

Eduardo R. Nouhra · Thomas R. Horton ·
Efren Cazares · Michael Castellano

Morphological and molecular characterization of selected *Ramaria* mycorrhizae

Received: 21 May 2003 / Accepted: 20 October 2003 / Published online: 27 January 2004
© Springer-Verlag 2004

Abstract *Ramaria* species are conspicuous mycorrhizal symbionts of conifers in the Pacific Northwest. Here we collected and identified sporocarps and associated ectomycorrhizae of *Ramaria acrisiccescens* Marr & Stuntz, *R. cyaneigranosa* Marr & Stuntz, *R. sandaracina* Marr & Stuntz, *R. celerivirescens* Marr & Stuntz, and *R. flavobrunnescens* var. *aromatica* Marr & Stuntz. An internal transcribed spacer (ITS)- restriction fragment length polymorphism pattern was observed for each of the *Ramaria* species and used as a diagnostic tool to support the identification of mycorrhizae occurring in mats below the sporocarps. We provide a description of ectomycorrhizae of *Ramaria*, which exhibit similar macro- and microscopic characteristics such as ramification pattern, coloration, abundance of mycelial strands and emanating hyphae, mantle morphology and chemical reactions of mantle and mycelial strands with KOH, FeSO₄ and Melzer's reagent. Sequences of the ITS region for each of the species are deposited in the GenBank.

Keywords *Ramaria* · Ectomycorrhizae · Internal transcribed spacer-restriction fragment length polymorphism · Morphology

E. R. Nouhra (✉)
IMBIV (CONICET/UNC),
C.C. 495, 5000 Córdoba, Argentina
e-mail: nouhra@imbiv.unc.edu.ar
Fax: +54-351-4332104

T. R. Horton
350 Illick Hall,
SUNY-ESF,
Syracuse, NY 13210, USA

E. Cazares
Department of Forest Science,
Oregon State University,
Corvallis, OR 97331, USA

M. Castellano
Pacific Northwest Research Station,
USDA Forest Service,
3200 Jefferson Way, Corvallis, OR 97331, USA

Introduction

Ramaria species are common and conspicuous fungi associated with conifers in the Pacific Northwest of North America (Marr and Stuntz 1973). They are usually known as coral fungi due to their colorful and finely branched sporocarps. Their macro and micro-morphological characters are quite diverse, as well as habitat conditions. Basidiocarps are either lignicolous or terricolous with many species forming a characteristic mycelial mat in the soil beneath the sporocarps. The mycelial mat consists of a compact hydrophobic aggregation of fungal strands, mycorrhizal roots and substrate. Similar mats are also formed by other ectomycorrhizal genera such as *Hysterangium* and *Gautieria*. In the case of *Hysterangium* and *Gautieria* these mats have been shown to play an important role in nutrient cycling (Entry et al. 1991, 1992; Griffiths et al. 1994). However, little data are available to suggest that different mat-forming species can be identified based on the mat structure alone.

Traditionally, the identification of the fungal symbionts of ectomycorrhizal roots has been determined by exhaustive observation of hyphal connections between the mantle and sporocarps (Agerer 1987–1998, 1991) or through pure culture synthesis of the symbiosis (Molina and Trappe 1982; Zak 1973, 1974). Ectomycorrhizae of *Ramaria aurea*, *R. largentii*, *R. spinulosa* and *R. subbotrytis* have been described using the former methodology (Agerer et al. 1996).

Molecular tools have been applied in mycorrhizal research to differentiate and identify symbionts (Erland et al. 1994; Gardes and Bruns 1993, 1996; Horton 2002; Kårén et al. 1997; Kernaghan et al. 1997; Kraigher et al. 1995). These techniques enable rapid and accurate identification of fungal taxa from mycorrhizal roots. Here, we characterize ectomycorrhizae occurring in mats associated with sporocarps of five *Ramaria* species and identify the species of fungus by matching internal transcribed spacer-restriction fragment length polymorphisms (ITS-RFLPs) from sporocarps to those from the ectomycorrhizae.

Material and methods

Specimens and associated mycorrhizae were collected from a forest stand of second growth Douglas-fir (*Pseudotsuga mensiezii*) and western hemlock (*Tsuga heterophylla*). The forest is between 80 and 120 years old, with herbaceous understory plants dominated by *Gaultheria shallon* and sword fern (*Polystichum* sp.). The area is located in Siuslaw National Forest, Mary's Peak trail (44°31'781"N, 123°32'960"W), Oregon.

