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Life cycle and mating compatibility in the Japanese white jelly mushroom, *Tremella yokohamensis*

Nanthawan Kaeoniwong[a], Kozue Sotome[b], Tsuyoshi Ichiyanagi[b], Norihiro Shimomura[b], and Tadanori Aimi[b*]

[a] *The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-cho Minami, Tottori 680-8553, Japan*
[b] *Faculty of Agriculture, Tottori University, 4-101 Koyama-cho Minami, Tottori 680-8553, Japan*

ABSTRACT

In this study, white jelly mushrooms that were collected in Tottori Prefecture, Japan, were identified as *Tremella yokohamensis* by phylogenetic analysis of the rDNA-ITS region. Fluorescent microscopic analysis using 4′,6-diamidino-2-phenylindole staining to visualize the nuclei in each cell revealed that basidiospores isolated from the fruiting body were monokaryotic. Furthermore, monokaryotic yeasts were germinated from these basidiospores and the resulting crossed mycelium was dikaryotic and bore clamp cells, suggesting a heterothallic lifecycle for this species. Crossing between compatible yeast strains, such as TUFC 101924 and TUFC 101925, that were isolated from the same fruiting body, successfully induced development of the filamentous stage bearing clamp connections after 7 d of incubation on Kagome vegetable juice agar medium. Mating compatibility tests employing 15 basidiospore isolates revealed that this fungus possess a bipolar mating system. The results indicated that *T. yokohamensis* is a heterothallic and bipolar mushroom.

Keywords: bipolar, *Cryptococcus*, DAPI, dimorphic fungus, heterothallic

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1. Introduction

The order Tremellales is the largest known group of fungi in the class Tremellomycetes (Basidiomycota). The order contains teleomorphic yeast species (*Tremella* Pers.), anamorphic yeast species (*Bullera* Derx, *Cryptococcus* Kütz., and *Fellomyces* Y. Yamada & I. Banno), taxa that completely lack a basidiocarp and grow in the hymenium of other fungi, and taxa associated with lichens (Bandoni & Boekhout, 2011; Millanes et al., 2011; Weiss et al., 2014). *Tremella* is a dimorphic genus that comprises two morphologically distinct life cycles: a teleomorphic state (diploid), in which basidiocarps resulting from the conjugation of yeast cells develop on appropriate substrates under suitable conditions, and an anamorphic state (haploid), in which basidiospores germinate by budding to establish a haploid yeast stage (Bandoni & Boekhout, 2011; Weiss et al., 2014). *Tremella* is a highly polyphyletic genus that contains a diverse array of teleomorphic yeast species and lichenicolous species. This categorization is supported by phylogenetic analyses of various *Tremella* species that are based on the D1/D2 region of the nuclear large subunit ribosomal rRNA (LSU) and the internal transcribed spacer (ITS) region (Alshahni et al., 2011; Scorzetti et al., 2002; Weiss *et al.*, 2014). *Tremella yokohamensis* (Alshahni, Satoh & Makimura) Yurkov was discovered in 2011 at the Kanazawa Zoological Park in Yokohama, Japan, was initially described as the anamorphic yeast species *C. yokohamensis* (Alshahni, Satoh & Makimura), a member of the Fuciformis clade (Alshahni et al., 2011; Scorzetti et al., 2002). In 2015, the sexual stage of *C. yokohamensis* (=*T. yokohamensis*) was discovered through corroboration with DNA sequence data. Interestingly, a specimen collected in the Russian Far East (VLA M-11700) formed a cluster in the same clade as *C. yokohamensis* (voucher JCM 16991). This evidence led to the fungus being reclassified as *T. yokohamensis* (Malysheva et al., 2015).

Tremella species are widely used in both food and medicine. For example, *T. fuciformis* Berk. and *T. aurantialba* Bandoni & M. Zang are both cultivated commercially for food. The fruiting bodies of these species are rich in immunostimulatory polysaccharides, triterpenoids, protein, dietary fiber, vitamins, minerals, and chitin (Zhang et al., 2011). In addition, polysaccharides extracted from *T. fuciformis* have been reported to be bioactive substances that possess a variety of antioxidant, anti-inflammatory, anti-tumor, anti-quorum sensing, and anti-aging effects, while also exhibiting immunomodulatory actions and contributing to blood sugar regulation (Wu et al., 2019; Xu et al., 2021; Yang et al., 2019; Zhang et al., 2014; Zhu & Sun, 2008). Park et al. (2007) reported that *T. fuciformis* could potentially be used as a preventative agent in neurodegenerative diseases.

