Symbiosis, 31 (2001) 213–229
Balaban, Philadelphia/Rehovot

Review article

Use of RAPD-PCR DNA Fingerprinting and Vegetative Incompatibility Tests to Investigate Genetic Variation within Lichen-Forming Fungi

PAUL S. DYER*, GARETH J. MURTAGH, and PETER D. CRITTENDEN
School of Life and Environmental Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK, Tel. +44-115-9513203, Fax. +44-115-9513251, E-mail. Paul.Dyer@Nottingham.ac.uk

Received August 23, 2000; Accepted April 25, 2001

Abstract

The use of the DNA fingerprinting technique randomly amplified polymorphic DNA (RAPD) analysis to investigate genetic variation in lichen-forming fungi is reviewed. Particular thermostable enzymes are recommended for RAPD-PCR together with the use of DNA extracted from axenic cultures of the mycobiont, thereby overcoming the problem of amplification of non-target contaminating DNA. The use of RAPD-PCR in phylogenetic reconstruction is described, with certain similarity coefficients and a resampling technique (bootstrapping or jackknifing) advocated. Investigations with a variety of lichen species have revealed genetic diversity values comparable with those detected in populations of free-living fungi. RAPD-PCR has also been used to investigate other aspects of lichen biology. Studies of breeding systems revealed homothallism in model species. Studies of thallus 'individuality' revealed that thalli may be composed of more than one genotype. The use of vegetative incompatibility tests to detect variation in lichen-forming fungi was also assessed using axenic cultures of *Graphis scripta* and *Phaeographis dendritica*. Pigmentation was observed at boundaries between colonies of different genotype unlike control tests. However, overall vegetative compatibility grouping results were inconclusive.

Presented at the Fourth International Association of Lichenology Symposium, September 3–8, 2000, Barcelona, Spain

*The author to whom correspondence should be sent.

0334-5114/2001/$05.50 ©2001 Balaban

Keywords: DNA fingerprinting, RAPD, lichen-forming fungi, vegetative incompatibility, lichen, individuality

1. Introduction

Many fungi exhibit an often unappreciated degree of genetic variation within a single species. A recent study by Purwantara et al. (2000) used molecular methods to show that all 100 isolates in a worldwide collection of the phytopathogen *Leptosphaeria maculans* were genetically unique. The presence of genetic variation within populations is important as it underlies the ability of a species to respond to evolutionary selective pressures (Milgroom, 1996).

Lichen-forming fungi are unusual in that three different levels of genetic variation may all be evident within a species. Firstly that between geographically isolated populations (Printzen et al., 1999), secondly that between discrete thalli within a population (Zoller et al., 1999), and finally variation within 'individual' thalli because these may be composed of two or more fungal genotypes (Laundon, 1978; Larson and Carey, 1986; Murtagh et al., 2000). The aim of the present review is to survey how novel methods have recently been used to investigate two outstanding questions in lichen biology; (a) the extent of genetic variability within lichen-forming fungi and (b) the nature of thallus individuality, focussing on the use of DNA fingerprinting and vegetative incompatibility tests.

2. Previous Investigations of Variability in Lichen-Forming Fungi

Evidence of genetic variation within lichens and lichen-forming fungi has previously come from studies using morphological, physiological or chemical markers, which have demonstrated variability both within and between species and populations as reviewed by Fahselt (1989, 1996). Observations of vegetative incompatibility, with the formation of demarcation boundaries between adjoining thalli of crustose lichens such as *Rhizocarpon geographicum* and *Graphis scripta* (Fig. 4A), have provided further evidence of genotypic diversity since this indicates the presence of somatically incompatible individuals (Rayner, 1991; Nauta and Hoekstra, 1996; Clayden, 1997).

More recently, molecular markers have been used to investigate variation within lichens and lichen-forming fungi. These have the advantages over secondary genetic markers of not being subject to environmental and cultural influences, and they require little starting material (Murtagh et al., 1999). Most studies have investigated the ribosomal DNA (rDNA) repeat unit,

analysing sequence divergence within the small subunit (SSU) rDNA and/or the internal transcribed spacer (ITS) region. Differences in SSU rDNA structure were used to detect variation between podetia of *Cladonia chlorophaea* (DePriest, 1993) and mats of *Cladina subtenuis* (Beard and DePriest, 1996). Genotypes of *Parmelia sulcata, Lobaria pulmonaria* and *Xanthoria elegans* have been distinguished on the basis of differences in size and sequence of their ITS region (Crespo et al., 1999; Zoller et al., 1999; Dyer and Murtagh, 2001). However, analysis of variation in rDNA lacks the sensitivity of some other molecular methods (Bridge and Hawksworth, 1998).

3. Use of DNA Fingerprinting to Detect Genetic Variation

The term 'DNA fingerprinting' was originally introduced by Jeffreys et al. (1985) to describe a method for the simultaneous detection of many variable DNA loci by hybridisation of multilocus probes to electrophoretically separated restricted DNA fragments. Since then it has been applied more widely to include any methods which use a multilocus approach to visualise DNA polymorphisms (Weising et al., 1995). These may be based on either hybridisation or polymerase chain reaction (PCR) techniques and in either case results in the production of a series of banding patterns for an individual specimen which may be used alone, or pooled with other data, to produce a 'DNA fingerprint' characteristic of a particular isolate or individual. The terms 'DNA profiling' and 'DNA typing' have also been applied to some of these methods. PCR-based methods are now used most frequently since they require little starting material and are, in general, faster and simpler than hybridisation-based techniques.

