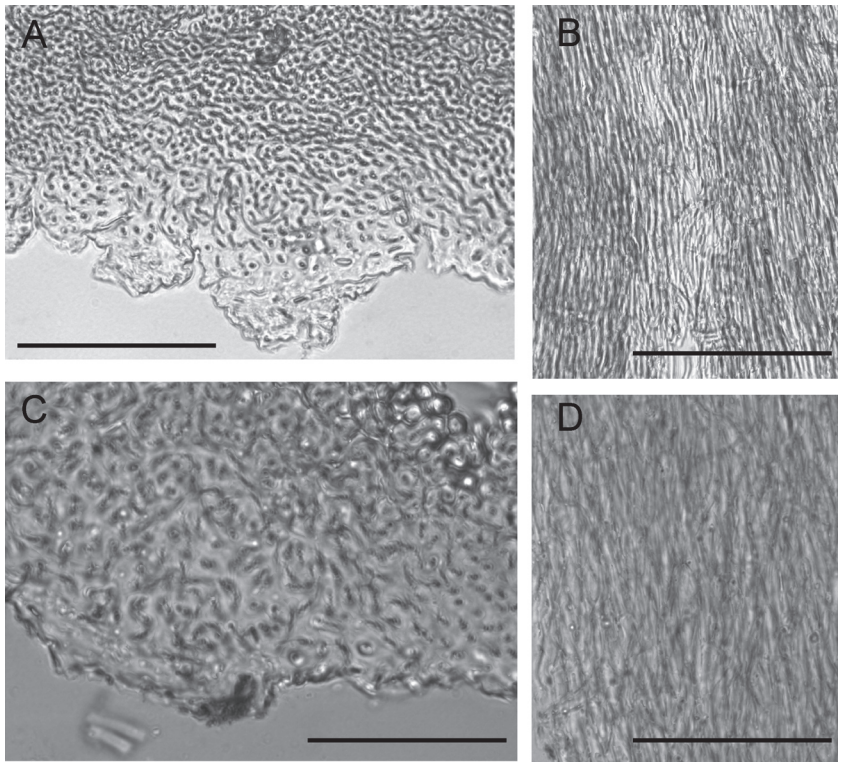


Fig. 4. Microtome sections of stereome under light microscope.
A) Transversal section of *C. subulata*. B) Lengthwise section of *C. subulata*.
C) Transversal section of *C. rei*. D) Lengthwise section of *C. rei*.
Bar = 50 μm.

Table 3. Results of the contingency table for *C. subulata* and *C. rei*.

| Character | *p* |
|---|---|
| Presence/absence of basal squamules | 0.035 * |
| Presence/absence of scyphi | 0.13 |
| Presence/absence of basal cortex | 0.00008 ** |
| Branching type I/branching type II | 0.196 |

*p*, significance level (* $p < 0.05$, ** $p < 0.01$).

The contingency table (Table 3) shows the correlation between the qualitative morphological characters previously used to distinguish these taxa and the clades implied by the phylogenetic analyses. Significant differences are observed, such as the presence/absence of squamules and the presence of basal cortex on the podetia, while there are no significant differences between both taxa in the podetial branching type. Significant statistical differences were found in the podetial anatomical characters (Table 4), with the podetial wall being thicker in *C. rei* than in *C. subulata*, as also the medulla and stereome layers are, with the stereome/medulla ratio higher in *C. subulata*. Also, the soredial granules are significantly larger in *C. rei* than in *C. subulata*.

Table 4. Statistical analyses for continuous characters.

| Character | *C. subulata* | *C. rei* | *p* |
|---|---|---|---|
| Soredium size | 17.5-80 (125) | (14.5) 20-65 (100) | $4.42e^{-8}$** |
| Podetium thickness | 115-310 (350) | (112.5) 130-400 (707.5) | 0.0054** |
| Medule thickness | 47.5-225 (250) | (22.5) 30-227.5 (260) | 0.0029** |
| Stereome thickness | 35-145 (187.5) | (14.5) 20-212.5 (400) | 0.0000** |
| stereome/medule ratio | 1.36-5.0 (5.70) | (1.22) 1.27-2.63 (3.08) | $6.57e^{-11}$** |

The minimum value corresponds to percentile 1 and the maximum to percentile 95. The absolute maximum and minimum values are in brackets.

*p*, significance level (* $p < 0.05$, ** $p < 0.01$).

TLC analyses revealed that 36 samples of *C. rei* contained homosekikaic acid together with fumarprotocetraric acid, while 24 samples contained only homosekikaic acid. In both cases, homosekikaic acid was accompanied by small amounts of sekikaic acid. Furthermore, in the samples of *C. rei* the accessory substance 4'-O-methylnorhomosekikaic acid was found. Frequently fumarprotocetraric acid is accompanied by protocetraric acid; besides, in 8 of the samples containing fumarprotocetraric acid, also confumarprotocetraric acid was detected. In all *C. subulata* samples fumarprotocetraric acid was present with protocetraric acid. In addition, in 34 of these samples the satellite substance confumarprotocetraric acid occurred.

