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FULL PAPER

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An aero-aquatic fungus, *Peyronelina glomerulata*, is shown to have teleomorphic affinities with cyphelloid basidiomycetes

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Abstract On decayed wood near a stream, tiny cyphelloid, hair-bearing, *Flagelloscypha*-like basidiomata were found coexisting with conidia of an aero-aquatic fungus, *Peyronelina glomerulata*. An isolate originating from the basidioma produced conidia of *P. glomerulata* by soaking the culture in water. Three additional strains originating from conidia of *P. glomerulata* produced immature basidiomata with basidium-like structures on the agar medium after about 4 months incubation. Fine structure of the hyphal septa of *P. glomerulata* was found to be of the dolipore type. Phylogenetic analysis based on sequences from the D1/D2 regions of the LSU rDNA showed that the strains from conidia and from a basidioma clustered together in the *Flagelloscypha* clade and nested within the *Nia* clade of Hymenomycetes. The culture studies and molecular phylogenetic analysis suggested that *P. glomerulata* has a *Flagelloscypha* teleomorph, a cyphelloid basidiomycete. The molecular data also indicate that *P. glomerulata* is phylogenetically related to the marine basidiomycetes, *Nia* and *Halocyphina*. Thus, this study revealed that cyphelloid basidiomycetes have evolved into both marine as well as freshwater habitats by morphological adaptations of the teleomorphs in the former and of the anamorph in the latter case.

Key words Aero-aquatic fungi · Cyphelloid fungi · *Flagelloscypha* · *Peyronelina glomerulata* · Teleomorph–anamorph relationship

Introduction

Peyronelina is a monotypic genus containing *Peyronelina glomerulata* Arnaud ex Fisher, Webster & Kane (Arnaud 1952; Fisher et al. 1976). This species inhabits submerged decaying twigs and litter in streams and ponds, although some records may suggest its fungicolous properties [Arnaud 1952; the Fungal Records Database of Britain and Ireland (FRDBI) managed by the British Mycological Society and hosted by CABI, UK; the online catalogue of International Collection of Micro-organisms from Plants (ICMP) culture collection managed by Landcare Research, New Zealand]. Crown-shaped conidia composed of central 20–30 subglobose cells and surrounding 7–17 arms are produced on the surface of the substrate in a moist atmosphere (Figs. 1–3). Ecologically, *P. glomerulata* belongs to the aero-aquatic fungi, because its conidia float on the surface of water by entrapping air inside the arms of the conidium (Fig. 3) (Fisher et al. 1976; Nakagiri and Ito 1997). The teleomorph of this species and its phylogenetic position are unknown and have not been studied so far.

Cyphelloid to disk-shaped basidiomata bearing basidia were found coexisting with conidia of *P. glomerulata* on decaying wood near a stream in Odawara, Kanagawa Prefecture, the mid-part of Japan, in April 2005 (Figs. 4–6). We tried to confirm the teleomorph–anamorph relationship of these fungi by culture studies and also from molecular data. The number of nuclei per cell and fine structures of the hyphal septa were examined to confirm the basidiomycete affinity of *P. glomerulata*.

The taxonomic position of *P. glomerulata* was investigated by phylogenetic analysis of the large subunit (LSU) rDNA (D1/D2 regions) by examining eight strains isolated from conidia of *P. glomerulata* and one strain from a cyphelloid basidioma (Table 1).

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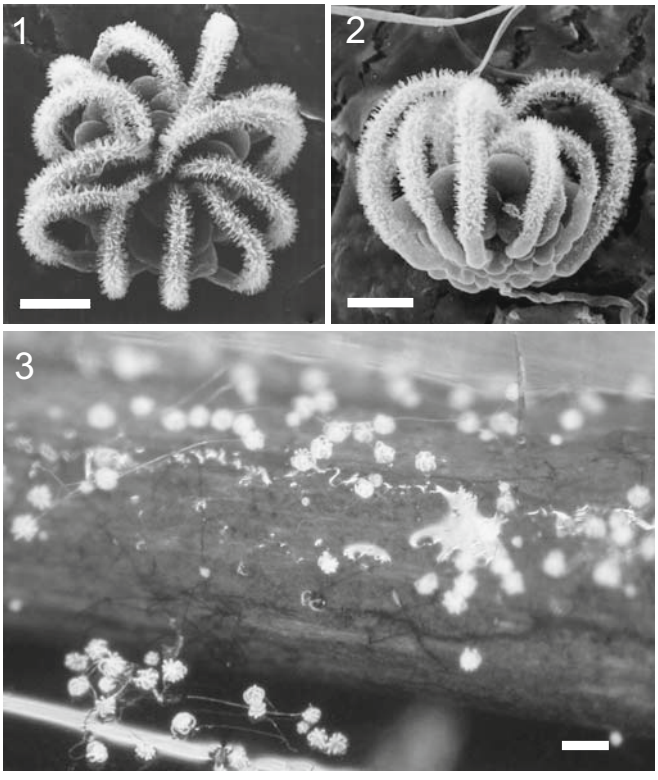
Materials and methods

Fungal materials and strains

Submerged twigs, litter, and wood were collected from streams and ponds in various parts of Japan (see Table 1) and incubated in a moist chamber at room temperature