PCR-based fingerprinting involves the amplification of particular DNA sequences mediated by arbitrarily chosen or specifically designed oligonucleotide primers and a thermostable DNA polymerase, followed by the electrophoretic separation of amplified products (amplicons), and the final detection of polymorphic banding patterns. Various techniques have been developed in the past decade and applied to filamentous fungi. These include Randomly Amplified Polymorphic DNA (RAPD) analysis (Williams et al., 1990), Arbitrarily Primed PCR (Welsh and McClelland, 1990), Amplified Fragment Length Polymorphism (AFLP) analysis (Vos et al., 1995), Microsatellite Primed PCR (Longato and Bonfante, 1997), Inter-Single-Sequence-Repeat anchored (ISSR) PCR analysis (Zietkiewicz et al., 1994; Grunig et al., 2001) (also termed Random Amplified Microsatellites [RAMS] by Hantula et al. [1996]), Universally Primed PCR (UP-PCR) (Lübeck et al., 1999) and consensus tRNA primed PCR-analysis (Carter et al., 2000). These techniques are of great benefit because they are able to reveal genotypic

differences which might not be apparent if only morphological, physiological, chemical or rDNA markers were used.

RAPD analysis of lichen-forming fungi

Of the DNA fingerprinting techniques available only RAPD analysis has, to our knowledge, been applied to lichen-forming fungi. The RAPD technique involves PCR amplification of genomic DNA with single decamer primers of arbitrary sequence (Williams et al., 1990). It has been used to discriminate between fungal species, populations, sub-populations and individuals providing greater sensitivity than molecular techniques such as rDNA analysis because markers are produced from many loci within the genome rather than single repeated sequences (Dyer et al., 1996; Wang et al., 1997; Munaut et al., 1998). It may result in the production of up to ten or more strongly staining DNA fragments per primer for lichen-forming fungi (Murtagh et al., 1999, 2000). There are several advantages in using this technique on lichens. It is quick, relatively simple and cheap, requires little starting material and no prior knowledge of the nucleotide sequence of the target genome is required (Newton and Graham, 1997). However, particular care must be taken with RAPD analysis. Because the technique is non-specific it will amplify any template DNA provided that the correct primer annealing sites are present. Therefore, RAPD-PCR is very susceptible to the presence of contaminating DNA sources which may produce amplicons and generate misleading results (Dyer and Leonard, 2000). This is a major consideration when working with lichens because DNA of both mycobiont and photobiont is present in whole thalli. In addition other contaminating microorganisms such as lichenicolous fungi or bacteria may be present, and yeasts isolated from lichen thalli have been shown to generate RAPD-banding patterns (Prillinger et al., 1997).

A detailed technical investigation of RAPD analysis in lichen-forming fungi was made by Murtagh et al. (1999) who applied the technique to the epiphytic species *Graphis scripta*, *G. elegans* and *Phaeographis dendritica*. The problem of contaminating photobiont and other microorganism DNA was overcome by extracting DNA from axenic cultures of the mycobiont so that only mycobiont DNA was present. Various DNA extraction techniques and PCR conditions were assayed to optimise the reliability and production of RAPD fingerprints. Key features of the final protocol were the extraction of DNA using a phenol-chloroform method, and amplification using DyNAzyme II DNA polymerase which was found to greatly increase the number of RAPD-bands generated compared to other thermostable enzymes. In addition, the quantity of template DNA was found to be a critical factor. Maximum number of bands were produced using 0.01–1 ng DNA per reaction, fewer bands being generated with higher

DNA concentrations. This emphasizes the importance of quantifying the amount of DNA used in RAPD-PCR (Weising et al., 1995). The protocol generated between four to ten strongly staining bands per primer for the three test species (Murtagh et al., 1999, 2000). RAPD analysis of five isolates of *P. dendritica* from a woodland revealed the presence of four different genotypes, whilst two isolates of *G. scripta* from the same woodland each had unique DNA fingerprints. RAPD-PCR was also performed with DNA extracted from whole thalli and, significantly, the resulting fingerprints were different from those produced by axenic cultures of the corresponding mycobionts. The extra bands observed in extracts from whole thalli were thought to arise from contaminating DNA other than that of the mycobiont, emphasising that difficulties of interpretation may arise when whole thalli are used in RAPD analysis (Murtagh et al., 1999). This conclusion confirmed the findings of Lohtander et al. (1998) who were unable to obtain informative genetic data for *Roccellinia capensis* using RAPD markers obtained with DNA from whole thalli or apothecial extracts. This was thought to be due to the presence of several homoplasious characters thought to have arisen from algal contamination.

RAPD analysis and phylogenetic studies with lichen-forming fungi

As well as being used to detect the presence or absence of genetic variation, RAPD data may also be used in phylogenetic studies to establish the genetic relatedness of particular individuals. However, care must again be taken in using RAPD analysis for such purposes. Landry and Lapointe (1997) assessed the use of RAPDs in phylogenetic reconstruction and concluded that RAPD-PCR was a powerful tool for determining relationships within species and genera but was not suitable for comparing taxa above the family level. They also concluded that both distance and parsimony methods could be used to determine genetic relatedness provided a minimum of six, though preferably 12 or more, primers were used in analyses. Furthermore they advised the use of Jaccard or Dice similarity coefficients in distance based analyses, and the use of a resampling procedure (bootstrapping or jackknifing) to obtain the most reliable results. Similarity values calculated using the Jaccard coefficient of band matching may result in relatively high values of genetic divergence between less closely related individuals. However, this technique has the advantage that it only takes into account positive band matching and is therefore recommended for RAPD analysis (Weising et al., 1995).

