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Intraspecific variation in *Armillaria* species from shrubs and trees in Northwestern Spain

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Summary. Until recently, the identification of *Armillaria* species relied upon morphological characteristics and mating tests, but now molecular techniques based on polymorphisms in the IGS region of the fungal rDNA are more commonly used, since these are more rapid and reliable. Differences found in RFLP patterns identifying *Armillaria* species have suggested the existence of intraspecific variation. In this work, 185 *Armillaria* isolates from different plant species (including fruit trees, broadleaf and coniferous trees, ornamental shrubs, kiwifruit and grapevine) affected by white root rot were analyzed by RFLP-PCR, in order to study intraspecific variation in *Armillaria* and the relationship with the plant host. *Armillaria mellea* was found in the majority of samples (71%), and was the most frequent *Armillaria* species in symptomatic ornamental shrubs, kiwifruit, grapevine, fruit trees and broadleaf trees. In conifers however white root rot was generally caused by *Armillaria ostoyae*. *Armillaria gallica* was identified, although with low incidence, in ornamental, coniferous, broadleaf and fruit hosts. Intraspecies variation was recorded only in *A. mellea*, for which RFLP patterns mel 1 and mel 2 were found. Most plants infected with *A. mellea* showed the mel 2 pattern. Further research is needed to study whether *Armillaria* RFLP patterns are specific to certain plant hosts, and whether intraspecific variation is related to differences in pathogenicity.

Key words: molecular diagnostic, RFLP patterns, white root rot.

Introduction

Species of *Armillaria* (*Agaricales*, *Basidiomycotina*) differ in their geographical and ecological distribution, host range, pathogenicity, and the type and frequency of rhizomorphs found in the soil (Guillaumin *et al.*, 1993). In Europe, *A. mellea* and *A. ostoyae* are aggressive pathogens, causing white root rot in a wide range of woody hosts, whereas four other species (*A. gallica, A. borealis, A. tabescens* and *A. cepistipes*) are considered only saprophytes or weak pathogens (Legrand and Guillaumin, 1993; Termorshuizen and Arnolds, 1994).

For many years, the identification of *Armillaria* species relied upon mating tests with haploid tester strains (Korhonen, 1978), a method that requires 6 to 8 weeks and sometimes gives ambiguous results (Guillaumin and Berthelay, 1981). More recently, molecular methods have been used based on variations in the ITS and IGS regions of ribos-

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omal DNA, among which RFLP-PCR provides rapid, easy and reliable identification of *Armillaria* species (Harrington and Wingfield, 1995; Schulze *et al.*, 1997; Chillali *et al.*, 1998; Mansilla *et al.*, 2000). Pérez-Sierra *et al.* (1999) found two RFLP patterns for *A. mellea* (mel 1 and mel 2), three for *A. gallica* (gal 1, gal 2 and gal 3), two for *A. cepistipes* (cep 1 and cep 2), and two for *A. borealis* (bor 1 and bor 2), suggesting the existence of intraspecies variation. Only one RFLP pattern has so far been described for *A. ostoyae* (pattern ost) and for *A. tabescens* (pattern tab).

The aim of this study was, using RFLP patterns, to identify *Armillaria* species causing white root rot in shrubs and trees in northwestern Spain, and to examine whether these species show intraspecific variation.

Materials and methods

Plants in vineyards, in kiwifruit and fruit tree plantations, and in forest stands, orchards and gardens in Galicia (NW Spain), and showing symptoms of white root rot (shoot decay, chlorotic leaves, poor shooting, and wilting), were inspected during 2001–2003. A small trench was dug around each plant to collect superficial roots with mycelia and/or rhizomorphs. Samples of fruiting bodies were also taken if they were found near symptomatic plants. A total of 185 samples were collected from coniferous, broadleaf and fruit trees, grapevine, kiwifruit and ornamental shrubs.

Armillaria species in samples were identified by RFLP patterns obtained after PCR-amplification and enzymatic digestion of a fragment of the IGS region of ribosomal DNA, following the method described by Mansilla *et al.* (2000). DNA was extracted from basidiospores, rhizomorphs found on roots and mycelium on bark, depending on the characteristics of each sample. DNA was extracted using the EZNA fungal miniprep kit (Omega Bio-Tek, Doraville, CA, USA). Ten to 40 mg of each fungal sample were needed to obtain 2–10 ng of DNA.