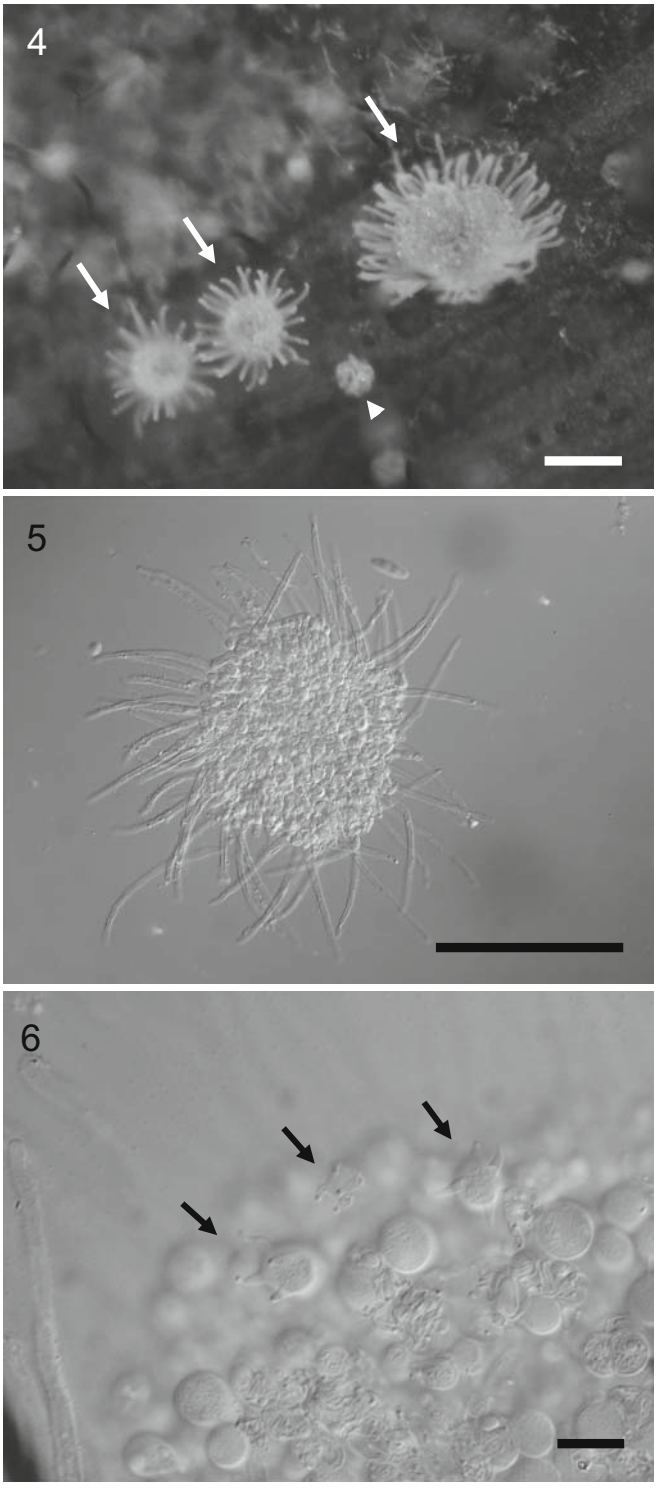


Figs. 1–3. Conidia of *Peyronelina glomerulata*. **1, 2** Crown-shaped conidium composed of central subglobose cells and arms. **3** Conidia produced on the surface of a twig. Note the released conidia floating on the water. **1, 2** Scanning electron micrographs; **3** dissecting micrograph. *Bars* **1, 2** 10 μm; **3** 50 μm

(~25°C) for at least 3 months. The material was examined under a dissecting microscope regularly once a week for the first month, then every 4 weeks for the following 2 months. Conidia and basidiomata that appeared on the substrates were picked up with a needle and inoculated onto YM agar (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1.5% agar, pH 5.6) containing tetracycline hydrochloride (50 mg/l). Nine strains [eight newly isolated strains (including one *Flagelloscypha* strain) and one strain (NBRC 32867) maintained in NITE Biological Resource Center (NBRC) collection] were used for this study (see Table 1). Strains were maintained by culturing on potato sucrose agar (PSA: extract from 200 g/l potato, 2% sucrose, 2% agar, pH 5.6) and potato carrot agar (PCA: extract from 20 g/l potato, extract from 20 g/l carrot, 2% agar, pH 6.0) at room temperature.

Culture studies to examine teleomorph–anamorph relationship

For inducing the teleomorph in culture, *Peyronelina* strains (NBRC 104517, NBRC 104518, NBRC 104521) were inoculated on sterilized twigs, which were stuck on 50 ml solid PCA medium in a 500-ml Erlenmeyer flask. Sterilized water was added in the flask to keep the twigs wet. Some flasks were incubated at room temperature for at least 4 months,



Figs. 4–6. Cyphelloid to disk-shaped basidiomata of *Flagelloscypha*-like teleomorph on natural substrates. **4** Cyphelloid basidiomata (*arrows*) and conidia of *Peyronelina glomerulata* (*arrowhead*) on decaying wood near a stream. **5** Cyphelloid hair-bearing basidioma. **6** Basidia bearing four sterigmata (*arrows*) produced on basidioma. **4** Dissecting micrograph; **5, 6** light micrographs. *Bars* **4** 0.1 mm; **5** 100 μm; **6** 10 μm

