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Phylogenetic analysis of cetrarioid lichens with globose ascospores

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Abstract. The group of cetrarioid lichens with globose ascospores includes 37 species from eight genera. Phylogenetic analysis, using program PAUP 3.1.1, was carried out on two different data sets – morphological (incl. anatomical and chemical) and molecular characters (ITS1, 5.8S, and ITS2 rDNA sequences) – to verify the correspondence of current taxonomy to the probable evolution of the taxa involved. The paraphyletic nature of the genus *Allocetraria* in regard to two species of *Dactylina* is assumed based on morphological characters. Still, the separation of these genera is strongly supported by the analysis of molecular data. The genus *Tuckermannopsis* (11 species) is paraphyletic in regard to *Esslingeriana idahoensis* and "*Nephromopsis" weii.* Monophyletic origin can be declared only for the *Tuckermannopsis ciliaris* group. The analysis based on molecular characters exhibits the monophyletic origin of the group including three *Tuckneraria* species. Monophyly of the genus *Ahtiana* was not supported by the analysis of morphological data.

Key words: cetrarioid lichens, Parmeliaceae, *Ahtiana*, *Allocetraria*, *Dactylina*, *Esslingeriana*, *"Nephromopsis" weii*, *Tuckneraria*, *Tuckermannopsis*, *Vulpicida*, ITS rDNA sequences, cladistic analysis.

INTRODUCTION

The group of cetrarioid lichens, comprising 23 genera and about 140 species within the family Parmeliaceae (Randlane et al., 1997; Randlane & Saag, 2000), is a very diverse and definitely polyphyletic assemblage. Phylogenetic affinities between several cetrarioid genera are rather obscure. The first review of the phylogeny of a major part of cetrarioid lichens was presented by Kärnefelt et al. (1992) where cladistic analyses were carried out for 50 species as terminal taxa. This analysis was mainly based on anatomical characters of reproductive structures

that had been revised originally. As a result, the analysed taxa were grouped into three separate aggregates, described by important anatomical characters: taxa with an “apical ring structure”, taxa with “uniseriate asci”, and taxa with “broadly clavate asci”. Another cladistic analysis of the same group of lichens was carried out independently (Saag & Randlane, 1995). In order to evaluate the systematic arrangement of the taxa 83 species were chosen for analysis from among approximately 120 cetrarioid lichen species known at the time. Morphological and chemical characters (character states) were identified or verified by the authors, while descriptions of anatomical characters were based mainly on literature data. Most of the species were grouped in a way that showed considerable similarity to the main three assemblages pointed out by Kärnefelt et al. (1992), while the taxonomy of several species of the genera *Nephromopsis*, *Cetreliopsis*, and *Cetrelia* remained unresolved. Similar results are particularly noteworthy because of the fact that the two analyses were based on different data matrices with emphasis on anatomical characters in one (Kärnefelt et al., 1992) and on morphological and chemical characters in the other (Saag & Randlane, 1995) study.

A few years later the three aggregates within the group of cetrarioid lichens referred to above were characterized in precise anatomical detail (Thell, 1996). The aggregates were called “evolutionary lines” although additional cladistic analyses were not performed. The first line, proposed as monophyletic, included species from seven genera: *Arctocetraria*, *Cetraria* s. str., *Cetreliopsis*, *Coelopogon*, *Flavocetraria*, *Masonhalea*, and *Nephromopsis*. They all were characterized by ellipsoid ascospores in narrowly clavate asci with a small axial body. Furthermore, an amyloid ring structure in tholus was pointed out as a significant character present in *Cetraria*, *Flavocetraria*, and occasionally in *Nephromopsis*. The second evolutionary line comprised six genera: *Ahtiana*, *Allocetraria*, *Dactylina*, *Esslingeriana*, *Tuckneraria*, and *Tuckermannopsis*. The species of these genera were anatomically characterized by subglobose to globose ascospores borne in narrowly clavate asci (earlier called “uniseriate asci”). The assumption of the monophyletic origin of this line was based on two earlier cladistic analyses of various species of cetrarioid lichens (Kärnefelt et al., 1992; Saag & Randlane, 1995). In both analyses, the clade comprising species with globose ascospores in narrowly clavate asci was clearly separated. The third line was represented by species of several genera and informal groups: *Asahinea*, the *“Cetraria” fendleri* group, *Cetrariella*, *Cetrelia*, *Cornicularia*, *Kaernefeltia*, the *Melanelia commixta* group, *Nimisia*, *Parmelaria*, *Platismatia*, and *Vulpicida*. Ellipsoid ascospores in broadly clavate asci with a broad axial body were pointed out as characteristic of these species. This heterogeneous assemblage is presumably paraphyletic as a number of parmelioid lichens most likely also belong here.

In further studies (Crespo & Cubero, 1998; Mattsson & Wedin, 1998; Thell, 1998, 1999; Thell & Miao, 1998; Thell et al., 1998, 2000; Crespo et al., 1999; Wedin et al., 1999; Kärnefelt & Thell, 2000, 2001), accumulation of molecular data has been the primary source which would allow improvement of investigations of phylogeny within the Parmeliaceae. Different regions of nuclear

rDNA (ITS, 5.8S, group I intron) have been studied in several taxa (including cetrarioid genera, e.g., *Ahtiana*, *Cetraria*, *Cetrariella*, *Dactylina*, *Platismatia*, *Tuckermannopsis*, *Vulpicida*). It has been presumed that there are uncertainties in the reliability of some of the sexual reproductive structures used to identify monophyletic groups in cetrarioid lichens (Mattsson & Wedin, 1998). Still, so far no serious phylogenetic hypotheses have been proposed based on sequences and comprising a large number of the members of this large family.

The group of cetrarioid species with globose ascospores is discussed here in more detail. This group covers the second evolutionary line referred to above in full and, in addition, includes some taxa (e.g., species of *Vulpicida*) from the third evolutionary line. Five of the seven genera, *Ahtiana*, *Allocetraria*, *Dactylina*, *Tuckneraria*, and *Vulpicida*, have recently been thoroughly revised (Mattsson, 1993; Randlane et al., 1994; Thell et al., 1995a, b; Kärnefelt & Thell, 1996). The sole species of the monotypic genus *Esslingeriana* was described in detail by Esslinger (1971). The genus *Tuckermannopsis* in the strict sense has also recently been circumscribed, both on morphological and molecular data (Kärnefelt & Thell, 2001). Another newly described species – "*Nephromopsis" weii* X. Q. Gao & L. H. Chen – has also been included in the ingroup because its ascospores are clearly globose (Chen & Gao, 2001).

Nevertheless, the data characterizing all these taxa are scattered in many papers and evolutionary affinities between the species included have not been evaluated.

MATERIAL AND METHODS

Material

Herbarium material from B, DUKE, FH, GZU, H, KW, LD, LE, M, MB, PC, S, TAIM, TNS, TU, UPS, US was used for this study. Morphology of the herbarium specimens was examined using stereomicroscopes Technival 2 and Olympus SZ40. The anatomical methods and equipment used are described in detail in Thell et al. (1995a). Secondary chemistry was investigated according to standardized methods of TLC (Culberson, 1972; Culberson et al., 1981).

