*Silva Lusitana* **12**(1): 67 - 75, 2004



# Identification of Portuguese *Armillaria* Isolates by Classic Mating-Tests and RFLP-PCR Analysis of the ITS1 Region of Ribosomal DNA

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**Abstract.** The diagnosis of *Armillaria*, a genus including distinct species of highly woody plant-pathogenic root-infecting fungi with worldwide distribution, is usually based on morphological characteristics and mating-tests, although the PCR-based restriction fragment length polymorphism (RFLP-PCR), specifically in nuclear rDNA spacers, have also been applied. In the present study, mating-tests and restriction analysis of Internal Transcribed Spacer 1 (ITS1) were used to identify 20 isolates of a Portuguese *Armillaria* collection. Although the majority of the diploid isolates (80%) could be identified in diploid-haploid pairings, the method is laborious, takes too much time (up to 2 months), and presents a high rate of inconclusive results. The ITS1 region showed to be a reliable molecular marker for *A. mellea*, in particular when *Hin*fI restriction analysis is applied, since two fragments with 245 bp and 125 bp have been obtained for this most aggressive species whereas 290 bp and 70 bp were produced from isolates of the other European species. As simple molecular techniques are involved and the whole procedure can be performed in one day, *A. mellea* identification by ITS1 analysis is a clearly accessible and more advantageous tool to plant pathology laboratories, mainly those involved on the control and preservation of forest trees.
**Key words:** RFLP-PCR; rDNA*; Armillaria mellea;* woody plants; cork oak

**Identificação de Isolados Portugueses de *Armillaria* por Testes Clássicos de Confrontação e Análise de RFLP-PCR da Região ITS1 do DNA Ribossomal**

**Sumário.** O género *Armillaria* inclui fungos de espécies muito agressivas que infectam raízes de plantas lenhosas em todas as regiões do globo. A identificação destas espécies tem sido efectuada com base em características morfológicas, em confrontações haplonte-diplonte ("mating-tests") e também em análise de DNA, nomeadamente análise de polimorfismos de restrição de fragmentos amplificados (RFLP-PCR) das regiões espaçadoras (ITSs) dos genes ribossomais (rDNA nuclear). Neste estudo, 20 isolados de uma colecção portuguesa de espécies de *Armillaria* foram analisados por "mating-tests" e por análise de restrição da região ITS1. Ainda que tenha sido possível identificar 80% dos isolados diplóides através das confrontações

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haplonte-diplonte, o método é laborioso, demorado (2 meses ou mais) e apresenta uma taxa elevada de resultados não conclusivos. A análise de restrição da região ITS1, com a enzima *Hinf*I, permitiu discriminar claramente a espécie *A. mellea* (245 bp e 125 bp), uma das mais agressivas, das outras espécies europeias deste género (290 bp e 70 bp).

A simplicidade das técnicas moleculares utilizadas, aliada à sua rapidez de execução (1 dia), torna a identificação de *A. mellea* por análise de ITS1 um método acessível e de grande utilidade em laboratórios de patologia vegetal, nomeadamente os envolvidos no controlo e preservação de florestas.

**Palavras-chave:** RFLP-PCR; rDNA*; Armillaria mellea;* plantas lenhosas; sobreiro

**Identification des Isolats Portugais d'*Armillaria* par Testes Classiques de Confrontation et Analyse de RFLP-PCR de la Région ITS1 des Gènes Ribosomiques**

**Résumé.** Le genre *Armillaria* est présente dans le monde entier par différentes espèces de champignons pathogéniques trés agressives qui se développent principalement au niveau des racines. L'identification de ces espèces a été effectuée avec des caractéristiques morphologiques, par confrontation ("mating-tests"), et aussi par l'analyse de l'ADN, surtout par des polymorphismes de restriction de fragments amplifiés (RFLP-PCR) des régions d'espacement (ITSs) des gènes ribosomiques (rDNA nucléaire). Dans ce travail, l'identification d'une collection de 20 isolats portugais de *Armillaria* a été effectuée en utilisant la méthode des confrontations et l'analyse de restriction de la région ITS1. Bien que la plupart des isolats diploïdes (80%) soit identifiée par confrontation diploïde-haploïde, la méthode s'avère laborieuse et longue (au moins 2 mois) et présente un taux élevé de résultats non concluants. Les résultats obtenus par la restriction de la région ITS1, en utilisant l'enzyme *Hinf*I, ont permit la discrimination nette de *A. mellea* (245 bp et 125 bp), une espèce des plus agressives, des autres espèces européennes du même genre (290 bp et 70 bp)**.** Les techniques moléculaires utilisées sont très simples et les procédés peuvent être exécutés en un jour, ce qui rend l'identification de *A. mellea* par l'analyse de ITS1 une méthode accessible et avantageuse pour les laboratoires de pathologie végétal, surtout ceux qui sont engagés dans le contrôle et préservation des fôrets.

