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PCR Applications in Studies of Lichen-forming Fungi

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5.1 Introduction

Analysis of DNA from lichens was rare prior to the development of PCR-based techniques. In early molecular applications, Blum and Keshevarov (1986, 1992) used total DNA hybridization to estimate the relationship of the genera *Umbilicaria* and *Lasallia*. Isolation and agarose gel electrophoresis of DNA from various cultured mycobionts was carried out by Ahmadjian *et al.* (1987). With the introduction of PCR (Mullis and Faloona, 1987) during the last decade the situation changed completely and nucleic acids became easily accessible sources of new characters. Today, PCR is an important tool for molecular investigations of mycobiont phylogeny and population genetics.

In this chapter we present an overview of the recent progress of PCR applications in the study of lichen-forming fungi. Because lichens are a symbiotic association of a fungus and an alga, they require special considerations for molecular studies. To date, most molecular work with lichens has been carried out with nuclear ribosomal genes of the fungal partner (mycobiont). This chapter, therefore, focuses on the mycobiont: in the first part we emphasize the specific features of lichens and their significance for DNA isolation and PCR techniques; an overview of phylogenetic approaches to lichens is followed by a section on insertions and introns in ribosomal genes of mycobiont DNA; and the remainder of our contribution concentrates on population studies and miscellaneous PCR applications.

 Applications of PCR in Mycology
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5.2 Characteristic Features of Lichens

As emphasized in the *Dictionary of the Fungi* (Hawksworth *et al.*, 1995), lichens are a biological and not a systematic group of fungi. Hawksworth and Honegger (1994) provided the definition of a lichen as 'an ecologically obligated, stable mutualism between an exhabitant fungal partner and an inhabitant population of extracellularly located unicellular or filamentous algal or cyanobacterial cells'. Lichens are not the only possible fungal/algal or fungal/cyanobacterial symbioses (Table 5.1) but they signify one of the most successful multibiont associations with a worldwide distribution.

Lichens are particularly remarkable in the diverse morphological features shown by the composite organism. The thalli of certain lichens are among the most complex structures to have evolved in fungi (Douglas, 1994). The fungal partners in lichens are not known to occur in a free-living form, rather they dominate the overall morphology of the vegetative and fertile structures of the symbiotic associations. Nevertheless, there is an interplay between the partners during ontogeny, as different morphologies of thalli may develop by the interaction of a single fungus with cyanobacteria or with green algae (James and Henssen, 1976). Another characteristic feature of almost all lichens is the longevity of the thalli and/or the fruiting bodies. Consequently, the long-term exposure to environmental conditions and the complex organization of thalli may result in a high degree of plasticity of lichens.

The scientific name that is applied to the lichen association strictly refers to the fungal partner alone (ICBN, art. 13.1d; Greuter *et al.*, 1994). Hence,

Table 5.1. Different forms of fungal/algal symbioses. From Hawksworth (1988).

Number of bionts	Examples
Two-biont symbioses	
Mycobiont as inhabitant	Mycophycobioses
	Fungal parasites of algae
Mycobiont as exhabitant	Lichens
Three-biont symbioses	
Two photobionts: one mycobiont	*Cephalodia*
	Blue-green/green morphotypes
	Algicolous lichens
	Bryophilous lichens
Two mycobionts: one photobiont	Lichenicolous fungi
	Mechanical hybrids
Four-biont symbioses	
Three photobionts: one mycobiont	*Cephalodia*
Two photobionts: two mycobionts	Lichenicolous lichens
Three mycobionts: one photobiont	Fungi on lichenicolous fungi
Five or more biont symbioses	Mechanical hybrids

the classification and systematics of lichens refer to the relationships among lichen-forming fungi, independent of their symbiotic photosynthetic partners, which have separate algal or cyanobacterial names. The combined association has no name and naming a lichen is synonymous to naming the fungal partner in a lichen association. As a consequence of this, molecular systematic studies have focused on the lichen mycobiont.

5.3 General Remarks on Molecular Work with Lichens

The molecular study of lichen symbioses has been impeded by several problems. Both mechanical isolation of sufficient starting material and axenic cultivation of lichen-forming fungi were cumbersome and impractical approaches for earlier molecular studies. Through the application of PCR, it is now possible to undertake investigations with minute amounts of living or even herbarium material. However, several points must be kept in mind in molecular studies: (i) lichens often grow in dense associations with other individuals or other species. Additionally, they may often be difficult to separate from their substrate. Therefore, lichen material intended for DNA isolations must be examined carefully to avoid exogenous contaminants, such as substrate particles (e.g. bryophytes) or DNA from other fungi (see Petrini *et al.*, 1990). In addition, an important source of undesired DNA may be the occurrence of inhabitant lichenicolous fungi. The algal or cyanobacterial partner is usually not problematic if discrimatory DNA isolation or PCR techniques are used; (ii) lichens are well known for their ability to synthesize high amounts of diverse secondary compounds including polysaccharides. High concentrations of various polysaccharides may inhibit the efficiency of PCR and so isolation techniques must be optimized to eliminate secondary compounds in general and polysaccharides in particular; (iii) PCR anomalies may occur when working with the rDNA of mycobionts. A pair of primers, which works well for many other isolations, may not always amplify the target region even if fresh material is used. Conversely, multiple PCR products may occur even when contamination by other fungal species can be excluded. These phenomena may be due to the presence of optional insertions at priming sites. Because insertions are frequently found in rDNA of mycobionts, they require further consideration and will be described in greater detail later.

5.4 DNA Isolation and PCR Amplification from Lichens

As indicated briefly above, careful dissection of lichen material is important to eliminate DNA of other organisms in general, and that of fungal cohabitants in particular. Both lichenicolous fungi, of which there are about 1000 described species, and lichenicolous lichens, frequently occur on or in

lichens as parasites or commensals (Hawksworth *et al.*, 1995). Microscopic examination prior to dissection of material may confirm the quality of the starting material. Amplification of genomic material from the photosynthetic partner can be circumvented by two approaches. First, fungal material may be isolated mechanically from the lichen thallus, or second, oligonucleotide primers are selected which exclusively amplify fungal DNA.

5.4.1 Mechanical isolation of fungal material

Purely fungal structures are almost always present in lichens. These include hymenia in fruiting bodies, or pycnidia, thalloconidia, rhizines, rhizinomorphs, cilia, medullae, and cortical structures (Fig. 5.1). Successful isolations from podetia (DePriest and Been, 1992), basidiomata (Lutzoni and Vilgalys, 1995a), ascomata (Grube *et al.*, 1995) and rhizines (Crespo *et al.*, 1997a) have been described.