Molecular methods

Sequences from the internal transcribed spacer 1 (ITS1), the 5.8S rDNA gene, and the internal transcribed spacer 2 (ITS2) of the nuclear rDNA gene were used for the molecular analysis.

Extraction

Fresh material, at least not older than three years, was used for extracting total DNA. Minute thallus fragments, 1–3 mm in diameter, were used for the extractions, which were performed according to the manufacturer's protocol with

one exception: the samples were carefully ground in 50 µL of the lysis buffer. As the next step, the rest of the lysis buffer, 350 µL, together with 10 µL of RNase A (100 mg/mL), was added to each sample. Finally, the extracted DNA was eluted in 100 µL of the elution buffer delivered with the NucleoSpin Plant Extraction Kit from MACHEREY-NAGEL GmbH & Co, which was successfully used for the amplification without further dilutions.

Amplification

The ITS regions and the 5.8S gene of the nuclear rDNA were amplified using a Perkin-Elmer Gene Amp PCR System 9700 thermal cycler. Ready-To-Go PCR beads in 0.2 mL tubes (Pharmacia Biotech Inc.) were used in amplification. The primers ITS1F and ITS4 were used for amplifying the ITS regions. A solution containing 11.8 µL of distilled water, 0.35 µL each of the primers at 16 pmol concentration, and 12.5 µL of DNA was added to the PCR beads (reaction size ca. 25 µL for 0.2 mL tubes). Following an initial 2-min template denaturing at 95 °C, a 30-cycle schedule was performed. The following cycling profile was used: denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were cleaned with QIAquick PCR purification kit (QIAGEN) and diluted in 50 µL of the elution buffer provided in the kit.

Sequencing and alignment

The sequencing reactions were prepared according to the protocol provided in the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). In a 20 µL reaction, 8 µL of BigDye and buffer (2.3 µL and 5.7 µL, respectively), 2 µL of the primers at 5 pmol concentration, 4–6 µL of the purified PCR product, and 4–6 µL of distilled water were mixed. A 25-cycle schedule including denaturation at 96 °C for 30 s, annealing at 50 °C for 15 s, and extension at 60 °C for 4 min was performed. The primers ITS1LM and ITS4 were used for the sequencing reactions above (Table 1). ITS1LM was here selected instead of ITS1F to avoid possible introns at the 3′ end of the 18S gene. The sequencing was performed with an automatic sequencer ABI Prism 377 (PE Biosystems).

The sequences were aligned with SeqApp/CAP2 (Huang, 1992; Gilbert, 1993), optimized by hand and transferred to PAUP 3.1.1 (Swofford, 1993).

Table 1. Primers used in the molecular analysis (IST1LM was used for the sequencing reaction only)

Primer	Sequence	Reference
ITS1F*	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns, 1993
ITS1LM	GAACCTGCGGAAGGATCATT	Myllys et al., 1999
ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990

* Fungal specific.

Phylogenetic analyses

The computer program for phylogenetic analysis using maximum parsimony as the optimization criterion, PAUP 3.1.1 (Swofford, 1993), was applied. Cladistic analyses were run on a Power Macintosh G3. The following heuristic search settings were applied: character-state optimization – DELTRAN; MULPARS; addition sequence – random; 1 tree held at each step during stepwise addition; tree-bisection-recollection (TBR) branch-swapping performed; all multi-state characters treated as unordered; when taxa are coded as having multiple states, multiple state interpreted as uncertainty; characters weighted equally. The same settings (except the last) were used in the successive approximations character weighting method. Tree support was investigated using Bremer support and bootstrap analysis. A hundred (for morphological data) or a thousand (for molecular data) bootstrap replicates were performed, with random addition sequence.

Separate analyses were carried out on two different data sets: morphological (incl. anatomical and chemical) data only and molecular data (ITS1, 5.8S, ITS2 sequences of rDNA gene) only.

Data

Taxa analysed

A total of 41 cetrarioid species were included in the morphological data matrix. The following 31 species from the above-listed genera were applied as terminal taxa of the ingroup: *Ahtiana aurescens* (Tuck.) Randlane & A. Thell, *A. pallidula* (Tuck. ex Riddle) Goward & A. Thell, *A. sphaerosporella* (Müll. Arg.) Goward, *Allocetraria ambigua* (Bab.) Kurok. & M. J. Lai, *A. endochrysea* (Lynge) Kärnefelt & A. Thell, *A. flavonigrescens* A. Thell & Randlane, *A. globulans* (Nyl.) A. Thell & Randlane, *A. madreporiformis* (Ach.) Kärnefelt & A. Thell, *A. oakesiana* (Tuck.) Randlane & A. Thell, *A. sinensis* X. Q. Gao, *A. stracheyi* (Bab.) Kurok. & M. J. Lai, *Dactylina arctica* (Richardson) Tuck., *D. ramulosa* (Hook.) Tuck., *Esslingeriana idahoensis* (Essl.) Hale & M. J. Lai, *"Nephromopsis" weii* X. Q. Gao & L. H. Chen, *Tuckermannopsis americana* (Spreng.) Hale, *T. chlorophylla* (Willd.) Hale, *T. ciliaris* (Ach.) Gyeln., *T. gilva* (Asahina) M. J. Lai, *T. inermis* (Nyl.) Kärnefelt, *T. microphyllica* (W. L. Culb. & C. F. Culb.) M. J. Lai, *T. orbata* (Nyl.) M. J. Lai, *T. platyphylla* (Tuck.) Hale, *T. platyphylloides* (Asahina) M. J. Lai, *T. subalpina* (Imshaug) Kärnefelt, *T. ulophylloides* (Asahina) M. J. Lai, *Tuckneraria ahtii* Randlane & Saag, *T. laureri* (Kremp.) Randlane & A. Thell, *T. laxa* (Zahlbr.) Randlane & A. Thell, *T. pseudocomplicata* (Asahina) Randlane & Saag, and *T. togashii* (Asahina) Randlane & A. Thell.

The cladograms were rooted using the genus *Vulpicida* (with *V. canadensis* (Räsänen) J.-E. Mattsson & M. J. Lai, *V. juniperina* (L.) J.-E. Mattsson & M. J. Lai, *V. pinastri* (Scop.) J.-E. Mattsson & M. J. Lai, *V. tilesii* (Ach.)

J.-E. Mattsson & M. J. Lai, *V. tubulosa* (Schaer.) J.-E. Mattsson & M. J. Lai, and *V. viridis* (Schwein.) J.-E. Mattsson & M. J. Lai in the outgroup). At first, the six species of *Vulpicida* were also included in the ingroup because the ascospores are mainly subglobose in this genus. Then several outgroup candidates, e.g. *"Cetraria" fendleri* (Nyl.) Tuck., *Cetraria islandica* (L.) Ach., *Flavocetraria cucullata* (Bellardi) Kärnefelt & A. Thell, and *Melanelia hepatizon* (Ach.) A. Thell, from different cetrarioid genera were tested. The analyses where *"Cetraria" fendleri*, *Cetraria islandica*, and *Flavocetraria cucullata* were separately used as outgroups resulted in much less resolution than the analysis with *Melanelia hepatizon* as an outgroup. On the strict consensus tree of that analysis, the species of *Vulpicida* appeared as a sister group to the rest of the species included. *Vulpicida* was therefore selected as the outgroup for further analyses. The genus *Vulpicida* is anatomically characterized by almost subglobose ascospores and broadly clavate asci of *Melanelia*-form with a large axial body (Thell et al., 1995b) while the taxa of the ingroup have globose to subglobose ascospores borne in narrowly clavate asci with a large axial body (*Tuckermannopsis*-form according to Thell et al., 1995b).