Understanding the life cycle and mating types of *Tremella* species is critical for their successful cultivation and breeding, as these characteristics can be used to optimize and control growth condi-

* Corresponding Author. Faculty of Agriculture, Tottori University, 4-101 Koyama-cho Minami, Tottori 680-8553, Japan
E-mail address: taimi@tottori-u.ac.jp

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tions, reproduction, and genetic variability of these taxa. The mating systems employed by the Tremellales are varied, and all known heterothallic *Tremella* species employ a tetrapolar mating system (Hanson & Wells, 1991; Wells, 1994). However, other teleomorphic species in the Tremellales, such as *Auriculibuller fuscus* J.P. Samp., J. Inácio, Fonseca & Fell (Sampaio et al., 2004), *C. neoformans* (San Felice) Vuill, and *C. gattii* (Vanbreus. & Takashio) Kwon-Chung & Boekhout have been reported to employ bipolar mating systems (Fraser et al., 2004; Keller et al., 2003; Kwon, 1975; Lengeler et al., 2002; Loftus et al., 2005; Nielsen et al., 2003).

In this study, fruiting bodies of a white jelly mushroom that were similar in morphological appearance to *T. fuciformis* were collected on the log of a broadleaved tree in central Tottori Prefecture, Japan. Isolates of the basidiospores were used to analyze the life cycle and mating compatibility of the collected fungi. This study was undertaken to develop and optimize the production and rearing of Japanese white jelly mushrooms, which are referred to as "*Shiro kikurage*" in Japanese.

2. Materials and methods

2.1. Fungal strains and culture conditions

Fruiting bodies of a white jelly mushroom were discovered on a log of a broadleaved tree in a kindergarten in Tottori College, Kurayoshi, Tottori Prefecture, Japan, in 2020. Fresh basidiocarp tissue was attached to the cover of a petri dish with Vaseline, and the spores were allowed to fall on the CJM-1 agar medium in the Petri dish below (2 g/L glucose, 2 g/L Difco Soytone, 15 g/L agar; Bandoni et al., 1975). The position of the fallen spores on the plate were checked using a microscope and spore locations were recorded by marking the bottom of the petri dish with a marker pen. Subsequently, small agar pieces containing a single spore, the locations of which had been marked using a marker pen, were excised from the gel and transferred to the new agar medium. The spores were successfully germinated, grown in yeast form, and 15 basidiospore isolates (A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O) were obtained and maintained on 2% malt agar medium at room temperature. Among the basidiospore isolates, isolates G and C, which were mating-compatible strains, were used for further analysis and deposited in the Tottori University Fungal Culture Collection (TUFC). The collection number of the voucher specimen is TUMH65418 and those of the monospore isolates G and C are TUFC101924 and TUFC101925, respectively.

2.2. Molecular phylogeny

2.2.1. DNA extraction and amplification

A single colony of yeast cells was inoculated into 20 mL minimal liquid medium [1.5 g/L $(NH_4)_2HPO_4$, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 1 mL/L thiamine hydrochloride (50 mg/mL), pH 5.6]. The yeast cells were grown at 25 °C with shaking at 120 rpm for 2 d, then harvested as yeast pellets by centrifugation. Genomic DNA was extracted from the yeast cells cultured in minimal media using the cetyltrimethylammonium bromide method (Dellaporta et al., 1983; Riffiani et al., 2021). The DNA was purified using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The purified DNA was used for polymerase chain reaction (PCR) amplification of ribosomal DNA. The partial small subunit ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and partial large subunit ribosomal RNA gene (i.e., the rDNA ITS region), which are considered to be effective for species identification and molecular phylogenetic analysis, were amplified with the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White et al., 1990). Each 20 μL PCR reaction mixture contained 20 ng of genomic DNA, 20 pmol of each primer, 2.5 mM dNTP mixture, 10 × Ex *Taq* buffer with 2 mM $MgCl_2$, and 0.5 U Ex *Taq* DNA polymerase (Takara Bio, Shiga, Japan). DNA amplification was performed using the following PCR protocol: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel in 1 × TAE buffer (40 mM Tris-acetate pH 8, 1 mM EDTA). The gel was stained with ethidium bromide and visualized under UV light. The PCR products were then purified using a QIAquick PCR Purification Kit (Qiagen, CA, USA), and analysis of the nucleotide sequences of the PCR products was outsourced (Eurofins Genomics Japan, Tokyo, Japan). The nucleotide sequence data of the rDNA ITS region of isolates G (TUFC 101924) and C (TUFC 101925), which are reported in this paper, were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC784256 and LC784255, respectively.

2.2.2. Phylogenetic analysis

Nucleotide sequence data were analyzed and edited with the GENETYX software package (version 13, GENETYX, Tokyo, Japan). The nucleotide sequences of the rDNA-ITS region of isolates G and C determined in this study, and nucleotide sequences of those of other *Cryptococcus* and related species extracted from the DDBJ database were aligned with MEGA 11 software (Tamura et al., 2021). The accession numbers of these sequences are shown in Table 1. A phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou & Nei, 1987). Data consistency was tested by bootstrapping the alignments using 1000 replicates. For the molecular phylogenetic analysis, DNA sequences downloaded from the National Center for Biotechnology Information (NCBI, USA; https://www.ncbi.nlm.nih.gov/) were added to the sequences obtained in this study, and *Ramaria pallidissima* Schild & G. Ricci ZT Myc 55616 (NR_171869.1) was used as an outgroup (Table 1).

