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First report of *Paraconiothyrium fuckelii* (*Didymosphaeriaceae*, *Pleosporales*), causing stem canker in *Rosa hybrida*, from Iran

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Jamali S. (2020): First report of *Paraconiothyrium fuckelii* (*Didymosphaeriaceae*, *Pleosporales*), causing stem canker of *Rosa hybrida*, from Iran. – Czech Mycol. 72(1): 71–82.

In spring 2016, stem canker symptoms were observed on most *Rosa hybrida* plants in greenhouses of Kermanshah Province in Western Iran. Initial symptoms of the disease were brown necrotic lesions, mostly 8–10 mm long, on stems. The lesions eventually depressed and expanded to a long ellipse, resulting in yellowing and wilting of the foliage. Fungal colonies resembling those of *Paraconiothyrium* species were obtained from diseased tissues. Based on morphological and DNA sequence analysis of the internal transcribed spacers ITS1 and ITS2 and the 5.8S gene the fungal isolates were identified as *Paraconiothyrium fuckelii*. This is the first report of stem canker disease caused by this species in Iran.

Pathogenicity tests were carried out on potted *Rosa hybrida* plants and detached branches under controlled conditions. Pathogenicity tests demonstrated that the fungus is able to infect and cause canker symptoms on inoculated branches of healthy plants after four weeks. The pathogen was re-isolated from all inoculated plants after observation of the symptoms, thus meeting Koch's postulates.

Key words: Dothideomycetes, fungal disease, internal transcribed spacers, Iran mycobiota, pathogenicity.

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Jamali S. (2020): První nález *Paraconiothyrium fuckelii* (*Didymosphaeriaceae*, *Pleosporales*), původce stonkové nekrózy růží, v Íránu. – Czech Mycol. 72(1): 71–82.

Na jaře 2016 byly pozorovány nekrotické symptomy na stoncích růží ve sklenících v provincii Kermanšah v západním Íránu. Prvotními symptomy napadení byly hnědé, 8–10 mm dlouhé nekrotické léze na stoncích; ty se časem prohlubovaly a rozšiřovaly do tvaru dlouhé elipsy, přičemž docházelo k žloutnutí a vadnutí listů. Z napadených pletiv byly získány kolonie připomínající druhy rodu *Paraconiothyrium*. Na základě morfologického studia a anylýzy sekvencí ITS1, ITS2 a 5.8S byly izoláty určeny jako *Paraconiothyrium fuckelii*. Jde o první záznam o výskytu tohoto druhu coby původce stonkové nekrózy v Íránu.

Na pěstovaných rostlinách a oddělených větvích růží byly následně provedeny testy patogenity v kontrolovaných podmínkách. Ty ukázaly, že houba je schopná během čtyř týdnů napadnout inokulované větve zdravých rostlin a způsobit na nich nekrózy. Z rostlin s objevenými symptomy se ve všech případech podařilo patogena opět izolovat, čímž jsou naplněny Kochovy postuláty.

INTRODUCTION

Rosa (rose) is a perennial woody flowering plant belonging to the *Rosaceae* family. Most species of *Rosa* are native to Asia and a few species are native to Europe, North America, and northwest Africa (Bruneau et al. 2007, Debener & Byrne 2014). The genus *Rosa* accommodates over 100 species and most of them are known as ornamental plants grown for their flowers in gardens and sometimes indoors. They have also been used for commercial perfumery and commercial cut flower crops (Debener & Linde 2009, Nadeem et al. 2011, Kobayashi et al. 2013, Debener & Byrne 2014).