5.4.2 Discriminatory molecular techniques

The most elegant and common approach to amplifying mycobiont DNA is by using fungal-specific oligonucleotides as PCR primers. Using this

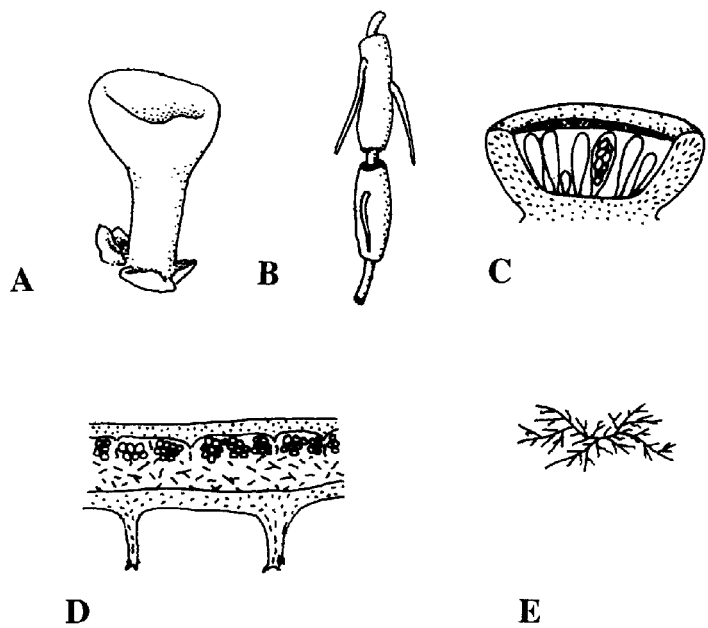

Fig. 5.1. Examples of algal-free material used for molecular studies with lichen mycobionts. (A) Decorticated podetium of *Cladonia*. (B) Chondroid medulla of *Usnea*. (C) Ascomatal structures. (D) Cortical layers, medulla, and rhizines. (E) Mycobiont cultures.

approach, it is possible to isolate DNA from the total lichen, including the algal/cyanobacterial partner without time-consuming dissection of fungal material. Under appropriate PCR conditions, it is often sufficient to have only one fungal-specific primer in the primer pair. About nine fungal-specific primers suitable for work with lichens have already been designed by comparative analysis of small subunit (SSU) rDNA sequences (Gargas and Taylor, 1992; Gardes and Bruns, 1993; Gargas and DePriest, 1996). These primers also allow selective amplification of fungal internal transcribed spacer (ITS) regions. To date, in the lichen-forming fungi, the nuclear large subunit (LSU) rRNA gene is currently being investigated with fungal-specific primers (P. Clerc, Genève, 1995, personal communication; M. Grube, unpublished work).

Standard DNA isolation protocols which are useful for a wide range of fungi, for example that described by Lee and Taylor (1990), may not be optimal for certain lichens. DNA extraction from lichens must consider the high concentration of polysaccharides present in many species. Armaleo and Clerc (1991) used column purification in their DNA isolation protocols to eliminate PCR and restriction enzyme inhibitors. Their method II also included an enzymatic treatment for the degradation of polysaccharides. More recently, addition of CTAB (*cetyl*-trimethyl ammonium bromide) to the extraction buffer has proved to be very efficient for DNA isolation from lichens (Armaleo and Clerc, 1995; Crespo *et al.*, 1997a). A protocol described by Grube *et al.* (1996) relies on the selective affinity of DNA to glass powder and was used to isolate DNA from ascomata.

Optimal yield of isolated DNA is generally achieved with fresh lichen material. However, fresh material is not always accessible and herbarium collections may provide important material for molecular studies. It has been demonstrated that intact DNA can be isolated from mushrooms that have been stored in herbaria for several decades (Bruns *et al.*, 1990; Taylor and Swann, 1994). The DNA degradation rate of herbarium material varies among different lichens and it is usually difficult to get sufficient DNA of *Usnea* species if the sample is more than 10 years old (data not shown), whereas it has been possible to get considerable amounts of PCR product from DNA of a 30-year-old specimen of *Multiclavula mucida* (GenBank acc. no. 23542; Gargas *et al.*, 1995a).

Usually, PCR conditions follow manufacturer's guidelines but they may be optimized depending on primer sequence and target sequence length. Addition of compounds such as dimethyl sulphoxide to the PCR buffer is not considered to improve results. A large number of primers are now available for the PCR amplification of nuclear ribosomal genes of fungi (Gargas and DePriest, 1996; Lutzoni and Vilgalys, 1995a) and a standardized nomenclature for these has been proposed by Gargas and DePriest (1996). Such primers are complementary to the conserved regions of the SSU rDNA, and also to the 5′ region of the LSU rDNA and all have successfully been applied to the PCR amplification of SSU, ITS regions and LSU rDNA of

lichen-forming fungi. However, 12 of the primers span positions of optional insertions and may fail to amplify if an insertion is disrupting a priming site (Gargas and DePriest, 1996). In such cases, it is advisable to amplify the target region with a different pair of primers. The primers used for PCR may also be applied for the direct sequencing of amplification products. Today, cycle sequencing has become the most popular technique for obtaining sequences from PCR products of lichen-forming fungi, and automated sequencing systems now allow sequence information to be obtained in a relatively short time.

5.5 Molecular Evolution in Lichens

About 13,500 or two-fifths of all ascomycetes occur as lichens and 13 of the 46 orders of ascomycetes include lichen-forming fungi (Hawksworth *et al.*, 1995). Investigation of phylogenetic relationships among lichen-forming fungi is therefore important for a better understanding of fungal evolution in general. The integration of lichen-forming mycobionts into the system of *Eumycota* has been particularly considered by molecular studies on the phylogenetic origins of the lichen symbioses, and an *Eumycota* phylogeny based on parsimony analysis of SSU rDNA sequences has been constructed by Gargas *et al.* (1995a). In this study five origins of lichenization were detected within the true fungi. Two origins of the lichen state were found in the ascomycetes where the lichen habit arose in groups with both saprophytic and parasitic relatives. Ongoing research is concentrating on a refinement of the *Eumycota* phylogeny by including other lichen groups (Gargas and DePriest, 1996).

Molecular-phylogenetic studies focusing on the SSU rDNA gene have also been carried out at various lower taxonomic levels. These are of special significance in groups where morphological characters are limited, or convergent morphological evolution is expected. Wedin (1996) has presented a phylogenetic hypothesis of four families of *Caliciales*. He demonstrated that representatives of *Sphaerophoraceae* form a monophyletic group within the *Lecanorales*. However, the core group of *Caliciales* including *Caliciaceae*, *Mycocaliciaceae* and *Sphinctrinaceae* was not monophyletic. Whereas *Caliciaceae* form a separate monophyletic group within the *Lecanorales*, the two other families appear as a monophyletic group basal to a clade formed by representatives of *Eurotiales* and *Onygenales*. Stenroos and DePriest (1996) provided a phylogeny of stipitate lichens encompassing *Cladoniaceae*, *Cladiaceae*, *Siphulaceae*, and *Stereocaulaceae*. According to their hypothesis, these families form a monophyletic group within the *Lecanorales*. Tehler (1995a, 1995b) and Myllis and Tehler (1996) carried out a phylogenetic analysis of *Arthoniales* and emphasized that some groups based on morphological data were corroborated by molecular results whereas others were clearly not. Eriksson and Strand (1995) studied the relationships of *Solorina*,

Peltigera and *Nephroma* and found that *Solorina* and *Peltigera* were closely related and should be kept in the same family, *Peltigeraceae*, whereas *Nephroma* was distinct and that its placement in a separate family, *Nephromataceae*, was supported by molecular characters. Marsh *et al.* (1994) investigated the cladistic relationships within the family *Ramalinaceae*. The preliminary analyses contributed to the understanding of generic concepts in *Ramalinaceae* and showed that *Dievernia* and *Ramalinopsis* were more closely related to each other than either is to *Nieblia*.

