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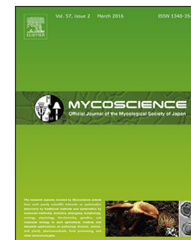

Full paper

Phylogenetic relationship of Japanese isolates belonging to the *Grosmannia piceiperda* complex (Ophiostomatales)

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ABSTRACT

This study was conducted to explore the morphological and phylogenetic relationships of the Japanese isolates tentatively identified as the *Grosmannia piceiperda* complex (Ophiostomatales, Ascomycota; asexual morph = *Leptographium* s.l.). The phylogenetic analyses of the internal transcribed spacer 2 and nuclear large subunit ribosomal RNA gene regions showed that the Japanese isolates belong to the *G. piceiperda* complex. The analyses of the partial β-tubulin and translation elongation factor-1 alpha (EF-1α) genes separated the species complex into nine and 13 lineages, respectively. Based on the β-tubulin sequences, morphologically distinct groups were included in the same clades. However, based on the EF-1α sequences, these groups could be separated into different lineages. Detailed morphological observations supported this result and confirmed that the Japanese lineages are distinct. Among the 13 recognized lineages, the Japanese isolates were separated into 11 lineages, including *G. aenigmatica*, *G. laricis*, *G. piceiperda* D, as well as eight lineages representing undescribed taxa.

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1. Introduction

The genus *Grosmannia* Goid. (Ophiostomatales, Ascomycota) is characterized by black perithecia with or without necks and that asexual morph is *Leptographium* Lagerb. & Melin (Zipfel et al. 2006). This genus had been treated as synonymous with *Ophiostoma* Syd. & P. Syd. and *Ceratocystis* Ellis & Halst. (Hunt 1956; Upadhyay 1981; Jacobs and Wingfield 2001).

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Zipfel et al. (2006) separated *Grosmannia* from *Ophiostoma* based on molecular phylogenetic analysis and characterized this genus based on the specific morphology of sexual and asexual morphs. In a recent phylogenetic study on Ophiostomatales, it was shown that *Grosmannia* and *Leptographium* do not form a well-supported monophyly, and they were together referred to as *Leptographium* s.l. (De Beer and Wingfield 2013). Species of these fungi are known to associate with conifer-infesting bark beetles (Coleoptera: Curculionidae, Scolytinae), and some species are causal agents of tree diseases (Harrington and Cobb 1988; Wingfield et al. 1993; Jacobs and Wingfield 2001).

Recently, Linnakoski et al. (2012) conducted phylogenetic analyses of *Grosmannia* and *Leptographium* based on the internal transcribed spacer (ITS) 2 and partial nuclear large subunit (LSU) ribosomal RNA (rRNA) gene regions, and concluded that nine species complexes exist within this group. In addition to this study, the most recent phylogenetic study included the *Raffaelea sulphurea* complex in *Leptographium* s.l. (De Beer and Wingfield 2013). Among the currently recognized species complexes in *Leptographium* s.l., the *G. piceiperda* complex is characterized by species with cucullate ascospores and leptographium-like asexual morphs (De Beer and Wingfield 2013). Recognized species in this complex are *Grosmannia aenigmatica* (K. Jacobs et al.) Zipfel et al., *G. europhioides* (E.F. Wright & Cain) Zipfel et al., *G. laricis* (K. van der Westh. et al.) Zipfel et al., and *G. piceiperda* (Rumbold) Goid. (De Beer et al. 2013).

