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Omphalina (Basidiomycota, Agaricales) as a model system for the study of coevolution in lichens

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SUMMARY

Although lichens represent the best example of mutualism, virtually nothing is known about the origin and evolution of the lichen symbiosis. In this paper we propose to use the genus *Omphalina* and its photobiont *Coccomyxa* as a model system for coevolutionary studies on mycobiont-photobiont mutualistic associations. As a first step toward subsequent studies on coadaptation and molecular genetics, phylogenetic relationships were examined among lichenized and non lichenized *Omphalina* using morphological, anatomical, and DNA sequence data. Phylogenetic conclusions based on preliminary molecular data from the nuclear-encoded 25S ribosomal RNA gene were congruent with anatomical and morphological evidence. Results of these phylogenetic analyses suggest that: 1) lichen-forming species of *Omphalina* are monophyletic and arose from a saprophytic lineage, 2) lichenization in the genus *Omphalina* is associated with the loss of clamps, the loss of a pure tetrasporic state, the loss of the ability to grow in axenic culture on MEA, and the development of a uninucleate teleomorph (which might correspond to a parthenogenetic mode of reproduction), and 3) globules (*Botrydina*) were the first evolutionary morphological innovation resulting from the symbiotic association, followed by squamules (*Coriscium*).

Introduction

Symbiotic interactions involving mutualism are much more the rule rather than the exception in nature (2, 25, 46). This is certainly the case in fungi, where obligately mutualistic lichen-forming and mycorrhizal species are associated with members of almost every other kingdom of life (6, 42, 56). As an example, more than 85% of all land plants (Archegoniatae) are associated with VAM (vesicular-arbuscular mycorrhizal) fungi in nature (28), and it has been suggested that the origin and evolution of the boreal forest are highly dependent on the presence of ectomycorrhizal fungi (45, 54, 57). Lichenization is one of the most successful ways whereby fungi fulfill their requirement for carbohydrates, with about 20% of the estimated 64,200 known species of fungi being lichenized (19).

Although the frequency of mutualism involving fungi is quite high, mycobiont-photobiont associations are not randomly distributed across all taxa. For example, the majority of fungi forming ectomycorrhizae involve members of the Basidiomycota (21, 28). Similarly, the widespread VAM fungi represent a rather small and homogeneous group (≈ 130 known species) within a single order, the Glomales (49, 55). Lichenization is almost restricted to the Ascomycota, which claims more than 98% of all lichen-forming species (19). Also, lichenization is restricted to specific lineages within the Ascomycota. Although approximately 50% of all known Ascomycota species are lichenized, only sixteen orders out of 37 include lichen-forming taxa (21), and only 5 orders are entirely lichenized (20). A similar pattern is present in the Basidiomycota, where lichenization is known to occur in only six of the estimated 1,100 genera of this division (21, 51). Such a strong concentration in the distribution of mutualistic associations must result from specific evolutionary mechanisms. Detailed studies of certain model lichen systems may shed light on mechanisms that play a major role in the evolution of other lichenized fungi and mycobiont-photobiont mutualistic associations.

Experimental approaches for elucidating mechanisms involved in the origin and evolution of lichenization will require appropriate groups of symbiotic organisms amen-

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able to laboratory study and whose phylogenetic relationships are relatively well known. In this paper, we describe such a model system for coevolutionary studies on lichens based on the basidiomycete genus *Omphalina* and its photobiont *Coccomyxa*. Previous taxonomic studies have often disagreed about the limits of the genus *Omphalina* (4, 5, 32, 34, 61). As an initial step in this project, we have attempted to estimate phylogenetic relationships among lichenized and non lichenized *Omphalina* based on morphological, anatomical, and DNA sequence data. Such a study is prerequisite to any subsequent molecular genetic and evolutionary studies on photobiont-mycobiont mutualistic interactions; it will be especially helpful in guiding the selection of appropriate taxa for experimental studies.

The objectives of this phylogenetic study were to determine: 1) How many lichenization events took place within the genus *Omphalina*? 2) What life history features are associated with transition to a lichenized state? 3) Wh t morphological transformations result during licheniz tion? Our results demonstrate the high potential of th s model system for studying the evolution of lichenizatio .

Materials and Methods

Morphological and anatomical study

Herbarium and field work

Herbarium specimens used in this study were borrowed fro O, F, TUR (24), and from Prof. Denise Lamoure's personal h barium (Table 1). Additional specimens of *Arrhenia* and *O phalina* were collected by the first author in the summer of 19 (Table 1). Notes on basidiomata characters were made within hours after collection using standard methods and color

Table 1. Collection data for populations of *Omphalina* species and *Arrhenia lobata* sampled for morphological-anatomical a molecular studies. All vouchers at DUKE unless otherwise noted. Study = (1) morphology and anatomy study, (2) molecular stuc

