Pythium kandovanense sp. nov., a fungus-like eukaryotic micro-organism (Stramenopila, Pythiales) isolated from snow-covered ryegrass leaves

Ali Chenari Bouket,1,2 Mahdi Arzanlou,1 Motoaki Tojo2 and Asadollah Babai-Ahari1

1Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Iran
2Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Japan

Pythium kandovanense sp. nov. (ex-type culture CCTU 1813T=OPU 1626T=CBS 139567T) is a novel oomycete species isolated from Lolium perenne with snow rot symptoms in a natural grassland in East-Azarbaijan province, Iran. Phylogenetic analyses based on sequence data from internal transcribed spacer (ITS)-rDNA, coxI and coxII mitochondrial genes clustered our isolates in Pythium group E as a unique, well supported clade. Pythium kandovanense sp. nov. is phylogenetically and morphologically distinct from the other closely related species in this clade, namely Pythium rostratilingens and Pythium rostratum. Pythium kandovanense sp. nov. can be distinguished from these two species by its cylindrical sporangia and lower temperatures for optimum and maximum growth rate. The development of zoospores released through a shorter discharge tube is an additional morphological feature which can be used to differentiate Pythium kandovanense sp. nov. from Pythium rostratilingens. Laboratory inoculation tests demonstrated the pathogenicity of Pythium kandovanense sp. nov. to L. perenne under wet cold (0–3 °C) conditions.

INTRODUCTION

The genus Pythium is highly diverse, containing more than 140 species, and has a cosmopolitan distribution present in terrestrial and aquatic habitats (Kageyama, 2014; Kirk et al., 2008; Tojo & Newsham, 2012). Members of this genus exhibit saprophytic and parasitic life styles (Hong & Moorman, 2005; Martin & Loper, 1999; Nechwatal et al., 2008). Parasitic species of the genus pose negative impacts on both plants and animals, including algae, mosses, humans, fish, crustaceans and mosquito larvae (Kawamura et al., 2005; Miura et al., 2010; Phillips et al., 2008; Van der Plaats-Niterink, 1981). Plant pathogenic species have a devastating impact on crops throughout the world, inciting a wide range of disease symptoms such as pre- and post-emergence damping-off, and seed and root rot. A number of species of the genus Pythium are known as causal agents of snow rot diseases on grasses in Japan, the USA (Takamatsu & Takenaka, 2001), Russia (Petrov, 1983) and Australia (Van der Plaats-Niterink, 1981). Disease symptoms usually appear as water-soaked, dark-green areas on leaves of winter cereals and grasses just after snow melt, and affected tissues eventually become grey and necrotic. Pythium iwayamai (Ito, 1935), Pythium okanoganense (Lipps, 1980) and Pythium paddicum (Hirane, 1960) are the most common snow-rot-causing species of the genus Pythium in Japan and the USA (Takamatsu & Takenaka, 2001). Pythium okanoganense has been recorded once from soil in the southern region of Iran (Mostowfizadeh-Ghalamfarsa & Banihashemi, 2005).

Here we describe a novel species of the genus Pythium from Lolium perenne under snow showing rot symptoms using a combination of morphology, cultural characteristics, multi-gene sequence data and pathogenicity to the host plant, referred to as the Consolidated Species Concept (Bakhshi et al., 2014, 2015).

METHODS

Isolation. All isolates were recovered from snow-covered grasslands in East-Azarbaijan province, Iran (37° 47' 42" N 46° 14' 55" E). Pieces of

Two supplementary tables and four supplementary figures are available with the online Supplementary Material.

The GenBank/EMBL/DDBJ accession numbers for the ITS sequences of the isolates are KP723165, KP723166, KP723167 and KP723168; those for the coxI gene sequences are KP938425, KP938426, KP938427 and KP938428; and those for the coxII gene sequences are KP723169, KP723170, KP723171 and KP723172.
of diseased leaves of *L. perenne* were washed in tap water and then dried on a paper towel, transferred onto two *Pythium*-selective media, namely NARM (Morita & Tojo, 2007) and VP₃ (Ali-Shtayeh et al., 1986), and incubated at 4 °C for 3 weeks. Pure cultures were established using a hyphal tip technique. Cultures were preserved on PCA (Van der Plaats-Niterink, 1981) slant vials at 4 °C in the dark until use. Detailed information of the isolates is provided in Tables S1 and S2 (available in the online Supplementary Material).