2.3. Mating compatibility test

The yeast cells of the 15 basidiospore isolates were crossed with each other by spotting a small amount of each inoculum on Kagome vegetable juice (KVJ) agar medium (50 mL/L Kagome Low Salt Vegetable Juice, 0.5 g/L KH_2PO_4, 40 g/L agar, pH 7.0) with a sterile loop and then mixing the spots, as described by Wickes et al. (1996). For this medium, "Kagome Low Salt Vegetable Juice" (Kagome Co. Ltd., Nagoya, Aichi, Japan) was used instead of "V8 juice" to observe mycelium development and clamp cell formation between compatible strains, such as G and C. Mycelial progression of compatible strains was observed by transferring one loop amount of each inoculate to minimal liquid medium, followed by mixing with a vortex mixer for a couple minutes. Subsequently, 10 μL of the mixed strains were applied onto KVJ agar medium by dropping with a micropipette and incubated at 25 °C.

2.4. Microscopic analysis

The mycelial progression resulting from the crossing of compatible strains (G and C) was observed at the edge of the colony on the KVJ agar plate using a light microscope (Eclipse 80i; Nikon, Tokyo) equipped with a digital camera (DS-L2; Nikon) following incuba-

Table 1. Nucleotide sequence with GenBank accession numbers used in the molecular phylogenetic analysis, ITS data were obtained from the National Center for Biotechnology Information website. Bold text indicates the species identified in this study.

Species	Strain/Voucher No.	GenBank accession No.
Tremella yokohamensis	KHU20230328_01	OR791291.1
Tremella yokohamensis	CBS:11776	KY105698.1
Tremella yokohamensis	**TUFC 101924**	**LC784256**
Tremella yokohamensis	**TUFC 101925**	**LC784255**
Cryptococcus yokohamensis	JCM 16990	HM222930.1
Cryptococcus yokohamensis	JCM 16989	HM222926.1
Tremella yokohamensis	VLA:M-11700	KP986529.1
Cryptococcus yokohamensis	JCM 16991	HM222928.1
Tremella fuciformis	CBS 6970	NR_155936.1
Tremella globispora	CBS 6972	NR_155889.1
Tremella mesenterica	CBS 6973	NR_155937.1
Tremella brasiliensis	CBS 6966	NR_119454.1
Cryptococcus amylolentus	CBS 6039	NR_111372.1
Cryptococcus wingfieldii	CBS:7118	KY105769.1
Cryptococcus neoformans	CBS:7827	KY102836.1
Cryptococcus gattii	AFLP4_CBS919	JN939459.1
Ramaria pallidissima	ZT Myc 55616	NR_171869.1

tion from days one to 21 after inoculation. The nuclear phase of the basidiospores, yeast cells, and hyphae was examined under a light microscope (Eclipse 80i; Nikon) fitted with a UV excitation apparatus (C-SHG1, Nikon) and a digital camera (DS-L2; Nikon). 4',6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Osaka, Japan) was used for nuclear staining according to the method described by Chen et al. (2021). Briefly, yeast cells were cultivated in liquid minimal medium at 25 °C for 2 d and harvested in microtubes. The yeast pellets were then fixed by suspending in Carnoy's solution (acetic acid: ethanol = 1: 3, v/v) for 20 min, followed by centrifugation at 10000 × *g* for 60 s and removal of Carnoy's solution. Then, one drop of DAPI (1 ppm) solution was to the pellet and the samples were incubated at room temperature for 30 min. A piece of mycelium that had been growing on KVJ agar medium for 14 d after crossing the compatible strains was then picked using a toothpick and transferred to a slide glass. Carnoy's solution was then dropped onto the mycelium to fix the sample, followed by one drop of DAPI solution (1 ppm) and one drop of Calcofluor White (Fluka, Buchs, Switzerland) solution (2 ppm) to stain the septa. The slide glass was then incubated overnight in the dark at room temperature before the nuclei were observed under a fluorescence microscope.

3. Results

3.1. Phylogenetic analysis

Nucleotide sequences of the rDNA-ITS region, which had an average length of 510 base pairs (bp), were aligned. A phylogenetic tree (Fig. 1) was constructed by the NJ method and based on the aligned sequences, shown that strain G, C, *T.* yokohamensis (KHU20230328, CBS:11776, and VLA: M-11700), *C. yokohamensis* (JCM16989 and JCM16991) were all grouped in the same clade as *C. yokohamensis* (JCM 16990). Moreover, a similarity search of the ITS regions using BLAST revealed that *C. yokohamensis* (JCM 16990) was most closely related to strains G and C, with only one nucleotide difference. Based on these findings, we classified the basidiospore isolate strains G (TUFC 101924) and C (TUFC 101925) as *T. yokohamensis*.

