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Bartheletia paradoxa is a living fossil on *Ginkgo* leaf litter with a unique septal structure in the *Basidiomycota*

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ABSTRACT

Bartheletia paradoxa, a basidiomycete growing on fallen leaves of *Ginkgo biloba*, is redescribed. In autumn a rapidly developing anamorph is formed on freshly fallen leaves and subsequently a teleomorph with hemispherical pustules of thick-walled resting spores (teliospores) that germinate after a resting period of one year with stipitate, longitudinally septate, statismosporic phragmobasidia. The basidia produce several basidiospores on each sporogenous locus. Inoculation experiments and observations in the field suggest that the basidiospores infect the freshly fallen leaves of *G. biloba* so that the life cycle is completed. The extraordinarily rapid development has also been confirmed in cultures on agar media and in inoculation experiments. Inoculation experiments also indicate that the fungus is specific to *G. biloba*. The septa of the hyphae have no central pores, but multiple plasmodesma-like perforations. The basidiospores and conidia are uninucleate, but an assessment of the karyology is still pending. A molecular phylogenetic hypothesis based on nuSSU rDNA sequences suggests that the fungus belongs to the *Agaricomycotina*, clustering in an unresolved position at the basal branching of the group. The family *Bartheletiaceae* fam. nov. is proposed to accommodate *Bartheletia paradoxa* in the *Agaricomycotina*. The name *B. paradoxa* is validated by a Latin diagnosis and by the designation of types.

Introduction

Ginkgo biloba, the maiden hair tree (*ginkyo* in modern Japanese, from Chinese 銀杏, *yín xìng* 'silvery apricot'), is probably the best-known example of a palaeoendemic and of a living plant fossil. It is the last representative of an otherwise extinct division of gymnosperms (*Ginkgophyta*), which was most broadly developed in the Mesozoic. Fossil records show that the geographical range of the genus had declined substantially by the late Tertiary. After the Pleistocene, only *G. biloba* survived in small relict areas in China. However, the species came to be a temple tree in eastern Asia. Since its introduction to European parks and gardens in the first half of the 18th century, *G. biloba* has become increasingly popular and is now

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planted globally in temperate regions. It is apparently the most ancient living seed plant and is important pharmacologically (e.g. van Beek 2000).

Some vesicular–arbuscular mycorrhizas are known from *Ginkgo* roots (Bonfante-Fasolo & Fontana 1985; Fontana 1985), but it might have been expected that such a phylogenetically ancient tree would have more host-specific fungal associates. However, resistance to fungal parasites might have contributed to its survival; it produces antifungal compounds (e.g. Major *et al.* 1960; Huang *et al.* 2000; Wang & Ng 2000; Krauze-Baranowska & Wiwart 2003; Sawano *et al.* 2007). The specificity of the few endotrophic fungi isolated from *Ginkgo* is poorly known (e.g. Kim *et al.* 1999), and a number of probably unspecific fungi are found on *Ginkgo* litter (Mel'nik & Scheuer 2007). Here we report on an apparently saprotrophic, but surprisingly host-specific and widespread fungal associate of *G. biloba*. The fungus has a unique set of phenotypic characters and a basal position in basidiomycete phylogeny. It was first collected in France by Jean Jules Barthelet in 1932, and the resting spores were later figured and briefly described by Arnaud (1954) as a new genus and species, *Bartheletia paradoxa*, but without a Latin diagnosis and, therefore, not validly. Although this collection from France is most probably lost (Nicot & Charpentié 1971; Carmichael *et al.* 1980), Arnaud's names are taken up here as his drawing of the characteristic resting spores (teliospores) evidently depicts the same fungus.

Material and methods

LM

Freshly collected samples and herbarium material were examined by LM using an Axioskop 50 (Zeiss, Vienna), with tap water, 0.2 % potassium hydroxide, or lactic acid as mounting media. Thin sections for LM were prepared by hand under a dissecting microscope or with a Kryomat (Leitz, Wetzlar) freezing microtome, and examined in tap water and lactic acid. Measurements were taken from dried herbarium material in tap water and in lactic acid. Macro- and microphotographs were taken with a Zeiss AxioCam MRc5 (Zeiss), under a Leica-Wild M3Z dissecting microscope (Leica) and an Axioskop 50 (Zeiss), and processed by the free online software CombineZ (http://www.hadleyweb.pwp.blueyonder.co.uk/CZ5/combinez5.htm).

