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Phylogeny and morphology of new species of *Globisporangium*

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pond water
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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.

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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 (*cox*1) 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, Múnera & 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 *cox*1 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 structure. 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 *cox*1 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 *cox*1. Amplification reactions and sequencing were conducted as reported previously (Uzuhashi *et al*. 2017).

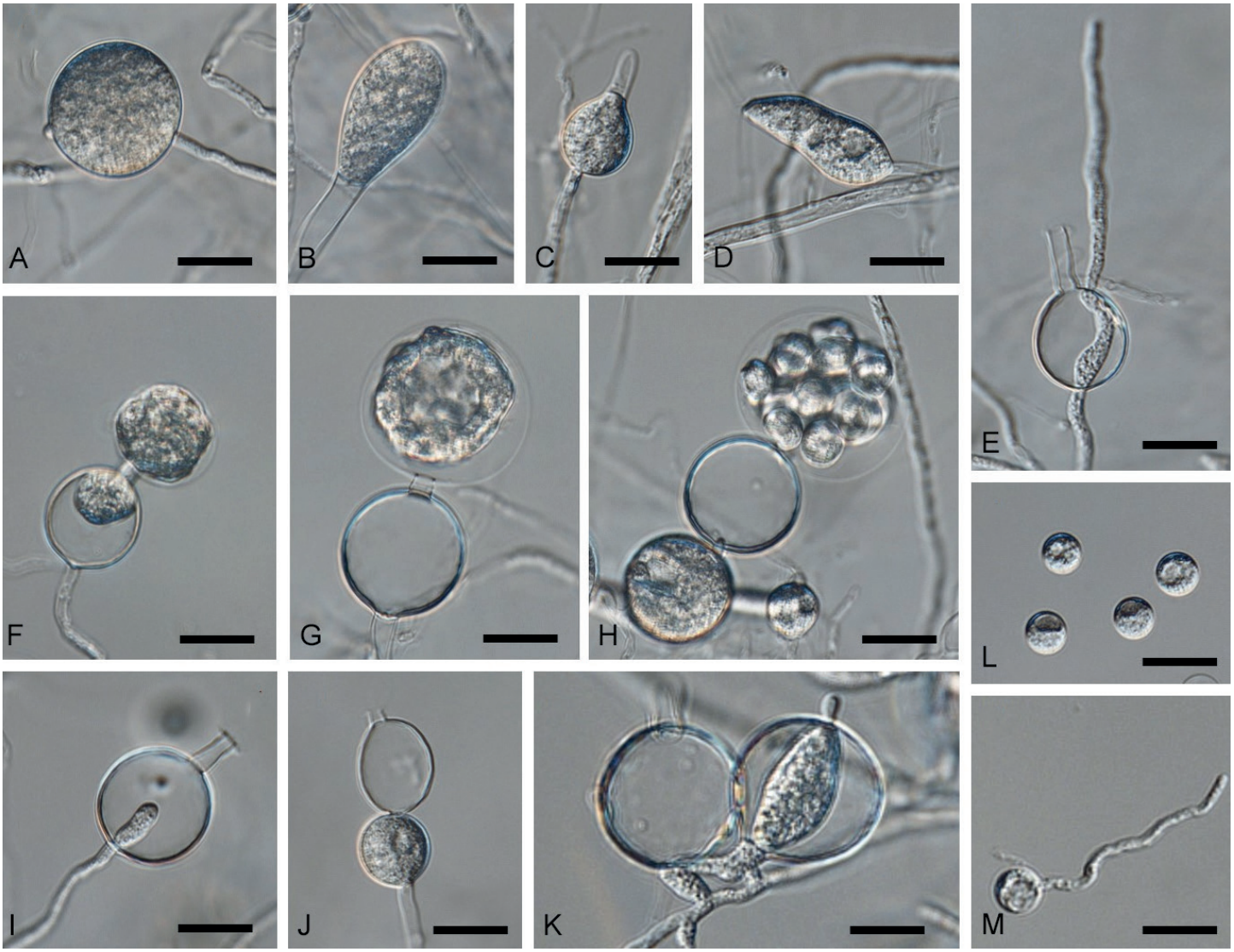

Fig. 1. Morphology of *Globisporangium lacustre*. **A–D.** Sporangia. **E.** Internally proliferation. **F.** Early stage of vesicle formation. **G, H.** Vesicle with zoospore development inside. **I.** Internal proliferation. **J.** Sporangium and empty sporangium. **K.** Empty sporangium and internal proliferation. **L.** Encysted zoospores. **M.** Germinated encysted zoospore. Scale bars = 20 µm.