Morphological characters

All in all, 37 morphological characters were used for the analyses (Table 2). They belong to three main groups, describing thallus morphology and general organization (characters 1–16), reproductive structures and their anatomy (17–27), and secondary chemistry (28–37). Lichen substances were not treated independently from each other but grouped into biochemically related sets, as suggested in a previous study (Saag & Randlane, 1995).

Character states were coded as 0, 1, 2, 3, and 4. The characters and character states were as follows.

1. Form of thallus: adnate (0), ascending (1)
2. Symmetry of thallus: dorsiventral (0), radial-symmetrical (1)
3. Interior of thallus: of densely arranged hyphae (0), of loosely arranged hyphae (1), becoming hollow (2)
4. Form of lobes: length of lobes ~ width of lobes (0), lobes longer than wide (1)
5. Width of lobes: up to 3 mm (0), up to 6 mm (1), up to 12 mm (2)
6. Colour of upper surface: yellow (0), brown (1), grey (2)
7. Colour of lower surface: whitish (0), yellow (1), brown (2), black (3)
8. Pseudocyphellae on upper side: absent (0), present (1)
9. Pseudocyphellae on lower side: absent (0), present (1)
10. Form of pseudocyphellae on lower side: spots (0), lines (1)
11. Cilia: absent (0), present (1)
12. Rhizines: absent (0), present (1)
13. Soredia: absent (0), present (1)

14. Isidia: absent (0), present (1)

15. Structure of the cortex (orientation of hyphae): both cortices para-plectenchymatous, i.e. hyphae randomly oriented (0); upper cortex palisade plectenchymatous, i.e. hyphae anticlinally oriented (1); both cortices palisade (2); both cortices prosoplectenchymatous, i.e. hyphae parallel to cortex (3)

16. Cell wall thickness (compared to cell lumina): leptodermatous, i.e. hyphae with thin cell walls and lumen is clearly larger in diameter than the adjacent cell walls (0); pachydermatous, i.e. hyphae with thick cell walls and lumen is smaller in diameter than the adjacent cell walls (1)

17. Position of apothecia: marginal only (0), marginal to submarginal (1), laminal (2), terminal (3)

18. Ascus shape: broadly clavate (0), narrowly clavate (1)

19. Ascus form according to Thell et al. (1995b): *Melanelia*-form (0), *Tuckermannopsis*-form (1), *Cetraria*-type (2)

20. Tholus: small (0), large (1)

21. Axial body: medium, 3–5 µm (0); broad, >5 µm (1); narrow, >1 and <3 µm (2), very narrow, ≤1 µm (3)

22. Shape of ascospores: globose (0), subglobose (1), broadly ellipsoid (2), ellipsoid (3)

23. Length or diameter of ascospores: short, ≤6 µm (0); long, >6 µm (1)

24. Position of pycnidia: marginal only (0), marginal and laminal (1), laminal only (2)

25. Emergence of pycnidia: emergent (0), immersed (1)

26. Shape of pycnoconidia: bacillariform (0), oblong citriform (1), dumb-bell shaped incl. disc-bar shaped (2), filiform (3), sublageniform (4)

27. Length of pycnoconidia: short, <7 µm (0); medium, 7–10 µm (1); long, >10 µm (2)

28. Usnic acid: absent (0), present (1)

29. Atranorin: absent (0), present (1)

30. Fatty acids: absent (0), present (1)

31. Substance of fatty acids: lichesterinic–protolichesterinic type acids (0), caperatic acid (1), rangiformic acid (2), unidentified (3)

32. Secalonic acids: absent (0), present (1)

33. Orcinol depsides and depsidones: absent (0), present (1)

34. Substance of orcinol depsides and depsidones: alectoronic and collatolic acids (0), physodic acid (1), olivetoric acid (2), microphyllinic acid (3), gyrophoric acid (4)

35. ß-Orcinol depsidones: absent (0), present (1)

36. Substance of ß-orcinol depsidones: fumarprotocetraric acid (0), physodalic acid (1)

37. Pulvinic acid derivates: absent (0), present (1)

Table 2. Morphological data matrix

Taxon	Character number and states																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Ahtiana aurescens	0	0	0	0	0	0	0	0	0	–	1	1	0	0&1	0	1	1	1
A. pallidula	1	0	0	0	2	0	1	0	0	–	0	1	0	0	0	1	1	1
A. sphaerosporella	0	0	0	0	0	0	0	0	0	–	0	1	0	0	0	0	2	1
Allocetraria ambigua	1	0	0	1	1	0	1	0	1	0	0	1	0	0	2	1	0	1
A. endochrysea	1	1	1	1	0	0	1	0	0	–	0	1	0	0	2	0	?	?
A. flavonigrescens	1	0	0	1	0	0	2/3	0	0	–	0	1	0	0	2	0	1	1
A. globulans	0	0	0	0	1	0/1	2	0	0	–	0	1	0	0	2	0	1	1
A. madreporiformis	1	0&1	1	1	0	0	1	0	0	–	0	0	0	0	2	0	3	1
A. oakesiana	0	0	0	0	1	0	2	0	0	–	0	1	1	0	0/2	0	1	1
A. sinensis	1	0	0	1	0	0	2	0	1	1	0	0	0	0	2	0	?	?
A. stracheyi	1	0&1	0	1	1	0	1/2	0	1	0	0	1	0	0	2	1	0	1
Dactylina arctica	1	1	2	1	2	0/1	1/2	0	0	–	0	0	0	0	2	0	3	1
D. ramulosa	1	1	2	1	0	1	1	0	0	–	0	0	0	0	2&3	1	3	1
Esslingeriana idahoensis	0	0	0	0&1	1	2	3	0	0	–	0	1	0	0	0	0	1	1
"Nephromopsis" weii	1	0	0	0	2	1	2	1	1	?	0	1	0	0	0	?	0	1
Tuckermannopsis americana	0	0	0	0	1	1	2	0	0	–	1	1	0	0	0	0	1	1
T. chlorophylla	0	0	0	0	1	1	2	0	0	–	0	1	1	0	0	0	0	1
T. ciliaris	0	0	0	0	1	1	2	0	0	–	1	1	0	0	0	0	1	1
T. gilva	0	0	0	0	1	1	2	0	0	–	1	1	1	0	0	0	?	?
T. inermis	0&1	0	0	0	0	1	0	0	1	1	0	1	0	0	0	1	1	1
T. microphyllica	0	0	0	0	1	1	2	0	0	–	0	1	0	0	0	1	1	1
T. orbata	0	0	0	0	1	1	2	0	0	–	1	1	0	0	0	0	0	1
T. platyphylla	0&1	0	0	0	2	1	2	1	0	–	0	1	0	1	0	1	1	1
T. platyphylloides	0	0	0	0	2	1	2	0	0	–	0	1	0	0	0	0	0	?
T. subalpina	1	0	0	1	1	1	2	0	1	1	0	0	0	0	0	0	0	1
T. ulophylloides	0	0	0	0	1	1	1/2	0	0	–	0	1	1	1	1	0	?	?
Tuckneraria ahtii	1	0	0	1	2	0	2/3	0	1	0	1	1	0	0	0	1	0	1
T. laureri	0&1	0	0	0	1	0	0/2	0	1	0	1	1	1	0	0	1	0	1
T. laxa	0	0	0	1	0	0	1	0	1	0	1	1	0	0	0	1	0	1
T. pseudocomplicata	1	0	0	0	2	0	0/2	0	1	0	1	1	0	0	0	1	0	1
T. togashii	0	0	0	0	1	0	1/2	0	1	0	1	1	0	1	0	1	0	1
Vulpicida canadensis	1	0	0	0	2	0	1	0	0	–	0	0&1	0	0	0	1	2	0
V. juniperina	0&1	0	0	0	1	0	1	0	0	–	0	1	0	0	0	1	1	0
V. pinastri	0	0	0	0	1	0	1	0	0	–	0	1	1	0	0	1	1	0
V. tilesii	1	0	0	0	0	0	1	0	0	–	0	0	0	0	0	1	1	0
V. tubulosa	1	0&1	0	1	0	0	1	0	0	–	0	0	0	0	0	1	1	0
V. viridis	0	0	0	0	1	0	1	0	0	–	0	1	0	0	0	1	2	0
"Cetraria" fendleri	0	0	0	0	0	1	0	0	0	–	0	1	0	0	0	1	1&2	0
Cetraria islandica	1	0	0	1	2	1	2	0	1	0	0	0	0	0	0&3	1	3	1
Flavocetraria cucullata	1	0	0	1	1	0	1	0	1	0	0	0	0	0	0/2	1	3	1
Melanelia hepatizon	0	0	0	0	0	1	3	1	0	–	0	1	0	0	0	?	1&2	0