Many fungal diseases of *Rosa* have been recorded throughout the world (Horst & Cloyd 2007). These include black spot caused by *Diplocarpon rosae* (McMaugh 2001), powdery mildew caused by *Podosphaera pannosa* (Kaufmann et al. 2012), botrytis blight caused by *Botrytis cinerea* (Hao et al. 2017), brown canker caused by *Cryptosporella umbrina*, rust caused by *Phragmidium mucronatum*, anthracnose caused by *Sphaceloma rosarum* (Jenkins 1932, Ross 1985) and downy mildew caused by *Peronospora sparsa* (Aegerter et al. 2002, López-Guisa et al. 2013). In addition to the mentioned diseases, roses are susceptible to canker disease. Ascomycetous fungi with coniothyrium-like asexual morphs cause cankers on *Rosa* and other plants including *Eucalyptus*, *Fraxinus excelsior*, *Malus sylvestris*, *Rubus*, *Pinus* and *Betula* (Michailides et al. 1994, Przybyl 2002, Cortinas et al. 2006, Damm et al. 2008).

The earliest record of pathogenic fungi on rose in Iran dates back to 1885, when Wettstein (1885) reported *Phragmidium mucronatum* as the causal agent of rose rust based on his observations. Other studies have been conducted to identify fungal pathogens on *Rosa* spp. in different provinces of Iran, the main pathogens being identified as *Armillaria mellea* (from Esfahan, Tabriz; Saber 1973), *Botryosphaeria dothidea* (Astara; Viennot-Bourgin 1958), *Botrytis cinerea* (Kashan; Jalali et al. 2004), *Botryotinia fuckeliana* (Esfahan, Karaj, Mahallat, Varamin; Mirabolfathy & Ershad 2004), *Coniosporium rosae* (Tehran; Khabiri 1952), *Diaporthe eres* (Esfahan, Karaj, Mahallat, Varamin; Mirabolfathy & Ershad 2004), *Marssonina rosae* (Ardebil, Astara, Gorgan, Rasht, Tonekabon; Scharif & Ershad 1966, Ershad 1995), *Mycosphaerella rosicola* (Ardebil, Karaj, Kashan, Khoy, Shiraz, Tabriz, Tehran, Tonekabon; Ershad 2009), *Phellinus punctatus* (Kashan; Nematollahi et al. 2004), *Phragmidium kupreviczii* (Lorestan;

Henderson & Jorstad 1996), *P. mucronatum* (Arasbaran, Kuhdasht, Kerman, Alvand, Caspian Sea area, Fasham, Khuzestan, Orumieh; Wettstein 1885, Magnus 1899, Ebrahimi & Minassian 1973, Tavanaei et al. 2004), *P. rosae-lacerantis* (Kerman and Ahvaz; Dietel 1905, Abbasi & Minassian 2005), *Phytophthora citrophthora* (Tehran; Mirabolfathy & Ershad 1998) and *Seimatosporium fusisporum* (Golzar and Lalehzar; Aminaee & Ershad 2008, Ershad 2009). *Podosphaera pannosa* has been reported from all over the country (Altman et al. 1972, Vaziri 1973, Jami et al. 2006). *Phytophthora citrophthora* has also been reported directly from *Rosa hybrida* (Mirabolfathy & Ershad 1998).

Roses are extensively grown throughout Iran, but comprehensive studies on pathogenic fungi causing stem canker in roses have not been reported yet from Kermanshah Province, western Iran. Therefore, this paper aims to report a new fungus, *Paraconiothyrium fuckelii*, causing stem canker on roses in this region. Morphological features and molecular identification of *Paraconiothyrium fuckelii* are described and discussed.

MATERIAL AND METHODS

Sampling and morphological characterisation. In spring 2016, ten isolates of *Paraconiothyrium* were obtained from branch and stem samples of *Rosa hybrida* plants showing canker symptoms in 10 greenhouses in the plant cultivation complex in the area of Dinawar, Kermanshah Province, western Iran. The infected samples were taken to the laboratory and examined using a standard light microscope (Olympus BX-51) and images were captured with a camera (Canon Powershot SX10). The fungus was isolated from conidia on stem and branch cankers from diseased rose plants and cultivated in vitro. Conidia were placed on 2% potato dextrose agar (PDA) plates supplemented with 20 µg/ml of streptomycin sulphate under aseptic conditions. Fungal colonies were grown at 25–27 °C in the dark for 14 days. For fungal identification, during the incubation period, plates were observed daily for the appearance of fungal colonies. Micromorphological features of the fungus such as size, shape, septation of conidia and conidiomatal type were recorded. Measurements were made using the BioloMICS Measure software (Robert et al. 2011); in total 50 conidia were measured. The identification was carried out comparing the information registered with that published in specialised literature (Damm et al. 2008, Verkley et al. 2004, 2014). A voucher specimen was deposited in the Fungal Reference Collection of the Ministry of Jihad-e-Agriculture at the Iranian Research Institute of Plant Protection, Tehran, Iran.

