

Phylogenetic and morphological re-evaluation of *Antherospora* on *Barnardia japonica* collected in Japan

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Abstract

Antherospora vaillantii is an anther smut fungus that was originally considered occurring on *Muscari* spp. around the world; however, cryptic species were revealed during recent taxonomic investigations, thus emphasizing the importance of host specificity. In Japan, *A. vaillantii* was identified on *Barnardia japonica* in 1936. Although the recent revelations indicate that it may be part of a complex based on the host plant genus, the phylogenetic placement of this Japanese species is currently unresolved. In this investigation, seven specimens of the *A. vaillantii* complex were collected from the Kanto area of Japan, and six new single teliospore isolates were established. Molecular phylogenetic analyses based on the internal transcribed spacer sequences and large subunit nuclear ribosomal DNA (28S) region indicated that the new samples formed a monophyletic clade with other *Antherospora* spp. among *Floromycetaceae* lineage. Our samples were separated from other known species of *Antherospora* and formed a robust monophyletic clade in any analysis. Detailed morphological observations revealed that the new samples could be distinguished from other known *Antherospora* species. As a result, it is proposed that *A. barnardiae* can accommodate *B. japonica* parasites. The evolutionary processes of *Floromycetaceae* in relation to their Asparagaceous host plants have also been carefully discussed. Based on the morphological observations of the basidiospore ontogenic patterns, the familial concept of *Floromycetaceae* was redefined.

Introduction

The anther smut fungus *Antherospora* Bauer, Lutz, Begerow, Piątek & Vánky (*Floromycetaceae*, *Urocystidales*; Vánky et al. 2008) was established to accommodate monocots-infected species by Bauer et al. (2008). Species of the genus were classified based on their small morphological differences and spore size and surface structures in a previous study (Bauer et al. 2008). More recently, the result of molecular analyses have revealed the existence of cryptic species for *A. vaillantii* (Tul. & C. Tul.) R. Bauer, M. Lutz, Begerow, Piątek & Vánky (Piątek et al. 2011). The type species *A. vaillantii* is originally described as "*Ustilago vaillantii* Tul. & C. Tul." by Tulasne and Tulasne (1847) and this species had been considered a causal species of anther smut from nine genera of the *Asparagaceae* subfamily *Scilloideae* (The Angiosperm Phylogeny Group et al. 2016) according to morphological observations (e.g., Vánky 1994). Genetic variation among the internal transcribed spacer (ITS) sequences and large subunit nuclear ribosomal DNA (28S; LSU) of *A. vaillantii* was revealed by Piątek et al. (2011), who resolved the species complex using host species and morphological characteristics. A subsequent study by Piątek et al. (2013) reclassified the *A. vaillantii* species complex into 6 species and emphasized that their host preference was for each host plant species.

The species diversity of *Antherospora* has been investigated using samples collected from both Europe and Africa (Piątek et al. 2011; Piątek et al. 2013; Bauer et al. 2008). *Antherospora vaillantii sensu lato* has been identified in Japan, and it occurs on another plant host *Barnardia japonica* (Thunb.) Schult. et Schult (*Scilloideae*, *Asparagaceae*) (Kakishima 1982, 2016; Ito 1936). However, its taxonomic validity has remained largely unexplored as previous reports have been limited to microscopic descriptions. In Japan, these previous reports were based on a herbarium specimen collected from the Shikoku region in 1921, and it has not been recorded again. During our taxonomic and systematic investigation of Ustilaginomycotinous fungi in Japan, we found more recent specimen that was identified as *A. vaillantii* collected from a Shimin no Mori, Chigasaki City, Kanagawa Prefecture, Japan in 2006 and preserved in the Kanagawa Prefectural Museum of Natural

History (KPM). We conducted a hearing survey from researchers who rediscovered this fungus at the time (Dr. Y. Degawa, personal communication). Based on this information, we investigated the distribution of the fungus in the Kanto area of Japan. As the host plant genus for the Japanese samples is different from those of African and European samples, the taxonomic status of Japanese *A. vaillantii sensu lato* requires further examination.

In this study, we conducted phylogenetic analysis of the *A. vaillantii sensu lato* samples collected in Japan using the nuclear rDNA ITS and LSU regions and performed a morphological examination to provide a robust taxonomic framework. Culture strains of the fungus were then established, and induced basidium formation to observe their ontogenic patterns; the results were compared with the previous report by Ingold (1984) and the phylogenetically closely related genus *Floromyces* Vánky, M. Lutz & R. Bauer that is known to have a holobasidium and phragmobasidium.

Materials And Methods

Specimens and field sampling

A specimen identified as *Ustilago vaillantii* (current name *Antherospora vaillantii*) was loaned from the Kanagawa Prefectural Museum of Natural History (KPM). Fresh samples were collected from six different urban grasslands (Tsukuba City, Ibaraki Prefecture), mantle communities (Chigasaki, Kanagawa), and meadows (Nagareyama, Chiba) in the Kanto plain of Japan, which is approximately 20 m above sea level, from September to October 2022. Each sample was placed in a paper bag and then transported back to the laboratory on the same day and kept at room temperature (approximately 20 °C). Spore isolation was performed within two days as described below.

Isolation

Teliospores were spread on 2% water agar (WA; FUJIFILM WAKO, Osaka, Japan). Germination of the basidiospores was confirmed using a × 40 objective lens. Handmade needles were used to obtain single- or multi-spore isolates, which were then transferred to yeast malt extract agar (YM, Yoshimura et al. 2002) consisting of 20 g of malt extract (Thermo Fisher Scientific, Detroit, MI, USA), 2 g of yeast extract (Becton, Dickinson and Company, Erembodegem, Belgium), 20 g of agar (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan), and 1000 mL of MilliQ water (Merck, Darmstadt, Germany), and then incubated at 20°C in the dark. Strains were then cultured on potato dextrose agar (PDA, BD Difco, Becton, Dickinson and Company, NJ, US) for 7, 10, and 30 d at 20°C in the dark. Images of the taxa presented in this study were taken on day 7 on the YM. Some of the cultures were deposited in the Japan Collection of Microorganisms (JCM), RIKEN BioResource Research Center, Tsukuba, Japan (Table 1). Specimens used in this study were deposited in the KPM herbarium and the isotype was deposited in Tochigi Prefectural Museum (TPM).