Table 2 continued

Taxon	Character number and states																		
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
Ahtiana aurescens	1	0	0/2	0	0	1	0	2	0	1	0	1	0&1	0	0	–	0	–	0
A. pallidula	1	0	0	0	0	1	1	2	0	1	0	1	0&1	0	0	–	0	–	0
A. sphaerosporella	1	0	0	0	0	1	0&1	2	0/1	1	0	1	0&1	0	0	–	0	–	0
Allocetraria ambigua	1	0	0	0	1	1	0&1	3	2	1	0	1	0	1	0	–	0	–	0
A. endochrysea	?	?	?	?	?	2	1	3	2	1	0	1	0	1	0	–	0	–	0
A. flavonigrescens	1	0	?	0	0	1	0&1	3	2	1	0	1	3	1	0	–	1	0	0
A. globulans	1	0	1	0	1	1	0	3	2	1	0	1	0	1	0	–	0	–	0
A. madreporiformis	1	0	0	1	1	2	1	3	2	1	0	1	0	0	0	–	0	–	0
A. oakesiana	1	0	1	0	0	0	0&1	3	2	1	0	1	0&1	1	0	–	0	–	0
A. sinensis	?	?	?	?	?	0	0	3	2	1	0	1	0	0	0	–	0	–	0
A. stracheyi	1	0	1	0	0	0	0&1	3	2	1	0	1	0	1	0	–	0	–	0
Dactylina arctica	1	0	0	0	0	2	1	1	0	1	0	0	–	0	1	1&4	1	1	0
D. ramulosa	1	0	0	0	0	2	1	1	0	1	0	0	–	0	1	1	1	1	0
Esslingeriana idahoensis	1	0	0	0	1	1	1	2	0	0	1	1	2	1	0	–	0	–	0
"Nephromopsis" weii	1	0	?	0	1	0	0	2	0	0	0	1	0&1	0	0	–	0	–	0
Tuckermannopsis americana	1	0	0	0	0	1	0	2	0	0	1	0	–	0	1	0	0	–	0
T. chlorophylla	1	0	0	0	0	0	1	2	0	0	1	1	0	0	0	–	0	–	0
T. ciliaris	1	0	0	0	0	1	0	2	0	0	1	0	–	0	1	1&2	0	–	0
T. gilva	?	?	?	?	?	?	?	?	?	0	1	0	–	0	1	0	0	–	0
T. inermis	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	–	0	–	0
T. microphyllica	1	0	?	0	1	0	0	3	2	0	1	0	–	0	1	3	0	–	0
T. orbata	1	0	0	0	0	1	0	2	0	0	0	1	0	0	0	–	0	–	0
T. platyphylla	1	0	0	0	0	1	1	2	0	0	1	1	3	1	0	–	0	–	0
T. platyphylloides	?	?	?	0	0	1	0	2	0	0	0	1	0	0	0	–	0	–	0
T. subalpina	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	–	0	–	0
T. ulophylloides	?	?	?	?	?	0	0	?	?	0	0	1	0	0	0	–	0	–	0
Tuckneraria ahtii	1	0	0/2	0	0	1	0	2	0	1	0	1	0&1	0	0	–	0	–	0
T. laureri	1	0	0	0	0	0	0	2	0	1	0	1	0	0	0	–	0	–	0
T. laxa	1	0	0	0	0	0	0	?	?	1	0	1	0	0	0	–	0	–	0
T. pseudocomplicata	1	0	0	0	0	0	0	2	0	1	0	1	0	0	1	0&1	0	–	0
T. togashii	1	0	0	0	0	1	0	2	0	1	0	1	0&1	0	0	–	0	–	0
Vulpicida canadensis	0	0	0	0	0	2	1	1	0	1	0	0	–	0	0	–	0	–	1
V. juniperina	0	0	0	0	0	1	0	4	1	1	0	–	–	0	0	–	0	–	1
V. pinastri	0	0	0	0	0	1	0	4	1	1	0	–	–	0	0	–	0	–	1
V. tilesii	0	0	0	0	0	1	0	4	1	1	0	–	–	0	0	–	0	–	1
V. tubulosa	0	0	0	0	0	1	0	4	1	1	0	0	–	0	0	–	0	–	1
V. viridis	0	0	0	0	0	?	?	1	0	1	0	0	–	0	0	–	?	–	1
"Cetraria" fendleri	0	0	0	1	1	1	1	2	0	0	0	1	0	0	0	–	0	–	0
Cetraria islandica	2	1	2	2	1	0	0	1	0	0	0	1	0	0	0	–	1	0	0
Flavocetraria cucullata	2	1	3	2	1	0	0	2	0	1	0	1	0	0	0	–	0	–	0
Melanelia hepatizon	0	0	1	2	1	1	0	2	0	0	0	0	–	–	0	–	1	2	0

Molecular data

In the molecular data set, sequences from the ITS1, 5.8S, and ITS2 regions of the nuclear rDNA gene for 23 specimens of 20 cetrarioid lichen species are presented. The matrix contains both sequences newly obtained and those published earlier (Table 3). Seventeen species with globose ascospores from six genera (*Ahtiana*, *Allocetraria*, *Dactylina*, *Esslingeriana*, *Tuckneraria*, and *Tuckermannopsis*) were applied as terminal taxa of the ingroup while three *Vulpicida* species were used as the outgroup.

The combined length of the sequences obtained from ITS1, 5.8S, and ITS2 was ca 500 nucleotides. In the parsimony analysis of molecular data, 116 informative characters were used.