Taxon	Study	Location
Arrhenia lobata	1	France: Savoie, Haute-Maurienne, Bonneval-sur-Arc, Lutzoni & Lamoure 910824-1
A. lobata	1	France: Savoie, Haute-Maurienne, Bonneval-sur-Arc, Lutzoni & Lamoure 910817-5
A. lobata	1	France: Savoie, Haute-Tarentaise, Vallon de La Sassière, Lutzoni & Lamoure 910820-2b
A. lobata	2	France: Savoie, Haute-Tarentaise, Vallon de La Sassière, Lutzoni & Lamoure 910828-2b
Omphalina epichysium	1	CSSR: Slovakia, Javorina, Siroké dolina, Singer C 5993 (F)
O. epichysium	1	CSSR: Nature Reserve on Mt. Boubin, Singer C 5200 (F)
O. epichysium	1	Canada: Newfoundland, Schefferville area, Kallio 518 (TUR)
O. epichysium	2	France: Savoie, Haute-Tarentaise, Courchevel, Lamoure 80.106 h.25 A2B1 (Personal herbarium)
O. ericetorum	1	Norway: Hedmark, Engerdal, Gulden 333/71 (O)
O. ericetorum	1	Norway: Hedmark, Alvdal, Gulden 395/71 (O)
O. ericetorum	1	Sweden: Abisko, Lamoure 28/7-79 (Personal herbarium)
O. hudsoniana	1, 2	Norway: Hordaland, Ullensvang, Gulden 621/71 (O)
O. hudsoniana	1	Norway: Hordaland, Ulvik, Finse, Gulden 582/81 (O)
O. hudsoniana	1	Finland: Kuusamo parish, Juuma, The Vuomas, Gulden 155/78 (O)
O. luteovitellina	1	Norway: Nordland, Vaerøy, Gulden 345/72 (O)
O. luteovitellina	1	Finland: Finnmark, Vadsø, Sarre & Høiland (O)
O. luteovitellina	1	Norway: Busk: Nore and Uvdal, Wischmann (O)
O. obscurata	1	Switzerland: Borgne de Ferpècle, Singer C 5462 (F)
O. obscurata	1	Switzerland: Valais, Glacier de Moiry, Singer C 5498 (F)
O. obscurata	1	Switzerland: Valais, glacier lake of Glacier de Moiry, Singer C 5499 (F)
O. obscurata	2	France: Savoie, Massif de l'Iseran, Petit-Plan, Lamoure 73-101 (Personal herbarium)
O. rivulicola	1	France: Savoie, Haute-Maurienne, Le Vallonnet de Bonneval, Lutzoni & Lamoure 910817-12
O. rivulicola	1, 2	France: Savoie, Haute-Tarentaise, Vallon de La Sassière, Lutzoni & Lamoure 910828-3a & -3d
O. rivulicola	1	France: Savoie, Haute-Tarentaise, Parc National de la Vanoise, Prarion, Lamoure 910821-2
O. velutina	1	Norway: Oppland, Vågå, Lange & Gulden 648/69 (O)
O. velutina	1	Norway: Oppland, Vågå, Lange & Gulden 727/69 (O)
O. grisella (= *O. velutina*)	1	Norway: Ahh, Nesodden, Gulden 64-1 (O)

ences (26, 27). Spore prints were collected and stored at 20 °C for later use. Dikaryotic cultures were obtained as either ltispore isolates or tissue isolates from basidioma. All isolates maintained as stock cultures on malt extract agar (MEA) at C.

Choice of taxa and outgroup

For this study, the genus *Omphalina* is considered in its broad nse to consist of approximately 40 species that can be grouped to three main stirps (*ericetorum, obscurata*, and *pyxidata*) and lditional minor *incertae sedis* stirps (Lamoure, pers. comm.). irps *ericetorum* consists entirely of lichenized species, and is aracterized by the absence of clamp connections and the presence of yellow pigments in the basidiomata. For phylogenetic studies, *O. ericetorum, O. hudsoniana*, and *O. luteovitellina* were selected from stirps *ericetorum*. Stirps *obscurata* and *pyxidata* are composed of both lichenized and non lichenized taxa characterized by dark brown-black basidiomata and by tan to reddish-brown basidiomata, respectively. Within these two groups, lichenized taxa lack clamp connections present in non lichenized species. Two non lichenized species, *O. obscurata* and *O. epichysium*, were chosen as representatives of stirps *obscurata*. Two species were selected from stirps *pyxidata*; one lichenized species, *O. velutina*, and one non lichenized species, *O. rivulicola*. In addition to these *Omphalina* taxa, another closely related species, *Arrhenia lobata*, was included in the study as an outgroup for phylogenetic analyses.

able 2. Characters used for parsimony analyses of 7 species of *Omphalina*, using *Arrhenia lobata* as an outgroup. Characters receded by an asterisk were not used in reconstructing the phylogeny; they were mapped subsequently onto the topology.

	Characters	Character states
*1.	lichenization	0 = absent, 1 = globular (*Botrydina* type), 2 = squamulose (*Coriscium* type)
2.	clamps at the base of basidium	0 = absent, 1 = present
3.	proportion of basidia with different numbers of spores	0 = 30% tetrasporic, 7.5% trisporic, 15% bisporic, 7.5% unisporic; 1 = 70% tetrasporic, 15% trisporic, 15% bisporic; 2 = 30% tetrasporic, 70% bisporic; 3 = 100% tetrasporic
*4.	reproduction	0 = potentially parthenogenic, 1 = sexual
5.	pileus surface	0 = free ends not forming scales, 1 = free ends forming scales
6.	cutis texture (based on Korf's [29] classification of fungal tissue texture)	0 = epidermoidea, 1 = intricata, 2 = porrecta, 3 = prismatica
7.	cutis free terminal cell	0 = absent, 1 = present
8.	pileus micro-incrustation on the cell wall	0 = absent, 1 = present
9.	pileus laciniate incrustation on the cell wall	0 = absent, 1 = present
*10.	axenic culture	0 = not possible on MEA, 1 = possible on MEA
11.	pileus colour	0 = reddish-brown, becoming dark brown, becoming yellowish brown, or ± red brown to grey brown to beige; 1 = pale yellow to brilliant orange yellow, or bright yellow, or olive brown when young becoming yellowish brown to yellow; 2 = dark grey brown to dark purplish brown, or smoky grey brown
12.	lamellae colour	0 = pale brown; 1 = bright yellow to bright orange yellow; 2 = dark greyish brown to brownish grey, or whitish to pale beige, or pale grey brown, or brownish, whitish, cream, yellowish, or pale greyish; 3 = grey-white to grey
13.	stipe base pubescence	0 = absent, 1 = present
14.	stipe interior	0 = hollow, 1 = solid
15.	stipe colour	0 = pale reddish-brown becoming medium brown, or ± red brown to grey brown to beige; 1 = white to pale orange yellow, sometimes with violet tinge, or bright yellow, or olive brown at apex when young becoming yellowish to yellow or only fading; 2 = dark grey brown to dark purplish brown, or smoky grey brown
16.	proportion of basidiospores with different numbers of nuclei	0 = 13% uninucleate, 60% binucleate, 13% trinucleate, and 13% > trinucleate; 1 = 100% binucleate; 2 = 100% uninucleate
17.	stipe	0 = absent, 1 = present