Table 1
Specimens, strains, and new nucleotide sequences obtained in this study

Species	Original specimen no.	Specimen no. ^{*1}	Isolate form	Strain no.	DDBJ accession no.
					ITS-LSU
<i>Antherospora barnardiae</i>	SahoUro1	KPM @ ^P	Single teliospore	SahoUro1s	@@@@
	SahoUro2	KPM @ ^T (= TPM @)	Single teliospore	SahoUro2s (= JCM @@@@)	@@@@
	SahoUro3	KPM @ ^P	Single teliospore	SahoUro3s (= JCM @@@@)	@@@@
	SahoUro4	KPM @ ^P	Single teliospore	SahoUro4s	@@@@
	SahoUro5	KPM @ ^P	Single teliospore	SahoUro5s	@@@@
	SahoUro6	KPM @ ^P	Single teliospore	SahoUro6s	@@@@
	-	KPM-NC 0015106	-	-	@@@@
*1 P: Paratypus, H: Holotypus					

Morphological Characterization

Fungal isolates were used for morphological observations in this study. The spores and mycelial structures were observed in preparations mounted in distilled water or oil (IO-8; Olympus, Tokyo, Japan). Teliospores were examined microscopically for 12–36 h on WA for time-lapse imaging of the basidiospore ontogeny. Field images were obtained using a mirrorless interchangeable lens camera LUMIX DMC-G2 (Panasonic, Tokyo, Japan) with H-FS014042 (Panasonic), X-M1 (FUJIFILM, Tokyo, Japan) with QZ-35M (TAMRON, Saitama, Japan) and a macro conversion lens (MSN202; Raynox, Saitama, Japan). The morphological characteristics of the samples were observed with a stereomicroscope SZ3-Tri (Olympus, Tokyo, Japan), EHT (Olympus), BHA (Olympus), and Optiphot2 (Nikon, Tokyo, Japan) using polarized light (AIP), light field observation (LF), and differential interference contrast observation (DIC). Images were captured using a digital camera with WRAYCAM-NOA630B (WRAYMER, Osaka, Japan) for SZ3-Tri and EHT, or Digital Sight DS-L1 (Nikon) for BHA or DS-L2 (Nikon) for Optiphot2.

Dna Extraction, Pcr, And Sequencing

DNA extraction from pure cultures was carried out using a Wizard® Genomic DNA Purification Kit (Promega, MAD, US) according to the manufacturer's protocol. Amplicons of the ITS and large subunit nrDNA (28S; LSU) were obtained by PCR with the primer pairs V9G/LR5 (Rehner and Samuels 1994; Vilgalys and Hester 1990). Amplifications were performed in 25 µL consisting of 2 µL of 2 ng/µL DNA extract, 12.5 µL of 2 × Buffer for KOD FX Neo, 5 µL of 2 mM dNTPs, 1 µL of each 20 pM primer, 3 µL MilliQ water, and 0.5 µL KOD FX Neo (TOYOBO, Tokyo, Japan). PCRs were carried out on a GeneAmp® PCR System 9700 (Applied Biosystems, California, US) as follows: 94°C for 2 min; followed by 38 cycles of 10 sec at 98°C, 30 sec at the designated annealing temperature 55°C, and 1.5 min at 68°C; and a final extension period of 7 min at 68°C. PCR products were purified using the FastGene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan), according to the manufacturer's protocol. Purified DNA was cycle-sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Warrington, UK) with the same primers as for the PCR or ITS2, ITS3 for ITS (White et al. 1990), LR3, LR4, LR5 for LSU (Vilgalys and Hester 1990). Sequencing was performed using the Applied Biosystems™ SeqStudio™ Genetic Analyzer with the default settings (ThermoFisher Scientific, Tokyo, Japan). Newly generated nucleotide sequences were deposited in DDBJ (Table 1).

Phylogenetic Analyses

Referring to Nasr et al. (2014), Piątek et al. (2013) and Nky et al. (2008), species in the families *Doassansiopsidaceae*, *Fereydouniaceae*, *Floromycetaceae*, and *Urocystidaceae* were used for the phylogenetic analysis, and *Thecaphora seminis-convolvuli* and *T. saponariae* (*Glomosporiaceae*) were used as the outgroup (Fig. 1). Nucleotide sequences for each data set were aligned using MAFFT version 7.429 as the default setting (Kato et al. 2017). Ambiguously aligned positions were manually removed using MEGA7 (Kumar et al. 2016). Phylogenetic analyses using ITS-LSU were conducted using ML and Bayesian methods. For the ML analysis, the GTR + CAT substitution model was selected for the ITS and LSU regions. Optimum substitution models for each data set were estimated using jModelTest (Tanabe 2011) based on the Bayesian information criterion (Schwarz 1978) for the Bayesian analysis. ML analysis was performed using the RAxML 8.2.9 (Stamatakis 2014). Bootstrap probability (BPs) was obtained using 1000 rapid bootstrap replications. Bayesian analysis was performed with MrBayes v. 3.2.7a (Ronquist et al. 2012) using substitution models with the BIC4 parameter (GTR + G was used for ITS and LSU). Two simultaneous and independent Metropolis-coupled Markov chain Monte Carlo (MCMC) runs were performed for one million generations with the tree sampled every 1000 generations of the analyses. Convergence of the MCMC procedure was assessed from the average standard deviation of split frequencies (< 0.01) and the effective sample size scores (all > 100) using MrBayes and Tracer v. 1.6 (Rambaut et al. 2014), respectively. The first 25% of the trees were discarded as burn-in, and the remainders were used to calculate the 50% majority-rule trees and to determine the posterior probabilities (PPs) for individual branches.

Result

Field sampling and isolation

We rediscovered *A. vaillantii sensu lato* from a Shimin no Mori. Additional five fresh samples were also collected from *B. japonica* in similar ecological environments with sunny but humid grasslands and a water

source around the Kanto area of Japan, which were suspected to be *A. vaillantii sensu lato*. Seven samples were collected from the six study areas in total, and six single teliospore strains were established (Table 1).

Phylogenetic Analyses

ML and Bayesian phylogenetic analyses were conducted using an aligned sequence dataset composed of 870 nucleotides from ITS and 565 from LSU. The alignment contained a total of 38 taxa, which consisted of 38 taxa (100%) in ITS and 37 (97.3%) in LSU (Table 1, 2). Of the 1435 characters included in the alignment, 633 were variable while 672 were conserved. The ML tree with the highest log likelihood (-7326.7656) is shown in Fig. 1. The topology recovered by the Bayesian analysis did not show any topological conflicts with the significant support ($\geq 70\%$ ML BP). The six isolates of *A. barnardiae* formed a robust clade (99% ML BP/1.00 Bayesian PP) and were grouped with *Antherospora* spp. with strongly supported values (100% ML BP/1.00 Bayesian PP, Fig. 1).

Table 2
List of *Antherospora* spp. with teliospore size and wall thickness, color, host plant species, country and references.

