PCR PRIMERS FOR THE AMPLIFICATION OF MITOCHONDRIAL SMALL SUBUNIT RIBOSOMAL DNA OF LICHEN-FORMING ASCOMYCETES

Stefan ZOLLER,★ Christoph SCHEIDEGGER★ and Christoph SPERISEN★

Abstract: Four primers for the amplification of mitochondrial DNA of lichen-forming ascomycetes are presented. The primers match the conserved regions U2, U4, and U6, respectively, of mitochondrial small subunit (SSU) ribosomal DNA (rDNA). Polymerase chain reaction using different combinations of the primers produced single amplification products from DNA of eight lichen-forming fungal species but did not amplify DNA of two axenic cultured algal species. The amplification product obtained from *Lobaria pulmonaria* was sequenced and the 894-bp sequence was compared with the mitochondrial SSU rDNA sequence of *Podospora anserina*. The two sequences revealed more than 76% identity in the conserved regions U3 to U5 demonstrating that we amplified mitochondrial DNA. The primers matching U2 and U6 yielded amplification products of 800–1000 bp depending on the species examined. The variation observed suggests that mitochondrial SSU rDNA may be useful for phylogenetic analyses of lichen-forming ascomycetes. © 1999 The British Lichen Society

Introduction

Analysis of nuclear ribosomal DNA (rDNA) has become an important tool in molecular studies of lichens. These studies have used rDNA to provide insight into the origin of lichens (Gargas *et al.* 1995), phylogenetic relationships (Lutzoni & Vilgalys 1995; Tehler 1995), and population structures (DePriest 1993). Application of mitochondrial rDNA for lichen studies has not been described although mitochondrial rDNA of fungal species often reveals high levels of nucleotide substitutions and length mutations (Hibbett & Donoghue 1995; Gryta *et al.* 1997; Bruns *et al.* 1998; Gonzales & Labarere 1998; Johnson 1999). In the basidiomycete order Boletales the variation of mitochondrial rRNA genes was even higher than that of nuclear rRNA genes (Bruns & Szaro 1992).

In this study, we present primers for the amplification of mitochondrial small subunit (SSU) rDNA of lichen-forming fungi. The primers match the universal regions U2, U4 and U6, respectively. These regions are part of eight universally conserved regions that form the minimal core secondary structure of mitochondrial SSU rRNA (Gray *et al.* 1984; Schnare *et al.* 1986; Cummings *et al.* 1989). We show that the novel primers produce amplification

*Swiss Federal Institute for Forest, Snow and Landscape Research, Zürcherstrasse 111, CH–8903 Birmensdorf, Switzerland. e-mail: stefan.zoller@wsl.ch

© 1999 The British Lichen Society

TABLE 1. *Primer sequences for amplification of mitochondrial SSU rDNA*

Primer	Primer sequence	Forward/reverse	Homologous to U region
mrSSU1	5′-AGCAGTGAGGAATATTGGTC-3′	Forward	U2
mrSSU2	5′-CTGACGTTGAAGGACGAAGG-3′	Forward	U4
mrSSU2R	5′-CCTTCGTCCTTCAACGTCAG-3′	Reverse	U4
mrSSU3R	5′-ATGTGGCACGTCTATAGCCC-3′	Reverse	U6

products from a broad range of lichen-forming ascomycetes and demonstrate that the amplification products reveal length differences among different species.

Materials and Methods

Lichen and algal material

The lichen species investigated were collected in Switzerland: *Lobaria pulmonaria* and *Peltigera praetextata* in Vordernwald, Canton of Aargau (co-ordinates 47°17 ± 20″N, 7°52 ± 30″E); *Lecanora allophana* and *Cladonia digitata* in Gurnigel, Canton of Bern (46°45 ± 00″ N, 7°26 ± 20″ E); *Leptogium saturninum* and *Graphis scripta* in Wägital, Canton of Schwyz (47°04 ± 00″ N, 8°54 ± 40″ E); *Parmelia pastillifera* and *Parmelia sulcata* in Bondo, Canton of Graubünden (46°20 ± 00″ N, 9°32 ± 00″ E).