RESULTS

Analyses based on morphological data

Parsimony analysis yielded 7436 equally parsimonious trees (all characters equally weighted, length of trees = 136 steps, consistency index CI = 0.382, homoplasy index HI = 0.618, retention index RI = 0.684). After reweighting the characters by maximum value of rescaled consistency indices 122 equally parsimonious trees (length of trees = 35 267 steps, CI = 0.677, HI = 0.323, RI = 0.882) were obtained. Successive weighting is particularly useful when applied to data sets with much homoplasy (Tehler & Egea, 1997). The successive approximations character weighting method generated remarkably low weights to some conspicuous morphological characters often used in key-books, such as presence of isidia and soredia (Table 4). This is justified as, in our opinion, these asexual structures are obviously independently derived. In other words, we suppose that sorediate and isidiate species did not share sorediate or isidiate ancestors but came from sexual species instead. The most highly weighted characters are either connected with the anatomy of the thallus (characters 2, 3, 15), anatomy of ascocarps (18–22), or with secondary compounds of both cortex (28, 29) and medulla (34, 36, 37). Still, some anatomical characters of the ascocarps (20, 22) and presence of medullary substances (36) appear to be uninformative in this analysis.

The majority rule consensus trees were obtained in both analyses – with characters equally weighted (Fig. 1) and after the reweighting of characters (Fig. 2). Two major monophyletic groups were found on both trees. Group A consists of 11 species of *Tuckermannopsis* together with *Esslingeriana idahoensis* and *"Nephromopsis" weii* while group B contains eight species of *Allocetraria* together with two *Dactylina* species. Furthermore, the clade including five *Tuckneraria* species (group C) was formed on the majority-rule consensus tree (supported by 95% of cladograms) with characters equally weighted. Still, this

Table 3. Sequenced taxa and their GenBank accession numbers (sequences produced for the present paper are in bold)

Species	DNA extr. No.	Specimen data	GenBank accession No.
Ahtiana sphaerosporella	DNA#AT 83	Canada, British Columbia, *Miao & Taylor* (TDI No 211)	AF072224
Allocetraria ambigua	DNA#AT 874	China, prov. Sichuan, *Obermayer 08141* (GZU)	**AF404128**
A. flavonigrescens	DNA#AT 873	China, prov. Sichuan, *Obermayer 08140* (GZU)	**AF404127**
A. globulans	DNA#AT 870	China, prov. Sichuan, *Obermayer 08137* (GZU)	**AF404126**
A. madreporiformis	DNA#AT 973	Austria, Tyrol, *Obermayer 7746* (M)	**AF416460**
A. madreporiformis	DNA#AT 974	Russia, Krasnoyarsk Terr., *Matveeva* (M)	**AF416461**
A. oakesiana	DNA#AT 136	Slovenia, Pokorje, *Kärnefelt SL-960306* (LD)	AF116179
A. sinensis	DNA#AT 868	China, prov. Sichuan, *Obermayer 08148* (GZU)	**AF404125**
A. stracheyi	DNA#AT 867	China, prov. Sichuan, *Obermayer 08147* (GZU)	**AF404124**
A. stracheyi	DNA#AT 875	China, prov. Sichuan, *Obermayer 08139* (GZU)	**AF404129**
A. stracheyi	DNA#AT 901	China, prov. Sichuan, *Obermayer 08143* (GZU)	**AF404130**
Dactylina arctica	DNA#AT 160	Canada, Alberta, *Miao* (TDI No 300)	AF115760
Esslingeriana idahoensis	DNA#AT 145	Canada, British Columbia, *Goward 961354* (UBC)	AF072227
Tuckermannopsis americana	DNA#AT 82	Canada, British Columbia, *Miao & Taylor* (TDI No 210)	AF072232
T. chlorophylla	DNA#AT 525	Chile, prov. de Magallanes, *Feuerer 29475* (TUR)	AF255620
T. platyphylla	DNA#AT 43	Canada, British Columbia, *Thell & Veer BC-9643* (LD)	AF072235
T. subalpina	DNA#AT109	Canada, British Columbia, *Thell BC-9606* (LD)	AF072237
Tuckneraria ahtii	DNA#AT 607	Bhutan, *Søchting* 8489 (LD)	**AF404122**
T. laureri	DNA#AT 610	Bhutan, Wangdi Distr., *Søchting 8124* (LD)	**AF404123**
T. pseudocomplicata	DNA#AT 907	China, prov. Sichuan, *Obermayer 08276a* (GZU)	**AF404131**
Vulpicida canadensis	DNA#AT 36	Canada, British Columbia, *Thell & Veer BC-96250* (LD)	AF072238
V. pinastri	DNA#AT 02	Canada, British Columbia, *Thell SK-9604* (LD)	AF139031
V. tubulosa	DNA#AT 933	Austria, Innsbruck, *Feuerer & Thell* (HBG)	**AF404132**

Table 4. Weights of all characters after the second reweighting according to maximum value of RC indices. Characters with weights over 500 are in bold

Character	Inform.	Weight	States
1. Form of thallus	Y	111	01
2. Symmetry of thallus	**Y**	**1000**	01
3. Interior of thallus	**Y**	**1000**	012
4. Form of lobes	Y	91	01
5. Width of lobes	Y	42	012
6. Colour of upper surface	**Y**	**611**	012
7. Colour of lower surface	**Y**	**508**	0123
8. Pseudocyphellae on upper side	Y	0	01
9. Pseudocyphellae on lower side	Y	175	01
10. Form of pseudocyphellae on lower side	Y	250	01
11. Cilia	Y	111	01
12. Rhizines	Y	125	01
13. Soredia	Y	0	01
14. Isidia	Y	0	01
15. Structure of cortex	**Y**	**1000**	0123
16. Cell wall thickness	Y	70	01
17. Position of apothecia	Y	314	0123
18. Ascus shape	**Y**	**1000**	01
19. Ascus form	**Y**	**1000**	01
20. Tholus	**N**	**1000**	0
21. Axial body	**Y**	**1000**	012
22. Shape of ascospores	**N**	**1000**	01
23. Length (diameter) of ascospores	Y	0	01
24. Position of pycnidia	Y	93	012
25. Emergence of pycnidia	Y	74	01
26. Shape of pycnoconidia	**Y**	**578**	0123
27. Length of pycnoconidia	Y	308	012
28. Usnic acid	**Y**	**1000**	01
29. Atranorin	**Y**	**1000**	01
30. Fatty acids	Y	273	01
31. Substance of fatty acids	Y	0	0123
32. Secalonic acids	Y	238	01
33. Orcinol depsides & depsidones	Y	222	01
34. Substance of orcinol depsides & depsidones	**Y**	**1000**	01234
35. ß-Orcinol depsidones	Y	250	01
36. Substance of ß-orcinol depsidones	**N**	**1000**	01
37. Pulvinic acid derivates	**Y**	**1000**	01

