Short paper

First report of *Phaeoacremonium rubrigenum*, associated with declining persimmon trees in Iran

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Abstract: The genus *Phaeoacremonium* is associated with decline disease of woody plants and with human infections. Members of this genus have broad host range and wide geographical distribution. During 2010, ten isolates of *Phaeoacremonium* were recovered from vascular tissues of persimmon (*Diospyros kaki*) trees, showing decline symptoms in Shiraz city. Anamorphic characteristics such as, conidiophore morphology, phialide type and shape, size of hyphal warts, conidial size and shape were investigated. Based on morphological characteristics the presence of *Pm. rubrigenum* in Iran was documented. To confirm morphological identification, DNA was extracted from isolates using a genomic DNA purification Kit. Region of internal transcribed spacers 1, 2 and 5.8S genes of rDNA were amplified using ITS4 and ITS1 universal primer set. Fragments of 630 bp were recovered from PCR, purified, sequenced, edited and deposited in GenBank. *Pm. rubrigenum* isolates had an average of 99% identity with all *P. rubrigenum* sequences compared. This species is a new report from Iran.

Keywords: *Phaeoacremonium rubrigenum*, persimmon trees, molecular identification

Introduction

*Phaeoacremonium*, a hyphomycetous genus, was introduced in 1996 with *Pm. parasiticum* as its type species (Crous et al., 1996). Members of the genus *Phaeoacremonium* are known to be cosmopolitan, having broad host range and wide geographical distribution. According to Mostert et al., (2006) of 22 *Phaeoacremonium* species that have been identified, nine of them, were isolated and reported from humans (Crous et al., 1996; Mostert et al., 2005) and 13 species were reported from various plants and humans (Mostert et al., 2006). *Phaeoacremonium* spp. have been reported from various woody plants that were associated with decline, wilting and dieback symptoms, including oak (*Quercus virginiana*) in Texas (Halliwell 1966), *Nectandra* sp. in Costa Rica (Hawksworth et al., 1976), *Prunus* spp. in South Africa (Damm et al., 2005), cherry in Greece (Rumbos 1986), kiwifruit vines in Greece, France and Italy (Di Marco et al., 2004), *Fraxinus pensylvanica* in USA (Hausner et al., 1992), apricot (Hawksworth et al., 1976), date palm (*Phoenix dactylifera*) in Iraq and other trees, although, originally some isolates were mis identified as another fungus. *Pm. scolyti* has been isolated from larvae of *Scolytus intericatus* (oak bark beetle) and adults of *Leperisinus fraxini* (*Fraxinus excelsior* bark beetle) in Czech Republic (Kubatova et al., 2004). These isolates had first been identified as *Pm. rubrigenum*, but later they were re-identified as *Pm. scolyti* by Mostert et al., (2005). To date, there is no available information about the occurrence of dieback of persimmon trees and associated *Phaeoacremonium* species in Iran.

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**Materials and Methods**

**Isolation**
Cross and longitudinal sections of woody persimmon branches were examined in order to check for woody discoloration symptoms. Isolation was made from different types of necrotic tissues. Small pieces, approximately 4 mm in size, of discolored or decayed tissues were surface disinfected by immersing in 1.5 % solution on NaOCl for 30 sec, rinsed in sterile distilled water and plated on potato dextrose agar (PDA) and malt extract agar (MEA) amended with chloramphenicol (25 µg/ml).

**Morphological and cultural studies**
Morphological and cultural characters of *Phaeoacremonium* isolates were studied on four media including MEA, PDA, WA and oatmeal agar (OA). The microscopic features were measured using water mounts of the specimens. Fifty measurements of each type of structure were made using BioloMICSMeasure software. Radial growth of the isolates was measured on MEA, PDA and OA after 16 days at 25 °C. All isolates were identified by morphological and molecular methods (Crous et al., 1996; Dupont et al., 2000; Alanize et al., 2007; Slippers et al., 2007).

**DNA extraction and amplification**
For DNA extraction, isolates were grown on PDA for 10 to 15 days at 25 °C in the dark. Total genomic DNA was extracted using a genomic DNA purification Kit (Fermentas, UK) according to the manufacturer's instruction. The ITS regions of nuclear rDNA were amplified with the universal ITS1 and ITS4 primers (White et al., 1990) on a CORBETT RESEARCH model CG1-96 thermocycler.

**Sequencing of the amplified ITS regions**
The amplification products of all specimens were purified with GeneJET PCR purification Kit (Fermentas, UK) to remove excess primers and nucleotides. Sequencing reactions were performed on purified PCR products in forward and reverse orientation using the primers used for amplification (ITS1 or ITS4). The sequence was determined with an ABI prism 377 DNA sequencer according to the manufacturers instruction. All DNA sequences of the ITS regions deposited at the National Center for Biotechnology Information GenBank (NCBI, http://www.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA).