Choice of characters

Characters for phylogenetic analyses were taken from the literature (3, 14, 15, 16, 23, 31, 32, 35, 36, 38, 39, 41, 50, 53, 58, 60, 63). In addition, several new characters were also evaluated. From a total of 93 potential characters for phylogenetic analyses, 76 were eliminated for at least one of the following reasons: 1) the description for a given character was too vague, 2) no variability in the data, 3) the impossibility of reliably describing a structure due to difficulties with its examination or to excessive variation within the same individual, or 4) absence of discrete character states. Nine of the 17 selected characters were observed directly on dried herbarium specimens (characters 1, 2, 5, 6, 7, 8, 9, 13, 17; Table 2). The remaining eight characters were taken from the literature; characters 11, 12, 14, 15 (Table 2) could not be recorded on dried specimens, and characters 3, 4, 10, 16 (Table 2) had been recorded previously on many specimens (35, 36, 38, 39). Except for the characters suspected to be linked with lichenization (characters 1, 4, 10; Table 2) and the basidioma colors (characters 11, 12, 15; Table 2), all characters were microscopic and were recorded using a Leitz HM-LUX compound microscope at 400X to 1000X magnification or a dissecting microscope with a magnification of 10X to 40X.

Parsimony analysis

Phylogenetic analyses based on morphological and anatomical data were performed using the exhautive search option of PAUP version 3.0s (61). The resulting trees were evaluated by 100 bootstrap replications (9) and by determining the decay index (47). The parsimony analyses were restricted to 14 of the 17 characters (Tables 2 and 3). Because 3 of the 17 characters (characters 1, 4, 10; Table 2) are directly linked with lichenization, these characters were only mapped subsequently onto the topology, along with the other characters, using the ACCTRAN character state optimization with MacClade version 3 (44). Taxa with polymorphic characters (Table 3) were coded using parentheses as suggested by Swofford (61), and Maddison and Maddison (44). Polarity of characters was established using Lundberg rooting (61) with *Arrhenia lobata* as an outgroup.

Molecular phylogenetics

As a source of additional independent characters, sequences of the nuclear encoded 25S rDNA genes were obtained from 5 of the 8 taxa studied in the morphological-anatomical study (Table 1). DNA was extracted from either axenic cultures or from ·erbarium specimens using SDS (43) or DTAB-CTAB minip eps (64). A region of about 1400 bp starting at the 5' end of the 25S rDNA was amplified by PCR using primers LR0R and LR7 (8). The amplified products were cleaned using Magic PCR I eps DNA Purification System (Promega), and sequenced directl by cycle sequencing (Promega).

The sequences were aligned manually using the Editor- IO computer program (52). Sequences for the 5 taxa overlappe for ≈700 bp. The only positions used in the phylogenetic ana ses were those where the nucleotides were determined for all ve taxa and their alignment was unambiguous (517 bp). Final a n-ment of sequences and positions used in the phylogenetic ana ses are given in Fig. 1.

The parsimony analysis of the molecular data was identic to the one for the morphological-anatomical data set. Selecte n-dels were used as characters and, therefore, treated as a th character state. The robustness of the internodes was assesse by 1000 bootstrap replications (9) and decay analyses (47). he most likely tree was generated using PHYLIP, DNAML ver n 3.5c (10). Felsenstein's generalized version of the Kimura 2-p a-meter model was used as a probabilistic model to estimate r a-tive rates of substitution; the transition/transversion ratio u d was 2.0; the jumble option was used with 10 different spe es orders.

Abbreviations

CTAB: hexadecyl trimethyl-ammonium bromide; DTAB: dodecyl trimethyl-ammonium bromide; ME : malt extract agar; PCR: polymerase chain reaction; bp: base pairs; VAM: vesicular-arbuscular mycorrhizae.

Results and Discussion

Phylogenetic studies of *Omphalina*

Initial phylogenetic analyses of morphological and anatomical characters were performed using PAUP (61). A phylogenetic tree summarizing the results (Fig. 2) shows the lichenized species of *Omphalina* and stirps *ericetorum* to be monophyletic and derived from non lichenized taxa.

Table 3. Morphological-anatomical data matrix for 7 species of *Omphalina* and *Arrhenia lobata* (outgroup). Unknown or non-applicable character states for taxa are indicated by a "?". Parentheses are used to accommodate taxa that were polymorphic for a given character (44, 61). Asterisks indicate those characters not used in reconstructing the phylogeny; they were only mapped subsequently onto the topology.