Table 1. Strains used in this study

Taxon	Strain	Strain data			Basidiomata production in culture	nuc-LSU rDNA (D1/D2 regions) accession no.
Peyronelina glomerulata	AN-1505 (= NBRC 32867)	Dec. 11, 1995	Pond; Kamegajyou-ike, Misaki-machi, Isumi-gun, Chiba Pref.	Submerged decaying culm of *Cyperus* sp.		AB455963
	6KY-12-10 (= NBRC 104517)	Mar. 5, 2003	Pond; Shinsekiya, Kimitsu-shi, Chiba Pref.	Submerged decaying pod		AB455955
	7KY-4-8 (= NBRC 104518)	Apr. 17, 2003	Pond; Jyouganji temple, Nakajima, Kimitsu-shi, Chiba Pref.	Submerged decaying twig	○	AB455956
	20KY-7-6 (= NBRC 104520)	Sep. 22, 2005	Stream; Fuchigasawa, Seiwa forest, Houei, Kimitsu-shi, Chiba Pref.	Submerged decaying twig	○	AB455957
	21KY-6-3 (= NBRC 102381)	Sep. 30, 2005	Waterfall; Sugadaira Kogen, Ueda-shi, Nagano Pref.	Submerged decaying litter		AB455958
	29KY-5-10 (= NBRC 104521)	Apr. 22, 2007	Stream; Goshouzawa, Iryuda, Odawara-shi, Kanagawa Pref.	Submerged decaying twig		AB455959
	30KY-8-2 (= NBRC 104128)	Apr. 28, 2007	Pond; Sugadaira Kogen, Ueda-shi, Nagano Pref.	Submerged decaying litter		AB455960
	36KY-8-3 (= NBRC 104522)	Sep. 26, 2007	Stream; Iriomote Island, Okinawa Pref.	Submerged decaying twig	○	AB455961
Flagelloscypha sp.	35KY-1-1 (= NBRC 104516)	Aug. 12, 2007	Stream; Goshouzawa, Iryuda, Odawara-shi, Kanagawa Pref.	Submerged decaying wood		AB455962
Flagelloscypha japonica	NBRC 101830 (= JCM 12855)	–	–	–	–	AB455964
Halocyphina villosa	NBRC 32086	–	–	–	–	AB455965
Halocyphina villosa	NBRC 32087	–	–	–	–	AB455966
Nia vibrissa	NBRC 32089	–	–	–	–	AB455967
Nia vibrissa	NBRC 32090	–	–	–	–	AB455968

while the others were incubated at 4°C for a month after preincubation at room temperature for a month, then transferred into the program incubator (CFH-300; Tomy Seiko, Tokyo, Japan). The following light–dark cycle and temperature were employed: 12 h light period at 20°C, 12 h dark period at 10°C, for simulating early spring natural conditions.

To induce conidium production, a strain isolated from the basidioma (NBRC 104516) was incubated as follows: the colony of NBRC 104516, which had been cultured on PCA at 20°C, was cut into 5 × 5 mm agar blocks, then the agar blocks were submerged into sterilized water and incubated for 2–3 weeks at room temperature.

Scanning electron microscopy

Small piece of the natural substrate and PCA agar blocks with conidia were fixed with 1% O_sO_4 at 4°C for 12 h or at room temperature for 2 h, then dehydrated in ethanol series and finally substituted with isoamyl acetate. After critical-point drying and coating with platinum-palladium, the specimens were observed with a JSM-6060 (JEOL, Tokyo, Japan) operated at 15 kV.

DAPI staining

Hyphal cells of the conidia-forming strains and conidial cells were treated with 4′,6-diamidino-2-phenylindole (DAPI) in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA) by following the manufacturer's protocol. The number of nuclei per cell was observed using an Axioplan2 imaging microscope (Carl Zeiss MicroImaging, Tokyo, Japan).

Observation of fine structure of hyphal septum

Mycelia cultured on PCA plates were submerged in 3.5% glutaraldehyde/1/15 M potassium phosphate buffer (pH 7.0) for 10–30 min for prefixation. Agar disks about 2–3 mm in diameter were cut out from mycelia and transferred to vessels containing 3.5% glutaraldehyde/1/15 M potassium phosphate buffer (pH 7.0) for 1 h at room temperature. After washing five times with 1/15 M potassium phosphate buffer (pH 7.0), the sample was fixed with 2% O_sO_4 solution for 1 h at room temperature. After washing three times with distilled water, the sample was dehydrated in ethanol series. Ethanol was substituted by acetone and then Spurr's resin

(Spurr 1969). The sample was embedded in the resin and solidified by the following resin polymerizing program: 50°C for 5 h, then 70°C for 48–60 h. Thin sections of mycelia were made using an ultramicrotome (Ultracut UCT; Leica Microsystems, Wetzlar, Hessen, Germany) equipped with a diamond knife, then picked up on the formvar-coated slot grids. Sections were stained with 3% uranyl acetate for 2 h followed by lead citrate (Reynolds 1963) for 5 min and examined under a H-7600 transmission electron microscope (Hitachi, Tokyo, Japan) operated at 100 kV.