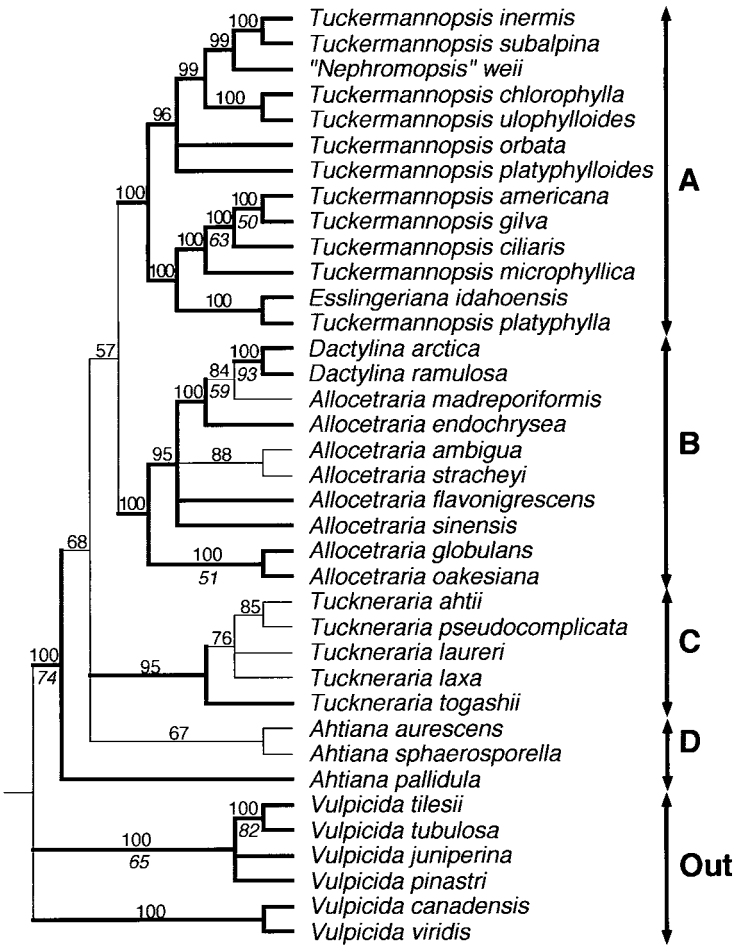

Fig. 1. Majority rule consensus tree of 7436 equally parsimonious cladograms (based on characters with equal weights), obtained as a result of the maximum parsimony analysis of the morphological data set. The length of the shortest trees 136, consistency index 0.382, retention index 0.684. The values above branches correspond to the percentages of equally most parsimonious trees with that specific bipartition; consensus 95% and more is shown by the thicker branches. Bootstrap values greater than 50% are shown below branches. Letters A–D indicate informal entities of the ingroup (see the text); Out indicates outgroup taxa.

clade did not reach the level of 50% on the consensus tree produced after the reweighting of characters. Three species of the genus *Ahtiana* (group D) did not form a separate clade at all.

Both Bremer support test and bootstrapping were used to find support for the obtained clades. In the Bremer support test, all trees are kept successively one step longer than the shortest tree until all the groups are lost in consensus.

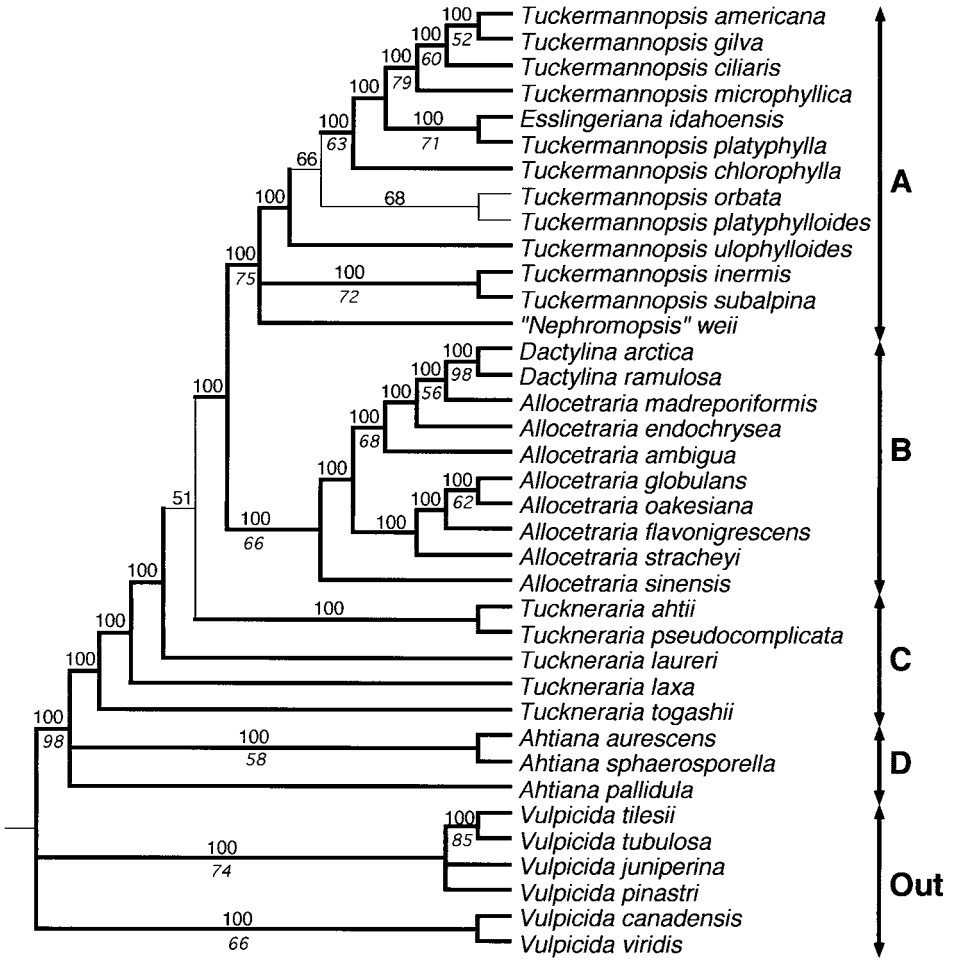

Fig. 2. Majority rule consensus tree of 122 equally parsimonious cladograms (based on reweighted characters), obtained as a result of the maximum parsimony analysis of the morphological data set. The length of the shortest trees 35267, consistency index 0.677, retention index 0.882. The values above branches correspond to the percentages of equally most parsimonious trees with that specific bipartition; consensus 95% and more is shown by the thicker branches. Bootstrap values greater than 50% are indicated below branches. Letters A–D mark informal entities of the ingroup (see the text); Out marks outgroup taxa.

Bremer's length difference has also been referred to as the decay test or decay index (Tehler & Egea, 1997). In our analysis, inclusion of trees one step longer than the shortest tree causes the majority of the tree to collapse into a polytomy and only the *Allocetraria–Dactylina* clade remains separate. This clade begins to decay at the Bremer support value of three.

Bootstrap support was not strong for any clades. In the analysis based on characters with equal weights the highest bootstrap values were obtained for the ingroup as a whole (74%) and two species of *Dactylina* (93%) (Fig. 1). Bootstrap values of 70% and more were obtained in the analysis with reweighted characters only for group A and for four small subclades (*Tuckermannopsis microphyllica–T. ciliaris*, *Esslingeriana idahoensis–Tuckermannopsis platyphylla*, *Tuckermannopsis inermis–T. subalpina*, *Dactylina arctica–D. ramulosa*) (Fig. 2).