Søchting and Lutzoni (1996) investigated the SSU rDNA variation in the *Teloschistaceae*. They showed that the genus *Xanthoria* is polyphyletic and includes lineages related to *Caloplaca* subgen. *Gasparrinia* and subfruticose *Caloplaca* species. A phylogenetic analysis of the *Umbilicariaceae* was undertaken by Bobrova and Ivanova (1996). According to their hypothesis, *Lasallia* does not form a sister group to *Umbilicaria*. The latter appears to be paraphyletic, contradictory to the current morphological concept. Lutzoni and Vilgalys (1995a) and Lutzoni (1996) undertook a phylogenetic analysis of *Omphalina*, including lichen-forming and free-living species. The sequence data based on ITS and LSU rDNA data suggested that the lichen-forming *Omphalina* species are a monophyletic group.

With the increasing number of sequences available, several other challenging issues of lichen evolution can be addressed by molecular methods. For example, the taxonomic position of sterile or otherwise poorly understood species can now be evaluated. Hoffmann (1996) analysed DNA from the sterile lichen *Normandina pulchella* and from co-occurring perithecia referred to as *Lauderlindsaya borreri*. The latter is sometimes regarded as the fertile stage of *Normandina*. The preliminary results suggest that *Normandina* is an ascomycete genus, but different from the lichenicolous *Lauderlindsaya*.

A classic debate in lichenology regarding the reproductive mechanisms and their impact on species evolution has been developed through the discussion about species pairs or 'Artenpaare' (Poelt 1970; Culberson and Culberson, 1973; Tehler 1982) and reviewed by Mattsson and Lumbsch (1989). Species pairs are parallel taxa with similar thallus morphology, where one taxon is fertile and the other is sterile, propagating via soredia, isidia or analogous organs. The relationships between species pairs have been investigated using ITS sequences (Lohtander and Tehler, 1996). A cladistic analysis of *Dendrographa* species suggests that the apomictic strains in this genus originated repeatedly from a fertile primary species (A. Tehler, Salzburg, 1996, personal communication).

Recently, efforts have been made to combine molecular and morphological evidence in lichen phylogenetic studies (Eriksson and Strand, 1995; Lutzoni and Vilgalys, 1995a, b; Tehler, 1995a, b; Lutzoni, 1997). The topology of phylogenetic trees from morphological and molecular data sets may differ significantly and there are several strategies that may be used to obtain single phylogenies. The taxonomic congruence method involves separate

analyses of different data sets and subsequent consensus analysis, whereas the method of total evidence uses both data sets in a combined analysis. Tehler (1995b) discussed the logical difficulties of applying taxonomic congruence and demonstrated the limitations of this method in a phylogenetic analysis of the *Arthoniales*. Alternatively, Lutzoni and Vilgalys (1995b) proposed that data sets should be analysed separately and be tested if any conflict arose between them. As trees are constructed from samples of data, one important reason for topological differences may be sampling error. If the differences can be explained by sampling error, the morphological and molecular data sets may be combined in phylogenetic analyses. Available tests for evaluating heterogeneity between data sets (e.g. Rodrigo's test, T-PTP test, and Kishino and Hasegawa test) have been compared by Lutzoni and Vilgalys (1995b). They showed that only the method of Rodrigo *et al.* (1993) explicitly addressed the question of sampling error.

5.6 Insertions and Introns

It has already been mentioned that PCR results are dependent on the presence or absence of insertions at oligonucleotide priming sites (Gargas and DePriest, 1996). Such insertion sequences, which may differ in length, are found at a high frequency in the ribosomal genes of the mycobiont and they may increase the length of the SSU rDNA by as much as 100%. To date, 17 insertion positions have been described from the nuclear SSU rDNA, and 13 of them are known from lichen-forming fungi (Table 5.2). Seven insertion positions are so far known from the nuclear LSU rDNA, although only one of them is also found in lichen mycobionts. Multiple insertions may be found in a single species, and seven insertions have been found in the SSU rDNA of *Lecanora dispersa* (Gargas *et al.*, 1995b).

Many of these insertions can be classified as group I introns (subgroup IC; Michel and Westhof, 1990) by sequence analysis. In a pioneering work, DePriest and Been (1992) showed that group I introns have a variable distribution in the rDNA of the *Cladonia chlorophaea* complex. As detailed later, this variability is of importance for lichen population studies.

When transcribed, group I introns share a common secondary structure and, by their intricate RNA folding, they may act as ribozymes, which catalyse their own excision from their host genes. DePriest and Been (1992) pointed out that the group I introns present in *C. chlorophaea* do not splice under standard *in vitro* conditions typically used for self-splicing experiments with other group I introns. This phenomenon is in accordance with a lack of structural elements that are usually found in self-splicing introns (Jaeger *et al.*, 1996; Lehnert *et al.*, 1996). For example, one characteristic feature of introns at positions 1046, 1199, 1210, 1389, and 1516 from *Cladonia* is a relatively short P5 region. This character is also found in homologous introns of other lichen-forming fungi, e.g. in

Table 5.2. Presence of insertions in nuclear SSU rDNA (based on Gargas *et al.*, 1995b; Grube *et al.*, 1996, and [a]unpublished data). Positions are according to *E. coli* numbering.

Insertion position	114	287	323	392	516	531	789	943	956	1046	1052	1199	1210	1389	1506	1512	1516
Lichen-forming fungi	+	+	–	+	+	–	+	+	–	+	–	+	+	+	+[a]	+	+
Other organisms	–	–	+	–	+	+	–	+	+	+	+	+	–	–	+	+	–

