World Applied Sciences Journal 8 (2): 217-223, 2010
ISSN 1818-4952

Introduction of New Hosts for *Armillaria mellea* and *Armillaria gallica* from North Forests of Iran

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Abstract: The genus *Armillaria* is a causal agent of a major root and crown rot disease of orchard (horticulture) and forest plant species. *Armillaria mellea* is the most important species of the genus that was reported from the different host plants in various regions of Iran. In this study, the hosts of *Armillaria* were investigated. Eighteen isolates of the genus *Armillaria* were obtained from *Fagus orientalis*, *Abies alba*, *Parrotia persica*, *Diospyros lotus*, *Quercus castaneifolia*, *Carpinus betulus*, *Alnus subcordata*, *Acer* sp., *Crataegus pentagyna*, *Picea abies* and *Gleditsia caspia* from northern forests of Iran. Two methods were used for identification of *A. mellea*: pairing-tests and the PCR-based restriction fragment length polymorphism (RFLP-PCR), specifically in nuclear rDNA spacers. In the present study, the 10 haploid and diploid isolates (55.6% isolates) could be identified in diploid-haploid and haploid-haploid pairings, the method was laborious and presented a high rate of inconclusive results. When HinfI was used to digest Internal Transcribed Spacer 1 (ITS1) amplicon, two clearly distinct patterns were obtained, one specific for all *A. mellea* isolates (mellea pattern, 230 bp and 130 bp) and the other common to the remaining *Armillaria* spp. (non-mellea pattern 290 bp and 70 bp). The seven new hosts of the genus *Armillaria* were introduced from the forest regions. *A. mellea* were obtained from *Carpinus betulus*, *Alnus subcordata*, *Gleditsia caspia*. *Diospyros lotus*, *Quercus castaneifolia* and *Fagus orientalis* were introduced as the new hosts of *A. gallica*. *Armillaria* sp. was isolated from *Picea abies*. The results showed that *Armillaria mellea* was the most prevalence species of the genus *Armillaria* in the northern forests of Iran.

Key words: RFLP-PCR · rDNA · Internal transcribed spacer 1 · *Armillaria mellea* · *Armillaria gallica* · New host

INTRODUCTION

Armillaria genus includes at least 36 species which were divided into two sections by the presence or absence of a vail: annulate and exannulate respectively [1-4]. The species are present in natural forests, orchards and parks, throughout the world, from the north temperate coniferous to tropical forests. They cause an important disease known as Armillaria root rot. The disease is well known to plant pathologists due to the substantial losses that it can cause in natural forests, commercial forest plantations and horticultural crops and in agriculture, where specifically cash crop plantations are damaged [5,6]. Pairing tests based on sexual behavior and *in vitro* somatic compatibility of isolates of *Armillaria* have been widely used to assess interspecific incompatibility in the genus *Armillaria*. Haploid monosporous cultures exhibit white and fluffy colony morphology, after compatible mating, the culture morphology changes to crustose, which is characteristic of diploid mycelium. Pairing tests have distinguished the biological species in Europe, America and Australia [7-10].

In Japan, five biological species of the fungus were recognized [11]. Six intersterility groups were recognized as distinct using isozyme analysis in annulated *Armillaria* spp. [12].

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Amplification of ITS1 and ITS2 has been useful in distinguishing *Armillaria* spp. [13-15]. Fungal primers ITS1 a nd ITS4 have been used to amplify the ITS1 region for direct sequencing analysis [16-18]. Species-specific primers were also developed for the ITS region of European *Armillaria* [14]. The ITS1 analysis was introduced for identification *A. mellea* [19].

In Iran *A. mellea* is widely distributed throughout the country and is a well known causal agent of root rot diseases [20-22]. Armillaria root disease was reported in association with many cultivated and forest tree species [23]. By the pairing method, 6 intersterility groups were recognized including *A. mellea, A. cepistipes, A. gallica, A. borealis, Armillaria* sp. as IISG5 and *Armillaria* sp. belong to IISG6 [20].

The objective of the present study was determination the host species of A. *mellea* in the north forest regions of Iran.

MATERIALS AND METHODS

Sampling and Fungal Isolation: Eighteen samples were collected from the roots, barks and woods of the trees with suspicious symptom of *Armillaria* infection, with mycelial fans or rhizomorph signs or basidiocarps, in the forest regions of Mazandaran province in north of Iran. Collections were made from the 11 different host species during 2006-2008.

The infected tissues or basidiocarps were sterilized in ethanol 96% for 1 min and small pieces from parts of the tissues were excised and placed on the Petri dishes including malt extract agar (20 g/l Malt extract, 16 g/l agar) amended with benomyl WP 50 (4 μg a.i./ml) and streptomycin sulfate (100 μg/ml) after autoclaving. The Petri dishes were incubated at 22±1°C [24].