Heibel et al. (1999) used RAPD data to investigate genetic variation within populations of the beard lichen *Usnea filipendula* found to be re-invading former polluted areas in western Germany. They used DNA extracted from the

central axis of thalli, thought to be free of contaminating alga and other microorganisms, in PCR. Phylogenetic analysis of 25 specimens showed no obvious clustering, suggesting that re-invading isolates were not derived from specific clones but instead came from different sources. They also suggested that most specimens of the species were genetically closely related, although no scale bar was provided on the associated dendrogram.

Printzen et al. (1999) investigated genetic variability of the crustose lichen *Biatora helvola* on spruce in Scandinavia and central Europe. They used DNA extracted from periclinal, supposedly algal free, apothecium sections for RAPD-PCR. However, such material may be dikaryotic and in the absence of knowledge about breeding systems it is not possible to distinguish heterozygotes from homozygotes (Grube and Kroken, 2000). The results revealed genetic differences between 17 samples of *B. helvola* from different regions and in general reflected the glacial disjunction of spruce in Europe. However, there was poor bootstrap support for most groups except for Fennoscandian collections. A maximum of approximately 55% genetic divergence was detected amongst samples as determined by Nei and Li's similarity index in a neighbour-joining analysis. ITS-sequencing was also used to assess variation in *B. helvola,* but much fewer intraspecific differences were apparent compared to those detected by the RAPD analysis.

Genetic variation between eight thalli of the crustose lichen *Graphis scripta* from different sites in the UK was investigated using RAPD fingerprinting by Murtagh et al. (2000). Five of the thalli were collected in the same Pembrokeshire woodland and three further thalli came from Somerset, Devon and Merioneth. DNA for PCR was extracted from *in vitro* cultures of the mycobiont and the resulting RAPD markers showed all isolates to have a unique DNA fingerprint (Fig. 1A). Phylogenetic analysis was performed using the Jaccard coefficient of band matching to calculate a pairwise similarity matrix and a bootstrapped dendrogram produced using the boot, neighbour-joining and NJ plot programs of PHYLIP 3.5 (Weising et al., 1995; Felsenstein, 1993). Neighbour-joining genetic divergence values were calculated, revealing up to 19% divergence between the five isolates from the Pembroke woodland and up to 57% divergence between isolates from the other UK sites (Fig. 2). The same experimental approach was then applied to assess genetic variation between isolates from 13 thalli of the foliose lichen-forming fungus *Xanthoria elegans* from north America, Europe and Antarctica (Murtagh, 1999; Murtagh, Dyer, Furneaux and Crittenden, unpublished results). All isolates yielded a unique genetic fingerprint (Fig. 1B) with between 6–79% genetic divergence. The RAPD analysis was able to separate the isolates far more effectively than data obtained from ITS sequencing, in agreement with results for other filamentous fungi (Dyer and Murtagh, 2001; Driver et al., 2000). A relationship between genetic relatedness and geographic origin was evident for most

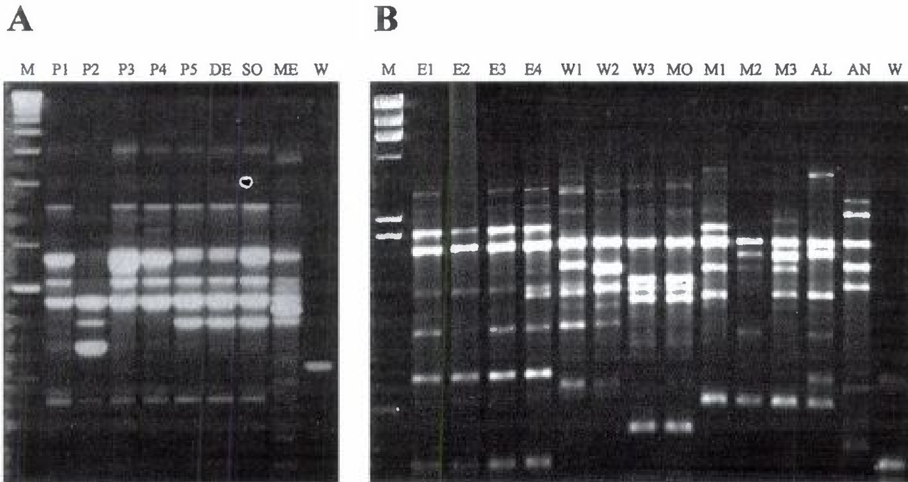

Figure 1. Use of RAPD markers to demonstrate variation in lichen-forming fungi. (A) Isolates of *Graphis scripta* from different locations in the UK following amplification with primer OPA-17; P1-P5 = Pembroke thalli, DE = Devon, SO = Somerset, ME = Merioneth. (B) Isolates of *Xanthoria elegans* from different worldwide locations following amplification with primer OPJA-01; E1-5 = England, W1-3 = Wyoming, MO = Montana, M1-3 = Maine, AL = Alaska, AN = Maritime Antarctica. M = molecular weight marker, W = water control.

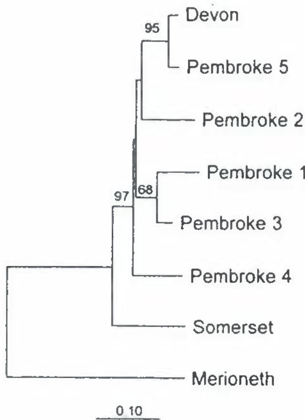

Figure 2. Genetic diversity of *Graphis scripta* isolates from Pembroke and other locations in the UK (Devon, 158 km SE of the Pembroke site; Somerset 160 km ESE; Merioneth 152 km NE). Horizontal branches of the tree are drawn to scale to reflect genetic distance according to the Jaccard coefficient of RAPD band matching (0.1 = 10% difference). Numbers at each node indicate the percentage of bootstrap samples (out of 100) in which the cluster to the right is supported (only values >60% are shown).