In conclusion, the analyses based on morphological, anatomical, and chemical characters alone were not supported by the decay test or bootstrapping.

Analyses based on molecular data

The ingroup of the phylogenetic analysis based on the molecular data set included 20 specimens representing 17 species (of the 31 taxa which were morphologically studied). This matrix is composed of complete sequences of the ITS1, 5.8S, and ITS2 genes. Parsimony analysis yielded 20 equally parsimonious trees (length of trees = 411 steps, consistency index CI = 0.640, homoplasy index HI = 0.360, retention index RI = 0.635). Within the ingroup, three monophyletic groups were revealed on the strict consensus tree as well as on the bootstrap 50% majority-rule consensus tree (Fig. 3). Group A includes a separately branched *Tuckermannopsis subalpina* and a polytomy consisting of two subclades (the genera *Tuckneraria* and *Tuckermannopsis* s. str.) and of three species from different genera (*Dactylina arctica*, *Esslingeriana idahoensis*, *Tuckermannopsis platyphylla*). Group B includes six *Allocetraria species* (*A. ambigua*, *A. flavonigrescens*, *A. globulans*, two specimens of *A. madreporiformis*, *A. sinensis*, and three specimens of *A. stracheyi*) while the seventh taxon from the same genus – *A. oakesiana* – remains unresolved as branch C.

DISCUSSION

The aim of this phylogenetic analysis was to search for monophyletic groups within the aggregate of cetrarioid lichens characterized by globose ascospores and to verify the correspondence of the current taxonomy to the probable evolution of the taxa involved.

The genus *Allocetraria*, which at first included only three species from high altitudes in south-east Asia, was introduced by Kurokawa & Lai (1991). It was separated from *Cetraria* because of the dichotomously branched lobes, the special appearance of pseudocyphellae, the palisade plectenchymatous cortex, and the unique chemistry. The authors did not pay attention to ascomatal and pycnidial characters. Later studies of these structures confirmed the necessity of a separate genus (Thell et al., 1995c). Today, ten species are combined in the genus; eight

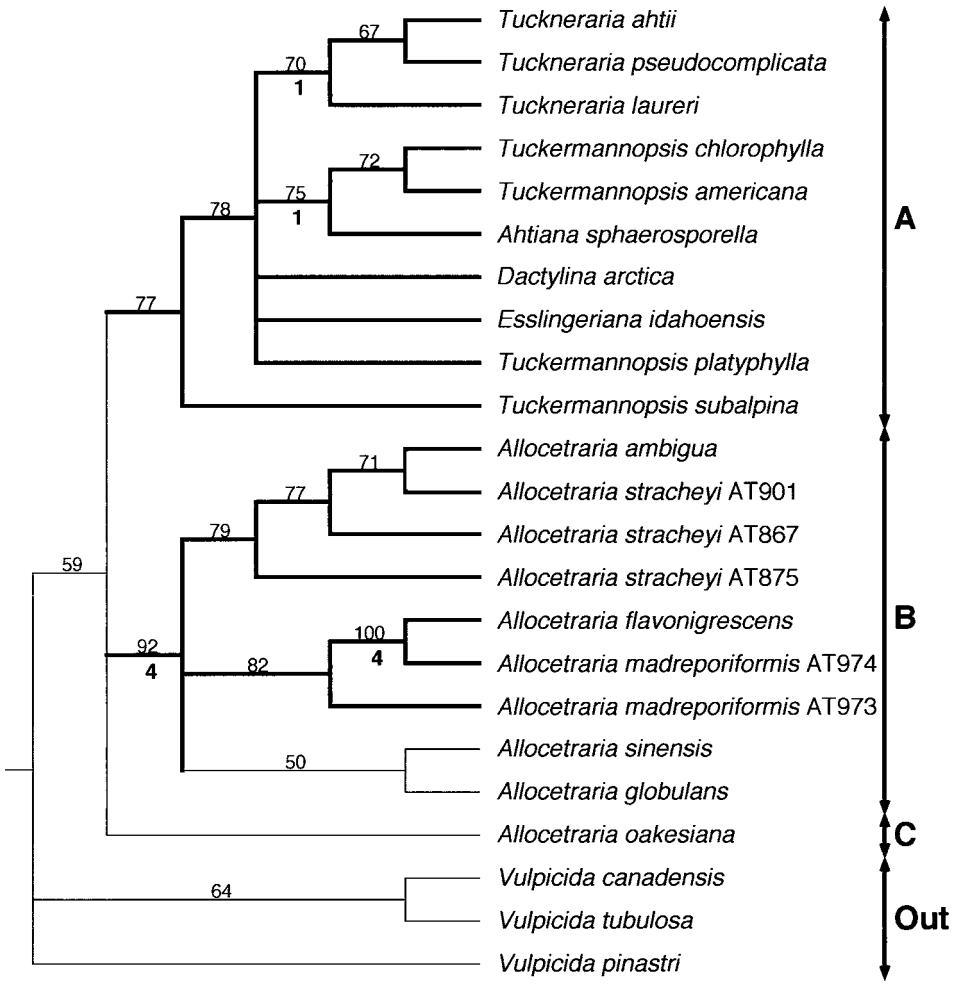

Fig. 3. Bootstrap majority-rule consensus tree, obtained as a result of analysing the molecular data set consisting of 17 species. Bootstrap values 50% and greater are indicated above branches. Clades supported by bootstrap values 70% and greater are shown by the thicker lines. Bremer support values are indicated below branches. Letters A–C mark informal entities of the ingroup (see the text); Out marks outgroup taxa.

of them were included in our analyses. We have not seen any herbarium material of *A. denticulata*, and only one specimen (an isotype material) of *A. isidiigera* was available. Unfortunately, the descriptions of these two species (Hue, 1899; Kurokawa & Lai, 1991) are too poor to present them properly in the data matrix. The value of both taxa is rather uncertain – the species are known from the type localities only; and, according to the literature, no fruiting bodies have been observed.

Six *Allocetraria* species out of the seven included in our molecular data set form a monophyletic group with high bootstrap and Bremer supports (group B in Fig. 3). The seventh taxon, *A. oakesiana*, remains separate in this analysis although its morphological, chemical, and anatomical characters show closeness to the other *Allocetraria* species.

The paraphyletic nature of the genus *Allocetraria* with respect to two species of *Dactylina* is assumed on the grounds of morphological characters (Figs. 1, 2). The closeness of these two genera was noticed quite recently, when two former *Dactylina* species – *D. madreporiformis* and *D. endochrysea* – were transferred to *Allocetraria* (Kärnefelt & Thell, 1996). The presence of filiform pycnoconidia and asci with an extremely broad axial body is essential for distinguishing *Allocetraria*. *Dactylina* is characterized by the radial-symmetrical thallus becoming hollow, the terminal position of apothecia, and also the secondary chemistry. The palisade plectenchymatous arrangement of cortical hyphae, which is rather unusual in the group of cetrarioid lichens, is characteristic of both *Allocetraria* and *Dactylina*. Still, the grouping of the species involved into two separate genera is strongly supported by our analysis of molecular characters (Fig. 3) and, therefore, the present nomenclature remains unchanged.