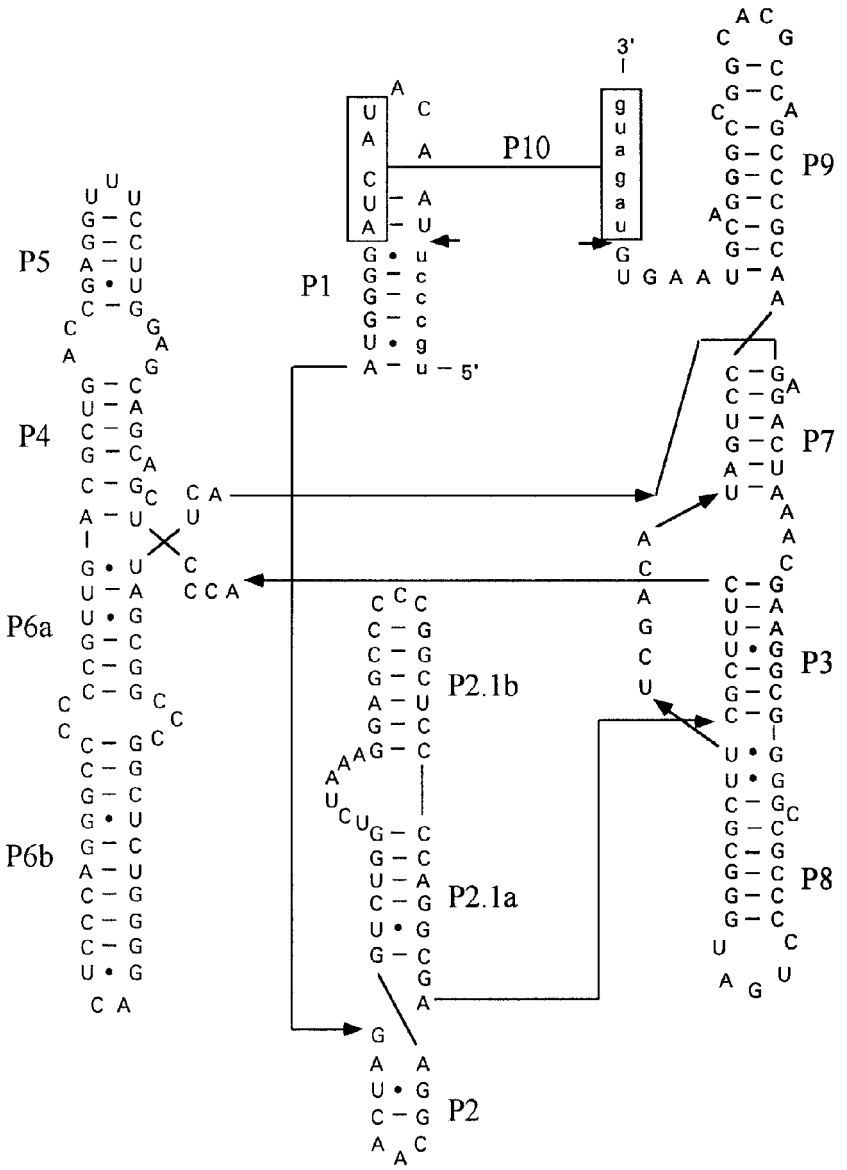

Fig. 5.2. Putative secondary structure of a group IC intron at position 1210 in the nuclear SSU rDNA of *Lecanora muralis*. Representation according to Cech *et al.* (1994).

Lecanora (Fig. 5.2). DePriest and Been (1992) suggested that *trans* acting factors are required for correct splicing because the *Cladonia* introns are missing in mature rRNA.

In addition to group I introns, an increasing number of relatively short insertions of less than 100 nucleotides in length has been described in lichen-forming and free-living fungi (Rogers *et al.*, 1993; Untereiner *et al.*, 1995; Grube *et al.*, 1996). These short insertions are located at known intron positions, and some of them disrupt essential regions of mature rRNA. Rogers *et al.* (1993) showed, by RT–PCR, that short insertions are cut out from mature transcripts, although the splicing mechanisms remain unclear. Gargas *et al.* (1995b) used the term degenerate introns for short insertions and Grube *et al.* (1996) suggested that they may be the vestiges of initially complete introns, which lack the P3–P9 regions.

Phylogenetic relationships of various insertion site lineages of introns have been investigated for green algae by Bhattacharya *et al.* (1994, 1996), who showed an apparent monophyly of introns at positions 1506 and 1512, respectively. Generally, the introns at homologous positions have almost identical sequences in the conserved regions of the catalytic core. Increased sequence variation is, however, observed at peripheral regions. The sequence of the P2 region, considered as a spacer by Peyman (1994), and the P9 region are the most variable parts of the introns. Additionally, by analysing the known lichen introns, Bhattacharya *et al.* (1996) suggested that the closer relationships of introns at position 1199 and 287 and of introns at position 1210 and 1389 may be due to an ancient lateral transfer of an intron. This view is underlined by similarities of the 5′ flanking regions and the P10 pairing in each of the lineages.

We prepared a preliminary phylogenetic analysis of introns at position 1516, which are frequently found in *Lecanorales* (Fig. 5.3). Introns at this position from various representatives of *Lecanorales* can be aligned and parsimony analysis shows partial incongruency with the current classification of the lichens studied. The *Cladonia* introns appeared together with introns from *Lecanora* and *Parmelia*. This branch represented a sister group to introns from *Physconia*. According to the current concept of the *Lecanorales*, *Cladonia* would be a basal clade in this selection of taxa. The incongruency might be due to horizontal transfer of introns within the *Lecanorales*. This is also consistent with the theory of 'late introns', i.e. introns which are inserted secondarily into a particular position during rDNA evolution (see DePriest 1995). As introns in each of the genera group together it is probable that horizontal transfer of divergent introns is an ancient or rare event in *Lecanorales*. Conversely, there is evidence for a considerable rate of intron mobility within populations of a single species (see below).

It is still unclear whether introns are evenly distributed throughout the ribosomal repeat. It has repeatedly been observed that more than one fragment is amplified even from DNA of a single thallus (unpublished work). That these phenomena are due to the presence of lichenicolous fungi can be

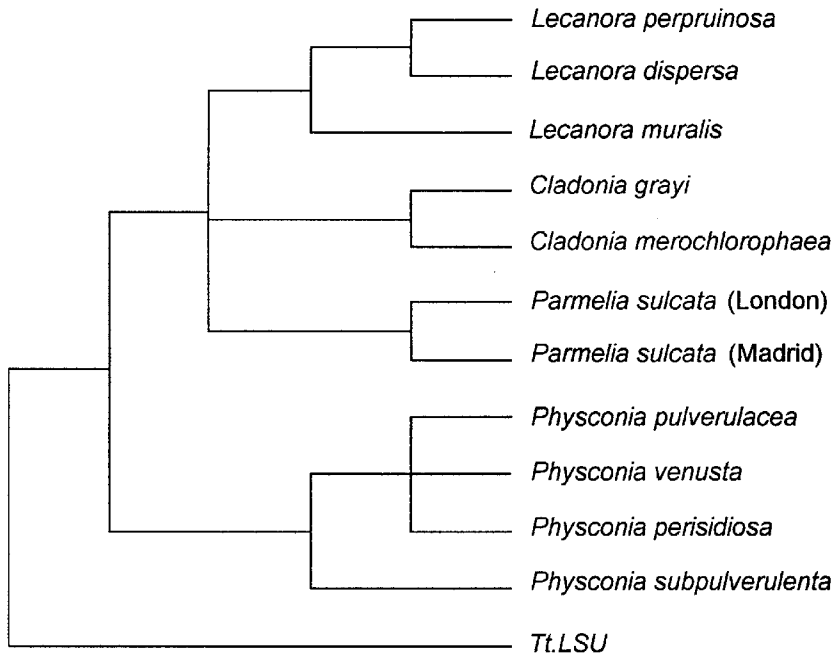

Fig. 5.3. Phylogenetic analysis of introns at position 1516 from various representatives of the *Lecanorales*. Strict consensus tree from three most parsimonious trees found using the branch and bound algorithm of the PAUP program (Swofford, 1991). The *Tetrahymena thermophila* intron (Tt.LSU) was used as an outgroup.