X. elegans, with isolates from the same location clustering more closely than those from more geographically disparate locations. Four isolates from England formed a distinct group as did three isolates from Maine. The Antarctic isolate showed relatively low similarity (maximum of 32% genetic relatedness) to all other isolates. The genetic divergence was also correlated with growth rate suggesting some biogeographical adaptation to localised environmental conditions.

The genetic divergence values obtained for these lichen-forming fungi may seem surprisingly high. However, they are comparable to those obtained for non-lichenised fungi using the Jaccard coefficient of RAPD band matching. Divergence values of up to 65% were reported for 38 worldwide isolates of the entomopathogen *Paecilomyces fumosoroseus* (Cantone and Vandenberg, 1998), up to 50% in 69 isolates of the tomato pathogen *Alternaria alternata* in California (Morris et al., 2000), 80–93% divergence between isolates of *Fusarium lateritum* from different plant hosts (Hyun and Clark, 1998) and up to 76% between 37 isolates of the cereal pathogen *Tapesia yallundae* from Europe and New Zealand (Dyer, Bradshaw and Meakin, unpublished results).

RAPD analysis of breeding systems in lichen-forming fungi

RAPD bands were used as genetic markers by Murtagh et al. (2000) in a study of breeding systems in lichen-forming fungi. Sets of 10 sibling ascospore progeny were collected from three apothecia from three different thalli of the crustose lichens *Graphis scripta* and *Ochrolechia parella*. These were used to establish *in vitro* cultures from which DNA was extracted for RAPD-PCR with a minimum of 30 primers. The resulting DNA fingerprints, based on 218–263 bands, showed complete genetic uniformity between sibling spores from the same ascoma (Fig. 3). This provided evidence of a homothallic (self fertilising) breeding system because variation amongst the offspring would have been expected if a heterothallic (obligate outcrossing) breeding system were present. Homothallism was considered to confer various biological advantages for lichens such as facilitating spore output without the need for outcrossing, and perpetuating successful genotypes adapted to prevailing environmental regimes.

If homothallism is a general characteristic of lichen-forming fungi, then this breeding system might be predicted to lead to reduced variation in populations as a result of inbreeding. However, the levels of variation detected above (section 3.2) might be explained by occasional outcrossing, because homothallic fungi are rarely obligately selfing (Burnett, 1975). Indeed, evidence of outcrossing has been provided by chemical analysis of progeny from the *Cladonia chlorophaea* and *Ramalina siliquosa* lichen complexes (Culberson

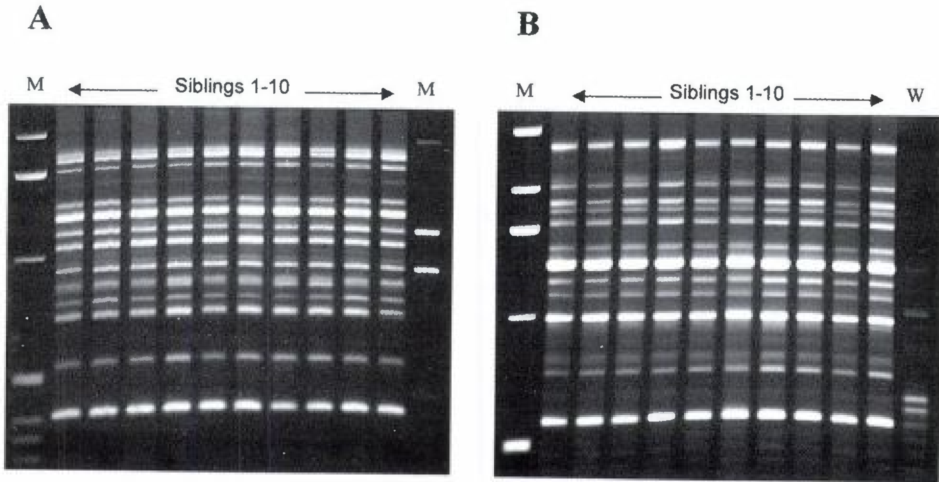

Figure 3. RAPD markers produced by sets of ten single-spore progeny from the same ascoma. (A) *Graphis scripta*, following amplification with primer OPAX-03, (B) *Ochrolechia parella* following amplification with primer OPJ-01. Note uniformity of fingerprint patterns indicating homothallism. M = molecular weight marker, W = water control.

and Culberson, 1994). Alternatively, variation may have also resulted from, and be sustained by, random mutational events.

RAPD analysis of individuality in lichen-forming fungi

Lichen thalli frequently harbour lichenicolous fungi and sometimes more than one 'primary' species of lichen-forming fungus (Hawksworth, 1982; Ott, 1987a, b). It has also been postulated that thalli may consist of more than one mycobiont genotype as a result of mechanical hybridizations between developing thalli, although supporting evidence is limited (Shaw, 1992; Jahns and Ott, 1997). This includes chemical analysis of thalli of *Umbilicaria vellea*, *U. mammulata* (Larson and Carey, 1986) and *Haematomma ochroleucum* (Laundon, 1978). Molecular investigations have also been made utilising rDNA markers. DePriest (1993) demonstrated that mats of *Cladonia chlorophaea* were comprised of many SSU rDNA genotypes and Crespo et al. (1998) estimated that 7% of 261 thalli of *Parmelia sulcata* contained more than one rDNA genotype. However, Beard and DePriest (1996) failed to detect any variation in SSU rDNA genotype within individuals from mats of *Cladina subtenuis*.