3.2. Mycelium induction

The morphology of the 15 basidiospore colonies were observed on KVJ agar medium; those of isolate C and isolate G are shown in Figs. 2A and 2B, respectively. Although this fungus produced a foliose, irregularly branched, and visible fruiting body, all of the basidiospore isolates germinated into the yeast form. Therefore, the condition of mycelial development from yeast was examined. Initially, basidiospore isolates C and G were maintained separately on CJM-1 agar medium, and no mycelium developed around each isolate's yeast colony. Then, the G and C isolates were mixed and inoculated on mycelium-inducing media, such as malt extract agar (20 g/L malt extract, 15 g/L agar, pH 5.6), minimal medium agar [1.5 g/L $(NH4)_2HPO_4$, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 1 mL/L thiamine hydrochloride (50 mg/mL), 15 g/L agar, pH 5.6], potato dextrose agar (200 g/L potato, 20 g/L glucose, 15 g/L agar, pH 5.6), yeast extract-peptone-dextrose agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar, pH 5.6), and KVJ agar medium. Unfortunately, mycelium development was observed only on the KVJ agar medium (Figs. 2C, 4E). Figure 3 shows mycelium extending beyond the edge of a yeast colony. When a mixture of two compatible strains (G and C) was inoculated on the KVJ agar medium, the mycelium began to extend beyond the edge of the yeast colony after 7 d. However, when these mycelia were transferred to fresh KVJ agar medium, the mycelia did not grow well any more, and the mycelia colony occasionally reverted to yeast form. Thus, other types of media need to be screened to determine whether the mycelial form of this fungus can be maintained. Moreover, developing mycelium from the homokaryotic yeasts without mating was difficult, and mating and heterokaryon formation were necessary in order to develop the mycelial form.

3.3. Nuclear phase

Basidiospores and yeast cells germinated from each basidiospore were observed under a light microscope (Figs. 4A and 4C, respectively). In order to clarify the life cycle of this fungus, the number of nuclei in the basidiospores, the yeast form of basidiospore isolates, and the developed mycelium were observed by fluorescent microscopy after staining with DAPI. The basidiospore (Fig. 4B) and yeast form of basidiospore isolates (Fig. 4D) were mononucleate (monokaryon). Germinated mycelium (Fig. 4G) from a mixed culture of strains C and G was binucleate (dikaryon), and clamp cells were observed (Figs. 4E, F). Based on these observations, it is strongly suggested that the isolates produced from basidiospores were homokaryotic monokaryons. Conversely, the my-