For DNA amplification, the primers used were LR12R: 5' CTG AAC GCC TCT AAG TCA GAA 3' (Veldman *et al.,* 1981) and O-1: 5' AGT CCT ATG GCC GTG GAT 3' (Duchesne and Anderson, 1990). Each PCR reaction contained a Ready-to-Go PCR bead (Amersham Biosciences, Little Chalfont, UK), 0.5 μl of each primer (10 pmol μl^{-1}), and sterile water (Sigma Chemical Co., Saint Louis, MO, USA) up to a final volume of 25 μl. Amplification was performed in a Mastercycler Personal thermocycler (Eppendorf, Hamburg, Germany) under the conditions described by Pérez-Sierra *et al.* (1999): initial DNA denaturation for 95 seconds at 95ºC, 35 cycles of annealing for 60 seconds at 60ºC, elongation for 120 seconds at 72ºC, chain denaturation for 60 seconds at 95ºC, and final elongation for 10 minutes at 72ºC.

PCR products were digested with three restriction enzymes, *Alu* I, *Nde* I and *Bsm* I (Roche Diagnostics, Indianapolis, IN, USA). For analysis with *Nde* I and *Bsm* I, PCR products were previously purified with the High Pure PCR Product Purification Kit (Roche Diagnostics). The digested DNA fragments were separated by electrophoresis on 3% agarose gel, in a 1× TBE buffer and stained with ethidium bromide. In each analysis, a negative control (without DNA) and a 100 bp-ladder (Marker XIV, Roche Diagnostics) were also included. The identification of *Armillaria* species was based on the size of digested fragments (pb) as compared to those reported by Harrington and Wingfield (1995) and Pérez-Sierra *et al.* (1999) for North American and European isolates.

Results and discussion

A DNA fragment of approximately 900 pb was obtained from all fungal samples (Fig. 1). Four restriction patterns, previously described by Pérez-Sierra *et al.* (1999), were found after digestion of the amplified rDNA region with *Alu* I. Three of these patterns allowed straightforward identification of the *Armillaria* species in 160 samples: mel 1 (320-180-155 bp) (Fig. 2) and mel 2 (320-155 bp) for *A. mellea* (Fig. 3), and gal 1 (400-240-190 pb) for *A. gallica*. The fourth restriction pattern (310-200-135 pb) (Fig. 4A) was observed in 25 samples, all but one from coniferous trees; this pattern was identified as *A. ostoyae* pattern ost, or *A. cepistipes* pattern cep 2, or *A. borealis* pattern bor 1. For these samples, further rDNA digestion with *Nde* I and *Bsm* I enzymes was needed, which led to the identification of *A. ostoyae* pattern ost in all these cases (Fig. 4B).

The *Armillaria* patterns of all fungal samples from the different plant hosts are shown in Ta-

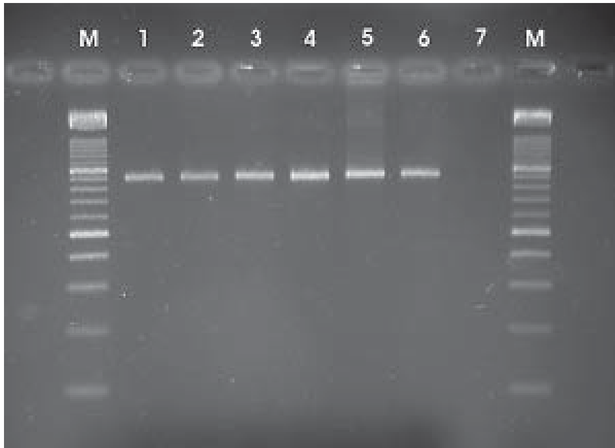

Fig. 1. Agarose gel showing PCR products from samples of *Castanea sativa*, *Magnolia grandiflora*, *Aesculus hippocastanum*, *Quercus robur*, *Pinus radiata* and *Thuja* sp. (lanes 1–6). Lane 7, negative control. The outside lanes (M) contain a 100 pb DNA molecular marker as a size standard.

