Annu. Rev. Microbiol. 2004. 58:273–301
doi: 10.1146/annurev.micro.58.030603.123730
First published online as a Review in Advance on May 21, 2004

EARLY MOLECULAR INVESTIGATIONS OF LICHEN-FORMING SYMBIONTS: 1986–2001*

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Key Words fungi, algae, symbiosis, coevolution, molecular systematics

■ **Abstract** From the mid-1980s the symbionts in lichen associations, heterotrophic fungi and photosynthetic algae or cyanobacteria, were the subject of increasing numbers of molecular investigations. Many of the studies examined the phylogenetic placement of the individual symbiotic partners with their free-living relatives, refining their nomenclature and classification. Resulting phylogenies permitted the mapping of transitions to and from lichenization and stimulated discussion of the relative ease of gaining and losing symbiotic lifestyles. Comparing symbiont phylogenies both rejected strict cospeciation and mirrored phylogenies and hinted at more complex forces of coevolution, including symbiont switching and selection. Studies at the species and population levels examined patterns of species delimitation and geographic dispersion and processes such as gene flow, self-fertilization, and founder effect. Significant genetic variation often was associated with mobile elements, group I and spliceosomal introns. This review examines the influence of molecular investigation on lichenology during this first 15 years.

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INTRODUCTION

Lichens are the symbiotic associations of fungi with photosynthetic algae or cyanobacteria. Typically, these filamentous and unicellular organisms associate to form undifferentiated plant-like structures referred to as thalli (Figure 1). The thalli, in varying morphological forms, colors, and sizes, are completely different from either symbiotic partner grown in axenic culture. Because of the uniqueness of these lichen morphologies, lichens traditionally are studied as a natural group and by specialists referred to as lichenologists. However, the scientific name frequently applied to lichens correctly refers to the dominant fungal partner, *Cladonia rangiferina*, which is a species of lichen-forming fungus. In the past century one of the challenges of lichenology has been to integrate these fungi and photosynthetic partners with their nonlichenized relatives. However, given the often-deceptive and plastic morphologies and well-known resistance to culturing and experimental crossing of these lichen symbionts, such an integration was difficult until the advent of modern molecular techniques. This review examines the first 15 years (1986–2001) of molecular investigations of lichens, which place the symbiotic partners with their free-living relatives.

THE NATURE OF THE LICHEN SYMBIOSIS

"Zusammenleben ungleichnamiger Organismen," literally differently named organisms living together, is the simple way Heinrich Anton DeBary defined symbiosis, a term coined by the lichenologist Albert B. Frank in 1877. Lichens are symbiotic associations in which lichen-forming fungi and algae live together, as was first noted in 1867 by Simon Schwendener. This definition of symbiosis encompasses a range of intimate relationships, with parasitism, commensalism, and mutualism providing benchmarks on a continuous scale. As Schwendener did in his original description, Ahmadjian (Reference 1 and citations therein) termed the association a "controlled parasitism" in which the photobiont is a "victim" rather than a partner. As a heterotroph, the fungal partner (mycobiont) depends on the

algal partner (photobiont), a photosynthetic autotroph, to provide fixed carbon. Benefit to the photobiont has not been demonstrated experimentally, but it is proposed to include protection by the surrounding fungal mantel, or mineral nutrition supplied by the fungal hyphae. Whether classified as a mutualism or a controlled parasitism, this association is stable, self-supporting, and self-reproducing.

DEVELOPMENT OF MOLECULAR TECHNIQUES IN LICHENOLOGY

Before the pivotal year 1995, fewer than 20 studies in lichenology had used molecular-genetic techniques. In the first published study in 1986, Blum & Kashevarov (16) used DNA:DNA hybridization to support the distinctiveness of the fungal genera *Umbilicaria* and *Lasallia* (15, 20, 73). In the next year Ahmadjian et al. (2; also see 1) described protoplast and DNA isolation from cultured lichen-forming fungi. During the next seven years many ongoing lines of research were initiated. Kardish et al. (70) and Leizerovich et al. (79) examined cyanobacteria in lichen associations. Armaleo (4) designed a culturing system to allow mutagenesis, selection, and transformation. Armaleo & Clerc (5) showed that a single fungal species forms the two morphologically different parts of a lichen chimera. DePriest (31, 32) developed nuclear ribosomal DNA (nu rDNA) group I introns as genetic markers. Gargas & Taylor (52; also see 120) proposed fungal-specific primers for the polymerase chain reaction (PCR) that are still in use today, and they produced the first nuclear small subunit (nuSSU) rDNA analyses of lichen-forming fungi. Niu & Wei (108) used nu rDNA internal transcribed spacer (ITS) sequences to compare species of lichen fungus *Lasallia*. Friedl & Zeltner (48) produced the first nuSSU rDNA phylogenies including lichen-forming algae.

The year 1995 was a watershed for molecular studies in lichenology, as both the number of studies and the amount of available data increased rapidly. The publication of the proceedings from two international meetings (128a, 138a) spurred a number of articles on lichenology (19, 33, 91, 92, 129, 130). In the same year Armaleo & Clerc (6) and Grube et al. (59) published improved techniques for isolating DNA from lichens. Gargas et al. (51) cataloged the 17 known positions of group I introns in the nuSSU rDNA and published the first large phylogenies to show the relationship of lichen-forming fungi to their nonlichenized counterpoints (50, 53). Friedl (44) did the same for lichen-forming algae. Eriksson & Strand (41), in a small phylogenetic study of the order Peltigerales, began the process of redefining the order Lecanorales, the major group of lichen-forming fungi.

After 1995 the number of molecular studies increased tremendously, with 90 studies in the next five years compared with 30 in the first ten. However, in this period most studies focused on producing rDNA sequences for phylogenetic analysis. In general, nucleotide sequences from four regions of the rDNA were examined and applied to studies at different taxonomic levels: (*a*) conserved nuSSU rDNA sequences for division to family level phylogenies, (*b*) conserved and

variable nuclear large subunit (nuLSU) rDNA sequences for order-to-species-level phylogenies, (*c*) variable nu rDNA ITS for species delimitations and population differentiation, and (*d*) optional group I introns and spliceosomal introns of the nuSSU rDNA for genus-to-population-level phylogenies. Subsequent studies examined sequences of mitochondrial SSU and LSU rDNA, protein-coding genes, and anonymous sequences. All these studies have addressed two major problems in applying molecular techniques to lichen symbionts: (*a*) extracting high-quality DNA from natural lichens or cultured symbionts (22, 25, 28, 94, 102, 117, 148) and (*b*) separating the fungal from algal DNA extracted from natural lichens (25, 36, 39, 49, 64, 112, 119, 121, 150).

FUNGAL PARTNERS IN LICHEN ASSOCIATIONS

The formation of lichen associations represents one of the most successful lifestyles among the fungi. Representing almost 20% of the 65,000 described fungi, species that form lichen associations equal or outnumber those that form parasitic associations (20%) or mycorrhizal associations (8%), and they are exceeded only by saprobic decomposers (50%). Almost all the 13,500 lichen-forming species are ascomycetes; only ~50 are basidiomycetes. (Although lichen-like associations have been reported in Actincomycota, Mastigomycota, and Myomycota, these groups are no longer considered true fungi and are not discussed here.) Given that lichen-forming fungi represent 40% of all described ascomycetes, the lichen-forming species are integral to understanding ascomycete relationships (Figure 2). Before the advent of molecular studies, ascomycetes were classified on the basis of their reproductive structures. This system divided fungi into traditional classes such as apothecial Discomycetes, cleiostothecial Plectomycetes, and perithecial Pyrenomycetes, with asexual forms classified as anamorphic Deuteromycetes. Classification using molecular phylogenies allows researchers to abandon these classes as paraphyletic or modify them to form monophyletic groups (i.e., 53, 83, 125), and a new phylogenetic system has been proposed by Eriksson & Winka (42).