**Mots clés:** RFLP-PCR; rDNA*; Armillaria mellea;* plantes ligneuses; chêne liège

## Introduction

*Armillaria* (Fr.: Fr.) Staude is a genus including distinct species of pathogenic root-infecting fungi with worldwide distribution. In Europe, there are seven intersterile groups or biological species (KORHONEN 1978; GUILLAUMIN *et al*., 1991, 1993): the exanulates *Armillaria tabescens* (Scopoli: Fries) Emel and *Armillaria ectypa* (Fries) Lamoure and the other species with annulate basidiomes, *Armillaria mellea* (Vahl: Fries) Kummer, *Armillaria gallica* Marxmuller and Romagnesi (synonymous: *Armillaria bulbosa* (Barla) Velenovsky), *Armillaria cepistipes* Velenovsky, *Armillaria borealis* Marxmuller and Romagnesi and *Armillaria ostoyae* (Romagnesi) Herink.

*Armillaria* root rot has been found in Portugal affecting forestry species, ornamental and fruits species, as well as vineyards. Regarding forest ecosystems, the stands of *Criptomeria japonica* (L. f.) D. Don, the most important forestry species of Azores islands, is threatened for a long time with high incidence rates (AZEVEDO, 1958; SANTOS and ALMEIDA, 1997). Besides the work further developed in *C. japonica* (AZEVEDO 1963, 1966, 1976a) and *Quercus suber* L. (AZEVEDO, 1976b), minor efforts had been made to clarify the *Armillaria* role in Portuguese forest ecosystems and to

evaluate the distribution and ecology of *Armillaria* species in Portugal.

The need for experienced staff and the time consuming identification procedures are long standing problems associated with the diagnosis of *Armillaria* (HARRINGTON and WINGFIELD, 1995; PÉREZ-SIERRA *et al*., 1999). In fact, as basidiomes are seasonal and rather uncommon, the identification of *Armillaria* biological species is usually performed with mycelium or rhizomorphs obtained from infected wood and currently involves analysis of characteristics in culture (GUILLAUMIN and BERTHELAY, 1981; RISHBETH 1986) and mating analysis with known haploid tester strains (KORHONEN, 1978; GUILLAUMIN and BERTHELAY, 1990; LEGRAND *et al*., 1996; TSOPELAS, 1999). Among the new alternative methods proposed for the identifycation of *Armillaria* isolates, the amplifycation and subsequent restriction analysis of the internal transcribed spacers (ITS) and intergenic spacers (IGS) of nuclear ribosomal RNA genes have shown high taxonomical potential (HARRINGTON and WINGFIELD, 1995; CHILLALI *et al*., 1998a; PÉREZ-SIERRA *et al*., 1999).

In the present study, mating analysis with known haploid tester strains and ITS1 restriction analysis (RFLP-PCR) were applied to 20 isolates of an *Armillaria* collection, obtained from several hosts and regions of Portugal. To assess the identification potential and the discriminative ability of the molecular method, isolates of known European species were used as reference.

## Material and methods

### *Fungal isolates and cultivation*

The twenty Portuguese isolates used in this study were obtained from a collection of *Armillaria* cultures maintainned at the Estação Florestal Nacional, Portugal (Table 1). Except for those obtained from basidiomes (*A. mellea*), all isolates had been identified as *A. mellea sensu lato* by the morphological patterns of pure culture.

Twenty-two isolates of known European species originating from several countries were also obtained from a collection of *Armillaria* cultures maintained at the INRA centre of Clermont-Ferrand, France (Table 1). Nine additional isolates used as haplont testers were obtained from J. Guillaumin: isolates A2 and A5 (*A. borealis*); B3 and B5 (*A. cepistipes*); C4 (*A. ostoyae*); D1 and D4 (*A. mellea*); E4 (*A. gallica*); and T3 (*A. tabescens*).