Molecular identification and phylogenetic analysis. For molecular identification, two isolates from rose plants were analysed. Total genomic DNA

was extracted from mycelia of the fungus grown on PDA medium by using the CTAB method (Gardes & Bruns 1993). The ITS1 (5'-CCGTAGGTGAACCTGCGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs (White et al. 1990) were used to amplify the ITS region including the 5.8S unit of the rDNA gene by polymerase chain reaction (PCR) with a T-personal thermocycler (Biometra, Analytik Jena, Germany) in a 25-µl reaction containing 20 ng genomic DNA, 1 µM of each primer, 100 µM of each dNTP, 0.5 U of Taq DNA polymerase (CinnaGen, Tehran, Iran), 1.5 mM of $MgCl_2$, 2.5 µl of 10× PCR buffer (CinnaGen, Tehran, Iran), and 14.5 µl of ddH_2O. PCR conditions were as follows: 30 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 80 s, elongation at 72 °C for 90 s, with initial denaturation of 5 min. at 95 °C before cycling and a final elongation of 10 min. at 72 °C after cycling. PCR products were sequenced using the ITS1 and ITS4 primers in a sequence analyser (ABI prism 377 DNA sequencer, Applied Biosystems, Foster City, USA). Sequences were edited using the BioEdit Sequence Alignment Editor version 7.2.5 software (Hall 1999), and sequence similarity searching was performed using BLAST (http://blast.ncbi.nlm.nih.gov). All DNA sequences of the ITS regions were deposited in the National Center for Biotechnology Information GenBank (NCBI, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/Entrez). The phylogenetic analysis was performed using MEGA5 (www.megasoftware.net; Tamura et al. 2011) by applying maximum-parsimony (MP) and neighbour-joining (NJ) methods. The robustness of the tree was evaluated by computing 1000 bootstrap resampling (Felsenstein 1985).

Pathogenicity test in laboratory. To examine the pathogenicity of the fungus, all isolates (ten in total) were used for laboratory pathogenicity testing on detached branches, under laboratory conditions. The middle part of each healthy rose branch (20 cm in length and 2 cm in diameter) was surface-sterilised with 75% ethyl alcohol. A superficial wound was made by thrusting a 10-mm-diameter cork borer into the bark to a depth of 0.5 cm, after which the tissue was removed, and a mycelium plug obtained from the margin of a growing fungal colony of a selected culture was placed in the wound and wrapped in with a Parafilm wrap. Inoculated branches were stored in a sterilised glass jar containing a moist sponge and incubated on a 12-12 h light-dark cycle in an incubator at 25 °C for 45 days until disease symptoms appeared (Sabernasab et al. 2019). A control wound on a different branch was seeded with a sterile PDA agar plug and sealed. Three replications for each isolate were used to demonstrate potential pathogenicity on detached rose branches.

Pathogenicity test in greenhouse. The pathogenicity tests were conducted in the greenhouse of the College of Agriculture, Razi University, Kermanshah. Two isolates (PaFu1 and PaFu2) were randomly selected and used for a pathogenicity test under greenhouse conditions using artificial inoculations on

potted plants (two plants for each isolate were inoculated and one control plant was treated with a sterile agar plug). Inoculations were made on the basal part of the stem (five cm above ground level). After disinfecting with 70% alcohol, a wound of five mm diameter was created with a cork borer in the bark of the plants, and plugs of mycelium five mm in diameter were placed into the wounds and covered with Parafilm. The non-inoculated plant served as a control.

