Phylogeny and morphology of new species of Globisporangium

S. Uzuhashi1,2, S. Nakagawa2, H.M.A. Abdelzaher3, M. Tojo2

1Genetic Resources Center, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
2Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Gakuen-cho 1-1, Naka-ku, Sakai, Osaka 599-8531, Japan
3Department of Botany and Microbiology, Faculty of Science, Minia University 61519, Minia city, Egypt

*Corresponding author: uzuhashi@affrc.go.jp

Abstract: An isolate originally obtained from pond water in Osaka in 1992 and identified as Pythium marsipium, was subsequently classified as Globisporangium marsipium. According to molecular phylogenetic analyses based on the internal transcribed spacer regions of the nuclear ribosomal RNA and mitochondrial cytochrome c oxidase subunit 1 genes, this isolate was shown to represent a new species, described here as G. lacustre sp. nov. In addition, two further new combinations are introduced in Globisporangium as G. camurandrum and G. takayamanum based on their DNA phylogeny.

Key words: Molecular phylogeny, new taxa, pond water, Pythium, re-identification

INTRODUCTION

Oomycetes are fungal-like organisms belonging to the kingdom Straminipila. The oomycete genus Globisporangium was segregated from the genus Pythium based on morphology and phylogeny in 2010 (Uzuhashi et al. 2010). Traditionally, species identification of Pythium s. lat., including Globisporangium, was based on morphological characteristics. Because the use of DNA for species identification is well established, many new species of Pythium s. lat have been described based on DNA sequences in addition to their morphology (e.g. Bouket et al. 2015, Uzuhashi et al. 2015, Ueta & Tojo 2016). In particular, the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene and the cytochrome c oxidase subunit 1 gene (cox1) are known as useful regions for species identification, and are recognised as DNA barcode markers for oomycetes (Robideau et al. 2011).

Proper identification of Globisporangium spp. is important not only for taxonomic studies but also biological studies, because the genus is widely distributed throughout the world, and some species have highly important ecological roles or economic impacts (e.g. Zhang & Yang 2000, Mùnera & Hausbeck 2016). Additionally, taxonomic evaluation of previously collected strains is also important, especially for those that have only been identified based on morphology. One strain, MAFF 236903, stored in NARO Genebank, Microorganisms Section (MAFF), Genetic Resources Center, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan, was obtained from pond water in Osaka, Japan, in 1992. It was initially identified as P. marsipium, which is now classified as G. marsipium, based on its morphology (Abdelzaher et al. 1994). We evaluated the identification of this strain using molecular phylogenetic analyses based on the ITS and cox1 regions. The result demonstrated that this strain is uniquely different from G. marsipium, and it is consequently redescribed here as Globisporangium lacustre sp. nov. We also provide updated temperature growth profiles and phylogenetic information for this new species. Furthermore, two new combinations in Globisporangium are introduced based on the molecular phylogenetic analyses generated here.

MATERIALS AND METHODS

Isolates and morphology

Strain MAFF 236903 and the reference strain of G. marsipium (CBS 773.81) were examined. Colony patterns of the two strains were recorded on potato carrot agar plates (PCA) prepared in accordance with van der Plaats-Niterink (1981), potato dextrose agar (PDA), and V8 juice agar (V8A) plates according to Miller (1995) after incubation for 8 d at 25 °C. Morphological characteristics were examined in a grass blade water culture (van der Plaats-Niterink 1981). At least 30 measurements were taken of each strain. Hyphal growth rate was also determined on PCA, as reported previously (Uzuhashi et al. 2017). The isolates were incubated on PCA at 3 °C intervals from 0–40 °C for 1–3 d. Hyphal growth was evaluated by measuring the average increase in colony diameter. The experiment had two replicates and was repeated twice.

DNA extraction and phylogenetic analysis

DNA extraction from the strain was performed as reported previously (Uzuhashi et al. 2017). The ITS and cox1 regions were amplified using the primer-pair ITS5/ITS4 (White et al. 1990) for ITS and OomCoxI-Levup/OomCoxI-Levlo (Robideau et al. 2011) for cox1. Amplification reactions and sequencing were conducted as reported previously (Uzuhashi et al. 2017).

