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Autor: Boyle, H. / Buscot, F.

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Differentiation of *Lactarius obscuratus* var. *radiatus* and *Lactarius omphaliformis* by amplified ribosomal DNA restriction analysis (ARDRA)

H. Boyle and **F. Buscot**

Staatliches Museum für Naturkunde, Görlitz, Am Museum 1, D-02826 Görlitz, Germany
Institut für Bodenbiologie, Bundesforschungsanstalt für Landwirtschaft, Bundesallee 50, D-38116 Braunschweig-Völkenrode, Germany

Summary – Restriction analyses of PCR-amplified ribosomal DNA internal transcribed spacer regions of the similar-looking ectomycorrhizal fungi *Lactarius obscuratus* var. *radiatus* and *L. omphaliformis* support the validity of their present status as separate taxa, as based on morphological characters.

Zusammenfassung – Restriktionsanalysen von PCR-amplifizierten Internal-Transcribed-Spacer-Regionen der ribosomalen DNA der morphologisch ähnlichen Ektomykorrhizapilze *Lactarius obscuratus* var. *radiatus* und *L. omphaliformis* unterstützen deren gegenwärtigen morphologisch begründeten Status als unterschiedliche Taxa.

Résumé – Des analyses de restrictions d´amplificats par PCR de la région ITS (internal transcribed spacer) au sein de l'ADN ribosomien confirment le statut taxonomique actuel de deux espèces mycorhiziennes proches, *Lactarius obscuratus* var. *radiatus* et *L. omphaliformis*, établi sur des bases morphologiques.

Introduction

The basidiomycetous fungus *Lactarius obscuratus* (Lasch) Fr., along with its var. *radiatus* (Lge.) Romagn., are known as specific ectomycorrhizal symbionts of certain *Alnus* species. Simultaneous occurrence of the *Alnus*-facultative *Lactarius omphaliformis* Romagn., especially on wet sites such as alder carrs, is a potential cause of confusion between the morphologically similar *L. obscuratus* var. *radiatus* and *L. omphaliformis.*

The latter are strikingly cognate in such subjective criteria as sporocarp size and colour range as well as in the taste, colour and translucence of the lactifer exudate that characterises the genus. Similarity of tangibly measurable features such as spore size, shape and ornamentation necessitates as a rule larger collections to ensure proper identification, especially when *L. omphaliformis* occurs concurrently with *L. obscuratus* var. *radiatus* in *Alnus* stands.

Taxonomy and systematics of *Lactarius* Section *Obscurati* are not as yet fully understood. According to Krieglsteiner (1984), *L. obscuratus* var. *radiatus* has been proposed to be identical with *Lactarius cupularis* ss. Bresadola, and thus a species in itself. Krieglsteiner (1984) suggested that there may be no species differentiation at all, but merely transitional forms between *L. obscuratus* var. *radiatus* and *L. omphaliformis,* however, in his distribution atlas (Krieglsteiner 1991), *L. omphaliformis* is treated as a species in its own right.

Comparative studies of different nucleic acid fragments have been increasingly used in the study of taxonomic and phylogenetic relationships of fungi (Bruns, White & Taylor, 1991; Egger, 1992; Henrion, Chevalier & Martin, 1994; Vilgalys, Hopple & Hibbett, 1994; Buscot *et al.,* 1995). The use of fungus-specific PCR-primers generally permits the investigation of field material of some species which cannot be cultured and therefore remain inaccessable to analysis with random primers. The ARDRA method is thus suitable with respect to its potential fungus-specificity for the investigation of species diversity in ecosystems. In addition, its relative simplicity could render it useful *i.e.* for comparative studies of ambiguous field collections with herbarium material and/or the holotypes.

This paper describes a differentiation between *L. obscuratus* ss. str., *L. obscuratus* var. *radiatus* and *L. omphaliformis* based upon DNA polymorphism as revealed by restriction analysis of the PCR-amplified internal transcribed spacer region (ITS) within the fungal ribosomal DNA.

