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Phylogenetic relationships and distribution of Karstenella (Pezizomycetes)

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Phylogenetic analyses of partial SSU and LSU rDNA sequences confirm that *Karstenella vernalis* is a member of the Pezizales. Substantiating this, we definitively report that the asci of *Karstenella* are operculate. *Karstenella vernalis* has been reported only from the type locality in Finland; we expand its known distribution to North America (New Mexico). The inconspicuous ascomata of *Karstenella* consist of very thin, resupinate apothecia borne on a subiculum. Hypotheses of a close relationship with *Pyronema* (Pyronemataceae) or other highly reduced apothecial forms are rejected. Combined analyses show that *Karstenella* constitutes an independent lineage within a highly supported group of the lineages B (Morchellaceae–Discinaceae and Helvellaceae–Tuberaceae) and C (Ascodesmidaceae, Pyronemataceae, Sarcoscyphaceae and Sarcosomataceae), Caloscyphaceae and Rhizinaceae. This corroborates recognition of a monotypic genus and family of *Karstenella*. A possible sister group relationship with Caloscyphaceae and/or Helvellaceae–Tuberaceae is suggested. The ascospores of *Karstenella* are shown to be bi- to multinucleate using DAPI staining. Excipulum structure and/or spore cytology support a shared origin with Caloscyphaceae and/or the Helvellaceae–Tuberaceae sublineage. *Karstenella* possesses the most reduced form of ascomata found so far outside lineages A (e.g. Ascobolaceae) and C (e.g. Ascodesmidaceae and Pyronemataceae).

Keywords: Karstenellaceae, Pezizales, cytology, LSU, SSU

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Introduction

Karstenella Harmaja (1969) is a monotypic, peculiar genus of the operculate discomycetes (Pezizales, Pezizomycetes). It produces resupinate, membranous (c. 0.4–0.7 mm thick in the center, getting thinner towards the edge) apothecia, circular to irregular in outline, 3–12 mm in diam. (Fig. 1), on bare, compacted soil and decaying twigs of *Corylus* and other litter. Young fruitbodies are pale greenish yellow; as they mature they change through a milky orange to light pinkish salmon or brownish red. The inconspicuous ascomata of *Karstenella vernalis* Harmaja are easily overlooked and have been reported only from the type locality in Finland. *Karstenella* has been considered to have an isolated position within Pezizales and its relationship to other members of the order has not been clear. Korf (1972) referred it to Pyronemataceae, subfamily Pyronematoideae together with *Pyronema*, because of the presence of a subiculum and hyaline, eguttulate spores. Nevertheless, he (Korf 1972) placed it in its own tribe, Karstenelleae, primarily based

on the consistently binucleate spores, unknown elsewhere in Pezizales. Harmaja (1974a) proposed the monotypic family Karstenellaceae in the suborder Pezizineae, and pointed out additional diagnostic characters, such as the lack of a cyanophilic perispore in all stages of spore development and the simple excipulum structure composed of *textura intricata* throughout. The inclusion of *Karstenella* in Pezizales has, however, been questioned (van Brummelen in Dissing & Schumacher 1994), as in fact no operculum had been observed.

In the present paper, we report the first collections of *K. vernalis* from North America (New Mexico) and address the evolutionary relationships of *K. vernalis* within Pezizales using phylogenetic analyses of the SSU and LSU rDNA.

Material and methods

Specimens. – The following specimens of *Karstenella vernalis* from New Mexico and Finland were studied and compared morphologically by Nancy S. Weber and Dr. Henry Dissing (University of Copenhagen, Denmark): Finland. Lohja, 20.VI.1973, H. Harmaja (C); USA. New Mexico, Bernalillo Co., Cibola National Forest, Cienega Springs, Sec. 22, T 11 N, R 5 E, gregarious to scattered, on moist, bare soil on inclined edge of path, 10.VIII.1990, N. S. Weber, NSW 6311 (OSC); ibid., scattered on bare damp soil under snowberry and herbaceous plants, 21.VIII.1992, N. S. Weber & Ellen Reed, NSW 6918 (OSC).

