

Incidence and phylogenetic analyses of *Armillaria* spp. associated with root disease in peach orchards in the State of Mexico, Mexico

By R. D. Elías-Román^{1,2}, R. A. Guzmán-Plazola³, N. B. Klopfenstein^{4,6}, D. Alvarado-Rosales³, G. Calderón-Zavala¹, J. A. Mora-Aguilera³, M.-S. Kim⁵ and R. García-Espinosa³

¹Colegio de Postgraduados, Campus Montecillo, Recursos Genéticos y Productividad-Fruticultura, Montecillo, Texcoco México;

²Fundación Salvador Sánchez Colín CICTAMEX S.C., Coatepec Harinas, Estado de México, México; ³Colegio de Postgraduados, Campus Montecillo, Fitosanidad-Fitopatología, Montecillo, Texcoco México; ⁴USDA Forest Service – RMRS, Moscow, ID 83843, USA;

⁵Kookmin University, Seoul, Korea; ⁶E-mail: nklopfenstein@fs.fed.us (for correspondence)

Summary

Incidence of peach [*Prunus persica* (L.) Batsch] tree mortality attributed to *Armillaria* root disease was assessed from 2009 to 2011 in 15 orchards in the State of Mexico, Mexico. Incidence increased gradually every year of assessment, reaching average values of 9.7, 15.3 and 20.3% tree mortality and 23.2, 24.7 and 28.3% disease-impacted area of the orchards during 2009, 2010 and 2011, respectively. The cultivars 'Nemaguard' and 'Criollo de La Goleta', a local rootstock used in the region, were both susceptible to the disease. To identify species of *Armillaria* isolated from infected peach trees, two nuclear rDNA regions (partial 5.8S-ITS2-LSU D-domains and partial 3' LSU-IGS1) and the translation elongation factor-1 α (*tef-1 α*) gene were sequenced and compared with sequences of known *Armillaria* species. DNA sequence analysis from 49 *Armillaria* isolates revealed that five isolates (10.2%) were *Armillaria mellea* and eight isolates (16.3%) were *Armillaria gallica*. DNA sequences from the remaining 36 isolates (73.5%) showed no close similarity to *Armillaria* sequences in GenBank, and apparently represent an undescribed *Armillaria* species. This undescribed species was the most widely distributed in the region of study. Separate phylogenetic analyses of the LSU region (D1–D3 domains concatenated with the partial 3' end) and the *tef-1 α* region show that the undescribed species is quite distinct from other *Armillaria* spp. reported in North America.

1 Introduction

In Mexico, peach [*Prunus persica* (L.) Batsch] is cultivated on approximately 45 000 ha, primarily in the State of Mexico, Michoacán, Morelos, and Puebla that together account for 65% of the country's domestic peach production (SIAP 2010). Peach orchards in these states have been established predominately in deforested areas. In the State of Mexico, disease has resulted in dead or dying trees within many peach orchards, with initial disease symptoms displayed as chlorotic leaves, trunk gummosis and patches of tree mortality. Disease incidence, disease dispersal patterns or potential causal agents have not been previously reported. Similar symptoms have been reported in association with root disease caused by different species of *Armillaria* on kiwi (*Actinidia deliciosa*), pear (*Pyrus communis* and *P. betulaeifolia*), peach, grape (*Vitis vinifera*) and cranberry (*Vaccinium corymbosum*) grown in diverse global regions, such as New Zealand, USA and Italy (Homer 1992; Rizzo et al. 1998; Cox et al. 2005; Pertot et al. 2008; Prodorutti et al. 2009). In peach orchards in Georgia, USA, *Armillaria* root disease is caused primarily by *Armillaria tabescens* and to a lesser degree by *A. mellea* (Schnabel et al. 2005). In Michigan, mortality of cherry (*Prunus cerasus*) and peach trees was associated with infection by *Armillaria solidipes* (reported as *Armillaria ostoyae*) and to a lesser degree with *A. mellea* (Proffer et al. 1987). These *Armillaria* species have been reported in forest trees in several regions of Mexico. In Chihuahua, Mexico, the presence of *A. solidipes* (reported as *A. ostoyae*) was detected and associated with mortality of Douglas-fir (*Pseudotsuga menziesii*) and Durango fir (*Abies durangensis*) (Shaw 1989). In central Mexico, particularly in the eastern State of Mexico and northeastern Morelos, *A. gallica* was identified on pines (*Pinus* spp.) and oaks (*Quercus* spp.), and *A. cepistipes* was detected on *Alnus* sp., whereas *A. mellea* has been found in pine-oak forests in northwestern Veracruz (Alvarado-Rosales and Blanchette 1994; Alvarado-Rosales 2007). Also, *A. tabescens* was previously found on the exotic monkey puzzle tree (*Araucaria araucana*) in Veracruz (Kim et al. 2010).

In a previous study based on the biological species concept, identification of *Armillaria* spp. from forests of central Mexico showed that some *Armillaria* isolates from Puebla State were incompatible with basidiospore-derived cultures of *A. mellea*, *A. tabescens*, *A. solidipes*, *A. gallica* or *Armillaria cepistipes* (Alvarado-Rosales and Blanchette 1994; Alvarado-Rosales 2007). These incompatibility tests raised the possibility that at least one *Armillaria* species found in Mexico is different from previously reported species. Although *A. gallica* has been previously associated with mortality of plum (*Prunus salicina*) grown on peach rootstock in the State of Morelos (Alvarado-Rosales 2007), the distribution and identification of *Armillaria* species are largely undetermined for fruit orchards in Mexico.

Because pathogenicity varies widely among different *Armillaria* species, it is critical to precisely identify which *Armillaria* species is the causal agent so that effective management strategies can be implemented (Ross-Davis et al. 2012). However, *Armillaria* spp. are most commonly found in a vegetative state and basidiomata are formed only sporadically. Even if they are found, the basidiomata of closely related species can be difficult to distinguish based on morphology (Kim et al. 2006; Brazee et al. 2011; Ross-Davis et al. 2012). Molecular techniques that rely on different rDNA regions, such as the internal transcribed spacer (ITS), intergenic spacer (IGS) and nuclear large subunit (LSU) of ribosomal DNA (rDNA)

(Anderson and Stasovski 1992; Kim et al. 2006), or the translation elongation factor 1 alpha (*tef-1 α*) gene (Brazee et al. 2011; Ross-Davis et al. 2012), have demonstrated utility to validate identification of *Armillaria* species from North America.

Towards an overall goal of developing a management strategy for *Armillaria* root disease in peach orchards of Mexico, the first objective of this study was to determine the incidence of mortality in peach trees within 15 orchards in the State of Mexico during 2009–2011. The second objective was to identify or characterize *Armillaria* spp. associated with disease in the peach orchards by obtaining partial sequences of nuclear rDNA regions (e.g. partial 5.8S-ITS2-LSU D1–D3 domains; and partial 3' LSU-IGS1) and the *tef-1 α* gene from representative *Armillaria* isolates for comparisons with *Armillaria* sequences deposited in GenBank and phylogenetic analyses.

2 Materials and methods

2.1 Study area

Peach orchards in Coatepec Harinas, Temascaltepec and Almoloya de Alquisiras, which represent the primary peach-producing regions of the State of Mexico, served as the study sites (Fig. 1). Nineteen orchards (0.2–1.7 ha) were included in the surveys, and the year of plantation establishment and rootstock type were recorded (Table 1).

2.2 Surveys, sampling and isolation of *Armillaria* within peach orchards

To survey peach trees for *Armillaria* root disease, the basal trunk and main lateral roots of at least 50% of the trees within patches of dead and symptomatic trees were inspected for signs of *Armillaria* (basidiomata, rhizomorphs and fan-shaped mycelial colonization). The fungus was isolated from infected roots and rhizomorphs following the procedure described by Harrington et al. (1992) in a Benomyl-Dichloran-Streptomycin (BDS; Worrall 1991) culture medium, and incubated for 3–5 weeks at 20–26°C.