Pairing Tests: Pairing tests of the Iranian diploid and haploid isolates were done by pairing method, using the known haploid test strains of *Armillaria* spp. [10]. Seven biological species were used Each isolate was paired with two or three different test strains of the known biological species. Haploid test strains from the species of *Armillaria*, their hosts and the geographic sources are indicated in Table 1.

The inoculum consisted of undifferentiated mycelium without crust or rhizomorph. Mycelial plugs (3 mm diam) were derived from the margin of a growing culture. These plugs were placed side by side. Each two different isolates pairs were placed in each Petri dish and each pairing was repeated twice. The Petri dishes with haploid-haploid or diploid-haploid pairings were incubated at 22±1°C and the evaluation was done after 6-8 weeks.

DNA Extraction: The isolates were grown in liquid MYE medium (2% Malt extract and 0.3% Yeast extract) at 22°C in the dark for 4 weeks. Mycelium was harvested by centrifugation (15300 *g* 20 min) and washed in sterile distilled water [17]. The freeze dried mycelia were mechanically disrupted by grinding it to a fine powder under liquid nitrogen using a mortar and pestle. DNA was extracted using hexadecyltrimethyl-ammonium bromide (CTAB) method and resuspended in 50 μL of TE (10 mM Tris-Base, 1 mM EDTA, pH 8,0) and stored at 4°C until use [25].

Table 1: Species, host, geographic location and source of the haploid testers of *Armillaria* spp

Taxonomic Name	Isolate Code	Isolate No	Collectors Name	Determined By	Geographic Location
Armillaria borealis Marxm. & Korhonen	99 68\4	B1	K. Korhonen	K. Korhonen	Finland
Armillaria borealis Marxm. & Korhonen	n, 2n	B2	M.R. Asef	M.R. Asef	Iran
Armillaria cepistipes Velen.	MB 79.23.1	C1	J.J. Guillaumin	K. Korhonen	Finland
Armillaria cepistipes Velen.	MB 79.24.1	C2	J.J. Guillaumin	K. Korhonen	Finland
Armillaria gallica Marmx. & Romagn.	ME 70.1.2	G1	J.J. Guillaumin	J.J.Guillaumin	France
Armillaria gallica Marmx. & Romagn.	n, 2n	G2	M.R. Asef	M.R. Asef	Iran
Armillaria mellea (Vahl) P. Kumm.	87 085\10	ME1	K. Korhonen	Grillo	Italy
Armillaria mellea (Vahl) P. Kumm.	90 254\3	ME2	K. Korhonen	Grillo	Italy
Armillaria mellea (Vahl) P. Kumm.	90260\1	ME3	K. Korhonen	Munda	Yugoslavia
Armillaria ostoyae (Romagn.) Herink	99 088\3	O1	K. Korhonen	K. Korhonen	Finland
Armillaria ostoyae (Romagn.) Herink	MC 79.27.1	O2	J.J. Guillaumin	K. Korhonen	Finland
Armillaria sinapina Bérubé & Dessur.	96-7-1	S1	Yuko Ota	Yuko Ota	Japan
Armillaria sinapina Bérubé & Dessur.	96-7-2	S2	Yuko Ota	Yuko Ota	Japan
Armillaria tabescens (Scop.) Emel	NT 1-9	T1	Yuko Ota	Yuko Ota	Japan
Armillaria tabescens (Scop.) Emel	NT 1-10	T2	Yuko Ota	Yuko Ota	Japan

Amplification of ITS: The ITS1 region, located between the 18S and the 5.8S ribosomal DNA genes, was amplified by PCR using primers ITS1 and ITS2 [26].

The PCR reaction mixture (50µL) included 80 ng of template DNA, 2 U of Taq DNA polymerase, 200 µM of each dNTP, 10 x PCR buffer supplied with the enzyme and 4mM $MgCl_2$ (Fermentas Inc., USA), 50 pmol of each primer. The final reaction volume was adjusted to 50 µL with H_2O [19].

Amplification was carried out using initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 30 sec, 58°C for 30 sec, for 2 min at 72°C and final cycle at 72°C for 10 min, run on 1.2% w/v agarose gel (Fermentas Inc., USA), in 0.5 x TAE at 100 V for 90 min, using 100 bp DNA Ladder as molecular size marker [12].