The UV test, traditionally used to detect the presence of homosekikaic acid, was applied to 60 samples; 87.5% of the samples where TLC detected homosekikaic acid gave a positive fluorescence. On the other hand, 96%

of the samples where TLC detected only fumarprotocetraric acid gave no fluorescence. The $FeCl_3$ test applied to 188 samples gave a positive reaction in 90% of the samples containing homosekikaic acid and was negative in 98% of the specimens containing only fumarprotocetraric acid.

## Discussion

### Evaluation of characters

Soredium size. Soredium size is one of the main characters used for species differentiation in many *Cladonia* species, as in the complex *C. chlorophaea* (Flörke ex Sommerf.) Spreng.–*C. fimbriata* (L.) Fr. (Hennings 1983). However, in *C. ochrochlora* Flörke the soredium size is variable (Hammer 1993). Statistically significant differences in soredium size were found in *C. subulata* and *C. rei,* with the soredial granules being bigger in *C. rei* (Table 4). As several factors (e.g., age, development stage, environmental conditions) probably affect soredium size (Paus et al. 1993), using this character to distinguish these species must be used with caution.

Cortex at the base of podetia. Earlier authors have discussed the utility of the podetial cortex to differentiate *C. rei* from *C. subulata.* Paus et al. (1993) and Spier & Aptroot (2007) consider it unreliable, while Syrek & Kukwa (2008) accept it as reliably diagnostic. Although a great many of the *C. rei* specimens studied were corticated, 40.62% of the *C. subulata* podetia also have corticate bases. The presence of this cortex was sometimes difficult to observe because it was covered by soredia and could be detected only by a transversal section of the podetium.

Squamules at the base of podetia. There are statistically significant differences between the *C. subulata* and *C. rei* clades related to the presence of squamules at the base of podetia (Table 4). However, as only 34.69% of *C. rei* podetia have squamules, possession of squamules cannot be used to differentiate these two species. In fact, Evans (1930) differentiated two forms of *C. nemoxyna* (Ach.) Arnold (a synonym of *C. rei*): *C. nemoxyna* f. *fibula* (Ach.) Vainio—lacking podetial squamules—and *C. nemoxyna* f. *phyllocephala* Arn.—with squamulose podetia. The presence/absence of squamules on the podetia is actually a variable character in many *Cladonia* species, e.g., *C. furcata* (Huds.) Schrad. and *C. rangiformis* Hoffm. (Burgaz & Ahti 2009).

Morphology of podetia. The presence of antler-like, irregularly branched podetia is one character attributed to *C. subulata* (Brodo 2001, Osyczka 2006, James 2009). In the material used for this paper, however, no significant differences were found between the podetia of *C. subulata* and *C. rei*. It is worth noting that much *C. subulata* material studied here was young and not well developed. Other authors (Paus et al 1993, Spier & Aptroot 2007) consider the

podetia morphology to be of little taxonomic value due to the wide variability (simple, cup-like, irregularly branched) that podetia show.

Anatomical characters. Statistically significant differences between *Cladonia subulata* and *C. rei* were found in the thickness of the podetial wall (Table 4). Nevertheless, as in soredium size, the thickness of the podetial wall and the thickness of each layer are widely variable in these two taxa, making it difficult to identify the two species based only on these characters. On the other hand, such anatomical features can be used to differentiate other similar taxa such as *C. mediterranea* P.A. Duvign. & Abbayes from *C. mitis* Sandst., *C. ciliata* Stirt. var. *ciliata* from var. *tenuis* (Flörke) Ahti (Burgaz & Martínez 2008), or the species within the *C. gracilis* (L.) Willd. group (Ahti 1980). In some cases, some taxonomic value is attributed to the stereome surface (Ahti 1980), which is different in *C. rei* and in *C. subulata*. Under the stereomicroscope, the reticulated stereome surface of *C. subulata* and the smooth stereome surface of *C. rei* can sometimes be observed. In most cases, however, a SEM is required to observe stereome surfaces, greatly limiting its utility for an everyday identification. Besides, the differing stereome hyphal thicknesses in those species may be responsible for the differences seen on the stereome surface.

Color of the podetia. The color of the podetia of *C. subulata* reportedly varies from whitish-greyish to bright green, up to brownish green, or at least with zones of brownish coloring, while in *C. rei* the podetia vary from brownish green to dirty brown (Suominen & Ahti 1966, Thomson 1968, James 2009); nevertheless color could turn out to be an ambiguous character due to the variation within either species (Paus et al. 1993, Spier & Aptroot 2007). In the present study we found that the podetia of *C. subulata* are often pale green or whitish (though some of them present brownish zones), while in *C. rei* they are green brownish.