In addition, RAPD-PCR has recently been used to investigate the nature of individuality in thalli of *Graphis scripta* and *Ochrolechia parella* (Murtagh, 1999; Murtagh et al., 2000; Murtagh, Dyer, Crittenden, Jayawardena and Ning, unpublished results). It was considered probable that ascospore discharge would reflect the genotype of the underlying vegetative mycelium because these species are known to have a homothallic breeding system (Murtagh et al., 2000; see above). Therefore, any variation in the DNA fingerprinting pattern of *in vitro* single spore cultures would be likely to arise from different genotypes present in thalli rather than from outcrossing events. Ascospore discharge was collected from three to four ascomata borne on the same thallus for three different thalli of both *G. scripta* and *O. parella*. Between 218–223 scorable RAPD markers were produced by the monospore cultures of *G. scripta*. For all three thalli it was found that the four ascomata from each thallus produced identical RAPD fingerprints i.e. there was no evidence of variation within the thalli. Therefore thalli of *G. scripta* may be assumed to consist of a single mycobiont genotype. However, it should be noted that the approach would not have detected other genotypes within the thalli which were not sexually active i.e. were not producing ascomata and subsequent ascospore discharge.

In contrast, RAPD analysis of *O. parella* demonstrated the presence of intra-thallus variation with at least two mycobiont genotypes resident in each of the three thalli studied. For each thallus it was found that three of the four ascomata examined produced identical RAPD fingerprints, whereas the fourth ascoma exhibited polymorphisms in approximately 7% of the total bands. Thus, these thalli may be better viewed as a fungal community rather than individual mycobionts. The difference between individual thalli of *G. scripta* and *O. parella* is probably related to habitat; *O. parella* occurs in densely occupied habitats with relatively high rates of spore deposition, so that coalescence of developing thalli is likely (Jahns and Ott, 1997). In contrast, *G. scripta* was collected from young trees where it is a typical pioneer species. Propagule deposition rates in such open habitats might be comparatively low and therefore thalli are likely to arise mainly from single spores, with little opportunity for merging of developing thalli (Murtagh et al., 2000).

4. Use of Vegetative Incompatibility Tests to Detect Genetic Variation

The phenomenon of vegetative incompatibility, referring to the formation of barrage zones between genetically incompatible individuals, has been well studied in fungi (Leslie, 1993). This has led to the identification of different vegetative compatibility groupings (VCGs) which have been of great use in studies of fungal populations. However, although lichen-forming fungi have

been cited as providing 'the most obvious example of somatic incompatibility' (Rayner, 1991), no work has been reported on the use of vegetative incompatibility markers to assess variation in axenic cultures of these fungi. Field observations clearly suggest that vegetative incompatibility exists in nature with mosaics of lichens being common (Laundon, 1978; Hawksworth and Chater, 1979; Pentecost, 1980; Clayden, 1997; Fig. 4A). As early as 1921 Smith reported interactions between thalli of *Rhizocarpon geographicum* on boulders, *Pyrenula nitida* on tree bark and *Arthopyrenia* spp. on acorn barnacles and seashells. Peripheral boundaries marked by black or white zones may arise when thalli are of a different genotype, with these pigmented borders thought to be analogous to somatic incompatibility reactions seen in other fungi (Hawksworth and Hill, 1984; Leslie, 1993).

To investigate whether vegetative incompatibility tests could be used to detect variation in lichen-forming fungi, we set up trials with axenic cultures of *Graphis scripta* and *Phaeographis dendritica* of known genotype from RAPD studies. Mycobiont mycelia from single spore progeny were harvested from liquid malt extract yeast extract (MEYE) medium by filtration and then gently macerated using a pestle and mortar. The macerate was used to inoculate 50 mm diameter membrane filters (Millipore Durapore filters, 0.22 µm pore size) (Oliver et al., 1989) on a bed of MEYE agar. Approximately 0.15 ml of macerate was inoculated at three points, all 1–1.5 cm from one another, in a triangular arrangement; this initiated the development of a three way confrontation. Three different levels of VCG test were set up. First 'control' reactions between three macerates of the same isolate of *G. scripta* or *P. dendritica*; second, 'intraspecific' reactions between three macerates of *G. scripta* or *P. dendritica* derived from three different thalli; finally an 'interspecific' reaction between three macerates of the species *G. scripta*, *P. dendritica* and *Ochrolechia parella*. A total of seven control tests (using two isolates of *G. scripta*, five isolates of *P. dendritica*), eight intraspecific tests (using three permutations of *G. scripta* isolates and five of *P. dendritica* from different thalli) and one interspecific test were set up. All tests were duplicated. Cultures were incubated at 18°C in the dark and inspected periodically over a 8 month period to observe whether VCG reactions with characteristic barrage line formation were produced between genetically distinct isolates.

Observations of the axenic cultures of *G. scripta* and *P. dendritica* revealed some pigmentation at the boundaries between growing colonies of different genotype. Pigmentation was less pronounced or absent in control tests with inoculum of the same isolate. In addition, pigmentation of whole colonies appeared to be much greater in intraspecies reactions between genotypically different isolates than in control reactions (Figs. 4B, 4C). However, no striking incompatibility reactions, such as barrage formation indicated by heavy pigmentation of the mycelium, were evident in any of the VCG test reactions.