Restriction Analysis of ITS1: To perform restriction analysis of ITS region, 5 µL sample of each PCR product was digested with 3 U of HinfI, in a final volume of 10 µL, according to manufacturer's instructions (Fermentas Inc., USA). After overnight incubation at 37°C, 1.5 µL of bromo-phenol blue solution (0.25% bromo-phenol 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 3% w/v agarose gel (Fermentas Inc., USA), in 0.5 x TAE at 100 V for 2 h 30 min, using 50 bp or 100 bp standard DNA ladder. The gels were stained with ethidium bromide solution (0.5 mg/ml) and visualized using UV light [27].

RESULTS

Mating Analysis: Eighteen isolates were obtained from 11 different host species in north forest of Iran. To identify the Iranian isolates, a total of 1080 pairings was performed. The majority of isolates were paired with two or three of the known test strains of one species but some of isolates showed unclear reaction (Figure 1).

The method identified 10 isolates (55.6%) of Armillaria belonged to different species. Seven isolates were identified as *A. mellea* from *F. orientalis*, *Abies alba*, *C. betulus* and *P. persica* and three isolates were identified as *A. gallica* which included of *F. orientalis*, *D. lotus* and *Q. castaneifolia*. The remaining isolates displayed unclear reactions with all test strains (Table 2). The method has several drawbacks, e.g. takes too much time (6 to 8 weeks) and presents a high rate of inconclusive results, especially when identifying diploid isolates.

Analysis of ITS1 Region: The amplification of ITS1 region with primers ITS1 and ITS2 resulted in a single fragment in the test strains and all Iranian 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 360 bp for *Armillaria* species.

When HinfI was used to digest the ITS1 amplicon, two clearly distinct patterns in the test strains were obtained (Figure 2), one specific for all *A. mellea* isolates

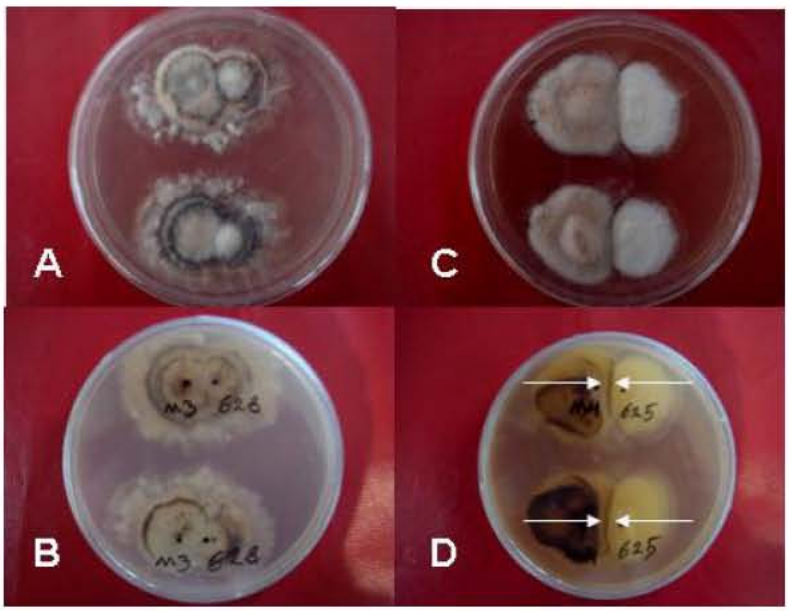

Fig. 1: Compatible (A, B) and incompatible (C, D) reactions and formation of black line in contact locality of two incompatible colonies

Table 2: Characteristics and results of the mating analysis and ITS1 RFLP-PCR patterns of the *Armillaria* isolates

