Characterisation of *Armillaria* species based on pectic isozyme analyses

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Armillaria spp. are the causal agents of Armillaria root rot on a wide variety of mainly woody plants. Identification of these fungi using morphological characteristics is complicated by the fact that fruiting structures are uncommon and often ephemeral. Pectic isozyme analysis has been successfully applied in taxonomic studies of *Armillaria* species. This technique, however, has never been used in a study that included a collection of species from across the world. In the present study, 36 *Armillaria* isolates, representing 17 *Armillaria* spp. from different hosts and geographic regions were characterised using isozyme patterns for pectin lyase (PL), pectin methylesterase (PME) and polygalacturonase (PG). Isozyme patterns were determined directly from culture filtrates through electrophoresis in polyacrylamide gels stained in ruthenium red. The majority of species could clearly be separated from one another and isolates belonging to the same species had similar banding patterns, grouping them together after cluster analysis. Results from this study showed that pectic enzyme analysis can be an effective tool in the identification of *Armillaria* species. Furthermore the isozyme analysis supports previous observations regarding the relationships between *Armillaria* species.

Key words: Basidiomycetes, isozymes, *Armillaria* root rot, taxonomy

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Introduction

Species of *Armillaria* are basidiomycetous root pathogens of a wide range of woody plants. These fungi have a wide global distribution and include some of the most important pathogens of trees. In this regard they are especially important in forests and fruit crops.

Various techniques have been used to identify and group *Armillaria* species. Earlier work relied exclusively on pairing tests and morphology. Morphological identification is based primarily on fruiting body characteristics (e.g. Watling *et al*., 1982; Bérubé and Dessureault, 1988, 1989). However, the seasonal nature and short life span of fruiting bodies limits the use of this method for identification. Mating tests, while not dependant on fruiting structures, rely on biological compatibility of isolates of the same species (Hintikka, 1973; Korhonen, 1978; Anderson and Ullrich, 1979). The tests, however, are time consuming, the results are often ambiguous and are only applicable to heterothallic and sexual species.

Qualitative DNA based methods including comparisons of sequences of the IGS-1 and ITS regions of the ribosomal DNA (Anderson and Stasovski, 1992; Chillali *et al*., 1997, 1998; Coetzee *et al*., 2000, 2001, 2003; Sekizaki *et al*., 2008), RFLPs (Jahnke *et al*., 1987; Anderson *et al*., 1989; Smith and Anderson, 1989; Sekizaki *et al*., 2008; Wingfield *et al*., 2009) and AFLPs (Pérez-Sierra *et al*., 2004; Wingfield *et al*., 2009) have recently been increasingly used to identify *Armillaria* species. Protein based techniques have also been employed in *Armillaria* taxonomy (Morrison, 1982; Morrison *et al*., 1985; Lin *et al*., 1989; Wahlström *et al*., 1991; Mwenje and Ride, 1996; Mwenje *et al*., 2006).

Despite limited resolution of protein-based methods in comparison to DNA techniques, they can potentially provide additional molecular characters for the identification and delineation of species, including those in *Armillaria*.

Isozymes are multiple forms of the same enzyme and which differ in molecular weight, regulation, isoelectric points and electrophoretic mobilities (D'Ovidio *et al*., 2004). Isozymes arise as a result of the presence of multiple genes coding for a protein or as a result of post-translational modification of the enzymes (D'Ovidio *et al*., 2004). Pectic isozymes have been widely used in fungal taxonomy (Johansson, 1988; Karlsson and Stenlid, 1991; Chang and Mills, 1992). The technique has also been applied successfully for the identification of *Armillaria* spp.