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 *cox*1.

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

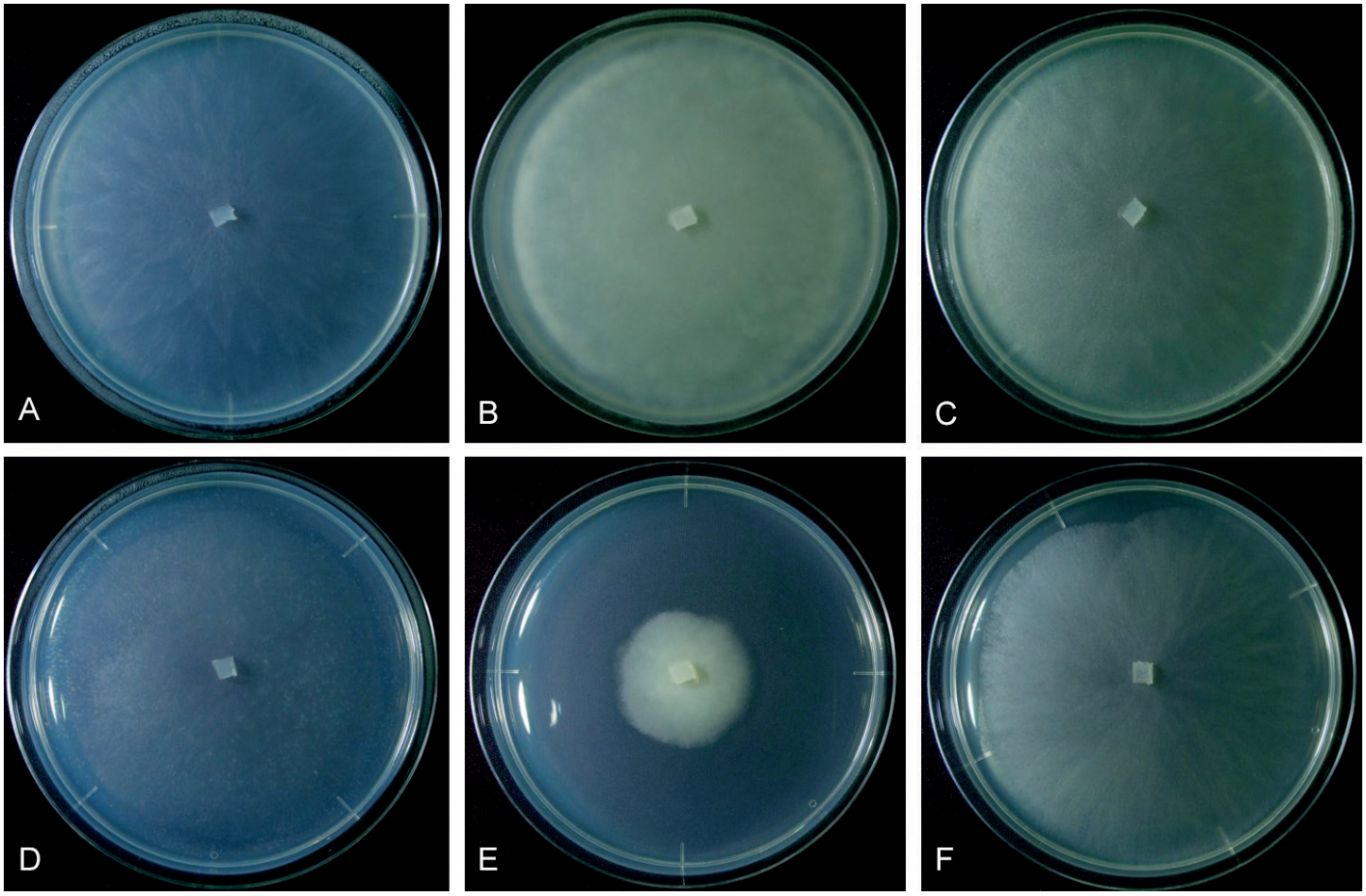

Fig. 2. Colony patterns of MAFF 236903 (*Globisporangium lacustre*) (**A–C**) and CBS 773.81 (*G. marsipium*) (**D–F**) at 25 °C on PCA (A, D), PDA (B, E), and V8A (C, F).

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).