Species	Teliospore size	Teliospore wall	Teliospore color	Host plant species (Subfamily)	Country	References
<i>A. albucae</i>	9.5–22.5 × 7–14.5 µm	ca. 0.5 µm	Yellowish brown	<i>Albuca</i> sp. (<i>Ornithogaloideae</i>)	Rwanda	Bauer et al. (2008)
<i>A. barnardiae</i>	7.9–12 × (5–)6–8.5 µm (av. 9.7 × 7.2 µm, n = 30)	0.6–1.1 µm thick	Yellowish brown to olivaceous-brown	<i>Barnardia japonica</i> (<i>Hyacintheae</i>)	Japan	This study
<i>A. eucomis</i>	5.5–8(–9) × 7–12(–13.5) µm	ca. 0.5 µm	Yellowish brown	<i>Eucomis punctata</i> (<i>Massonieae</i>)	South Africa	Ványkó (2009)
<i>A. hortensis</i>	(5.5–)6–10.5(–13.0) × 5.0–8.5(–9.5) µm	ca. 0.5–0.7 µm	Olive-brown	<i>Muscari armeniacum</i> , <i>M. schliemannii</i> , <i>M. cyaneoviolaceum</i> (<i>Hyacintheae</i>)	Germany, UK	Piątek et al. (2013)
<i>A. muscari-botryoidis</i>	(5–)6–10.5(–11.0) × (4.5–)5.0–8.5(–9.5) µm	ca. 0.5–0.8 µm	Olive-brown	<i>Muscari botryoides</i> (<i>Hyacintheae</i>)	Europe, New Zealand	Piątek et al. (2013)
<i>A. peglerae</i>	9–24(–27) × 7–12 µm	ca. 0.5 µm	Yellowish or pale olivaceous brown	<i>Ornithogalum lacteum</i> (<i>Ornithogaloideae</i>)	South Africa	Bauer et al. (2008)
<i>A. scillae</i>	7–11.5(–13) × (6–)6.5–9.5(–10) µm	ca. 0.5 µm	Olive-brown	<i>Scilla bifolia</i> (<i>Hyacintheae</i>)	Germany	Bauer et al. (2008)
<i>A. sukhomlyniae</i>	(6.5–)7–10 × 7.5–13(–14) µm	ca. 0.8 µm	Yellowish brown	<i>Hyacinthella</i> sp. (<i>Hyacintheae</i>)	Ukraine	Savchenko (2015)
<i>A. tourneuxii</i>	7–16 × 6–10 µm	No data	No data	<i>Bellevallia trifoliata</i> (<i>Hyacintheae</i>)	Egypt	Bauer et al. (2008)

Species	Teliospore size	Teliospore wall	Teliospore color	Host plant species (Subfamily)	Country	References
<i>A. tractemae</i>	6.5–14.5(–16.5) × (5.5–)6.0–10.5(–11.5) μm	ca. 0.5–1.3 μm	Olivaceous or yellowish-brown	<i>Tractema verna</i> (Hyacintheae)	UK	Piątek et al. (2011)
<i>A. urGINEA</i>	9.5–15(–17.5) × 7–12 μm	ca. 0.5–0.8 μm	Yellowish brown	<i>Urginea maritima</i> (Urgineoideae)	Morocco	Bauer et al. (2008)
<i>A. vaillantii</i>	(5.5–)6–10.5(–13) × 5–8.5(–9) μm	ca. 0.5–0.7 μm	Olive-brown	<i>Muscari comosum</i> , <i>M. tenuiflorum</i> (Hyacintheae)	Europe, Africa, North America	Piątek et al. (2013)
<i>A. vindobonensis</i>	(6–)7–11(–15) × (6–)7–9(–10) μm	ca. 0.5 μm	Olive-brown	<i>Scilla vindobonensis</i> , <i>S. vindobonensis</i> subsp. <i>borhidiana</i> (Hyacintheae)	Europe	Bauer et al. (2008)

Morphological And Ontogenic Observations

Morphological characterization was conducted using a mirrorless interchangeable lens camera and stereo microscope for field and macro morphological observations, respectively (Fig. 2) and light microscopy for microscopic (Fig. 3) and time-lapse (Fig. 4) observations.

Spore germination resulted in long-ellipsoidal, broadly fusiform to cylindrical, 1–4-celled phragmobasidia, usually on a narrow pedicel (Fig. 4A–P). Basidiospores were produced on sterigmata both laterally, apically, and basally on the basidia (Figs. 3O–R, 4Q–S). Young basidia appeared to be easily misidentified as holobasidia (Fig. 4D–G), but the growth observations for the basidia confirmed that they were phragmobasidia (Fig. 4H–K). Mature basidiospores were 1–3 celled, long ellipsoidal or broadly fusiform, giving rise to secondary yeast-like cells (Figs. 3W, 4T–Y). Overmature basidia after forming basidiospores blastically formed yeast-like cells (Fig. 3X). For the matured basidiospore and overmature basidia, yeast-like cells were similar to the basidiospore but could be distinguished from the latter by their budding scar, smaller spore size, and central septum position when compared with the eccentric septum position in the basidiospore. Yeast-like cells appeared to produce daughter cells by polar budding (Fig. 4S–W) or hyphae (Fig. 4I–K) in one day.

Taxonomy

Antherospora barnardiae S. Shibata & A. Hashim., **sp. nov.** Figures 2–5

MycoBank MB@@@

Etymology: Named after the host plant genus.

Typus: Japan: Kanagawa, Chigasaki, Tsutsumi, Shimin no Mori (24m), 23 Sept 2022, Collector Akira Hashimoto (SahoUro2 = KPM @ holotypus designated here, TPM @ isotypus)

ITS/LSU nucleotide sequences GenBank accession no. [DNA extracted from culture SahoUro2s (= JCM @) that isolated from SahoUro2]

Sori in flowers of an inflorescence, in the inner floral organs filled with a blackish-brown, powdery mass of spore.

Peridium absent. Teliospore circular or elliptic, in side view elliptic, $7.9\text{--}12 \times (5\text{--})6\text{--}8.5\text{ }\mu\text{m}$ (av. $9.7 \times 7.2\text{ }\mu\text{m}$, $n = 30$), l/w $1.0\text{--}1.9(-2.4)$ (av. 1.4 , $n = 30$), in planeview elliptic, sometimes subpolygonally, concave in oil mount, yellowish brown to olivaceous-brown; wall $0.6\text{--}1.1\text{ }\mu\text{m}$ thick (av. $0.8\text{ }\mu\text{m}$, $n = 5$), nailheaded, finely, densely, uniformly verruculose. Teliospore germination results in phragmobasidia. Phragmobasidia broadly fusiform, $(1\text{--})3$ -septated, $24.5\text{--}76 \times 3.5\text{--}7.5(-9)\text{ }\mu\text{m}$, hyaline, smooth, usually on a narrow pedicel composed of $1.8\text{--}3.5 \times (1.7\text{--})2\text{--}2.8\text{ }\mu\text{m}$. Matured basidia produce basidiospores from laterally or apically developed sterigmata and then produce yeast-like cells. Basidiospores 1-septated, eccentric, ellipsoidal or broadly fusiform, $19.5\text{--}26.5 \times 3.5\text{--}4.5\text{ }\mu\text{m}$ (av. $23.7 \times 3.9\text{ }\mu\text{m}$, $n = 20$), hyaline, smooth. The basidiospores bud and eventually form hyphae or yeast-like cells from both ends. Yeast-like cells aseptate to 1-septated, ellipsoid, $11\text{--}21.5 \times 2\text{--}3.5\text{ }\mu\text{m}$ (av. $15.5 \times 3.2\text{ }\mu\text{m}$, $n = 20$), hyaline, smooth, thin-walled (on WA). Matured yeast-like cells inflated, aseptate to 1-septated, central, budding to produce daughter cells and also developing hyphae (on WA).