	Characters																
Species	1*	2	3	4*	5	6	7	8	9	10*	11	12	13	14	15	16	17
A. lobata	0	(01)	3	1	1	(12)	0	1	1	1	2	2	?	?	?	2	0
O. epichysium	0	1	?	1	(01)	2	1	(01)	0	1	2	3	?	?	2	2	1
O. ericetorum	1	0	0	0	0	(12)	0	0	0	0	1	2	1	(01)	1	0	1
O. hudsoniana	2	0	1	0	0	(01)	1	0	0	0	1	1	1	(01)	1	2	1
O. luteovitellina	1	0	1	0	0	1	0	0	0	0	1	1	0	1	1	1	1
O. obscurata	0	1	3	1	1	(12)	1	1	1	1	2	2	0	1	2	0	1
O. rivulicola	0	1	3	1	0	(123)	0	1	1	1	0	0	(01)	0	0	2	1
O. velutina	1	0	2	0	(01)	1	0	(01)	0	0	0	2	1	1	0	?	1

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ARRHENIA     1 GTGTACAAGTCTC-TGGA-TAG-GCGTCATAGAGGGTGAGAATCCCTGTCTTTGCCACGGACTG
EPICHYSIUM     GTGTACAAGTCTCTTGGAACAGAGCGTCATAGAGGGTGAGAATCCC-GTCTTTGACACGGACTA
HUDSONIANA     G?G?ACAAG?C?C??GGAAAAGAGCA?CATAGAGGGTGAGAA?CCC-G?CTTTGAC?CGGACTG
OBSCURATA      GTGTACAAGTCTCTTGGAATAGAGCGTCATAGAGGGTGAGAATCCC-GTCTTTGACACGGACTA
RIVULICOLA     GTGTACAAGTCTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCCGTCTTTGACACGGACTA
               * * ***** * *  *********** *************** ***** ******* *******

ARRHENIA    65 CCAGTGC-TTGTTATGCGCTCTCAAC?AGTGCGAGTTGTTTGGGAATGCAGCTCAAA/
EPICHYSIUM     CCAGTGCATTGTGATGCGCTCTCAACGAGT-CGAGTTGTTTGGGAATGCAGCTCAAA/
HUDSONIANA     CCAA?GCA?TGTGATGCGC?C?CGATGAG?-C?AGTTGTTTGGGAATGCAGCT???T/
OBSCURATA      CCAGTGCATTGTGATGCGCTCTCAACGAGT-CGAGTTGTTTGGGAATGCAGCTCAAA/
RIVULICOLA     CCAGGGCTTTGTGATGCGCTCTCAAAGAGT-CGAGTTGTTTGGGAATGCAGCTCAAA/
               **** *** ********** * **** ** ** ********************   *

ARRHENIA   330 T-AGTTTTGACTGTTGGAATAAAAGCTGGAGGAATGTGGC-TCTCC--GGATGTGTTATAGCCT
EPICHYSIUM     TCAATTTTGATCGTTGG-ATAAAAGCGAGAGGAATGTGGCATCTTC--GGATGTGTTATAGCCT
HUDSONIANA     TCAG-TTC-A-TGTTGG-ACAAAGGTCAGAGGAATGTGACATCTTTTTGGATGTGTTATAGCCT
OBSCURATA      TCAATTTCGACTGTTGG-ATAAAAGCGGGAGGAATGTGGCATCTCC--GGATGTGTTATAGCCT
RIVULICOLA     TCAATTTTGACCGTTGG-AAAAAAGTTGGGGGAATGTGGCATCTTC--GGATGTGTTATAGCCC
               ****************************************************************

ARRHENIA   394 CTGGTTGCATACAACAGTTGGGATTGAGGAACTCAGCA?GC?G-?AA-GG-C?GGGGTTTTA/
EPICHYSIUM     TT?-TTGCATGCAACGGTTGGGATTGAGGAACTCAGCACGCCG-CAA-GG-CCGGGATTT-A/
HUDSONIANA     CGGGTCGCATGCAACGGTTGGGATTGAGGAACTCAGCACGCCTTCATTGG-CCGAGG--C-A/
OBSCURATA      CTCGTTGCATGCAACAGTTGGGATTGAGGAACTCAGCACGCCG-CAA-GG?CCGGGGTTCGC/
RIVULICOLA     CTGATTGTATACAACGGTTGGGATTGAGGAACTCAGCATGCCG-CAA-GGA-CGGGTTTT-A/
               ** ********************************** ** ***** * *********

ARRHENIA   616 CCAG-ACCAGACCTTTTGTGACGGATCTGCGGTTGAGCGTGTATGTTGGGACCCGAAAGATGGT
EPICHYSIUM     CCGGAACCAGACCTTTTGTGACGGATCCGCGGTGGAG?GTGTATGTTGGGACCCGAAAGATGGT
HUDSONIANA     CCGG-ACCAGACCTTTTGTGACGGATCCGCGGTAGAGCGTGCATGTTGGGACCCGAAAGATGGT
OBSCURATA      CCAG-ACCAGACCTTTTGTGACGGATCTGCGGTAGAGCGTGTATGTTGGGACCCGAAAGATGGT
RIVULICOLA     CCGG-ACCAGACCTTTTGTGACGGTCCCGCGGTAGAGCATGTATGTTGGGACCCGAAAGATGGT
               ************************************ **************************

ARRHENIA   680 GAACTATGCCTGAATAGGC-GAAGCCA?AGGAAACTCTGGTGGA?GCTCGTAGC?ATTCT?ACG/
EPICHYSIUM     GAACTATGCCTGAATAGGC-GAAGCCAGAGGAAACTCTGGTGGAGGC?CGTAGC?ATTCT?ACG/
HUDSONIANA     GAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGC?CGTAGC?AATCTGA?G/
OBSCURATA      GAACTATGCCTGAATAGGC-GAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTGACG/
RIVULICOLA     GAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTGACG/
               ************************** **************** ** ****** ***** * *

ARRHENIA   976 ATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAAC?C?AGGTTAAGGTGCCG?AATTT
EPICHYSIUM     ATTTTT?GTAAGCAGAACTGGCGATGCGGGATGAACCGAAC?C?AGGTTAAGGTGCCGGAATTT
HUDSONIANA     ATTTTTGGTAAGC?GAACTGGCGATGC?GGATGAACCGAAC?CGAGGTTAAGGTGCCGGAATTC
OBSCURATA      ATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGCCGGAATTC
RIVULICOLA     ATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAAC?C?AGGTTAAGGTGCCGGAATTT
               ****** ****** ************* ************ * ************** *****