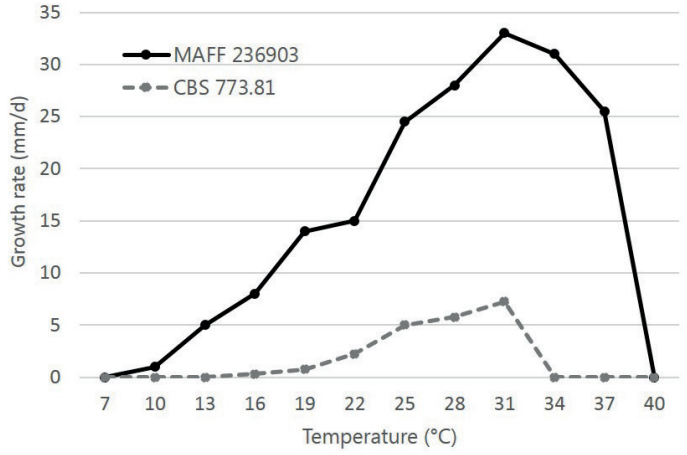

Fig. 3. Effect of temperature on the growth rate of MAFF 236903 (*Globisporangium lacustre*) and CBS 773.81 (*G. marsipium*) on PCA.

Sequencing and phylogeny

The ITS and *cox*1 sequences of MAFF 236903 had 85 % and 93 % similarities with those of *G. marsipium* CBS 773.81, respectively. For both the ITS or *cox*1 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-

Table 1. Morphology and hyphal growth temperature of *Globisporangium marsipium* and MAFF 236903.

	***G. marsipium*[1]**	**MAFF 236903[2]**
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
Oospore 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 oospores	Aplerotic	Aplerotic, usually yellowish
Number of oospores/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.

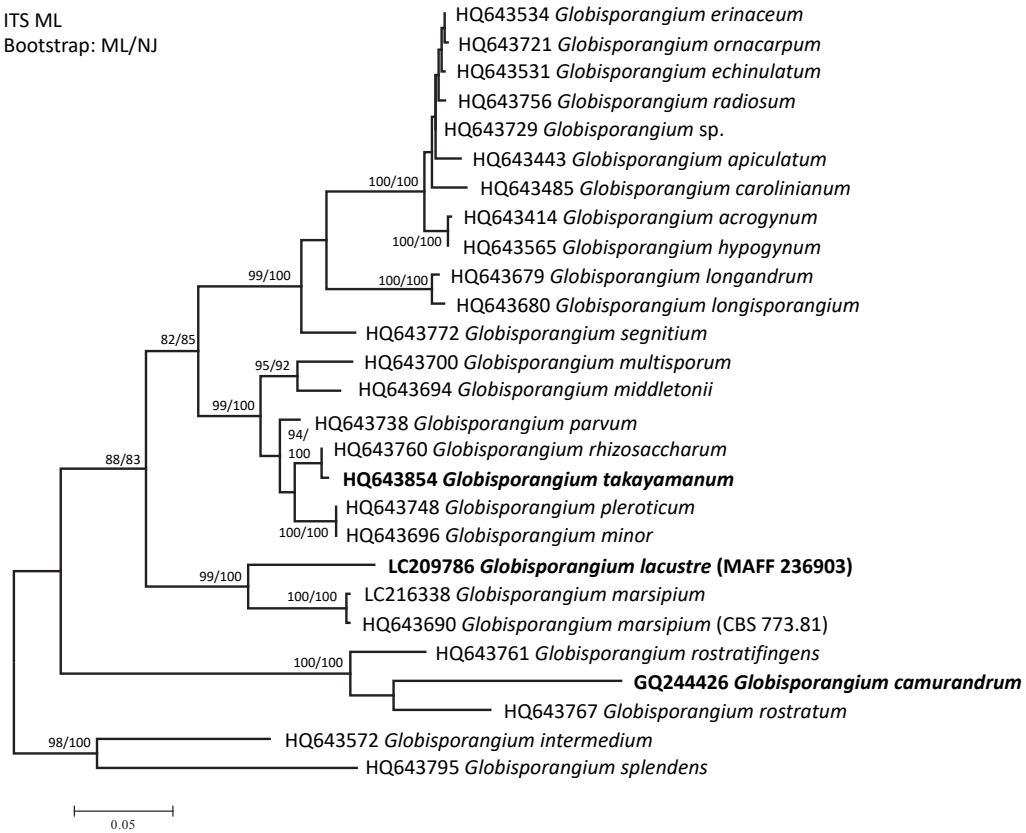

Fig. 4. Maximum Likelihood (ML) tree based on ITS sequences showing the relationship between MAFF 236903 (*Globisporangium lacustre*) and other species in clade E (Lévesque & de Cock 2004). *Globisporangium intermedium* and *G. splendens* were used as outgroups. Numbers along the nodes indicate bootstrap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