Culture characteristic: Colonies on PDA attaining $28\text{--}34\text{ mm}$ diam within 30 d at 20°C in the dark, velvety, plane, pale pink (Fig. 5D, E); asexual morph form. Hyphae septate, $2.5\text{--}3.5\text{ }\mu\text{m}$ wide, hyaline, smooth, thin-walled, sometimes sympodially or holoblastically produce yeast-like cells at the tip. Yeast-like cells aseptate to 1-septated, ellipsoid, $11.5\text{--}14.5 \times 2.5\text{--}4\text{ }\mu\text{m}$ (av. $12.6 \times 3.2\text{ }\mu\text{m}$, $n = 16$), hyaline, smooth, thin-walled. Matured yeast-like cells inflated, aseptate to 1-septated, $16\text{--}23 \times 3\text{--}4.5\text{ }\mu\text{m}$ (av. $18.4 \times 4\text{ }\mu\text{m}$, $n = 18$), budding to produce daughter cells and also developing hyphae.

Additional specimens examined (paratypes): JAPAN, Ibaraki, Tsukuba, Tennodai, Tsukuba University (27m), 17 Sept 2022, on *Barnardia japonica*, Collector S. Shibata, Y. Takashima, A. Hashimoto, SahoUro1 (= KPM @); Ibaraki, Tsukuba, Matsushiro, Kajihata Children's Park (25m), 25 Sept 2022, on *B. japonica*, Collector A. Hashimoto, SahoUro3 (= KPM @); Ibaraki, Tsukuba, Koyadai, around RIKEN BioResource Research Center (23m), Oct 3 2022, on *B. japonica*, Collector S. Shibata, Y. Takashima, A. Hashimoto, SahoUro4 (= KPM @); Chiba, Nagareyama, Nagasaki, around Saka river (18m), 3 Oct 2022, on *B. japonica*, Collector A. Hashimoto, SahoUro5 (= KPM @); Chiba, Nagareyama, Nishifukairi, around Tone Canal (21m), 3 Oct 2022, on *B. japonica*, Collector A. Hashimoto, SahoUro6 (= KPM @); Kanagawa, Chigasaki, Tsutsumi, Shimin no Mori (24m), 13 Sep 2007, Collector T. Yasui, KPM-NC 0015106.

Note: The abovementioned seven specimens were identified as *A. barnardiae* and were collected from September to October. They were all obtained from broadly similar habitats with sunny but humid grasslands and a water source, such as a pond or brook. The spore sizes were almost identical amongst the samples. We hypothesized that the specimen previously reported as "*Ustilago vaillantii*" that occurred on *B. japonica* from Shikoku Island Japan is the same species as was identified in this investigation, because the teliospore size,

host, and seasonal characteristics were identical (vs. $7\text{--}12 \times 7\text{--}9\text{ }\mu\text{m}$, on *B. japonica*, at October 1921; Kakishima 1982; Ito 1936).

The phylogenetic analysis showed that the six identified strains of this species completely matched each other and were phylogenetically different from the six known species. *Antherospora barnardiae* is similar to *A. scillae* (Cif.) R. Bauer, M. Lutz, Begerow, Piątek & Vánky in teliospore size ($7\text{--}11.5\text{--}13 \times (6\text{--})6.5\text{--}9.5\text{--}10\text{ }\mu\text{m}$; Bauer et al. 2008) but can be distinguished from the latter species by its yellowish brown to olivaceous-brown spores and wider spore wall when compared with the previously reported olive–brown colour and approximately 0.5- μm -thick spore in Bauer et al. (2008). ITS nucleotide sequences between these two species differed in 21 positions with 31 gaps. Although *A. barnardiae* is also morphologically similar to *Antherospora vaillantii* and its segregates by Piątek et al. (2013), i.e. *muscari-botryoidis* (Cif.) Piątek & M. Lutz, *A. hortensis* Piątek & M. Lutz and *A. vaillantii*, these species can be separated from *A. barnardiae* by 20–25 bp differences in the ITS region and their tendency for somewhat smaller spores and host plant genera (Table 2). The five species without DNA sequence data could be distinguished using their spore size and the different plant genera (Table 2).

Discussion

Most Asian species of *A. vaillantii sensu lato* have been identified based on their similarities with previous European reports in terms of host plants. In this study, morphological and molecular phylogenetic analyses were used to resolve the validity of the previous reports of *A. vaillantii sensu lato* on *B. japonica* in Japan. The Floromycetous anther smuts were used as representative examples and the diversification of *Asparagaceae* was previously found to play a dominant role in the evolution of fungi (Bauer et al. 2008; Piątek et al. 2013; Vánky et al. 2008). The molecular phylogenetic analyses in this study suggest that Japanese species are phylogenetically distinct from the known species on other Asparagaceous host species that have been reported from Africa, Europe, and North America. Detailed morphological comparisons revealed that the parasitic species of *B. japonica* were neither *A. vaillantii* nor *A. scillae*. Consequently, we have proposed that *A. barnardiae* can accommodate *B. japonica* parasites.