All isolates were grown in Petri dishes containing MEA medium (2% w/v malt extract; 1.5% w/v agar) at 24ºC.

### *Pairing tests*

The Portuguese diploid isolates were identified in compatibility tests with known haploid tester strains of the European *Armillaria* species according to KORHONEN (1978) and GUILLAUMIN *et al*. (1991).

Every diploid isolate was paired with three different tester strains of each *Armillaria* species (original reference: *A. mellea* – D1, D4, D5; *A. borealis* - A1, A2, A5; *A. ostoyae* - C2, C4, C5; *A. gallica* – E4, E5, E6; *A. cepistipes* – B2, B3, B5 and *A. tabescens* – T2, T3, T4). The inoculum for pairing consisted of undifferentiated mycelium without crust or rhizomorphs, cut from the margin of a growing culture. These plugs, 3 mm side, were placed side by side. Two different pairings were performed in each Petri

dish and each pairing was repeated twice. The plates with diploid-haploid pairings were checked after days 15, 25, 35 and until two months.

*DNA extraction*

The mycelium was collected from 3-week growing cultures and stored at –80ºC. Total DNA was extracted from 0.1 g of frozen mycelium, using the method described by CENIS (1992), resuspended in 50 µL of TE (10 mM Tris, HCl, 2 mM EDTA, pH 8,0) and stored at 4ºC until use.

*Amplification of ITS1*

The ITS1 region, located between the 18S and the 5.8S ribosomal RNA genes, was amplified by PCR using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; annealing in the 3' terminal region of 18S rDNA) and ITS2 (5'-GCTGCGTTCTTCATC GATGC-3'; annea-ling in the 5' terminal region of 5.8S rDNA) described by WHITE *et al*. (1990).

The PCR reaction mixture (50µL) included 400 ng of template DNA, 2 U of Taq DNA polymerase (Gibco-BRL, Paisley, UK), 50 pmol of each primer (Gibco-BRL), 1 x PCR buffer supplied with the enzyme, 4mM $MgCl_2$ and 200 µM of each dNTP (Gibco-BRL). To each PCR tube *ca* 50 µL of mineral oil were added and amplification occurred in a RoboCycler 96 (Stratagene, La Jolla, CA, USA), according to the following amplification program: 4 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C; 5 min at 72°C.

Each reaction sample was run on a 1.2% w/v agarose gel, in 0.5 x TBE (50 mM Tris, 45 mM boric acid, 1 mM EDTA) at 100 V for 2 h, using 100 bp DNA Ladder (Gibco-BRL) as molecular size marker. After ethidium bromide staining, the gels were analysed with KODAK 1D 2.0 software (Gibco-BRL).

*Restriction analysis of ITS1*

The amplified DNA was purified before restriction enzyme digestion using the procedure described by JOHNSTON and JONES (1997).

To perform restriction analysis of ITS1 regions, 5 µL samples of each purified PCR product were digested with 3 U of each one of two restriction endonucleases, *Alu*I and *Hin*fI (New England Biolabs, Beverly, MA, USA), in a final volume of 10 µL, according to manufacturer's instructions. After overnight incubation at 37°C, 1.5 µL of bromophenol blue solution (0.25% bromophenol blue, 0.25% xylene cyanol, 10 mM EDTA, 15% Ficoll in water) were added to each sample to stop the reaction. Each reaction sample was run on a 3% w/v agarose gel (2% agarose standard; 1% agarose 1000, Gibco-BRL), in 0.5 x TBE at 100 V for 2 h 30 min, using 50 bp or 100 bp standards (Gibco-BRL) as molecular size markers. After ethidium bromide staining, the gels were analysed with KODAK 1D 2.0 software.

## Results and discussion

*Mating analysis*

To identify the Portuguese isolates, a total of 720 pairings was performed using a set of three testers from each of the six *Armillaria* species. Using the criteria defined by GUILLAUMIN and BERTHELAY (1981), successful identification was assumed whenever compatible reactions were only obtained with testers

from a sole species.

The results from the pairing-test method allowed the identification of sixteen isolates (Table 1). Fourteen isolates were identified as *A. mellea*, one as *A. gallica* and the other as *A. tabescens*. Among the unidentified isolates, two of them (304 and 311) displayed unconclusive reactions with all testers and the other two (319 and 357) showed some compatible pairings with *A. mellea* testers not associated with clear incompatibility with all other species.