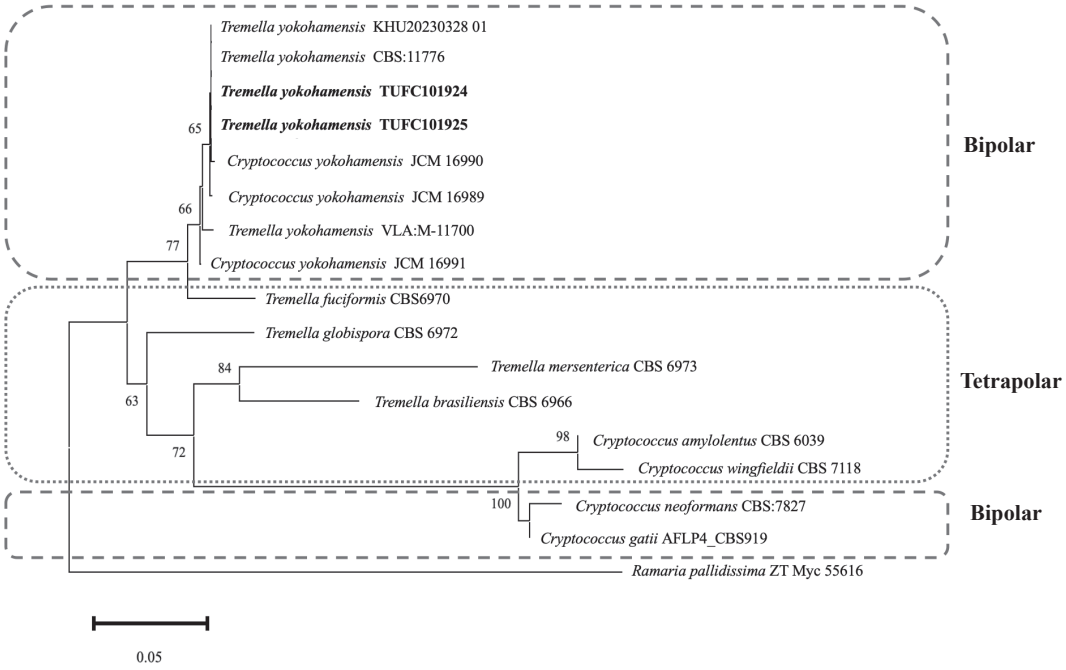

Fig. 1 – Phylogenetic tree constructed from the internal transcribed spacer sequences of *Tremella yokohamensis* and closely related species using the Neighbor-joining method implemented in the MEGA software package (version 11). Bootstrap values were calculated from 1,000 replicates (≥ 50%). The scientific names and the accession numbers of the sequences of the species used in this study are indicated in bold. *Ramaria pallidissima* (ZT Myc 55616) was used as an outgroup. The dashed box shows the bipolar mating type and the dotted box shows the tetrapolar mating type. The bar indicates a patristic distance of 0.05 substitutions per site.

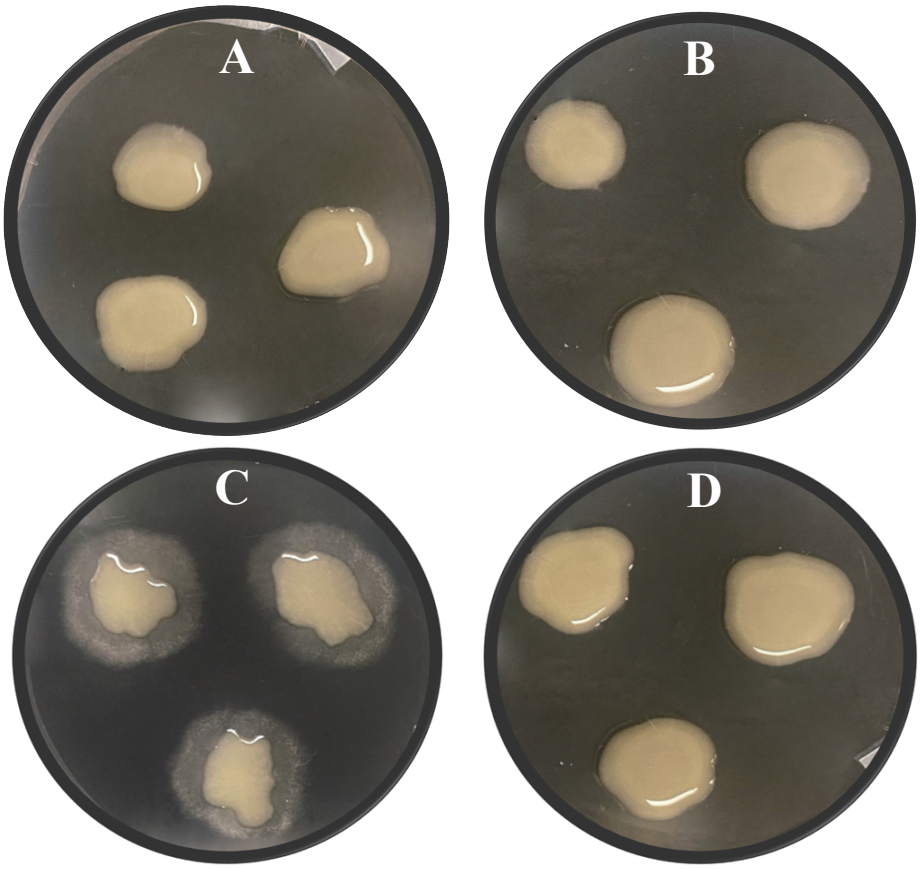

Fig. 2 – Colony morphologies of *Tremella yokohamensis* cultured on KVJ agar medium. A, B: Basidiospore isolates TUFC101925 and TUFC101924, respectively. C: Cross between compatible yeast cells (TUFC101924 × TUFC101925), with whitish hyphae appearing at the periphery of the yeast colony. D: Cross between incompatible yeast cells; no hyphae appear at periphery of the yeast colony.

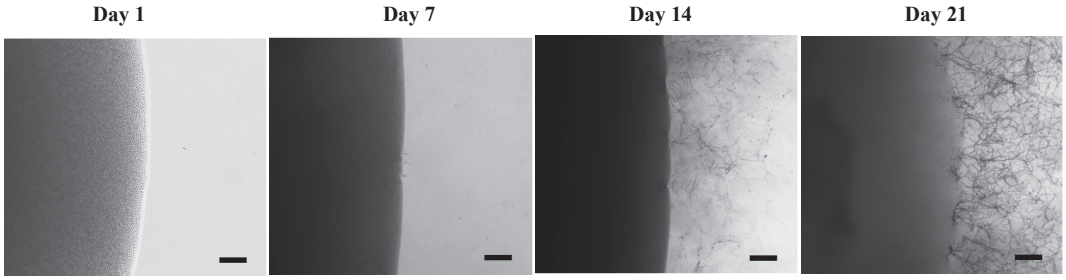

Fig. 3 – Mycelial elongation in a cross of *Tremella yokohamensis* TUFC101924 × TUFC101925 strains on KVJ agar medium observed under a light microscope. Bars: 100 μm.