Morrison *et al*. (1985) used esterase and polyphenol oxidases to study isolates from British Columbia and separated the isolates into *A. bulbosa* [= *A. gallica*], North American Biological species (NABS) IX [= *A. nabsnona*], NABS X, and group F clustered with NABS V [= *A. sinapina*]. Esterase patterns were also used to differentiate four North American Biological Species of *Armillaria* by Lin *et al*. (1989). Whalström *et al*. (1991) analysed the pectic esterase and polygalacturonases isozyme patterns of five European species and found that the patterns differed among the species. *Armillaria mellea* had two specific polygalacturonase bands, which were absent in the other species. *Armillaria ostoyae* and *A. borealis* had very similar profiles. In Japan, researchers differentiated Japanese biological species of *Armillaria* based on isozyme patterns from selected enzymes (Cha and Igarashi, 1995; Matsushita *et al*., 1996). *Armillaria* isolates from Zimbabwe have been shown to reside in three groups using isozymes (Mwenje and Ride, 1996). Mwenje and Ride (1997) also identified four taxonomic groups in Africa based on pectin lyase and pectin methylesterase isozyme patterns. More recently, Mwenje *et al*. (2006) employed isozyme patterns from these enzymes together with DNA sequence data to elucidate the *Armillaria* species causing disease on tea in Kenya. Despite the usefulness of pectic enzymes in distinguishing some species of *Armillaria*, a comparison of the pectic enzyme profiles of a collection of the well-known species is not available.

The aim of this study was to analyse the relationships between a wide range of *Armillaria* spp. using pectin lyases, pectin methylesterases and polygalacturonases isozyme profiles. These relationships were then compared with those of previously published molecular based comparisons. In this way, the value of the enzyme profiles in the taxonomy of a global collection of *Armillaria* species could be assessed.

Materials and methods

Origin of Isolates

In total, 17 species of *Armillaria* represented by 36 isolates from many different origins were considered in this study (Fig. 1). *Armillaria mellea* was represented by eight isolates; two from each of the four geographical clades as indicated by Coetzee *et al*. (2000), while one to three isolates were included for the other *Armillaria* species. The isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI). A representative set of isolates has also been deposited with the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Cell wall preparation

In order to produce cell walls, fresh stem segments of Msasa (*Brachystegia spiciformis*) were ground into fine sawdust using a mill as described by Mwenje and Ride (1996). This sawdust was soaked for one hour in 100% ethanol, filtered through Whatman filter paper (#1) and rinsed in 100% ethanol for 10 minutes. This was followed by washing twice in acetone (10 minutes per wash) after which it was air dried. The crude extract was then stored at room temperature until further use.

Enzyme production

Isolates were grown in duplicate at 25°C under stationary conditions in 250 mL conical flasks containing 50 mL Vogel's medium (Vogel, 1956) amended with one gram cell walls. After 30-35 days of incubation in the dark, the cultures were harvested by filtration