Ramaria sporocarps were collected in autumn 2000. Fresh characters were recorded and the sporocarps were identified following Marr and Stunz (1973). Sporocarps were dried and deposited in the Oregon State University Herbarium.

Soil samples were taken by cutting out a 15-cm³ block of soil directly beneath *Ramaria* sporocarps. Soil samples were placed in plastic bags and stored at 4°C. Soil samples were examined with a Zeiss stereo microscope at 10–40× for hyphal connections leading from sporocarps to fungal mantles of mycorrhizal tips (Agerer 1991).

Mycorrhizal roots and mycelial strands, were cleaned of soil particles and stored for few days in water at 4°C, until microscopic examination. Mycorrhizae were characterized following a modified approach of that developed by Agerer (1991). Chemical reactions on the mantle surface and mycelial strands were tested with 15% KOH, FeSO₄ and Melzer's reagent (Largent et al. 1977).

Subsamples of ectomycorrhizal roots were cleaned of soil and debris in running tap water by wet sieving, using a series of mesh sizes (2 mm, 850 μm, 250 μm) and stored in 2×CTAB buffer solution at 4°C for further analysis (100 mM TRIS pH 9.0, 1.4 M NaCl, 20 mM EDTA, 2%CTAB).

DNA was extracted individually from one to three mycorrhizal root tips and from sporocarp branch tissue, as described by Gardes and Bruns (1993). The ITS region of the nuclear rDNA repeat was amplified by polymerase chain reaction (PCR) using ITS-1F and ITS-4 primers (Gardes and Bruns 1993; White et al. 1990).

Variation in the ITS region was characterized by RFLP patterns. RFLP reactions from mycorrhizal and sporocarp samples, digested by the same enzyme, were run in adjacent lanes on a 3% agarose gel for direct comparison. A 100-base pair DNA ladder was used to determine fragment size (New England Biolabs). Gels were stained in ethidium bromide and observed under UV light. Band size estimations were made with Scanalytics, Gene Profiler 3.56 software. We consider ITS-RFLPs from two independent restriction reactions using *Alu* I and *Hinf* I diagnostic for a species (Gardes and Bruns 1993; Kärén et al. 1997).

DNA products were purified with the QIAquick PCR purification kit, following the manufacturer's protocol. The purified PCR products were sequenced with the primer ITS-1F on an ABI model 373A (Perkin-Elmer) automated DNA sequencer at the Center of Gene Research and Biotechnology at Oregon State University. DNA Sequencing Analysis (version 2.01) and Sequence Navigator software were used to process the raw data.

Results

All *Ramaria* sporocarps were identified to species (see Table 1 for names). Mats beneath sporocarps consisted of abundant mycelial strands in aggregation with soil par-

ticles, occurring mostly in the mineral soil layer, and included dense clusters of mycorrhizae. The soils associated with the mats appeared hydrophobic and lighter in color than the surrounding moist, humic soils. Mycorrhizal root tips appeared turgid and active, with a whitish uniform mantle and numerous hyphal strands intermingled with numerous root tips that appeared senescent. Some mycorrhizae were brownish olive to dark brown to almost black, probably an indication that they were old and inactive. In most cases, it was not possible to trace rhizomorphs connecting the base of the sporocarp to the mycorrhizal root tips, although mycelial strands in the mats were abundant. A general description of *Ramaria* mycorrhizae is given below, with exceptions unique to some of the species in our study noted.

Morphotype description

Type irregular, monopodial pinnate or monopodial pyramidal. Length of system: 4.5–15 mm (20×); length of tips 1.5–4.5 mm (20×); width of tips 250–600 (–750) μm (20×); tip shape mostly straight and bent, some slightly tortuous. Color: white to silvery white due to air trapped in mantle hyphae, yellowish at the end. Surface: smooth, some areas wooly (Fig. 1A, C), mostly with hyphal fans.

Emanating hyphae

Mostly aggregated as hyphal fans and strands along the mantle surface, then projecting into the substrate, usually white to cream colored (Fig. 1D).

Rhizomorphs

Dimensions variable due to the presence of numerous ramifications, flat to rounded in cross section, smooth (Fig. 1B).