The algae *Trebouxia species* and *Dictyochloropsis reticulata*, the photobiont of *L. pulmonaria*, were isolated using the procedure developed by Yamamoto (1987) and cultured on Bold's Basal Medium BBM (Bischoff & Bold 1963).

Extraction of total DNA

Total DNA of lichens was extracted as described by Ziegenhagen *et al.* (1993) and purified using the QIAamp DNA Blood Mini Kit (QIAGEN). Air-dried thallus pieces (up to 70 mg) were cleaned by hand, transferred into a 2-ml precooled microfuge tube containing an agate ball (7 mm in diameter), and ground to fine powder in a shaking mill (Micro-Dismembrator II, Braun) for 2 min at full speed. The powder was dispersed in 350 µl of extraction buffer [100 mM sodium acetate pH 5·5, 50 mM EDTA, 500 mM NaCl, 2% (w/v) polyvinyl pyrrolidone, 1·4% (w/v) sodium dodecyl sulphate supplemented with 0·5% (w/v) sodium bisulphite], incubated at 65°C for at least 20 min, and centrifuged for 15 min at 20 000 *g*. The supernatant (*c.* 300 µl) was transferred to a new 1·5-ml tube and 300 µl of AL buffer (provided with the kit) was added. The mixture was thoroughly shaken and incubated at 70°C for 10 min. Three hundred microlitres of absolute ethanol were added and the mixture was transferred onto a spin column placed on a 2-ml collection tube (provided with the kit) and centrifuged for 1 min at 6800 *g*. The column was washed by addition of 400 µl of AW washing buffer (provided with the kit) and centrifugation for 1 min at 6800 *g*. The washing step was repeated with a final centrifugation for 1 min at 20 000 *g*. The spin column was placed onto a new 1·5 ml microfuge tube and incubated for 10 min at 70°C to dry the membrane. DNA was eluted by addition of 300 µl TE (1 mM EDTA, 10 mM Tris-HCl pH 9·0, preheated to 70°C), incubation for 5 min at 70°C in an oven, and centrifugation for 1 min each at 6800 *g* and 20 000 *g*. Finally, 5 µl RNAse A (10 mg ml^{-1}) was added to each sample.

Primer construction and PCR

Four primers for amplification of fungal mitochondrial SSU rDNA were designed (Table 1). The primer sequences were derived from conserved regions among the ascomycetes *Neurospora crassa* (GenBank accession number: J05254), *Aspergillus terricola* (U29212), *Trichophyton rubrum* (X88896), *Beauveria bassiana* (U91338), *Petromyces albertensis* (U29229), and *Podospora anserina* (X14734). A BLAST search on the GenBank database was performed to verify specificity to
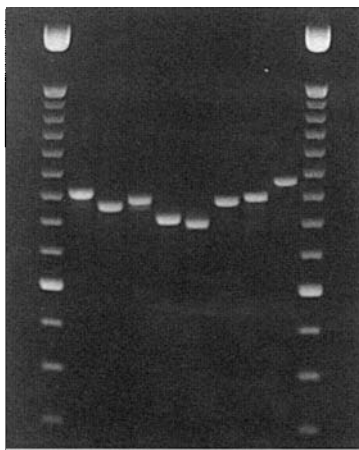
Fig. 1. Agarose-gel electrophoresis of PCR products obtained with primers matching the conserved regions U2 and U6 of the mitochondrial SSU rDNA. Lanes 1 and 10: 100-bp DNA ladder with brighter 600-bp band (GIBCOBRL). Lanes 2–9 contain 5 µl of PCR product of the following taxa: *Cladonia digitata* (2); *Peltigera praetextata* (3), *Graphis scripta* (4), *Lecanora allophana* (5), *Leptogium saturninum* (6), *Lobaria pulmonaria* (7), *Parmelia pastillifera* (8), and *Parmelia sulcata* (9).

fungal sequences. Amplification of fungal and algal DNAs was performed in 50 µl reaction volumes [1 × PCR buffer (GIBCOBRL), 1 mM dNTPs, 2 mM MgCl$_2$, 2 µM of each primer, 2·5 U Taq DNA polymerase (GIBCOBRL), 2 µl DNA extract] in a thermal cycler (PTC-100, MJ RESEARCH, INC.) using a standard cycling protocol: denaturation at 94°C for 3 min; 35 cycles with 94°C denaturation for 1 min, annealing at 52°C for 1 min, and extension at 72° for 1 min; followed by a final extension at 72°C for 7 min. PCR products were analysed on 2% TBE-agarose gels. The two primers MS1 and MS2 (White *et al.* 1990) were tested using the same PCR procedure with DNA of *L. pulmonaria*.