excluded by microscopic examination. On the other hand it is possible that a lichen thallus is occasionally composed of more than one individual since fusion of different lichen thalli and mechanical hybridization can be observed occasionally in nature (see below). Alternatively, DePriest (1993a, b), Gargas *et al.* (1995b) and Crespo *et al.* (1997a) mentioned that more than one repeat type had been found even in single individuals of mycobionts. Gargas *et al.* (1995b) stated that the variable occurrence of insertions in the rDNA-repeat may produce anomalous PCR amplifications. We occasionally observe that even in those cases where a single intron-containing product was amplified, an intron-less product is obtained when a primer is used that spans the intron position, and which does not, therefore, amplify repeat units that contain the intron (unpublished results; see Gargas and DePriest, 1996). The reasons for these phenomena are unclear; however this is a challenging issue for future research. It is also important in this context to consider that the number of rDNA repeat units may change during vegetative growth (Pukkila and Skrzynia, 1993) and that meiotic recombination of rDNA is suppressed in certain fungi (Cassidy *et al.*, 1984; Russell *et al.*, 1988).

5.7 Genetic Variability of Populations

Lichen species may exhibit a high degree of morphological variation (Poelt, 1994). In many cases, this variation is apparently due to different ecological conditions. On the other hand, numerous examples are known where significantly different morphotypes of the same species grow side-by-side (Poelt, 1994). This indicates that certain phenotypic variation is also inherited as a genetic trait, which is also the case for chemical polymorphisms (Culberson *et al.*, 1988). However, whereas there is now evidence that secondary compounds are produced by the mycobiont alone, it has not been estimated how much of the morphological variation may be attributed to different populations of photobionts within a lichen thallus. Independent genetic characters are therefore of great importance for lichen population studies.

Ribosomal DNA offers characters for direct investigation of mycobiont populations. Great variability of fungal rDNA within a lichen population was first demonstrated by DePriest and Been (1992). A single mat (about the size of a hand) of *C. chlorophaea* was composed of 13 different genotypes. A detailed analysis of these genotypes was presented by DePriest (1994) by using restriction site patterns and Southern hybridization. The sharing of restriction patterns suggests that some chemotypes may be polymorphisms of a single species in the *C. chlorophaea* complex. Significant size polymorphism of PCR products already show that this tremendous variation is due to the variable occurrence of insertions. Sequence analysis indicates that most insertions are group I introns in the coding regions of the rDNA.

DePriest (1995) used the information of the different genotypes to address questions of intron mobility in the *C. chlorophaea* complex. The phylogenetic hypothesis based on rDNA types suggests that it is most parsimonious to consider that both insertion and deletion of group I introns occur in rDNA of *C. chlorophaea*.

In different species of lichens, the heterogeneity of populations may vary. Whereas single mats of *C. chlorophaea* consist of many SSU rDNA genotypes, individual mats of *Cladina subtenuis* are genetically uniform (Beard and DePriest, 1996). However, different mats of *Cladina* may be assigned to various genotypes. Three size classes of PCR products from the 3′ region of the SSU rDNA were detected among separate mats due to the presence of optional group I introns. Different PCR size-classes have also been described in a population study of *Parmelia sulcata* (Crespo *et al.*, 1997b, 1998). In this study, PCR products from the ITS regions including the terminal region of the SSU and LSU rDNA were amplified. A significant increase in product size – of *c.* 200 nucleotides – was due to the presence of an intron at position 1516 in *P. sulcata*. Similar size variations were also observed in the related species *Parmelia saxatilis* (Fig. 5.4). In *Physconia*, the frequency of group I introns at position 1516 varied among different species (Cubero and Crespo, 1996). However, no correlation has yet been observed

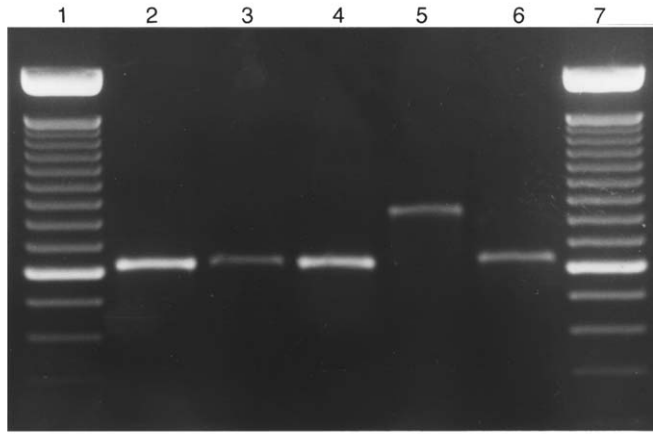

Fig. 5.4. Length variation of PCR products from *Parmelia saxatilis*. Lanes 1 and 7, 100 bp ladder; lane 2, a specimen from Spain (Casa de Campo); lanes 3 and 4, material from England (New Forest, Hampshire); lanes 5 and 6, specimens from England (Sheffield). Lane 5 shows a significantly larger product (850 nucleotides) due to the presence of a group I intron.

between the different intron frequencies and morphological variation, sexual mechanisms or ecological amplitude of species.

LaGreca (1996) presented a population study on the *Ramalina americana* complex, which included morphologically identical but chemically different races. Cladistic analysis of ITS sequence data suggested that the species complex consisted of two distinct groups. This grouping was also correlated with differences in chemical diversity and geographic distribution. The two groups were also supported by analyses of a second data set based on sequences of introns at position 1516 in the nuclear SSU rDNA.

An alternative approach to the study of lichen populations is possible using the random amplification of polymorphic DNA (RAPD) technique (Williams *et al.*, 1990). Short primers are used to randomly amplify fragments which are interpreted as genetic markers. This technique may be applied to estimate variation over complete genomes. The only available RAPD study with lichens (Cubero *et al.*, 1995) showed complex banding patterns with significant differences between populations from distant sites (Fig. 5.5). Further work is needed to evaluate the significance of RAPD analysis for population studies in lichens.