2.3 Disease incidence

During June and July 2009, 2010 and 2011, surveys were conducted to assess the percentage of dead and symptomatic trees in each orchard. Disease severity was measured with a severity scale similar to that reported by Amiri and Schnabel

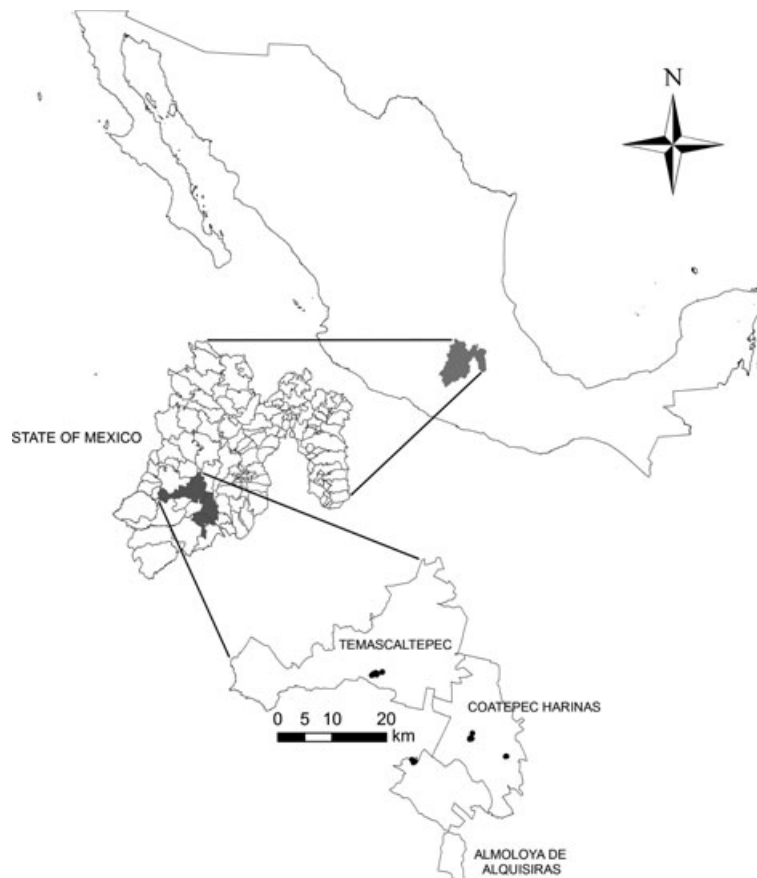


Fig. 1. Map showing the locations of *Armillaria* collection sites within the State of Mexico, Mexico.

Table 1. Accumulated percentage of mortality and accumulated percentage of disease-impacted area in 15 commercial peach orchards with *Armillaria* root disease observed from 2009 to 2011 across three municipalities of the State of Mexico, Mexico.

Orchard #	Year of plantation established	Rootstock	Accumulated mortality (%)			Accumulated disease-impacted area (%)		
			2009 ¹	2010	2011	2009 ¹	2010	2011
1	2007	'Criollo'	0.8	1.5	3.1	3.2	5.1	8.1
2	2006	'Criollo'	NA	2.4	3.8	NA	5.5	8.9
3	2001	'Nemaguard'	NA	3.4	3.9	NA	6.5	6.9
4	1997	'Nemaguard'	2.6	3.4	6.6	5.4	12.9	17
5	2006	'Criollo'	NA	5.8	8	NA	8.8	10.2
6	2001	'Nemaguard'	NA	7.2	8.7	NA	11.3	12.4
7	2000	'Nemaguard'	NA	10.6	16.7	NA	16.6	23
8	1991	'Criollo'	11.5	13.7	23.1	17.7	20.3	26.8
9	2002	'Nemaguard'	NA	14.6	18	NA	29.1	29.5
10	2002	'Criollo'	11.1	15.7	23.7	33.2	33.8	38.4
11	1999	'Criollo'	9.2	17.1	21.3	31.2	31.2	32.8
12	1996	'Criollo'	NA	23.9	30.8	NA	33.9	39.5
13	1999	'Nemaguard'	23.3	26.7	38.6	48.7	50	54.3
14	2003	'Nemaguard'	NA	31.3	39.9	NA	44.8	51.3
15	2001	'Criollo'	NA	52	59.2	NA	60.5	65.7

¹Only orchards # 1, 4, 8, 10, 11, and 13 were assessed in 2009.

(2012) with modified categories 1 to 3: 1 = healthy tree; 2 = symptomatic tree with chlorotic foliage and gummosis at the trunk base; and 3 = dead tree. The percentage of disease-impacted area was estimated for each orchard using the number and location [(UTM, WGS-84, 14N zone, obtained with GPS eTrex[®], Garmin International Inc., Olathe, KS, USA)] of trees affected during 2009, 2010 and 2011. The location of trees in each orchard was coded in a point-type shapefile, with the severity attributes associated with each tree, using ArcGIS (ver. 9.3; Environmental Systems, Research Institute, Inc., Redlands, CA, USA). In the vector layer generated, a buffer area was drawn around the points corresponding to each tree or set of diseased trees to estimate the disease-impacted area. Buffer width was defined as the average distance between plants in each orchard. Once the buffers were generated, the proportion of disease-impacted area was calculated for each orchard. In 2009, mortality and disease-impacted area were recorded for six orchards (#1, 4, 8, 10, 11 and 13), but in 2010–2011, data from nine additional orchards (#2, 3, 5, 6, 7, 9, 12, 14 and 15) were also recorded for both variables, giving a total of 15 orchards evaluated of 19.

2.4 DNA amplification and sequencing

In preparation for DNA extraction, *Armillaria* isolates were subcultured to sterile, 0.2-µm pore, nylon filters (Millipore Corp., Billerica, MA, USA) overlaying 3% malt-agar medium (3% malt extract, 3% dextrose, 1.0% peptone and 1.5% agar) in Petri dishes and maintained at ca. 22°C in the dark for 2 weeks. Mycelia were scraped from the filter and ground in extraction buffer using a FastPrep[®] FP120 cell disruptor (Qbiogene, Carlsbad, CA, USA), and DNA was purified using DNeasy[®] Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. DNA quantity and quality were verified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Polymerase chain reaction (PCR) mixtures contained ca. 100 ng template DNA, 2.5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA), PCR buffer, 4 mM MgCl₂, 200 µM dNTPs and 0.5 µM of each primer in a total volume of 50 µl.

Amplification of the ITS1-5.8S-ITS2 (hereafter called ITS), partial 5.8S-ITS2-LSU D-domains and partial 3' LSU-IGS1 regions followed the protocol of Kim et al. (2006). For the ITS region, primers ITS-1F and ITS-4 were used (White et al. 1990; Gardes and Bruns 1993) with PCR conditions of 94°C for 2 min 30 s, 35 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min 30 s, and finally 72°C for 10 min. Amplification of the partial 5.8S-ITS2-LSU D-domains used 5.8SR and LR7 primers (Moncalvo et al. 2000) with PCR conditions of 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 2 min, and finally 72°C for 5 min. For the partial 3' LSU-IGS1 region, primers LR12R and O-1 (Veldman et al. 1981; Duchesne and Anderson 1990) were used with PCR conditions of 95°C for 1 min 30 s, 35 cycles of 95°C for 1 min 30 s, 60°C for 40 s and 72°C for 2 min, and finally 72°C for 10 min. Amplification of *tef-1α* followed the protocol of Ross-Davis et al. (2012), except that primers were replaced by ARMEF-F3A (5' CGT GAY TTY ATC AAG AAC ATG 3') and ARMEF-R (5' TAC CCG TTC GGC GAT CAA TCT 3') designed by J.W. Hanna (USDA Forest Service, RMRS) with PCR conditions of 94°C for 2 min; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min and 30 s; then 72°C for 7 min.

The sequences were determined in two directions with a Prism[®] 3700 DNA Sequencer at the Biotechnology Center of the University of Wisconsin (Madison, WI, USA). The same primers used for initial amplification of ITS, partial 3' LSU-IGS1 regions, and *tef-1α* were used for sequencing, whereas the LR0R, LR15 and LR5 primers (Moncalvo et al. 2000) were used for sequencing of the partial 5.8S-ITS2-LSU D-domains.