EM

For TEM, samples were fixed with 2 % glutaraldehyde in 0.1 M Sodium cacodylate buffer (pH 7.2) at room temperature overnight. Following six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1 % osmium tetroxide in the same buffer for 1 h in the dark, washed in distilled water, and stained in 1 % aqueous uranyl acetate for 1 h in the dark. After five washes in distilled water, samples were dehydrated in acetone, using 10 min changes at 25, 50, 70, 95 %, and three times in 100 % acetone. Samples were embedded in Spurr's plastic and sectioned with a diamond knife. Ultrathin serial sections were mounted on formvar-coated, single-slot copper grids, stained with lead citrate at room temperature for 5 min, and washed with distilled water. They were examined using a Zeiss transmission electron microscope (Zeiss) operating at 80 kV.

For SEM, leaves were fixed in 2.5 % glutaraldehyde (pH 7.2, phosphate buffer), washed in buffer, dehydrated (acetone series), critical point dried (Baltec, CO_2), mounted on aluminium stubs with double-sided tape and sputter coated with gold (AGAR sputter coater, Christine Gröpl Elektronenmikroskopie, Tulln). The samples were investigated with a FEI XL30 ESEM (Philips, Austria) in high vacuum mode (20 kV, secondary electron detection).

Molecular analysis

Total genomic DNA was extracted from *Bartheletia* material collected in the botanical gardens in Graz and/or Tübingen (voucher specimens in GZU and/or TUB), according to Cubero *et al.* (1999) or with the QIAGEN Plant Mini Kit (Qiagen, Vienna). PCR reactions were prepared for a 30 µl final volume containing 4 µl double-distilled water, 3 µl 10 × *Taq* polymerase reaction buffer (10 mM Tris pH 8,3), 3 µl of 2.5 mM dNTPs, 0.15 µl *Taq* DNA polymerase, 1.5 µl for each of the 10 µM primers. PCR amplifications were: initial 2 min at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 52 °C, and 90 s at 72 °C, and a final extension of 7 min at 72 °C. PCR products were cleaned using Qiaquick spin columns (Qiagen, Vienna) and both strands were sequenced (BigDye Cycle Sequencing Ready Reaction Kit, Applera, Vienna). Sequences were run on an ABI310 sequencer (Applera). To test the anamorph–teleomorph connection we sequenced the 5′ end of nuLSU rDNA using primers LR0 and LR3 (http://www.biology.duke.edu/fungi/mycolab/primers.htm) from harvested teliospores, basidiospores, conidia, and cultures derived from basidiospores.

The phylogenetic position was studied with full-length sequences of the nuSSU rDNA. The primers used were NS1 and NS8 for PCR, and NS1, NS2, NS3, NS4, NS5, NS6, and NS8 for sequencing (see White *et al.* 1990). The alignment included 41 sequences from GenBank, was produced automatically with ClustalW as implemented in BioEdit 5.0.6 (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html, Hall 1999), and then manually adjusted. As an outgroup sequence we used *Taphrina pruni*. The GenBank accession nos are included with the tree in Fig 5.

The phylogenetic analysis was carried out using MP (as implemented in PAUP 4.0b10; Swofford 2001) and a Bayesian approach as implemented in the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2003; Ronquist *et al.* 2005: MrBayes 3.1 Manual, http://mrbayes.csit.fsu.edu/mb3.1_manual.pdf). MP analysis was based on a heuristic search with 500 replicates of random addition sequences and tree bisection–reconnection as a branch-swapping algorithm. BS values were estimated from 1 K replicates. In the Bayesian analysis the General Time Reversible substitution model (Rodriguez *et al.* 1990) with estimation of invariant sites and assuming a gamma distribution with four categories (GTR + I + G) was used for likelihood calculations. This model was selected after analysis of the data with the program MrModeltest v3.7 (J.A.A. Nylander, http://morphobank.ebc.uu.se/mrbayes/). The Metropolis Coupled MCMC analysis was run for 2 M generations, with six chains starting from a random tree and using the default temperature of 0.2. Every 100th tree was sampled, and the first 150 K generations were discarded as burn-in.

Cultures

Pure cultures of *Bartheletia* were grown on malt extract agar (15 g agar, 20 g malt extract, 20 g glucose, 1 g universal peptone per litre distilled water) and on malt–yeast–peptone agar (15 g agar, 7 g malt extract, 1 g universal peptone, 0.5 g yeast extract per litre distilled water). Deposits of basidiospores for starting the cultures were obtained by fixing little pieces of freshly collected leaves with *Bartheletia* on the underside of the lid of a petri dish. After 1 h of incubation at room temperature, the leaves were removed and the germination of the basidiospores was checked microscopically. Normally cultural growth could be observed after 1 d. Conidia were taken in small quantity from freshly collected leaves and spread over the media with a very fine needle.