To test hypotheses regarding relationships of *Karstenella*, the LSU and SSU rDNA sequences were analyzed with 101 species of the Pezizales (data set from Marek et al. 2008) representing all known sublineages within Pezizales, 83 genera and 15 of the 16 families currently recognized in the order. The analyses placed *Karstenella* unresolved among the Morchellaceae–Discinaceae, Helvellaceae–Tuberaceae, and Rhizinaceae lineages, as sister groups to the C lineage of Pezizales (see Hansen and Pfister 2006), data not shown. Based on these results a pruned data set was constructed including 40 pezizalean species retrieved from GenBank (LSU/SSU): *Ascobolus carbonarius* AY500526/AY544720; *Ascobolus crenulatus* AY500527/AY544721; *Ascodesmis* spp. (represented by *Ascodesmis sphaerospora*–/U53372; *Ascodesmis nigricans* DQ168335/ –); *Balsamia magnata* U42683/U42656; *Barssia oregonensis* U42684/U42657; *Byssonectria terrestris* AY500531/Z30241; *Caloscypha fulgens* (1) DQ247799/DQ247807; *Caloscypha fulgens* (2) –/U53374; *Choiromyces venosus* U42688/U42661; *Dingleya verrucosa* U42686/U42659; *Discina macrospora* U42678/U42651; *Disciotis venosa* U42670/U42643; *Eleutherascus lectardii* DQ168334/DQ062997; *Fischerula subcaulis* U42673/U42646; *Gyromitra californica* U42677/U42650; *Gyromitra melaleucoides* U42680/U42653; *Helvella* cf. *compressa* AY544655/AY544699; *Helvella lacunosa* U42681/U42654; *Hydnotrya cerebriformis* U42676/U42649; *Iodophanus carneus* AY500534/U53380; *Labyrinthomyces varius* U42689/U42662; *Lasiobolidium orbiculoides* DQ062995/DQ063000; *Leucangium carthusianum* U42674/U42647; *Morchella elata* U42667/U42641; *Morchella esculenta* AF279398/U42642; *Peziza badiofusca* AF335132/DQ646542; *Peziza vesiculosa* AY500552/AFTOL –202; *Psilopezia deligata* DQ220390/DQ646547; *Psilopezia juruensis* DQ220391/DQ646548; *Psilopezia* cf. *nummularialis* EU722509/EU722510; *Pyronema domesticum* DQ247805/DQ247813; *Reddellomyces donkii* U42687/U42660; *Rhizina undulata* DQ220410/U42664 ; *Sarcoscypha austriaca* AY945856/AF006318; *Sarcosoma globosum* –/U53386; *Tuber gibbosum* U42690/U42663; *Underwoodia columnaris* U42685/U42658; *Verpa bohemica* U42672/U42645; *Verpa conica* U42671/U42644; *Wynnella silvicola* U42682/U42655. *Neolecta vitellina* (AF279401/Z27393) was used as outgroup.

Morphological methods. – For general morphological methods see Korf (1973) and Weber (1995). For observation of the number of nuclei in spores, herbarium material of *K. vernalis* was rehydrated for 15–30 min. in dH_2O and then stained with DAPI (90% glycerol, 10% PBS, 2.5% DABCO and 0.5–1 mg/ml DAPI). The material was studied with a Zeiss Axioskop 2 microscope with UV illumination.

Molecular methods and analyses. – DNA was extracted and sequenced twice from coll. no NSW 6918 (OSC), on two independent occasions (in the laboratory of Prof. O. Eriksson, Umeå University, Sweden and the laboratory of Prof. M. Berbee, University of British Columbia, Canada), to verify the SSU rDNA sequence of *K. vernalis*. Using separate kits and chemicals at both occasions, DNA was extracted using Dynabeads DNA Direct (DYNAL, Oslo, Norway) and PCR amplification and sequencing followed Landvik et al. (1998). The 5' end of the LSU rDNA was subsequently obtained in the laboratory of D.H. Pfister (Harvard University Herbaria, USA) generally following the procedure in Hansen et al. (2005). The LSU region was amplified with primers LROR and LR5 (Moncalvo et al. 2000) and the following PCR conditions: a hot start at 94°C for 5 min, then 38 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1:30 min, and a final extension of 72°C for 7 min with a 4 ºC soak. Sequencing primers included those used for PCR as well as LR3R and LR3 (Moncalvo et al. 2000). DNA extraction was attempted from a collection of *Karstenella* from the type locality (Finland, 6.VI.1972, H. Harmaja, H), but without success.