**Morphology and growth temperature relations.** Mycelial patterns of all isolates were recorded 15 days after inoculation at 22 °C on CMA (Becton Dickinson), PDA (Sigma Aldrich), PCA and V8A (Miller, 1955). Morphological observations were made on structures produced on sterile grass blades floated in sterile pond water (Martin, 1992). Thirty measurements were made for each microscopic structure including hyphae, sporangia, oogonia and oospores. Photographs were taken using an Olympus-BX43 microscope with a digital camera system (DP2-ASL) (Olympus). The cultures were deposited in the Culture Collection of Tabriz University (CCTU), Tabriz, Iran, Osaka Prefecture University (OPU) and CBS-KNAW Fungal Biodiversity Centre (CBS). The cardinal temperatures were determined 3 days after inoculation on PCA at temperatures ranging from 0 to 31 °C, with three replicates.

**DNA extraction and amplification.** DNA was extracted from mycelia grown on PDA with a manual process described by Möller et al. (1992). The internal transcribed spacer (ITS) of the rDNA was amplified by PCR using primers ITS1F (TCTCCTCCGTTATGATGC) and ITS2 (GGGATTCAACCTGCTATGGA) (White et al., 1990). Primers FM52 (TTAGAATGGAATTAGCACAAC) and FM55 (GGCATACAGCTAAACCTAA) were also used to amplify the mitochondrial cytochrome oxidase subunit I gene (coxI) (Martin, 2000). Primers FM58 (CCCAAAATTTCTACATTAGAT) and FM66 (TAGGATTGCAAGATCTG) were also used to amplify the mitochondrial cytochrome oxidase subunit II gene (coxII) (Villa et al., 2006). All reactions were carried out in a total volume of 50 µl, containing 5 µl 10× Ex Taq buffer (20 mM Tris/HCl, pH 8.0, 100 mM KCl), 4 µl 2.5 mM dNTP mixture, 0.5 µM of each primer, 1.25 U Taq DNA polymerase (Takara Bio) and 10 ng DNA. Amplifications were performed using a PerkinElmer 9700 thermal cycler (PerkinElmer) with the following cycling profile: 95 °C for 5 min followed by 30 cycles including denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min for ITS and for 5 min for 5 s followed by 40 cycles including denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1 min. A final extension step at 72 °C for 7 min for *coxI* and *coxII*. PCR products were purified with a GenElute PCR Clean-Up kit (Sigma-Aldrich) based on the instructions of the manufacturer and then used for sequence analysis.

**DNA sequencing and phylogenetic analysis.** Amplicons were sequenced in both directions using the same PCR primers as for amplification and a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems) as recommended by the manufacturer and subsequently analysed on a 3130xl Genetic Analyzer (Applied Biosystems). Raw sequence files were edited manually using SeqManII (DNASTAR) and a consensus sequence was generated for each sequence. The raw trace files were inspected and edited with MEGA v. 6 software (Tamura et al., 2013), and consensus sequences were generated manually from the forward and reverse sequences. The consensus sequence for each genomic region was blasted against the NCBIs GenBank sequence database using Megablast to identify the closest neighbours. For each gene, the sequences obtained from GenBank together with sequences generated in this study were aligned using the multiple sequence alignment online interface MAFFT (Katoh & Toh, 2008) and, if necessary, adjusted by eye in MEGA v. 6. For phylogenetic comparison, Bayesian inference (BI) analyses on individual and concatenated ITS and *coxI* loci were performed with MrBayes v. 3.2.1 (Ronquist & Huelsenbeck, 2003). The best evolutionary model for each data partition was obtained using the software MrModelTest v. 2.3 (Nylander, 2004). An initial BI analysis was restricted to an individual alignment of ITS, *coxI* or *coxII* data. Subsequently, a second BI analysis was run using a concatenated alignment for ITS and *coxI*. The heating parameter was set at 0.15 and the Markov Chain Monte Carlo analysis of four chains was started in parallel from a random tree topology and lasted until the average standard deviation of split frequencies came below 0.01. Trees were saved each 100 000 000 generations, and the first 25 % of saved trees were discarded as the ‘burn-in’ phase and posterior probabilities determined from the remaining trees. The resulting phylogenetic tree was printed with Geneious v. 5.6.7 (Drummond et al., 2012). Sequences derived from this study were lodged at NCBI’s GenBank nucleotide database (http://www.ncbi.nlm.nih.gov; Tables S1 and S2).