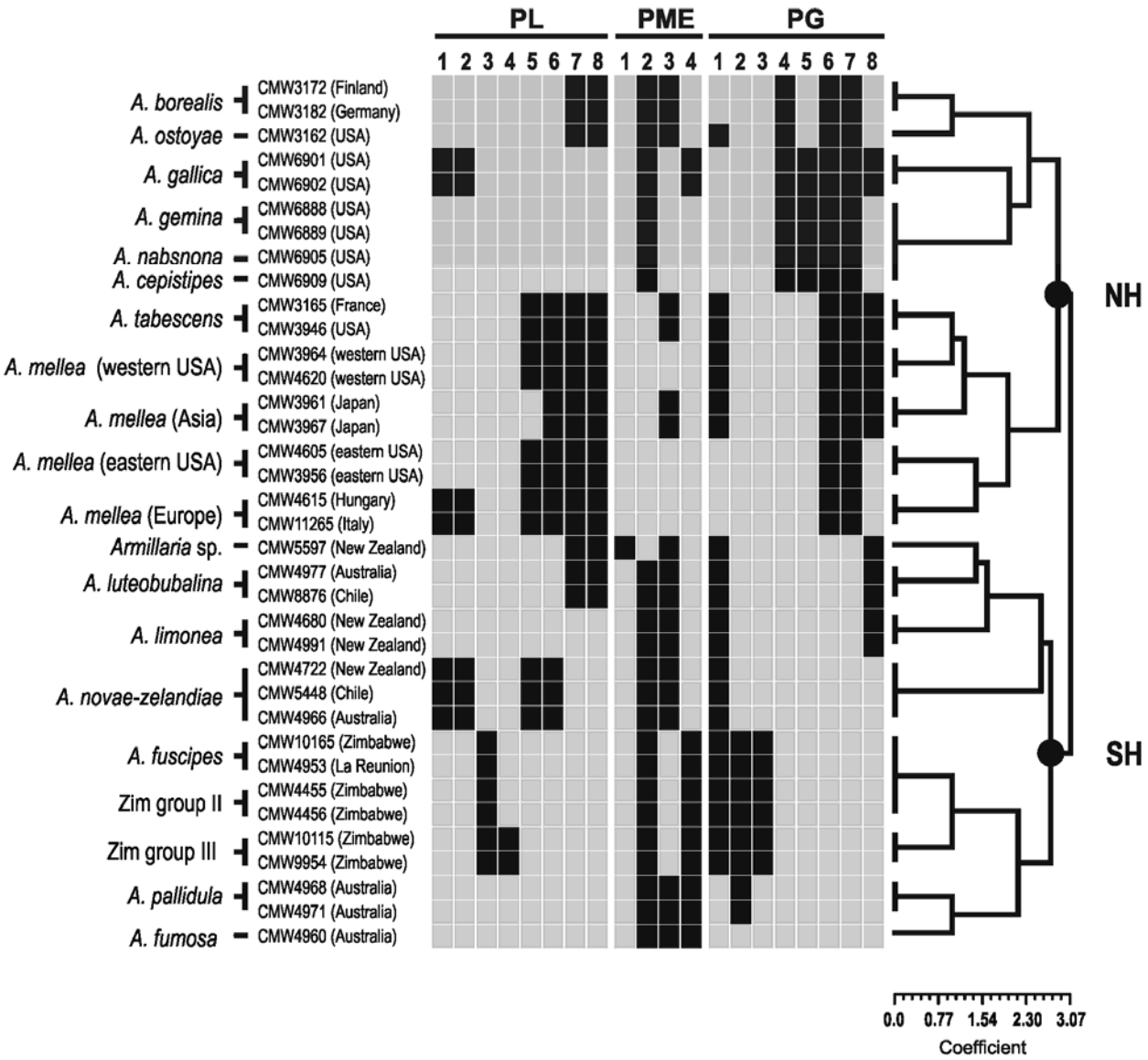

Fig. 1. Diagram showing the type and number of pectin lyase (PL), pectin methylesterase (PME) and polygalacturonase (PG) patterns as well as a dendrogram generated from the isozyme profiles after cluster analysis for the *Armillaria* isolates. Each column represents an isozyme and a black block represents the presence of a corresponding isozyme in an isolate. The country of origin is indicated within the brackets for each isolate. NH and SH next to the closed circles indicate the nodes that are shared by the species occurring in the Northern hemisphere and those from the Southern hemisphere, respectively. Dissimilarity values are shown on the scale.

using Whatman filter paper (#1). Filtrate (30-40 mL) was concentrated overnight by dialysis to approximately one mL using 12.5% (w/v) polyethylene glycol dissolved in sodium acetate buffer (pH 5.5). The concentrate was stored at –20ºC.

Native gel electrophoresis

Electrophoresis was performed in 10% polyacrylamide resolving gels, using a high pH, non-dissociating discontinuous system (Hames, 1987). Pectin was incorporated at 0.5% (w/v) into resolving and plug gels, but not in the stacking gels, to enable the detection of pectic enzymes. Equal amounts of double strength buffer and enzyme solution were mixed prior to loading the gel.

Detection of Pectin Lyases (PL) and Pectin Methylesterases (PME)

The method used by Mwenje and Ride (1996) was employed to detect PLs and PMEs. Gels were incubated for 10 minutes at 5°C in 10 mM $CaCl_2$ after electrophoresis. They were then incubated for 40 minutes at room temperature in 20 mM Tris-HCl (pH 8.5)

containing 10 mM $CaCl_2$. Gels were stained overnight in 0.03 (w/v) ruthenium red. Excess ruthenium red was removed using at least three changes of distilled water. Pectin lyase bands appeared as white bands, while PME bands appeared as dark, red/ purple bands.