DNA isolation and PCR amplification

Mycelia cultured on PSA or PCA plates were harvested using a spatula and put into 2-ml plastic tubes. DNA was extracted using the Nucleon PhytoPure Genomic DNA Extraction Kit (Amersham Biosciences, Piscataway, NJ, USA). Polymerase chain reaction (PCR) was performed by using the *TaKaRa Ex Taq* Hot Start Version Kit (Takara Bio, Otsu, Shiga, Japan). LSU rDNA (D1/D2 region) fragments were amplified using primers NL1 and NL4 (O'Donnell 1993). A total 50 µl mixture [33.7 µl distilled water, 5 µl DNA extract (template), 5 µl PCR buffer, 4 µl dNTP, 0.3 µl Taq DNA polymerase, 1 µl each primer (final conc., 1 µM)] was employed for PCR. Amplification of the DNA fragments was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the following thermal cycling program: an initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, a final extension at 72°C for 5 min, and a 4°C soak. Amplified DNA was purified by using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences).

DNA sequencing

Sequencing reactions were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers NL1 and NL4 on the GeneAmp PCR System 9700 (Applied Biosystems) under the following program: an initial denaturation at 96°C for 3 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, extension at 60°C for 4 min, and a 4°C soak. Sequencing reaction products were purified with the Agencourt CleanSEQ Kit (Agencourt Bioscience, Beverly, MA, USA) and sequenced with the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contiguous sequences were assembled by using the Sequencher ver. 4.7 (Gene Code, Ann Arbor, MI, USA). Sequences obtained in this study were deposited at DDBJ/EMBL/GenBank.

Phylogenetic analysis

Forty-eight sequences from 38 taxa of homobasidiomycetes including cyphelloid fungi (by referring to Bodensteiner et al. 2004) were downloaded from DDBJ/EMBL/GenBank. The data set (42 taxa) including our own sequences of *Peyronelina*, *Flagelloscypha*, *Nia*, and *Halocyphina* strains (see Table 1) was aligned using Clustal X ver. 1.83 (Thompson et al. 1997). The resulting alignment was manually refined and gap positions were removed using Se-Al v2.0a11 (Rambaut 2007). The alignment was deposited at TreeBASE (http://www.treebase.org/treebase/index.html; study No. S2217, matrix No. M4214). A phylogenetic tree was constructed based on the neighbor-joining (NJ) method (Saitou and Nei 1987) and the K_{nuc} value (Kimura 1980) by using Clustal X. The topology of the tree was evaluated by the bootstrap resampling method (Felsenstein 1985) with 1000 replicates. The NJplot program (Perrière and Gouy 1996) was used for plotting the phylogenetic tree.