Sequences were edited using Sequencher 3.0 (Gene codes, Ann Arbor, Michigan). The three new sequences determined in this study are deposited in GenBank: *Karstenella vernalis*, NSW 6918 (OSC), LSU FJ499391/SSU FJ499392; *Caloscypha fulgens* JV94 –112 (C), LSU FJ499390; and *Sarcosoma globosum*, KH.07.04 (S), LSU FJ499393. Nucleotide sequences were aligned by hand using Se –Al v. 2.0a11 (Rambaut 2002). The combined LSU and SSU alignment is available from TreeBASE as accession number M4227. Phylogenetic analyses were performed using PAUP 4.0b10 for Unix (Swofford 2002). Parsimony (MP) analyses with heuristic searches consisted of 1000 random sequence addition replicates with TBR branch swapping, MULPARS in effect, and saving all equally most parsimonious trees (MPTs). All characters were equally weighted and unordered. Support for individual branches was estimated by parsimony boot-

strap (PB) analyses, using 500 bootstrap replicates, each consisting of a heuristic search with 100 random addition sequence replicates, TBR branch swapping and MAXTREES unrestricted.

Prior to combined analyses the combinability of the data was explored by visual inspection of the individual PB values. We considered the phylogenies to be incongruent only if they displayed strong PB supported (PB>70%) incongruence; that is, clades that are strongly supported in analysis of the one gene region that conflict with different and strongly supported clades in analysis of the other gene region.

ML and Bayesian analyses were performed on the combined data set. The GTR + I + G model of sequence evolution was found to fit each of the individual datasets best, using a hierarchical likelihood ratio test as implemented in the program MrModeltest 2.2 (Nylander 2004). The ML model parameters were calculated from the MPT recovered in the MP analysis of the combined data described above. The ML analysis consisted of heuristic searches with 100 random stepwise sequence addition replicates, and TBR branch swapping. Maximum likelihood bootstrap (MLB) proportions were generated using 500 bootstrap replicates of "fast" stepwise sequence addition and the model parameters estimated for the ML analysis entered manually into PAUP.

Bayesian analyses were performed using Metropolis-coupled MCMC (MCMCMC) methods as implemented in MrBayes 3.1.1 (Huelsenbeck & Ronguist 2001) using uniform prior probabilities and the GTR + I + G model. The LSU and SSU data sets were specified as distinct partitions. Analyses consisted of two parallel searches, with four simultaneous chains of MCMCMC, run for 5000000 generations, starting from random trees. The chains were sampled every 100 generations for a total of 50000 trees each, sampled from the posterior distribution. Those trees sampled prior to the chains reaching a split deviation frequency of 0.02 were discarded from the sample as the 'burn-in', while the remaining trees were used to calculate the Bayesian PPs of the clades.

Results

Morphological features. – Side-by-side comparisons of Finnish and American material of *Karstenella vernalis* did not show any significant differences between the collections at either the microscopic or macroscopic level. Differing slightly from the original description (Harmaja 1969) the young apothecia in the New Mexican material are pale greenish yellow, and change through a milky orange (Fig. 1) to light pinkish salmon as they mature. Harmaja (1969) described the edge of the apothecia as paler (orange-colored) and the extreme margin as greenish yellow, especially in young specimens. The mature apothecia were however, described as mostly brownish red. We attribute some of the color differences to age. Two or more nuclei per spore of *Karstenella* were detected using DAPI staining and UV illumination. Operculate asci were observed on rehydrated material of NSW 6311. Spores were forcibly discharged from fresh specimens of NSW 6918 onto cover slips.

Fig. 1. *Karstenella vernalis*, New Mexico (NSW 6918), very thin, resupinate apothecia on thin subicula, on mildly compacted, exposed soil, along a woodland path. ×2.5 cm. Photo James A. Weber.