Chemistry. Secondary metabolites were confirmed as the only reliable characters to distinguish *C. rei* and *C. subulata*. A negative *p*-phenylenediamine (Pd) reaction is still useful in diagnosing specimens as *C. rei*. But a positive reaction is not reliable (Pišút 1961, Paus et al. 1993, Spier & Aptroot 2007), because many *C. rei* samples contain fumarprotocetraric acid in addition to homosekikaic acid, although Suominen & Ahti (1966) note that the *C. rei* Pd reaction is slow, being yellow at first, while in *C. subulata* it is normally instantly red, due to different fumarprotocetraric acid concentrations. Specimens containing homosekikaic acid do appear white under UV, but our results have shown small errors occur in detecting the presence of homosekikaic acid using the UV test. Nonetheless, we find the UV test useful in differentiating the species in most cases. Homosekikaic acid can also be detected by the ferric chloride test, which produces a violet spot when it is positive (Huneck &

Yoshimura 1996). Although this reaction is not used in the keys, we consider it useful for differentiating *C. rei* from *C. subulata*, and it should be included in the identification keys.

**Delimitation of the taxa**

Despite the high phenotypic similarity of *C. subulata* and *C. rei*, the phylogenetic analyses of the ITS rDNA, *rpb2* and *ef1α* regions show two strongly supported monophyletic clades. These clades agree with the chemical variability of the *C. subulata-C. rei* complex. All the specimens included in the *C. rei* clade contain homosekikaic acid with fumarprotocetraric acid as a frequent accessory substance, while in the *C. subulata* clade no specimens with homosekikaic acid were found. If the taxa belonged to a single species with two (to three) chemotypes, it should be expected that the chemotypes would appear intermingled, which is not true. Besides, each clade is associated with a different set of morphological characters.

In addition, the two species have obviously different ecological requirements. *Cladonia rei* is a terricolous species growing in open areas with low humus content and subneutrophilous substrate. It may sometimes grow on impoverished soils with high heavy metal content (Hajdúk & Lisická 1999). *Cladonia subulata* grows on humus-rich acidophilous substrates and even in shady areas (Sipman 1977, Paus et al. 1993, Hammer 1995, Syrek & Kukwa 2008). However, both species do occasionally grow on wood or bare rocks (Spier & Aptroot 2007). Both taxa are broadly distributed in Europe, Asia, and North America and have also been found in Australasia. However, *C. rei* has not been reported for South America or the Antarctic, while *C. subulata* grows in Argentina and Chile. In general *C. subulata* has a wider distribution, although absent in warm areas, while *C. rei* is more common in temperate or sub-arid areas, being absent in Arctic and Antarctic zones (Ahti in litt.).

Suominen & Ahti (1966) found that the *C. rei* chemotypes usually did not appear intermingled, suggesting that the chemotypes are genetically, not environmentally, determined. But the incongruities detected among the different data sets within the *C. rei* clade shows that phylogenetic relationships within this clade are not fully resolved (Wiens 1998).

Our results support *C. subulata* and *C. rei* as two independent phylogenetic species. This conclusion is founded on: 1) the genealogic concordance of the three gene regions; 2) the existence of a correlation between clades and morphological characters; and 3) the fact that both species have different habitats. Our data corroborate the results obtained in the phylogenetic study of *Cladonia* by Stenroos et al. (2002) and Dolnik et al. (2010) where *C. subulata* and *C. rei* appear in separate clades. Spier & Aptroot (2007) pointed out that the Canadian specimen of *C. rei* (AF455191) analyzed by Stenroos et al. (2002)

possibly belongs to another taxon than the European ones. Our ITS analysis, which included this sequence, shows it grouping with the other *C. rei* samples.

*Cladonia glauca* is morphologically similar to *C. rei,* sharing grey brownish podetia and squamules at the podetia base (Brodo et al. 2001, Syrek & Kukwa 2008, James 2009, Burgaz & Ahti 2009). However, they contain different lichen substances representing different biosequential groups. *Cladonia glauca* has squamatic acid or (rarely) thamnolic and barbatic acids (Burgaz et al. 1999, Burgaz & Ahti 2009). In addition, *C. glauca* presents a very peculiar groove along the podetium that distinguishes it from *C. rei*, and it is fully unable to produce cups (scyphi), which occur in mature specimens of *C. rei* and *C. subulata.* Our phylogenetic analyses clearly separate *C. glauca* from *C. rei. Cladonia glauca* seems to be related to *C. cenotea* (in some areas they can be difficult to distinguish), and Stenroos et al. (2002) cite *C. cenotea* as phylogenetically related to *C. crispata* (Ach.) Flot. and *C. subsubulata* Nyl. Nevertheless, further studies including additional taxa are necessary to establish the phylogenetic relationships of *C. glauca*.

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