Effectively published online: 28 November 2018.
The sequences of the two regions were aligned separately with relevant *Globisporangium* sequences obtained from the GenBank database using the ClustalW program included in MEGA7 (Kumar et al. 2016). Because *G. marsipium* is known to be located in clade E described by Lévesque & de Cock (2004) (e.g. Lévesque & de Cock 2004, Uzuhashi et al. 2010), sequence data of *Globisporangium* species in clade E were included for phylogenetic analyses as well as *G. splendens* and *G. intermedium* as outgroups. All alignments were submitted to TreeBASE under accession number 20573. Phylogenetic analyses based on these regions were conducted using MEGA7 with the Neighbour-Joining (NJ) and Maximum Likelihood (ML) phylogenetic methods, as reported previously (Uzuhashi et al. 2017). All of the positions containing gaps and missing data were eliminated. The strength of the internal branches in the trees obtained were tested by bootstrap analysis using 1 000 replications. The sequences of MAFF 236903 were deposited in DDBJ under the accession numbers LC209786 for ITS and LC209787 for cox1.

**RESULTS**

**Morphology and hyphal growth**

Morphological characteristics of MAFF 236903 have been described in detail previously (Abdelzaher et al. 1994). In this study, sexual structures such as oogonia, antheridia, and oospores were not formed in any artificial cultures, although sporangia and zoospores were abundantly produced in water culture (Fig. 1). Additionally, reference strain *G. marsipium* CBS 773.81 formed no spores in water culture as well as agar cultures. The morphological characteristics of MAFF 236903 were quite similar to those of *G. marsipium* (Table 1). The main difference was the production of yellowish oospores by MAFF 236903. Additionally, MAFF 236903 produced slightly larger oogonia and oospores and greater numbers of antheridia (Table 1).

The colony patterns of MAFF 236903 were different from those of *G. marsipium* CBS 773.81 on all three agar plates, especially on PCA and PDA (Fig. 2). Colonies of MAFF 236903 were submerged with a chrysanthemum pattern on PCA and coarse rosette pattern with aerial mycelium on PDA, but no specific pattern on V8A. On the other hand, CBS 773.81 showed
no specific patterns on any agar plates (Fig. 2D–F). The hyphal growth rate of MAFF 236903 determined in this study was completely different from that of CBS 773.81. In this study, MAFF 236903 grew at 10–37 °C with an optimum temperature of 31 °C. On the other hand, CBS 773.81 could be grown at 16–31 °C, also with an optimum temperature at 31 °C. Additionally, the radial growth rate of MAFF 236903 on PCA at 25 °C was 25 mm in this study, but just 5 mm in CBS 773.81 (Fig. 3).

Sequencing and phylogeny

The ITS and cox1 sequences of MAFF 236903 had 85 % and 93 % similarities with those of G. marsipium CBS 773.81, respectively. For both the ITS or cox1 regions tree topologies obtained through NJ and ML analyses were similar. In phylogenetic analyses based on these two regions, MAFF 236903 was located in a sister-group position to the clade of G. marsipium in both trees (Figs 4, 5).

TAXONOMY

**Globisporangium lacustre** Uzuhashi & Tojo, *sp. nov*. MycoBank MB819698. Figs 1, 2.

*Etymology*: lacustre refers to the origin (pond water) of the isolate.

*Colonies* submerged, forming a rosette pattern on PDA, submerged, with a radiate pattern on potato-carrot agar (PCA). Daily growth at 25 °C on PCA 25 mm. *Cardinal temperatures* minimum 10 °C, optimum 31 °C, maximum 37 °C. *Main hyphae* up to 7 µm wide. *Appressoria* club-shaped. *Sporangia* terminal, occasionally intercalary, subspherical, pyriform, irregular longitudinal or often unsymmetrically utriform, papillate. Subspherical, 20–70 µm diam, pear-shaped, 16–25 × 10–15 µm diam, utriform 25–68 × 20–45 µm, internally proliferating. *Encysted zoospores*, 9–12 µm diam. *Oogonia* produced in single culture, globose, smooth-
|                         | **G. marsipium** | **MAFF 236903** |
|-------------------------|------------------|-----------------|
| Cardinal temperature for hyphal growth (°C) | 16–31            | 10–37           |
| Daily growth at 25 °C on PCA (mm)               | 5                | 25              |
| Width of hyphae (µm)                              | Up to 7.5        | Up to 7        |
| Sporangium (Sp) production                        | Globose or asymmetrically utriform, papillate, beaked, often bent, transversely attached on hypha branches | Subspherical, pyriform, irregular longitudinal, often unsymmetrically utriform, papillate |
| Diameter of Sp (µm)                               | 20–100 × 3–4 (terminal) | 20–70 (subspherical) |
|                                                      | 25–70 (intercalary) | 16–25 × 10–15 (pear-shaped) |
|                                                      | 25–68 × 20–45 (utriform) |               |
| Position of Sp                                     | Mostly terminal, occasionally intercalary | Terminal, occasionally intercalary |
| Zoospore production                               | Produced         | Produced        |
| Oogonium diameter (µm)                            | (23–)27–36 (~39) (av. 31) | 26–39 (av. 33) |
| Position of oogonia                               | Mostly intercalary, occasionally catenulate, sometimes subterminal | Terminal or subterminal, mostly intercalary, occasionally catenulate |
| Oogonium ornamentation                             | Smooth           | Smooth          |
| Oospare diameter (µm)                             | (19–)23–31 (~33) (av. 26) | 20–35 (av. 28) |
| Oospore wall thickness (µm)                        | Up to 2.8        | 1.5–2.5         |
| Plerotic or aplerotic oosposes                    | Aplerotic        | Aplerotic, usually yellowish |
| Number of oosposes/oogonia                        | 1                | 1               |
| Number of antheridia/oogonia                      | 1–4              | 1–8             |
| Monoclinous or diclinous antheridium              | Diclinous        | Diclinous       |
| Antheridia                                         | 10–20 × 8–12, making broad apical contact with the oogonium | 10–27 × 8–12, apical contact with the oogonium, often persisting after fertilization |