RESULTS AND DISCUSSION

Symptoms and morphology

The canker disease was observed in most *Rosa hybrida* plants in greenhouses of Kermanshah Province (West Iran). A total of ten *Paraconiothyrium* isolates were obtained from ten diseased rose plants (Fig. 1A) displaying external symptoms such as brown to black cankers on stems and crown, and internal dark brown vascular necrosis in cross sections of the stems and branches. On the surface of the infected stems, conidiomata were observed, which were pycnidial, scattered, and immersed in the host tissue (Fig. 1B).

Colonies on PDA after three days were light to dark grey in all isolates. Hyphae were mid-brown to dark brown and smooth; pycnidia uni-loculate, ostiolate and globose to subglobose (Fig. 1D); conidia hyaline to light brown, smooth, aseptate, spherical to subspherical, ellipsoidal, 3–4 × 2–3 µm (average 3.7 × 2.6 µm) (Fig. 1E).

Material examined

I r a n. Kermanshah Province, Dinawar District, greenhouses near Dinawar Road between the villages of Kang and Cheshmeh Qolam Weish, 34°35' N, 47°26' E, elevation 1350 m, 22 June 2016, leg. & det. S. Jamali (voucher specimen: IRAN 17672F).

Molecular identification and phylogenetic analysis

Based on a BLASTn search of the NCBI GenBank nucleotide database, the closest sequence to our isolates sequenced (ITS accession nos KX264328 and KX264329) was *Paraconiothyrium fuckelii* (JX496096, CBS 508.94; Verkley et al. 2014). The phylogenetic reconstruction of 41 sequences of ca 550 bp covering the ITS1 + 5.8S + ITS2 regions was inferred by using distance-based (neighbour-joining) and cladistic (maximum parsimony) methods. The final aligned data matrix comprised 429 characters including alignment gaps, of which 96 characters were parsimony-informative, 124 variable characters, and 305 characters were conserved. The ITS phylogenetic trees inferred with both distance-based (data not shown) and cladistic methods (Fig. 2) showed the same topology, although there were differences in bootstrap value between equivalent branches. In the maximum