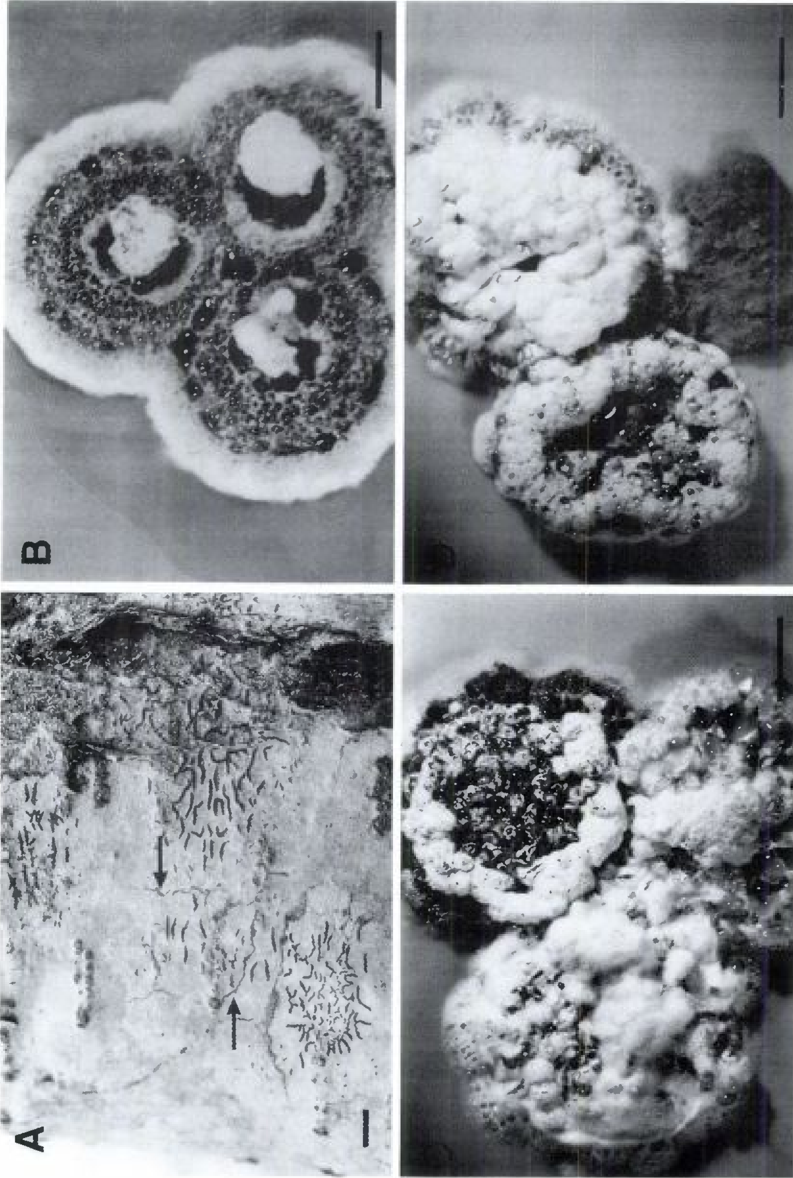

Figure 4. Vegetative incompatibility reactions in lichen-forming fungi. (A) Pigmented demarcation boundaries (arrowed) formed between adjoining thalli of *Graphis scripta* on tree bark. (B) Control reaction between inoculates of the same isolate of *Phaeographis dendritica* showing merger of colonies. (C) Intraspecies reaction between inoculates of different isolates of *G. scripta* showing distinct colony morphologies. (D) Interspecies reaction between inoculates of *G. scripta*, *P. dendritica* and *Ochrolechia parella* with no merger of colonies. Scale bar indicates 0.5 cm.

Growth of *O. parella* was poor on both replicate plates in the interspecies reactions whilst *P. dendritica* developed a thickened margin when challenged by *G. scripta* (Fig. 4D). Other differences in thallus morphology were not markedly greater than those seen in VCG reactions between different genotypes of the same species.

Overall the vegetative incompatibility results were inconclusive with difficulties encountered in discerning clear mycelial interactions. This was due mainly to the growth characteristics of lichen-forming fungi in pure culture. Isolates typically exhibited profuse aerial growth rather than the flat radial growth generally preferred for VCG tests. However, vegetative incompatibility tests may yet be feasible if improved media for the cultivation and manipulation of growth characteristics of lichen-forming fungi become available. These tests may complement RAPD testing, although VCG tests lack the resolution of RAPD analysis (Cantone and Vandenberg, 1998). Some variation in the gross morphology of stock cultures of *G. scripta* on agar slopes was observed. However, the amount of variation between genotypically distinct isolates was generally found to be no greater than between genotypically identical isolates from ascomata on a single thallus (Murtagh, 1999).

5. Conclusions and Future Prospects

We have described how the DNA fingerprinting technique RAPD-PCR and vegetative incompatibility tests have been used to investigate genetic variability and the concept of 'individuality' in lichen-forming fungi. These experimental methods may also have other uses in research involving lichen biology. One topic of current debate concerns the particular criteria used to define species of lichen, and fungal species in general (Grube and Kroken, 2000). These are currently defined on either morphological or biological (ability to mate) grounds. However, Taylor et al. (2000) have argued that such criteria may overlook the presence of 'cryptic species' which are genetically distinct. They recommended the use of a phylogenetic approach to investigate species concepts, involving the sequencing and then pooling of data from multiple gene loci. They argued that the presence of strongly supported branches within any resulting dendrogram could be used as an indication of speciation. This approach was used by Kroken and Taylor (2001) to investigate species boundaries in the lichenised fungus *Letharia*. On the basis of pooled data from six loci they concluded that the *Letharia* complex consisted of six phylogenetic species rather than the two species identified by traditional means. RAPD data may be able to complement this approach, being used to test whether

putative species are indeed supported by groupings arising from RAPD analysis and *vice versa*.