Inoculation experiments (see also supplementary material online)

Conidia were obtained either from *Bartheletia* cultures on malt extract agar, or from freshly collected leaves colonized by the fungus. In either case conidial masses were transferred to Eppendorf tubes and suspended in tap water. Basidiospores were obtained by vigorously shaking freshly collected *Bartheletia* teliospores forming basidia in tap water. Concentrations were determined using a counting chamber and adjusted to 10^8 spores ml^{-1}. Freshly fallen or still attached leaves for inoculation were collected, carefully preventing any contact to rotting *Ginkgo* leaves of the previous growing season on the ground, where basidiospores could be present. For the inoculation of leaves, we spread 40 μl of the inoculum with the aid of a small brush over the upper side or the underside, placed them in petri dishes with moistened crepe paper, and incubated them at room temperature. Every 2 d the leaves were moistened with a spray bottle. The inoculated leaves and the control samples were observed under a dissecting microscope (×40) at regular intervals for up to 25 d.

(1) To test whether *Bartheletia* could be an endotrophic fungus that becomes apparent on senescing or fallen leaves at the end of the growing season regularly, or whether the fungus colonizes the freshly fallen leaves by its basidiospores and conidia, we collected such leaves from two trees (Botanical Garden, Graz; Old Botanical Garden, Tübingen). We inoculated ten leaves from each locality with 40 μl of (a) a suspension of conidia obtained from freshly collected leaves, (b) a suspension of conidia obtained from a culture, and (c) a suspension of basidiospores. Twenty leaves from each locality were treated only with tap water instead of a spore suspension and incubated as control. This experiment was conducted with newly fallen leaves in 2004, and repeated with still attached (but already yellow) leaves in 2005.

(2) To test whether the cycle of colonization (mycelial growth in the leaves, mass reproduction by conidia, and formation of dormant stages/telia) depends on temperature and light conditions, we inoculated in each case ten *Ginkgo* leaves on the underside with a suspension of conidia obtained from a culture (as described in 1) and incubated them (a) in the dark at room temperature, (b) in constant light at room temperature, (c) in the dark at 22 °C, and (d) in the dark at 6 °C.

(3) To test whether *Bartheletia* is restricted to *Ginkgo*, we inoculated leaves of 55 vascular plant species (mainly trees and shrubs) of various taxonomic groups. Two leaves per species were inoculated with a suspension of conidia obtained from pure culture, one leaf on the upper side, the other on the underside. Inoculated *Ginkgo* leaves were incubated as control.

Results

EM

TEM revealed that the septa between vegetative cells in telia and conidial sori, as well as the septa at the base of teliospores, are without a central pore. Instead, they are traversed by multiple plasmodesma-like perforations, approximately 8 nm diam (Fig 1A–E). Under optimal sectioning conditions it appears that the plasma membrane is continuous through the perforations (Fig 1D).

The most striking features observed under SEM are the conidiogenous cells (Fig 2B–E) and the basidia (Figs 3B–D, 4). The scars of conidial secession are very broad and shallow, and their marginal frills are usually obscured by some dense verruculose ornamentation. We frequently found sympodially (and clearly distichously) arranged secession scars at the tip of the conidiogenous cell, where the marginal frills neither run around the whole girth of the conidiogenous cell, nor around the proximate younger scar (Fig 3C–D). Conversely, we found ring-like structures, which run around the whole girth of the conidiogenous cell. These ring-like marginal frills are certainly remnants of percurrent proliferation (annellidic *s. lat.*, Fig 3E). The longitudinal septa of the basidia are well discernible as blunt longitudinal ridges in SEM. All four cells have a typical sporogenous locus at the apex. These loci are at first flat, with crowded secession scars of basidiospores. Later on, the sporogenous locus starts to bulge, and finally it grows out to form a conspicuous protrusion of somewhat irregular shape. Often two (rarely three) young basidiospores at different stages of development can be observed on the same sporogenous locus (Fig 4B).

Molecular analysis

Sequence data from the 5′ end of nuLSU rDNA of teliospores, basidiospores, conidia, and basidiospore-derived cultures were identical, which clearly confirms the anamorph–teleomorph connection of the observed asexual and sexual structures. Both parsimony and Bayesian analyses confirmed major lineages of *Basidiomycota*, i.e. *Agaricomycotina*, *Pucciniomycotina*, and *Ustilaginomycotina* (Fig 5). In all analyses, the relationship of *Bartheletia* with known groups (e.g. *Agaricomycetidae*, *Tremellomycetidae*, including *Cystofilobasidiales* and *Filobasidiales*) was not significantly supported. Rather, *Bartheletia* appeared in an unresolved position at the basal branching within the *Agaricomycotina*.