Single emanating hyphae

Present on some areas of the mantle surface, 2–5 μm in diameter, hyaline, not ornamented or finely incrustated, septate, contents clear, ramifications rare, thin-walled or slightly thickened up to 1 μm, anastomoses of the H type

Table 1 Restriction fragment band size expressed as number of base pairs (bp), for *Ramaria* spp., obtained with restriction digests of the internal transcribed spacer region using *Hinf* I and *Alu* I. OSC Oregon State University Herbarium

Taxon	Collection	GenBank accession no.	<i>Hinf</i> I	<i>Alu</i> I
<i>R. acrisiccescens</i>	OSC 87690	AY102857	363/258	509/187
	OSC 87692	AY102858		
<i>R. cyaneigranosa</i>	OSC 87689	AY102859	355/180/168	282/243/186
<i>R. sandaracina</i>	OSC 87695	AY102860	366/172/112	702
<i>R. celerivirescens</i>	OSC 87688	AY102861	337/177/164	673
<i>R. flavobrunnescens</i> var. <i>aromatica</i>	OSC 87693	AY102864	391/240/114	545/184

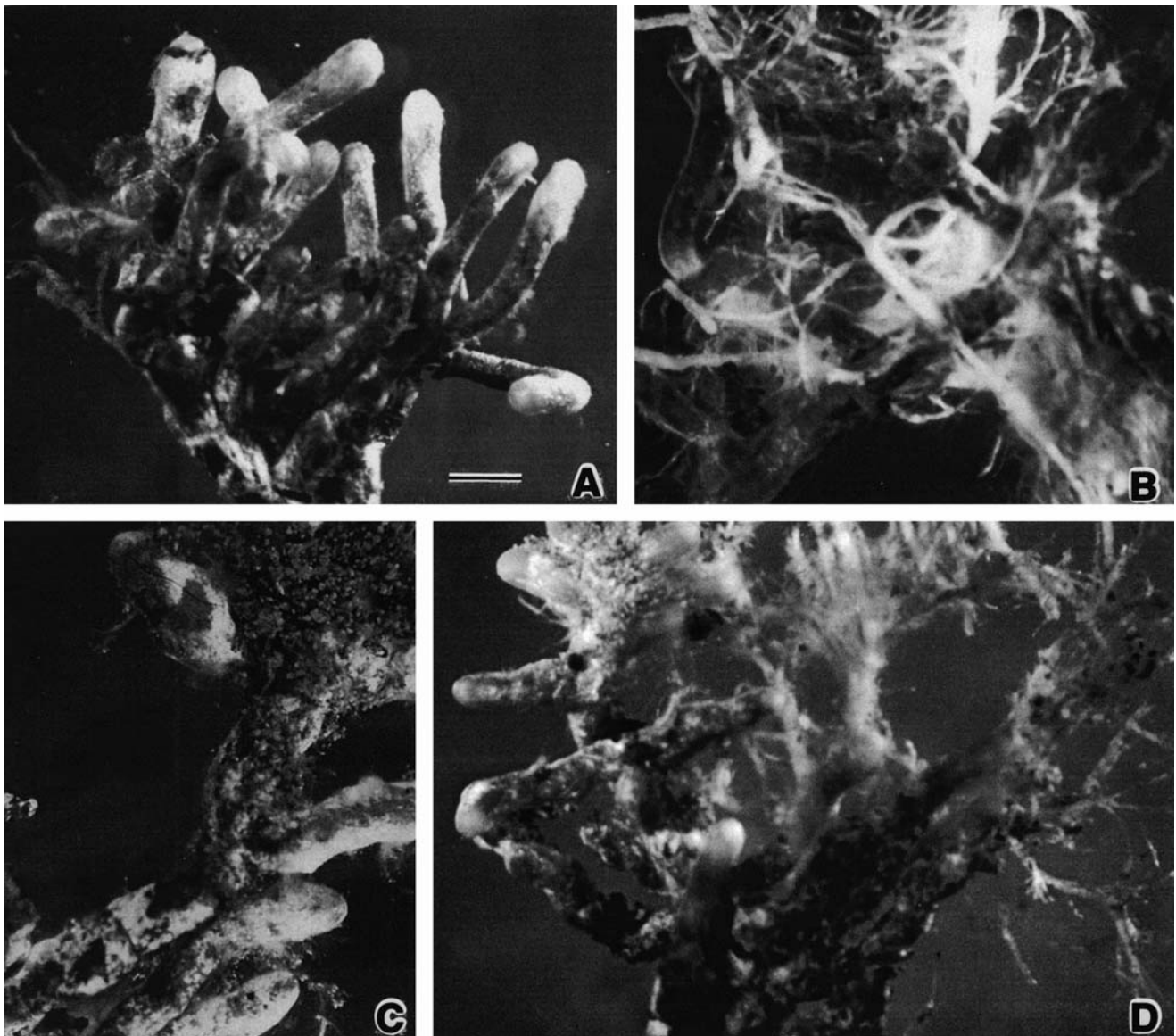


Fig. 1A–D Important features of *Ramaria* mycorrhizal morphology viewed with a dissecting microscope. **A** *R. celerivirescens* ectomycorrhizae; **B** rhizomorphs and mycelia of *R. cyaneigranosa*;