Origins of Lichenization in the Fungi

One major question asked with molecular data has been, How many times does the lichen association originate in the fungi? In the first parsimony analysis to include nuSSU sequences from lichen-forming taxa within a comprehensive sampling of ascomycete and basidiomycete fungi, Gargas et al. (50) resolved five lineages of lichen-forming fungi interpreted as independent origins of lichen symbiosis. Three of these independent origins occurred among the few lichen-forming basidiomycetes represented by *Multiclavula mucida*, *Omphalina umbellifera*, and *Dictyonema pavonia* and seem to reflect recent and opportunistic shifts in lifestyle. (There may be additional origins; other basidiolichens such as the algal parasitic

Athelia remain to be examined.) In the ascomycetes, Gargas et al. (50) noted a phylogenetic separation of two groups forming lichens: (*a*) the Arthoniales and allies and (*b*) the Lecanorales and allies. On the basis of equal costs for gain and loss, they proposed that these two lineages, and potentially others, represent independent origins of lichenization.

In response, Aptroot (3) tallied as many as 12 independent origins and 24 losses of lichenization among the pyrenomycetous lichen fungi. Using additional lichen taxa, new gene sequences, nuLSU sequences, and new phylogenetic methods, Lutzoni et al. (90) subsequently agreed with Gargas et al. (50) on the separation of the Arthoniales and Lecanorales, and they recognized a number of additional phylogenetically related lichenized groups as noted in other analyses (35, 83, 87, 113, 114, 122, 123, 125, 127, 132, 145, 146). However, Lutzoni et al. (90) interpreted that it was easier (more common) to lose than to gain the lichen habit, which had been gained once [or almost as likely, twice (as described in Reference 50)] and lost at least four or five times among the sampled lineages. In this interpretation lichen-forming fungi were ancestral and paraphyletic—giving rise to nonlichenized groups. However, some phylogenetically intermediate, nonlichenized lineages—Dothidiomycetes, Dothidiomycetes incertae sedis, and Mycocaliciales (see 123)—were not included. Furthermore, this interpretation requires that equally specialized fungal lifestyles, i.e., plant pathogens, animal pathogens, and mycorrhizae, be more frequently and more easily gained because they are derived multiple times from lichenized ancestors.

Phylogenetic Classification

Traditionally, systematics encompasses two related activities: the taxonomy of homologous units (i.e., species) on the basis of their differences and the classification of hierarchical groups (classes, order, families, etc.) on the basis of their shared similarities. Molecular techniques have contributed to both of these activities, but especially to classification through phylogenetic analysis. Phylogenetic concepts demand that groups formally recognized and named in classification schemes be monophyletic, i.e., they must include all descendants of a common ancestor. Phylogenetic techniques such as cladistics and likelihood analysis find the most parsimonious trees, and bootstrap, jackknife, and Bayesian analyses provide comparative support. To apply names to well-supported monophyletic groups, it is critical that nomenclatural types be represented in the analyses. Therefore, most meaningful are those phylogenies that use type genera and type species (if possible), that use specimens that have been expertly identified and available for study through deposit in herbaria, and that use multiple and diverse representative taxa.

STATISTICAL SUPPORT FOR CLASSES AND ORDERS Some studies (91, 130) have discussed the problems of basing ascomycete classification primarily on parsimony analysis of a single gene, the nuSSU rDNA, that may not have sufficient information content to fully resolve relationships. Subsequent analyses using more

variable nuLSU sequences alone (84) or in combination with nuSSU and protein-coding genes have supported class and order relationships found in the nuSSU phylogenies (90, 113, 114). However, even when groups are recognized in many independent analyses, bootstrap or jackknifing methods may not provide strong statistical support. For example, Tehler et al. (132) reported phylogenetic analyses of large fungal rDNA data sets from the Ribosomal Database Project (485 sequences) and rRNA Web server (785 sequences) on the basis of parsimony jackknifing analyses. These analyses supported as monophyletic many traditional fungal groups. However, neither the Ascomycetes nor the Lecanoromycetes and Lecanorales (including a group now classified as the Mycocaliciales) were supported with the Ribosomal Database Project data set. In contrast, using a Bayesian approach with maximum likelihood analysis, Lutzoni et al. (90) found strong support (>95%) for the order Lecanorales, but not the class Lecanoromycetes. Re-examination using Bayesian analysis of the alignment generated by Gargas et al. (50) produced support (100%) for the Lecanorales and the Lecanoromycetes (P.T. DePriest & A. Gargas, unpublished results). The question remained, What type and level of statistical support is required to recognize a monophyletic group?

CLASSIFICATION OF LICHEN-FORMING ASCOMYCETES nuSSU rDNA, more recently supplemented by nuLSU, has provided a preliminary outline of phylogenetic relationships and classifications of lichen-forming ascomycetes (reviewed in 83). Lichen-forming fungi occur in five classes of ascomycetes (42) and at least three orders that cannot yet be placed in the class system. These classes are the Arthoniomycetes, including Opegraphales (81, 104, 106, 129, 130, 133); the Chaetothyriomycetes, represented by the Verrucariales [found with ITS (65)] and, possibly, by the Pyrenulales (90); the Dothidiomycetes, represented by *Arthrorhaphis* of the Patellariales, and by incertae sedis genera *Eopyrenula* and *Pyrenocollema*; the Orbiliomycetes, represented by a few lichenized species of *Orbilia*; and the Lecanoromycetes, representing the largest class of lichen-forming fungi (127).

The incertae sedis orders that include lichen-forming fungi are the Licheniales, Trichotheliales, and Umbilicariales. The Licheniales was examined with nuSSU rDNA (122) and represent a monophyletic group separate from the Lecanorales. On the basis of analysis of nuSSU and nuLSU sequences, they may be basal to the Arthoniomycetes and Sordariomycetes (90). The order Trichotheliales, represented by only one nuSSU rDNA sequence from *Porina guentheri*, is of uncertain placement in the Dothidiomycetes et Chaetothyriomycetes incertae sedis. The Umbilicariales has been excluded from the Lecanoromycetes on the basis of nuSSU and nuLSU sequence analysis (35, 90, 123, 127), and its two genera have been paraphyletically intermixed on the basis of ITS sequences (67, 68, 108). Most analyses place this group as basal to the lineage containing the Eurotiomycetes, Chaetothyriales, and some Dothidiomycetes or, more rarely, as basal to the Lecanoromycetes. The nonlichenized Mycocaliciales (139), previously placed in the Caliciales, is also a member of the Eurotiomycetes-Chaetothyriomycetes lineage (50, 52, 144–146).

CLASSIFICATION OF THE CLASS LECANOROMYCETES On the basis of a phylogenetic analysis by Stenroos & DePriest (127), Eriksson & Winka (42) recognized the class Lecanoromycetes as including members of the lichen-forming orders Lecanorales, Agyriales, Ascarosporales, Gyalectales, Ostropales, and Pertusariales (85, 87, 90, 113, 114, 127, 147; but see 132). The latter five orders are either a monophyletic sister group or paraphyletic and basal to the Lecanorales (or the Eurotiomycetes lineage). The class Lecanoromycetes is often found to be monophyletic in both parsimony and likelihood analyses, and it is strongly supported by Bayesian analysis (90), but not by bootstrap or jackknife analysis (127, 132). It is a sister clade to a lineage typically including the Chaetothyriomycetes, Eurotiomycetes, Mycocaliciales, and Umbilicariales (127).

Resolution of the relationship of the Lecanorales to the other orders of class Lecanoromycetes requires more analysis. The resurrected order Agyriales was supported as a separate order within the Lecanoromycetes with analyses of nuSSU rDNA (87) and ITS sequences (86), although its classification and nomenclature could not be resolved without sequences from the type genus *Agyrium*. The orders Ascarosporales and Gyalectales were placed in the Lecanoromycetes on the basis of the few nuSSU and nuLSU rDNA sequences available by 2001 (90, 127); at that time the size and relationships of these groups were largely unknown. The order Ostropales, comprising lichenized and nonlichenized species, examined with nuSSU rDNA sequences includes the Graphidales (147), and species relationships within its genus *Diploschistes* have been examined with nuLSU sequences (95). For the order Pertusariales, phylogenetic analyses of the nuSSU and nuLSU rDNA (85, 90, 113, 114, 126) required a new circumscription to include the Icmadophilaceae (including *Dibaeis* and the asexual *Siphula* and *Thamnolia*) (90, 127) and the Baeomycetaceae (113, 114). Both these groups were previously placed in the Helotiales (Leotiales), although they are now recognized as phylogenetically divergent.