2.5 DNA sequence comparisons and phylogenetic analyses

DNA sequences were edited and aligned manually with BioEdit (ver. 7.1.3; Ibis Biosciences, Inc., Carlsbad, CA, USA; <http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Polymorphic sites were coded using the IUPAC codes for ambiguous nucleotides. The partial 5.8S-ITS2-LSU D-domains, partial 3' LSU-IGS1 and *tef-1α* sequences from *Armillaria* spp. isolates obtained from infected peach trees in the State of Mexico were compared with those available in the database of the National Center for Biotechnology Information (NCBI) using a nucleotide search with BLAST® (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

For phylogenetic analyses, sequences of the LSU region (D1–D3 domains concatenated with the partial 3' end) and *tef-1α* were analyzed with the Maximum Parsimony method in the software Phylogenetic Analysis Using Parsimony, PAUP* (ver. 4.0b10; Sinauer Associates, Inc., Publishers, Sunderland, MA, USA) and by Bayesian inference with the software MRBAYES (ver. 3.1.2; Huelsenbeck and Ronquist 2007). Both analyses incorporated the events of nucleotide insertion or loss (gaps) using the simple codification method proposed by Simmons and Ochoterena (2000), implemented in the software SeqSTATE (ver. 1.4.1; Müller 2005).

The Maximum Parsimony analyses for LSU and *tef-1α* regions were performed with the following adjustments: heuristic search, MaxTrees (number of trees stored) with automatic increments. The bootstrap method was applied also with heuristic search and 1000 replications with the tree-bisection-reconnection (TBR) procedure. The starting trees were obtained using stepwise with the random addition of 10 sequences.

For the Bayesian analyses of the LSU and *tef-1α* regions, the best nucleotide substitution model was chosen based on the AIC (Akaike Information Criterion) obtained with the software JMODELTEST (ver. 0.1.1; Posada 2008). For LSU, the JmodelTest selected the model GTR+G (nst = 6 rates = gamma; Rodriguez et al. 1990), and for *tef-1α*, the model GTR+I+G (nst = 6; Rodriguez et al. 1990). The simple-substitution, F81-like model (nst = 1; Felsenstein 1981) was assigned to the binary characters that represent losses/insertions of nucleotides in each alignment of both analyses (LSU and *tef-1α*).

For both LSU and *tef-1α* regions, four chains with 3×10^6 generations were analyzed and two files were generated with 30 001 trees. The first 7500 trees of each file were discarded with the 'burnin' option and the rest (45 002) were analyzed in PAUP* (Swofford 2002) to make the 75% majority rule consensus tree for LSU and *tef-1α*. The aforementioned phylogenetic analyses were also attempted for the ITS and IGS1 regions, but could not be completed because of ambiguous alignments (see Results and Discussion).

For the phylogenetic analyses, the sequences of various regions (LSU, IGS1 and *tef-1α*) from well-characterized isolates of 10 North American *Armillaria* spp. (Kim et al. 2006; Ross-Davis et al. 2012) were used for comparison with isolate sequences from the State of Mexico. *Flammulina velutipes* (GenBank accession number HQ660197) was used as the outgroup for the LSU analysis, and *Pleurotus ostreatus* (AY883432) was used as the outgroup for *tef-1α* analysis, based on coverage and similarity to *A. tabescens* sequences.

The edited 5.8S-ITS2-LSU D-domains, partial 3' LSU-IGS1 and *tef-1α* DNA sequences data have been deposited in GenBank (Table 2). And, LSU (D1–D3 domains concatenated with the partial 3' end) and *tef-1α* DNA sequences data used to generate the 50% majority-rule bootstrap consensus have been deposited into TREEBASE (www.treebase.org; study accession number 13552).

Table 2. Representative isolates of *Armillaria* obtained from peach orchards in the State of Mexico, Mexico.

Species	Isolate	GenBank accession no.		
		5.8S-ITS2-LSU D domain ¹	Partial LSU -IGS1 ²	<i>tef-1α</i> ³
<i>Armillaria mellea</i>	MEX72	JX281806	JX281796	–
	MEX74	JX281807	JX281797	KC111011
	MEX100	JX281808	JX281798	KC111012
<i>Armillaria gallica</i>	MEX55	JX281809	JX281799	KC111014
	MEX61	KC111010	KC111006	KC111015
	MEX77	JX281810	JX281800	–
<i>Armillaria</i> sp.	MEX90	JX281811	JX281801	KC111013
	MEX40	KC111007	KC111002	KC111016
	MEX43	JX281813	JX281802	–
	MEX46	JX281812	JX281803, JX281804	–
	MEX47	KC111008	KC111003, KC111004	KC111017
	MEX57	JX281814	JX281805	–
	MEX60	KC111009	KC111005	KC111018

¹5.8S partial-internal transcribed spacer 2 (ITS2)- large subunit (LSU) D-domains.

²Partial 3' LSU- intergenic spacer 1 (IGS1).

³Translation elongation factor-1α.

3 Results

3.1 Detection of *Armillaria* root disease in peach trees and collection of *Armillaria* isolates

In 15 (78.9%) of the 19 peach orchards assessed, symptoms and signs typical of *Armillaria* root disease were observed, and 49 isolates were obtained. Results from the 15 orchards with *Armillaria* root disease are presented (Table 1).

3.2 Disease incidence (%)

Disease incidence was associated with *Armillaria* root disease in almost all of the trees assessed. Disease incidence increased yearly during 2009–2011, and reached average values of 20.3% mortality and 28.3% impacted area (Fig. 2). In every orchard surveyed, mortality increased gradually during the assessment period (Fig. 3).

Accumulated mortality varied widely in each year of assessment (Table 1). In 2011, accumulated mortality in the 15 orchards ranged from 3.1 to 59.2% (Table 1; Fig. 3). Orchards # 1, 2 and 5, with trees <5 years old had lower mortality than orchards with trees older than 8 years (denominated orchards #7–15). In 2011, accumulated mortality in orchards with 4- to 5-year-old peach trees was 5.0% on average, whereas nine orchards with peach trees older than 7 years had an average mortality of 30.1%. However, the trend in tree age effect on mortality was variable. For example, orchards #3, 4 and 6, which had trees more than 10 years old, had average mortality rates of 6.4% in 2011, similar to 5.0% mortality in orchards with 4- to 5-year-old trees.

Armillaria root disease affected trees established on both 'Nemaguard' and the generic, local 'Criollo' peach rootstock, which represent the only rootstocks used in the State of Mexico. During 2009, 2010 and 2011, orchards using 'Nemaguard' rootstock reached average values of 12.9, 13.9 and 18.9% mortality, respectively, and orchards using local 'Criollo' rootstock

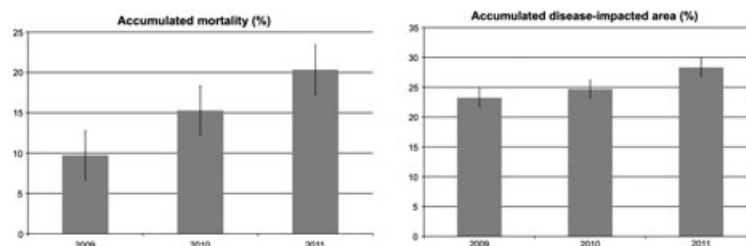


Fig. 2. Accumulated average mortality and disease-impacted area (%) in 15 commercial peach orchards with *Armillaria* root disease across three municipalities of the State of Mexico, Mexico ($\pm$ typical error). Please note: only orchards # 1, 4, 8, 10, 11, and 13 were assessed in 2009.