Materials & methods

Specimens of *Lactarius obscuratus* ss. str., *L. obscuratus* var. *radiatus* and *L. omphaliformis* were collected beneath *Alnus glutinosa* (L.) Gärtn. in three alder carrs in Northern Germany (Bornhöved, Schleswig-Holstein and two near Braunschweig, Lower Saxony) during the fruiting season 1994. *Lactarius obscuratus* was found in both forms between June and October, thus enabling numerous (at least 10) collections of each form. *Lactarius omphaliformis* could only be collected three times in the month of September. Fruit bodies were identified using Moser (1983) and Kreisel (1983). Trama of the fresh sporocarps was either immediately analysed or conserved at –70°C until use.

Polymerase chain reaction

Extraction of the whole-cell DNA was performed according to the small scale procedure described by Henrion, Le Tacon & Martin (1992). The resulting DNA was resuspended in 50 µl Tris/HCl buffer containing 2.5 mM EDTA (pH 8.0) and stored at 4°C until use (normally within 24h).

The internal transcribed spacer region (ITS) of the fungal rDNA was amplified by the polymerase chain reaction, using the primer pair ITS1 and ITS4 described by White *et al.* (1990). The primers were supplied by MWG-BIOTECH (Ebersberg, Germany). The amplification reactants were combined in 0.5 ml polypropylene tubes to a net volume of 100 µl comprising: 10 mM Tris/HCl (pH 8.8 at 25°C), 50 mM KCl, 0.5% Triton X100, 0.01% gelatine, 0.02 µM of each primer, 200 µm each of dATP, dTTP, dCTP and dGTP and 0.05 to 0.5 ng DNA template (200 to 500-fold aqueous dilution of the small-scale DNA extract).

PCR was carried out in an «Omnigene» (Hybaid) thermocycler operating under tube temperature control mode. The thermal cycling parameters were an initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 40 s and extension at 70°C for 40 s, with a subsequent final extension at 70°C for 10 min. In all cases a DNA-free control was included among the samples in order to verify the absence of contaminants in the reagents.

10 µl each of the resulting ITS amplification products were electrophoresed on an 1.5% agarose gel against a DNA size standard (ϕX174 digested by *Hae III*, Boehringer, Mannheim, Germany) and visualised after staining with ethidium bromide under a uv light source.

Restriction analysis

The ITS fragments were digested by seven different type-II restriction endonucleases *(Alu I, Eco R I, Hae III, Hind III, Msp I, Nde II* and *Xba I).* For each digest, 10 µl amplification product was combined with 5 U of the respective enzyme and incubated at 37° C for 1 h according to the manufacturers instructions. The restriction fragments were size-fractionated against the above-mentioned DNA size standard using a 2% agarose gel and visualised in the same manner as the ITS fragments.

Number of replicates

Each step described above was independently repeated at least once to ensure uniformity and reproducibility of the analyses.

Results

The ITS fragment length of *Lactarius obscuratus* ss. str., *L. obscuratus* var. *radiatus* and *L. omphaliformis* was *ca* 740 base pairs (Figure 1), which also was true for five other species of *Lactarius* (data not shown). This necessitated further attempts at species differentiation by means of restriction analysis.

One of the seven employed restriction endonucleases *(Nde II)* revealed qualitative fragment length polymorphism as shown in Figure 2 between *Lactarius obscuratus* and *L. omphaliformis* (lane b and c), not, however, between *L. obscuratus* ss. str. (lane a) and *L. obscuratus* var. *radiatus* (lane d).

No variation between taxa of differing geographical origin could be detected.

