Discrimination of Czech *Armillaria* Species Based on PCR Method and High Performance Liquid Chromatography

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Abstract

The genus *Armillaria* belongs to basidiomycetes and has been known to induce root rot disease and to cause extensive economic losses to a forest crop. We analysed about 40 isolates of *Armillaria* collected in Czech Republic by PCR and restriction analysis using gel electrophoresis and ion-exchange HPLC. Restrictase Hinf I was able to discriminate all investigated *Armillaria* species. The sensitivity and resolution of HPLC method was better than that performed by gel electrophoresis. HPLC was able to detect some heterozygous. The results prove the similarity of the species *A. borealis, A. cepistipes, A. gallica, A. ostoyae* in difference of *A. mellea* and *A. tabescens*.

Keywords: *Armillaria*; PCR; forest pathogen; basidiomycete; root rot disease

INTRODUCTION

Some species of the genus *Armillaria* has been known to induce root rot disease, which can result in extensive economic losses to a forest crop. The main function of this fungus in the ecological system is the decomposition of wood waste, but it can very often turn to the necrotrophic parasitism and attack a wide range of woody species. The seven species of *Armillaria* have been identified in Europe up to now: *A. borealis, A. cepistipes, A. ectypa, A. gallica, A. mellea, A. ostoyae* and *A. tabescens*. The individual species have different pathogenic behaviour and thus the forest management necessitates confirming the presence of virulent *Armillaria* species. The current identification of *Armillaria* have included observation of cultural characteristics, pairing tests based on both sexual and somatic incompatibility, isozyme analysis and immunological techniques (CHILLALI 1998a,b). Recently, molecular-biological technique has been used to identify *Armillaria* species. The advantage of this technique, as against the methods mentioned above, are very good reproducibility and the rapidity of the analysis.

The aim of this study was to introduce the molecular-biological techniques of *Armillaria* species identification to the laboratory practice in Czech Republic and either confirm or revalue the previous classification of that species collected by Phytopathological laboratory of Masaryk University in Brno and Mendel University of Agriculture and Forestry Brno. The results and sequences of ITS region were compared with the published data.

MATERIALS AND METHODS

Source of isolates

Isolates of *Armillaria* strains were obtained from the collection of Department of Forest Protection, Faculty of Forestry, Mendel University of Agriculture and Forestry Brno and as a generous gift of Collection of Microbiological Institute of Czech Academy of Sciences, Prague. Some isolates were a gift of

dr. Guillaumin, INRA Clermond-Ferrand, France. About 40 mycelia strains were analysed (number of isolates in parentheses): *A. borealis* (3), *A. cepistipes* (7), *A. gallica* (8), *A. mellea* (5), *A. tabescens* (6), *A. ostoyae* (10). All strains were previously identified by pairing tests.

DNA isolation and amplification

DNA from mycelium was isolated by using Kit Nucleospin Plant (Macherey-Nagel) according to the manufactures instructions.

Amplification of ITS region was carried out by PCR using primers ITS1 and ITS4 in a cycler Techne (Progene) under conditions described by WHITE *et al*. (1990).

RFLP analysis

Aliquots of the amplified DNA were digested for 5 h with the restriction endonucleases Alu I, Hinf I and Mbo I (Fermentas) according to the manufactures instructions. The restriction fragments were analysed both on 3% agarose gel stained with ethidium bromide and by HPLC (HP 1100 Series) on the ion exchange column TSK-gel DEAE-NPR (Supelco). Elution solution was a gradient of 0.3–0.8 M NaCl in 10 mM Tris/HCl (pH 9.0) during 10 min followed by 2 min washing period with 0.8 M NaCl (KATZ & DONG 1990).

Sequencing of ITS region

The ITS amplified products of eight isolates were purified using a precipitation in polyethyleneglycol 8000. The concentration of DNA was determined by HPLC and the sequencing of purified products was carried out by Laboratory of functional genomics, Masaryk University, Brno.

RESULTS AND DISCUSSION

Parameters of HPLC system

The resolution and sensitivity achieved on the HPLC was comparable or better than on 3% agarose gel. The amount lower than 380 ng of 24 base length DNA could be detected and the resolution of peaks was better than 3% of the fragments length for the fragments to 500 bp (Figure 1). The increase in temperature from 22 to 26°C during the analysis cause the prolongation of the retention time up to 2 min.