CLASSIFICATION OF THE ORDER LECANORALES The Lecanorales with ~8000 species is the largest order of lichen-forming fungi and one of the largest orders of fungi. Gargas et al. (50, 53) and later Wedin et al. (141, 144–146) demonstrated that a monophyletic Lecanorales includes representatives of the Sphaerophoraceae and Caliciaceae formerly placed in the order Cladiciales, but it excludes the group now called Mycocaliciales (families Mycocaliciaceae and Sphinctrinaceae) (see 139). Stenroos & DePriest (127) identified a core group of Lecanorales including the Peltigerales and Teloschistales, but they excluded representatives of three of its suborders (131): Acarosporales, Agyriales, and Umbilicariaceae. Although the original analyses had low bootstrap and jackknife support for this order, it was strongly supported on the basis of a Bayesian analysis of the original data set (P.T. DePriest & N. Hoffmann, unpublished results) and combined nuSSU and nuLSU sequences (90).

Several families in the Lecanorales have been examined in molecular-phylogenetic studies. The family Lecanoraceae is paraphyletic on the basis of nuSSU

rDNA sequence analysis (40), and on the basis of ITS sequence analysis it includes the monotypic Australian genus *Ramalinora* despite their different ascal morphologies (78). The type genus *Lecanora* includes the lobate *Placodium* species, but not the asexual *Lecanora demissa*, now called *Caloplacda demissa* (8, 9). Although members of the family Bacidiaceae have been synonymized with Lecanoraceae, on the basis of nuSSU rDNA sequence analysis it is monophyletic but unrelated to the Lecanoraceae (40). The fruticose families Cladoniaceae, Cladiaceae, and Stereoculoaceae have been examined in nuSSU rDNA sequence phylogenies. Stenroos & DePriest (127) demonstrated that each of these families is paraphyletic without reclassification and exclusion of some taxa. Wedin et al. (141, 142) proposed that Cladoniaceae should include the Cladiaceae and Heterodeaceae, the genus *Pilophorus*, and possibly the family Stereocaulaceae, but should exclude the genera *Neophyllis* and *Austropeltum*.

Members of the family Parmeliaceae have been examined by ITS sequences alone (24, 26, 71, 96, 97, 134, 136, 138) and in combination with nuSSU rDNA (143) or homologous group I intron sequences (135, 137). Mattsson & Wedin (96) and Wedin et al. (143) supported the monophyly of the Parmeliaceae including representatives of the Alectoriaceae, the Hypogymniacea, and the genus *Usnea*. Both the parmelioid genera, previously classified as *Parmelia* s. lat. (24, 26), and the cetrarioid genera, previously classified as *Cetraria* s. lat. (71, 134–138), appear polyphyletic. Eriksson & Strand (41) used nuSSU sequences to place three representatives of Peltigeraceae as basal to the Lecanorales and proposed that *Peltigera* and *Solorina*, but not *Nephroma*, are closely related. Subsequently, ITS sequences (54, 55, 56) and a combined data set of chemical, morphological, and nuLSU sequence (98) have been used to examine relationships within the genus *Peltigera*. Members of the family Physciaceae have been examined with ITS sequences (58, 80) with some genera shown to be monophyletic (*Physcia*, *Phaeophyscia*, and *Physconia*) and others paraphyletic (*Anapthycia*, *Buellia*, and *Rinodina*). Members of the family Teloschistaceae were examined with nuSSU rDNA analysis, which showed that the family is not related to either the Physciaceae (144) or the Umbilicariaceae (127). Relationships among the genera *Xanthoria* and *Fulgensia* have been examined with ITS sequences alone (43) and in combination with nuLSU sequences and their spliceosomal introns (72), respectively.

CONGRUENCE OF MORPHOLOGICAL CHARACTERS Some morphological characters have been rejected as homologous on the basis of molecular-phylogenetic analyses. In the simplest example, Stenroos & DePriest (127; also see 8, 104) showed that the division into simple growth forms, i.e., crustose, foliose, and fruticose, is not phylogenetically meaningful. Furthermore, for fruticose forms, structures analogous to podetia are of independent origins, and the term true podetia—lichenized generative tissues supporting reproductive structures—should be limited to a few genera in Cladoniaceae (127, 141, 142). In particular, fungal reproductive structures have features traditionally used for classification that are not supported as homologous. The presence of boundary tissue, a pigmented tissue between

generative and vegetative tissue in the reproductive structures, is not homologous, although a later study proposed its use in a restricted way (37, 142). Similarly, the reproductive structures previously used to define the polyphyletic Caliciales, mazadia with passive dispersal of spores from thin-walled prototunicate asci, have been derived on at least three occasions from typical dehiscent, spore-shot asci (144–146). Ascal-tip types have played important roles in the classification of genera families and suborders of the Lecanorales. However, some unrelated groups share ascal types, for example, the families Lecanoraceae and Bacidiaceae (40). Furthermore, ascospore types proposed for classification within the Physciaceae were of limited use in only a few groups (58).

Delimiting Species: Relationships and Identity

Many species in lichenology, predating the concept of Darwinian evolution, reflect phenetic and not modern species concepts. Before the era of molecular systematics, few lichenologists explicitly stated a species concept for fungal symbionts outside of the "species counterparts" [or "Artenpaare" (here called species pair)] and the chemospecies concepts, both of which identify characters that qualify a species for recognition—reproductive propagules or secondary product chemistry, respectively. Species pairs and chemospecies have been supported as distinct on the basis of edaphic preferences (an ecological species criterion) and as conspecific and interbreeding on the basis of gene flow among sympatric chemospecies (a biological species criterion). Whereas DePriest (31–33) applied a species diagnosability criterion, Kroken and colleagues (62, 76) used a genealogical criterion. With the increasing availability of molecular sequence data, a monophyly criterion has been applied to species pairs, chemospecies, and even cryptic species. Bridge & Hawksworth (18; also see 17) and Grube & Kroken (62) have reviewed the use of molecular approaches for delimiting lichen species.

ITS AS A PHYLOGENETIC SPECIES MARKER Ribosomal ITS has been the main tool used to examine relationships at the species level. The first published ITS1 sequences from lichen symbionts were those by DePriest & Been (34) from the *Cladonia chlorophaea* complex and its algal partner *Trebouxia ericii*, and Niu & Wei (108) were the first to use ITS in a systematic study comparing ITS2 sequences among two species of *Lasallia* in the Umbilicariales. In this period, researchers have examined several genes: (*a*) variable rDNA genes including group I introns (31), nuclear rDNA (149), and mitochondrial rDNA (149, 150) as well as (*b*) protein coding genes including β-tubulin (107; also see 68), histone 3 (149), chitin synthase I (76), and anonymous markers (76). Yet few genes have demonstrated ITS's level of reliability and variability. However, excessive ITS sequence variation among species, genera, and families has created problems with ambiguous alignment of sequences and finding outgroups for rooting phylogenetic analyses (76, 90). Although one approach is to exclude the ambiguous regions (88, 93), Myllys et al. (106) demonstrated that even these regions contain phylogenetic structure;

similar results were reported in other studies of alignments (86, 95, 132). Additionally, different coding of the alignment gaps, as missing or fifth-character states, did not substantially alter phylogenetic results (29, 80, 86).

An early study by DePriest & Been (34) reported substantial (10%) nucleotide sequence variation in ITS1 between chemospecies in the *Cladonia chlorophaea* complex. However, studies of subsequent ITS phylogenies (112) suggest that these chemospecies are actually phylogenetically distant despite apparent evidence for their interbreeding. Similarly, the highly variable ITS sequences from *Omphalina* reflect, in part, its polyphyletic nature; the genus encompasses at least three widely divergent lineages (101).