Isolate No.	Derivation	Host	Localities	Ploidy	Pairing-test results	ITS RFLP patterns
M1	Wood fragment	*Abies alba* Mill.	Mazandaran(Neka)	D	*A.mellea*	I
M2	Basidiocarp	*Parrotia persica* C.A. Mey	Mazandaran(Neka)	N	*A.mellea*	I
M3	Basidiocarp	*Parrotia persica* C.A. Mey	Mazandaran(Neka)	N	*A.mellea*	I
M4	Basidiocarp	*Parrotia persica* C.A. Mey	Mazandaran(Neka)	N	*A.mellea*	I
M5	Basidiocarp	*Picea abies* (L.) Karst.	Mazandaran(Sangedeh)	D	*Armillaria sp.*	II
M6	Wood fragment	*Carpinus betulus* L.	Mazandaran(Behshar)	D	*Armillaria sp.*	I
M7	Wood fragment	*Crataegus pentagyna* Walds. et Kit.	Mazandaran(Behshar)	D	*Armillaria sp.*	I
M8	Basidiocarp	*Alnus subcordata* C.A. Mey	Mazandaran(Chamestan)	D	*Armillaria sp.*	I
M9	Basidiocarp	*Carpinus betulus* L.	Mazandaran(Sangedeh))	D	*Armillaria sp.*	I
M10	Wood fragment	*Fagus orientalis* Lipsky	Mazandaran(Behshar)	N	*A.mellea*	I
M11	Wood fragment	*Carpinus betulus* L.	Mazandaran(Behshar)	D	*A.mellea*	I
M12	Basidiocarp	*Carpinus betulus* L.	Mazandaran(Sangedeh))	D	*A.mellea*	I
M13	Wood fragment	*Carpinus betulus* L.	Mazandaran(Chamestan)	D	*Armillaria sp.*	I
M14	Wood fragment	*Gleditsia caspia* Desf.	Mazandaran(Chamestan)	D	*Armillaria sp.*	I
M15	Wood fragment	*Acer* sp.	Mazandaran(Chamestan)	D	*Armillaria sp.*	I
M16	Rhizomorph	*Fagus orientalis* Lipsky	Mazandaran(Neka)	D	*A.gallica*	II
M17	Rhizomorph	*Diospyros lotus* L.	Mazandaran(Neka)	D	*A.gallica*	II
M18	Rhizomorph	*Quercus castaneifolia* C.A. Mey	Mazandaran(Sari)	D	*A.gallica*	II

*Hinf I RFLP-PCR patterns: I = 130 bp + 230bp; II = 70 bp + 290 bp

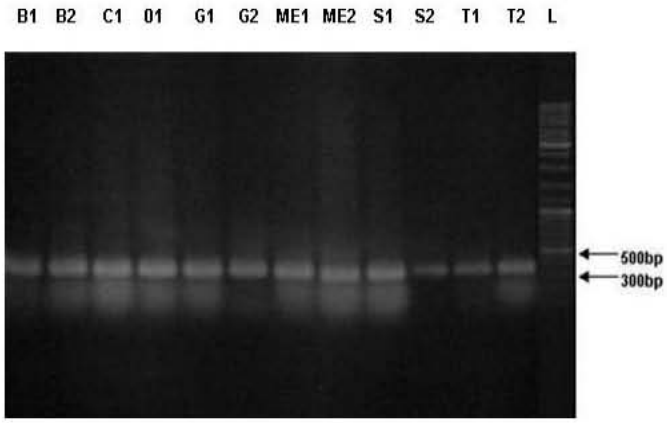

Fig. 2: ITS1 amplicons of *Armillaria* test strains; B = *A. borealis*, C = *A. cepistipes*, O = *A. ostoyae*, G = *A. gallica*, ME = *A. mellea*, T = *A. tabescens*, (L: 100 bp DNA ladder)

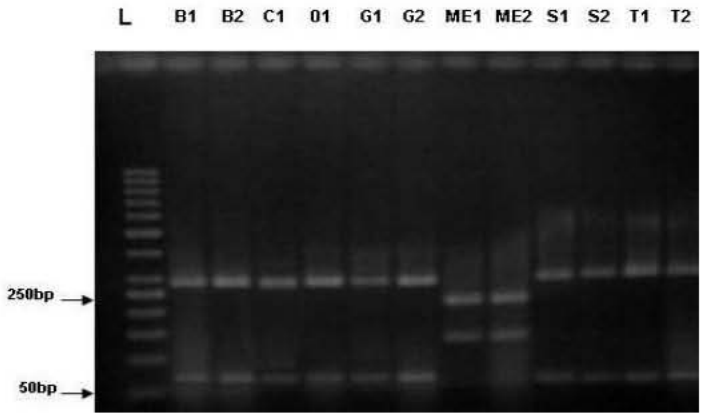

Fig. 3: Restriction profiles of ITS1 region of test strains with HinfI. Lanes L 50 bp DNA ladder; B = *A. borealis*, C = *A. cepistipes*, O = *A. ostoyae*, G = *A. gallica*, ME = *A. mellea*, T = *A. tabescens*