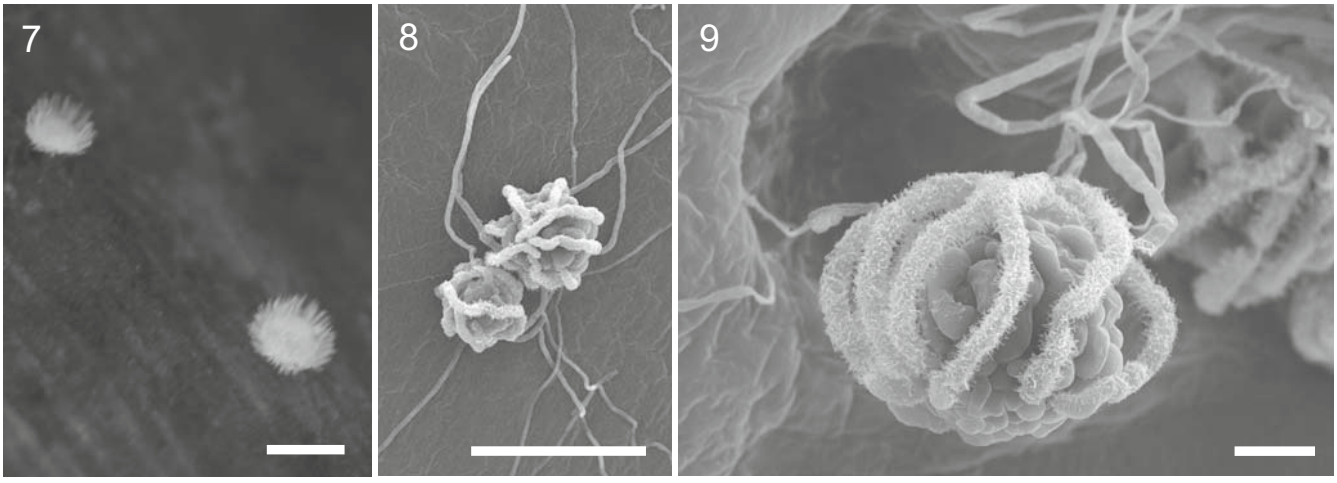
Results

Teleomorph–anamorph relationship

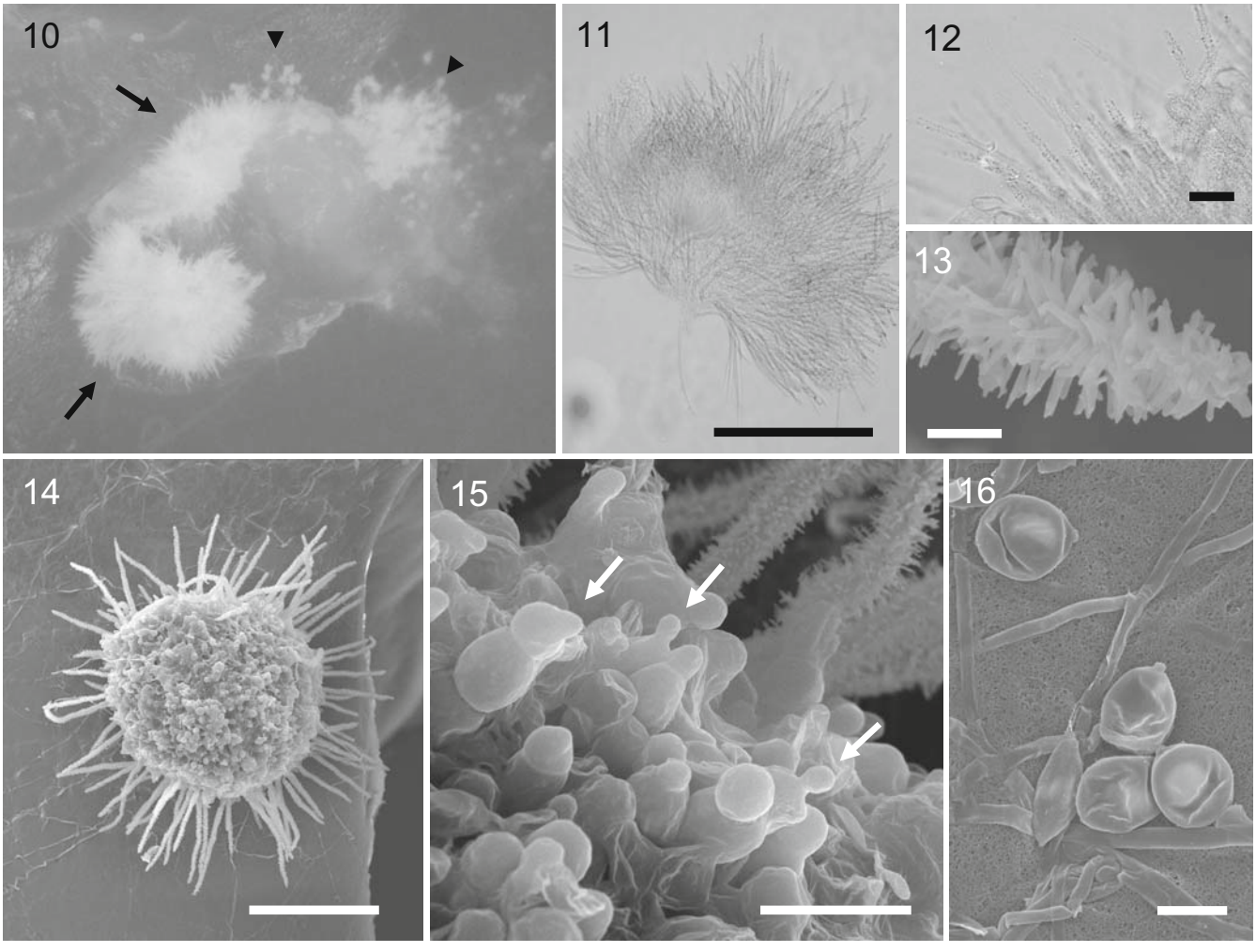
Cyphelloid hair-bearing basidiomata about 0.1 mm in diameter, which resembled apothecia of discomycetes, were found coexisting with conidia of *Peyronelina glomerulata* on decaying wood near a stream in Odawara, Kanagawa Prefecture in April 2005 (Fig. 4). Basidia bearing four sterigmata of the tapering spore-ejecting type were observed at the upper part of the basidioma, but basidiospores were not found (Fig. 6). The cyphelloid basidiomata were again found on submerged decaying wood at a stream in Kanagawa Prefecture in August 2007 (Fig. 7). The strain (NBRC 104516) was obtained by isolating a culture from a single basidioma. The culture of NBRC 104516 was found to produce conidia of *P. glomerulata* after submerging PCA agar blocks containing mycelia in water (Figs. 8, 9).

Isolates from conidia (NBRC 104518, NBRC 104520, NBRC 104522) of *P. glomerulata* produced cup- to disk-shaped immature basidiomata on PCA after incubation for about 4 months at room temperature (Figs. 10–14). The basidiomata show morphology of the genus *Flagelloscypha*, *Lachnella*, or *Pseudolasiobolus* (Agerer 1983). The characteristics of the surface hairs, i.e., tapering to the apex and encrusted with finely or coarsely acicular crystals or rhombic crystals (Figs. 10–14), indicate assignment of the fungus to *Flagelloscypha* (Agerer 1975, 1979). Although the basidiomata on the agar media were further incubated for an additional 2–3 months, basidia and basidiospores were not found on them, whereas deformed basidia-like structures and ejected basidiospores on the media around the basidioma were observed (Figs. 15, 16). Attempts to induce sexual reproduction by culturing on twigs with PCA medium in the program incubator were unsuccessful as well, even after the vernalization treatment (keeping the culture at 4°C for a month).