More recently Groner & LaGreca (57) found no nucleotide sequence differences in ITS between *Ramalina panizzei* and *R. fastigiata* and only two nucleotide differences (<1%) between chemotypes of *R. siliquosa*. Ivanova et al. (68) reported <1% variation between samples of *Umbilicaria deusta*, compared with 3 to 14% among species within the family Umbilicariaceae. Lohtander et al. (81) reported <2% variation in ITS sequences for geographically dispersed individuals of *Roccellina capensis*. Mártin et al. (95) demonstrated on the basis of ITS comparisons that *Diploschistes ocellatus* var. *almeriensis* was an extreme morphological modification within a monophyletic *D. ocellatus*. However, other studies (29) have reported phylogenetically defined species with significant variation, up to 16%.

PHYLOGENETIC ANALYSIS OF CHEMOSPECIES AND SPECIES PAIRS Phylogenetic analysis of molecular characters has shown that species defined by either chemospecies or species-pair concepts do not always represent separate species. Using phylogenetic analysis of 13 nuSSU rDNA repeat types, DePriest (33) examined sympatric and interbreeding chemotypes of *C. chlorophaea*. Likewise, using analysis of ITS sequences, LaGreca (77) examined eight morphologically indistinguishable chemical races of *Ramalina americana*. In both studies, monophyletic species consisted of multiple chemical races, a finding consistent with chemical polymorphism among interbreeding populations. Lohtander et al. (80, 82) and Myllys et al. (105–107) analyzed variable sequences from sexually and vegetatively reproducing forms of *Dendrographa leucophaea* and *Roccellina capensis* as well as species pairs of *R. canariensis* and *R. tuberculata*, *Physcia distorta* and *Physcia detersa*, and *Physcia aipolia* and *Physcia caesia*. They supported the repeated, even frequent, origin of the vegetatively reproducing forms with species pairs interpreted as members of a single phylogenetic species. For the *P. aipolia* and *P. caesia* pair, combined analysis of ITS, group I intron, and partial β-tubulin sequences supported that vegetative forms had evolved at least twice; and for *R. capensis*, analysis of ITS sequences and randomly amplified polymorphic DNA (RAPD) patterns supported that vegetatively reproducing forms were derived in parallel at different geographical locations. Kroken & Taylor (76) used a genealogical criterion to examine species boundaries in the sympatric species pair *Letheria columbiana* and *L. vulpina*. Combined analysis of nucleotide sequence data from 12 loci, including ITS, rDNA intron, chitin synthase I, and 10 anonymous

loci, predicted at least five strongly supported species and one paraphyletic metaspecies.

GENOTYPIC MARKERS FOR SPECIES AND POPULATIONS Molecular characters provide discrete genetic markers to examine variability and polymorphism within species, interbreeding groups, and populations. DePriest (31, 32) reported rDNA length and restriction site variation due to optional group I introns (34) and high levels of gene diversity (average heterozygosity = 0.931) within populations of four sympatric chemotypes of the *Cladonia chlorophaea* complex. Even small colonies or mats had distinct genetic individuals and moderate gene diversity (average heterozygosity = 0.38). In contrast, Beard & DePriest (10) found that mats of *Cladonia subtenuis* have a single rDNA repeat type (average heterozygosity = 0) and most likely are a single genetic individual. Crespo et al. (21, 23) reported similar rDNA variation within *Parmelia sulcata* from 32 collecting sites and later (22) across a single lichen thallus. Therefore, rDNA length variation cannot be used to discriminate species, despite a proposal to use this characteristic in diagnostic keys (63).

RAPD markers, which are more sensitive than sequence markers, have been used to examine issues of population variation (62, 81). However, it is difficult to verify homology of RAPD bands and to separate the fungal and algal contributions in samples of thalli. Murtagh et al. (102) published a protocol for identifying algal contributions to RAPD fingerprints of lichen thalli by comparing DNA extracted from axenic mycobiont cultures against the whole thalli from which the cultures were isolated. Murtagh et al. (103) used this protocol to suggest that two lichens, *Graphis scripta* and *Ochrolechia parella*, were self-fertilizing (homothallic) because single-spore progeny from the same ascoma, which must share at least the same maternal individual, were typically monomorphic. Variances in RAPD fingerprints among sporelings from different ascomata of a single thallus were interpreted as support for the presence of multiple fungal genotypes in the thallus tissue, but they also seem consistent with outcrossing among genetically distinct thalli.

GENETICS OF CONSERVATIONS AND REINVASION Such sequence variation provides a much-needed genetic marker for studies of threatened lichens. Zoller et al. (149) examined ITS and nuLSU sequence variation in six populations of a threatened lichen, *Lobaria pulmonaria*, that has suffered decline in Switzerland. This study suggested that conservation priority be given to even small sexually reproducing populations because sexual reproduction is correlated with increased genetic variation. Similarly, Dyer & Murtagh (38) used ITS sequence variation to show the minimal variation (0.2% and 0.1% divergence) between populations of two lichen species, *Buellia frigida* and *Xanthoria elegans*, from continental Antarctica, an ecosystem currently subject to climate changes. The authors suggested that the limited genetic variation within populations may affect their survival ability.

Genetic markers have also been used to date invasions and dispersions, whether historical or geological. Heibel et al. (64) used RAPD markers on fungal tissue

to suggest that *Usnea filipendula* reinvading formerly polluted areas was not limited in genetic variation but reinvaded from heterogeneous sources. In contrast, Crespo et al. (23) found in *Parmelia sulcata* a lower genetic diversity (only the most common genotype was present) in recolonizing sites compared with long established sites, consistent with a bottleneck or founder event. This reduced diversity was interpreted as a genetic response to environmental pollution. Printzen et al. (115, 117) used molecular-genetic markers to predict that the distribution of *Biatora helvola* tracked the glacier-associated retreats and advances of European *Picea abies* forest in the late Cretaceous and Tertiary periods. Additionally, ITS sequences from this species and subtropical *Phyllopsora* (116), whose divergence presumably dates to the separation of Laurasia from Gondwana, were used to calibrate diversification of arctic-alpine *Biatora* to the mid-Tertiary.

ALGAL PARTNERS IN LICHEN ASSOCIATIONS

Because lichens were recognized as a dual organism in the nineteenth century, microscopic examinations suggested that relatively few but diverse algae formed lichen symbioses. At present, an estimated 100 species in 40 genera are reported to form lichen symbioses. They are placed with free-living relatives in at least five phylogenetically divergent classes: the prokaryotic Cyanophyceae, the eukaryotic Tribophyceae (Xanthophyceae), Fucophyceae (Phaeophyceae), Chlorophyceae, and Trebouxiophyceae (46). As with lichen-forming fungi, algal lichenization apparently has arisen independently in each of these unrelated groups. The term photobiont is used here for all photosynthetic lichen partners; phycobiont is reserved for the eukaryotic algae and cyanobiont for the prokaryotic cyanobacteria.

Cyanobionts of Lichen Associations

An estimated 10% of the lichen-forming fungi (~150 species in 58 genera) form associations with cyanobionts, which provide fixed nitrogen along with photosynthetically fixed carbon. Lichen cyanobionts are classified in 14 to 16 genera in four diverse orders (46, 140), including *Nostoc*, which forms symbiotic associations with bryophytes, ferns, cycads, and *Gunnera*. However, modifications in morphology and life cycles of cyanobionts in lichen associations, including an increase from 10% to 35% in heterocysts that fix nitrogen, make axenic culturing and molecular techniques necessary for their comparison with symbiotic and free-living cyanobacteria. Early comparisons by Kardish et al. (70) and Leizerovich et al. (79) using Southern hybridization detected differences between cultured and symbiotic *Nostoc* of a single lichen thallus and complex hybridization patterns consistent with multiple genotypes in lichen associations. Using SSU rDNA sequences, Miao et al. (100) demonstrated that the cultured *Nostoc* cyanobionts from colormorphs of *Peltigera membranacea* were different from those detected in the symbiotic thallus. These studies suggest that individual lichen associations with

Nostoc may contain a major and a minor cyanobiont or contaminating cyanobacteria.