Sequence analysis

The PCR-product of *L. pulmonaria* was ethanol precipitated and sequenced with the Applied Biosystems Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI). Nucleotide base detection was performed on an Applied Biosystems 310 genetic analyser. Cycle sequencing was carried out with the PCR primers. Sequences were assembled using Sequencher 3·0 (Gene Codes Corporation) and manually adjusted. The alignment of *L. pulmonaria* and *P. anserina* sequences was done with GAP of the GCG 9·1 software package (Genetics Computer Group) by using a gap creation penalty of 5·00 and a gap extension penalty of 0·30. An unambiguous alignment of the two sequences was not possible. We did not do any visual adjustments although in several regions more matches could have been obtained.

Results and Discussion

The primers MS1 and MS2, previously described for the amplification of fungal mitochondrial small subunit rDNA (White *et al.* 1990) did not amplify DNA of *Lobaria pulmonaria*. However, the primers we designed on the basis of conserved ascomycete sequences yielded single amplification products from all eight lichen-forming fungal species examined. The primers did not amplify DNA from axenic cultures from the two algae *Trebouxia* species and *Dictyochloropsis reticulata*, the photobiont of *L. pulmonaria*. Moreover, a GenBank

```
                        mrSSU1          U2
P. anserina    AC AGCAGTGAGGAATATTGGTC AATGGCCTAACGGCTGAACTGGTAACTTGGA................  70
                             ||| |||||||||||||||| | |||||||
L. pulmonaria                AATAGCCTAACGGCTGAACCAGCAACTTGGAGGAATGAAAGGGCAATA

P. anserina    .......................AGAATGGCTCCATAAAAATGGAGTTAATCGATTTATCGAATAAA  140
                                      || |   |   |  |||| ||||| |  ||         ||
L. pulmonaria  GCTGATTGCATTAAATTTAAAATTAGAGTTTTTTTTTATTTTTAATGTAGTTACTACTTTGTAGTTTAAAT

                                                               U3
P. anserina    ATTCTGTATACATACCTATAATGACGGTATGTACATCTT.GTCTTGACC.AATTGCGTGCCAGCAGTCGC  210
                |   |   ||    || ||||| ||| || | ||||||||| |||| |||||||||||||||||
L. pulmonaria  TTCTAGGTAGAATTATGATGATGACATTATCTATCTATAGGTCTTGACCAAATTACGTGCCAGCAGTCGC

                      U3
P. anserina    GGCAATACGTAAGAGACTAGTGTTATTCATCTTAAATAGGTTTAAAGGGTACTTAGACG...........  280
               || |||||||  |||| ||||||||||||||| | |||||||||| |||| |||||
L. pulmonaria  GGTAATACGTAGAAGACAAGTGTTATTCATCTTTATTAGGTTTAAAGAGTACCAAGACGGTTAATAAAGC

                      U3
P. anserina    .GTAGAATTTGCCTTAACGGGAACAATTTTACTAGAGTTTTATATAAG.....AAGATCTTACTCTAGAG  350
                ||  |||||| |||  || ||||||| ||  ||||| |||    |||       |||| |||| ||
L. pulmonaria  CCAAAATACATGAGCACAGGGGACACATTAACTAGAGTTATATGAAAGGAAGCATTAAGGTACTGTTGAG

P. anserina    TGTAGAGTTGTAATTCATTAATACC.....TAGGGGACTGGTAAAGGCGAAGGCA.ATCTTTTATATAAA  420
               |||||| ||| |     ||||| |         | | ||| |  | |  |||||||   | | ||| |||