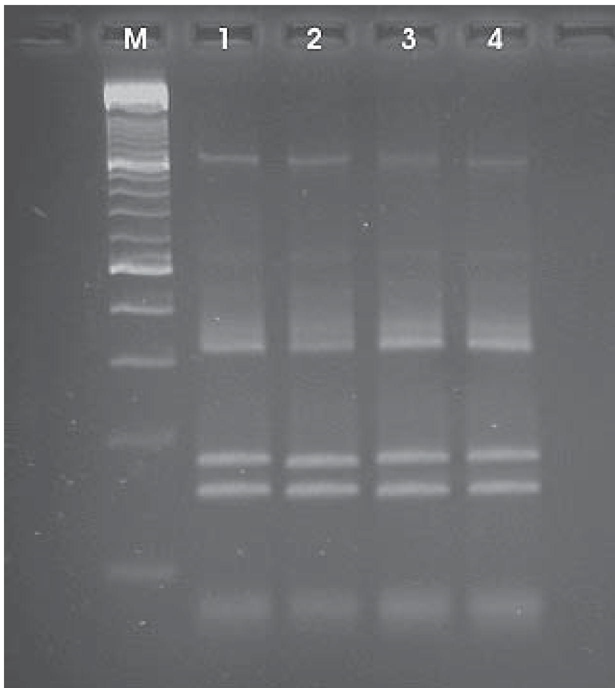

Fig. 2. Restriction analysis of the IGS region of the rDNA amplified by PCR, digested with *Alu* I and electrophoresed in 3% agarose gel stained with ethidium bromide. Lanes 1–4: *Armillaria mellea* pattern mel 1 obtained from *Vitis* sp., *Malus domestica*, *Camellia* sp. and *Olea europaea*. The outside lane (M) on the left contains a 100 pb DNA molecular marker as a size standard.

ble 1. Ornamental shrubs, kiwifruit, grapevine and all trees but conifers were mostly affected by *A. mellea*. *A. gallica* was found in some samples from *Acacia melanoxylum*, *Acer pseudoplatanus*, *Aesculus hippocastanum*, *Camellia* sp., *Castanea sativa*, *Citrus limon*, *Citrus sinensis*, *Corylus avellana*, *Eucalyptus globulus*, *Hydrangea macrophylla*, *Laurus nobilis*, *Prunus avium*, *Rhododendron* sp., and *Robinia pseudoacacia*. Only one sample, from *Salix babylonica*, yielded *A. ostoyae*.

In *Pinus* species, white root rot was generally caused by *A. ostoyae*, as was also reported by Legrand and Guillaumin (1993) and by Termorshuizen and Arnold (1994), although *A. ostoyae* has also been found in other plant species (*Corylus*

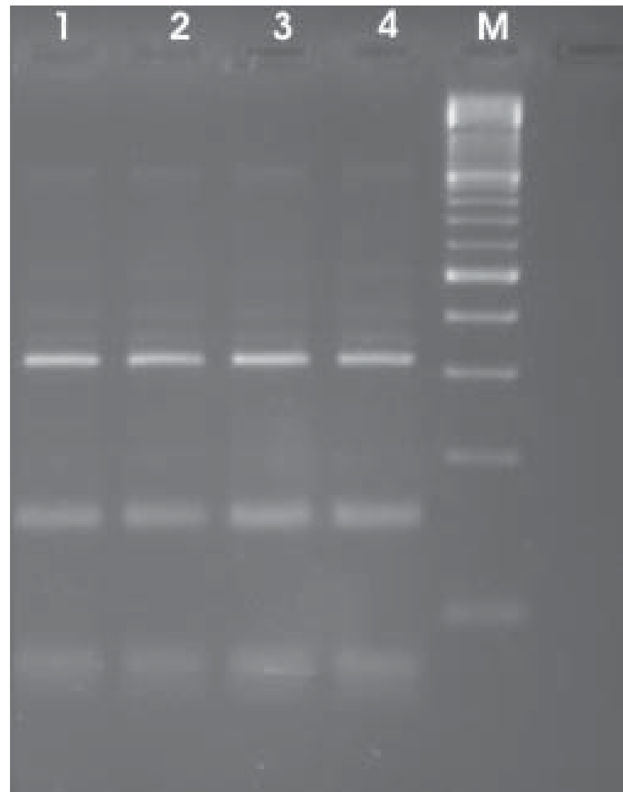

Fig. 3. Restriction analysis of the IGS region of the rDNA amplified by PCR, digested with *Alu* I and electrophoresed in 3% agarose gel stained with ethidium bromide. Lanes 1–4, *Armillaria mellea* pattern mel 2 obtained from *Vitis* sp., *Actinidia deliciosa*, *Castanea sativa* and *Pinus pinaster*. The outside lane (M) contains a 100 pb DNA molecular marker as a size standard.