Bipartite and Tripartite Associations

Cyanobionts occur in two types of lichen associations: a two-part symbiosis, called bipartite, with a continuous layer of photosynthetic and nitrogen-fixing cyanobionts and a three-part symbiosis, called tripartite, with warty, nitrogen-fixing cephalodial cyanobionts. In the latter, a second eukaryotic photobiont forms the continuous photosynthetic layer. Initially studies using $tRNA_{UAA}^{Leu}$ group I introns suggested that each thallus, whether bipartite or tripartite, had a single cyanobiont and was a single symbiotic individual (109, 110). Subsequently, Paulsrud et al. (111) showed that cephalodia from a single thallus had different cyanobionts. They (111) and Miao et al. (100) also showed that lichen fungi form associations with distinct cyanobionts in colormorph thalli. Although initial studies reported different cyanbionts forming bipartite and tripartite association (109), further studies by Paulsrud et al. (110, 111) demonstrated the same cyanobiont formed bipartite and tripartite associations, even in connected bipartite and tripartite lobes called photosymbiodemes, or chimera. This suggests that, under the influence of the fungal partner or environmental conditions, cyanobionts switch their functional roles in symbioses.

Phycobionts in Lichen Associations

The majority of lichen-forming fungi form associations with eukaryotic phycobionts that are classified in 25 to 28 genera from four diverse classes: Tribophyceae, Fucophyceae, Chlorophyceae, and Trebouxiophyceae (46). The most common phycobiont genus, *Trebouxia*, is present in approximately 20% of all lichen species. The second–most common genus, *Trentepholia*, is present especially in members of the orders Arthoniales, Ostropales, and Pyrenulales (118) and is more limited in its substrate preference for bark and living leaves. Unlike the cyanobionts, these algae are not known from other symbiotic associations, although *Trentepholia* is often free living. The genera are morphologically distinctive, e.g., *Trebouxia* has a lobed or star-pointed central chloroplast that contains several pyrenoids. However, within these lichen associations some morphologies are altered and sexual stages suppressed. In fact, culturing is often required for species identification. More recently, design of algal specific primers for nu rDNA (12, 36, 66, 112; but see 121), nuclear actin gene (75), and chloroplast rubisco gene (119) has allowed molecular-genetic analysis of photobionts from natural lichen associations.

PHYLOGENETIC RELATIONSHIPS OF *TREBOUXIA* In the first molecular-phylogenetic study to include a lichen phycobiont, Kantz et al. (69) used partial nuSSU and nuLSU rDNA sequences to show that *Trebouxia gigantea* (as *Pseudotrebouxia*) was a sister taxon to the soil alga *Myrmecia israelensis* (as *Friedmannia*). Expanding this study, Friedl & Zeltner (48) used complete nuSSU rDNA sequences to

define a monophyletic Lichen Algae Group. Friedl (44), Friedl & Rokitta (47), and Bhattacharya et al. (13) recognized three lineages within this group formally described as the order Trebouxiales: *Trebouxia* and *Myrmecia*, *Dityochloropsis* and *Chlorella* p.p. (121), and *Leptosira* (as *Pleurastrum*). Each lineage was proposed as an independent origin of lichenization, with the soil alga *M. israelensis* representing a loss of the symbiotic state. The genus *Trebouxia* was revised on the basis of nuSSU and nuLSU rDNA phylogenies to include the autospore-forming *Pseudotrebouxia*. Some species excluded from *Trebouxia* were placed morphologically with *Asterochloris phycobiontica* (44, 47, 48, 118), a placement supported by the ITS phylogeny of Piercey-Normore & DePriest (112). To examine further relationships within *Trebouxia* s. str. Beck and colleagues (11, 12) and Helms et al. (66) used ITS sequences, whereas Bhattacharya et al. (13) and Friedl et al. (45) used ITS and nuSSU rDNA group I introns sequences.

PHYCOBIONT DIVERSITY WITHIN LICHEN GROUPS A number of studies have examined the genotypic diversity of photobionts in association with particular lichen-forming fungi or lichen communities. Piercey-Normore & DePriest (112) identified 24 distinct Asterochloris ITS genotypes associated with a worldwide sample of 46 fungal species, most of them members of the Cladoniaceae. Similarly, Helms et al. (66) identified 12 *Trebouxia* s. str. ITS genotypes in association with 20 species of the Physciaceae. Kroken & Taylor (75) added an actin gene sequence to ITS to identify seven cryptic species among the highly variable *Trebouxia jamesii* photobionts associated with *Letharia*. Beck and colleagues (11, 12) used culturing studies and ITS sequences to identify five photobiont species associated with ten lichen-forming fungi from a bark-inhabiting community and two species associated with nine fungi from a rock community. In the latter study, each lichen species showed a selective preference for a single photobiont species, although in this and other studies photobiont genotypes were shared among fungal species. Furthermore, each thallus had a single photobiont, in contrast with reports of multiple algal genotypes in a single thallus (13, 66).

Coevolution of the Lichen Symbionts

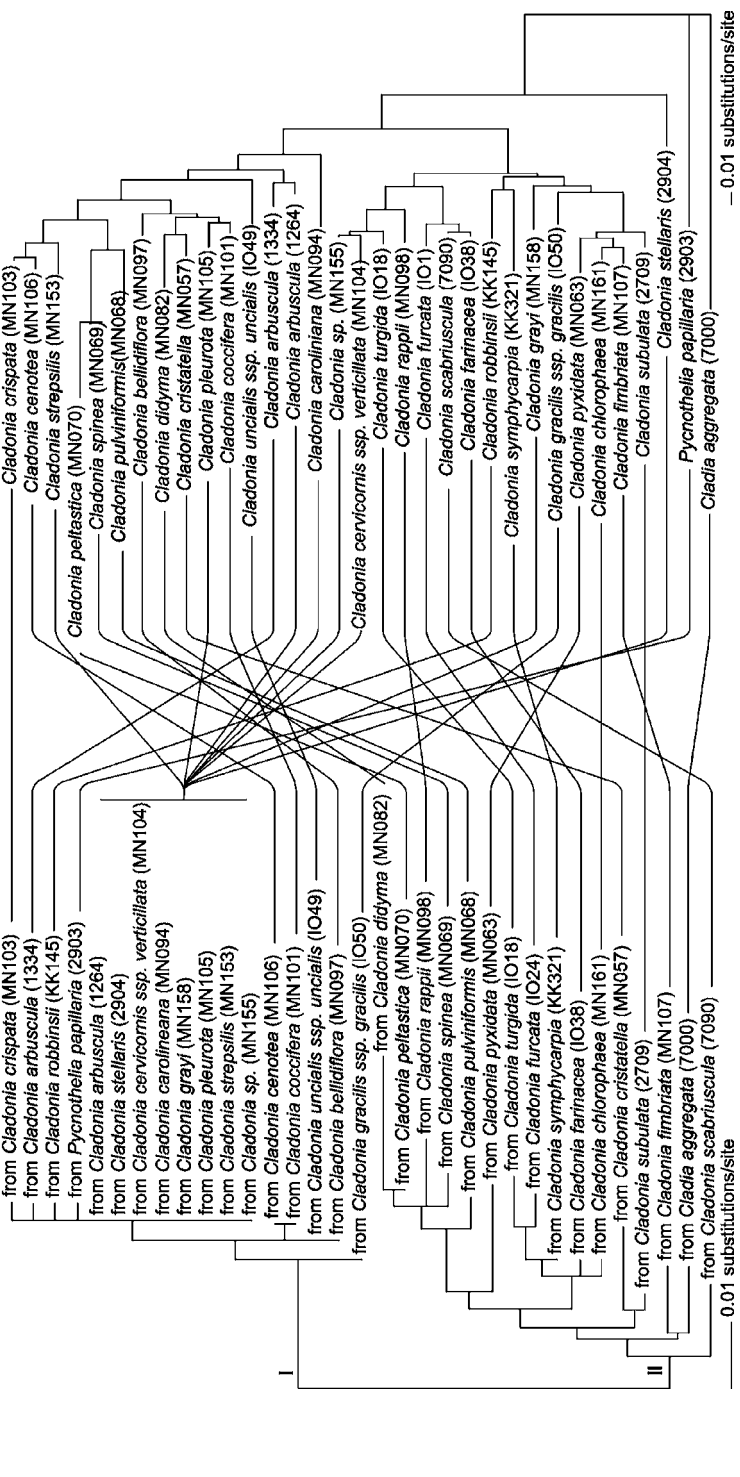
Long-term and intimate symbioses, such as lichen associations, are often hypothesized to have undergone coevolution, i.e., reciprocal genetic change. In this definition, coevolution does not necessarily culminate in one-for-one specificity of symbiotic partners, a possibility already eliminated by the 100-fold excess of lichen fungi compared with their photobiont partners. Acceptance of coevolution requires the direct demonstration of increased fitness in the form of differential survival; symbionts undergoing reciprocal genetic change will have increased survival relative to their unchanged relatives. Without effective methods for controlled crosses and artificial lichenization, such fitness cannot be measured in lichen symbionts. Therefore, coevolution of lichen symbionts at present can be tested only with indirect measures: specificity and selectivity by one or both symbionts through