L. pulmonaria  TGTAGAGATAAAATTCAGTCATACCAGATGAAAGGCACAGATTTATGCGAAGGCATCTTTCTTTATGTAAT

                    mrSSU2/mrSSU2R          U4
P. anserina    AA CTGACGTTGAAGGACGAAGG CTTAGGTCACAAAAAGGATTAGATACCCAAGTAGTCTTAGCAGTCAAT  490
                  |||||||| ||||||||||||  |        | ||||||||||| ||| |||||||| || |
L. pulmonaria  AACTGACGTTGTAGGACGAAGGCTTTTGAAGTGAAAAGG.TTAGATACCCTTGTAATTTAAGCAGAAAAT

P. anserina    TATGAATGCCATAGGTTAGATATAGTTAAATACAAACAAGGGCGTAAGCTCGTATTAAATTTTACCATCT  560
                |||||| ||      ||| |   |  |   |  |  |         | | | |       | | | |
L. pulmonaria  GATGAATGTTA..........TAGGTTTAATATATACAGCCCAAAAAAAAAAGGGTCAATGTTTTATTG

                                                  U5
P. anserina    AGTCTATAAATGAAAGTGTAAGCATTTCACCTCAAGAGTAATGTGGCAACGCAGGAACTGAAATCACTAG  630
                ||||||||| ||||||  ||||| |||  |  ||||||||  || ||||| || ||||| |||| ||
L. pulmonaria  GGTCTATAAATGAAATTGTAAGCATCCCTCCTCAAAATTAATTGGCAACATTGGAACTGAAATCATTAA

P. anserina    ACCGTTTCTGACACCAGCAGTGAAGTATGTTATTTAATTCGATGACCCACGAAAAACCTTACCACAACTT  700
               |||||||||| || |||||| ||||||||||| | ||||||  |||| |||   ||| |||||||||
L. pulmonaria  ACCGTTTCTGAAACCAGTAGTGAAGTATGTTGTTTAATTAGATGATACACAAAAAACCTTACCACAAT..

P. anserina    GAATATAAAAAAATGCTATCTACTACCATCACTTATTTAAAGGTAGCGGCGCGGGGCGGCGAAAGCTCCT  770
L. pulmonaria  .......................................................................

P. anserina    CCTCGCTCTAAATTTAAGTGTTAGGCGCAAGCTCTAAGATATATAGCTGGCTTAAATATTATTTAGCTAA  840
L. pulmonaria  .......................................................................

P. anserina    CTATGCTTTTAGGTTATAGATTAATGCAAGTATTATACAAGTGTTGCACGGCTGTCTTCAGTTGATGTTG  910
                |  |   |||| |      |||| ||||||  ||||| ||||||||||||||||||||||  |||||
L. pulmonaria  .........TTGAATATATATCATTAATGATATT.TACAAGCGTTGCACGGCTGTCTTCAGTTAATGTCG

P. anserina    TGAAACTGTGGTTCGGTCCATGGAATTAACGAAAACCCTTGCTTTATTTGTAAATTCTAAAATAAAGCAG  980
               ||||| | ||||| ||||| |  ||||||||||| |||| ||| |||||||     ||||| | |
L. pulmonaria  TGAAATTTTGGTTAGTTCCATAAAATTAACGTAAACCCTCGTTTTATTTATAAA...TAAAATTTACAAT

                                                       U6            mrSSU3R
P. anserina    TTCACCTGTATATGGGTTATGATAAAAGGGATCAAGACAAGTCCTCATGGCCTTGATGTTGT GGGCTATA  1050
                | ||| |    | |   | |  | | |||| || |  | |||| |  ||||||||| ||| |||
L. pulmonaria  CTGTTTCTAAAATTTGATTTAAAAAAGG..GACTAAACAATTCACATGGCCTAAATATTGT

                    mrSSU3R
P. anserina    GACGTGCCACAT GTACCTAAACAAAGAG   1078
```
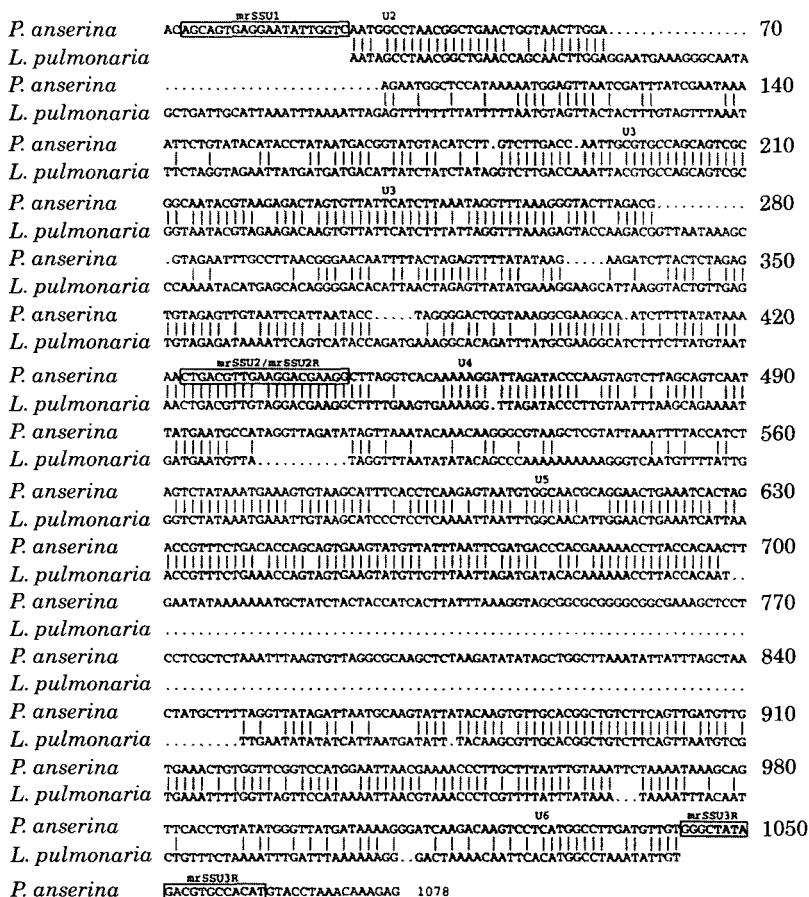
FIG. 2. Sequence comparison of mitochondrial SSU rDNA of *Podospora anserina* (GenBank Accession X14734) and *Lobaria pulmonaria*. The mitochondrial sequence of *L. pulmonaria* was deposited in the GenBank database (Accession Nr. AF069541). U2 to U6 denote the universally conserved regions and are shaded. Primer sequences are given in boxes.