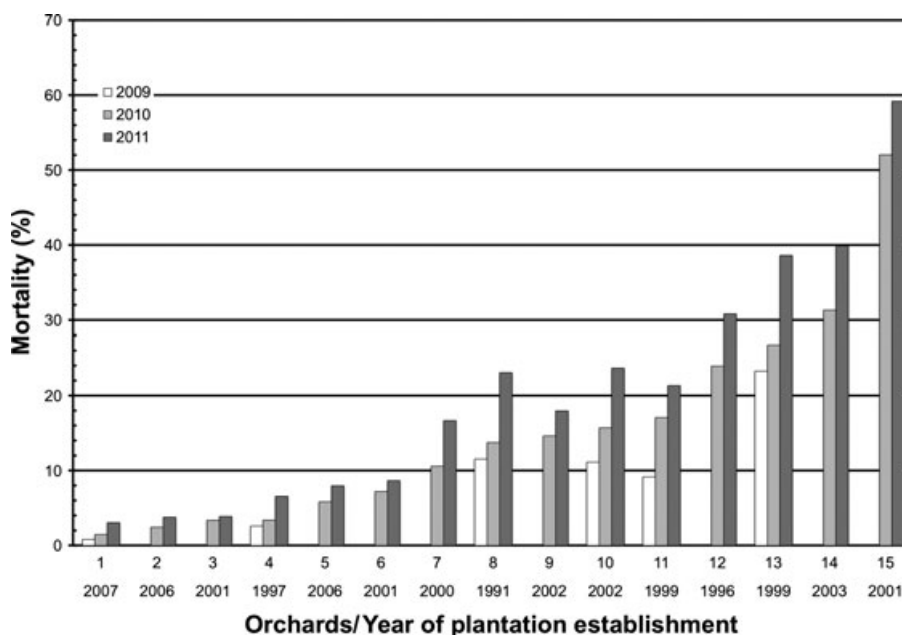


Fig. 3. Accumulated percentage of mortality observed from 2009 to 2011 in 15 commercial peach orchards across three municipalities of the State of Mexico, Mexico. Please note: only orchards # 1, 4, 8, 10, 11, and 13 were assessed in 2009.

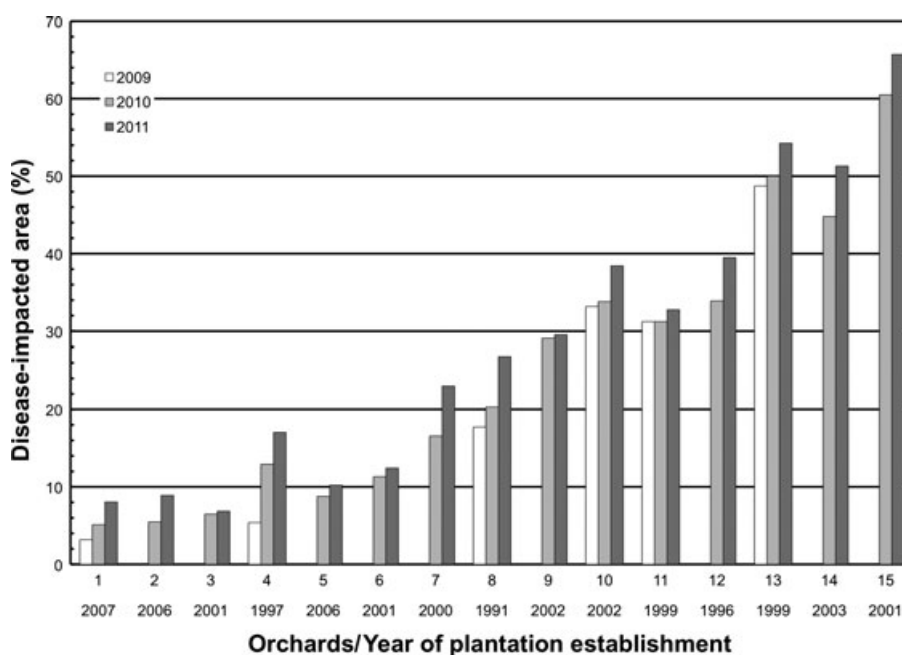


Fig. 4. Accumulated percentage of disease-impacted area observed from 2009 to 2011 in 15 commercial peach orchards across three municipalities of the State of Mexico, Mexico. Please note: only orchards # 1, 4, 8, 10, 11, and 13 were assessed in 2009.

averaged 8.1, 16.5 and 21.6% mortality, respectively (Table 1). For the period 2009–2011, mortality reached average values of 15.4% and 15.2% in orchards using ‘Criollo’ and ‘Nemaguard’ rootstocks, respectively.

Armillaria root disease occurred in patches of trees located in the interior or on the edges of the orchards. In each assessment period, the area of Armillaria root disease patches expanded as additional dead and symptomatic trees appeared on the periphery of those recorded in previous years (data not shown). In 2011, accumulated disease-impacted area in the 15 orchards ranged from 6.9 to 65.7% (Table 1; Fig. 4).

3.3 DNA sequence analyses

Based on the alignment of 49 partial 3' LSU-IGS1 sequences, three distinct groups were obtained (Table 2). The first group (Group 1) comprised five *Armillaria* isolates (10.2%) from two orchards (#10 and 11) in Coatepec Harinas. Partial 3' LSU-IGS1 sequences from Group 1 isolates (represented by MEX72, MEX74 and MEX100) were 98–100% similar to each other and had 97% similarity with *A. mellea* (accession number AY509188; based on 95% coverage) from USA. The second group (Group 2) comprised eight isolates (16.3%) collected from three orchards (#9, 12 and 14) located in three municipalities. Partial 3' LSU-IGS1 sequences from Group 2 isolates (represented by MEX55, MEX61, MEX77 and MEX90) were 99–100% similar to each other and had 99% similarity with sequences of *A. gallica* (AY509173), *Armillaria sinapina* (AY509168) and *A. calvescens* (AY509163) based on 98% coverage. The third group (Group 3) comprised 36 isolates (73.5%) collected from ten orchards (# 1–8, 13 and 15) in three municipalities. Partial 3' LSU-IGS1 sequences from Group 3 isolates (represented by MEX40, MEX43, MEX46, MEX47, MEX57 and MEX60) were 99–100% identical to each other. Based on 32% coverage that included partial 3' LSU, Group 3 isolate sequences showed 94–95% similarity to *Armillaria* sp. (AB510832), *A. cepistipes* (AB510825) and *A. gallica* (JN657431). However, IGS1 sequences (excluding partial 3' LSU) of Group 3 isolates did not have >5% similarity with any sequences on GenBank (November 2012).

The ITS region (ITS1-5.8S-ITS2) sequences of representative isolates of Group 3 isolates were complex due to intragenomic sequence heterogeneity. For example, the ITS sequences from this group possessed multiple insertions of variable size. Because sequences of this region were often ambiguous (data not shown), the ITS sequences were not useful for similarity comparisons.

Comparisons of partial 5.8S-ITS2-LSU D-domain sequences from representative *Armillaria* isolates also revealed the same three groups that were defined by partial 3' LSU-IGS1 sequences. Group 1 was defined with sequences from four isolates (represented by the isolates MEX72, MEX74 and MEX100). Based on 100% coverage, Group 1 sequences had 98% similarity with *A. mellea* (AY213584). Group 2 was defined with four isolates (represented by the isolates MEX55, MEX61, MEX77 and MEX90). Based on 100% coverage, Group 2 sequences were 99% similar to *A. gallica* (AY213569), *A. calvescens* (AY213559) and *A. sinapina* (AY213563). Group 3 was defined with 13 isolates (represented by MEX40, MEX43, MEX46, MEX47, MEX57 and MEX60). Group 3 sequences for 5.8S-ITS2-LSU did not possess high similarity with any sequences on GenBank, and the closest species-level matches for Group 3 isolates were *A. mellea* (AY213584, AY213586, AY213587) from USA and Mexico (MEX72, MEX74 and MEX100) with only 93% similarity based on 80–86% coverage (Table 2).

For analysis of *tef-1 α* sequences, (i) Group 1 isolates (represented by MEX74 and MEX100) had 99% *tef-1 α* similarity and 100% coverage with *A. mellea* (JF313137); (ii) Group 2 isolates (represented by MEX55, MEX61 and MEX90) were identified as *A. gallica* based on 99% *tef-1 α* similarity and 100% coverage with *A. gallica* (FJ313125); and (iii) Group 3 isolates (represented by MEX40, MEX47 and MEX60) had no close *tef-1 α* matches in GenBank, but showed 91% *tef-1 α* similarity and 100% coverage with *A. mellea* (JF313137).

Thus, GenBank Blasts based on partial 3' LSU-IGS1, 5.8S-ITS2-LSU D-domain and *tef-1 α* sequences of the peach orchard isolates identified the same three groups of *Armillaria* species: Group 1 was a close match with *A. mellea*, Group 2 was a close match with *A. gallica* and Group 3 showed no close matches on GenBank.