**Table 1** - Summary characteristics and results of the mating analysis and ITS1 RFLP-PCR patterns of the *Armillaria* isolates used in this study

| Reference[1] | | Species | Host[2] | Geographical origin | Year of isolation | Collector | Pairing-tests results[3] | ITS1 RFLP-PCR pattern[4] |
|---|---|---|---|---|---|---|---|---|
| 270 | | *Armillaria* sp. | *Quercus suber* (M) | Portugal, Sant.Cacém | 1991 | Fonseca | *A. mellea* | I |
| 303 | | *Armillaria* sp. | *Ulmus sp.* (M) | Portugal, Buçaco | 1998 | Bragança | *A. gallica* | II |
| 304 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1958 | Azevedo | NC | I |
| 305 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1958 | Azevedo | *A. mellea* | I |
| 306 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1958 | Azevedo | *A. mellea* | I |
| 307 | | *Armillaria* sp. | *Ficus elastica* (M) | Portugal, St Tirso | 1998 | Chicau | *A. mellea* | I |
| 308 | | *Armillaria* sp. | *Vitis vinifera* (nr) | Portugal, Pen.Castelo | 1998 | Bragança | *A. mellea* | I |
| 309 | | *Armillaria* sp. | *Prunus persica* (nr) | Portugal, Alcobaça | Unknown | Sousa | *A. mellea* | I |
| 310 | | *Armillaria* sp. | *Olea europaea* (nr) | Portugal, Alcobaça | Unknown | Sousa | *A. mellea* | I |
| 311 | | *Armillaria* sp. | *Citrus sinensis* (nr) | Portugal, Alcobaça | Unknown | Sousa | NC | I |
| 312 | | *Armillaria* sp. | *Quercus suber* (nr) | Portugal, Alcobaça | Unknown | Sousa | *A. tabescens* | II |
| 313 | | *A. mellea* | *Quercus suber* (B) | Portugal, Grândola | 1997 | Bragança | *A. mellea* | I |
| 314 | | *A. mellea* | *Quercus suber* (B) | Portugal, Grândola | 1997 | Bragança | *A. mellea* | I |
| 315 | | *A. mellea* | *Quercus suber* (B) | Portugal, Grândola | 1997 | Bragança | *A. mellea* | I |
| 317 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1997 | Melo | *A. mellea* | I |
| 318 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1997 | Melo | *A. mellea* | I |
| 319 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1956 | Azevedo | NC | I |
| 328 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1958 | Azevedo | *A. mellea* | I |
| 339 | | *Armillaria* sp. | *Cryptomeria japonica* (M) | Portugal, Açores | 1960 | Azevedo | *A. mellea* | I |
| 357 | | *Armillaria* sp. | *Ulmus pumila* (nr) | Portugal, Chamusca | 1964 | Azevedo | NC | I |
| B1 | (PA84-5) | *A. borealis* | *Betula verrucosa* (M) | France | 1984 | Guillaumin | | II |
| B2 | (A1) | *A. borealis* | *Betula* sp. (B) | Finland, Ruovesi | 1979 | Guillaumin | HT | II |
| C1 | (PB71-1) | *A. cepistipes* | *Tilia platyphyllus* (M) | France | 1971 | Guillaumin | | II |
| C2 | (PB93-9) | *A. cepistipes* | *Pinus sylvestris* (R) | Sweden, Uppsala | 1993 | Guillaumin | | II |
| C3 | (B2) | *A. cepistipes* | *Alnus* sp. (B) | Finland, Tampere | 1979 | Guillaumin | | II |
| G1 | (PE80-37) | *A. gallica* | *Aesculus hippocastanum* (nr) | Germany, Munchen | 1980 | Marxmüller | HT | II |
| G2 | (PE88-23) | *A. gallica* | *Populus nigra* (nr) | Italy, Piemont | 1988 | Anselmi | | II |
| G3 | (E5) | *A. gallica* | *Corylus* sp. (B) | France,Puy-de-Dôme | 1980 | Guillaumin | HT | II |
| G4 | (E6) | *A. gallica* | *Unknown* (B) | France, Corrèse | 1981 | Guillaumin | HT | II |
| M1 | (PD82-68) | *A. mellea* | *Unknown* (nr) | Unknown | 1982 | Unknown | | I |
| M2 | (PD69-2) | *A. mellea* | *Prunus persica* (M) | France, Vénegen | 1969 | Guillaumin | | I |
| M3 | (PD84-78) | *A. mellea* | *Opuntia ficus-indicae* (M) | Italy, Sicily | 1984 | San Lio | | I |
| M4 | (PD-92-3) | *A. mellea* | *Abies cephallonica* (nr) | Greece, Iti | 1992 | Tsopelas | | I |
| M5 | (D5) | *A. mellea* | *Prunus persica* (B) | France, Perpignan | 1979 | Guillaumin | HT | I |
| O1 | (PC79-4) | *A. ostoyae* | *Pinus pinaster* (M) | France, Gironde | 1979 | Lung-Escarmant | | II |
| O2 | (PC82-4) | *A. ostoyae* | *Picea omorika* (M) | German, Gottingen | 1982 | Siepmann Korhonen | | II |
| O3 | (C2) | *A. ostoyae* | *Pinus sylvestris* (B) | Finland, Nurmijarvi | 1979 | Guillaumin | HT | II |
| O4 | (C5) | *A. ostoyae* | *Pinus uncinata* (B) | France, Font-Romeu | 1982 | Guillaumin | HT | II |
| T1 | (PT84-12) | *A. tabescens* | *Eucalyptus gummii* (M) | France, Boissède | 1984 | Guillaumin and Berthelay | | II |
| T2 | (PT90-5) | *A. tabescens* | *Prunus amygdalus* (nr) | Spain, Malaga | 1990 | Lopez Herrera | | II |
| T3 | (T2) | *A. tabescens* | *Quercus robur* (B) | France,Puy-de-Dôme | 1983 | Guillaumin | HT | II |
| T4 | (T4) | *A. tabescens* | *Quercus robur* (B) | France, Gers | 1985 | Guillaumin | HT | II |