search did not reveal any significant similarities between the primer and algal sequences.

Primers matching the universally conserved regions U2 and U6 (see Table 1) produced single amplification products of approximately 800–1000 bp depending on the species examined (Fig. 1). Primers homologous to U2 and U4, and U4 and U6, respectively, resulted in amplification products of 400–500 bp. Five of the seven species could clearly be distinguished based on PCR-fragment-length differences. The two species *Graphis scripta* and *L. pulmonaria* gave similar fragment lengths.

The amplification product of *L. pulmonaria* was sequenced and the 894-bp sequence was compared with the mitochondrial SSU rDNA sequence of *Podospora anserina* (Cummings *et al.* 1989). The two sequences revealed 76%

to 89% identity in the regions U3 to U5 demonstrating that mitochondrial DNA was amplified (Fig. 2). From U2 and U6, only segments were amplified revealing 88% and 71% identity, respectively. The sequences between the conserved regions showed considerable length differences. To align these sequences, a total of nine gaps of 1 to 131 bp length had to be introduced (Fig. 2). Parts of these regions could not be aligned unambiguously.

Our findings are consistent with results obtained from basidiomycetes, often showing species-specific length mutations in mitochondrial rDNA (Bruns & Szaro 1992; Hibbett & Donoghue 1995; Bruns *et al.* 1998; Gonzales & Labarere 1998). This variation together with nucleotide substitutions has been proved to be useful for phylogenetic studies. By analogy with the basidiomycetes the findings of this study shows promise that mitochondrial SSU rDNA may also be useful for studying the phylogeny of lichen-forming ascomycetes. Mitochondrial rDNA may be valuable for phylogenetic analyses for several reasons. First, mitochondrial rDNA appears to reveal high rates of nucleotide substitutions in many fungal species, in the order Boletales higher than that observed in its nuclear counterpart (Bruns & Szaro 1992). A reduced constraint on mitochondrial rRNA has been proposed as an important factor contributing to the high rates of nucleotide substitutions observed although increased mutation rates could not be excluded. Second, mitochondrial DNA is uniparentally inherited in most ascomycetes studied so far (Röhr *et al.* 1999). Variation uniparentally inherited is more prone to genetic drift and founder events than variation of nuclear DNA, both resulting in reduced diversity and higher rates of molecular divergence (Moritz *et al.* 1987). Finally, concerted evolution might be rather different in mitochondrial rDNA because the mitochondrial genomes are partitioned into many mitochondria and are separated mitotically. In summary, mitochondrial rDNA appears to show different modes and rates of evolution than nuclear rDNA and may thus represent an additional marker to the widely used nuclear rDNA.