Amplification and restriction analysis

The amplification of isolated DNA resulted in an approximately 870 bp fragment. No length variation of ITS region were observed among isolates. The restrictase HinfI allowed to discriminate all six analysed species. The restrictase AluI did not enable the discrimination of *A. borealis, A. cepistipes, A. gallica* and *A. ostoyae* and restrictase MboI did not discriminate the species *A. gallica* and *A. cepistipes*.

Polymorphism in ITS region

The results of the restriction analysis of all *Armillaria* strains were compared with data published earlier by CHILLALI *et al*. (1998a,b) and SCHMIDT (2001). Three types of results were obtained: (1) restriction fragment analysis corresponded to published sequence data, (2) the results corresponded to the other species

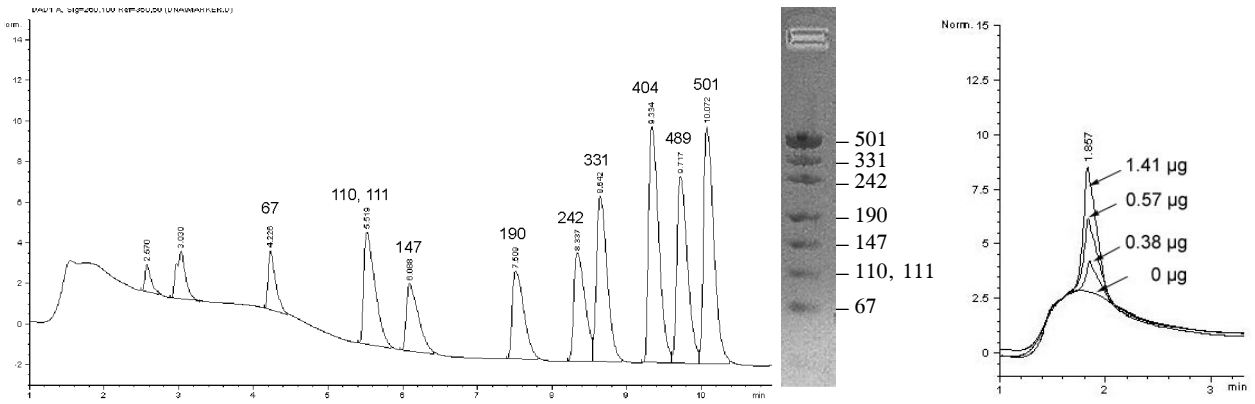

Figure 1. The analysis of length marker pUC 19 on HPLC and comparison with 3% agarose, quantitative analysis of 24 base length primer

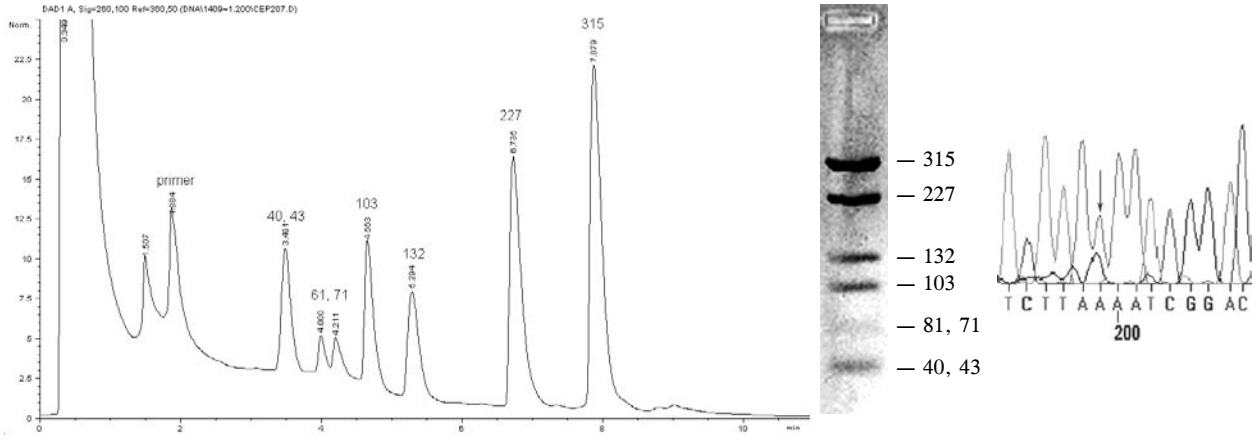

Figure 2. The analysis of restriction fragments after cleavage with restrictase Hinf I of *A. cepistipes* 207 and the part of the sequence of the reverse strand. The arrow shows the mutation that resulted in the new restriction site

so that the classification obtained by classical pairing test had to be revised, (3) in fragments profiles were some anomalies, i.e. presence of additional peaks thanks to a mutation in a sequence.