Further insights may come from the use of other DNA fingerprinting techniques. AFLPs is a very powerful method, likely to reveal more genetic polymorphisms than might be detected with RAPD analysis. Meanwhile, other novel techniques may reveal further aspects of lichen biology. For example, preliminary work has identified the possible presence of plasmid-like DNA in nucleic acid extracts of certain isolates of *Graphis scripta* and *Ochrolechia parella* (Murtagh, 1999). Such vectors might be important in DNA transfer and the evolution of the lichen symbiosis (Ahmadjian, 1987).

Acknowledgements

PSD thanks the Biotechnology and Biological Sciences Research Council (UK) for a David Phillips University Research Fellowship. GJM and PDC acknowledge financial support from the Natural Environment Research Council (UK) and the British Antarctic Survey. We also thank P.C. McClure, C.A. Jayawardena and B. Ning for technical assistance, and R. Lewis Smith for helpful discussions.

REFERENCES

Ahmadjian, V. 1987. Coevolution in lichens. In: *Endocytobiology*. Vol. 3. J. Lee and J.F. Fredrick, eds. New York Academy of Sciences, New York, pp. 307–315.

Beard, K.H. and DePriest, P.T. 1996. Genetic variation within and among mats of the reindeer lichen, *Cladina subtenuis*. *Lichenologist* **28**: 171–182.

Bridge, P.D. and Hawksworth, D.L. 1998. What molecular biology has to tell us at the species level in lichenized fungi. *Lichenologist* **30**: 307–320.

Burnett, J.H. 1975. *Mycogenetics: An Introduction to the General Genetics of Fungi*. Wiley, London.

Cantone, F.A. and Vandenberg, J.D. 1998. Intraspecific diversity in *Paecilomyces fumosoroseus*. *Mycological Research* **102**: 209–215.

Carter, A.T., MacKenzie, D.A., Pearson, B.M., and Archer, D.B. 2000. PCR fingerprinting with a consensus tRNA primer enables strain identification of *Mortierella alpina*. *Mycological Research* **104**: 794–799.

Clayden, S.R. 1997. Intraspecific interactions and parasitism in an association of *Rhizocarpon lecanorinum* and *R. geographicum*. *Lichenologist* **29**: 533–545.

Crespo, A., Bridge, P.D., Hawksworth, D.L., Grube, M., and Cubero, O.F. 1999. Comparison of rRNA genotype frequencies of *Parmelia sulcata* from long established and recolonizing sites following sulphur dioxide amelioration. *Plant Systematics and Evolution* **217**: 177–183.

Crespo, A., Cubero, O.F., and Grube, M. 1998. PCR applications in studies of lichen-forming fungi. In: *Applications of PCR in Mycology*. P.D. Bridge, D.K. Arora, C.A. Reddy, and R.P. Elander, eds. CAB International, Wallingford, pp. 85–105.

Culberson, W.L. and Culberson, C.F. 1994. Secondary metabolites as a tool in Ascomycete systematics: lichenized fungi. In: *Ascomycete Systematics: Problems and Perspectives in the Nineties*. D.L. Hawksworth, ed. Plenum Press, New York, pp. 155–163.

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

Driver, F., Milner, R.J., and Trueman, J.W.H. 2000. A taxanomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycological Research* **104**: 134–150.

Dyer, A.T. and Leonard, K.J. 2000. Contamination, error, and nonspecific molecular tools. *Phytopathology* **90**: 565–567.

Dyer, P.S. and Murtagh G.J. 2001. Variation in the ribosomal ITS-sequence of the lichens *Buellia frigida* and *Xanthoria elegans* from the Vestfold Hills, eastern Antarctica. *Lichenologist* **33**: 151–159.

Dyer, P.S., Nicholson, P., Lucas, J.A., and Peberdy, J.F. 1996. *Tapesia acuformis* as a causal agent of eyespot disease of cereals and evidence for a heterothallic mating system using molecular makers. *Mycological Research* **100**: 1219–1226.

Fahselt, D. 1989. Enzyme polymorphism in sexual and asexual umbilicate lichens from Sverdrup Pass, Ellesmere island, Canada. *Lichenologist* **21**: 279–285.

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

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Grube, M. and Kroken, S. 2000. Molecular approaches and the concept of species and species complexes in lichenised fungi. *Mycological Research* **104**: 1284–1294.

Grunig, C.R., Sieber, T.N., and Holdenrieder, O. 2001. Characterisation of dark septate endophytic fungi (DSE) using inter-simple-sequence-repeat-anchored polymerase chain reaction (ISSR-PCR) amplification. *Mycological Research* **105**: 24–32.

Hantula, J., Dusabenyagasani, M., and Hamelin, R.C. 1996. Random amplified microsatellites (RAMS) – a novel method for characterising genetic variation within fungi. *European Journal of Forest Pathology* **26**: 159–166.

Hawksworth, D.L. 1982. Secondary fungi in lichen symbioses: parasites, saprophytes and parasymbionts. *Journal of the Hattori Botanical Laboratory* **52**: 357–366.

Hawksworth, D.L. and Chater, A.O. 1979. Dynamism and equilibrium in a saxicolous lichen mosaic. *Lichenologist* **11**: 75–80.

Hawksworth, D.L. and Hill, D.J. 1984. *The Lichen-Forming Fungi*. Blackie, Glasgow and London.

Heibel, E., Lumbsch H.T., and Schmitt, I. 1999. Genetic variation of *Usnea filipendula* (Parmeliaceae) populations in western Germany investigated by RAPDs suggest reinvasion from various sources. *American Journal of Botany* **86**: 753–757.

