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Two plant pathogenic species of *Phytophthora* associated with stem blight of Easter lily and crown rot of lettuce in Japan

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ABSTRACT

New diseases of Easter lily and lettuce were noticed in Japan and the pathogens were identified initially as *Phytophthora megasperma* and *Phytophthora* sp., respectively, based on morphological characteristics. We re-examined these isolates using phylogenetical and morphological analysis. In a detailed phylogenetic analysis of all clade members that was based on the sequences of eight genomic regions, Easter lily isolates formed a monophyletic group with high bootstrap support. The unique colony pattern supported this result. In separate analyses of *Phytophthora* clade 8 based on rRNA gene ITS and the *cox*1 gene sequences, the lettuce isolates grouped with *P. lactucae* (described from lettuce in Greece) in the rRNA gene ITS tree but formed a distinct group in the *cox*1 tree. The sporangia of Japanese lettuce isolates germinated directly, an uncommon feature in clade 8b. These results suggested that the Easter lily and lettuce isolates are novel species that we formally describe as *Phytophthora lilii* sp. nov. and *P. pseudolactucae* sp. nov., respectively. Moreover, *P. lilii* appears to represent a new *Phytophthora* clade.

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1. Introduction

Easter lily (*Lilium longiflorum* Thunb., Liliaceae) is one of the economically most important ornamentals in Japan. Easter lily bulbs are the most frequently exported materials for plant propagation in Japan. Ever since the Meiji period, Easter lilies have been cultivated extensively on the Okinoerabu Island of Kagoshima Prefecture (Kimishima et al. 1988). Stem blight of Easter lily was reported by Kimishima and colleagues in 1988 (Kimishima et al. 1988). Pathogenic isolates from affected plants were identified as *Phytophthora* based on serological and ELISA (Enzyme Linked Immune Sorbent Assay) tests, and as *P. megasperma* Drechsler based on morphological and physiological characteristics. The pathogen showed a narrow host range limited to Easter lily in pathogenicity tests (Kimishima et al. 1988). Twenty-two hosts from 11 families were inoculated with the Easter lily isolates and only *Tulipa gesneriana* L. (Liliaceae) showed slight infection symptoms. Villa et al. (2006) included the Easter lily isolate NBRC 32176 in their phylogenetic analysis in which it was positioned on an isolated branch. In a recent study, the Easter lily isolates were considered to represent a new taxon (Rahman et al. 2014).

In lettuce (*Lactuca sativa* L., Asteraceae), the most consumed vegetable in Japan, various pathogens are causing huge losses every year. A new disease called "lettuce Phytophthora wilt" was noticed by Kanto in Hyogo Prefecture in 2004 (Kanto et al. 2004). The causal agent was temporarily identified as *Phytophthora* sp. based on complex morphological characteristics; it was considered closely related to *P. brassicae* de Cock & Man in't Veld. More recently, this isolate has been identified as a new taxon (Rahman et al. 2014). Moreover, Bertier et al. (2013) described several other lettuce isolates collected from Greece as new species.

In oomycetes such as *Phytophthora*, species identification based on morphological characteristics alone is not always reliable, because morphological characters are variable and overlapping among species. Modern molecular techniques facilitate the accurate differentiation of *Phytophthora* species. According to the molecular phylogenies produced by Cooke et al. (2000), Kroon et al. (2004) and Blair et al. (2008), the genus *Phytophthora* consists of 10 clades. In the first of these comprehensive phylogenetic analyses, Cooke et al. (2000) established two sub-clades of clade 8, namely 8a and 8b; sub-clade 8b initially comprised *P. porri* Foister (Foister 1931), *P. primulae* J.A. Tomlinson (Tomlinson 1952), and *P. syringae* (Klebahn) Klebahn (Klebahn 1909). Man in't Veld et al. (2002) segregated *P. brassicae* from *P. porri*, increasing the number of species in sub-clade 8b from three to four. Bertier et al. (2013) further expanded sub-clade 8b by adding three new species (*P. lactucae* Bertier, H. Brouwer & de Cock, *P. dauci* Bertier, H. Brouwer & de Cock, *P. cichorii* Bertier, H. Brouwer, de Cock & D.E.L. Cooke) and two taxa (*P.* taxon castitis, *P.* taxon parsley).

The aim of this study was to resolve the taxonomic ambiguities of the Japanese Easter lily and lettuce isolates, using morphological and physiological characteristics as well as a molecular phylogenetic analyses.

2. Materials and methods

2.1. Oomycete isolates

The Easter lily isolates (Table 1) were obtained from diseased stems using a selective medium according to Masago et al. (1977). The lettuce isolates (Table 1) from Kagawa Prefecture were obtained from diseased crowns. The diseased crowns were disinfected with 70% ethanol for 10 s, followed by rinsing three times with sterilized water. Crown segments were dried on sterilized filter paper and then placed on corn meal agar (CMA; 20 g corn meal in 1 l of water were steamed for 30 min, the extract was filtered, 20 g agar and water was added to adjust the volume to 1 l, and the solution was autoclaved for 1 h at 121 °C). The specimen from Hyogo Prefecture (Table 1) was disinfected with 70% ethanol for 30 s and 1% sodium hydrochloride solution for 2 min, followed by rinsing three times with sterile water. The segments were placed on 1% water-agar medium after they were dried on sterilized filter paper. All isolates were maintained on CMA slopes at 20–25 °C, at Gifu University, Japan. Representative cultures are also available at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands (CBS), the NITE Biological Resource Centre, Kisarazu, Japan (NBRC), and the NIAS Genebank, Microorganism Section, Genetic Resources Center, National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan (MAFF).

2.2. Molecular phylogeny

2.2.1. DNA isolation

The isolates (Table 1) were allowed to grow for 7 d on V8A (100 ml V-8 juice, 2.5 g $CaCO_3$, 20 g agar and 900 ml distilled water). Most of the isolates were grown at 25 °C, while some were grown at between 20 °C and 25 °C. For each DNA sample, a small amount of mycelial mat from an advanced growth area was collected in 100 μl of 50% PrepMan Ultra Reagent (Applied Biosystems, Foster city, CA, USA) and heated to 100 °C for 10 min. After 3 min at room temperature, the sample was centrifuged at 21,880 *g* for 3 min. The supernatant was transferred to another tube and 100 μl TE buffer (10 mM Tris–HCl, pH 7.5 and 0.1 mM EDTA) were added.

2.2.2. DNA amplification

The sequences of the rRNA gene internal transcribed spacer (ITS) region, rRNA gene large subunit (LSU), translation elongation factor 1 alpha (*EF1-α*), β-tubulin, 60S ribosomal protein L10 (60SL10), heat shock protein 90 (HSP90), triose phosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase (tigA), and the mitochondrial *cox*1 genes were amplified using the primer sets listed in Table 2. We used 25 μl reaction mixtures containing 1 μl DNA, 0.2 μM of each primer for rRNA gene regions and 2.0 μM of each primer for other genes, 0.4 mg/ml BSA, 0.2 mM dNTP for rRNA gene regions and 0.4 mM dNTP for other genes, 0.625 units of rTaq DNA polymerase (Takara Bio, Otsu, Japan), and PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). The PCR reactions were carried out using a 2700 DNA thermal cycler (Applied Biosystems). The reaction