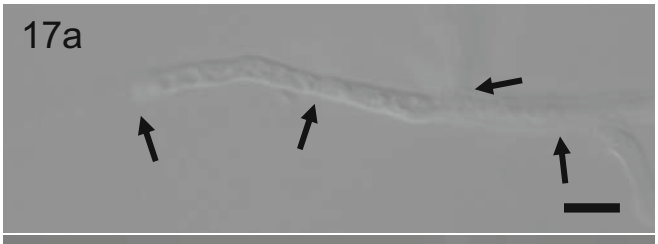
The number of nuclei per cell was examined by DAPI staining in the strains of *P. glomerulata*-producing conidia. One or two (mainly two, rarely three or four) nuclei were observed in each hyphal cell, and one to four nuclei were observed in the subglobose cells and the arm cells of the conidia (Figs. 17, 18).

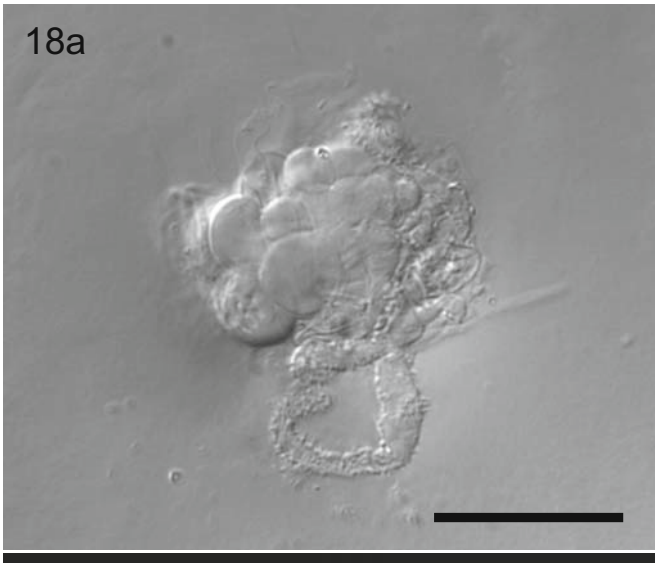


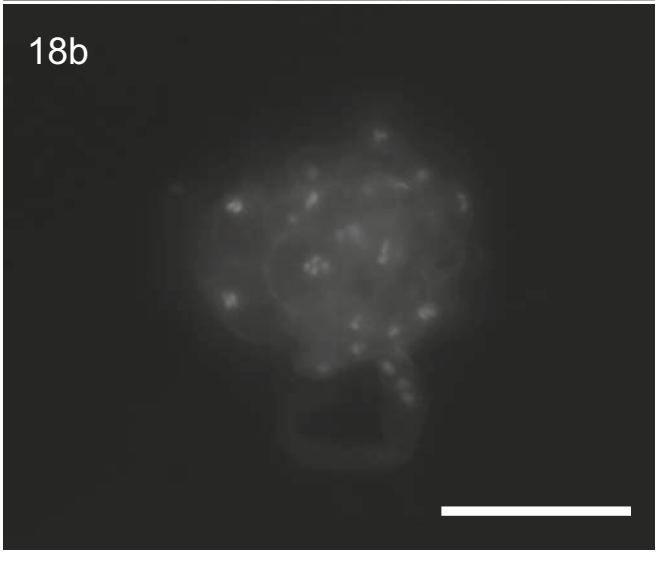
Figs. 7–9. Cyphelloid to disk-shaped basidiomata of *Flagelloscypha* sp. (NBRC 104516) and conidia of *Peyronelina glomerulata* produced by the strain. **7** Basidiomata on decaying wood submerged in a stream. **8, 9** Conidia produced on submerged potato carrot agar (PCA) blocks containing mycelia. **7** Dissecting micrograph; **8, 9** scanning electron micrographs. *Bars* **7** 0.2 mm; **8** 50 µm; **9** 10 µm



Figs. 10–16. Basidiomata and basidium-like structures produced by *Peyronelina glomerulata* strains. **10** Basidiomata (*arrows*) produced on potato carrot agar (PCA) after incubating for about 4 months. Note coexisting conidia of *Peyronelina glomerulata* (*arrowheads*). **11, 14** Cup- to disk-shaped basidiomata with hairs. **12** Tapering hairs around basidioma. **13** Surface of hairs. **15** Immature or deformed basidium-like structures with sterigma-like projections (*arrows*) on the upper surface of basidioma. **16** Discharged basidiospores scattered around basidioma on agar. **10–12** NBRC 104518; **13–16** NBRC 104522. **10** Dissecting micrograph; **11, 12** light micrographs; **13–16** scanning electron micrographs. *Bars* **11** 200 µm; **12** 20 µm; **13** 2 µm; **14** 100 µm; **15** 10 µm; **16** 5 µm







Figs. 17–18. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) of *Peyronelina glomerulata* strain (NBRC 104518). **17a–c** Vegetative hypha with hyphal septations (*arrows*). **18a,b** Globose cells and arm cells of a conidium. **17a, 18a** Nomarski optics; **17b,c, 18b** DAPI staining. *Bars* **17** 5 μm; **18** 20 μm