taxonomic and demographic methods, and parallel cladogenesis and cospeciation through phylogenetic methods.

SPECIFICITY AND SELECTIVITY Selectivity and specificity are related as process and pattern. In the selection process, a symbiont identifies and associates with the most favorable partner available temporally and spatially. In the specificity pattern, a symbiont associates with a particular partner whether that association is due to strong selectivity or strict vertical transmission of the symbiotic partnership. Strong selectivity may lead to new associations during development, as the selection by *Diploschistes muscorum* of a new photobiont during its transition from a lichen parasite to a symbiont suggests (46). Not all possible combinations of lichen fungus and photobiont are observed in nature. Rambold et al. (118) suggested that some fungal suborders of the Lecanorales are specific for particular algal genera. *Trebouxia* (with few exceptions) is associated with the suborder Lecanorineae (11, 12, 66, 76) and *Asterochloris* with the suborder Cladoniineae (112). However, Piercey-Normore & DePriest (112) found that *Asterochloris* also formed associations with *Anzina* in the order Agryiales.

Likewise, fungi of the same genus (12, 66) and species (76, 109, 111, 112) as well as colormorphs (5, 54, 55, 100, 111) form associations with phylogenetically divergent genotypes of photobionts. Some studies suggest that multiple genotypes may form lichenized associations simultaneously in a lichen thallus (13, 66) or among cephalodia in tripartite associations (111). With multiple acceptable partners, specificity and selectivity may also be determined by geographic distribution and/or ecology. For two communities Beck et al. (11, 12) demonstrated photobiont specificity and inferred strong selectivity despite the availability of other photobionts. For the pioneering fungal species that arrive as photobiont-free ascospores, the question remains whether their partners will differ between communities with distinct photobiont populations. However, Goffinet & Bayer (Reference 54 and citations therein) suggested that cyanobacterial and eukaryotic green algal colormorphs of the Peltigeraceae may be a response to environmental cues such as humidity and light. Under some environmental conditions the fungus may select cyanobacteria for their appropriate photosynthetic and nitrogen-fixation rates.

PARALLEL CLADOGENESIS AND COSPECIATION Coevolution may be demonstrated indirectly by showing parallel cladogenesis or cospeciation. In strict parallel cladogenesis the symbiotic partners should have mirrored phylogenetic relationships. In cospeciation symbiotic partners should have coordinated speciation (divergence). However, a number of evolutionary processes can obscure this relationship: natural selection, population processes, and taxon sampling. Recent studies (75, 112) have examined parallel cladogenesis and cospeciation indirectly using comparative phylogenetic methods. Piercey-Normore & DePriest (112) compared the ITS phylogenies of *Asterochloris* algal and Cladoniaceae fungal partners and, using a number of statistical methods, rejected cospeciation and strict parallel cladogenesis (see Figure 3). They proposed that switching of highly selected algal genotypes,



termed algal switching, occurs repeatedly among lichen associations. In a similar study Kroken & Taylor (75) visually compared phylogenies of *Trebouxia* algal and *Letheria* fungi, rejected their cospeciation, and proposed switching. These results are consistent with the domestication model (see Reference 4), analogous to human agriculture, in which the fungal partner selects the best available algal partner and thereby genetically shapes the algal populations.

MOLECULAR EVOLUTION OF rDNA INSERTIONS AND INTRONS

In 1991 Ahmadjian (1) suggested that lichen symbionts might exchange genetic material. Although such exchange has not been detected, analogous gene transfer has been proposed for autonomous sequence elements, group I and spliceosomal introns, found in lichen symbionts. The earliest molecular examinations of the nu rDNA of lichen-forming fungi detected unexpected length and restriction site variation (5, 34, 52). DePriest (31–33) and DePriest & Been (34) characterized this variation as due to optional group I introns, autocatalytic sequence elements typically 200 or 400 nucleotides in length. Gargas et al. (51, 53) reported group I introns or sequence insertions from a diversity of lichen-forming fungi and their allies. Group I introns were also reported in the nuSSU rDNA of lichen-forming green algae (13, 45), and from the $tRNA_{UAA}^{Leu}$ intron of lichen-forming cyanobacteria (109). Other nu rDNA insertions have been identified as spliceosomal introns (mRNA introns) (14, 27, 105; also see 60, 126) or complex nested insertions (105; also see 10, 31–33). Gargas & DePriest (49) published PCR primers and techniques for amplifying intron-containing rDNA. Analyses of introns and insertions in the nuSSU rDNA and, more recently, in the nuLSU rDNA have been applied to questions of phylogeny and evolution for fungal (27, 33, 61, 107, 126, 135, 137), green algal (13, 45), and cyanobacterial partners (109–111). Because of the early focus on introns in lichen systematics, lichenology has been a leader in the field of intron evolution.

Positions of Insertions and Introns in rDNA

In 1992 when DePriest & Been (34) reported seven introns at five rDNA positions for the lichen-forming *Cladonia chlorophaea* complex, only seven other introns

Figure 3 Algal switching shown by incongruence between phylograms of algal and fungal partners from lichen associations. The most likely phylogenies of the Asterochloris algal partners (*left*) and the Cladoniaceae and *Anzia* fungal partners (*right*) are shown. Partners from the same lichen association are connected by lines between the phylograms; their crossing shows no overall parallel cladogenesis. The two major algal clades, Clade I and Clade II, are labeled adjacent to their nodes. Modified from Reference 112.

and four other intron positions had been published. In 1995 Gargas et al. (51) mapped 17 insertion positions in conserved regions of the nuSSU, most from lichen symbionts, and developed a stable position-naming scheme. Subsequently, Bhattacharya et al. (14) compiled 18 and 9 positions of smaller spliceosomal introns in both the nuSSU rDNA and nuLSU rDNA, respectively. By 2001 group I introns or spliceosomal introns were reported in at least 52 unique positions in the 1800-nucleotide nuSSU rDNA: group I introns at 33 positions and spliceosomal introns at 22 positions (Figure 4). Lichen-forming fungi have introns in at least 38 of these nuSSU rDNA positions, 19 (60%) of the group I intron positions and 22 (100%) of the spliceosomal intron positions. Reverse PCR (13, 27, 34, 61) supports the finding that most of the insertions are removed by splicing, although a spliceosomal intron at one position is retained in the mature rRNA (105).