C details of mycorrhizal root tips, mantle and emanating hyphae (*R. celerivirescens*); **D** hyphal fans and mycorrhizae of *R. flavobrunnescens* var. *aromatica*. Bar = 500 μ m (A–D)

present but rare (Fig. 2D), clamps not observed except in *R. sandaracina* (Fig. 2D).

Mantle in plane view

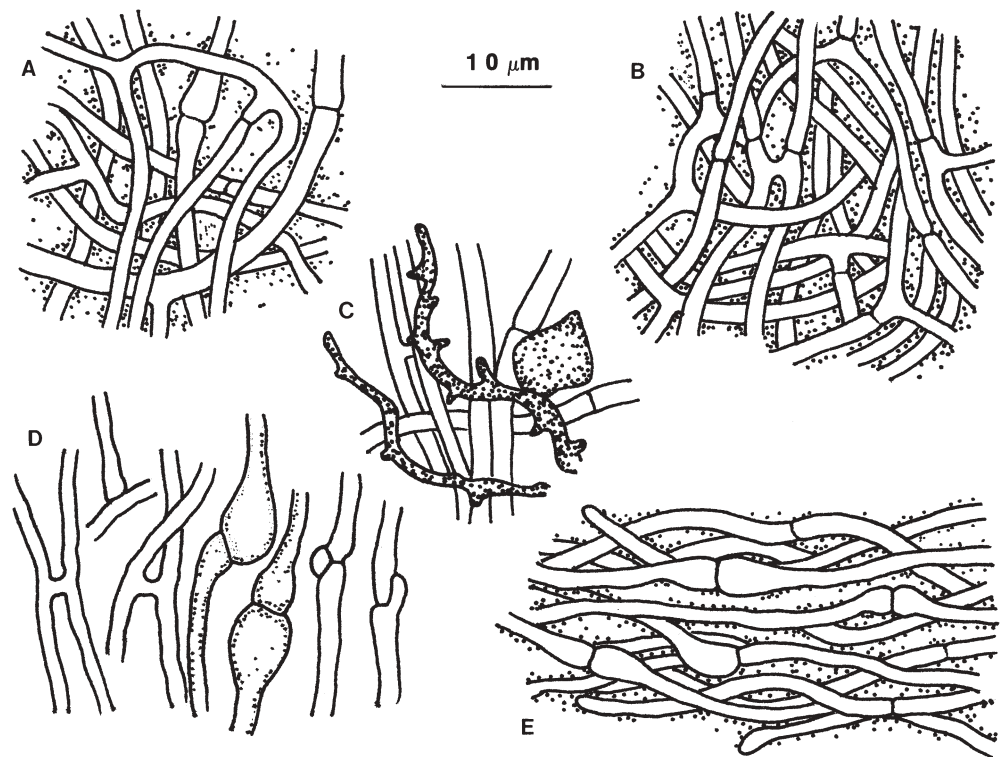
Plectenchymatous, outer layer of hyaline, interwoven, cylindrical hyphae, 2–5 μ m in diameter, with slightly gelatinous matrix (Fig. 2A), without ornamentation, ramifications common, septate, inflated septa common, up to 12 μ m in diameter, mostly thin walled, some thickened, up to 1.5 μ m in diameter, acanthohyphae with oleiferous contents observed in *R. cyaneigranosa* and *R. sandaracina* (Fig. 2C), clamps present (occasionally) in *R. flavobrunnescens* var. *aromatica* and *R. sandaracina*, not observed in the remaining species. Anastomoses rare

to common, H type without clamps (Fig. 2D), not observed in *R. sandaracina*.

Inner layer of mantle

Plectenchymatous of cylindrical hyphae but arranged more compactly in semicircular structures of almost parallel hyphal bundles (Fig. 2B), 2–4 μ m in diameter, hyaline, non-ornamented, thin-walled, septate, clamps not observed, anastomoses of the H type present, oleiferous hyphae of granular content and yellowish brown coloration present in *R. sandaracina*. Hartig net present.