ARRHENIA  1040 AC?CTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGTGGCCATGGAAGT
EPICHYSIUM     AC?CTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT?GCCATGGAAGT
HUDSONIANA     ACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGTGGCCATGGAAGT
OBSCURATA      ACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGTGGCCATGGAAGT
RIVULICOLA     AC?CTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGTCGCCATGGAAGT
               ** ************************************************* ***********

ARRHENIA  1104 CGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAGCCTGAA
EPICHYSIUM     CGGAACCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATCAACTAGCCTGAA
HUDSONIANA     CGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAGCCTGAA
OBSCURATA      CGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAGCCTGAA
RIVULICOLA     CGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAGCCTGAA
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Fig. 1. Aligned sequences of four portions of the 5′ end of the 25S rDNA. Individual indels marked by hyphens; unknown marked by "?". The 517 nucleotide positions used in the phylogenetic analyses are indicated an asterisk.

Overall support for the topology in Figure 2, as represented by boostrap replications and decay index, is rather weak, thus any conclusions based on this topology should be regarded as hypotheses to be tested. It is worth noting, however, that the lichen clade is also the most robust branch of the tree (Fig. 2).

To test the phylogeny based on morphological and anatomical data, a parallel study was initiated using DNA sequence data from the nuclear-encoded 25S ribosomal RNA gene. The results of two different phylogenetic analyses employing parsimony (61) and maximum likelihood (10) are shown in Figure 3A and B. Except for differences in branch lengths, topologies based on parsimony and the maximum likelihood method are in exact agreement. This topology is essentially congruent with the one based on morphological-anatomical data (Fig. 2). The only topological difference is that when using DN‹ sequences, the *Arrhenia lobata* root occurs on the d ect lineage of O. *obscurata*, rather than on the internode ık-ing the common ancestor of O. *rivulicola* and licher ·ed *Omphalina* with the common ancestor of O. *obscu ıta* and O. *epichysium* (Fig. 3C).

The results of our phylogenetic analyses (Figs. 2 an 3) support the following interpretation for the origin ıd evolution of lichenization in *Omphalina*: 1) licheniza ɔn occurred only once during the evolution of *Ompha na* and has been retained by subsequent generations ıd species, 2) the transition occurred from a saprotrophic › a mutualistic nutritional mode, 3) morphological inn(a-tions resulting from the mycobiont-photobiont coev(u-tion are the formation of a globular crustose thallus ›l-lowed by the formation of a squamulose thallus.