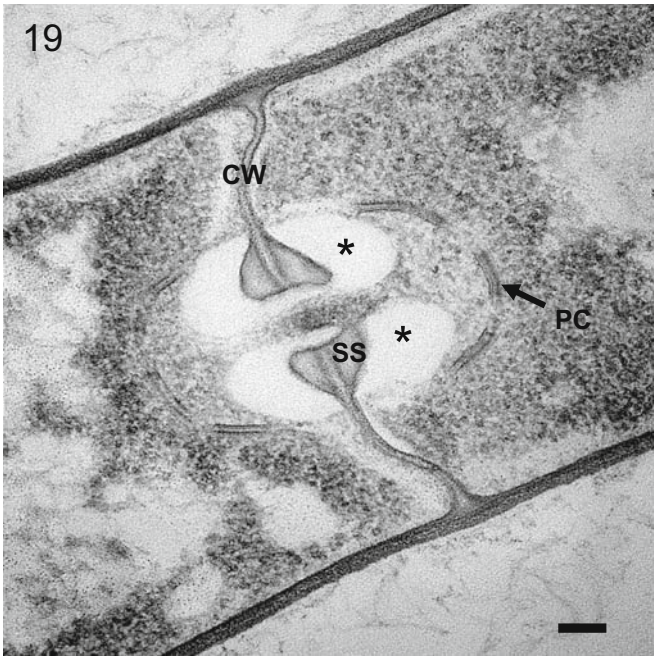


Fig. 19. Transmission electron micrograph of dolipore/parenthesome septum of *Peyronelina glomerulata* strain (NBRC 104517). Pore cap or parenthesome (*PC*), septal swelling (*SS*), electron-transparent zone (*asterisks*), and cross wall (*CW*). *Bar* 100 nm

The ultrastructure of hyphal septa of *P. glomerulata* strain (NBRC 104517) was observed to be a dolipore septum surrounded by a pair of the perforated pore cap or parenthesome (Fig. 19), which is the typical type of septa of agaricalean basidiomycetes (Kahn and Kimbrough 1982; Markham 1994).

Phylogenetic analysis

Phylogenetic analysis of LSU rDNA (D1/D2 regions) sequence data showed that eight strains from conidia and one strain from a basidioma are all located within the homobasidiomycetes and nested within the *Nia* clade containing marine species (*Nia*, *Halocyphina*, and *Calathella mangrovei*) and terrestrial species (*Flagelloscypha*, *Lachnella*, *Cyphellopsis*, etc.) (Fig. 20). The *Peyronelina* strains clustered together with *Flagelloscypha minutissima* (Burt) Donk and *F. japonica* T. Handa & Y. Harada. These topologies were supported by high (91%) and moderate (74%) bootstrap values (Fig. 20; bootstrap values with boldface).