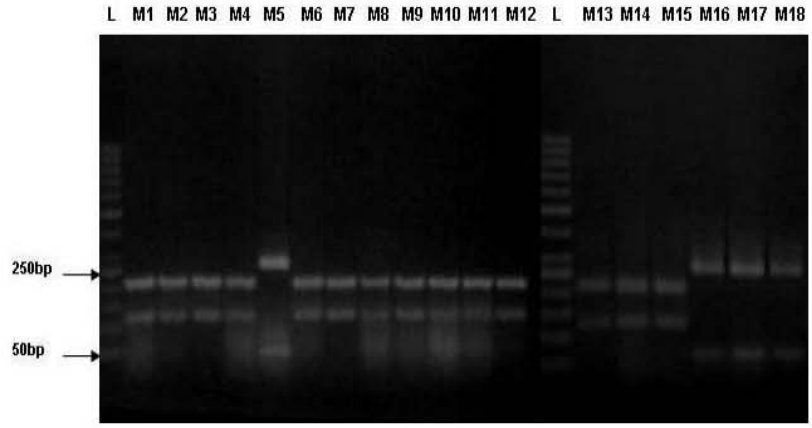

Fig. 4: Restriction profiles of ITS1 region with HinfI. Lanes L 50 bp DNA ladder; M1, M2, M3, M4, M6, M7, M8, M9, M10, M11, M12, M13, M14, M15 (mellea pattern), M5, M16, M17, M18 (non-mellea pattern)

(mellea pattern) and the other common to the remaining *Armillaria* spp. (non-mellea pattern). Both patterns consisted of two fragments as follows: fragments with 230 bp and 130 bp in length (mellea pattern; ME1, ME2) and fragments with 290 bp and 70 bp in length (non-mellea pattern; B1, B2, O1, C1, G1, G2, S1, S2, T1, T2 in Figure 3).

Fourteen isolates were identified as *A. mellea*. *F. orientalis*, *Abies alba*, *P. persica*, *C. betulus*, *Alnus subcordata*, *Acer* sp., *C. pentagyna* and *G. caspia* were the hosts of *A. mellea*. *Alnus subcordata*, *C. betulus* and *G. caspia* were identified as new hosts of *A. mellea* in the northern forest of Iran. Three isolates (M16, M17 and M18) were showed pattern non-mellea species which identified using pairing tests as A. gallica (Fig. 4).

DISCUSSION

Eighteen isolates of *Armillaria* spp. were obtained from the forest regions north of Iran. The isolates recovered from the East, West and Center of Mazandaran Province. The methods identified 94.4% of the isolates. Fourteen isolates belonged to *A. mellea*. The species was the most commonly found species, representing 77.8 % of collections. The fungus infected 8 the plant species. Some of plant species such as *Alnus subcordata*, *C. betulus* and *G. caspia* were identified as new hosts of *A. mellea* from Iran.

Armillaria mellea on *C. betulus* were reported from Serbia and Montenegro [28]. *Armillaria mellea* was isolated on the different conifers and hardwoods from Japan [29]. The species introduced as the main species in Kenya [30]. *Armillaria mellea* was most common in milder temperate regions, particularly Mediterranean climates, where it was a common cause of mortality of ornamental, fruit and forest trees [8,31,32].

The species was mainly a pathogen of broadleaved trees in ornamental parklands, natural woodlands, fruit orchards, etc at Malawi, but it can kill young coniferous trees (pines, spruce, *etc*.) planted in sites where the broadleaved species were felled [33]. *Armillaria mellea* was reported in central and south Europe, but is common only in the southern and western parts of this area [34].

Armillaria mellea was previously reported from the different regions and hosts from Iran [20-23]. The genus *Armillaria* were isolated from *Ulmus minor, Rosa* sp., *Populus nigra, Amygdalus communis, Cerasus avium, Quercus macranthera, Platanus orientalis, P. persica, Acer* sp. and *F. orientalis* in Iran [20].

Armillaria gallica was the second most commonly collected species, constituting 16.6% of the collections. The species were isolated from *Q. castaneifolia*, *C. betulus* and *F. orientalis*. This is the first report of *A. gallica* on the hosts from Iran. Moreover, *A. mellea* and *A. gallica* were isolates from *F. orientalis*.

Armillaria mellea and *A. gallica* were identified and reported from the Missouri Ozark Mountains that *A. mellea* has predominated on both white and red oaks. The results showed nearly all recent oak mortality caused by *A. mellea* [35]. *Armillaria gallica* was common in coniferous and broad-leaved forests in the high altitudes of central and northern Greece, predominating in the beech forests. The fungus was a weak parasite or a saprophyte of forest trees and was occasionally found on cultivated plants [36]. *Armillaria gallica* has been reported from North America [7]. In Japan *A. gallica* was the prevalence species of the genus *Armillaria* [29]. The species was the second most commonly collected species in Wisconsin that was found on Angiosperms [37]. *Armillaria gallica* was previously reported only from stumps in northern of Iran [20].

The results showed *A. mellea* was one of the prevalence species of *Armillaria* in the northern forests of Iran. By attention to the pathogenic characteristics of *A. mellea* on forest plant species, evaluation of the importance and wood destroying activity on the different forest plants species in the Northern forest of Iran will be necessary.

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