**Pathogenicity assay.** Pathogenicity was studied using the isolates CCTU 1811, CCTU 1813 and CCTU 1814 in pot experiments. Inoculum was prepared by growing the isolates on autoclaved *Poo pratensis* leaves at room temperature for 3 weeks. Each of nine 1 cm diameter plastic pots containing potting mixture (Aisai I, Katakura Chikkarin) received 0.8 g of *L. perenne* ‘Reg-Z’ seeds (Snow Brand Seed) and was inoculated for 3 weeks in a greenhouse. Plant tops were cut to 1.5 cm high and inoculated with 4 g of the inoculum on the leaves. Inoculated plants were covered with a wet cotton sheet, enclosed in a plastic bag and incubated at 0–3 °C for 8 weeks in darkness. After the incubation, plants were transferred to the greenhouse and incubated for 2 weeks to observe the regrowth of leaves. The severity of leaf damage was recorded with Motic Image Plus 2.2 S software (Shimadzu Rika) as the percentage of leaves showing symptoms. Colonization of the isolates on *L. perenne* and formation of oogonia within leaf tissues was examined by optical microscopic observation (×1000) (Fig. S1). The inoculated isolates were reisolated from infected leaf tissue using selective VP₃ (Ali-Shtayeh et al., 1986) or NARM (Morita & Tojo, 2007) media. The means of the data were compared by least significant difference analysis using the Tukey–Kramer honestly significant difference (HSD) test (*P*<0.05) using JMP software (version 8; SAS Institute). There were nine replications per isolate with an equal number of control plants inoculated with autoclaved leaves.

**RESULTS AND DISCUSSION**

**Morphology and growth temperature response**

All four isolates of *Pythium kandovanense* sp. nov. had globose, cylindrical, intercalary and terminal lemon-shape sporangia, and oogonia with two or rarely one antheridia that contained one or two plerotic oospores (Fig. 1). Such morphological features of *Pythium kandovanense* sp. nov. resembled those of *Pythium rostratifingens* de Cock & Lévesque and *Pythium rostratum* Butler. *Pythium kandovanense* sp. nov. differed from *Pythium rostratifingens* in shorter discharge tubes of sporangia and could be distinguished from both *Pythium rostratifingens* and *Pythium rostratum* by the presence of cylindrical sporangia and lower optimum and maximum growth temperatures (Table 1). *Pythium kandovanense* sp. nov. isolates were able to produce zoospores only at 0 to 5 °C. This feature might be a unique feature of *Pythium kandovanense* sp. nov.; however, there is no data available on this feature.
for *Pythium rostratifingens* and *Pythium rostratum* for comparison. The main hyphae were up to 4–7 mm wide. All isolates of *Pythium kandovanense* sp. nov. had similar mycelial patterns, i.e. chrysanthemum pattern on V8A, submerged to stellate on CMA and PCA, and rosette pattern on PDA (Fig. 1). Optimum growth occurred at 22°C on PCA, with the mean daily growth rate of 5.5 mm. The maximum growth temperature was 28°C (Fig. S2).

**Phylogenetic position**

Four isolates of *Pythium kandovanense* sp. nov. were subjected to DNA sequence analyses.

**Single gene phylogeny.** The final aligned ITS and *coxI* dataset contained 31 ingroup taxa with a total of 837 and 549 characters, containing 441 and 107 unique site patterns, respectively. While the *coxII* dataset contained 19 ingroup taxa with a total of 562 characters, containing 81 unique site patterns. *Phytophthora capsici* (GenBank accession HQ643188) served as the outgroup taxon. The heating parameter was set to 0.15. The results of MrModeltest recommended a HKY + G model with a gamma distributed rate variation for ITS and GTR + G model with gamma-distributed rates for *coxI* and *coxII*, and Dirichlet base frequencies. During the generation of the ITS tree, a total of 1792 trees were saved, and consensus trees and posterior probabilities were calculated from the remaining 1344 (75 %) trees. For the *coxI* tree, a total of 1552 trees were saved, and consensus trees and posterior probabilities were calculated from the remaining 1164 (75 %) trees. For the *coxII* tree, a total of 2402 trees were saved and consensus trees and posterior probabilities were calculated from the remaining 1802 (75 %) trees. Based on the results of the ITS and *coxI* sequence data, all of the *Pythium kandovanense* sp. nov. isolates obtained in this study resided in the unique well-supported clade in the *Pythium* group E clade (Figs S3 and S4). The topology of the *coxII* tree was in congruence with ITS and *coxI*. *Pythium kandovanense* sp. nov. isolates clustered in a unique clade (data not shown).