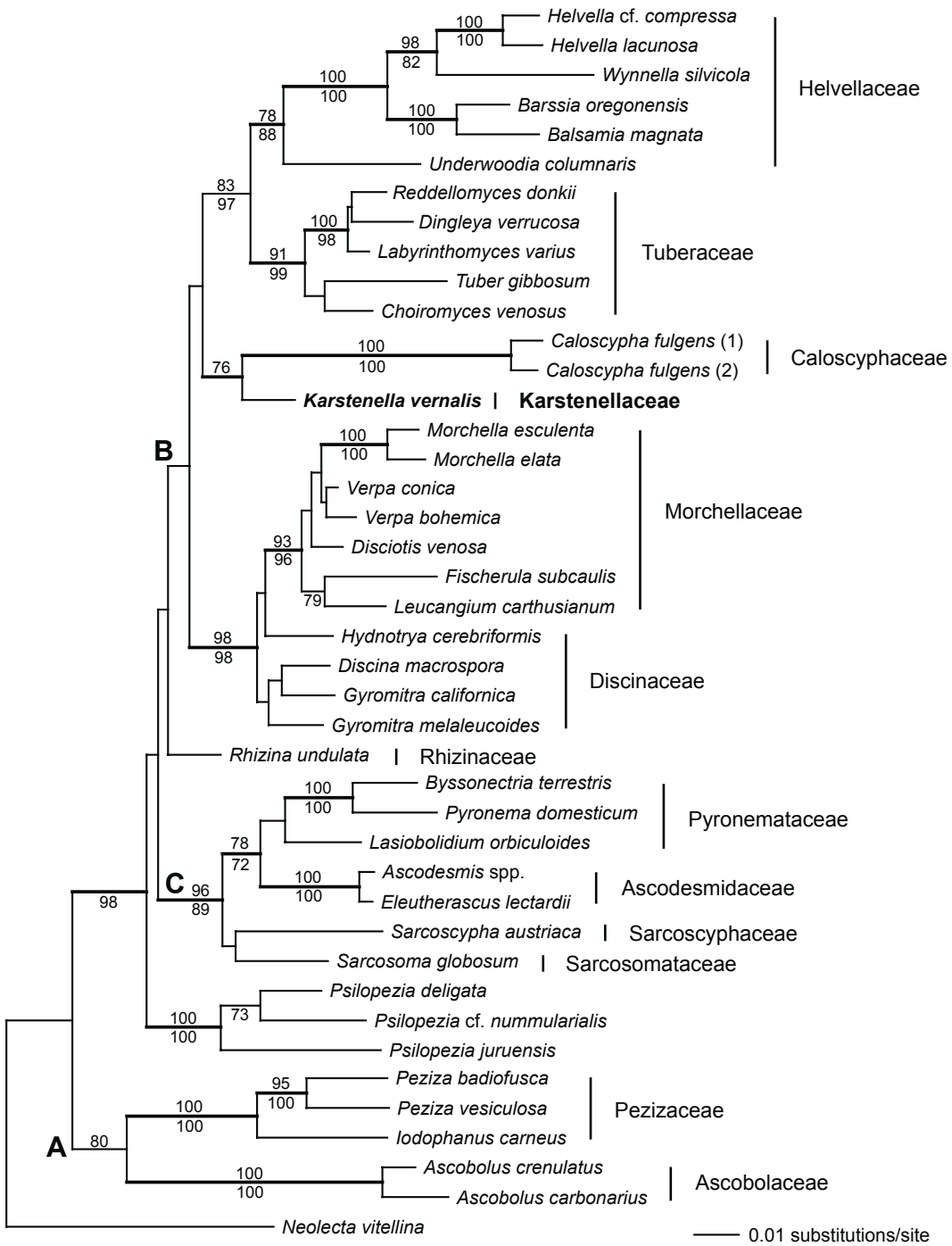

Fig. 2. Phylogenetic relationships of *Karstenella vernalis*, Karstenellaceae, among members of Pezizomycetes (taxon selection are based on a large-scale molecular phylogeny of Pezizomycetes: Marek et al. 2008) inferred from LSU and SSU rDNA sequences. The tree with the highest likelihood (-lnL = 15837.87780) obtained from a maximum likelihood analysis. Bayesian PP>95% are indicated by thickened branches, numbers above branches are MLB>70% and below branches are PB>70%.

Phylogenetic relationships of Karstenella. – The combined SSU and LSU rDNA alignment included 2391 characters, with 735 variable positions including 539 that were parsimony informative; 1778 characters of SSU with 272 being parsimony informative; and 613 of LSU with 267 being parsimony informative. Most aligned sequences of the LSU region were only 613 bp and the alignment was therefore pruned accordingly.

Parsimony analyses of the SSU resulted in 12 MPTs (1006 steps) and of the LSU in 4 MPT (1509 steps). Parsimony bootstrap analyses identified more strongly supported clades in the SSU gene tree than in the LSU tree. Nevertheless, none of the single gene trees showed support for the exact placement of *Karstenella*. The strict consensus trees of the individual SSU and LSU MPTs, placed *Karstenella* unresolved among the B sub-lineages, and Caloscyphaceae (SSU), or Rhizinaceae (LSU), respectively (trees not shown).