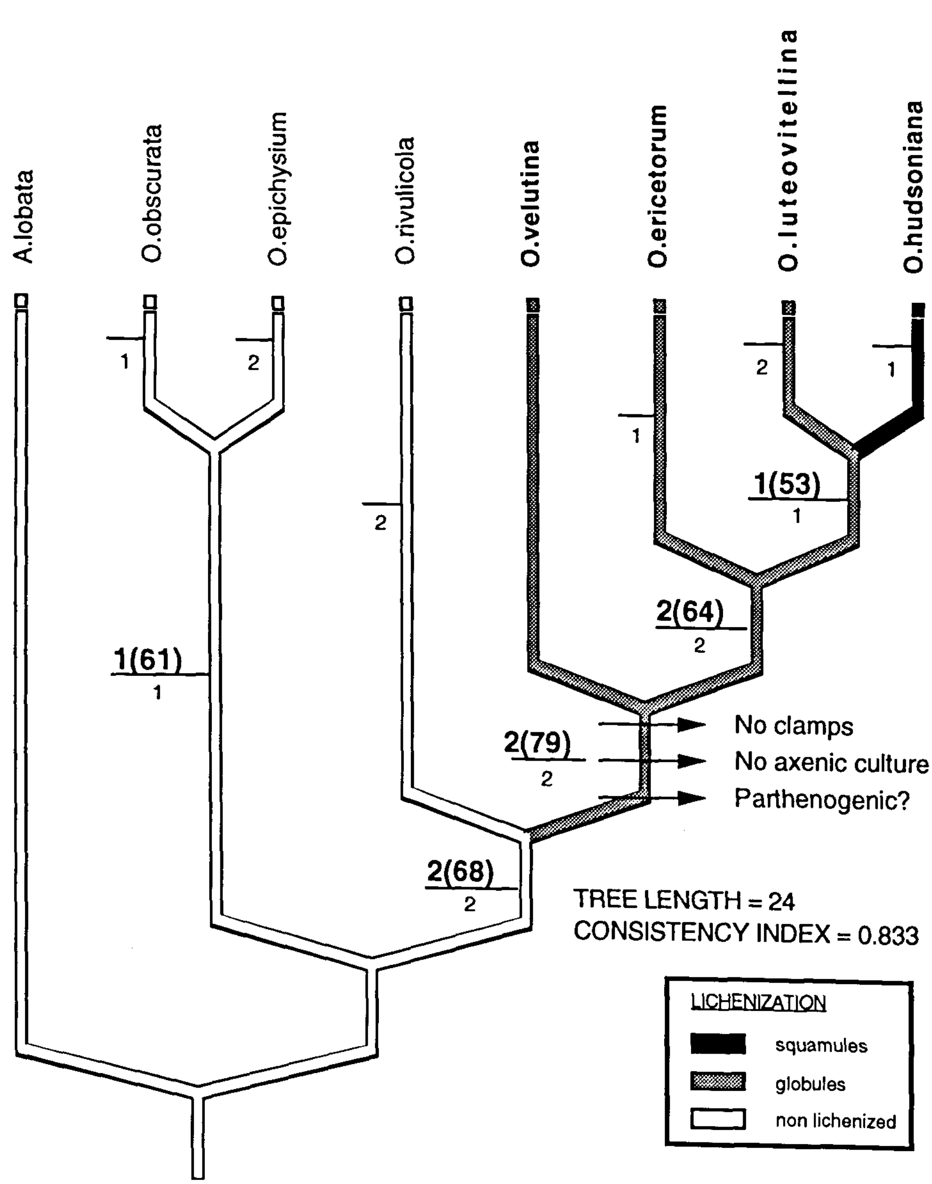

Fig. 2. Evolution of lichenization and related characters in the genus *Omphalina*. Single most parsimonious tree generated by an exhaustive search using PAUP (61) based on 14 morphological and anatomical characters. The lichenized taxa are in bold. *Omphalina ericetorum*, O. *hudsoniana*, and O. *luteovitellina* represent stirps *ericetorum*; O. *epichysium* and O. *obscurata* represent stirps *obscurata*, and O. *rivulicola* and O. *velutina* are representatives of stirps *pyxidata*. Only unambigious transformational changes in characters used to build the tree are mapped; these changes are shown as denominators for each branch. The first numerator (not included in parentheses) is the decay index (47). The numerator in parentheses is the percentage of bootstrap replications (100 replications) supporting each branch (9).

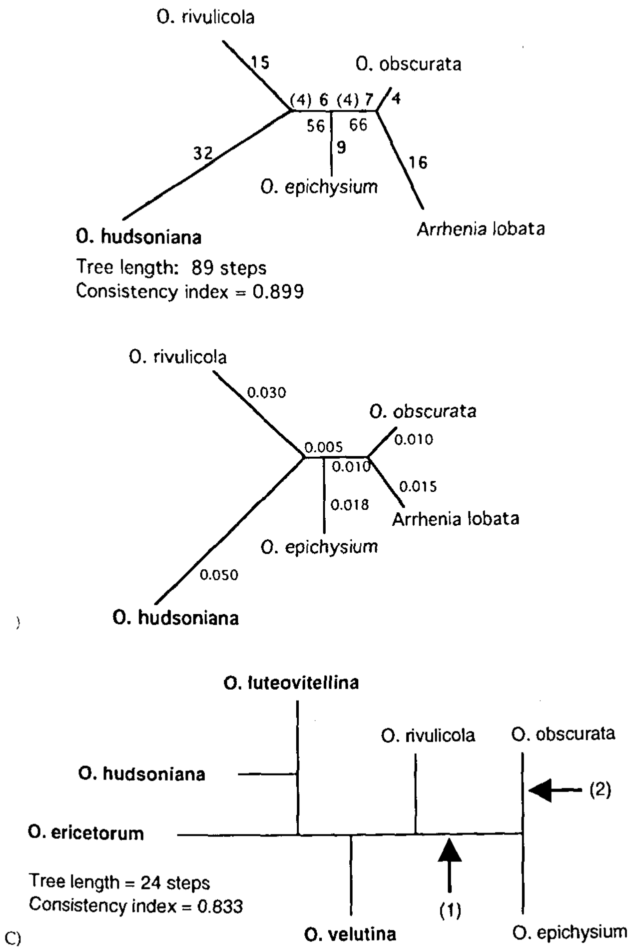

Fig. 3. A) Single most parsimonious tree generated by an exhaustive search using PAUP (61) based on nuclear-encoded 25S rDNA sequences. Numbers above the internodes and in parentheses are the decay indices (47), numbers below the internodes are the percentage of bootstrap replicates (9) for which these internodes did not collapse (1000 replications), and those in bold are the number of transformational changes. B) Most likely tree (Ln Likelihood = −1073.44581) obtained using maximum likelihood method (10) based on the same nucleotide positions as in "A". Numbers along the branches are the branch lengths recorded as expected number of nucleotide substitutions per site. C) Wagner network representation of the tree shown in Figure 2, based on morphology and anatomy, for comparison. Arrow 1 indicates where the network was rooted based on morphological data, and arrow 2 indicates where the network would have been rooted based on nuclear rDNA sequences. *Arrhenia lobata* was chosen as the outgroup in all analyses. Lichenized taxa are in bold.

Requirements for an ideal model system to study the evolution of lichenization

To understand how lichenization evolved, it is critical to determine the time and place when fungi made their transition to a mutualistic mode of nutrition. This transition can be identified by reconstructing the evolutionary history of a group of fungi that includes lichenized and non lichenized taxa. To do this, the taxa under study should be readily available and should possess sufficient numbers of variable characters. To gain confidence in a given topology and to enhance the possibility that a gene tree corresponds to the organismal tree, independent data sets for both morphological and molecular characters should be used to develop phylogenetic hypotheses. The use of a group of closely related species within which lichenization occurred recently is essential, so as to minimize extinction events and the number of incidental differences between organisms with different nutritional modes. The potential of these differences to be associated with the transition from a non mutualistic state to an obligatory mutualistic state would therefore be maximized. Ideally, the species involved should be amenable to study at the molecular, population, and interspecific levels.

From an experimental vantage, a model system should be easily adopted for laboratory studies. It should be possible to synthesize the mutualistic association artificially *in vitro* and have readily observable morphological markers for detecting a mutualistic interaction. The latter requirement is essential to develop any biological assay for molecular genetic studies. Large numbers of meiospores per sporocarp should be readily obtainable from natural populations of the mycobiont. When grown separately, each symbiont should grow rapidly in axenic culture and be easily handled *in vitro*. Simple induction of fructification would make possible genetic analyses. The generation time of both symbionts should be as short as possible. From a practical point of view, molecular work such as DNA isolation should be easily carried out without risking contamination by the other symbiont and without requiring growth in axenic cultures. When necessary, DNA extraction should also be possible directly from field samples or herbarium material.