Group I Introns

Group I introns are autocatalytic sequence elements that are precisely removed by splicing when the coding region is transcribed to RNA. In 2001 lichen introns were not reported to be self-splicing in vitro (32), but they seemed to be removed by splicing in vivo (13, 32, 61; but see 105). Most introns from lichen-forming fungi have the structure and sequence motifs of group IC1. However, one intron from the lichen-forming *Cladonia chlorophaea* has been classified as group IE (128). Introns from the lichen-forming algae belong to either group IC or group IB; introns from the cyanobacteria belong to the former. Some lichen introns were reported to have an exceptional flanking region with a G at the 5′ junction (61). Phylogenetic analysis suggests that most introns at the same position are homologous and vertically transferred (33, 61, 105, 107, 135, 137). However, their optional occurrence suggests they are mobile by insertion and deletion (34). In addition, Bhattacharya et al. (13) suggested that some introns had been transferred laterally among positions in an rDNA gene (but see 34, 50). Researchers proposed that these introns were transferred laterally among different algal (13, 48) or fungal (107, 135) lineages. Some studies have suggested that symbiosis would provide the opportunity for transfer between fungal and algal symbionts (33, 45), and Friedl et al. (45) further suggested that this could be mediated by viruses, although currently no observations support this intersymbiont transfer.

Spliceosomal Introns

Spliceosomal introns are a second type of sequence element that is removed by splicing that is catalyzed by a spliceosomal complex. The earliest reports from lichen-forming fungi classified the small introns of the rDNA (less than ~100 nucleotides) as degenerate group I introns (51, 60). Subsequently, these small insertions were reported from a number of lichen-forming fungi (27, 67, 72, 105, 126, 147). Myllys et al. (105) identified them as spliceosomal introns and adjusted the splicing site to form the conserved junctions. They also identified putative branch motifs of this intron type. Later Cubero et al. (27) described 24 spliceosomal

introns at seven positions in the nuSSU rDNA, and Bhattacharya et al. (14) tabulated 69 rDNA spliceosomal introns at 27 positions, 18 in nuSSU and 9 in nuLSU rDNA. The latter study refined the conserved donor, branch, and acceptor sites and, based on statistical tests, proposed a proto-slice site. Bhattacharya et al. (14) also suggested that introns were inserted into their rDNA positions rather recently and thus are restricted to a monophyletic group of ascomycetes. Stenroos & DePriest (126) supported the notion that these small insertions are homologous and provide evolutionary information. As with group I introns, spliceosomal introns appear to be mobile by insertion and deletion (14, 27).

Nested Insertions and Deletions

Comparison of related series of group I and spliceosomal introns suggest that they evolve by the insertion and, more likely, deletion of stem/loop segments that represent function units. For example, Myllys et al. (105) showed that a spliceosomal intron was present in three different lengths among closely related species: 66, 146, and 199 nucleotides. The 66-nucleotide core intron was homologous to the 5′ and 3′ ends of the longer introns. Interestingly, reverse transcription experiments demonstrated that the small intron was present in the mature RNA, although the longer variants of this intron had been removed. Similar precise insertion/deletions of 180 nucleotides and 168 nucleotides in otherwise homologous group I introns are known from studies with *Cladonia chlorophaea* (34) and *C. subtenuis* (10), respectively. It is possible that the inserted sequences represent other sequence elements, such as spliceosomal introns, because complex introns composed of a spliceosomal intron in a group I intron have been reported in nuSSU rDNA.

THE EVOLUTION OF SYMBIOSIS

The most important lichenological questions to be addressed with molecular tools are, What and how do genes and gene products control the symbiotic interaction between lichen fungi and algae? These questions can ultimately be addressed with two different approaches. In the first approach, changes in symbiosis-related phenotypes and genotypes can be mapped on phylogenies to show phyletic change and identify major transitions. In the second, genes and gene families predicted to influence symbiosis can be studied through genomic sequence annotation and gene identification, characterization, and expression in vitro and in vivo. One study has already examined RNA turnover as a measure of gene activation and expression without showing a response to environmental cues (30; also see 19).

The Transition to Lichenization

In the first approach, researchers try to determine whether the transition into lichenized fungi is a transformative event (exemplified in References 7, 90) or whether the lichen fungi are simply opportunists that acquire and lose the lichen habit

(exemplified in References 34, 50, 83). Using the basidiolichen *Omphalina* as a model of lichen evolution, Lutzoni and colleagues (88, 89, 91, 92) proposed that the evolution of lichenization was associated with some phenotypic changes, e.g., difficulty in culturing, loss of dikaryotic state, and high mutation rate. Kranner & Lutzoni (74) attributed the latter to production of thymine dimers that increase formation of mutagenizing free radicals in response to higher levels of solar radiation, desiccation, and ambient oxygen generated by the photobiont. However, re-examination of *Omphalina* (compare Reference 90 with 101) suggests that the lichen-forming species are paraphyletic and that the mutation rate should be recalculated.

In the second approach, Armaleo & Miao (7) proposed a higher rate of DNA methylation in lichenized thallus tissues compared with algal-free reproductive structures and nonlichenized sporeling cultures on the basis of differential restriction enzyme activity. Because such methylation is thought to modify gene expression and phenotype, they proposed that increases and decreases in methylation mark the "transitions to and from symbiosis" and dramatically affect morphology and function. (However, care is needed when comparing sporelings and vegetative thalli because methylation is lost during meiosis.) In addition, Armaleo & Miao (7) showed that polyketide synthetases genes, encoding biosynthetic pathways for production of secondary compounds important in lichen chemotaxonomy, had greater methylation in DNA from lichenized tissue compared with DNA from axenic cultures. They used this finding to suggest that secondary compounds are expressed as a consequence of lichenization (also see 1). Further adding to the research in this approach, Sinnemann et al. (124) studied expression of a polyketide synthetase gene pyrG from the lichen *Solorina crocea* in the model *Aspergillus nidulans*, and Miao et al. (99) provided a review of polyketide pathways genes in lichens.

Beyond Mutualism: Parasitic Associations

Compared with the genetic and phenotypic changes proposed for the transition to lichenization, is the loss of lichenization less costly? Lutzoni et al. (90) suggested that some of the 2000 species of lichenicolous fungi (e.g., the parasitic, pathogenic, commensalistic, or saprobic fungi living on lichens) represent a special evolutionary state in the loss of lichenization (see references in 123). Grube et al. (59) demonstrated the DNA isolation and PCR amplification of nuLSU rDNA from isolated ascomata of *Arthonia molendoi* growing parasitically on *Xanthoria elegans*. Wedin and colleagues (139, 145, 146) included the lichen parasite *Sphinctrina* in their phylogenetic analysis of nonlichenized Mycocaliciales. DePriest et al. (35) amplified and characterized psychrophilic (cold-loving) basidiomycetes and ascomycetes saprobically colonizing 1500-year-old subfossil lichens. Sikaroodi et al. (123) showed that axenic cultures of several lichenicolous species of *Hobsonia*, *Illosporium*, and *Marchandiomyces* that have been taxonomically linked in the past are largely unrelated ascomycetes and basidiomycetes. Therefore by 2001 a small sample of lichen parasites included a number of lineages and origins of

lichen parasitism, of which only one, *Arthonia molendoi*, appeared closely related to lichen-forming fungi.

CONCLUSIONS

During the first 15 years that molecular-genetic techniques were applied in lichenology, molecular observations changed our views regarding lichen symbiosis in various ways: (*a*) Molecular phylogenies demonstrate that analogous lichen lifestyles have been gained and lost within fungal, green algal, and cyanobacterial symbionts. (*b*) Chemotype or species-pairs concepts alone cannot delimit symbiont species; additional examination of phylogenetic, genealogical, and interbreeding relationships is required. (*c*) Reproductive strategies and population-level processes have to be considered when interpreting and conserving genetic variation within symbiont species and populations. (*d*) Coevolution of lichen symbionts is not a matter of strict cospeciation and mirrored phylogenies, but rather is most likely a matter of selection for optimal symbiotic phenotypes. (*e*) Significant amounts of variation are due to optional group I and spliceosomal introns that are mobile genetic elements. Lichenization may or may not be a transformative event for the fungi. Thus molecular-genetic techniques and molecular systematics and phylogenetic approaches have transformed lichenology.

ACKNOWLEDGMENTS

My greatest thanks go to the fellows, students, and interns who enriched my understanding of lichenology: Andrea Gargas, Michele Piercey-Normore, Soili Stenroos, Mary Carmen Molina, Abdurahimova Zuvaydajan, Rebecca Yahr, Nikolaus Hoffmann, Masoumeh Sikaroodi, Ilona Oksanen, Kati Kärkkainen, Martin Grube, Janet Marsh, Oscar Cubero, Karen Beard, and Natalia Ivanova.