**Phylogenetic analysis**
Sequences of the internal transcribed spacer regions including the 5.8S gene of rDNA were used to study phylogenetic relationships of the studied isolates. The internal transcribed spacer sequences of rDNA generated in this study were compared to those of other isolates obtained from GenBank (Fig. 3). Multiple alignments were performed with CLUSTALW (Thompson et al., 1994) using default settings and were manually optimized with BIOEDIT v.7.0.9 (Hall 1999). Phylogenetic analyses were performed by means of distance and maximum parsimony (MP) methods. Distances were calculated according to the Kimuras 2 parameter model (K2P). Tree topology was inferred by the Neighbor-Joining (NJ) method (Saitou and Nei 1987). The confidence of branching was assessed by computing 1000 bootstrap resamplings (Felsenstein, 1985). Maximum parsimony (MP) trees were inferred with the Close-neighbor-interchange (CNI) method with the aid of MEGA 4 software. The bootstrap method (Felsenstein, 1985) was performed with 1000 replications to evaluate the reliability of tree topologies. Other statistics, including tree length, consistency index, retention index and rescaled consistency index were calculated. *Pleurostomophora richardsiae* (GenBank accession no. AB364701) was selected as outgroup taxa based on their phylogenetic position in Essakhi et al., (2008).

**Results**
Fifty fungal isolates were recovered from persimmon trees showing decline and dieback symptoms. The most common fungi isolated from most diseased persimmons were *Pm. rubrigenum*, *Acrostalagmus luteoalbus* and *Lecanicillium lecanii* with the frequency of 25.2 7.3 and 6.3 percentage respectively. Numerous isolates of
Aspergillus spp., Penicillium spp., Acremonium spp., Fusarium spp. and Rhizopus sp. were always associated with diseased persimmons in Shiraz city. *Pm. rubrigenum* was isolated mainly from brown and black vascular tissues of branches.

**Morphological identification**

Ten isolates of *Phaeoacremonium* were obtained from discolored vascular system of persimmon trees. These isolates were characterized as follows: color of colony of isolates on malt extract agar (MEA), was medium pink and radial growth more than 9 mm after 7 d at 25 °C in the dark. Minimum temperature for growth 12 °C, optimum 27 °C, maximum 33 °C. Colonies on PDA flat, appressed and woolly to powdery. Colonies on OA flat and felty (Fig. 1). Phialides terminal or lateral, mostly monophialidic, smooth to verruculose, pale brown to subhyaline. Type I phialides subcylindrical, or elongated ampulliform, attenuated at the base, or constricted, 8–11 × 2.5 μm (Fig. 2 A); type II phialides mostly subulate, some navicular, tapering towards the apex, 15–18 × 2–3 μm (Fig. 2 B and C). Type III phialides subulate, tapering towards the apex, 25–28 × 2–3 μm (Fig. 2 D). Conidia hyaline, mostly ellipsoidal, some cylindrical, 3.5–4 × 1–2 μm (Fig. 2 E).

**Molecular study**

All *Phaeoacremonium* isolates previously identified based on morphological and culture characters, were amplified using the primers pair ITS1 and ITS4. A amplicon of about 600 bp was obtained for all of the *Phaeoacremonium* isolates. Through Blast search in GenBank all isolates were identified as *Pm. rubrigenum*. All DNA sequences of *Phaeoacremonium* isolates (accession numbers: JQ387572 and JQ387573) showed 100 % homology with valid sequences previously identified and deposited in GenBank. According to DNA sequence analyses and morphological characters, our isolates recovered from wood of *Diospyros kaki* showing dieback symptoms could be assigned to *Pm. rubrigenum* (Fig. 3).

**Phylogenetic analysis**

For the ITS data set, 71 sequences of ca 600 bp covering the ITS1 + 5.8S + ITS2 regions were used. The *Pleurostomophora richardsiae* was used as out group. Both distance-based and cladistic methods were applied for phylogenetic reconstruction of 71 specimens. The ITS phylogenetic trees inferred by both distance-based (Fig. 3) and cladistic methods (data not shown) showed the same topology, although there were differences in percent bootstrapping. In cladistic method, the tree length was 3958 with a CI of 0.507; RI = 0.82; RCI = 0.47 for all sites; iCI = 0.53 for parsimony informative sites and iRI = 0.82. With this, 47 trees were retained. Our isolates were clustered in a distinct monophyletic clade related to *Pm. rubrigenum* from other authors (Fig. 3). *Pm. rubrigenum* was a sister taxon of *Pm. scolyti* (98 % NJ and 99 % MP). Our *Pm. rubrigenum* isolates had an average of 99.5 % similarity with a range of 98.5-100 % similarity between themselves and only an average of 98 with a range of 93-100 % similarity between all *Pm. rubrigenum* sequences analyzed.