1 van der Plaats-Niterink (1981) except for cardinal temperature for hyphal growth and daily growth at 25 °C on PCA obtained from this study.

2 Abdelzaher et al. (1994) except for cardinal temperature for hyphal growth and daily growth at 25 °C on PCA obtained from this study.

---

**Fig. 4.** Maximum Likelihood (ML) tree based on ITS sequences showing the relationship between MAFF 236903 (*Globisporangium lacustre*) and other species in clade E (Levesque & de Cock 2004). *Globisporangium intermedium* and *G. splendens* were used as outgroups. Numbers along the nodes indicate bootstrap support values above 80% for ML/NJ, respectively.
Species of *Globisporangium*

**Editor-in-Chief**
Prof. dr P.W. Crous, Westerdijk Fungal Biodiversity Institute, P.O. Box 85167, 3508 AD Utrecht, The Netherlands. E-mail: p.crous@westerdijkinstitute.nl

Description and illustration of MAFF 236903 by Abdelzaher morphological comparisons are mainly conducted based on the sexual structures in artificial conditions, respectively. Therefore, ability to produce sexual structures, and both asexual and growth. Moreover, the ITS and cox1 sequences of *G. marsipium* demonstrated that it is a different species. This was also indicated in the phylogenetic analyses (Figs 4, 5).

Notes: In this study, MAFF 236903 and CBS 773.81 lost the ability to produce sexual structures, and both asexual and sexual structures in artificial conditions, respectively. Therefore, morphological comparisons are mainly conducted based on the description of MAFF 236903 by Abdelzaher et al. (1994) and description of *G. marsipium* by van der Plaats-Niterink (1981).

**Typus:** Japan, Sakai, Osaka, pond water, 12 Oct. 1992, H.M.A. Abdelzaher (holotype, TNS-F-53297; ex-type strain, MAFF 236903 = UOP 406).

*Globisporangium camurandrum* (Bala et al.) Uzuhashi, *comb. nov.* MycoBank MB828474.

Basionym: *Pythium camurandrum* Bala et al., *Persoonia* 25: 26. 2010.

Description and illustration: Bala et al. (2010).

*Globisporangium takayamanum* (Senda & Kageyama) Uzuhashi, *comb. nov.* MycoBank MB828473.

Basionym: *Pythium takayamanum* Senda & Kageyama, *Mycologia* 101: 446. 2009.

Description and illustration: Senda et al. (2009).

**DISCUSSION**

*Globisporangium lacustre* is morphologically similar to *G. marsipium*, although colony patterns, hyphal growth speed, and its response to temperature were significantly different. Hyphal growth of *G. marsipium* has never been described previously (Drechsler 1941, van der Plaats-Niterink 1981). The present result demonstrated that *G. lacustre* is also distinguished from *G. marsipium* by its wide temperature range and rapid hyphal growth. Moreover, the ITS and cox1 sequences of *G. marsipium* were sufficiently different from those of *G. marsipium* to demonstrate that it is a different species. This was also indicated in the phylogenetic analyses (Figs 4, 5).

In culture collections, some strains are used as reference strains for studies, so it is important to maintain strains that are correctly identified. Some new techniques including molecular analyses, and new morphological or physiological characteristics, could provide new information to re-identify species. Therefore, taxonomic re-evaluation of previously collected isolates will be needed at some point. Here, we evaluated the species identification of strain MAFF 236903 in the NARO Genebank based on molecular analyses, because the strain was initially identified as *G. marsipium* by morphology alone in 1994. In this study, MAFF 236903 never formed sexual organs, and the reference strain, CBS 773.81, also lost the ability to sporulate under artificial conditions. Without morphological information, species identification or re-evaluation of species is usually difficult. However, molecular analyses could clearly indicate the difference between them. Finally, we concluded that MAFF 236903 should not be identified as *G. marsipium*, and it was re-described as *G. lacustre* sp. nov., because of differences in the molecular phylogenetic relationships.
in particular, as well as hyphal growth rate and colony pattern. Most of the description of \textit{G. lacustre} sp. nov. is based on the initial observations by Abdelzaher \textit{et al}. (1994). Our result indicated that taxonomic re-evaluation of strains is sometimes needed even if the strain becomes sterile in culture. If there are previously published detailed morphological descriptions as in this case, re-designation of isolates should be attempted.