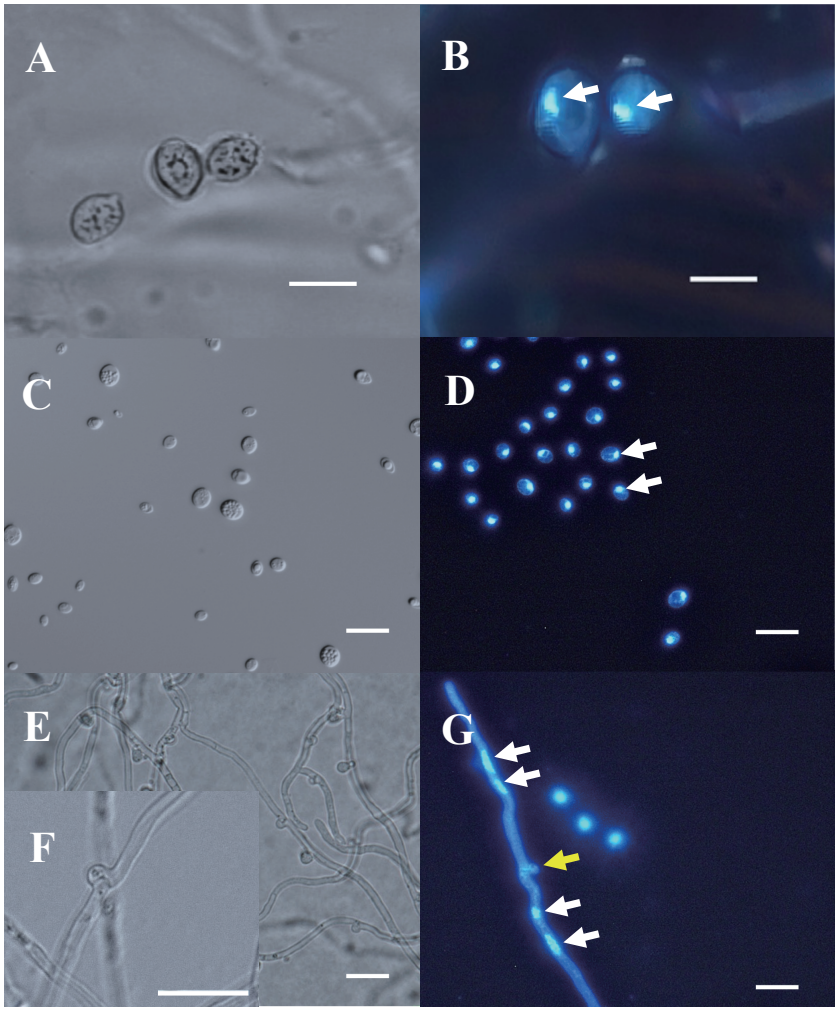

Fig. 4 – Morphological characteristics of *Tremella yokohamensis*. A: Light micrograph of basidiospores. B: Fluorescence micrograph of basidiospores after staining with DAPI, the white arrows indicate the position of nuclei. C: Light micrograph of yeast cells. D: Fluorescence micrograph of yeast cells, the white arrows indicate the position of nuclei. E, F: Light micrograph of secondary mycelia with clamp connection after crossing between *T. yokohamensis* TUFC101924 × TUFC101925. G: Fluorescence micrograph of staining with DAPI and Calcofluor White, the yellow arrow indicates the position of a septum and the white arrows indicate the position of nuclei. Bars: 10 μm.

celium that germinated from the mixed culture was a heterokaryotic dikaryon. Furthermore, strains C and G were identified as being sexually compatible and the proposed life cycle is shown in Fig. 5. The transition from the nuclear phase in basidiospores, through the germinated yeast form of basidiospore isolates, to the development of dikaryotic mycelium with clamp cells in *T. yokohamensis* (=*C. yokohamensis*), collectively indicates a heterothallic life cycle for this species.

3.4. Polarity of mating type

To investigate the mating system of this fungus, we crossed 15 basidiospore isolates (A-O) with each other and examined clamp cell formation under a light microscope. In the case of compatible yeast cell combinations, hyphal filaments were observed at the periphery of the yeast colony (Fig. 2C). Conversely, incompatible yeast cell combinations remained as yeast cells (Fig. 2D). The re-