The *Annual Review of Microbiology* is online at http://micro.annualreviews.org

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Figure 1 Reindeer lichen composed of the fungal partner *Cladonia rangiferina* and the algal partner Asterochloris. The three-dimensional fruticose thallus is a true podetium, lichenized generative tissues supporting fungal apothecial reproductive structures. Illustration by Wm. Keith Harrison, © Smithsonian Institution.

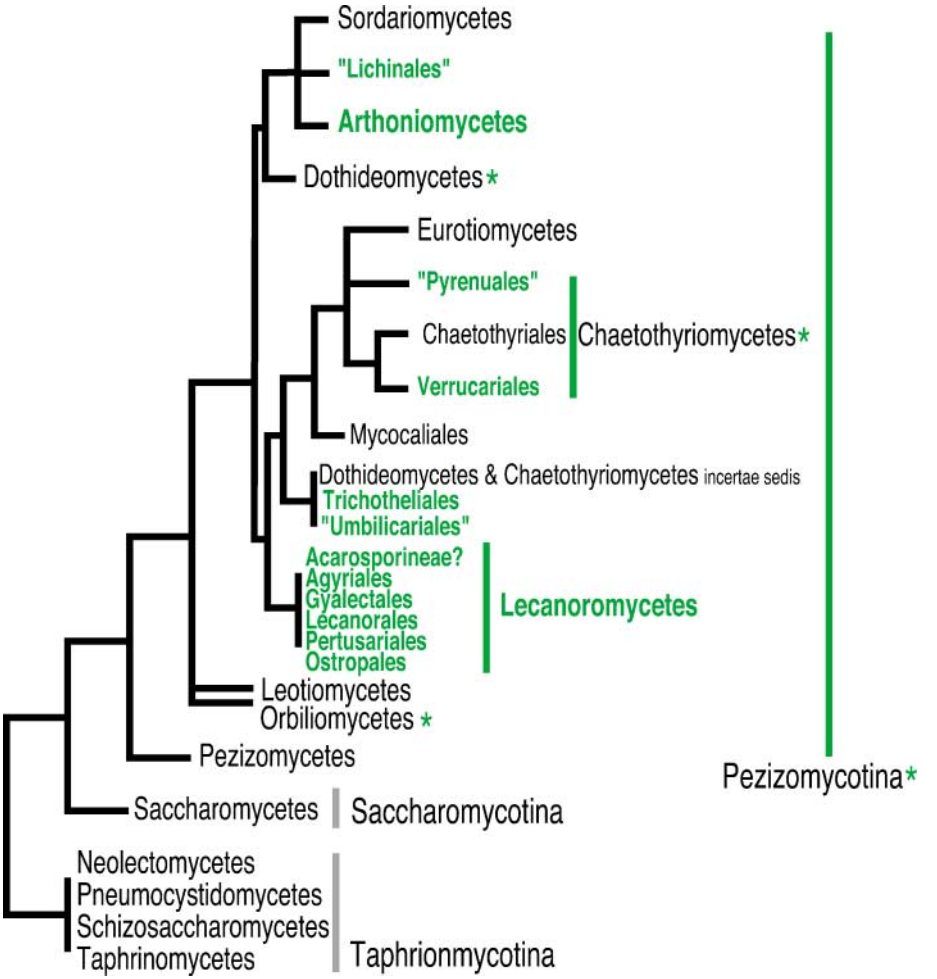

Figure 2 A phylogenetic scheme for the Ascomycota. Lichen-forming fungi occur in five classes: Arthoniomycetes; Dothidiomycetes; Chaetothyriomycetes, including Verrucariales and, possibly, Pyrenulales; Lecanoromycetes; and Orbiliomycetes. Lecanoromycetes is the largest class of lichen-forming fungi with five orders, possibly including Acarosporineae. The three orders that cannot be placed in the class system are Licheniales, Trichotheliales, and Umbilicariales. Groups that are predominately lichenized are noted in green; groups that include lichenized fungi, i.e., Pezizomycotina, are indicated with a green asterisk.

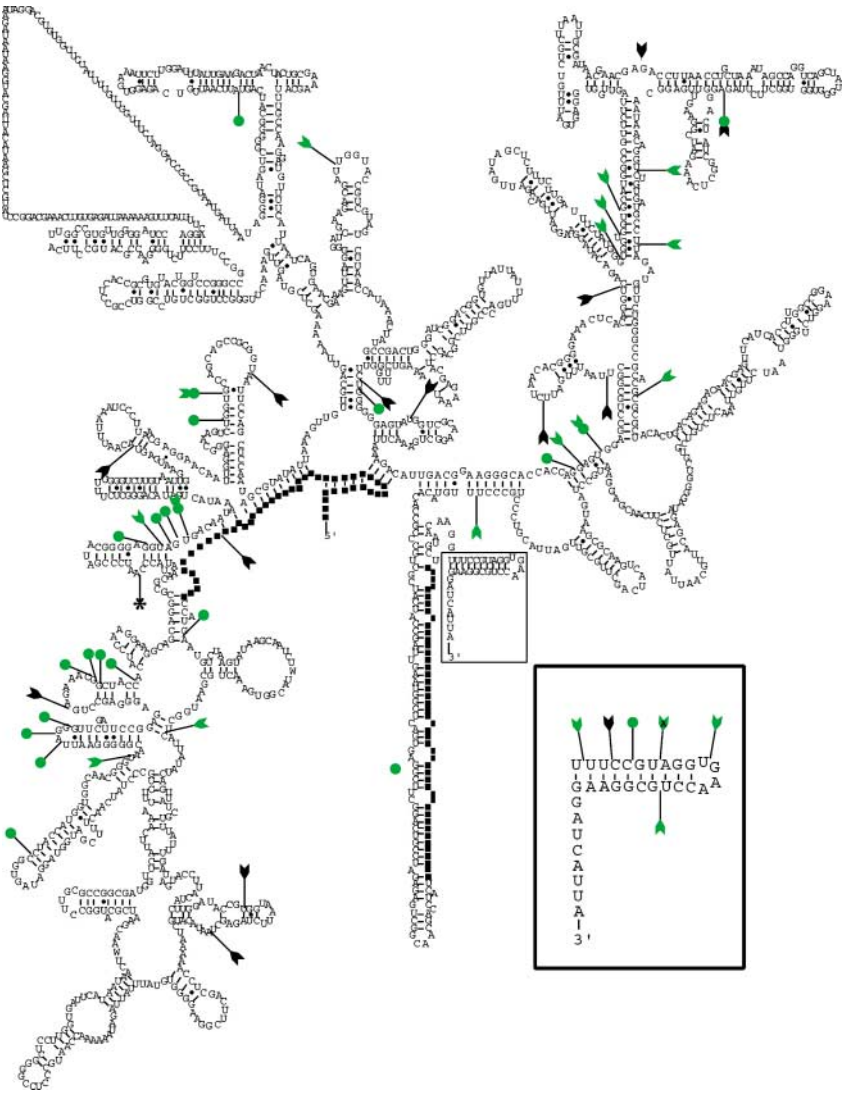

Figure 4 Secondary structure model for the lichen fungus *Lecanora dispersa* nuSSU rDNA with approximate positions of 52 group I and spliceosomal introns. Lines indicate the approximate positions of introns; arrow tips, the positions for group I introns; and filled circles, the spliceosomal introns. Positions at which both group I and spliceosomal introns are reported have combined arrow tips and circles. Positions for unclassified introns are indicated by an asterisk. Two additional introns, a spliceosomal intron and an unclassified intron, cannot be placed and are show in their approximate region. Introns reported from lichen-forming symbionts, fungi or alga, are indicated by green. Canonical base pairs are indicated by dashes and the noncanonical base pairings of G and U are indicated by dots. Unknown nucleotides are indicated by small squares.

Annual Review of Microbiology
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