[1] The original reference in Guillaumin's collection is presented in parenthesis.
[2] Derivation of isolates: B = basidiome; M = mycelium; R = rhizomorph; nr = no data available
[3] NC: no conclusive ; HT: haplont tester
[4] *Hin*f I RFLP-PCR patterns: I = 125 bp + 245 bp; II = 70 bp + 290 bp

Although the majority of our diploid isolates (80%) could be identified in diploid-haploid pairings, the method is laborious, takes too much time (up tp 2 months) and presents a high rate of inconclusive results. In fact, only 55% of the performed pairings produced clear results of compatibility or incompatibility, with the remainder displaying weak or equivocal reactions. This is a usual difficulty, specially in initial experiments (GUILLAUMIN *et al*. 1991; TSOPELAS, 1999), that restrains this identification approach to laboratories with the necessary expertise.

*Analysis of ITS1 region*

The amplification of the ITS1 region with primers ITS5 and ITS2 resulted in a single fragment in all Portuguese and European reference isolates, that includes a small portion of the 3'end of the 18S rDNA and of 5' end of the 5.8S rDNA. The length of the amplicon was estimated as 370 bp for *A. mellea* and 360 bp for the remainder *Armillaria* species. This size difference can be used for the direct identification of *A. mellea*, as shown in Figure 1a (lanes 3, 4 and 7) and Figure 1c (lanes 7 and 8, since some minor amount of ITS product is still present above *Hin*fI restriction fragments, due to an incomplete digestion).

Nevertheless, the reliability of the difference as a molecular marker is reduced, since it will depend on the achieved electrophoretic separation in this molecular size range. In fact, this difference was not even detected in the amplification of the overall ITS region, producing a *ca.* 840 bp fragment (CHILLALI *et al*., 1998a) and led to the misinterpretation of absence of ITS rDNA length variation among European *Armillaria* species.