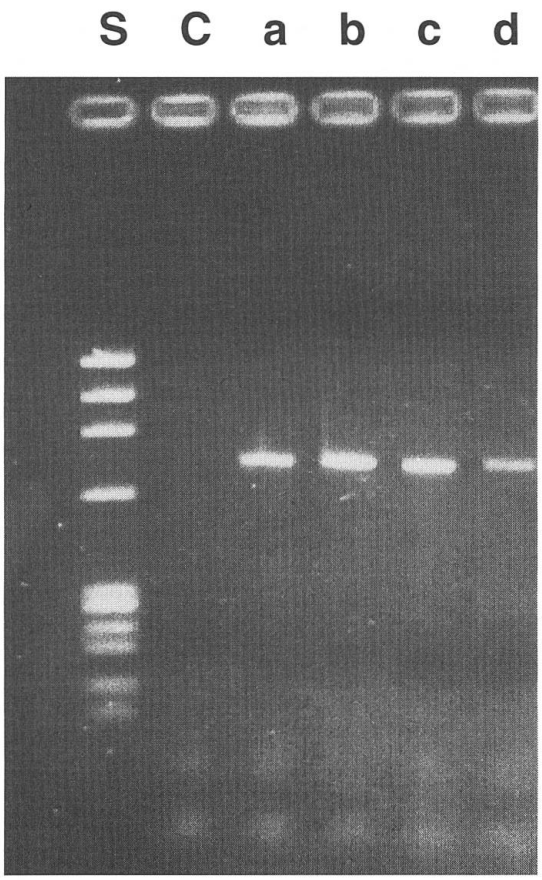

Fig. 1: Agarose electrophoresis gel of the rDNA ITS of three Lactarius *taxa. C, control; S, DNA size standard (X174 digested by Hae III); a,* L. obscuratus *ss. str.; b,* L. omphaliformis; *c,* L. omphaliformis; *d,* L. obscuratus *var.* radiatus

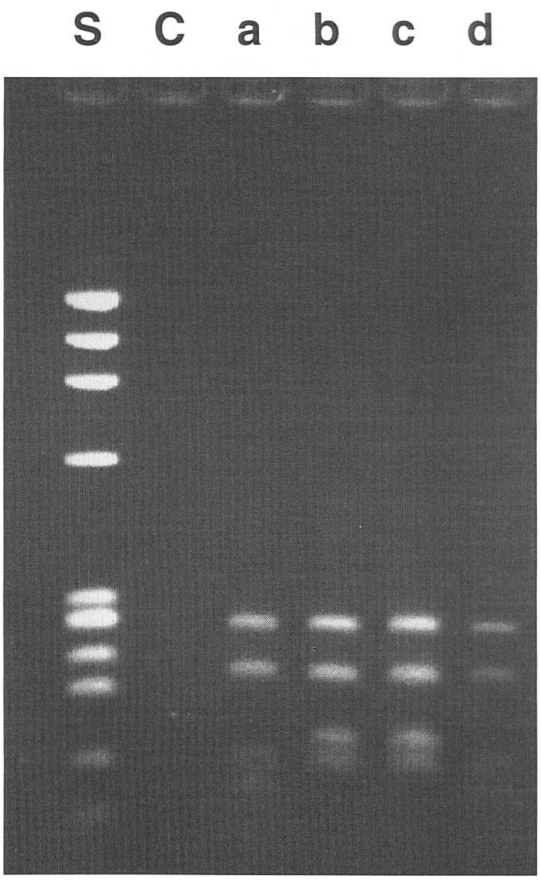

Fig. 2: Agarose electrophoresis gel of the ITS-restriction digest by Nde II *of three* Lactarius *taxa. S, DNA size standard ((X174 digested by Hae III); a,* L. obscuratus *ss. str.; b,* L. omphaliformis; *c,* L. omphaliformis; *d,* L. obscuratus *var.* radiatus

Discussion

Species differentiation in fungi as based upon classical morphological criteria remains controversial. For the genus *Lactarius,* between 69 (Neuhoff 1956) and 200 (Hesler & Smith 1979) species are recognised by different authors. Such variety of opinion is evidence for the present scope of interpretation of species delimitation.

In spite of existing difficulties on the interpretational level, the classical approach to taxonomy is by no means obsolete, as it provides a basis of reference for relevant data of many different kinds. Since molecular techniques have become available, they have been able to supplement taxonomic descriptions with further-reaching information, quite often supporting morphological diagnoses and/or supplying cladistic character states.

Comparative PCR/RFLP analysis is able to discriminate at the interspecific level as shown for example by Egger (1992), Feibelman, Bayman & Cibula (1994) and Henrion et al. (1994), as well as in this study. Buscot et al. (1995) have even demonstrated intraspecific DNA polymorphism in different geographical strains.

The above results tend to support the status of *L. obscuratus* var. *radiatus* as a subspecific taxon and weaken arguments for its classification as a species, as both varieties of *L. obscuratus* were indiscernable from one another with the enzymes used. On the other hand, the morphologically very similar, ecologically somewhat broader-ranged *L. omphaliformis* is clearly discernable from both varieties of *L. obscuratus.* A close relationship, as can be expected within a section, is however suggested by the fact that six of seven enzymes found the same recognition sites within the ITS of the three taxa. The results presented here should be understood as an incentive to carry out further studies of section Obscurati, which will be necessary to clarify the actual taxonomic status.

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