3.4 Phylogenetic analyses

DNA sequences of ITS2 and IGS1 (excluding partial 3' LSU region) from *A. mellea*, *A. tabescens* and isolates MEX40, MEX43, MEX46, MEX47, MEX 57 and MEX60 (*Armillaria* sp.) could not be aligned unambiguously with each other nor with other species; therefore, these species were excluded from IGS1 analysis. However, a phylogenetic analysis using Maximum Parsimony (50% majority-rule consensus) was performed based on the IGS1 region of closely related *Armillaria* species. In this analysis, the Group 2 isolates from the State of Mexico clustered within a clade containing *A. gallica* and related species (100%, 100%) with high support values. This clade contained three subclades, one of which (93%, 100%) contained Group 2 *Armillaria* isolates (MEX55, MEX61, MEX77 and MEX90) and one isolate of *A. gallica* (ST23). The second subclade was separated into one *A. sinapina* isolate (ST12) with 55% bootstrap support and another group (75%, 100%) that included *A. calvescens* isolates (ST3, ST17) and an *A. gallica* isolate (ST22). The third subclade (68%, 97%) included only *Armillaria altimontana* isolates (=NABSX, Brazee et al. 2012). Isolates of *A. gallica* (M70), *A. sinapina* (ST13, M50), *A. cepistipes* (M110, S20, W113) and *A. calvescens* (ST18) were not separated within the *A. gallica* clade (data not shown).

Forty LSU sequences (D1–D3 domains concatenated with the partial 3' end) were analyzed, each with 1094 traits, 49 variable sites and 85 informative sites for the parsimony analysis. The heuristic search resulted in 600 trees with a length of 180 steps with consistency indexes (CI) of 0.906 and retention indexes of 0.952. In the 50% majority rule consensus tree resulting from the parsimony analysis in which *F. velutipes* was used as the outgroup (Fig. 5), Bayesian analysis posterior probability values obtained from the 75% majority rule consensus tree are reported. The parsimony analysis grouped all of the *Armillaria* species into three main clades (Fig. 5): (i) One clade (100% bootstrap support and 100% posterior probability) contained only the Group 3 isolates (MEX40, MEX47 and MEX60), which corresponded to the species found most frequently in the peach orchards of the State of Mexico (Fig. 5). Group 3 isolates could not be identified based on LSU sequences, which indicates that isolates within this well-defined clade represent an undescribed *Armillaria* species. (ii) A second clade (100%, 100%) comprised isolates of *A. mellea* from the USA with a subclade (96%, 86%) containing Group 1 isolates (MEX72, MEX74 and MEX100) from the State of Mexico. (iii) A third clade contained two subclades, one subclade (94%, 99%) comprised *A. tabescens* and the other subclade (77% bootstrap support) comprised eight other *Armillaria* species from North America (Fig. 5). This larger clade contained Group 2 *Armillaria* isolates (MEX55, MEX61 and MEX90) from peach trees, and these isolates were not separated from *A. altimontana* (NABSX), *A. cepistipes*, *A. calvescens*, *A. sinapina* and isolates of *A. gallica* from the USA and Canada. However, this larger clade contained two subclades. One subclade (59% bootstrap support) contained the three *Armillaria nabsnona* isolates and the other subclade (60%, 92%) contained *A. solidipes* and *Armillaria gemina* (Fig. 5).

The *tef-1 α* data comprised 39 sequences with 919 traits, of which 97 sites were variable and 217 were informative for the parsimony analysis. The heuristic search resulted in 900 trees with a length of 632 steps, a CI of 0.815 and RI of 0.887. In the 50% bootstrap consensus tree resulting from the parsimony analysis (Fig. 6), Bayesian analysis posterior probability values are reported (derived from the 75% majority rule consensus tree). The parsimony analysis clustered the *Armillaria* species into two primary clades. The first primary clade (100%, 100%) contained only *A. tabescens* and the second primary clade (95% bootstrap support) comprised 10 *Armillaria* species divided into two major subclades. The first of these major subclades contained Group 3 (MEX40, MEX47 and MEX60) unidentified *Armillaria* sp. (100%, 100%) and *A. mellea* isolates (100%, 94%), which were well separated into two different subclades. The *A. mellea* subclade contained at least two groups, with the Group 1 isolates (MEX74 and MEX100) separated into a distinct group. The second major subclade (82%, 79%) comprised *A. solidipes*, *A. gemina*, *A. sinapina*, *A. cepistipes*, *A. nabsnona*, *A. altimontana* (NABSX), *A. calvescens* and *A. gallica* (Fig. 6). The isolates representing each species were well separated within subclades, with the exception of a single *A. gallica* isolate (M70) derived from Canada. Within this second subclade, *A. solidipes* and *A. gemina* were clustered as sister subclades (99%, 99%), and *A. sinapina* formed an independent subclade (100%, 100%). Another subclade (63%, 89%) within this major subclade comprised well-separated minor subclades representing *A. cepistipes*, *A. nabsnona*, *A. altimontana* (NABSX), *A. calvescens* and the single *A. gallica* isolate (M70) as a separate entity. An ancillary subclade (97%, 95%) contained the remaining *A. gallica* isolates, with the Group 2 isolates (MEX55, MEX61 and MEX90) clustered together as a subgroup of the *A. gallica* subclade (98%, 100%). Thus, *tef-1 α* -based phylogenetic analysis was very effective at separating *Armillaria* species, and indicates that Group 3 *Armillaria* isolates from peach orchards represents an undescribed species.

4 Discussion

The annual increase in peach tree mortality and area associated with *Armillaria*-infected trees in most of the surveyed orchards strongly indicates that *Armillaria* spp. are the principal pathogenic agents responsible for peach tree mortality in this region of Mexico. The undescribed *Armillaria* sp. isolates (Group 3) were recovered from 75% of the diseased trees