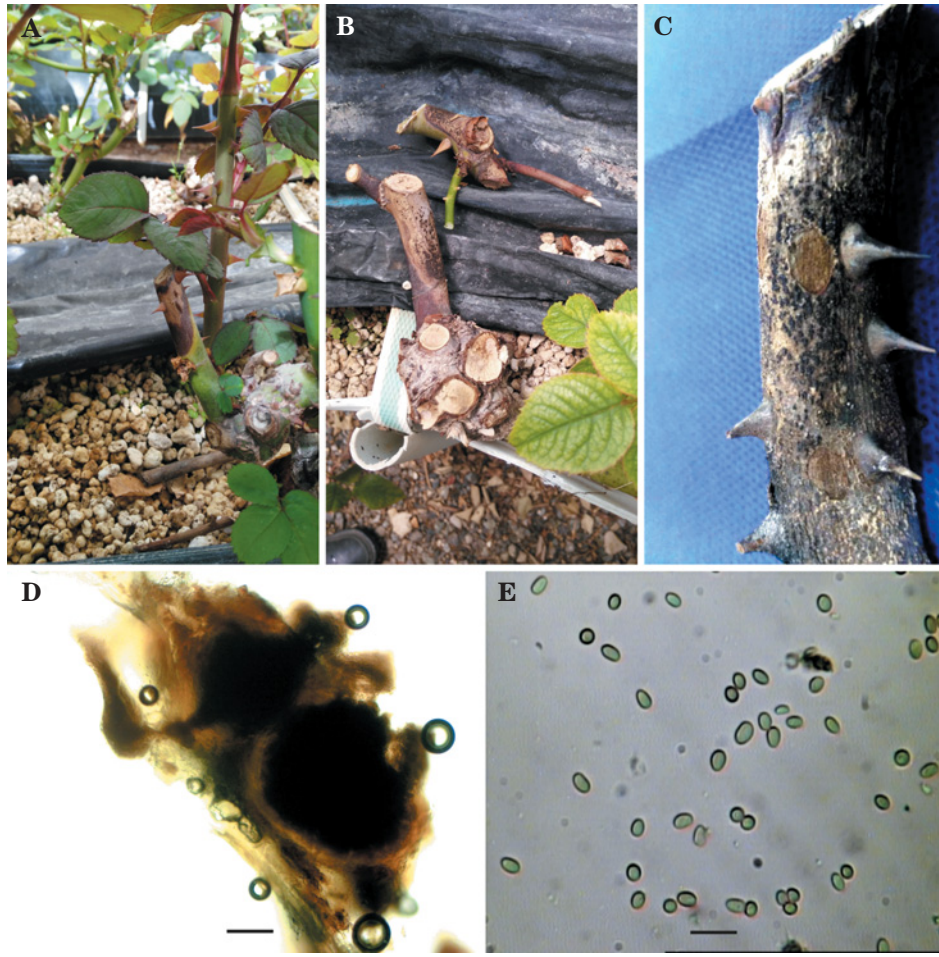
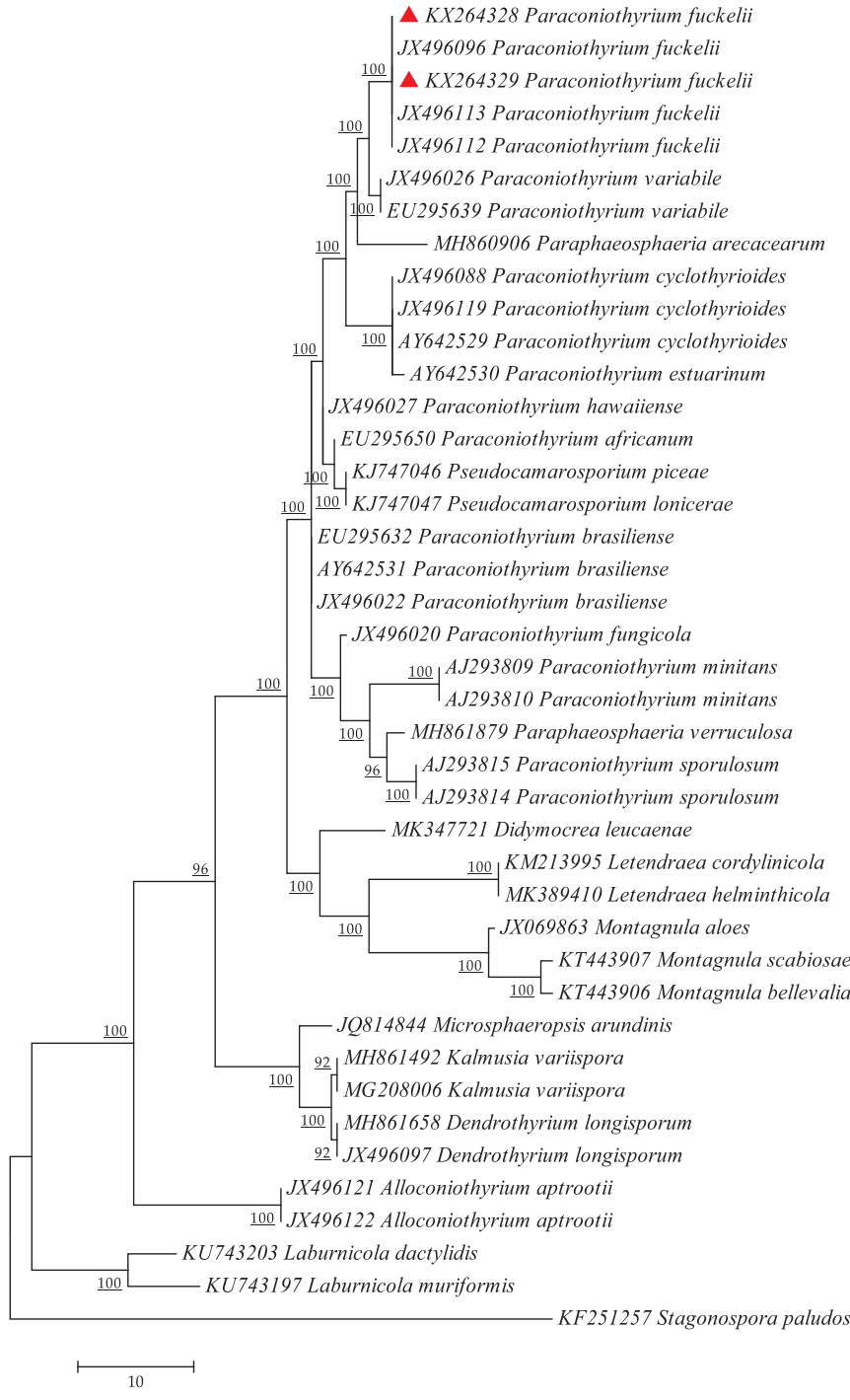