Most recently, with the advent of molecular phylogenetic analysis, it was suggested that co-evolution promoted fungal species diversification in *Ustilaginomycotina* (Begerow et al. 2000; Bauer et al. 2006). Piątek et al. (2013) estimated that a common ancestor of the genus *Antherospora* was *Muscari* or *Scilla* anther smuts, based on investigation using African and European samples. In the present study, the phylogenetic position of *A. barnardiae* resolved as basal lineages of *Antherospora* and did not cluster with species on African or European *Scilloideae* host plants. The genus *Antherospora* was reconstructed the strongly supported clade with *Floromyces anemarrhenae* (C.H. Chow & Chi C. Chang) Vánky, M. Lutz & R. Bauer from China that is parasitic on *Anemarrhena asphodeloides* (*Agavoideae*, *Asparagaceae*) (98% ML BP/1.00 Bayesian PP in Fig. 1). ML analysis of the chloroplast genomes by Givnish et al. (2018) showed a close relationship with the *Agavoideae* and *Scilloideae* subfamilies among *Asparagaceae*. The phylogenetic relationship among these two plant subfamilies suggests diversification of the fungal lineages of the genera *Antherospora* and *Floromyces*. According to the organelle and nuclear genome ancestral reconstructions for *Hyacinthoideae*, the first major radiations occur on the crown nodes of the tribes *Hyacintheae* and *Massonieae* (Ali et al. 2012). The radiation of the genus *Barnardia* suggested that the early lineages of *Hyacintheae* had East Asian origins, and the diversification of *Muscari*, *Scilla*, and *Tractema* was estimated in the second major extinction during the

expansion from the Mediterranean to West Asia via Northern Europe. A Mediterranean origin for the *Scilla sensu lato* (including *Muscari*, *Scilla*, and *Tractema*) was suggested using the chloroplast DNA regions in another study (Özüdoğru et al. 2022). The genus *Antherospora* reportedly has 12 species (Table 2) (Bauer et al. 2008; Piątek et al. 2011; Piątek et al. 2013; Vánky 2009; Savchenko 2015). It is thought that *A. tractemae* Piątek & M. Lutz, known as a parasite of *Tractema verna* (*Hyacintheae* basal lineages; Ali et al. 2012), might have close affinities to *A. barnardiae* based on their host phylogenetic relationships, although the phylogenetic information for *A. tractemae* is currently lacking. In addition to this, if the co-phylogenetic relationship of the genus *Antherospora* with their host plants *Hyacinthaceae* is reliable, the relationships of the other subfamilies such as *Antherospora albucae* (Syd. & P. Syd.) R. Bauer, M. Lutz, Begerow, Piątek & Vánky and *A. peglerae* (Bubák, Syd. & P. Syd.) R. Bauer, M. Lutz, Begerow, Piątek & Vánky occurred on *Ornithogaloideae* (*Albuca* sp. and *Ornithogalum lacteum*), *A. eucomis* Vánky on *Massonieae* (*Eucomis punctata*), and *A. urginea* (Maire) R. Bauer, M. Lutz, Begerow, Piątek & Vánky on *Urgineoideae* (*Urginea maritima*) (Table 2) require research. The molecular phylogeny analysis for *Floromycetaceae* raised new questions, and further investigation will be required to determine if the evolution of *Floromycetaceae* is as simple as previously discussed by Piątek et al. (2013). According to their hypothesis, the evolutionary order among the family should be parallel to that of the host plants subfamily/tribe/species. It is possible that another phylogenetic hypothesis has been overlooked due to the phylogenetic position of the Asian taxa *A. barnardiae* and *Floromyces*. There may be a more complex evolutionary history, such as how the ancestor of *Floromycetaceae* acquired parasitism to *Asparagaceae* after host species diversification (not co-evolution). To resolve these questions, further studies with a focus on the evolution of Asian *Asparagaceae* and the discovery of the new *Floromycetaceae* lineages may contribute to an improved understanding of the natural relationship of anther smut fungi in *Ustilaginomycotina*.

Our microscopic observations agreed with those of Ingold (1984), who conducted time-lapse observations for *A. vaillantii sensu lato* (as *U. vaillantii*). He considered that the germination pattern was similar to that of “*Ustilago violacea* (Pers.) Roussel [current *Microbotryum violaceum* (Pers.) G. Deml & Oberw.]” as there was a dissociable basidium, but it could be distinguished from the latter as *A. vaillantii sensu lato* did not bud yeast-like cells through the wall of the teliospore (Ingold 1983). Subsequently, Deml and Oberwinkler (1982) transferred *U. violacea* to the genus *Microbotryum* Lév. and the proposal was supported by molecular phylogenetic evidence (Roux et al. 1998). The fact that the two phylogenetically distant anther smuts showed similar basidium development is not only of interest evolutionarily, from the perspective of the basidiomycetous basal group, but is also important as it is taxonomically informative and helps to distinguish these groups, as suggested by Ingold (1984). Another genus of *Floromycetaceae*, *Floromyces* also has the same ontogenic pattern as the *Antherospora*, according to the illustration provided by Vánky et al. (2008). However, their description seems to be problematic, i.e., *Floromyces* can be characterized as having holobasidia and phragmobasidia. Considering our morphological observations and the report by Ingold (1984), they may have misinterpreted the short pedicel as a holobasidium and immature aseptate basidia as a basidiospore (like our Fig. 30) due to the nature of the dissociability from the teliospore. Our morphological observations confirmed that these aseptate basidium-like cells (Fig. 30) developed to a phragmobasidium (Fig. 3P–R) and the time-lapse observations strongly supported this (Fig. 4; Ingold 1984). Thus, we concluded that holobasidia were misinterpreted and were actually immature aseptate basidia, which led us to re-circumscribe the familial concept of the *Floromycetaceae* as follows:

Floromycetaceae M. Lutz, R. Bauer & Vánky, Mycotaxon 104: 181 (2008)

Sori in host plant anthers in *Agavaceae* forming a dark coloured. Peridium, columella and sterile cells lacking. Infection systemic. Spores single or in balls, sterile cells lacking. Spores subabgular, ellipsoidal, slightly irregular. Spore germination results phragmobasidia or long with short pedicel. Basidiospores usually septate, produced on sterigmata, germinating by secondary sporidia or hyphae. Host-parasite interactions by haustoria. Septal pore simple.

Notes: The family *Floromycetaceae* was established to accommodate *Antherospora* and *Floromyces*, which were found on the anther smut of *Agavaceae* and originally characterized by a holobasidia and phragmobasidia (Vánky et al. 2008). According to our morphological observations and those of Ingold (1984), the basidium-type should be interpreted as phragmobasidium. As the genera of the family can be characterized by anther smut, spore single or in balls, and lacking sterile cells, it can be distinguished from the family in *Urocystidales*. Although it was not noted or mentioned in previous studies (Ingold 1984; Vánky et al. 2008), the formation of the metabasidium on a short pedicel seems to be a common derived feature of this family.

Compared to *Microbotryales* (*Microbotryomycetes*), the basidiospore ontogenic pattern of the Urocystidalean anther smut has not previously been assessed from an evolutionary or phylogenetic perspective, except for a few studies (Bauer et al. 2008; Piątek et al. 2011; Piątek et al. 2013). Originally, Ingold (1983, 1984) only investigated the biological cell perspective but unintentionally revealed the taxonomically important differences in the ontogenic patterns, which can be used to distinguish *Urocystidales* and *Microbotryales*. To understand morphologically or ecologically convergent evolution, host interactions (Bauer et al. 2008; Piątek et al. 2011; Piątek et al. 2013) and anatomical features (Bauer et al. 2008) as well as sexual reproductive processes may also be required.