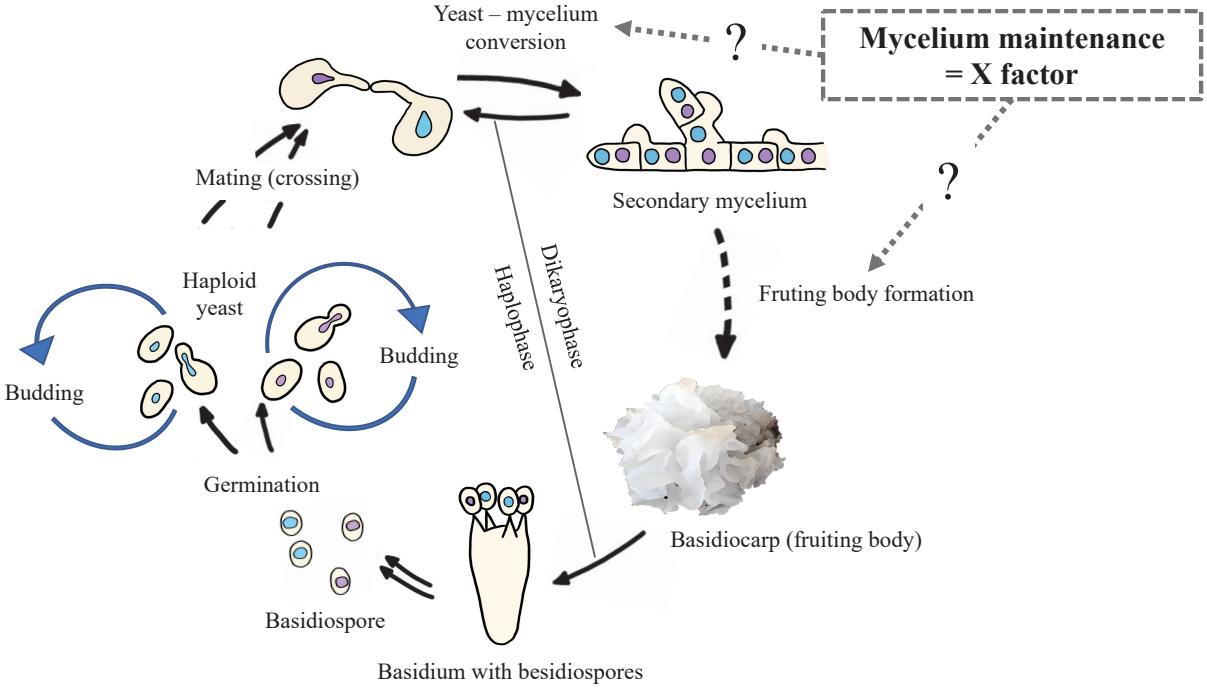

Fig. 5 – Proposed life cycle of *Tremella yokohamensis*.

sults of the mating compatibility tests involving the 15 basidiospore isolates are shown in Table 2. The 15 basidiospore isolates could clearly be divided into two incompatibility groups depending on the production of clamp cells after crossing with each other. Group 1 comprised isolates A, C, E, F, H, K, L, M, and O, while Group 2 comprised isolates B, D, G, I, J, and N. Based on this grouping, we concluded that *T. yokohamensis* is a heterothallic bipolar mushroom, and the mating types of Group 1 and Group 2 were defined as "a" and "α", respectively.

The polarity of mating type in each species is also shown in Fig. 1. Previous studies have been conducted on the mating behavior of several Tremellales yeast species, and their *MAT* loci structures have been investigated (Guerreiro et al., 2013; Hsueh et al., 2011). These studies showed that tremellaceous yeasts, including *C. amylolentus* (Van der Walt, D.B. Scott & Klift) Golubev, *C. heveanensis* (Groen.) Baptist & Kurtzman, *Kwoniella mangroviensis* Statzell, Belloch & Fell, and *T. mesenterica* Retz, employ tetrapolar mating systems (Hsueh et al., 2011). This is the first report of a bipolar mating system in the genus *Tremella* and the second in the genus *Cryptococcus*. The latter genus includes *C. neoformans* and *C. gattii*, both of which are reported to employ bipolar mating systems comprising two opposite mating types, defined as a and α (Fraser et al., 2003; Keller et al., 2003; Kwon, 1975, 1976a, 1976b; Nielsen et al., 2003).

Table 2. Results of the mating compatibility test among 15 basidiospore isolates (A–O) obtained from the same fruiting body of *Tremella yokohamensis*.