Figure 1 *Phaeoacremonium rubrigenum*. 21-day-old colonies on MEA (A), PDA (B) and OA (C).

Figure 2 *Phaeoacremonium rubrigenum*. type I phialide (A), type II phialide (B and C), type III phialide (D), conidia (E). Bars = 25.6 μm (Figs A, B, C); 16.7 μm (Fig D); 25.6 μm (Fig. E).
Figure 3 Neighbor joining phylogram generated in Mega from the alignment of 71 combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA sequences of *Phaeoacremonium* species using Kimura 2 parameter model with complete deletion gap handling and 1000-replication bootstrapping.
Discussion

Based on morphological characteristics 10 isolates of recovered fungi from diseased persimmons belonged to the genus *Phaeoacremonium*. But due to the overlappings in several characters among *Phaeoacremonium* species, some misidentifications have been made when using these characteristics and it seems that the use of molecular methods is needed in order to identify and separate different species correctly. Using PCR with the primers ITS1 and ITS4, a fragment of about 600 bp was obtained for *Pm. rubrigenum* isolates. Based on ITS gene sequences, these isolates showed 100 % homology with *Pm. rubrigenum* isolates deposited in GenBank. Subsequently, both phenotypical and molecular data confirmed the identification of the *Phaeoacremonium* isolates as *Pm. rubrigenum*. Analysis of sequence alignment shows that, in 71 *Phaeoacremonium* species compared in addition to *Pleurostomophora richardsiae* as an outgroup, there are 540 potentially phylogenetic informative sites which are mainly comprised of substitutions, deletions and insertions. Dupont et al., (2000) collected several *Phaeoacremonium* isolates and based on their morphology as well as DNA phylogeny of the transcribed spacers, 5.8 rRNA gene region and β-tubulin gene, designated these isolates as *Pm. viticola* (Dupont et al., 2000). Different molecular methods have been used for identification of *Phaeoacremonium* spp. such as; RFLP patterns, direct PCR based on specific primers and phylogenetic analysis data (Mostert et al., 2006). Tegli et al., (2000) used the RFLP patterns of ITS region for separation and identification of *Pm. inflatipes*, *Pm. aleophilum* and *Pm. rubrigenum* (Tegli et al., 2000). Later Dupont etal. (2002), used PCR-RFLP markers of ITS regions and analysis of the partial β-tubulin gene to distinguish five *Phaeoacremonium* species of *Pm. aleophilum*, *Pm. rubrigenum*, *Pm. inflatipes*, *Pm. viticola* and *Pm. parasiticum*. This is the first report of *Pm. rubrigenum* with morphological and molecular details in Iran. Previous studies show that *Pm. parasiticum*, *Pm. aleophilum*, and *Pm. inflatipes* are reported from Iran (Mohammadi and Banihashemi 2010). Pathogenicity of this species on persimmon trees in greenhouse is under investigation.

References


اولین گزارش از Phaeoacremonium rubrigenum از زوال حالت در خرمالوی درختان با همراهی Jamali1 و ضیاءالدین بین‌هاشemi ایران

چکیده

یک گونه هیپومیتیکی می‌باشد که به‌عنوان عامل زوال میزان‌های چوبی و بافت‌کننده انسان توصیف شده است. هدایت از این گونه درختان خرمالوی در حال زوال از مناطق مختلف شیراز جداسازی شد. خصوصیات ریخت‌شناسی‌های شامل ریخت‌شناسی گونه‌های شناسی کنیدی‌فرچه‌ها، شکل و نوع فلاین، اندازه‌ی زیگ‌های هیپ‌ها و شکل و اندازه‌های کنیدی‌ها مورد بررسی قرار گرفت. براساس خصوصیات ریخت‌شناسی حضور گونه‌های فارقی Phaeoacremonium در ایران مشخص شد، ضمن اینکه با استفاده از آنالیز فیلوژنیکی نواحی پایان‌های جداینده‌ای نشان‌داده و سطح دو و زن 8/6 آس‌دی آی ای رپوزومی صحبت تشخیص براساس خصوصیات ریخت‌شناسی تأیید شد. این اولین گزارش از حضور این گونه برای ایران می‌باشد.

واژگان کلیدی: درختان خرمالوی، تشخیص مولکولی Phaeoacremonium rubrigenum