In this investigation, we have succeeded in establishing a pure *Floromycetaceae* culture, which, to the best of our knowledge, is only the second time this has been reported (only one strain in IMI; WGCM; <https://gcm.wdcm.org/>, accessed 2022 December). This situation may be due to the fact that previous studies have focused exclusively on a specimen-based approach, not including field sampling. In addition to this, the establishment of pure culture strains from *Urocystidales* from the teliospore is often difficult due to the difficulties in germination. To date, many species are still uncultured. We hope that our pure culture strains may help to elucidate biological properties such as the formation of teliospore *in vitro* (like Enomoto 1953) and the induction of basidiospores *in vitro* for repeatable observations.

Declarations

Ethics approval and Consent to participate: The authors declare that there is no Ethical statement. This article does not contain any studies with animals performed by any of the authors.

Consent for publication: Not applicable.

Availability of data and materials: The DNA sequences have been deposited at DDBJ under accession number @-@.

Competing interests: The authors declare they have no competing interests.

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Authors' contributions: Research Proposals: SS. Conceived and designed the experiments: AH; SS. Field Research: AH; SS. Performed the experiments: AH. Methodology: AH. Review and editing: SS; AH; MO. Supervision: MO. Wrote paper: AH; SS

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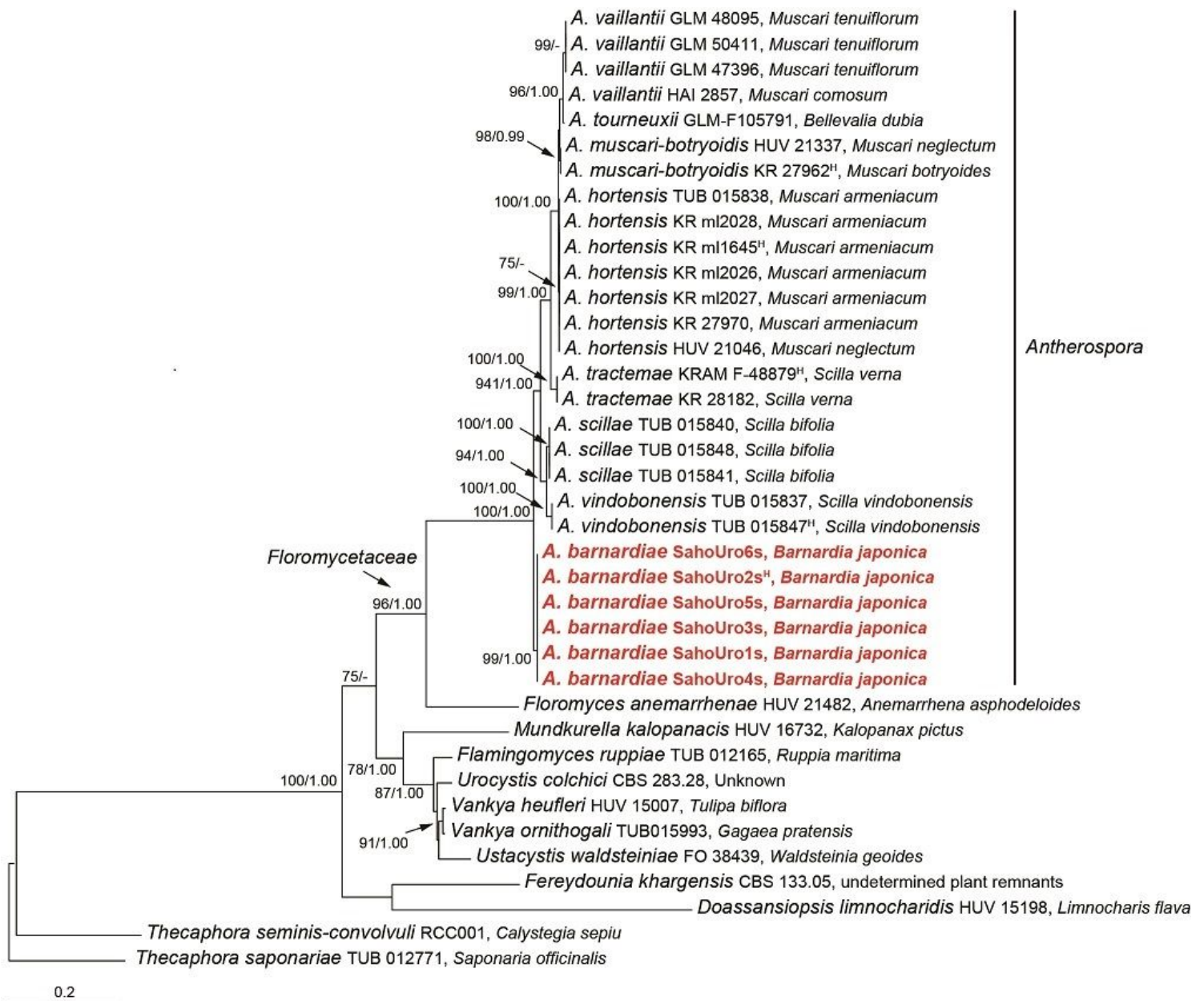
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Figures



Shibata and Hashimoto et al. (2023)