Detection of Polygalacturonases (PGs) and Pectin Methylesterases (PMEs)

After electrophoresis, gels were washed briefly in water and incubated for 1.5 hour at 25°C in 100 mM malic acid without shaking. Gels were rinsed in water for 5 min and stained overnight in ruthenium red (0.03 w/v in water). Stained gels were washed in water to remove excess ruthenium red. Polygalacturonases were visualised as white bands and PMEs appeared as dark bands.

Numerical analyses

Bands that were clearly visible on the zymograms were scored as 1 and 0 representing present and absent bands, respectively. The combined data set for polygalacturonase, pectin lyase and pectin methylesterase was analysed using the Numerical Taxonomy and Multivariate Analysis System version 2.1 (NTSYSpc) (Exeter Software, New York, USA). Euclidean distances were calculated and a dendrogram generated using an unweighted pair group method with arithmetic mean (UPGMA).

Results

Isozyme patterns for various pectic enzymes

A total of eight different PL isozymes, designated PL1 – PL8, were detected (Fig. 1). European *A. mellea* strains (CMW4615 and CMW11265) yielded six PL bands, and thus the largest number of PL isozymes. PL1 and PL2 bands were present in these isolates, as well as in *A. novae-zelandiae* and *A. gallica* isolates. PL3 was present only in the species from Africa. Band PL4 was detected only in Zimbabwean group III isolates (CMW9954 and CMW10115). All *A. mellea* isolates except one from Japan had band PL5, which they shared with *A. novae-zelandiae* and *A. tabescens* isolates. Band PL6 was common in all *A. mellea* isolates and was also detected in *A. novae-zelandiae* and *A. tabescens*. Bands PL7 and PL8 were common in all *A. mellea* isolates but were also detected in *A. tabescens*, *A. luteobubalina*, *A. ostoyae*, *A. borealis* and the unnamed *Armillaria* sp. from New Zealand (CMW5597).

Four different PME bands, designated PME1 – PME4, were found in this study (Fig. 1). PME1 was detected only in the isolate of an unnamed *Armillaria* sp. from New Zealand (CMW5597). Band PME2 was absent in *A. tabescens*, the unnamed species from New Zealand and all *A. mellea* isolates. Band PME3 was detected in *A. borealis*, the unnamed species from New Zealand, *A. limonea*, *A. luteobubalina*, *A. novae-zelandiae*, *A. pallidula*, *A. tabescens*, *A. mellea* from Japan, *A. fumosa* and *A. ostoyae*. Band PME4 was present in all isolates from Africa, *A. gallica*, *A. fumosa* and *A. pallidula*.

Polygalacturonase isozyme analysis resulted in the production of eight different bands (PG1 – PG8) (Fig. 1). All African isolates were identical in their PG banding patterns; having PG1, PG2 and PG3. The PG1 band was also present in *A. luteobubalina*, *A. novae-zelandiae*, *A. limonea*, *A. tabescens*, *A. mellea* isolates from North America and Japan, *A. ostoyae* and unnamed isolate (CMW5597) from New Zealand. Band PG2 was present in the African isolates and *A. pallidula*, and PG3 was exclusive to the African isolates. Band PG4 was detected in *A. cepistipes*, *A. borealis*, *A. nabsnona*, *A. ostoyae*, *A. gemina* and *A. gallica* and band PG5 was present in *A. gallica*, *A. gemina*, *A. nabsnona*, and *A. cepistipes*. Bands PG6 and PG7 were present in *A. borealis*, *A. tabescens*, *A. ostoyae*, *A. cepistipes*, *A. nabsnona*, *A. gemina*, *A. gallica* and all *A. mellea* isolates. Band PG8 was present in unnamed species from New Zealand, *A. gallica*, *A. limonea*, *A. luteobubalina*, *A. tabescens* and North American and Japanese *A. mellea*.