No supported conflict (PB>70%) was found between the individual LSU and SSU gene trees and the data was therefore combined. Parsimony analysis of the combined SSU and LSU rDNA data produced a single MPT (2542 steps). The ML analysis found a single optimal tree (-lnL = 15837.87780; Fig. 2). Bayesian analysis reached an average standard deviation of split frequencies below 0.02 after approximately 155000 generations, and the first 1550 trees were excluded as the 'burn-in.' Three main lineages are identified by ML analysis that overall correspond to the A, B and C lineages resolved previously (see Hansen & Pfister 2006) (Fig. 2). Lineage A (Pezizaceae–Ascobolaceae) is resolved as a sister group to the rest of the Pezizales, which form a highly supported monophyletic group as measured by Bayesian PP (99%) and PB (98%) (Fig. 2). The lineage C (Ascodesmidaceae–Pyronemataceae–Sarcoscyphaceae–Sarcosomataceae) is highly supported in all analyses (MLB 96%, PP 100%, PB 89%). Lineage B (Morchellaceae–Discinaceae–Helvellaceae–Tuberaceae), however, is without support and is not resolved in the parsimony analyses. The Helvellaceae–Tuberaceae and Morchellaceae–Discinaceae sub-lineages, identified previously (O'Donnell et al. 1997, Hansen & Pfister 2006), are highly supported in all analyses (83–98%, 100%, 97–98%). *Rhizina* and *Psilopeziza* are not resolved as a monophyletic group, as found previously (Hansen & Pfister 2006). *Karstenella vernalis* forms a moderately supported monophyletic group with *Caloscypha fulgens* in ML and Bayesian analyses (MLB 76%, PP 100%). The *Karstenella–Caloscypha* clade is nested within the B-lineage, resolved as a sister group to Helvellaceae and Tuberaceae (Fig. 2). Relationships among *Karstenella–Caloscypha*, Helvellaceae–Tuberaceae, Morchellaceae–Discinaceae, lineage C, *Rhizina* and *Psilopezia* are, however, without support. In the single MPT *Karstenella* constitute a separate distinct lineage, as sister to the Helvellaceae–Tuberaceae clade, while *Caloscypha* is resolved as a sister group to the rest of the Pezizales (excluding lineage A), but without support.

Discussion

Distribution and localities. – The disjunct Finnish-American distribution of *Karstenella* is surprising, but because of the inconspicuous ascomata it is likely overlooked and may have a much wider distribution. The type locality in Finland is calcareous soil in rich deciduous forest with predominantly *Corylus avellana*, *Populus tremula*, *Tilia cordata* and the herb *Aegopodium podagraria*. In New Mexico *Karstenella* was collected at c. 2256–2290 m elevation in the foothills on the east side of Sandia Mountain, on soil in and near a path along a small stream that flowed over sedimentary rocks. Along the path were a wide variety of trees, such as *Quercus gambelii* (shrubby oak), *Acer negundo*, *Pinus ponderosa, Abies concolor*, *Salix* spp. and *Pseudotsuga menziesii*. This area is predominantly influenced by the southern Rocky Mountain regional flora, but also contains floristic elements from the adjacent Chihuahuan Desert, Great Plains and Colorado Plateau ecoregions (Sivinski 2007). In Finland *K. vernalis* has been collected in the spring (end of May and June), whereas in New Mexico it was collected in late summer (mid August) after the monsoon rains started. The monsoon season is the period with the highest precipitation in this part of New Mexico (Sivinski 2007), and the diversity of fruiting macrofungi and the numbers of sporocarps both peak at this time. Several species found in the spring in other areas, e.g., members of *Helvella acetabulum* s. l., commonly fruit in August. The fruiting time of *Karstenella* in the mountains of New Mexico may thus be comparable to its fruiting time in southernmost Finland.