5.8 Miscellaneous Molecular Studies

Some miscellaneous studies address further issues of molecular lichenology. Glacier-covered lichens may serve as a source of ancient DNA. Gargas *et al.*

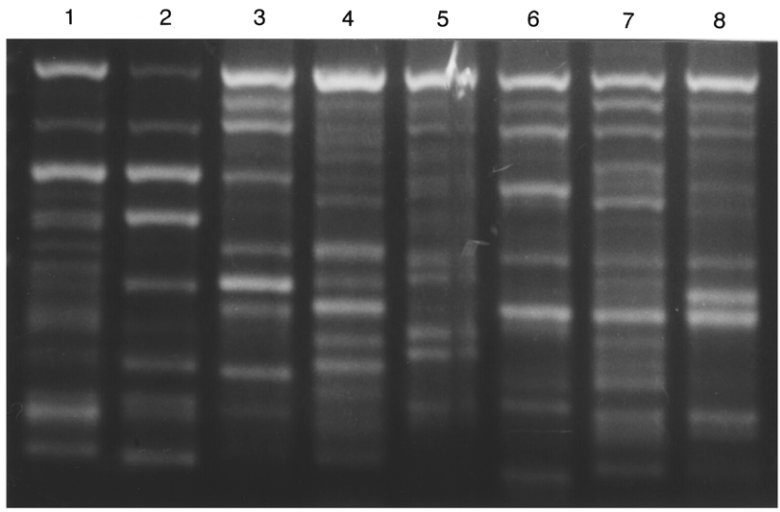

Fig. 5.5. Random amplification of polymorphic DNA (RAPD) from *Parmelia saxatilis* (Cubero *et al.*, 1995). Lanes 1 and 2, specimens from Antarctica (South Settlands, Livingston Island); lanes 3, 4 and 5, specimens from Spain (Sierra de Aillón); lanes 6, 7 and 8, specimens from Spain (Ávila, Sierra de Gredos).

(1994) have isolated DNA from specimens of *Umbilicaria cylindrica* that were recently exposed after the glacier receded. Radiocarbon dating suggested that this material had been under the ice for 1300 years. Successful PCR amplification of DNA was possible from such apparently well preserved material. Therefore mummified lichens can be used to investigate sequence variation over historical time periods.

Armaleo and Clerc (1991) analysed different photomorphs of single lichen-forming fungi in the genera *Pseudocyphellaria* and *Sticta*. Fragments 2.2 kb in length were amplified from the 5′ region of nuclear LSU rDNA. The products were then digested by restriction enzymes. Subsequent agarose gel analysis of the restriction fragments showed a 'near identity' of the different photomorphs. The similarity of the restriction patterns can be interpreted as molecular evidence that the corresponding photomorphs belong to the same fungal species.

PCR product length may also be used to detect distinct genetic individuals in lichen associations. Both partners of the lichen symbiosis may consist of more than one individual. This was suggested by indirect evidence from morphological and isozyme analyses (summarized by Fahselt, 1996) and mechanical hybridization between different initial thalli is a common explanation for such multisymbioses (see Fig. 5.10 in Honegger, 1996). Fahselt (1996) suggested that if intrathalline electrophoretic differences are generated by multiple symbionts, it is likely that they are related to the mycobiont, since

fungal biomass predominates in lichen thalli. Further evidence for this hypothesis is found by rDNA size polymorphisms within thalli of *P. sulcata*. Multiple amplification products were found with a frequency of 7% ($n = 261$ thalli; A. Crespo, unpublished work) in single thalli using fungal-specific primers (ITS1F and ITS4; Gardes and Bruns, 1993). PCR products of different length have been amplified from DNA isolations along a gradient through a thallus (Fig. 5.6). These data may indicate that more than one fungal rDNA genotype is present in a single thallus of *P. sulcata*.

5.9 Conclusions

PCR is a powerful technique for the investigation of the genetic variation in nuclear rRNA genes of lichen-forming fungi. The newly available sequence analyses contribute much to our understanding of lichen phylogeny. More representatives of the different orders of ascomycetes will be sequenced and this will clarify our views on the evolutionary origins of lichenization.

Investigations at lower taxonomic levels will improve our knowledge about evolution within and among lichen populations, species, genera, and families. It is now possible to link genetic information with that of morphological and chemical characters to study convergent evolution in different groups of lichen-forming fungi.

The frequency of insertions and introns is regarded as a useful character for population studies on lichens. The sequence diversity of mycobiont introns will also offer new information about the molecular evolution of ribozymes.

Future work will include investigations of various, yet poorly exploited

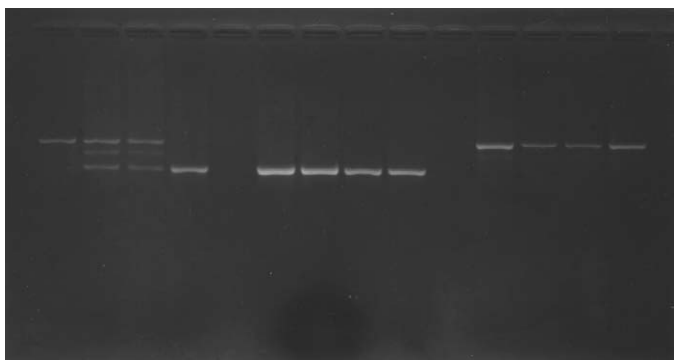

Fig. 5.6. PCR products of different length within a single thallus of *Parmelia sulcata*. Lanes 1–4 show PCR products from isolations along a gradient through a thallus 3 cm in diameter. Lanes 5 and 10 are used as spacers. Lanes 6–9 show uniform PCR products from four isolations along a gradient through a second thallus. Lanes 11–14 show uniform but longer PCR products from four isolations along a gradient through a third thallus. The three specimens were from Casa de Campo, Madrid, Spain.

genes. The ITS regions, LSU rDNAs and protein genes will be important additional sources of characters for phylogenetic studies. Beside further studies on ribosomal genes, a major challenge for the next years will be to study directly the genes which contribute to the tremendous chemical diversity of lichen compounds. Armaleo and Miao (1996), Miao and Davies (1998) and Miao *et al.* (1996) have already made advances in this direction.

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References

Ahmadjian,V., Chadeganipour, M., Koriem, A.M. and Paracer, S. (1987) DNA and protoplast isolations from lichens and lichen symbionts. *Lichen Physiology and Biochemistry* 2, 5–15.

Armaleo, D. and Clerc, P. (1991) Lichen chimeras: DNA analysis suggests that one fungus forms two morphotypes. *Experimental Mycology* 15, 1–10.

Armaleo, D. and Clerc, P. (1995) A rapid and inexpensive method for the purification of DNA from lichens and their symbionts. *Lichenologist* 27, 207–213.

Armaleo, D. and Miao, V. (1996) Towards a molecular analysis of lichen polyketide biosynthesis. *IAL3 Abstracts*, Salzburg, 92 pp.

Beard, K.H. and DePriest, P.T. (1996) Genetic variation within and among mats of the reindeer lichen, *Cladina subtenuis* (des Abb.) Hale and W.Culb. *Lichenologist* 28, 171–182.

Bhattacharya, D., Surek, B., Rüsing, M., Damberger, S. and Melkonian, M. (1994) Group I introns are inherited through common ancestry in the nuclear-encoded rDNA of Zygnematales (Charophyceae). *Proceedings of the National Academy of Sciences USA* 91, 9916–9920.