REFERENCES

Bischoff, H. W. & Bold, H. C. (1963) Physiological studies: IV. Some soil algae from enchanted rock and related algal species. *University of Texas Publications No. 6318, Physiological Studies* **4**: 1–95.

Bruns, T. D. & Szaro, T. M. (1992) Rate and mode differences between nuclear and mitochondrial small-subunit rRNA genes in mushrooms. *Molecular Biology and Evolution* **9**: 836–855.

Bruns, T. D., Szaro, T. M., Gardes, M., Cullings, K. W., Pan, J. J., Taylor, D. L., Horton, T. R., Kretzer, A., Garbelotto, M. & Li, Y. (1998) A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. *Molecular Ecology* **7**: 257–272.

Cummings, D. J., Domenico, J. M., Nelson, J. & Sogin, M. L. (1989) DNA sequence, structure and phylogenetic relationships of the small subunit rRNA coding region of mitochondrial DNA from *Podospora anserina*. *Journal of Molecular Evolution* **28**: 232–241.

DePriest, P. T. (1993) Small subunit rDNA variation in a population of lichen fungi due to optional group I introns. *Gene* **134**: 67–71.

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

Gonzales, P. & Labarere, J. (1998) Sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6, and V9 domains reveal highly species-specific variations within the genus *Agrocybe*. *Applied and Environmental Microbiology* **64**: 4149–4160.

Gray, M. W., Sankoff, D. & Cedergren, R. J. (1984) On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Research* **12**: 5837–5852.

Gryta, H., Debaud, J. C., Effosse, A., Gay, G. & Marmeisse, R. (1997) Fine-scale structure of populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum* in coastal sand dune forest ecosystems. *Molecular Ecology* **6**: 353–364.

Hibbett, D. S. & Donoghue, M. J. (1995) Progress toward a phylogenetic classification of the Polyporaceae through parsimony analysis of mitochondrial ribosomal DNA sequences. *Canadian Journal of Botany* **73**(Suppl. 1): 853–861.

Johnson, J. (1999) Phylogenetic relationships within *Lepiota* sensu lato based on morphological and molecular data. *Mycologia* **91**: 443–458.

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

Moritz, C., Dowling, T. E. & Brown, W. M. (1987) Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics* **18**: 269–292.

Röhr, H., Kües, U. & Stahl, U. (1999) Recombination: Organelle DNA of plants and fungi: Inheritance and recombination. In *Progress in Botany 60* (K. Esser, J. W. Dadereit, U. Lüttge & M. Runge, eds): Berlin: Springer.

Schnare, M. N., Heinonen, T. Y. K., Young, P. G. & Gray, M. W. (1986) A discontinuous small subunit ribosomal RNA in *Tetrahymena pyriformis* mitochondria. *Journal of Biological Chemistry* **261**: 5187–5193.

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

White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols, a Guide to Methods and Applications* (M. A. Innis, D. H. Gelfan, J. J. Sninsky & T. White, eds): 315–322. San Diego: Academic Press.

Yamamoto, Y. (1987) Tissue culture of lichens. In *Tissue Cultures of Lichen and Bryophyte* (Y. Yamada et al., eds): 14–25. Osaka: Nippon Paint Company.

Ziegenhagen, B., Guillemaut, P. & Scholz, F. (1993) A procedure for mini-preparations of genomic DNA from needles of silver fir (*Abies alba* Mill.). *Plant Molecular Biology Reports* **11**: 117–121.