Fig. 2A–E Important features of *Ramaria* mycorrhizal morphology viewed with a compound microscope. **A** Plectenchymatous outer layer of mantle; **B** inner layer of mantle, of ring-like arranged hyphae; **C** acanthohyphae and exudates within inner layer; **D** from left to right anastomoses of the H type, inflated septa and clamp connections; **E** mycelial strand in plan view, possessing numerous inflated septa on hyphae at the core



Mycelial strands

In plane view, slightly differentiated due to the presence of some hyphae with inflated septa mainly at the core (Fig. 2E). Hyphae: cylindrical, 2–5 μm in diameter, mostly thin-walled, some up to 1 μm thick, hyaline, septate, inflated septa common, up to 10 μm in diameter, (Fig. 2D), not ornamented, oleiferous hyphae (cyanophilic) of tortuous shape observed in *R. flavobrunnescens* var. *aromatica*. Hyphal ramifications common, clamps not seen except in *R. sandaracina*. Anastomoses present and common of the H type (Fig. 2D).

Chemical reactions

KOH, 15%

Mantle changes from whitish to orange brown in all mycorrhizae. Hyphal strands remained whitish.

*FeSO*₄

Mantles of *R. cyaneigranosa* and *R. sandaracina* ectomycorrhizae become dark brown. Mantles of *R. acrisiccenscens*, *R. flavobrunnescens* var. *aromatica* and *R. celerivirescens* become dark green, then blackish. No color reactions in the mycorrhizal mycelial strands.

Melzer's reagent

No color reactions in all mycorrhizae and mycelial strands.

RFLP analysis

All of our *Ramaria* species could be differentiated by RFLP analysis of the amplified ITS region. Ectomycorrhizae from the soil cores were matched with *R. acrisiccenscens* (two columns), *R. cyaneigranosa*, *R. flavobrunnescens* var. *aromatica*, *R. celerivirescens* and *R. sandaracina*. Restriction fragment band sizes for mycorrhizal morphotypes are shown in Table 1, as well as GenBank accession and herbaria collection numbers. Bands <90 base pairs are not included. The sequences obtained from two sporocarps for *R. acrisiccenscens* were identical without the need for editing, thus we were confident that the base calling by the machine was repeatable. While we checked the chromatograms of all the sequences, we found the base calling unambiguous and accepted the sequence output from the sequencer other than truncating the sequences at the both ends.

Discussion

The general appearance of the mycorrhizae and morphological characters of the root tips as well as the microscopic features of mantle and mycelial strands were similar in all our *Ramaria* species, and quite similar to the

morphotypes of *R. aurea*, *R. largentii*, *R. spinulosa* and *R. subbotrytis* described by Agerer et al. (1996). Agerer et al. (1996), also mention the lack of distinguishing characters in *Ramaria* ectomycorrhizae. Specifically, they describe some differences in the diameter of rhizomorphs and outer mantle hyphae, as well as some cap-like thickenings of short mantle hyphae, different color reactions with guaiac, formol and FeSO₄ and the presence of crystals in the rhizomorphs.

We observed the characteristic aggregation of mycorrhizae that Agerer et al. (1996) describe as dense nest-like clusters. These extensive mycorrhizal “mats” are produced by other members of the Gomphales such as *Gomphus*, *Gautieria* and *Hysterangium*, but also by members of the Thelephorales such as *Hydnellum*, *Sarcodon*, *Phellodon*, *Bankera*, *Boletopsis*, and in a member of the Corticiaceae, *Piloderma* (Agerer 2001). The mats of *Gautieria* and *Hysterangium* in particular seem to have a modified soil chemistry and microorganism biomass (Cromack et al. 1979; Entry et al. 1991, 1992; Griffiths et al. 1994). We are unaware of soil chemistry analyses for the other mat formers.

PCR amplification failures with some root tips sampled at the beginning of the experiment were correlated with older looking mycorrhizae as described. Later, successfully amplified products were obtained when only turgid “fresh looking” mycorrhizae were used. Similar observations have been made for monotropoid mycorrhizae produced by *Rhizopogon ellenae* (Smith) and *Sarcodes sanguinea* (Kretzer et al. 2000).