Digestion of ITS1 amplicon with *Alu*I produced the same restriction pattern for all isolates of *A. borealis*, *A. cepistipes*, *A.gallica*, *A. ostoyae* and *A. tabescens*, consisting of two fragments with 215 bp and 145 bp, respectively (Figure 1b). Regarding the *A. mellea* isolates, four apparently distinct profiles were observed, consisting of two to four fragments (see lanes 7 to 9 in Figure 1b) with a size between 40 bp and 225 bp. A comparison of the observed profiles with the expected ones, taking in account the location of *Alu*I restriction sites (CHILLALI *et al*., 1998a; 1998b), showed that the differences could be explained by partial restriction digestion and that a sole real pattern for *A. mellea* would be found with four fragments containing 145 bp, 95 bp, 90 bp and 40 bp in length. Although *Alu*I restriction of ITS1 could be used for identification of *A. mellea* isolates, the possible occurrence of undetected partial restriction digestion and the high similarity of some *A. mellea* partial digestion profiles with the restriction pattern obtained for other *Armillaria* spp. (see lanes 6 and 9 in Fig. 1b, where larger fragments are 215 bp and 225 bp in length, respectively) makes this approach not advisable.

When *Hin*fI was used to digested the ITS1 amplicon, two clearly distinct patterns were obtained (Figure 1c and Table 1), one specific for all *A. mellea* isolates (further referred as the mellea pattern) and the other common to the remaining *Armillaria* spp. (non-mellea pattern). Both patterns consisted of two fragments as follows: fragments with 245 bp and 125 bp in length (mellea pattern; see lanes 6 and 7 in Figure 1C) and

fragments with 290 bp and 70 bp in length (non-mellea pattern; see for example, lanes 8, 9 and 10 in Figure 1C).

The non-mellea pattern was unexpected as, according to the ITS sequence analysis performed by CHILLALI *et al*. (1998a; 1998b), no *Hin*fI sites were detected in the ITS1 rDNA of *A. borealis*, *A. cepistipes*, *A. gallica*, *A. ostoyae* , *A. tabescens* and *A. ectypa*. Restriction mapping of this *Hin*fI site using reference isolates (data not shown), allowed its location in the 5' terminal region of the amplicon (inside ITS1 region) and upstream to the available ITS sequences, thus enlightening the discrepancy.

Taking in account the distinctiveness of the mellea and the non-mellea patterns above mentioned and the easy detection of incomplete restriction digestions (as could be detected by a sole fragment with 360 or 370 bp in length) *Hin*fI restriction of ITS1 revealed to be a reliable method to distinguish *A. mellea* (one the most aggressive species) from other European species.