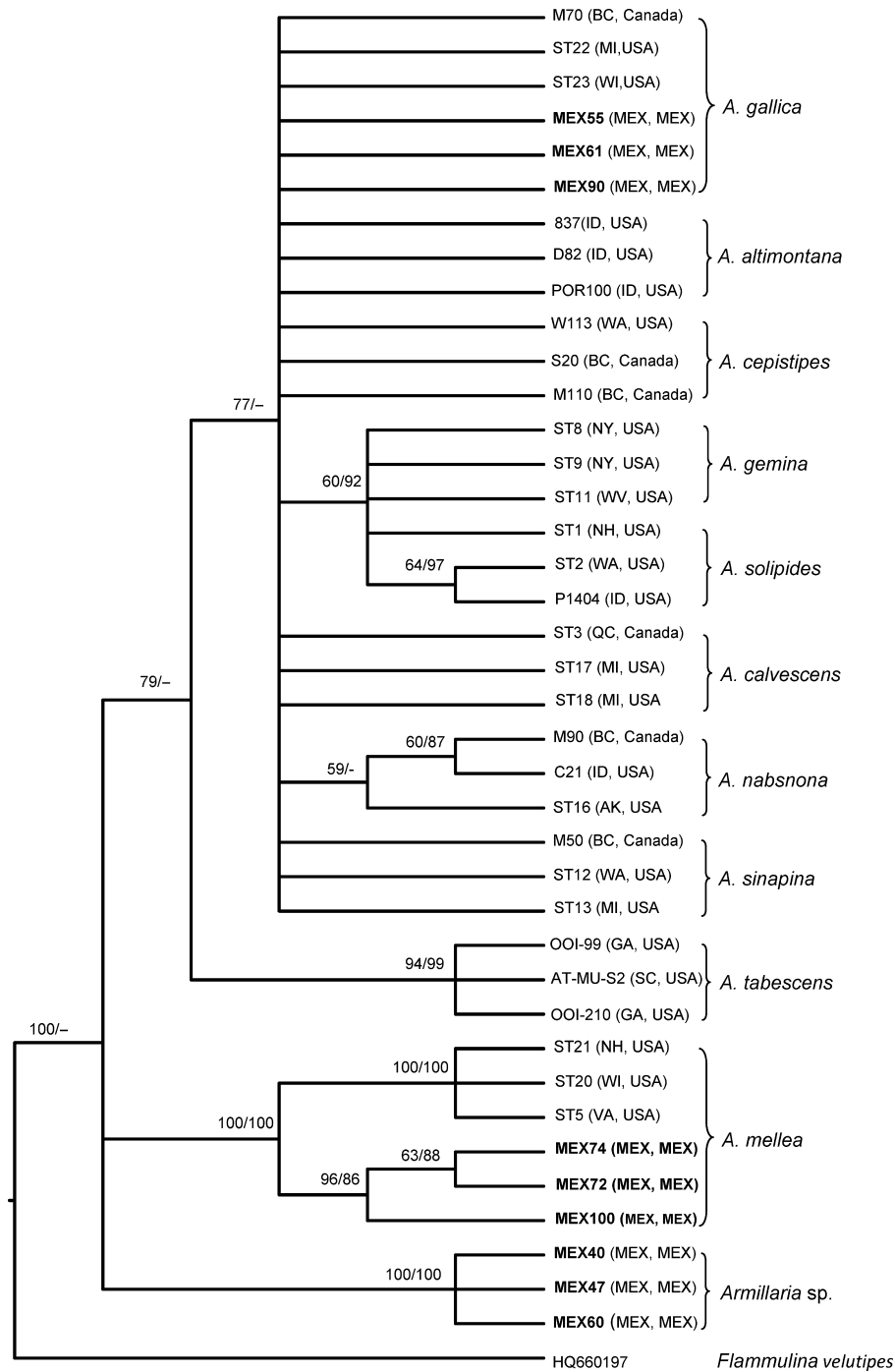


Fig. 5. 50% majority-rule bootstrap consensus tree generated from nuclear large subunit (LSU: D-domain concatenated with the partial 3' end) sequences. The values of bootstrap support and posterior probability are shown above the nodes. GenBank information of Mexican *Armillaria* (MEX) is described in Table 2. GenBank and isolate information of North American *Armillaria* species used in this analysis are described in Kim et al. (2006).

sampled, and thus, it is considered the main cause of mortality in these orchards in the State of Mexico. The broad variation in mortality rates could be attributed to differences in pathogenicity among the *Armillaria* species that were isolated in this study. Significant variation in pathogenicity among *Armillaria* species has been documented on *Prunus* spp. (Cox et al. 2005). Other factors, however, could have also contributed to peach tree mortality in this study. For example, variation in mortality caused by *Armillaria* root disease could also be related to inoculum density (Lung-Escarment and Guyon 2004).

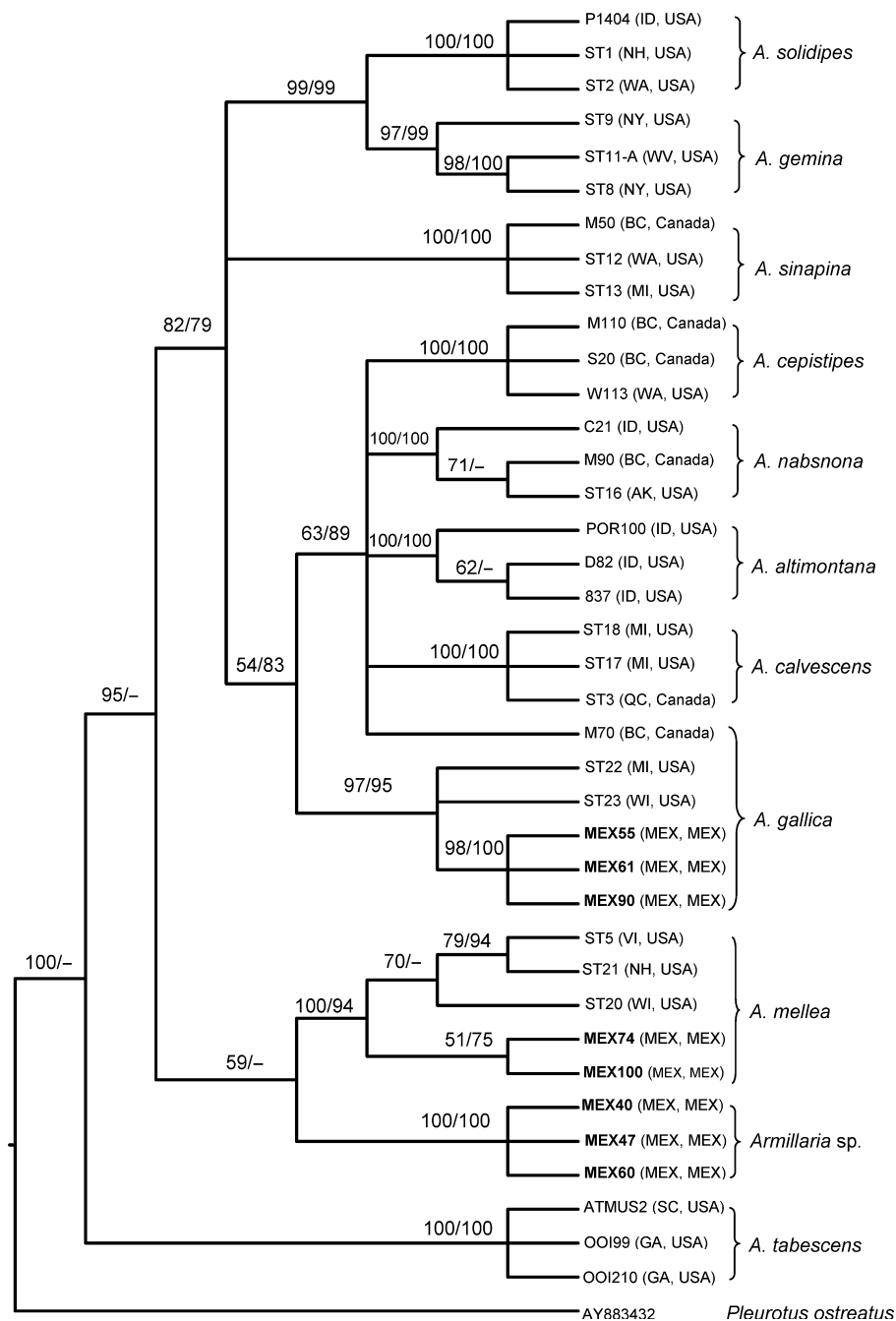


Fig. 6. 50% majority-rule bootstrap consensus tree generated from translation elongation factor-1 α (*tef-1 α*) sequences. The values of bootstrap support and posterior probability are shown above the nodes. GenBank information of Mexican *Armillaria* (MEX) is described in Table 2. GenBank and isolate information of North American *Armillaria* species used in this analysis are described in Ross-Davis et al. (2012).

Variation among rootstocks or peach genotypes for tolerance to *Armillaria* root disease is another consideration. For example, in orchards infested by *A. tabescens*, 'Nemaguard' was among most susceptible rootstocks to *Armillaria* root disease (Beckman et al. 1998). In the orchards that were surveyed in this study, the generic, local 'Criollo' rootstock tended to show average mortality similar to mortality in 'Nemaguard' rootstock. However, these results may have been influenced by variation in spatial patterns of pathogen distribution or inoculum density, and the age of the orchards. Furthermore, mortality levels in orchards using 'Criollo' rootstock may be influenced by the genetic variation associated with seed-derived rootstock.

Mortality increased continually during 2009–2011 in all of the surveyed orchards, and incremental increases in the size of mortality patches or centres of infection were also evident (Table 1; Figs 3 and 4). Annual increases in the size of

Armillaria infection centres are generally attributable to pathogen spread via infected roots and/or rhizomorphs. Thus, spread of *Armillaria* spp. is favoured by the short distances among plants, which is common within peach orchards in this region of Mexico where spacing averages ca. 3.1 m between plants and 4.3 m between rows. An abundance of rhizomorphs was observed in samples from most of the orchards, which suggests a role of these structures in *Armillaria* spread. Previous studies have indicated that *A. mellea* disperses mainly by rhizomorphs in pear orchards (Rizzo et al. 1998). Furthermore, infection centres comprising several trees have been reported in kiwi, peach and cranberry orchards, among others, in New Zealand, USA, Italy and other countries (Homer 1992; Cox et al. 2005; Prodorutti et al. 2009).