The morphological features of *Ramaria* ectomycorrhizae are so similar that the species cannot be readily separated based on morphological characters alone. However, the variation in the ITS sequences was high enough to produce distinctive RFLP patterns for each species. Because we included only one or two specimens per species, we do not know if polymorphic RFLP patterns will be observed with a larger sample size. Intraspecific variation in the ITS region has been reported in species occurring in a variety of genera, especially at regional scales and larger (Horton 2002; Kårén et al. 1997), making RFLP patterns a poor choice for comparison outside our study area. However, the sequence data will be useful as a diagnostic tool for interested researchers. Here we provide ITS sequences for all species, giving researchers the ability to assess the similarity of their putative mycorrhizal species to those given here with sequence comparison.

Further work should be done to investigate the soil chemistry associated with these mats and the specificity of the fungi to their plant hosts.

Acknowledgement The Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, provided a research scholarship to E. R. N.

References

- Agerer R (ed) (1987–1998) Colour atlas of ectomycorrhizae, 1st–11th edn. Einhorn, Schwäbisch Gmünd
- Agerer R (1991) Characterization of ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Techniques for the study of mycorrhiza. (Methods in microbiology, vol 23) Academic Press, London, pp 25–73
- Agerer R (2001) Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza* 11:107–114
- Agerer R, Danielson R, Egli S, Ingleby K, Luoma D, Treu R (1996) Descriptions of ectomycorrhizae, vol 1. Einhorn, Schwäbisch Gmünd, p 183
- Cromack K, Sollins P, Graustein WC, Speidel K, Todd AW, Spycher G, Chingy LI, Todd RL (1979) Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hysterangium crassum*. *Soil Biol Biochem* 11:463–468
- Entry J, Rose C, Cromack K (1991) Litter decomposition and nutrient release in ectomycorrhizal mat soils of Douglas-fir ecosystem. *Soil Biol Biochem* 23:285–290
- Entry J, Rose C, Cromack K (1992) Microbial biomass and nutrient concentration in hyphal mats of the ectomycorrhizal fungus *Hysterangium setchellii* in a coniferous forest soil. *Soil Biol Biochem* 24:447–453
- Erland S, Henrion B, Martin F, Glover L, Alexander I (1994) Identification of the ectomycorrhizal basidiomycete *Tylospora fibrillosa* Donk by RFLP analysis of the PCR-amplified ITS and IGS regions of ribosomal DNA. *New Phytol* 126:525–532
- Gardes M, Bruns T (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Mol Ecol* 2:113–118
- Gardes M, Bruns T (1996) ITS-RFLP matching for identification of fungi. *Meth Mol Biol* 50:177–186
- Griffiths R, Baham J, Caldwell B (1994) Soil solution chemistry of ectomycorrhizal mats in forest soils. *Soil Biol Biochem* 26:331–337
- Horton TR (2002) Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. *Plant Soil* 244:29–39
- Kårén O, Högborg N, Dahlberg A, Jonsson L, Nylund JE (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. *New Phytol* 136:313–25
- Kernaghan G, Currah R, Bayer R (1997) Russulaceous ectomycorrhizae of *Abies lasiocarpa* and *Picea engelmannii*. *Can J Bot* 75:1843–1850
- Kraigher H, Agerer R, Javornik B (1995) Ectomycorrhizae of *Lactarius lignyotus* on Norway spruce characterized by anatomical and molecular tools. *Mycorrhiza* 5:175–180
- Kretzer A, Bidartondo M, Grubisha L, Spatafora J, Szaro T, Bruns T (2000) Regional specialization of *Sarcodes sanguinea* (Ericaceae) on a single fungal symbiont from the *Rhizopogon ellenae* (Rhizopogonaceae) species complex. *Am J Bot* 87:1778–1782
- Largent D, Johnson D, Watling R (1977) How to identify mushrooms to genus. III. Microscopic features. Mad River Press, Eureka, Calif.
- Marr C, Stuntz D (1973) *Ramaria* of Western Washington. *Bibliotheca mycologica*, vol 38. Cramer, Germany
- Molina R, Trappe JM (1982) Lack of mycorrhizal specificity by the ericaceous hosts *Arbutus menziesii* and *Arctostaphylos uva-ursi*. *New Phytol* 90:495–509
- White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D, Sninsky J, White T (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315–322
- Zak B (1973) Characterization of ectomycorrhizae. In Marks GC, Kozłowski TT (eds) *Ectomycorrhizae—their ecology and physiology*. Academic Press, New York, pp 43–78
- Zak B (1974) Ectomycorrhiza of pacific madrone (*Arbutus menziesii*). *Trans Br Mycol Soc* 62:201–206