Figure 1

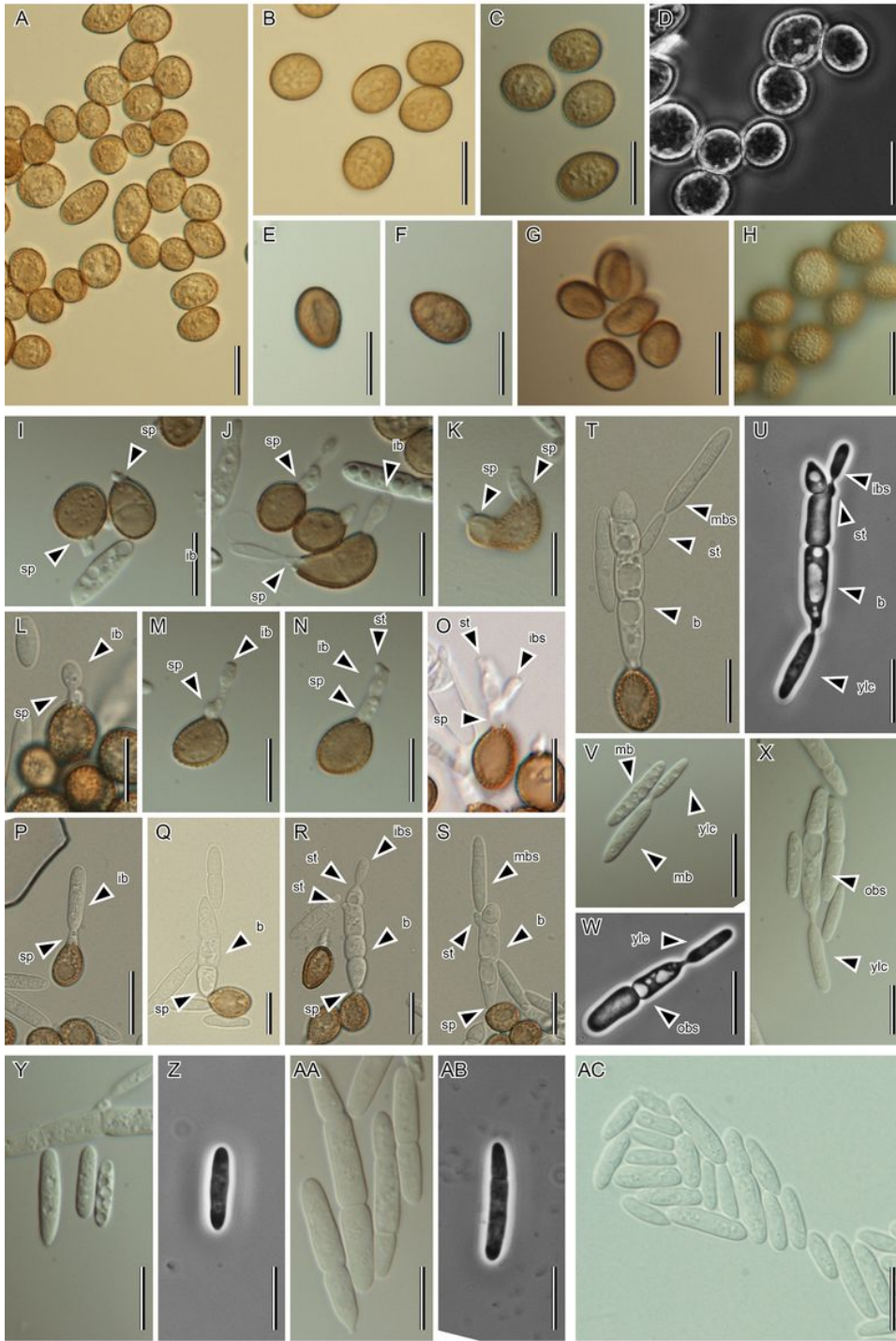
Maximum-likelihood (ML) tree of *Floromycetaceae* based on ITS-LSU sequences. ML rapid bootstrap proportion (BP) > 70 % and Bayesian posterior probabilities (PP) > 0.95 are presented at the nodes as ML BP/Bayesian PP. A hyphen ('-') indicates values < 70 % BP or 0.95 Bayesian PP is shown with 'x'. Ex-holotype strains or holotypes are indicated with a superscript H. The newly obtained sequences are shown in **bold** and red. The scale bar represents nucleotide substitutions per site.



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Figure 2

Habits of *Antherospora barnardiae* on *Barnardia japonica*. **A–G** Habitats (Arrowhead indicates infected plants). **H, I** Healthy inflorescences (I indicates anthers covered with white pollen for comparison with sori). **J–O** Sori of *Antherospora barnardiae* in all flowers of an inflorescence of *Barnardia japonica*. Note that the buds were filled with teliospores in M–O. A, F, G, J–O SahoUro1; B, D, E, H, I SahoUro3; C SahoUro4. Scales I, J = 5 mm, K = 500 μ m, L = 200 μ m, M–O = 1 mm.



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Figure 3

Morphology of *Antherospora barnardiae*. **A–H** Teliospores (H indicates ornamentation of teliospores), **I–X** Germination of Teliospores (b = basidium, ib = immature basidium, ibs = immature basidiospore, obs = overmature basidiospore, mbs = matured basidiospore, sp = short pedicel, st = sterigma and ylc = yeast-like cell; Note U indicates dissociable basidium). **Y–AB** Basidiospores. **AC** Yeast-like cells. A–D, G, H–K, M, N, U–X–AC from SahoUro3 (= KMP @), O from SahoUro1 (= KMP @), L, P–T from SahoUro2 (= KMP @), E, F from KPM-NC 0015106. Scales A–AC = 10 μm.