Numerical analyses and grouping of isolates

All isolates included in this study could be grouped according to the *Armillaria* spp. that they represented in the dendrogram generated based on combined data of pectin lyase, polygalacturonases and pectin methylesterases (Fig. 1). Isolates also resided in two major groups. One of these included all *Armillaria* spp. found only in the Northern hemisphere and the second group included all species from the Southern hemisphere.

Armillaria borealis grouped very close to *A. ostoyae*, forming one group within the Northern hemisphere collection of isolates. This group was connected to a cluster that included *A. gallica*, *A. gemina*, *A. nabsnona* and *A. cepistipes*. A second major cluster within the Northern hemisphere group was formed by *A. mellea* and *A. tabescens*. *Armillaria mellea* isolates formed clusters, comprising of isolates from Japan, Europe, eastern and western North America, respecttively. Isolates representing *A. tabescens* were connected to the cluster comprising of Japanese *A. mellea* isolates.

The Southern hemisphere group included isolates representing the unnamed isolate from New Zealand, *A. limonea*, *A. luteobubalina*, *A. novae-zelandiae*, *A. pallidula*, *A. fumosa*, *A. fuscipes* and the Zimbabwean groups II and III as defined by Mwenje and Ride (1996). Two major groups were observed within the Southern hemisphere cluster. One of the groups included *A. novae-zelandiae*, *A. limonea*, *A. luteobubalina*, and the unknown species from New Zealand. Within this group, the unnamed species was closely related to *A. luteobubalina* and together formed a cluster closely related to *A. limonea*. The other group included the African taxa together with *A. pallidula* and *A. fumosa* to form the second major cluster. Within this group, *A. pallidula* and *A. fumosa* were closely related and the African taxa formed a distinct group.

Discussion

In this study, we have shown that the pectic enzymes PL, PG and PME can be used to separate most species of *Armillaria*. These included 17 species and the majority of those that are commonly encountered. We have further been able to show that Northern hemisphere isolates are completely different to those from the Southern hemisphere. Southern hemisphere species have been found to group basally in a DNA based phylogeny, hence ancestral to those from the Northern Hemisphere (Coetzee *et al*., 2001; Dunne *et al*., 2002). DNA sequence data have shown that species from the Southern hemisphere are more closely related to each other and very distantly to those from the Northern hemisphere. Isozyme comparisons in this study, confirm this view.

Isolates from Zimbabwe representing *Armillaria* group II (Mwenje and Ride, 1996; Mwenje *et al*., 2003) and *A. fuscipes* had identical banding patterns. These isolates also clustered together in the dendrogram. This result is in contrast to IGS-1 sequence data (Mwenje *et al*., 2003), which separates these isolates into different groups. Isolates of African *Armillaria* group III (Mwenje and Ride, 1996; Mwenje *et al*., 2003) differed slightly in their enzyme profile from groups I and III. This is also consistent with analyses of IGS-1 sequence data, where these isolates have been shown to represent different, but closely related groups (Mwenje *et al*., 2003).

Armillaria fumosa and *A. pallidula* differ at only one isozyme and thus grouped closely in cluster analysis. Previous reports have indicated that these two species are phylogenetically closely related. For example, Coetzee *et al*. (2001) could not differentiate between the two species based on sequences of the ITS region. The species could also not be separated based on EF 1-α sequence data in a previous study (Maphosa *et al*., 2006). However, *A. fumosa* and *A. pallidula* were previously shown to be distinct species based on morphology and mating type tests (Kile and Watling, 1988).