Hyun, J.-W. and Clark, C.A. 1998. Analysis of *Fusarium lateritum* using RAPD and rDNA RFLP techniques. *Mycological Research* **102**: 1259–1264.

Jahns, H.M. and Ott, S. 1997. Life strategies in lichens – some general considerations. *Bibliotheca Lichenologia* **67**: 49–67.

Jeffreys, A.J., Wilson, V., and Thein, S.L. 1985. Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**: 67–73.

Kroken, S. and Taylor, J.W. 2001. A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*. *Mycologia* **93**: 38–53.

Landry, P.A. and Lapointe, F.J. 1997. RAPD problems in phylogenetics. *Zoologica Scripta* **25**: 283–290.

Larson, D.W. and Carey, C.K. 1986. Phenotypic variation within 'individual' lichen thalli. *American Journal of Botany* **73**: 214–223.

Laundon, J.R. 1978. *Haematomma* chemotypes form fused thalli. *Lichenologist* **10**: 221–225.

Leslie, J.F. 1993. Fungal vegetative compatibility. *Annual Review of Phytopathology* **31**: 127–150.

Longato, S. and Bonfante, P. 1997. Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycological Research* **101**: 425–432.

Lohtander, K., Källersjö, M., and Tehler, A. 1998. Dispersal strategies in *Roccellinia capensis* (Arthoniales). *Lichenologist* **30**: 341–350.

Lübeck, M., Alekhina, I.A., Lübeck, P.S., Jensen, D.F., and Bulat, S.A. 1999. Delineation of *Trichoderma harzianum* into two different genotypic groups by a highly robust fingerprinting method, UP-PCR and UP-PCR product cross-hybridization. *Mycological Research* **103**: 289–298.

Milgroom, M.G. 1996. Recombination and the multilocus structure of fungal pathogens. *Annual Review of Phytopathology* **34**: 457–477.

Morris, P.F., Connolly, M.S., and St. Clair, D.A. 2000. Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RAPDs. *Mycological Research* **104**: 286–292.

Munaut, F., Hamaide, N., Vanderstappen, J., and Maraite, H. 1998. Genetic relationships among isolates of *Colletotrichum gloeosporioides* from *Stylosanthes* spp. in Africa and Australia using RAPD and ribosomal DNA markers. *Plant Pathology* **47**: 641–648.

Murtagh, G.J. 1999. Sex and variation in lichen-forming fungi. Ph.D. Thesis. University of Nottingham.

Murtagh, G.J., Dyer, P.S., McClure, P.C., and Crittenden, P.D. 1999. Use of randomly amplified polymorphic DNA markers as a tool to study variation in lichen-forming fungi. *Lichenologist* **31**: 257–267.

Murtagh, G.J., Dyer, P.S., and Crittenden, P.D. 2000. Sex and the single lichen. *Nature* **404**: 564.

Newton, C.R. and Graham, A. 1997. *PCR*. Second edition. Bios Scientific Publishers, Oxford.

Nauta, M.J. and Hoekstra R.F. 1996. Vegetative incompatibility in ascomycetes: highly polymorphic but selectively neutral? *Journal of Theoretical Biology* **183**: 67–76.

Oliver, E., Crittenden, P.D., Beckett, A., and Brown, D.H. 1989. Growth of lichen-forming fungi on membrane filters. *Lichenologist* **21**: 387–392.

Ott, S. 1987a. Sexual reproduction and developmental adaptations in *Xanthoria parietina*. *Nordic Journal of Botany* **7**: 219–228.

Ott, S. 1987b. Reproductive strategies in lichens. *Bibliotheca Lichenologica* **25**: 81–93.

Pentecost, A. 1980. Aspects of competition in saxicolous lichen communities. *Lichenologist* **12**: 135–144.

Prillinger, H., Kraepelin, G., Lopandic, K., Schweigkofler, W., Molnar, O., Weigang, F., and Dreyfuss, M.M. 1997. New species of *Fellomyces* isolated from epiphytic lichen species. *Systematic and Applied Microbiology* **20**: 572–584.

Printzen, C., Lumbsch H.T., Schmitt, I., and Feige, G.B. 1999. A study on the genetic variability of *Biatora helvola* using RAPD markers. *Lichenologist* **31**: 491–499.

Purwantara, A., Barrins, J.M., Cozijnsen, A.J., Ades, P.K., and Howle, B.J. 2000. Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. *Mycological Research* **104**: 772–781.

Rayner, A.D. 1991. Challenge of the individualistic mycelium. *Mycologia* **83**: 48–71

Shaw, A.J. 1992. The evolutionary capacity of bryophytes and lichens. In: *Bryophytes and Lichens in a Changing Environment*. J.W. Bates and H.M. Farmer, eds. Oxford, Clarendon Press, pp. 362–379.

Smith, A.L. 1921. *Lichens*. Cambridge University Press, Cambridge.

Taylor, J.W, Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., and Fisher, M.C. 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**: 21–32.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA-fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Wang, X.R., Ennos, R.A., Szmidt, A.E., and Hansson, P. 1997. Genetic variability in the canker pathogen fungus, *Gremmeniella abietina*. 2. Fine scale investigation of the population genetic structure. *Canadian Journal of Botany* **75**: 1460–1469.

Weising, K., Nybom, H., Wolff, K., and Meyer, W. 1995. *DNA Fingerprinting in Plants and Fungi*. CRC Press, Boca Raton.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**: 7513–7214.

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

Zietkiewicz, E., Rafalski, A., and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain-reaction amplification. *Genomics* **20**: 176–183.

Zoller, S., Lutzoni, F., and Scheidegger, C. 1999. Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. *Molecular Ecology* **8**: 2049–2059.