Figure 2 shows typical results of the HPLC analysis compared with gel electrophoresis of *A. cepistipes* 207 (restrictase Hinf I). The length of typical restriction fragments should be 40, 43, 103, 132, 227 and 315 bp. The figure shows a splitting of the peak of 132 bp into two peaks of 61 and 71 bp. The retention times are proportional to the length of the fragments and the heights are proportional to the amounts of DNA. It is evident that HPLC detected only about one half heights of the fragments of 132, 61 and 71 bp. Therefore, the strain 207 was classified as a heterozygous. For this purpose, we proceeded to the sequencing of ITS region. The sequence showed, that one point mutation of thymin for cytosine resulted in a new restriction site splitting the fragments of 132 bp. The sequencing confirmed that strain is heterozygous.

As mentioned above, the restriction analysis of some species proved the presence of additional bands or disappearance of other restriction fragments caused by mutation or deletion in a restriction site. Figure 3 shows polymorphism in ITS region of several isolates.

Dendogram analysis

The cluster dendograms derived from ITS region divided the six investigated species into three groups. The similarity between *A. borealis, cepistipes, gallica* and *ostoyae* was very high, about 98%. On the other hand, the similarities between *A. tabescens, A. mellea* and other four species were lower. In the case of *A. tabescens* it was about 87%. In the case of *A. mellea* it was about 50% and 75% in ITS 1 and ITS

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                    43  222 240 464 467 475 512 636 715 745 759
A.gallica          ---a--t--t--g--g--t--c--g--t--a--t
A.gallica 173      ---*--t--t--g--g--t--c--a--c--t--c
A.gallica E4       ---*--c--a--t--c--g--g--g--c--t--c

                    152 170 463 574 633 634 700
A. cepistipes      ---t--c--t--g--g--a--t
A. cepistipes 207  ---c--t--c--a--g--a--y
A. cepistipes 182  ---y--y--y--a--r--r

                    248 499 623 702 726
A.ostoyae          ---c--g--g--c--g
A.ostoyae 151      ---y--r--g--s--g
A.ostoyae C2       ---c--a--g--c--a
A.ostoyae 209      ---c--g--*
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Figure 3. The examples of polymorphism in ITS region at three species of *Armillaria* (*deletion, y = c/t, s = g/c, r = a/g)

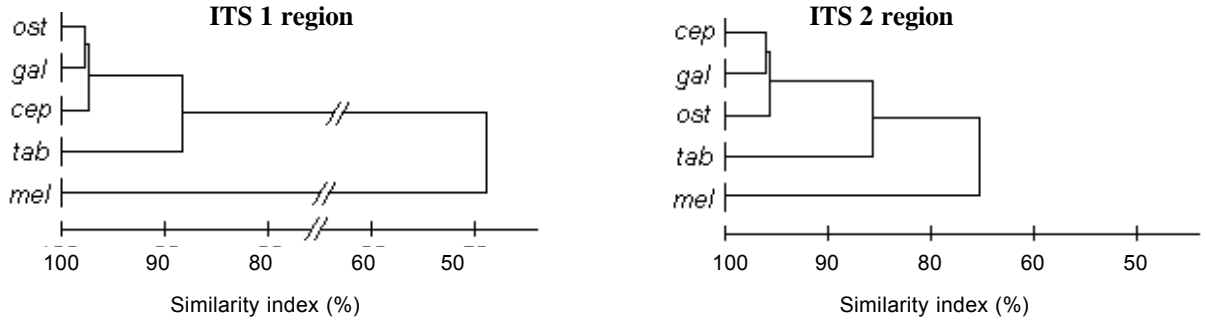

Figure 4. Dendograms of ITS 1 and ITS 2 regions showing similarities among the five analysed Armillaria species. The trees are based on data obtained from sequences

2 regions, respectively (Figure 4). The 5.8S rDNA region was very conserved because the similarity was 100% among all species. Homology of all sequenced isolates within one species was 98–99%.

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