Although most mutualistic fungi do not conform to all the requirements described above, progress toward developing experimental approaches is still possible. Lichens offer several advantages as experimental systems for coevolutionary studies. One advantage is that most lichens form a well-delimited thallus, which permits easier identification of the symbionts than in other systems, such as mycorrhizae. Since the photobiont of lichens is microbial, this is advantageous for laboratory work compared to the vascular plant photobiont of mycorrhizae. The most serious problem in working with lichens, however, is the generally slow growth of the mycobiont in culture, which requires extended periods of time for resynthesis experiments. This presents a difficulty in obtaining sufficient pure material for analysis. Recent advances have been reported, however, which permit analysis of very small samples for the determination of lichen secondary metabolites (e. g. 7, 8). DNA sequences of the mycobiont can also be obtained from small samples of the lichen thallus, which includes both the mycobiont and the photobiont (12).

The *Omphalina / Coccomyxa* model system

The lichenized Ascomycota represent the largest group of lichen-forming fungi. For this reason it could be argued that the ideal system should be an ascolichen. However, after comparing different ascolichen and basidiolichen systems, *Omphalina* was found to be the best system we can work with at the present. As an experimental system, evolutionary study of ascolichens is likely to be complicated by their ancient origin relative to basidiolichens and subsequent complex phylogenetic history. Within the basidiolichens, the genus *Omphalina* (Agaricales, Tricholomataceae) satisfies many of the requirements of a model system discussed above. Lichenization is believed to have evolved only recently within the Basidiomycota (20), where only 50 of the 16,000 known species of basidiomycetes are lichenized (19). The simplicity of morphological structures within the lichenized species (globules and squamules) also suggests a relatively recent origin of lichenization within *Omphalina*. For these reasons, we chose to study the genus *Omphalina* whose smaller numbers and more recent evolution might facilitate phylogenetic study and permit us to examine lichenization more directly.

As model systems, basidiolichen associations such as those in *Omphalina* may be more similar to ascolichens than they might first appear. For example, *O. hudsoniana* shares the same photobiont (*Coccomyxa icmadophilae* Jaag.) as two well-established ascolichens, *Baeomyces roseus* and *Icmadophila ericetorum* (62). Tschermak-Woess (62) also reported that *Coccomyxa* has been found associated with other well-known ascolichens, including *Nephroma, Peltigera*, and *Solorina*, and is also associated with the lichenized basidiomycete, *Multiclavula*. Thus, the photobiotic part of *Omphalina* is virtually identical to that found in well established ascolichens. These data, along with the ultrastructural characterization of the mutualistic mycobiont-photobiont interaction in lichenized *Omphalina* by Oberwinkler (51), suggest that the mutualistic interaction between lichenized *Omphalina* and the photobiont is similar in many ways to the typical mutualistic association between lichenized Ascomycota and unicellular Chlorophyceae. Phylogenetic studies and molecular genetic experiments on *Omphalina* should, therefore, advance our understanding of the origin and evolution of lichens in general.

In addition to six lichenized species, the genus *Omphalina* includes saprophytic, parasitic, and bryophilous species (20). The basidioma has a typical mushroom shape with decurrent gills. Most lichenized species of *Omphalina* form a crustose globular thallus that has been referred to the lichen genus *Botrydina* (11). Another species, *O. hudsoniana*, forms a squamulose thallus previously referred to the lichen genus *Coriscium* (11).

Acton (1) was the first to propose that the green globular thallus of *Botrydina* was composed of an alga and a fungus. Half a century later, Gams (11) suggested that the fungal partner was a basidiomycete, since he repeatedly observed the presence of the *Botrydina*-type thallus at the base of *Omphalina* basidiomata. Lamoure (33) later demonstrated the presence of dikaryotic hyphae in the mycobiont of the crustose globular thallus similar to those found in basidiomata of *O. ericetorum*. Anatomical features of lichenized *Omphalina* were studied in detail by Oberwinkler (51) using scanning and transmission electron microscopy. He demonstrated the presence of dolipore septae, a uniquely basidiomycete feature, in both the globular thallus of *O. ericetorum* and the squamulose thallus of *O. hudsoniana*.

In pure-culture studies of *Omphalina/Coccomyxa*, species representing both fungus and photobiont have been successfully obtained in axenic culture. The photobiont *Coccomyxa* is easily isolated from the thallus of lichenized *Omphalina* and grows well in axenic culture. This has permitted us to extract DNA from the photobiont without contamination. Although it is not possible to grow the *Omphalina* mycobiont in axenic culture on MEA, most non lichenized *Omphalina* grow quite well on this medium. For molecular analyses, it is possible to obtain sufficient pure fungal tissue of the mycobiont, free of the photobiont partner, from basidiomata collected in nature. We have used this approach to obtain mycobiont DNA for sequence analyses. Because only a small fraction of the basidioma is required for DNA extraction, the rest can be used for morphological and anatomical studies. The ability to analyze naturally occuring basidiomata and lichen thalli also makes it possible to separately analyze genomes of *Omphalina* and *Coccomyxa* arising from a single thallus. For genetic analyses, both lichenized and non lichenized species of *Omphalina* produce large numbers of meiospores relative to ascoma of lichenized Ascomycota, which is a major advantage for genetic and *in vitro* resynthesis experiments. Our preliminary experiments suggest that *in vitro* resynthesis of lichenized species of *Omphalina* is possible and results in the production of a typical globular thallus structure.