Armillaria luteobubalina and *A. limonea* grouped in the same cluster and had slightly different banding patterns. *Armillaria luteobubalina* is of Australian origin and *A. limonea* originates from New Zealand. Using compatibility tests, Kile and Watling (1988) concluded that these represent different biological species. The grouping of these species based on pectic enzymes in this study is consistent with the findings of Coetzee *et al*. (2003), who showed that these two species are phylogenetically closely related based on their ITS sequence data. Also present in this cluster was an isolate of an unnamed species from New Zealand. The fact that this isolate grouped with isolates representing *A. luteobubalina* indicated that it might be related to *A. luteobubalina*. Coetzee *et al*. (2003) showed that this isolate has ITS sequences that are not identical, but that it is phylogenetically related to *A. luteobubalina*. This unnamed isolate also has distinct EF 1-α sequences (Maphosa *et al*., 2006) providing

further evidence that it represents a previously undescibed species.

Armillaria novae-zelandiae isolates from Chile, New Zealand and Australia grouped together regardless of geographic origin. This result is consistent with the report of Kile and Watling (1983) who showed that *A. novae-zelandiae* from Australia and New Zealand are sexually compatible and belong to the same biological species. Using ITS sequence data, Coetzee *et al*. (2001) showed that these isolates grouped in a single clade further confirming that they represent a single species.

Armillaria mellea isolates had differing isozyme banding patterns and formed four sub-clusters corresponding to their biogeographic distributions. Thus, isolates from Japan, Europe, western and eastern North America formed separate sub-clusters, which are consistent with those emerging from DNA sequence data. The separation of *A. mellea* isolates according to their geographic origin is supported by a number of studies. Anderson *et al*. (1989) using RFLP data from the rRNA operon showed that *A. mellea* from Europe and North America have different restriction patterns. Harrington and Wingfield (1995) also showed that North American and European isolates of *A. mellea* have different IGS-1 RFLP profiles after digestion with *Alu*I. Likewise, Coetzee *et al*. (2000) using ITS and IGS-1 data separated *A. mellea* isolates according to geographic origin.

Isolates representing *A. tabescens* had banding patterns different, yet closely related to those of *A. mellea* from western North America. This grouping is consistent with the finding of Coetzee *et al*. (2000) who showed that *A. tabescens* resides in a clade basal to those of *A. mellea*. It has similarly been shown, using DNA re-association data, that *A. tabescens* is very closely related to *A. mellea* (Miller *et al*., 1994). Although these species are clearly different, we have added evidence that *A. tabescens* and *A. mellea* are closely related species.

Armillaria ostoyae had a unique banding pattern. The profile of this species was most closely related to that of *A. borealis* and they clustered closely together on the dendrogram obtained in this study. The IGS-1 RFLP patterns for these two species are very similar (Harrington and Wingfield, 1995) and they have previously been shown to be closely related based on their isozyme profiles (Wahlström *et al*., 1991). Furthermore, Anderson *et al*. (1989) also concluded that these two species are closely related using rDNA operon data and Anderson and Stasovski (1992) showed that the intergenic region sequences for *A. borealis* and *A. ostoyae* are very similar.

Armillaria gemina, *A. nabsnona* and *A. cepistipes* had identical banding profiles and these species clustered together in the dendrogram. They shared most of the bands with *A. gallica*, although the latter species was clearly different. The grouping of *A. gallica*, *A. nabsnona* and *A. cepistipes* but not *A. gemina* concurs with previous studies indicating that these species are phylogenetically closely related (Anderson and Stasovski, 1992; Miller *et al*., 1994; Chillali *et al*., 1998). The grouping of *A. gemina* within this group was unexpected as it has been shown to be more closely related to *A. ostoyae* in IGS-1 DNA sequence and rDNA data (Smith and Anderson, 1989; Anderson and Stasovski 1992), RFLP patterns (Harrington and Wingfield, 1995) and morphology (Bérubé and Dessureault, 1989).

Results of this study have demonstrated that combined pectin lyase, polygalacturonase and pectin methylesterase data can be used to differentiate between many *Armillaria* species. The technique, however, failed to separate some closely related species, reflecting findings from earlier studies based on morphology and DNA sequence data. The relationships among species based on the combined isozyme profiles is in agreement with results from previous phylogenetic studies. Results from this study also support previous suggestions that species from the Northern and Southern hemispheres reside in two distinct groups.

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