The formation of *Armillaria* infection centres and identification of *Armillaria* species associated with disease have not been previously documented in the peach orchards of the State of Mexico. *Armillaria* infection centres, like those observed in peach orchards in the State of Mexico, are not commonly observed in natural forests of Central Mexico (Cannon et al. 2008), which may suggest that *Armillaria* root disease existed, but was not predominant on these formerly forested sites before the establishment of peach orchards. Monocultures of a closely spaced, susceptible species, such as peach (Beckman et al. 1998), likely facilitate the spread of *Armillaria* spp. within the orchards. To maintain productivity in established peach orchards, it is critical to develop and adopt management practices to diminish impacts of *Armillaria* root disease, such as reducing inoculum sources before planting or in established orchards (Onsando et al. 1997; Cox et al. 2005; Prodorutti et al. 2009) and development of resistant rootstock, among other measures. Although management strategies for *Armillaria* root disease in peach orchards have not been implemented in this region of Mexico, this study contributes to the development of appropriate management strategies by characterizing the *Armillaria* root disease pathogens.

DNA sequence analysis of the partial 5.8S-ITS2-LSU D-domain, partial 3' LSU-IGS1 and *tef-1 α* indicate that three *Armillaria* species are present in the surveyed orchards: *A. mellea*, *A. gallica* and an undescribed *Armillaria* species that does not closely match any sequences of *Armillaria* species reported in the GenBank database. ITS sequences had little utility for characterizing *Armillaria* isolates from this study due to intragenomic sequence heterogeneity (data not shown). Such intragenomic variation within ITS sequences of North American *Armillaria* spp. was previously observed by Kim et al. (2006). Because of intragenomic variation within the ITS, this region was not useful for phylogenetic analysis, which concurs with a similar study on *Laetiporus* spp. (Lindner and Banik 2011).

The phylogenetic analyses revealed the presence of a subclade of *A. mellea* formed by the isolates from the State of Mexico. The subgroups of *A. mellea* have been documented with isolates from USA, Europe and Asia (Coetzee et al. 2000; Baumgartner et al. 2010; Brazee et al. 2011), suggesting a process of speciation (Coetzee et al. 2000). Similar results are illustrated in the phylogenetic tree based on *tef-1 α* sequences (Fig. 6) in which the *A. gallica* clade had a subgroup made up of the Group 2 isolates from Mexico (MEX55, MEX61 and MEX90). In this analysis, a reference genotype of *A. gallica* (M70) could not be conclusively identified using the *tef-1 α* sequences; this coincides with the results of Ross-Davis et al. (2012), who considered the possibility that *A. gallica* could contain cryptic genotypes or species. Although the presence of genetically distinct groups of *A. gallica* in North America has not been conclusively demonstrated (Brazee et al. 2011), limited evidence by Kim et al. (2006, 2012) and Ross-Davis et al. (2012) suggest their existence. The *tef-1 α* sequences have been used to identify *A. gallica* isolates, which could not be separated from closely related species on the basis of ITS, LSU and IGS1 sequences (Anderson and Stasovski 1992; Kim et al. 2006; Brazee et al. 2011; Ross-Davis et al. 2012). However, the Group 2 isolates from the State of Mexico that clustered within an *A. gallica* subclade were also similarly separated with high support values (93%, 100%) by a focused phylogenetic analysis of the IGS1 region of closely related species (data not shown). Phylogenetic analyses from this study and other previous studies provide evidence that *A. gallica* comprises multiple species and the *A. gallica* derived from peach orchards in Mexico may represent a distinct cryptic species within the *A. gallica* species complex. Continued studies are needed to better define phylogenetic and taxonomic relationships within the *A. mellea* and *A. gallica* species complexes.

A previous study identified *A. gallica* and *A. mellea* in native forest species of the central region of Mexico (Alvarado-Rosales and Blanchette 1994), located more than 70 km from the study area. This previous study demonstrated that some isolates obtained in the State of Puebla exhibited incompatibility reactions with known North American Biological Species (NABS) of *Armillaria*, which suggests that the isolates are distinct from *Armillaria* species that were previously known to occur in Mexico (*A. solidipes*, *A. cepistipes*, *A. mellea*, *A. gallica* and *A. tabescens*) (Alvarado-Rosales 2007; Kim et al. 2010). Perhaps these incompatible isolates could also belong to the same undescribed species of *Armillaria* (Group 3) that was frequently isolated from peach trees in this study, for which no similar sequences are available in GenBank. Although GenBank does not contain DNA sequences for all known fungal pathogens of peach (Brock et al. 2009), sequences of currently recognized *Armillaria* species in North America are well represented on GenBank. The macro- and micro-morphological characteristics of the undescribed species and accompanying DNA sequence data will provide the basis for a formal description of this species.

An undescribed species of *Armillaria* (whose partial 5.8S-ITS2-LSU D-domain, partial 3' LSU-IGS1 and *tef-1 α* sequences were quite distinct from any of those available in the GenBank) was isolated more frequently from symptomatic and dead trees in peach orchards in southern State of Mexico that had a significant annual rate of mortality, although *A. gallica* and *A. mellea* were occasionally isolated. Because this undescribed *Armillaria* species appears very different from known *Armillaria* species typically associated with root disease in peach orchards in USA and Mexico (Beckman et al. 1998; Cox et al. 2005; Schnabel et al. 2005; Alvarado-Rosales 2007), the development of specific rootstock may be required for resistance to this undescribed species. The genetic analyses of the *Armillaria* isolates obtained from this study contributes additional information on the diversity of this fungal genus in North America and on the usefulness of the LSU, IGS1 and *tef-1 α* regions in separating the unidentified species of *Armillaria*.

Acknowledgements

The first author thanks CONACyT (Consejo nacional de Ciencia y Tecnología) for the scholarship that supported this study. All authors appreciate the support provided by Grupo Produce Estado de Mexico, and the USDA Forest Service Special Technology Development Program, S&PF Forest Health Monitoring, and Western Wildlands Environmental Threat Assessment Center. We also thank Dr. Amy Ross-Davis, John W. Hanna, Lily Zelaya-Molina, Sara Ashiglar and Anna E. Case for technical assistance and reviews of earlier versions of this manuscript.

Dedication

This article is dedicated to the memory of Dr. Roberto García-Espinosa (1944–2012). Roberto was a visionary and an endless generator of great ideas. He lived convinced that chemical management of soil-borne diseases is ecologically impractical. Furthermore, he was a strong proponent of concepts pioneered by J.E. van der Plank and Raul Robinson on horizontal resistance to plant diseases. Dr. García is greatly missed by his family, friends, colleagues and students.