Other characteristics of *Omphalina* species may also provide an ideal system for studies on the evolution of sex (asexual versus sexual reproduction) in a mutualistic environment. Law and Lewis (42) concluded that selection against sex for one of the symbionts is intrinsic to mutualism, just as selection for sex in both symbionts is inherent to parasitism. Using the exhabitant-inhabitant principle, they concluded that the selective forces on the inhabitant are mainly, if not exclusively, from the exhabitant, which tends to hold the inhabitant's genome constant by selection against sex. The exhabitant, being subjected to antagonistic environmental selective forces, is selected for sex. These conclusions by Law and Lewis (42) assume that both taxa are initially reproducing sexually and only subsequent to the symbiotic interaction and differential selection (inhabitant versus exhabitant) would the inhabitant evolve toward asexual reproduction. It also implies that clonal reproduction in one of the two symbionts is a condition for mutualism. The possibility of clonal (parthenogenetic) behavior in lichenized *Omphalina* was discussed by Lamoure (34). Further work would present an opportunity to test Law and Lewis' hypotheses.

Little is known about symbiont specificity and dependence of lichenized *Omphalina* species. However, different *Coccomyxa* species are reported as the photobiont of the *Botrydina* and *Coriscium* thallus types (62). Basidiomata of stirps *ericetorum*, for example, are consistently found to be associated with lichenized thalli over l: e geographic areas (11). The inability of lichenized :phalina to be grown in axenic culture (22, 37) might gest some dependence by the mycobiont on its photo- ıt. Of the lichenized species, *O. ericetorum* seems to be htly less dependent on its photobiont than other *Om- ılina* species, since individuals of it growing on *Sphag- n* in coniferous forests often have sparse globules at the e of the basidioma (17, 23).

Far less is known about the biology of *Coccomyxa*. ecies of *Coccomyxa* are known to reproduce asexually ly and to exist both in the free-living aerophytic state d the lichenized state (13, 62). Tschermak-Woess (62) ed twelve species of *Coccomyxa* occurring in lichen alli; three are associated with lichenized *Omphalina* ecies.

Shifts in rates of evolution associated with ·chenization

Beyond estimating phylogenetic relationships, it is also esirable to determine the relative rates of evolution. In- ormation about rates of nucleotide substitutions are es- ential to determine if different fungal or algal lineages volve at different rates, and if these differences correlate with the lichenization process. For the algal and fungal ineages that are cospeciating, it is possible to compare the rates of evolution between the alga and the fungus. The estimation of expected number of nucleotide substitutions for each lineage along with the ability to test constancy of the rate of nucleotide substitutions is critical in this context. Combined with other approaches in comparative biology, maximum likelihood methods seem destined to play an important role in understanding coevolutionary processes (18).

Life history features associated with lichenization

A number of life history features of *Omphalina* species appear to be associated with the transition to a lichenized state (Fig. 2). These include the loss of clamp connections and tetrasporic basidia, and the loss of the ability to grow in axenic culture on MEA (35, 36, 38). Another life history pattern associated with lichenization is the transition from a typically dikaryotic to a uninucleate state (Table 4). The life cycles of non lichenized *Omphalina* include a dikaryotic stage, whereas, there appears to be a trend within the lichenized *Omphalina* toward losing this stage since basidiomata are often uninucleate (38, 39). Populations of *O. ericetorum* have the highest proportion of dikaryotic individuals, with *O. luteovitellina* being intermediate and *O. hudsoniana* populations having the lowest proportion (Table 4). Phylogenetic analyses (Figs. 2 and 3) suggest that the dikaryotic stage is an ancestral state within the *ericetorum* group, and that its evolution is towards uninucleate basidiomata. The loss of a dikaryotic stage is more accentuated in alpine and subarctic populations than in high arctic populations (Table 4).

The presence of uninucleate basidiomata in lichenized species of *Omphalina* raises the question of whether these strictly uninucleate individuals are haploid or diploid. If uninucleate individuals are haploid, parthenogenesis may have evolved due to selective pressure associated with the symbiotic interaction, as suggested in Figure 2. This would agree partly with the hypothesis by Law and Lewis (42) that asexual reproduction within at least one symbiotic partner is required to maintain mutualism, whereas sexual reproduction is required in both partners for parasitism to be maintained in the host/parasite interactions. In the case of *Omphalina*, however, both symbionts (the inhabitant and exhabitant *sensu* Law and Lewis, 42) might be reproducing asexually.

Table 4. Transition from a dikaryotic state to a uninucleate state within the stirps *ericetorum* of alpine and subarctic populations versus high arctic populations (based on data from Lamoure, 39); "n" corresponds to the number of specimens sampled. The evolutionary polarity was revealed by parsimony analysis on the morphological and anatomical data (Fig. 2). The positions of *O. hudsoniana* and *O. luteovitellina* along the evolutionary axis are interchangeable since they are sister species.

	Species	Localities	One nucleus		Two nuclei	
			n	%	n	%
EVOLUTION ↑	*O. hudsoniana*	Alpine and subarctic	76	100.0	0	0.0
		High arctic	27	93.1	2	6.9
	O. luteovitellina	Alpine and subarctic	33	82.5	7	17.5
		High arctic	15	42.9	20	57.1
	O. ericetorum	Alpine and subarctic	137	76.1	43	23.9
		High arctic	93	37.2	157	62.8
	Non lichenized *Omphalina*			0		100

The *Omphalina*/*Coccomyxa* model system has many desirable characteristics for coevolutionary studies on mutualism. It offers the potential to address a broad range of fundamental questions at the molecular, population, and species levels that are critical for a thorough understanding of the evolutionary processes associated with the origin and evolution of lichenization. Coupled with a comparative approach (18), phylogenetic studies of both symbionts will generate new hypotheses and suggest appropriate taxa for future molecular genetic studies.

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