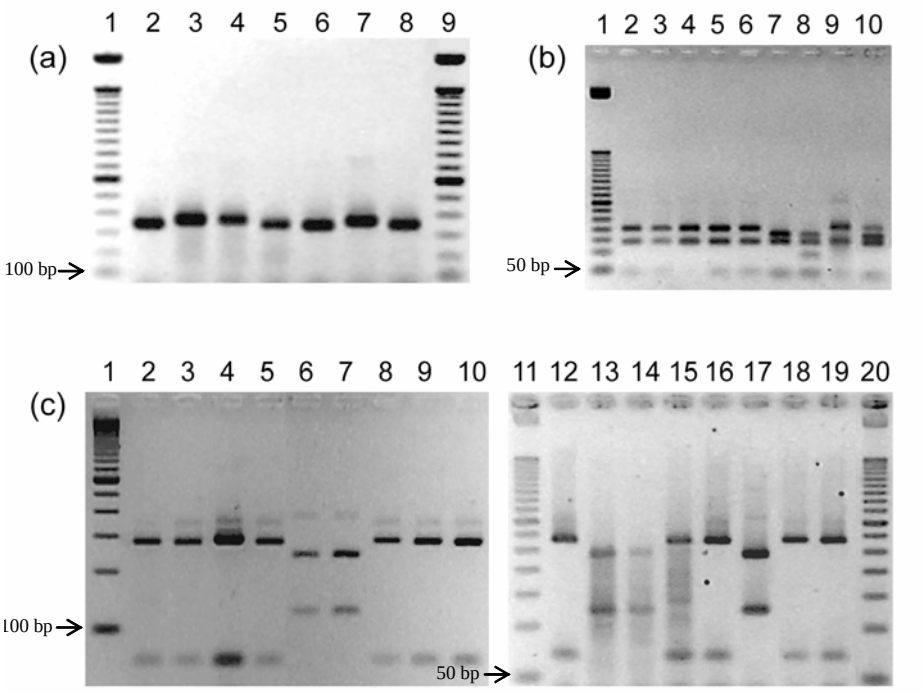


**Figure 1 -** ITS1 RFLP-PCR analysis of *Armillaria* isolates used in this study.
**(a)** Intact ITS1 amplicons of *Armillaria* isolates. Lanes: 1,9, 100 bp DNA ladder; 2, 303 (G); 3, 304 (M); 4, 308 (M); 5, T2; 6, O3; 7, M5; 8, G3.
**(b)** Restriction profiles of ITS1 region with *Alu*I. Lanes: 1, 50 bp DNA ladder; 2, G3; 3, G2; 4, 303 (G); 5, T3; 6, O3; 7, M5; 8, M4; 9, 357 (M); 10, 314 (M).
**(c)** Restriction profiles of ITS1 region with *Hin*fI. Lanes: 1, 100 bp DNA ladder; 11, 20, 50 bp DNA ladder; 2, T4; 3, T3; 4, O3; 5, B2; 6, M2; 7, M1; 8, O2; 9, O1; 10, C1; 12, 303 (G); 13, 304 (M); 14, 308 (M); 15, 312 (T); 16, O3; 17, M5; 18, G2; 19, G3. (B = *A. borealis*, C = *A. cepistipes*, G = *A. gallica*, M = *A. mellea*, O = *A. ostoyae*, T = *A. tabescens*).

The identification obtained with pairing-tests and the ITS1 RFLP-PCR analysis showed total agreement (see Table 1), as all identified isolates displayed the expected restriction pattern. Regarding the unidentified isolates, all of them could be identified as *A. mellea* by the molecular approach.

In spite of the close association of *A. ostoyae* with coniferous species, all isolates obtained from *Criptomeria japonica* in Azores were clearly identified as *A. mellea*. The work developed on *A. mellea* - *C. japonica* relationships (under ecological conditions in Azores) makes clear that this fungus behaves as primary pathogen (AZEVEDO, 1958, 1963, 1966, 1976a,b).

As it concerns to cork oak, a species with considerable social economical and ecological importance in the Mediterranean basin, *A. mellea* has been found as the most frequent *Armillaria* species (see Table 1), mainly in decaying stands. This suggests that *A. mellea* is probably involved in cork oak decline such as reported by BRUHN *et al*. (2000) for two other oak species (*Quercus coccinea* and *Quercus velutina*), and COETZEE *et al*. (2001) for dying oaks.

## Conclusions

The comparison made in this study between mating analysis and Internal Tanscribed Spacer ITS1 restriction analysis, as tools for the identification of *Armillaria* isolates, confirmed the potential of the latter for reliably discriminate *A. mellea* from the other European species.

As simple molecular techniques are involved (DNA extraction, PCR, enzymatic restriction and agarose gel electrophoresis) and the whole procedure can be performed in one day, *A. mellea* identification by RFLP-PCR based ITS1 analysis is clearly accessible and more advantageous to forest pathology laboratories.

## Acknowledgments

We are grateful to Dr. J.-J. Guillaumin of INRA, Clermont-Ferrand, France for providing the European isolates and for helpful suggestions and discussions during the course of the investigation.

The research was partially supported by Fundação para a Ciência e Tecnologia (Program PRAXIS XXI).

## References

AZEVEDO, N., 1958. Uma Armilariose em *Cryptomeria japonica* (L.f) D. Don. Separata das publicações da *Dir. Geral dos Serviços Florestais e Aquicolas* XXV (I,II) : 59-75.

AZEVEDO, N., 1963. Nitrogen utilization by four isolates of *Armillaria mellea*. *Trans. Brit. Mycol. Soc.* **46** : 281-284.

AZEVEDO, N., 1966. Ácerca da *Armillaria mellea* (Vahl Ex Fr.) Kummer. Tese de Investigação. Lisboa, Instituto Superior de Agronomia, 129 pp.

AZEVEDO, N., 1976a. Significance of rhizomorph formation of *Armillaria mellea* (Fr.) Kum. on *Cryptomeria japonica* (L. f.) D. Don in Azores. *Phytopathologia Mediterranea* **15** : 73-77.

AZEVEDO, N., 1976b. Ecologie des souches de l'Armillaria du *Quercus suber*. *Poljoprivredna znanstvena smotra* **39** : 485-493.

BRUHN, J.N., WETTEROFF Jr, J.J., MIHAIL, J.D., KABRICK, J.M., PICKENS, J.B., 2000. Distribution of *Armillaria* species in upland Ozark Mountain forest with respect to site, overstory species composition and oak decline. *Eur. J. Forest Pathol*. **30**(1) : 43-60

CENIS, J.L., 1992. Rapid extraction of fungal DNA for PCR. *Nucl. Acid Res*. **20** : 2380.