**Multi-gene phylogeny.** In the multi-gene analyses (gene boundaries of ITS: 1–837 and *coxI*: 848–1396) of 27 isolates of *Pythium* clade E and four isolates from this study, 1386 characters including the alignment gaps were used and these characters contained 548 unique site patterns (441 and 107 for ITS and *coxI*, respectively). *Phytophthora capsici* (CBS 111333) was used as an outgroup in the phylogenetic analyses on the basis of its position as a sister genus to *Pythium*. The results of MrModeltest recommended a HKY + G and GTR + G with

**Fig. 1.** *Pythium kandovanense* sp. nov. Colony morphology of isolate CCTU 1813T on V8A (a), CMA (b), PDA (c) and PCA (d). (e) Oogonium with one antheridium. (f) Globose sporangium. (g, h) Terminal and intercalary lemon-shape sporangium. (i) Plerotic oospore. (j) Oogonium with two monoclinous antheridia. (k) Oogonium with diclinous antheridium. (l) Encysted zoospore. (m) Cylindrical sporangium. (n) Oogonium with two oospores. (o) Sporangium with papilla-like protuberance that subsequently develops into a discharge tube. (p) Empty sporangium after discharge of zoospores. (q) Zoospores released from a vesicle. (r) Internal proliferation of sporangium. Bars 10 μm (e–k and m) and 20 μm (n–r and l).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th><em>Pythium kandovanense</em> sp. nov. CCTU 1813&lt;sup&gt;T&lt;/sup&gt;</th>
<th><em>Pythium rostratifingens</em> (de Cock &amp; Lévesque, 2004)</th>
<th><em>P. rostratum</em> (Van der Plaats-Niterink, 1981)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily growth on PCA</td>
<td>5.5 mm</td>
<td>9 mm</td>
<td>8 mm</td>
</tr>
<tr>
<td>Minimum temperature for mycelial growth</td>
<td>Below 0 °C</td>
<td>Below 3 °C</td>
<td>Below 5 °C</td>
</tr>
<tr>
<td>Optimum temperature for mycelial growth</td>
<td>22 °C</td>
<td>27 °C</td>
<td>25 °C</td>
</tr>
<tr>
<td>Maximum temperature for mycelial growth</td>
<td>28 °C</td>
<td>33–36 °C</td>
<td>35 °C</td>
</tr>
<tr>
<td>Width of main hyphae</td>
<td>4–7 μm</td>
<td>up to 7 μm</td>
<td>up to 8 μm</td>
</tr>
<tr>
<td>Globose sporangium</td>
<td>Intercalary and terminal, 18–34.5 μm in diam.</td>
<td>Intercalary, occasionally terminal, occasionally oval, (11–)16–27 μm in diam.</td>
<td>(17–)19–32 μm</td>
</tr>
<tr>
<td>Cylindrical sporangium</td>
<td>33–42 μm length, 17.5–24 μm width</td>
<td>Not formed</td>
<td>Not formed</td>
</tr>
<tr>
<td>Ellipsoidal sporangium</td>
<td>Not formed</td>
<td>Present (oval shape)</td>
<td>Present</td>
</tr>
<tr>
<td>Lemon-shape sporangium</td>
<td>10–45 μm length, 17.5–38 μm width, with one or two protuberances</td>
<td>Not formed</td>
<td>Present, but without protuberance</td>
</tr>
<tr>
<td>Proliferation of sporangia</td>
<td>Internally extended</td>
<td>Many sporangia do not develop spores but may germinate directly by one or more hyphae</td>
<td>Non-proliferating</td>
</tr>
<tr>
<td>Zoospores</td>
<td>Formed at 0–5 °C, encysted zoospores 9–12 μm in diam.</td>
<td>Up to 30 μm length, 5–10 μm width</td>
<td>Variable length, about 5 μm width</td>
</tr>
<tr>
<td>Discharge tube</td>
<td>5–8 μm length, 2.5–4 μm width</td>
<td>Intercalary, occasionally terminal, globose, smooth, colourless, 11–22 μm in diam.</td>
<td>Smooth, (sub)globose, mostly intercalary, occasionally terminal, (17–)19–24(–26) μm in diam., often in chains</td>
</tr>
<tr>
<td>Oogonia</td>
<td>Globose, smooth, intercalary or terminal, 20–32 μm in diam.</td>
<td>1–4, mostly two per oogonium, monoclinous, occasionally diclinous, sessile, on a short stalk or hypogynous</td>
<td>1–2 per oogonium, monolinous, mostly sessile and arising immediately below the oogonium or hypogynous</td>
</tr>
<tr>
<td>Antheridia</td>
<td>Usually two and rarely one per oogonium, diclinous or monoclinous</td>
<td>Plerotic</td>
<td>Plerotic</td>
</tr>
<tr>
<td>Oospore</td>
<td>Plerotic, one or two per oogonium, 12.5–25 μm in diam.</td>
<td>Up to 1.5 μm</td>
<td>Up to 2 μm</td>
</tr>
<tr>
<td>Wall thickness of oospore</td>
<td>Up to 1 μm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
gamma rate variation for ITS and coxl, respectively. All partitions had Dirichlet base frequencies. The Bayesian analysis lasted 245,000 generations and saved a total of 492 trees. After discarding the first 25% of sampled trees for burn-in, the consensus trees and posterior probabilities were calculated from the remaining 370 trees. Phylogenetic inference based upon combined sequence data of the ITS and coxl region further ascertained the phylogenetic position of Pythium kandovanense sp. nov. as a unique clade in the Pythium E group. The situation was the same for the other closely related species, each of which clustered in well-supported separate clades (Fig. 2).