Fig. 1. *Paraconiothyrium fuckelii* (IRAN 17672F): **A** – canker on stem of *Rosa hybrida* in greenhouse; **B** – canker and scattered pycnidia on crown and stem; **C** – symptom of *P. fuckelii* infection on detached branch under laboratory conditions; **D** – pycnidia; **E** – conidia. Scale bars: D – 100 µm, E – 10 µm. Photo S. Jamali.

Fig. 2. Maximum parsimony phylogram generated in MEGA5 from the alignment of 41 combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal DNA sequences of *Paraconiothyrium* species and related taxa with complete deletion gap handling and 1000-replication bootstrapping. Red triangles refer to Iranian isolates. Other sequences used for analysis were retrieved from the GenBank database. ▶


KX264328 Paraconiothyrium fuckelii
JX496096 Paraconiothyrium fuckelii
KX264329 Paraconiothyrium fuckelii
JX496113 Paraconiothyrium fuckelii
JX496112 Paraconiothyrium fuckelii
JX496026 Paraconiothyrium variabile
EU295639 Paraconiothyrium variabile
MH860906 Paraphaeosphaeria arecacearum
JX496088 Paraconiothyrium cyclothyrioides
JX496119 Paraconiothyrium cyclothyrioides
AY642529 Paraconiothyrium cyclothyrioides
AY642530 Paraconiothyrium estuarinum
JX496027 Paraconiothyrium hawaiiense
EU295650 Paraconiothyrium africanum
KJ747046 Pseudocamarosporium piceae
KJ747047 Pseudocamarosporium lonicerae
EU295632 Paraconiothyrium brasiliense
AY642531 Paraconiothyrium brasiliense
JX496022 Paraconiothyrium brasiliense
JX496020 Paraconiothyrium fungicola
AJ293809 Paraconiothyrium minitans
AJ293810 Paraconiothyrium minitans
MH861879 Paraphaeosphaeria verruculosa
AJ293815 Paraconiothyrium sporulosum
AJ293814 Paraconiothyrium sporulosum
MK347721 Didymocrea leucaenae
KM213995 Letendraea cordylinicola
MK389410 Letendraea helminthicola
JX069863 Montagnula aloes
KT443907 Montagnula scabiosae
KT443906 Montagnula bellevaliae
JQ814844 Microsphaeropsis arundinis
MH861492 Kalmusia variispora
MG208006 Kalmusia variispora
MH861658 Dendrothyrium longisporum
JX496097 Dendrothyrium longisporum
JX496121 Alloconiothyrium aptrootii
JX496122 Alloconiothyrium aptrootii
KU743203 Laburnicola dactylidis
KU743197 Laburnicola muriformis
KF251257 Stagonospora paludosa
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parsimony analysis, the tree length was 201 with a consistency index (CI) of 0.62, a retention index (RI) of 0.79; RCI = 0.49 for all sites; iCI = 0.56 for parsimony-informative sites and iRI = 0.79 for parsimony-informative sites. With this, 25 trees were retained. One of the most parsimonious trees is presented in Fig. 2. The phylogenetic analysis of the sequenced ITS fragment positioned our isolates within the *P. fuckelii* clade defined by other authors (100% MP).