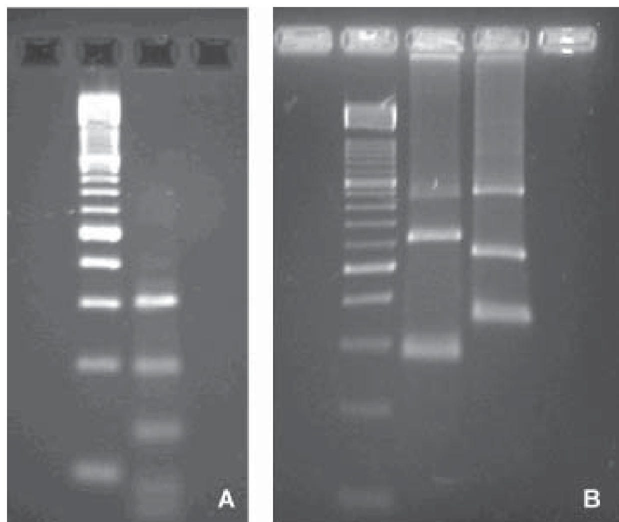

Fig. 4. Restriction analysis of the IGS region of the rDNA amplified by PCR of a *Pinus radiata* sample, digested with *Alu* I (A) or with *Nde* I and *Bsm* I (B), and electrophoresed in 3% agarose gel stained with ethidium bromide. A) lane 3, pattern obtained with *Alu* I; B) lanes 3 and 4, patterns obtained with *Bsm* I and *Nde* I, respectively. Lane 2 both in A and B contains a 100 pb DNA molecular marker as a size standard.

avellana, Castanea sativa, Quercus robur, Crataegus sp.*, Rubus* sp.) (Guillaumin *et al.,* 1993). *A. mellea* was also detected in *Pinus* sp., *P. pinaster*, *Cedrus* sp., *Cupressus sempervirens* and in *Thuja* sp. These results are consistent with those of Rishbeth (1982), who found that white root rot in conifers was also caused by *A. mellea*. A few conifer samples (from *Abies*, *Pseudotsuga*, *Pinus* and *Thuja*) were affected by *A. gallica***,** which has been frequently reported as a saprophyte or weak parasite in the *Abietaceae* and *Cupressaceae* (Guillaumin *et al.*, 1993).

Intraspecies variation was recorded only for *A. mellea*, which yielded the RFLP patterns mel 1 and mel 2 (Table 1). The predominant RFLP pattern for *A. mellea* was mel 2, which was identified in 111 samples, whereas mel 1 occurred in 21 samples. Chestnut and grapevine were mainly affected by the *A. mellea* mel 2 pattern. The greatest incidence of mel 2 in grapevine cultivated in NW Spain (as compared to mel 1) was previously found in samples collected from 1998 to 2000 (Aguín *et al.*, 2004), but to our knowledge the incidence of these RFLP patterns on other plant species have not been studied.

When conifers were infected with *A. mellea*, only the mel 2 pattern was detected (Table 1). For fruit trees, broadleaf trees, grapevine, kiwifruit and ornamental shrubs, the majority of samples also presented only one pattern per species. However both mel 1 and mel 2 patterns were found in *Castanea sativa*, *Aesculus hippocastanum*, *Malus domestica*, *Olea europea* and *Vitis* sp. Pérez-Sierra *et al*. (1999) likewise found both mel 1 and mel 2 patterns in symptomatic samples of *Betula*, *Acer*, *Ligustrum* and *Prunus*. A more extensive survey of kiwifruit, ornamental shrubs, coniferous, broadleaf and fruit trees infected with white root rot might reveal whether there is a specific relation between the two *Armillaria* patterns and certain plant hosts.

In *A. gallica*, only the gal 1 pattern was detected. This pattern seems to be the most common in Europe since Pérez-Sierra *et al*. (1999) also found it in most of their samples of *Euonymus*, *Corylopsis*, *Quercus*, *Picea*, *Pinus* and *Corylus* infected with *A. gallica*. They found gal 2 in only two samples from an unknown plant host, and gal 3 in one sample from *Rubus*. *A. ostoyae* in conifer samples was of the ost pattern, the only pattern so far described for this fungus.