**Pathogenicity**

The results of pathogenicity assays revealed Pythium kandovanense sp. nov. isolates being pathogenic on L. perenne. Disease symptoms initially appeared as water-soaked lesions on leaves. The lesions then coalesced and gave the leaves a blighted appearance. Leaves failed to resume growth on inoculated plants; however, regrowth was observed on control plants. Disease rates for CCTU 1811, CCTU 1813T and CCTU 1814 in inoculated plants were 73.8 ± 16.5%, 74.4 ± 16.6% and 77.7 ± 19.2%, respectively. No significant differences were observed in the disease rates induced by different isolates according to a Tukey–Kramer honestly significant difference (HSD) test (P < 0.05). Oospores of Pythium kandovanense sp. nov. were frequently found within cells of inoculated plants (Fig. S1). Pythium kandovanense sp. nov. was consistently reisolated from inoculated plants (100%) but not from the control plants (0%).

Species of the genus Pythium belonging to clade E have been mostly isolated from water, debris or soil. Some of

![Fig. 2. Bayesian inference phylogenetic tree of 31 isolates of Pythium clade E. The tree was reconstructed using concatenated sequences of the ITS and coxl gene each with a separate model of DNA evolution. The tree was rooted to Phytophthora capsici CBS 111333. Bar, number of expected changes per site.](image-url)
them occur worldwide, but have not been known as devastating pathogens of crop plants (Lévesque & de Cock, 2004).

**Description of Pythium kandovanense** Chenari Bouket, Arzanlou, Tojo & Babai-Ahari sp. nov. *Pythium kandovanense* (kan.do.van.en.'se. kandovanense named after the region where the type was isolated, Kandovan, East-Azarbaijan, Iran).

MycoBank number: MB 811554

Colonies on V8A, CMA, PCA and PDA chrysanthemum, submerged to stellate and rosette patterns, respectively. Daily growth at 22 °C on PCA 5.5 mm. Cardinal temperatures on PCA: minimum below 0 °C, optimum 22 °C, and maximum between 28 and 31 °C. Main hyphae hyaline, aseptate, up to 4–7 μm wide. Globose sporangia intercalary or terminal, 18–34.5 μm (mean, 26 μm) in diameter; cylindrical sporangia 33–42 μm (mean, 33 μm) length and 17.5–24 μm (mean, 20 μm) width; and lemon-shaped sporangia 10–45 μm (mean, 36 μm) length and 17.5–38 μm (mean, 22 μm) width. Sporangia with internally extended proliferation. Discharge tubes 5–8 μm length, 2.5–4 μm width. Zoospores form at 5 °C and lower. Encysted zoospores 9–12 μm in diameter. Oogonia produced in single culture, globose, smooth, intercalary or terminal, 20–32 μm (mean, 26 μm) in diameter. Antheridia, usually two and rarely one per oogonium, diclinous or monoclinois. Oospores plerotic, one or two per oogonium, 12.5–25 μm (mean, 19 μm) in diameter. Wall thickness up to 1 μm.

Material examined: Iran, East-Azarbaijan province, Kandovan, leaves of snow-covered *Lolium perenne* (Poaceae), April 2014, culture preserved as ex-type CCTU 1813T = OPU 1626T = CBS 139567T.

**ACKNOWLEDGEMENTS**

Special thanks are due to the Research Deputy of the University of Tabriz and the Studienstiftung für mykologische Systematik und Ökologie for funding, National Institute of Polar Research (research project 25–25) and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (research nos. 23510302 and 23247012). We acknowledge Dr Mounes Bakhshi for valuable help in phylogenetic analysis. The first author also thanks Mr Amin Khodabandeh, Mr Abolfazl Narmani, Mrs Shoko Ueta and Mr Xiaodong You for their great technical assistance.

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