Grosmannia piceiperda was described by Rumbold (1936) as a blue-stain fungus of *Picea glauca* infested with the bark beetle *Dendroctonus rufipennis* (as *D. piceaperda*) in Canada. Later, this species was isolated from many other bark beetle species infesting conifers (especially *Pinus* spp., *Picea* spp.) in various countries (Jacobs and Wingfield 2001; De Beer et al. 2013). *Grosmannia europhioides* was described by Wright and Cain (1961) from *Picea* spp. and *Pinus* spp. in Canada. The taxonomic treatments of these two species have been controversial. Some studies proposed that *G. piceiperda* and *G. europhioides* are conspecific (Upadhyay 1981; Jacobs et al. 2000), while Solheim (1986), Harrington and Cobb (1988), and Yamaoka et al. (1997) treated them as distinct. *Grosmannia laricis* was described by Van der Westhuizen et al. (1995) from *Ips subelongatus* (as *I. cembrae*) infesting *Larix kaempferi* in Japan. This species is characterized by curved ascospores instead of cucullate ascospores. *Grosmannia aenigmatica* was described by Jacobs et al. (1998) from *I. typographus japonicus* infesting *Pic. jezoensis* and is characterized by short perithecial necks. *Grosmannia laricis* and *G. aenigmatica* have been reported only from Japan and have been treated as individual species in the *G. piceiperda* complex (Linnakoski et al. 2012; De Beer and Wingfield 2013).

Phylogenetic analyses of the *G. piceiperda* complex based on the partial β-tubulin and translation elongation factor-1 alpha (EF-1α) genes showed that the complex could be separated into seven lineages (Linnakoski et al. 2012). Two lineages represented *G. laricis* and *G. aenigmatica*, and the remaining five lineages were composed of three North American lineages, one Russian lineage, and one European lineage. Linnakoski et al. (2012) suggested that these lineages might include novel cryptic taxa.

Isolates collected during previous studies from bark beetle infesting conifers in Japan morphologically resemble species belonging to the *G. piceiperda* complex (Yamaoka et al. 1998, 2004, 2009). However, the phylogenetic relationships of these isolates remain unknown and their taxonomic positions unresolved. The aims of this study were to clarify the taxonomic placement of these isolates based on sequences of the ITS2 and LSU rRNA gene regions, partial β-tubulin, and EF-1α genes, and also to characterize their morphology.

2. Materials and methods

2.1. Fungal isolates

A total of 53 isolates resembling species of the *G. piceiperda* complex were used in this study (Table 1). These isolates were obtained from 15 different bark beetle species (in seven genera), infesting 11 species of conifers (in four genera), and were collected between 1989 and 2008 in Hokkaido, Iwate, Tochigi, Yamanashi, and Nagano prefectures in Japan. Some isolates have been reported previously (Yamaoka et al. 1997, 1998, 2004, 2009).

2.2. Phylogenetic analyses

2.2.1. DNA extraction, PCR amplification, and sequencing

All 53 isolates were used for molecular phylogenetic analyses. Isolates were cultured on 2% malt extract Ebios agar (MEBA) [2% MEBA; 20 g Difco malt extract, 1 g Ebios (Brewer's yeast preparation; Asahi food and healthcare Co., Tokyo, Japan), 15 g agar, 1000 ml distilled water] for 2 wk. DNA was extracted as described by Uzuhashi et al. (2008).

The ITS and LSU rRNA gene regions and portions of the β-tubulin and EF-1α genes were amplified by polymerase chain reaction (PCR) using the primers ITS5 and ITS4 (White et al. 1990) for ITS; ITS3 (White et al. 1990) and LR3 (Vilgalys and Hester 1990) for ITS2-LSU; NL1 and NL4 (O'Donnell 1993) for LSU; T10 (O'Donnell and Cigelnik 1997) and BT12 (Kim et al. 2003) for β-tubulin; and EF-1F and EF-2R (Jacobs et al. 2004) for EF-1α. Amplification was performed in a PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the following protocol; initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s (ITS), 58 °C for 45 s (ITS2-LSU), 57 °C for 30 s (LSU), 55 °C for 60 s (β-tubulin), or 60 °C for 50 s (EF-1α), extension at 72 °C for 1 min, and a final extension at 72 °C for 8 min.

PCR products were separated on 1% agarose gels stained with ethidium bromide and visualized under UV light. PCR products were purified using Wizard® SV Gel and PCR Clean-Up Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Sequencing was performed using the BigDye™ Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems) following the manufacturer's instructions using both forward and reverse primers and analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited with BioEdit ver. 7.1.9 (Hall 1999), and were deposited in GenBank (Table 1).

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