As shown in the phylogenetic analyses presented here, \textit{Pythium camurandrum} and \textit{P. takayamanum} were known to be located in the clade E in the phylogenetic trees of Bala \textit{et al}. (2010) and Senda \textit{et al}. (2009), respectively. Because all species of clade E were transferred to the genus \textit{Globisporangium} by Uzuhashi \textit{et al}. (2010), we decided to also transfer \textit{P. camurandrum} and \textit{P. takayamanum} to \textit{Globisporangium}.

\section*{REFERENCES}

Abdelzaher HMA, Ichitani T, Elnaghy MA (1994). \textit{Pythium marsipium} from pond water in Osaka. \textit{Mycological Research} 98: 920–922.

Bala K, Robideau GP, Désaulniers N, \textit{et al}. (2010). Taxonomy, DNA barcoding and phylogeny of three new species of \textit{Pythium} from Canada. \textit{Persoonia} 25: 22–31.

Bouket AC, Arzanlou M, Tojo M, \textit{et al}. (2015). \textit{Pythium kandovanense} sp. nov., a fungus-like eukaryotic micro-organism (\textit{Stramenopila, Pythiales}) isolated from snow-covered ryegrass leaves. \textit{International Journal of Systematic and Evolutionary Microbiology} 65: 2500–2506.

Drechsler C (1941). Three species of \textit{Pythium} with proliferous sporangia. \textit{Phytopathology} 31: 478–507.

Kumar S, Stecher G, Tamura K (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. \textit{Molecular Biology and Evolution} 33: 1870–1874.

Lévesque CA, de Cock AWAM (2004). Molecular phylogeny and taxonomy of the genus \textit{Pythium}. \textit{Mycological Research} 108: 1363–1383.

Miller PM (1995). V-8 juice agar as a general purpose medium for fungi and bacteria. \textit{Phytopathology} 45: 461–462.

Múnera JDC, Hausbeck MK (2016). Characterization of \textit{Pythium} species associated with greenhouse floriculture crops in Michigan. \textit{Plant Disease} 100: 569–576.

Robideau GP, de Cock AWAM, Coffey MD \textit{et al}. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. \textit{Molecular Ecology Resources} 11: 1002–1011.

Senda M, Kageyama K, Suga H, \textit{et al}. (2009). Two new species of \textit{Pythium, P. senticosum} and \textit{P. takayamanum}, isolated from cool-temperate forest soil in Japan. \textit{Mycologia} 101: 439–448.

Ueta S, Tojo M (2016). \textit{Pythium barbulae} sp. nov. isolated from the moss, \textit{Barbula unguiculata}; morphology, molecular phylogeny and pathogenicity. \textit{Mycoscience} 57: 11–19.

Uzuhashi S, Hata K, Matsuura S, \textit{et al}. (2017). \textit{Globisporangium oryzicola} sp. nov., causing poor seedling establishment of directly seeded rice. \textit{Antonië van Leeuwenhoek} 110: 543–552.

Uzuhashi S, Okada G, Ohkuma M (2015). Four new \textit{Pythium} species from aquatic environments in Japan. \textit{Antonië van Leeuwenhoek} 107: 375–391.

Uzuhashi S, Tojo M, Kakishima M (2010). Phylogeny of the genus \textit{Pythium} and description of new genera. \textit{Mycoscience} 51: 337–365.

van der Plaats-Niterink AJ (1981). Monograph of the genus \textit{Pythium}. \textit{Mycoscience} 51: 337–365.

van der Plaats-Niterink AJ (1981). Monograph of the genus \textit{Pythium}. \textit{Mycoscience} 51: 337–365.

White TJ, Bruns T, Lee S, \textit{et al}. (1990). Amplification and direct sequencing of fungal ribosomal RNA gene for phylogenetics. In: PCR protocols: a guide to methods and applications (Innes MA, Gelfand DH, Sninsky JJ, White TJ, eds). USA, NY, Academic Press: 315–322.

Zhang BQ, Yang XB (2000). Pathogenicity of \textit{Pythium} populations from corn-soybean rotation fields. \textit{Plant Disease} 84: 94–99.