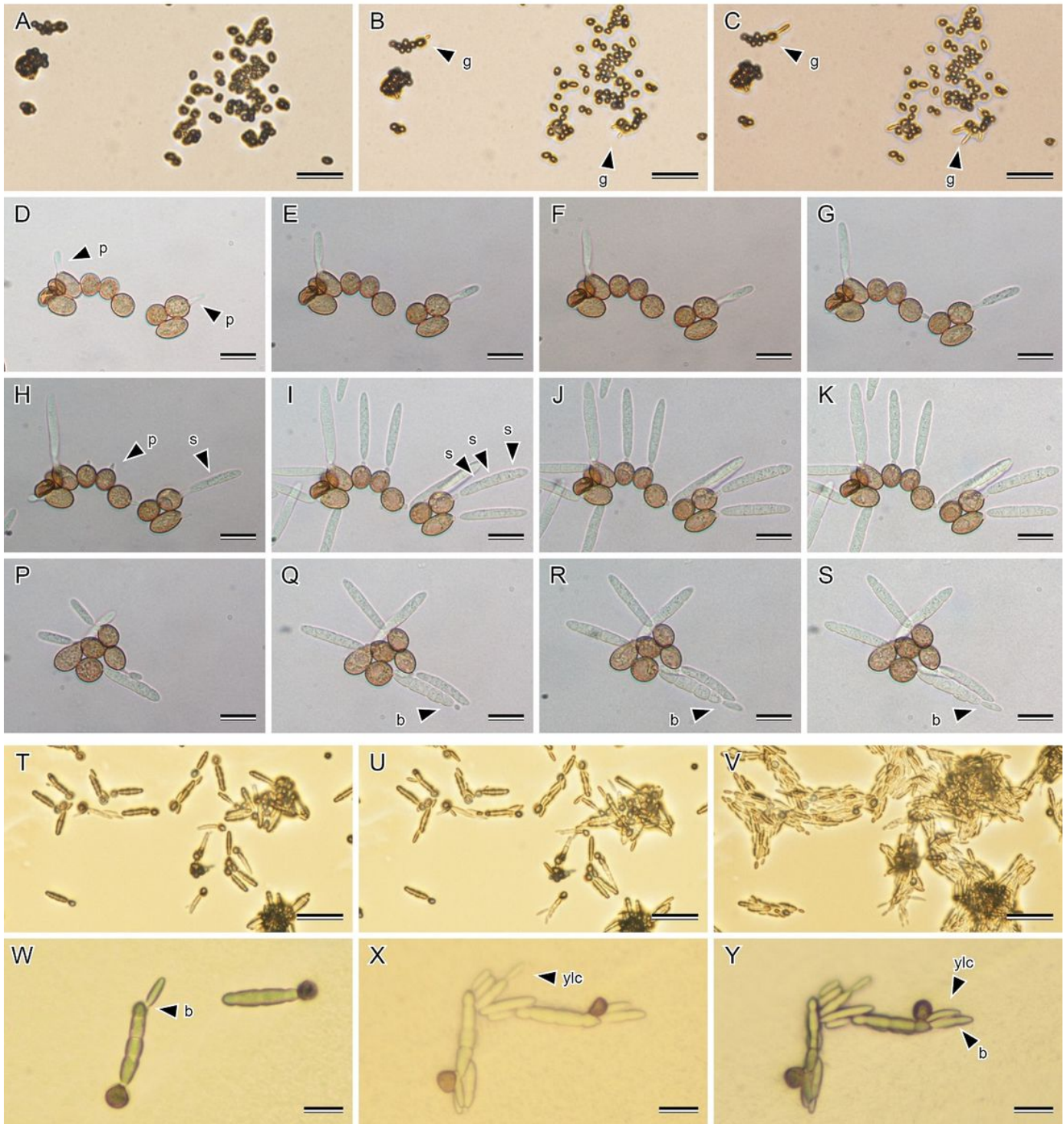


Figure 4

Time-lapse ontogeny of basidia, basidiospores and yeast-like cells of *Antherosporea barnardiae*. **A–C** Overview of germination of teliospores (g indicates germinated teliospora). **D–K** Germination of spore and development of basidia (p indicates pedicel, s indicates septation). **P–S** Development of basidiospore (b indicates basidiospore). **T–V** Overview of budding of yeast-like cells. **W–Y** Budding of yeast-like cells (ylc indicates yeast-like cell). A–S SahoUro6 (= KMP @). T–Y SahoUro3 (= KMP @). Scales A–C, T–V = 50 μ m, D–S, W–Y = 10 μ m.

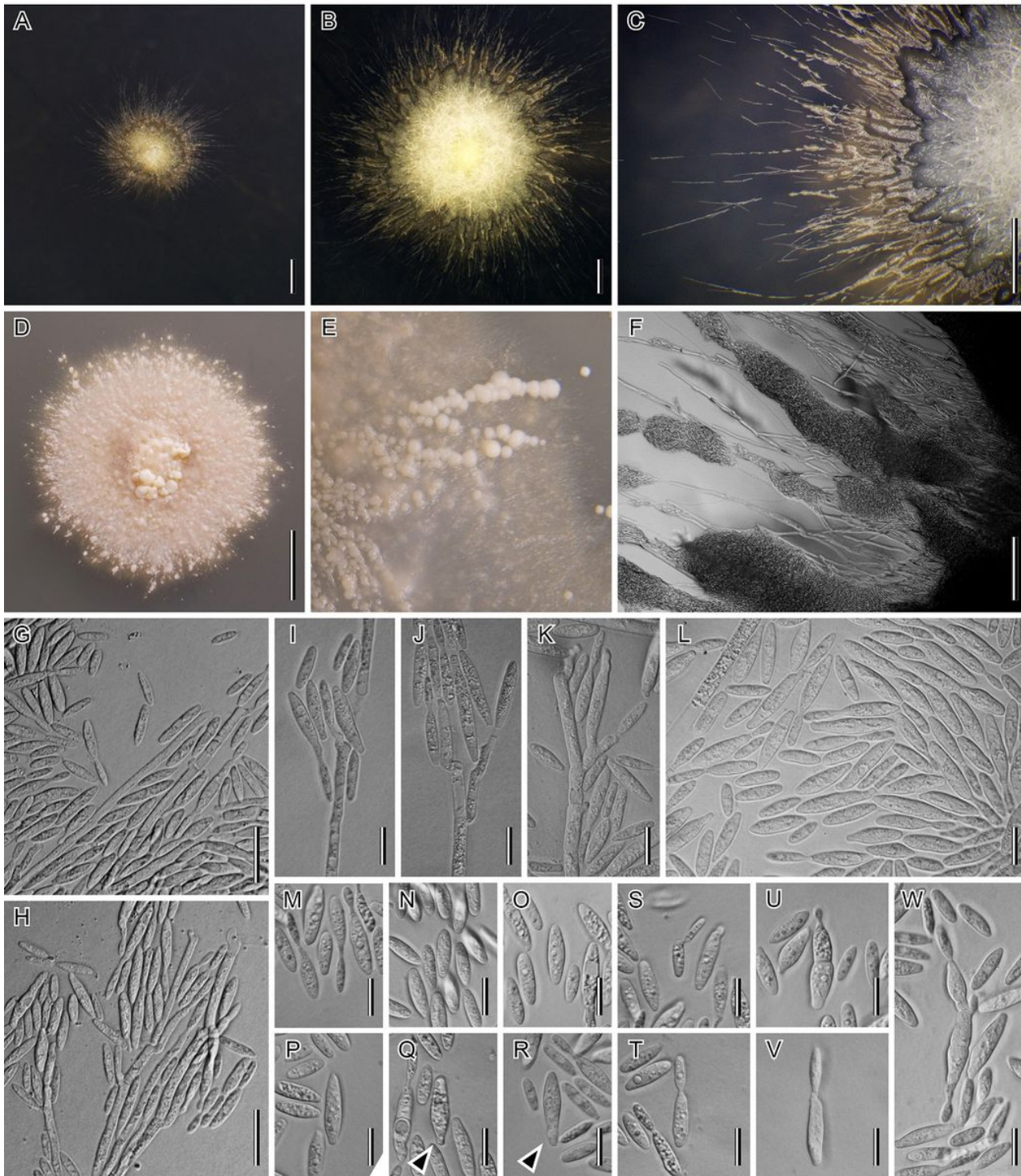


Figure 5

Culture characteristics of *Antherospora barnardiae*. **A** Seven-day colony on YM after isolate from WA. **B, C** Ten-day colony on YM after isolate from WA. **D, E** Colony characters on PDA within 1 month at 20 °C in the dark. **F** Overview of hyphae with yeast-like cells. **G–K** Yeast-like cells (ylc) (I–K indicate budding of ylc from hyphae). **L–W** Yeast-like cells (arrowhead indicates a scar of budding in Q, R, T–W indicate ylc budding from overmature ylc). A–C, G–W from Culture SahoUro1s. D–F from Culture SahoUro2s (=JCM @). Scales A–C = 1mm, D = 1cm, F, H = 100 μm, I–W = 10 μm.