Further research is needed to study whether intraspecific variation in *A. mellea*, as detected by RFLP analysis, is host-related and whether such variation involves differences in pathogenicity.

The intraspecific variation could also point out the existence of two species, which is further supported by the fact that we noted morphological differences in the colour and consistency of colonies between mel 1 and mel 2 isolates. Pérez Sierra *et al*. (1999) also found morphological differences between mel 1 and mel 2 isolates. However, to establish whether mel 1 and mel 2 isolates belong to different species, tests for intersterility must be conducted. Up to now, delimitation of species in *Armillaria* has been done based on tests for sexual compatibility (Korhonen, 1978). In fact, intraspecific variation within *A. mellea* isolates, related with virulence, basidiome morphology, and rhizomorph production, was not used for species delimitation unless differences in sexual compatibility were also found (Harrington and Rizzo, 1999).

Table 1. Host species, number of samples, host origin, geographical localization and identification of *Armillaria* species according to RFLP patterns.

Host species	No. of samples	Host origin/ geographical localization	*Armillaria* RFLP pattern			
			mel 1	mel 2	ost	gal 1
Broadleaf hosts						
Acacia melanoxylum	1	Garden/Galicia	0	0	0	1
Acacia sp.	1	Garden/Galicia	0	1	0	0
Acer pseudoplatanus	1	Garden/Galicia	0	0	0	1
Actinidia deliciosa	8	Plantation/Galicia	0	8	0	0
Aesculus hippocastanum	4	Garden/Galicia	2	1	0	1
Alnus sp.	2	Forest stand/Galicia	0	2	0	0
Camellia sp.	7	Garden/Galicia	4	0	0	3
Castanea sativa	9	Forest stand/Galicia	1	7	0	1
Citrus limoni	3	Orchard/Galicia	0	2	0	1
Citrus sinensis	3	Orchard/Galicia	0	2	0	1
Corylus avellana	1	Forest stand/Galicia	0	0	0	1
Crataegus azarolus	1	Garden/Galicia	0	1	0	0
Escallonia macrantha	1	Garden/Galicia	0	1	0	0
Eucalyptus globulus	1	Forest stand/Galicia	0	0	0	1
Euonymus japonica	3	Garden/Galicia	0	3	0	0
Hydrangea macrophylla	2	Garden/Galicia	0	1	0	1
Laurus nobilis	2	Garden/Galicia	1	0	0	1
Ligustrum sp.	1	Garden/Galicia	0	1	0	0
Magnolia grandiflora	3	Garden/Galicia	0	3	0	0
Malus domestica	5	Plantation/Galicia	2	3	0	0
Morus sp.	1	Garden/Galicia	1	0	0	0
Olea europaea	3	Orchard/Galicia	2	1	0	0
Prunus armeniaca	1	Orchard/Galicia	0	1	0	0
Prunus avium	10	Orchard/Galicia	0	5	0	5
Prunus persica	1	Orchard/Galicia	0	1	0	0
Quercus robur	2	Forest stand/Galicia	0	2	0	0
Quercus suber	1	Forest stand/Galicia	0	1	0	0
Robinia pseudoacacia	1	Garden/Galicia	0	0	0	1
Rhododendron sp.	3	Garden/Galicia	0	2	0	1
Salix babylonica	4	Garden/Galicia	0	3	1	0
Syringa vulgaris	1	Garden/Galicia	0	1	0	0
Tibouchina urvilleana	1	Garden/Galicia	0	1	0	0
Vitis sp.	61	Vineyard/Galicia	8	49	0	4
Coniferous hosts						
Abies sp.	1	Forest stand/Galicia	0	0	0	1
Cedrus sp.	1	Garden/Galicia	0	1	0	0
Cupressus sempervirens	2	Garden/Galicia	0	2	0	0
Pinus pinaster	8	Forest stand/Galicia	0	1	7	0
Pinus radiata	6	Forest stand/Galicia	0	0	6	0
Pinus sp.	14	Forest stand/Galicia	0	2	11	1
Pseudotsuga menziesii	1	Forest stand/Galicia	0	0	0	1
Thuja sp.	3	Garden/Galicia	0	2	0	1
Total	185		21	111	25	28

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