CHILLALI, M., IDDER-IGHILI, H., GUILLAUMIN, J.-J., MOHAMMED, C., LUNG-ESCARMANT, B., BOTTON, B., 1998a. Variation in the ITS and IGS regions of ribosomal DNA among the biological species of European *Armillaria*. *Mycol. Res.* **102**(5) : 533-540.

CHILLALI, M., WIPF, D., GUILLAUMIN, J.J., MOHAMMED, C., BOTTON, B., 1998b. Delineation of the European *Armillaria* species based on the sequences of the internal transcribed spacer (ITS) of ribosomal DNA. *New. Phytol.* **138** (3) : 553-561.

COETZEE, M.P., WINGFIELD, B.D., HARRINGTON, T.C., STEIMEL, J. COUTINHO T.A., WINGFIELD, M.J., 2001. The root rot fungus *Armillaria mellea* introduced into South Africa by early Dutch settlers. *Mol. Ecol.* **10**(2) : 387-396**.**

GUILLAUMIN, J.-J., BERTHELAY, S., 1981. Détermination spécifique des armillaires par la méthode des groupes de compatibilité sexuelle. Spécialisation écologique des espèces françaises. *Agronomie* **1** : 897-908.

GUILLAUMIN, J.-J., BERTHELAY, S., 1990. Comparaison de deux méthodes d'identification des clones chez le Basidiomycète parasite *Armillaria obscura*. *Eur. J. Forest Pathol.* **20** : 257-268.

GUILLAUMIN, J- J., ANDERSON, J., KORHONEN, K., 1991. Life cycle, infertility, and biological species. In *Armillaria* Root Disease. SHAW, C. G.; KILE, G. A. (ed). Washington, United States Department of Agriculture, Forest Service pp. 10-20.

GUILLAUMIN, J-J., MOHAMMED C., ANSELMI, N., COURTECUISSE, R., GREGORY, S. C., HOLDENRIEDER, O., INTINI, M., LUNG, B., MARXMULLER, H., MORRISON, D., RISHBETH, J, TERMORSHUIZEN, A., TIRRO, A., VAN DAM, B., 1993. Geographical distribution and ecology of the six *Armillaria* species in Western Europe. *Eur. J. Forest Pathol*. **23** : 321-341.

HARRINGTON, T.C., WINGFIELD, B.D., 1995. A PCR-based identification method for species of *Armillaria*. *Mycologia* **87** : 280-288.

JOHNSTON, P.R., JONES, D., 1997. Relationship among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. *Mycologia* **89**(3) : 420-430.

KORHONEN, K., 1978. Interfertility and clonal size in the *Armillariella mellea* complex. *Karstenia* **18** : 31-42.

LEGRAND, P., GHAHARI, S., GUILLAUMIN, J.-J., 1996. Occurrence of genets of *Armillaria* spp. in four mountain forests in Central France: the colonization strategy of *Armillaria ostoyae*. *New Phytol*. **133**(2) : 321-332.

PÉREZ SIERRA, A., WHITEHEAD, D.S., WHITEHEAD, M.P., 1999. Investigation of a PCR-based method for the routine identification of British *Armillaria* species. *Mycol. Res.* **103**(12) :1631-1636.

RISHBETH, J., 1986. Some characteristics of English *Armillaria* species in culture. *Trans. Br. Mycol. Soc.* **85** : 213-218.

SANTOS, R., ALMEIDA, H., 1997. Identificação de alguns elementos condicionantes do repovoamento florestal nos Açores. In *Proc. I Congreso Florestal Hispano Luso*., Pamplona, Spain, June, 23-27, pp. 639-644.

TSOPELAS, P., 1999. Distribution and ecology of *Armillaria* species in Greece. *Eur. J. Forest Pathol.* **29**(2) : 103-116.

WHITE, T.J., BRUNS, T., LEE, S., TAYLOR, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols. *A Guide to Methods and Applications.* INNIS, M.A., GELFANG, D.H., SNINSKY, J.J., WHITE, T.J. (ed.). Academic Press, pp. 315-322.

*Entregue para publicação em Maio de 2003*
*Aceite para publicação em Outubro de 2003*