	Mating type	a									α					
Mating type	strain	A	C	E	F	H	K	L	M	O	B	D	G	I	J	N
a	A		-	-	-	-	-	-	-	-	+	+	+	+	+	+
	C	-		-	-	-	-	-	-	-	+	+	+	+	+	+
	E	-	-		-	-	-	-	-	-	+	+	+	+	+	+
	F	-	-	-		-	-	-	-	-	+	+	+	+	+	+
	H	-	-	-	-		-	-	-	-	+	+	+	+	+	+
	K	-	-	-	-	-		-	-	-	+	+	+	+	+	+
	L	-	-	-	-	-	-		-	-	+	+	+	+	+	+
	M	-	-	-	-	-	-	-		-	+	+	+	+	+	+
	O	-	-	-	-	-	-	-	-		+	+	+	+	+	+
α	B	+	+	+	+	+	+	+	+	+		-	-	-	-	-
	D	+	+	+	+	+	+	+	+	+	-		-	-	-	-
	G	+	+	+	+	+	+	+	+	+	-	-		-	-	-
	I	+	+	+	+	+	+	+	+	+	-	-	-		-	-
	J	+	+	+	+	+	+	+	+	+	-	-	-	-		-
	N	+	+	+	+	+	+	+	+	+	-	-	-	-	-	

+: clamp cells were observed
−: clamp cells were not observed

4. Discussion

Considering the results of the phylogenetic analysis and the polarity of the mating type, although *T. yokohamensis* has a very similar life cycle to *Tremella* and is a member of the Fuciformis clade (Alshahni et al., 2011; Scorzetti et al., 2002), which contains tetrapolar mushrooms (Chang & Miles, 2004), *T. yokohamensis* employs a bipolar mating system. Maia et al. (2015) investigated the mating system of the yeast *Leucosporidium scottii* Fell, Statzell, I.L. Hunter & Phaff, and concluded that the tetrapolar mating type is the ancestral state of all basidiomycetes. Hsueh et al. (2011) reported that *C. amylolentus* and *C. heveanensis* employ tetrapolar mating systems, which provides additional support for the tetrapolar-to-bipolar evolutionary model. Fraser et al. (2004) conducted a study on the *MAT* structure of *C. neoformans* and proposed an evolutionary model of the *MAT*-specific regions and linkage of the two loci from a hypothetical tetrapolar ancestor to form the large, derived locus of the bipolar *Cryptococcus* species. Other studies have examined the composition and function of the mating-type loci in bipolar basidiomycetes. Aimi et al. (2005) characterized the bipolar mating system of the mushroom *Pholiota microspora* (Berk.) Sacc. (synonym *P. nameko* (T. Itô) S. Ito & S. Imai) ("nameko" in Japanese) by linkage mapping and DNA sequencing. Although both *A* and *B* mating-type homologs are found in bipolar mushrooms, they are found on different chromosomes, and only the *A* mating-type homologs are related to mating compatibility. Yi et al. (2010) investigated the role of homeodomain protein genes in regulating clamp formation in the bipolar basidiomycete *P. microspora*. Their results suggested that the high expression levels of these genes are necessary for clamp formation, and that altered expression of the *A* mating-type genes alone can drive true clamp formation. According to James et al. (2006), these occurrences were caused by a loss of a mating-type-specific pheromone receptor function.

The basidiospore of *T. yokohamensis* germinates into the yeast form, but cannot develop into mycelia by themselves; however, mixed cultures of compatible basidiospore isolates can develop into mycelia on a limited medium. Wickes et al. (1996) reported that the concentration of agar was crucial for mycelium formation and that a concentration of 4% was optimal. According to Kent et al. (2008), no single factor is responsible for the effects of the V8 juice medium. Rather, the unique composition of V8 juice medium provides the proper nutrient composition for promoting and preserving complete sexual development, with copper playing a vital role in the activation of the mating pheromone genes *MFa* and *MFα* in *C. neoformans*. In this study, maintaining the mycelium form during subculture failed on agar plates, as KVJ medium is deficient in nutrients and cannot support and maintain mycelium growth. Nevertheless, some rich-nutrient media, such as potato dextrose agar and yeast extract-peptone-dextrose agar, can induce an increase in the yeast form, but inhibit yeast-mycelium conversion. In order to maintain and enhance the mycelium form of this fungus, other media and supplements must be investigated. For example, supplementing media with inositol has been demonstrated to increase the likelihood of inducing mating structure development, successful sexual reproduction, and mycelium growth. The presence of quorum sensing peptides, as well as using glucosamine as the sole carbon source in nutrient-rich conditions, has been demonstrated to induce filamentation in *Cryptococcus* (Kent et al., 2008; Tian et al., 2018; Xu et al., 2017; Xue et al., 2007). Enjalbert and Whiteway (2005) reported that quorum sensing molecules, such as farnesol in the growth medium of *Candida albicans* (C.P. Robin) Berkhout cells, play a significant role in triggering the induction of hyphal formation during the resumption of growth. In addition, two hormones, tremerogens A-9291-1 and A-9291-11, were isolated from the culture filtrate of *A*-type cells of *T. brasiliensis* (Möller) Lloyd. These pheromones have been shown to induce conjugation tube formation in *a*-type cells of the same species (Ishibashi et al., 1983). To gain a deeper understanding of this mushroom's growth and life cycle, we propose conducting future experiments where we will cultivate dikaryotic hyphae formed by mating compatible strains. These hyphae will then be maintained on a specific nutrient medium before being introduced for the cultivation of the fruiting body.

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