Bhattacharya, D., Friedl, T. and Damberger, S. (1996) Nuclear-encoded rDNA group I introns; origin and phylogenetic relationships of insertion site lineages in the green algae. *Molecular Biology and Evolution* 13, 978–989.

Blum, O.B. and Kashevarov, G.P. (1986) The DNA homologies as a proof of the legitimacy of the establishment of the lichen genus *Lasallia* Merat (Umbilicariaceae). *Doklady Akademii Nauk Ukrainskoi SSR, Ser. B* 1986, 61–64 (in Russian).

Blum, O.B. and Kashevarov, G.P. (1992) The DNA homologies as a proof of the legitimacy of the establishment of the lichen genera *Lasallia* Merat, *Cladina*

(Nyl.) Harm. and *Pseudevernia* Zopf. In: Karnefelt, I. (ed.) *IAL2 Abstracts*, Lund, p. 1.

Bobrova, V.K. and Ivanova, N.V. (1996) The distribution of group I introns in the SSU rDNA among the representatives of the lichen family Umbilicariaceae. *IAL3 Abstracts*, Salzburg, p. 98.

Bruns, T.D., Fogel, R. and Taylor, J.W. (1990) Amplification and sequencing of DNA from fungal herbarium specimens. *Mycologia* 82(2), 175–184.

Cassidy, J.R., Moore, D., Lu, B.C. and Pukkila, P.J. (1984) Unusual organization and lack of recombination in the ribosomal RNA genes of *Coprinus cinereus*. *Current Genetics* 8, 607–613.

Cech, T.R., Damberger, S.H. and Gutell, R.R. (1994) Representation of the secondary and tertiary structure of group I introns. *Structural Biology* 1, 273–280.

Crespo, A., Bridge, P.D. and Hawksworth, D.L. (1997a) Amplification of fungal rDNA-ITS regions from non-fertile specimens of the lichen-forming genus *Parmelia*. *Lichenologist* 29(3), 275–282.

Crespo, A., Bridge, P.D., Cubero, O.F. and Hawksworth, D.L. (1997b) Determination of genotypic variability in the lichen-forming fungus *Parmelia sulcata*. *Bibliotheca Lichenologica* (in press).

Crespo, A., Bridge, P.D., Hawksworth, D.L., Grube, M. and Cubero, O.F. (1998) Recolonization by one of three rRNA genotypes of *Parmelia sulcata* following SO_2 amelioration. *Botanical Journal of the Linnaean Society* 68, 73–79.

Cubero, O.F. and Crespo, A. (1996) Genetic variability in populations of *Physconia enteroxantha* (Nyl.) Poelt. *IAL3 Abstracts*, Salzburg, p. 151.

Cubero, O.F., Crespo, A. and Ochando, M.D. (1995) Aplicación de la técnica de RAPD a estudios en líquenes. *Resumenes del XI Simposium Nacional de Botánica Criptogámica*, Santiago de Compostela, p. 231.

Culberson, W.L and Culberson, C.F. (1973) Parallel evolution in lichen-forming fungi. *Science* 180, 196–198.

Culberson, C.F., Culberson, W.l. and Johnson, A. (1988) Gene flow in lichens. *American Journal of Botany* 75, 1135–1139.

DePriest, P.T. and Been, M.D. (1992) Numerous group I introns with variable distribution in the ribosomal DNA of a lichen fungus. *Journal of Molecular Biology* 228, 315–321.

DePriest, P.T. (1993a) Small subunit rDNA variation in a population of lichen fungi due to optional group I introns. *Gene* 134, 67–74.

DePriest, P.T (1993b) Molecular innovations in lichen systematics: The use of ribosomal and intron nucleotide sequences in the *Cladonia chlorophaea* complex. *The Bryologist* 96, 314–325.

DePriest, P.T. (1994) Variation in the *Cladonia chlorophaea* complex II: ribosomal DNA variation in a southern Appalachian population. *The Bryologist* 97, 117–126.

DePriest, P.T. (1995) Phylogenetic analyses of the variable ribosomal DNA of the *Cladonia chlorophaea* complex. *Cryptogamic Botany* 5, 60–70.

Douglas, A.E. (1994) *Symbiotic Interactions*. Oxford University Press, Oxford.

Eriksson, O.E. and Strand, A. (1995) Relationships of the genera *Nephroma*, *Peltigera* and *Solorina* (Peltigerales, Ascomycota) inferred from 18S rDNA sequences. *Systema Ascomycetum* 14(1), 33–38.

Fahselt, D. (1996) Individuals, populations and population ecology. In: Nash III, T.H. (ed.) *Lichen Biology*. Cambridge University Press, Cambridge, pp. 181–198.

Gardes, M. and Bruns, T.D. (1993) ITS primers with enhanced specificity for

basidiomycetes. Application to the identification of mycorrhizae and rust. *Molecular Ecology* 2, 113–118.

Gargas, A. and DePriest (1996) A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. *Mycologia* 88, 745–748.

Gargas, A. and Taylor, J.W. (1992) Polymerase chain reaction (PCR) primers for amplifying and sequencing nuclear 18S rDNA from lichenized fungi. *Mycologia* 84, 589–592.

Gargas, A., DePriest, P.T. and Ivanova, N. (1994) Glacier-covered lichens: sources of ancient DNA. *IMC5 Abstracts*. Vancouver, p. 69.

Gargas, A., DePriest, P.T., Grube, M. and Tehler, A. (1995a) Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* 268, 1492–1495.

Gargas, A., DePriest, P.T. and Taylor, J.W. (1995b) Positions of multiple insertions in SSU rDNA of lichen-forming fungi. *Molecular Biology and Evolution* 12, 208–218.

Greuter, W., Barrie, F.R., Burdet, H.M., Chaloner, W.G., Demoulin, V., Hawksworth, D.L., Jørgensen, P.M., Nicolson, D.H., Silva, P.C., Trehane, P. and McNeill, J. (1994) *International Code of Botanical Nomenclature (Tokyo Code). Adopted by the Fifteenth International Botanical Congress, Yokohama. August–September 1993*. [Regnum Vegetabile 131] Koeltz Scientific Books, Königstein.

Grube, M., DePriest, P.T., Gargas, A. and Hafellner, J. (1995) DNA isolation from lichen ascomata. *Mycological Research* 99, 1321–1324.

Grube, M., Gargas, A. and DePriest, P.T. (1996) A small insertion in the SSU rDNA of the lichen fungus *Arthonia lapidicola* is a degenerate group I intron. *Current Genetics* 29, 582–586.

Hawksworth, D.L. (1988) The variety of fungal–algal symbioses, their evolutionary significance, and the nature of lichens. *Botanical Journal of the Linnaean Society* 96, 3–20.

Hawksworth, D.L. and Honegger, R. (1994) The lichen thallus: a symbiotic and phenotype of nutritionally specialized fungi and its response to gall producers. In: Williams, M.A.J. de. (ed.) *Plant Galls: Organisms, Interactions, Populations*. Clarendon Press, Oxford.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N. (1995) *Ainsworth and Bisby's Dictionary of the Fungi*. CAB International, Wallingford.