Evolutionary relationships. – Here we definitively report that *Karstenella* produces operculate asci and belongs to Pezizomycetes. Harmaja (1969) was convinced that *K. vernalis* is an operculate discomycete, because the spores were forcibly discharged (with a puff), but noted, nevertheless, that no opened persisting lid had been observed. Our phylogenetic analyses show that *K. vernalis* constitutes an independent lineage within a highly supported monophyletic group of the lineages B and C of Pezizales (Fig. 2). The results corroborate the erection of a monotypic genus and family for *K. vernalis* (Harmaja 1969, 1974a). *Karstenella* is neither closely related to *Pyronema* or *Byssonectria* (also possessing a subiculum), nor a member of the Pyronemataceae. The exact placement of *Karstenella* is uncertain, but a possible sister group relationship with *Caloscypha* and/or Helvellaceae and Tuberaceae (of lineage B) is suggested. This grouping may at first seem rather surprising considering the discrepancies in fruiting body morphology, color and size. Species in Helvellaceae and Tuberaceae, however, already demonstrate an extreme variation in ascomata types (Weber et al. 1997), including hypogeous forms, stereothecia and infolded, chambered or solid ptycothecia, and a diverse array of apothecia, e.g. sessile to stipitate, cup-shaped, saddle-shaped, ear-shaped (*Wynnella*) and clavate (*Underwoodia*). Ascomata in the lineage B are in general large and fleshy, but for example those of *Helvella terrestris* do not reach more than 3–5 mm in height (Landvik et al. 1999). The relatively bright colors of *Karstenella* ascomata are not common in lineage B, but *Caloscypha* likewise possess brightly colored apothecia, being orange-yellow, turning green or bluish with age or when touched or broken. The genus *Wynnella* (Helvellaceae) also produces apothecia with reddish-brown colors.

Several important characters are indicative of a relationship of Karstenellaceae with Caloscyphaceae, Rhizinaceae and members of lineage B, such as spore cytology and excipulum structure. *Karstenella vernalis* has been reported to have consistently bi-nucleate spores (Korf 1972), but using DAPI staining we observed the spores to be bi- to multinucleate. The spores in lineage B are tetra- or multinucleate, with a variable number of nuclei (from one to 18:Vizzini 2003) in Tuberaceae. Lineage A and C of Pezizales have uninucleate spores, with the exception of Sarcoscyphaceae, Sarcosomataceae and Chorioactidaceae which have multi-nucleate spores. *Caloscypha* has likewise uni-nucleate spores, whereas the number of nuclei in spores of *Rhizina* still is to be documented. The excipulum and margin of *Karstenella* ascomata are exclusively composed of interwoven, filamentous hyphae (*textura intricata*) and are, as is also typical for *Caloscypha*, *Rhizina* and the lineage B members, without any inflated cells (*textura globulosa*) (scattered versiform inflated cells are present in *Rhizinaceae*). Ascomata in Helvellaceae and Tuberaceae typically have a distinctly stratified excipulum, with a medullary layer of *textura intricata* and an outer layer of *textura intricata* to *textura angularis*. The ascomata of *Caloscypha* and *Rhizina* share a non-stratified excipulum with *Karstenella*. In contrast, in many species of Pezizales with small ascomata, including *Pyronema* and *Byssonectria*, the excipulum is composed of globose cells throughout or at least one layer composed of globose to angular cells.

In the descriptions of the families Karstenellaceae and Caloscyphaceae, Harmaja (1974a, 2002, respectively) emphasized the spores are without a cyanophilic secondary wall at any stage of development ('perisporeless' type, Harmaja 1974b). In the B sub-lineages, however, both 'perisporeless' (*Morchella*, *Disciotis* and *Verpa*) and 'persistently perisporous' spore types (*Gyromitra*, *Helvella*) occur (Harmaja 1974b).

The great diversity in ascomata forms and sizes in Pezizales has inspired several hypotheses of trends in the evolution. Hansen and Pfister (2006) suggested that highly reduced ascomata of e.g. *Eleutherascus*, *Ascodesmis* (Ascodesmidaceae) and *Pyronema* are evolutionary derived structures within the Pezizales rather than representing an ancestral state. The large and complex ascomata of lineage B have been regarded as highly derived forms (Nannfeldt 1937, Dissing 1966, Kimbrough 1994), based in part on their tetra- to multinucleate spores, and elaborate septal pore plugs at the base of the asci in taxa of Helvellaceae and Morchellaceae. As pointed out by Landvik et al. (1997) and Hansen and Pfister (2006) minute or reduced ascomata forms have been lacking so far in the lineage B, which is noteworthy since both lineage A and C includes minute forms. *Karstenella* possesses the most reduced form of ascomata found so far among members of lineage B, Caloscyphaceae and

Rhizinaceae. Because of the lack of support for the exact placement of *Karstenella*, however, it remains a question if the thin, resupinate apothecium of *Karstenella* represents an ancestral or highly derived form.

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