References

- Alvarado-Rosales, D., 2007: Pudrición de raíz por *Armillaria*/*Armillaria* root rot. *Armillaria* (Fr.) Staude. (Agaricales, Marasmiaceae). In: Enfermedades forestales en México/Forest Diseases in Mexico. Ed. by Cibrián-Tovar, D.; Alvarado-Rosales, D.; García-Díaz, S. E. UACH, Chapingo, México: Mundi-Prensa, pp. 211–215.
- Alvarado-Rosales, D.; Blanchette, R. A., 1994: *Armillaria* species from forest of central Mexico. *Phytopathology* **84**, 1106.
- Amiri, A.; Schnabel, G., 2012: Persistence of propiconazole in peach roots and efficacy of trunk infusions for *Armillaria* root rot control. *Int. J. Fruit Sci.* **12**, 437–449.
- Anderson, J. B.; Stasovski, E., 1992: Molecular phylogeny of Northern Hemisphere species of *Armillaria*. *Mycologia* **84**, 506–516.
- Baumgartner, K.; Travadon, R.; Bruhn, J.; Bergemann, S. E., 2010: Contrasting patterns of genetic diversity and population structure of *Armillaria mellea* sensu stricto in the eastern and western United States. *Phytopathology* **100**, 708–718.
- Beckman, T. G.; Okie, W. R.; Nyczepir, A. P.; Pusey, P. L.; Reilly, C. C., 1998: Relative susceptibility of peach and plum germplasm to *Armillaria* root rot. *HortScience* **33**, 1062–1065.
- Brazee, N. J.; Hulvey, J. P.; Wick, R. L., 2011: Evaluation of partial *tef1*, *rpb2*, and *nLSU* sequences for identification of isolates representing *Armillaria calvescens* and *Armillaria gallica* from northeastern North America. *Fungal Biol.* **115**, 741–749.
- Brazee, N. J.; Ortiz-Santana, B.; Banik, M. T.; Lindner, D. L., 2012: *Armillaria altimontana*, a new species from the western interior of North America. *Mycologia* **104**, 1200–1205.
- Brock, P. M.; Döring, H.; Bidartondo, M. I., 2009: How to know unknown fungi: the role of a herbarium. *New Phytol.* **181**, 719–724.
- Cannon, P.; Klopfenstein, N. B.; Kim, M.-S.; Hanna, J. W.; Medel, R.; Alvarado-Rosales, D., 2008: An *Armillaria* survey in Mexico: a basis for determining evolutionary relationships, assessing potentially invasive pathogens, evaluating future impacts of climate change, and developing international collaborations in forest pathology. In: Proceedings of the 55th Western International Forest Disease Work Conference, 2007 October 15–19, Sedona, AZ, USA. Ed. by McWilliams, M.; Palacios, P. Salem, OR, USA: Oregon Department of Forestry, pp. 28–39.
- Coetzee, M. P. A.; Wingfield, B. D.; Harrington, T. C.; Dalevi, D.; Coutinho, T. A.; Wingfield, M. J., 2000: Geographical diversity of *Armillaria mellea* s.s. based on phylogenetic analysis. *Mycologia* **92**, 105–113.
- Cox, K.; Scherm, H.; Beckman, T., 2005: *Armillaria* root and crown rot. In: Southeastern Peach Growers Handbook. Ed. by Horton, D.; Johnson, D. Athens, GA, USA: Cooperative Extension Service, University of Georgia, pp. 162–166.
- Duchesne, L. C.; Anderson, J. B., 1990: Location and direction of transcription of the 5S rRNA gene in *Armillaria*. *Micol. Res.* **94**, 266–269.
- Felsenstein, J., 1981: Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**, 368–376.
- Gardes, M.; Bruns, T. D., 1993: ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rust. *Mol. Ecol.* **2**, 113–118.
- Harrington, T. C.; Worrall, J. J.; Baker, F. A., 1992: *Armillaria*. In: Methods for Research on Soilborne Phytopathogenic Fungi. Ed. by Singleton, L. L.; Mihail, J. D.; Rush, C. M. St. Paul, MN, USA: APS Press, pp. 81–85.
- Homer, I. J., 1992: Epidemiology of *Armillaria* root rot of Kiwifruit. *Acta Hort. (ISHS)* **297**, 573–578.
- Huelsenbeck, J. P.; Ronquist, F., 2007: MrBayes, version 3.1.2. Bayesian analysis of phylogeny. Application program distributed by the authors on the GNU General Public License, website <http://mrbayes.csit.fsu.edu>.
- Kim, M.-S.; Klopfenstein, N. B.; Hanna, J. W.; McDonald, G. I., 2006: Characterization of North American *Armillaria* species: genetic relationships determined by ribosomal DNA sequences and AFLP markers. *For. Path.* **36**, 145–164.
- Kim, M.-S.; Klopfenstein, N. B.; Hanna, J. W.; Cannon, P.; Medel, R.; López, A., 2010: First report of *Armillaria* root disease caused by *Armillaria tabescens* on *Araucaria araucana* in Veracruz, México. *Plant Dis.* **94**, 784.
- Kim, M.-S.; Stewart, J. E.; Ota, Y.; Hanna, J. W.; Ross-Davis, A. L.; Klopfenstein, N. B., 2012: Phylogenetic relationships among northern hemisphere *Armillaria* species based on the *tef1-α* locus. *Phytopathology* **102**(S4), 63–64.
- Lindner, D. L.; Banik, M. T., 2011: Intragenomic variation in the ITS rDNA obscures phylogenetic relationships and inflates estimates of operational taxonomic units in the genus *Laetiporus*. *Mycologia* **103**, 731–740.
- Lung-Escarmant, B.; Guyon, D., 2004: Temporal and spatial dynamics of primary and secondary infection by *Armillaria ostoyae* in a *Pinus pinaster* plantation. *Phytopathology* **94**, 125–131.
- Moncalvo, J. M.; Lutzoni, F. M.; Rehner, S. A.; Johnson, J.; Vilgalys, R., 2000: Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Syst. Biol.* **49**, 278–305.
- Müller, K., 2005: Primer design and sequence statistics for phylogenetic DNA datasets. *Appl. Bioinformatics* **4**, 65–69.
- Onsando, J. M.; Wargo, P. M.; Waudu, S. W., 1997: Distribution, severity, and spread of *Armillaria* root disease in Kenya tea plantations. *Plant Dis.* **81**, 133–137.
- Pertot, I.; Gobbin, D.; De Luca, F.; Prodanutti, D., 2008: Methods of assessing the incidence of *Armillaria* root rot across viticultural areas and the pathogen's genetic diversity and spatial-temporal pattern in northern Italy. *J. Crop Prot.* **27**, 1061–1070.
- Posada, D., 2008: jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.* **25**, 1253–1256.
- Prodanutti, D.; Vanblaere, T.; Gobbin, D.; Pellegrini, A.; Gessler, C.; Pertot, I., 2009: Genetic diversity of *Armillaria* spp. infecting highbush blueberry in northern Italy (Trentino region). *Phytopathology* **99**, 651–658.

- Proffer, T. J.; Jones, A. L.; Ehret, G. R., 1987: Biological species of *Armillaria* isolated from sour cherry orchards in Michigan. *Phytopathology* **77**, 941–943.
- Rizzo, D. M.; Whiting, E. C.; Elkins, R. B., 1998: Spatial distribution of *Armillaria mellea* in pear orchards. *Plant Dis.* **82**, 1226–1231.
- Rodriguez, F.; Oliver, J. F.; Marín, A.; Medina, J. R., 1990: The general stochastic model of nucleotide substitution. *J. Theor. Biol.* **142**, 485–501.
- Ross-Davis, A. L.; Hanna, J. W.; Kim, M.-S.; Klopfenstein, N. B., 2012: Advances toward DNA-based identification and phylogeny of North American *Armillaria* species using elongation factor-1alpha gene. *Mycoscience* **53**, 161–165.
- Schnabel, G.; Ash, J. S.; Bryson, P. K., 2005: Identification and characterization of *Armillaria tabescens* from the southeastern United States. *Mycol. Res.* **109**, 1208–1222.
- Shaw, C. G., 1989: *Armillaria ostoyae* associated with mortality of new hosts in Chihuahua, Mexico. *Plant Dis.* **73**, 775.
- SIAP, 2010. Cierre de la producción por cultivo. SAGARPA. Revisado 27 junio 2012. www.siap.gob.mx/index.php/agricultura/produccion-anual/cierre-de-la-produccion-agricola-por-cultivo.html.
- Simmons, M. P.; Ochoterena, H., 2000: Gaps as characters in sequence based phylogenetic analyses. *Syst. Biol.* **49**, 369–381.
- Swofford, D. L., 2002: PAUP 4.0b10: Phylogenetic Analysis Using Parsimony Sunderland, MA, USA: Sinauer Associates.
- Veldman, G. M.; Klootwijk, J.; de Regt, V. C. H. F.; Rudi, J., 1981: The primary and secondary structure of yeast 26S rRNA. *Nucleic Acids Res.* **9**, 6935–6952.
- White, T. J.; Bruns, T. D.; Lee, S. B.; Taylor, J. W., 1990: Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal DNA genes. In: *PCR Protocols: A Guide to Methods and Applications*. Ed. by Innis, M. A.; Gelfand, D. H.; Sninsky, J. J.; White, T. J. New York: Academic Press, pp. 315–322.
- Worrall, J. J., 1991: Media for selective isolation of hymenomycetes. *Mycologia* **83**, 296–302.