Hoffmann, N. (1996) SSU rDNA sequence data support *Normandina pulchella* as a sterile ascomycete. *IAL3 Abstracts*. Salzburg. p. 106.

Honegger, R. (1996) Morphogenesis. In: Nash III, T.H. (ed.) *Lichen Biology*. Cambridge University Press, Cambridge, pp. 65–87.

Jaeger, L., Michel, F. and Westhof, E. (1996) The structure of group I ribozymes. *Nucleic Acids and Molecular Biology* 10, 33–51.

James, H.M. and Henssen, A. (1976) The morphological and taxonomic significance of cephalodia. In: Brown, D.H., Hawksworth, D.L. and Bailey, R.H. (eds) *Lichenology: Progress and Problems*. Academic Press, London.

LaGreca, S. (1996) A phylogenetic evaluation of chemical variation in the *Ramalina americana* complex and a preliminary molecular phylogeny for the genus *Ramalina*. *IAL3 Abstracts*. Salzburg. p. 112.

Lee, S.B. and Taylor, J.W. (1990) Isolation of DNA from fungal mycelia and single spores. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds) *PCR Protocols: a Guide to Methods and Applications*. Academic Press, San Diego, pp. 282–287.

Lehnert, V., Jaeger, L., Michel, F. and Westhof, E. (1996) New loop–loop tertiary interactions in self-splicing introns of subgroup IC and ID: a complete 3D model of the *Tetrahymena thermophila* ribozyme. *Chemistry and Biology*, 993–1009.

Lohtander, K. and Tehler, A. (1996) Species pair studies in *Dirina* and *Dendrographa* based on molecular data. *IAL3 Abstracts*. Salzburg, p. 114.

Lutzoni, F. and Vilgalys, R. (1995a) *Omphalina* (Basidiomycota, Agaricales) as a model system for the study of coevolution in lichens. *Cryptogamic Botany* 5, 71–81.

Lutzoni, F. and Vilgalys, R. (1995b) Integration of morphological and molecular data sets in estimating fungal phylogenies. *Canadian Journal of Botany* 73 (suppl. 1), S649–S659.

Lutzoni, F. (1996) Phylogeny of lichen- and non-lichen-forming omphalinoid mushrooms and the utility of testing for combinability among multiple data sets. *Systematic Biology* 46, 373–406.

Marsh, J., DePriest, P.T., Gargas, A. and Nash III, T. (1994) A cladistic analysis of the genera of the lichen family Ramalinaceae based on 18S rDNA sequences. *IMC5 Abstracts*. Vancouver. p. 133.

Mattsson, J. and Lumbsch, H.T. (1989) The use of species pair concept in lichen taxonomy. *Taxon* 38(2), 238–241.

Miao, V. and Davies, J. (1998) Molecular diversity of polyketide biosynthesis genes in lichens. In: Baltz, R.H. and Hegeman, G.D. (eds) *Proceedings of GMBIM-6, American Society for Microbiology* (in press).

Miao, V., Glass, N.L. and Lee, A. (1996) A genetic approach to understand polyketide metabolism in lichens. *IAL3 Abstracts*. Salzburg. p. 36.

Michel, F. and Westhof, E. (1990) Modelling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis. *Journal of Molecular Biology* 216, 581–606.

Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Methods in Enzymology* 155, 355–350.

Myllis, L. and Tehler, A. (1996) A phylogeny of *Arthoniales* based on SSU rDNA data. *IAL3 Abstracts*. Salzburg. p. 120.

Petrini, O., Hake, U. and Dreyfuss, M.M. (1990) An analysis of fungal communities isolated from fruticose lichens. *Mycologia* 82, 444–451.

Peyman, A. (1994) P2 functions as a spacer in the *Tetrahymena* ribozyme. *Nucleic Acids Research* 22, 1383–1388.

Poelt, J. (1970) Das Konzept der Artenpaare bei den Flechten. *Vorträge aus dem Gesamtgebiet der Botanik herausgegeben von der deutschen botanischen Gesellschaft, n.s.* 4, 187–198.

Poelt, J. (1994) Different species types in lichenized ascomycetes. In: Hawksworth, D.L. (ed.) *Ascomycete Systematics: Problems and Perspectives in the Nineties*. Plenum Press, New York.

Pukkila, P.J. and Skrzynia, C. (1993) Frequent changes in the number of reiterated ribosomal RNA genes throughout the life cycle of the basidiomycete *Coprinus cinereus*. *Genetics* 133, 203–211.

Rodrigo, A.G., Kelly-Borges, M., Bergquist, P.R. and Bergquist, P.L. (1993) A randomisation test of the null hypothesis that two cladograms are sample estimates of a parametric phylogenetic tree. *New Zealand Journal of Botany* 31, 257–268.

Rogers, S.O., Yan, Z.H., Shinohara, M., LoBuglio, K.F. and Wang, C.J.K. (1993)

Messenger RNA intron in the nuclear 18S ribosomal RNA gene of deuteromycetes. *Current Genetics* 23, 338–342.

Russell, P.J., Petersen, R. and Wagner, S. (1988) Ribosomal DNA inheritance and recombination in *Neurospora crassa*. *Molecular General Genetics* 211, 541–544.

Søchting, U. and Lutzoni, F. (1996) Phylogenetic relationships among members of the Teloschistaceae based on the nuclear ribosomal small subunit. *IAL3 Abstracts*. Salzburg. p. 126.

Stenroos, S. and DePriest, P.T. (1996) Phylogeny of the lichen families Cladoniaceae, Cladiaceae, Siphulaceae and Stereocaulaceae: evidence from nuclear rDNA sequences. *IAL3 Abstracts*. Salzburg. p. 88.

Swofford, D.L. (1991) *PAUP: Phylogenetic Analysis using Parsimony, Version 3.1*. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Taylor, J.W. and Swann, E.C. (1994) DNA from herbarium specimens. In: Herrman, B. and Hummel, S. (eds) *Ancient DNA*. Springer-Verlag, Berlin.

Tehler, A. (1982) The species pair concept in lichenology. *Taxon* 31, 708–714.

Tehler, A. (1995a) Arthoniales phylogeny as indicated by morphological and rDNA sequence data. *Cryptogamic Botany* 5, 82–97.

Tehler, A. (1995b) Morphological data, molecular data, and total evidence in phylogenetic analysis. *Canadian Journal of Botany* 73 (suppl. 1), S667–S676.

Untereiner, W.A., Straus, N.A. and Malloch, D. (1995) A molecular-morphotaxonomic approach to the systematics of the *Herpotrichiellaceae* and allied black yeasts. *Mycological Research* 99, 897–913.

Wedin, M. (1996) Phylogeny and evolution of Caliciales s.lat. as shown by ribosomal DNA sequences. *IAL3 Abstracts.* Salzburg. p. 10.

Williams, J.G.K., Kubelik, A.R., Lavak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18, 6531–6535.