The genus *Tuckermannopsis* was described by Gyelnik (1933) in a very brief manner: "Affinis generi Nephromopsi sed thallus subtus pseudocyphellis deficientibus". Only two species (*T. ciliaris* (Ach.) Gyeln. and *T. endoxantha* (Hue) Gyeln.) were recognized in the genus at that time. *T. ciliaris* is the type species of the genus while *T. endoxantha* is not accepted nowadays as its type material was found to comprise two different species, both included in the genus *Nephromopsis* today (Lai, 1981; Kärnefelt & Thell, 2001). The genus *Tuckermannopsis* remained unaccepted for a long time, until the 1980s when Lai (1981) resurrected it. Although the description of the genus was still rather poor, several new combinations were soon proposed (Hale in Egan, 1987; Kurokawa, 1991; Weber in Egan, 1991) to accommodate various cetrarioid species in this genus. Several of those have later been combined into other genera (*Ahtiana*, *Allocetraria*, *Kaernefeltia*, *Melanelia*, *Vulpicida*). After numerous rearrangements eleven species are still recognized in *Tuckermannopsis* (Randlane et al., 1997; Randlane & Saag, 1998). At present it is generally accepted that globose ascospores in narrowly clavate asci, large axial body, dumb-bell shaped pycnoconidia, and moderately small foliose brown to greenish thallus (indicating the absence of usnic acid in the cortex) are the important characters in delimiting the genus. The defining character proposed by Gyelnik – presence of pseudocyphellae on the lower surface of thallus – is not considered to be of importance any longer. A recent review of the genus (Kärnefelt & Thell, 2001) indicates that only seven species (*T. americana*, *T. chlorophylla*, *T. ciliaris*, *T. gilva*, *T. microphyllica*, *T. orbata*, and *T. ulophylloides*) should be included in the genus s. str. while the positions of *T. inermis*, *T. platyphylla*, *T. platyphylloides*, and *T. subalpina* are doubtful. Still, molecular data are available for four

Tuckermannopsis species (*T. americana*, *T. chlorophylla*, *T. platyphylla*, and *T. subalpina*) only.

According to our phylogenetic analysis based on morphological characters (Figs. 1, 2), the genus *Tuckermannopsis*, as it is generally accepted (including eleven species), should be considered paraphyletic. *Esslingeriana idahoensis*, the sole species of the genus *Esslingeriana*, is predominantly connected with *Tuckermannopsis platyphylla* and this pair of species always branches out next to the so-called *Tuckermannopsis ciliaris* group. Monophyletic origin can be declared only for this group. The subclade consisting of four apparently closely related species (*T. americana*, *T. ciliaris*, *T. gilva*, *T. microphyllica*) is also supported by bootstrapping (bootstrap value 79%).

"Nephromopsis" weii, recently described from China (Chen & Gao 2001), is another taxon which is included in the clade mainly consisting of *Tuckermannopsis* species (group A in Figs. 1 and 2). There is no doubt that this lichen does not belong to the genus *Nephromopsis*, which is characterized by rather large foliose thalli of yellowish colour (containing usnic acid), the presence of pseudocyphellae only on the lower surface, marginal apothecia situated on the lower side, and narrowly clavate asci with ellipsoid ascospores. *"Nephromopsis" weii* has olive brown thallus, pseudocyphellae present on both surfaces, and ascospores clearly globose (6–7 µm in diameter) (Chen & Gao, 2001). A new combination for this taxon can be presented after additional studies have been carried out on the type specimen.

The phylogenetic analysis based on molecular characters includes only four *Tuckermannopsis* species out of eleven. It reveals the closeness of *T. americana* and *T. chlorophylla* while *T. subalpina* branches out separately and the position of *T. platyphylla* remains unresolved in a polytomy together with *Dactylina arctica* and *Esslingeriana idahoensis* (Fig. 3). In our opinion, any further taxonomical rearrangements in this genus would not be justified until additional molecular research has been carried out.

The recently proposed genus *Tuckneraria* (Randlane et al., 1994) includes five species, which predominantly grow in eastern and south-eastern Asia. They were transferred mainly from *Nephromopsis* to the new genus because of important anatomical characters (globose ascospores and asci of the same form as in *Tuckermannopsis*). Later the idea of close affinities between *Tuckneraria* and *Ahtiana* was proposed (Thell et al., 1995a). The monophyletic origin of the genus *Tuckneraria* is supported in one series of current analyses based on the morphological data set (with equally weighted characters) (group C in Fig. 1); in the other series (after the reweighting of characters), the species of *Tuckneraria* do not form a separate clade but branch out successively (group C in Fig. 2). Phylogenetic analysis based on molecular characters exhibits the monophyletic origin of all three *Tuckneraria* species (*T. ahtii*, *T. laureri*, and *T. pseudocomplicata*) included in the analysis (Fig. 3).

The originally monotypic genus *Ahtiana* was segregated from *Parmelia* s. lat. on the basis of emergent pycnidia, globose ascospores, leptodermatous cortex,

and the presence of medullary caperatic acid (Goward, 1985). Despite its parmelioid habit with laminal apothecia and pycnidia, *Ahtiana sphaerosporella* was shown to be closely allied to *Cetraria pallidula* (Goward, 1985). Today the genus includes three species (Thell et al., 1995a). In our present analysis based on morphology, two species, *A. sphaerosporella* and *A. aurescens*, form a pair while *A. pallidula* branches out separately (group D in Figs. 1 and 2). The analysis based on ITS sequences indicates a close relationship between the genera *Ahtiana* (represented by the type species *A. sphaerosporella*) and *Tuckermannopsis* (Fig. 3) while *Tuckneraria* species form a separate subclade.

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Ümmarguste kotteostega tsetrarioidsete samblike fülogeneetiline analüüs

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Ümmarguste kotteostega tsetrarioidsete samblike rühma kuulub 37 liiki kaheksast perekonnast. Arvutiprogrammi PAUP 3.1.1 abil tehti selle rühma fülogeneetiline analüüs kasutades kahte andmemaatriksit – morfoloogilisi (sh anatoomilisi ja keemilisi) tunnuseid sisaldavat maatriksit ning molekulaarseid tunnuseid (ITS1, 5.8S ja ITS2 rDNA nukleotiidide järjestusi) sisaldavat maatriksit – eesmärgiga kontrollida praegu kasutusel oleva süsteemi vastavust nimetatud taksonite võimalikule evolutsioonile. Morfoloogiliste tunnuste põhjal võib oletada, et perekond *Allocetraria* on parafüleetiline kahe *Dactylina* liigi suhtes. Nimetatud perekondade eristamist toetavad tugevalt siiski molekulaarandmed. Perekond *Tuckermannopsis* laias mahus (sisaldab 11 liiki) on parafüleetiline liikide *Esslingeriana idahoensis* ja *"Nephromopsis" weii* suhtes. Morfoloogiliste tunnuste põhjal saab selles klaadis monofüleetiliseks pidada üksnes perekonnasisest mitteformaalset rühma, mis sisaldab liiki *Tuckermannopsis ciliaris* ja talle lähedasi taksoneid. Molekulaarsed tunnused viitavad perekonna *Tuckneraria* kolme uuritud liigi monofüleetilisusele. Oletus perekonna *Ahtiana* ühtsest päritolust ei leia morfoloogiliste andmete põhjal toetust.