Pathogenicity tests

The results of the pathogenicity tests conducted under laboratory conditions revealed that all ten isolates of *Paraconiothyrium* in this study were able to form characteristic canker symptoms, and pycnidia were evident on stems of inoculated detached branches (Fig. 1C). After inoculation of *Paraconiothyrium* isolates on detached branches, re-isolation was performed to fulfill Koch's postulates. The results of the pathogenicity test under greenhouse conditions showed that *P. fuckelii* isolates (PaFu1 and PaFu2) were pathogenic to young seedlings. Four weeks after inoculation, cankers extended upward and downward from the point of inoculation on stems of inoculated seedlings. *Paraconiothyrium fuckelii* was re-isolated from the inoculated seedlings, thus fulfilling Koch's postulates. No symptoms were observed on the non-inoculated controls.

Notes

The genus *Paraconiothyrium* was introduced by Verkley et al. (2004) to accommodate some species with *Coniothyrium* anamorphs, including *C. minitans* and *C. sporulosum* (Damm et al. 2008). Members of the genus *Paraconiothyrium* are known to be cosmopolitan, having a broad host range and broad geographical distribution. They are reported from rose and other woody plants from different countries including South Africa, Brazil, Turkey, the USA and most European countries (O'Gara 1911, Pole-Evans 1928, Alfieri 1969, Whipps & Gerlagh 1992, Verkley et al. 2004, Göre & Bucak 2007, Riccioni et al. 2007).

Identification of *Paraconiothyrium* species by morphological examination is challenging. Due to the overlap in several characters of morphologically similar *Paraconiothyrium* species, some misidentifications have been made when using these characteristics (Crous et al. 2012). Therefore, application of molecular detection procedures, such as PCR and sequencing analysis, is critical for an accurate determination of the identity of *Paraconiothyrium* species. In recent years, several studies have focused on *Didymosphaeriaceae* with the objective of providing a robust classification of this family, which have made it possible to clearly define the main species (Verkley et al. 2004, Ariyawansa et al. 2014, Verkley et al. 2014, Crous et al. 2015, Tennakoon et al. 2016). In most studies, ITS rDNA sequence data has been used for delimitation of the species. Based on ITS gene

sequences, our isolates showed 99% homology with *P. fuckelii* (JX496096, CBS 508.94; Verkley et al. 2014). Phylogenetic analysis based on internal transcribed spacers of the rRNA genome placed them into one distinct monophyletic clade (Fig. 2). This group comprises our isolates with authentic isolates of *P. fuckelii*. Our results show that *Paraconiothyrium* is paraphyletic. This is in concordance with the results of a study conducted by Verkley et al. (2014), which revealed that *Paraconiothyrium* is paraphyletic and separated the genus from *Alloconiothyrium*, *Dendrothyrium* and *Paraphaeosphaeria*. The internal transcribed spacer alone may identify most species accurately, as it is sufficiently variable in most closely related taxa in the *Didymosphaeriaceae*, but it fails to distinguish all species (Verkley et al. 2014). Most species with identical ITS sequences can also be distinguished by colony features and conidial characters, when careful morphological analyses are conducted on fresh isolates under standard conditions and on common media (Verkley et al. 2014).

Paraconiothyrium fuckelii has been reported from different plant families, such as *Fabaceae*, *Rosaceae*, *Pinaceae* and *Sapindaceae* (Kuter 1986, Mulenko et al. 2008, Kowalski & Andruch 2012). In Iran, no previous records of *Paraconiothyrium* on rose plants exist. This is the first report of *P. fuckelii* infecting *Rosa hybrida* in Iran, which may have an important economic impact on rose growing. Therefore, monitoring and control strategies are to be developed to prevent economic losses from stem canker caused by *P. fuckelii*.

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