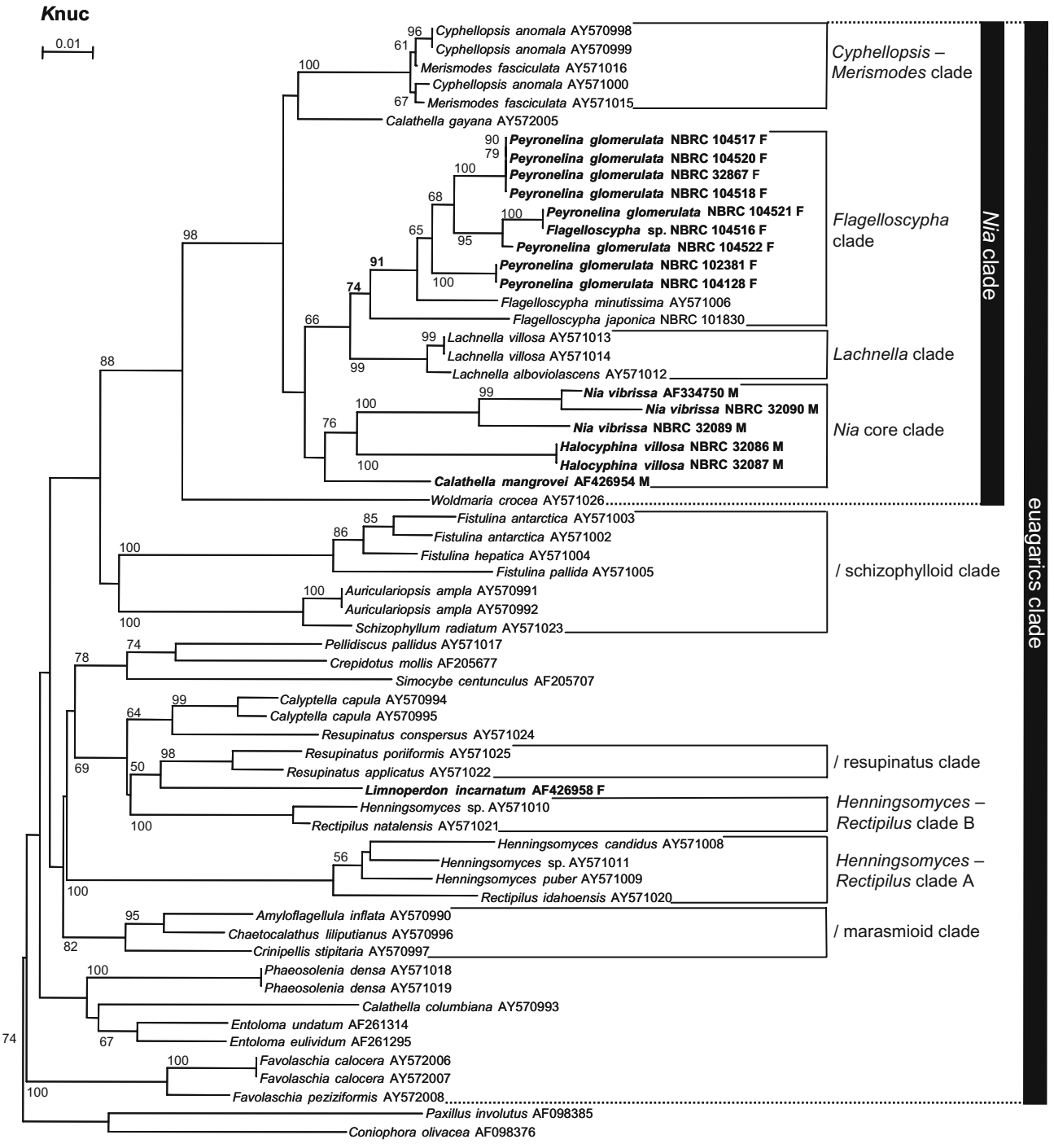


Fig. 20. Neighbor-joining phylogenetic tree inferred from large subunit (LSU) rDNA (D1/D2 regions) sequence data (513 bp). Names of aquatic taxa are in *boldface*. *M*, marine; *F*, freshwater. Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. The names of clades refer to Bodensteiner et al. (2004). Samples of which new sequences had been generated in this study are given with strain numbers. Sequences downloaded from GenBank are given with accession numbers

Discussion

The culture studies and phylogenetic analysis of the LSU rDNA (D1/D2 regions) sequence data revealed that *Peyronelina glomerulata* has a *Flagelloscypha*-like teleomorph.

The morphology of basidiomata on decayed wood and of those induced from *P. glomerulata* strains in culture corresponds with that of the genus *Flagelloscypha* (see Figs. 4–7, 10–14). However, basidiomata produced by *P. glomerulata* strains did not mature in culture. Our attempts to induce maturation of basidiomata were unsuccessful. For species-level identification of the teleomorph, therefore,

further research on mature specimens formed on the natural substrates or in culture is required.

One to four (mainly two) nuclei were observed in each cell of *P. glomerulata* strains by DAPI staining (Figs. 17, 18). This observation indicates that conidia-forming hyphae are probably dikaryotic (secondary hyphae). Further studies on nuclear behavior are required to clarify whether mating or self-duplication of nuclei is involved in the development of dikaryons. *Peyronelina glomerulata* strain (NBRC 104517) was found to have hyphae with dolipore septa surrounded by a perforated parenthesome, which has been typically observed in homobasidiomycetes (Fig. 19) (Kahn and Kimbrough 1982; Markham 1994). This dolipore structure is characteristic in having an electron-transparent zone around a septal swelling, which was also observed in the marine basidiomycetes *Nia vibrissa* and *Digitatispora marina* (Brooks 1975). These findings of dikaryotic cells and the dolipore structure of the hyphal septa are clear proof that *P. glomerulata* is a basidiomycetous anamorphic fungus.

Most aero-aquatic fungi have been known as ascomycetous anamorphs (Webster and Weber 2007). Only two aero-aquatic fungi have been known to have basidiomycetous teleomorphs, i.e., *Aegerita candida* Persoon and *Aegeritina tortuosa* (Bourdot & Galzin) Jülich, the teleomorphs of which are *Bulbillomyces farinosus* (Bresàdola) Jülich (Kendrick and Watling 1979) and *Subulicystidium longisporum* (Patouillard) Parmasto (Kendrick and Watling 1979), respectively. The *Peyronelina–Flagelloscypha* connection is the first case in which the teleomorph is a cyphelloid basidioma-forming basidiomycete, as the teleomorphs of *Aegerita* and *Aegeritina* produce corticioid basidiomata (Kendrick and Watling 1979).

Molecular phylogenetic analysis showed that *P. glomerulata* was nested within the cyphelloid homobasidiomycetes and clustered with *Flagelloscypha*, terrestrial cyphelloid basidioma-forming fungi (Fig. 20). *Peyronelina glomerulata* strains were separately clustered into several clades (Fig. 20). This separation partly corresponds to the geographic origins of the strains. Because we could not find any differences among the strains in morphology and other phenotypic characters, we consider that all these strains belong to a single species, *P. glomerulata*.

This study also showed that *P. glomerulata* is phylogenetically related to the marine basidiomycetes *Nia vibrissa* R.T. Moore & Meyers and *Halocyphina villosa* Kohlmeyer & E. Kohlmeyer (Fig. 20). *Nia* and *Halocyphina* are considered to have evolved from terrestrial cyphelloid fungi and adapted to marine habitats by forming gasteroid basidiomata and appendaged or nondischarged basidiospores (Nakagiri and Ito 1991; Jones and Jones 1993; Hibbett and Binder 2001; Bodensteiner et al. 2004). Thus, these cyphelloid basidiomycetes succeeded in evolving into marine habitats by modifying their teleomorph structures. No anamorph is known from these marine basidiomycetes. On the other hand, *Peyronelina–Flagelloscypha* showed apparent adaptation to freshwater habitats by its aero-aquatic anamorph instead of its *Flagelloscypha* teleomorph, which has the terrestrial type of discharging basidia and basidiospores. These phenomena may indicate that the terrestrial cyphelloid basidiomycetes have adapted to marine and freshwater environments by different strategies, i.e., by modifying teleomorphs and anamorphs, respectively.

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