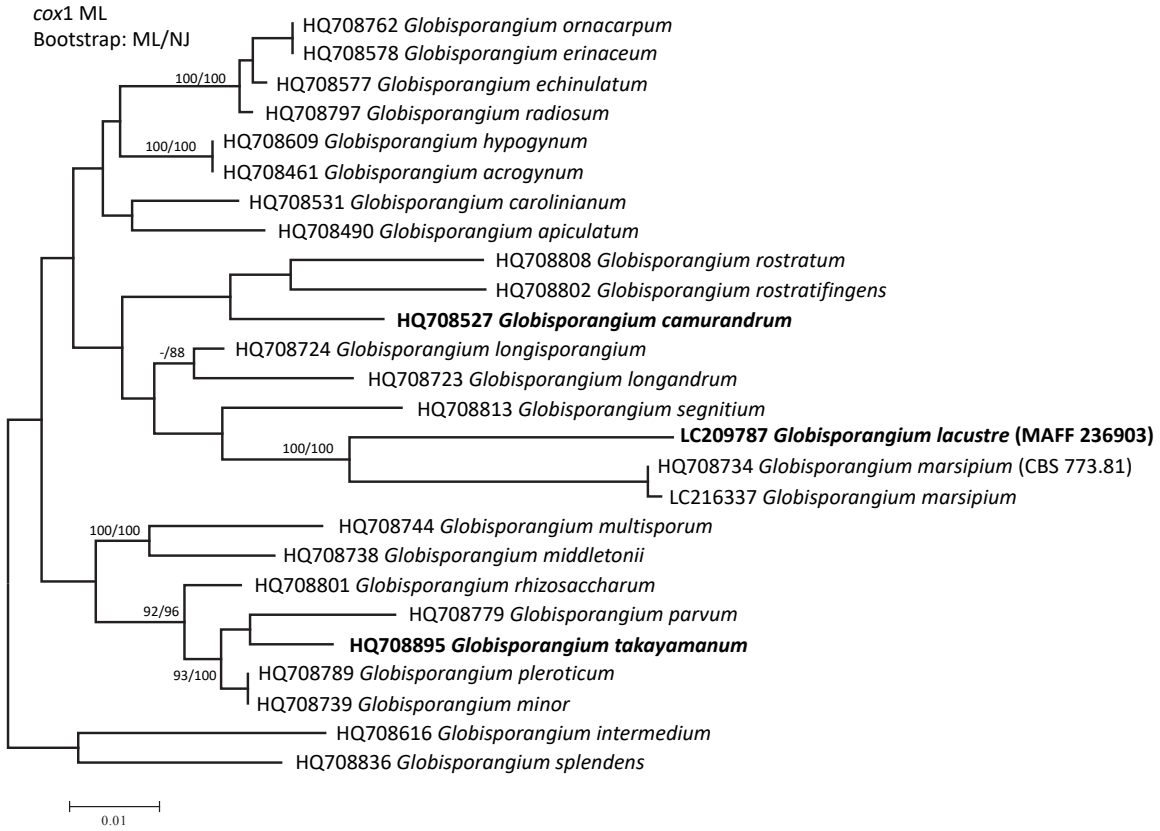

Fig. 5. Maximum Likelihood (ML) tree based on *cox*1 sequences showing the relationship between MAFF 236903 (*Globisporangium lacustre*) and other species in clade E (Lévesque & de Cock 2004). *Globisporangium intermedium* and *G. splendens* were used as outgroups. Numbers along the nodes indicate bootstrap support values above 80 % for ML/NJ, respectively.

walled, terminal or subterminal, mostly intercalary, occasionally catenulate, 26–39 µm (av. 33 µm) diam. *Antheridia* ellipsoidal, cupulate or bell-shaped, diclinous, 1–8 per oogonium, antheridial stalks mostly branched, or unbranched, often persisting after fertilisation. *Oospores* aplerotic, usually yellowish, spherical to subspherical, 20–35 µm (av. 23 µm) diam, wall 1.5–2.5 µm thick.

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 *cox*1 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 *G. lacustre* sp. nov. is based on the initial observations by Abdelzaher *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, *Pythium camurandrum* and *P. takayamanum* were known to be located in the clade E in the phylogenetic trees of Bala *et al*. (2010) and Senda *et al*. (2009), respectively. Because all species of clade E were transferred to the genus *Globisporangium* by Uzuhashi *et al*. (2010), we decided to also transfer *P. camurandrum* and *P. takayamanum* to *Globisporangium*.

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