Cultures

Colonies on agar plates white to pale brown, without aerial mycelium. Abundant slimy conidia were produced already

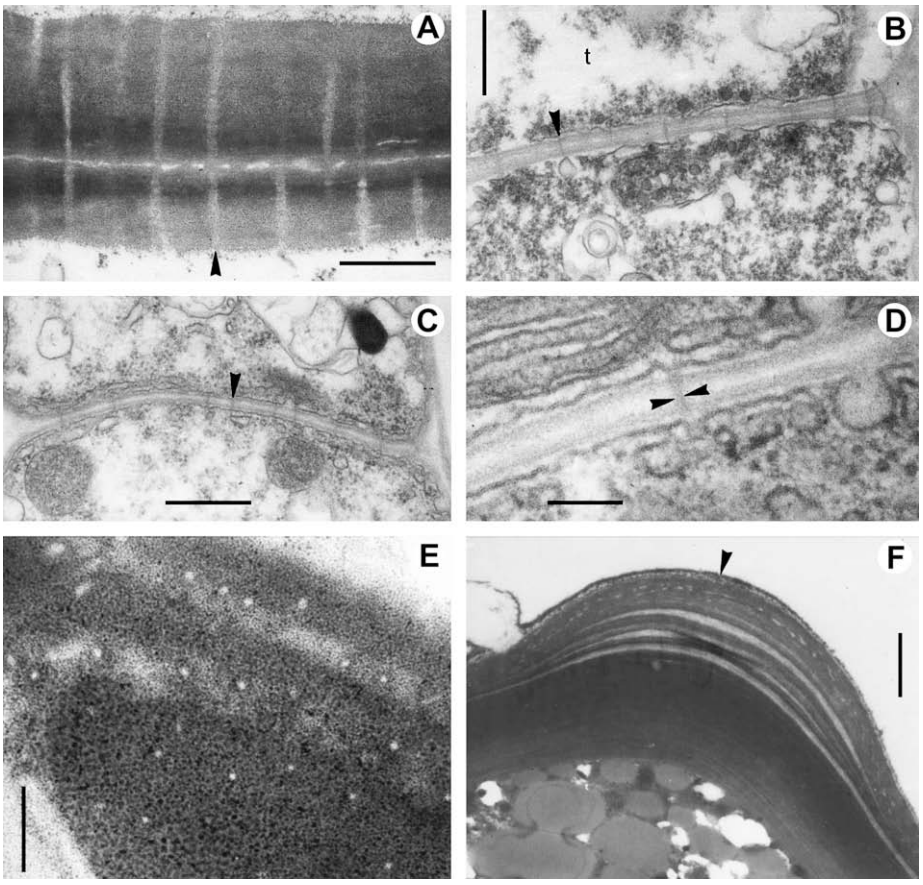

Fig 1 – Ultrastructure of *Bartheletia paradoxa*. (A) Section through a septum of an old, thick-walled vegetative hypha in a telium showing multiple plasmodesma-like perforations (one is indicated by an arrowhead). Bar = 0.3 μm. (B) Section through a septum between a young teliospore (t) and its sporogenous hypha showing multiple plasmodesma-like perforations (one is indicated by an arrowhead). Bar = 0.5 μm. (C) Section through a septum of a hypha in a conidial sorus showing multiple plasmodesma-like perforations (one is indicated by an arrowhead). Bar = 0.5 μm. (D) One plasmodesma-like perforation of a septum of a hypha in a conidial sorus at high magnification. Note that the pore membrane (arrowheads) is continuous with the plasma membrane at least of the upper cell. Bar = 0.1 μm. (E) Transversal section through a septum between an old teliospore and its sporogenous hypha showing multiple plasmodesma-like perforations in more or less cross section. Bar = 0.2 μm. (F) Section through a teliospore showing the complex wall layering, especially at the apex (arrowhead). Bar = 1 μm.

one day after inoculation. Teliospores were not formed in these cultures.

Inoculation experiments (see also supplementary material online)

All observations reported here refer to conidial sori and telia visible under the dissecting microscope (×40).

(1) Control samples of freshly fallen (or senescing but still attached) *Ginkgo* leaves treated only with tap water instead of a spore suspension remained free of conidial sori and telia. Both basidiospores and conidia were able to infect the inoculated leaves. The conidial sori developed before the telia, but the phase of mass reproduction by conidia was short and the abundance of conidial sori started to decrease after two to three weeks (Fig 6).

(2) The light and temperature conditions tested had no substantial influence on the time and quantity of telia formation. Only the formation of conidial sori was slowed down by low temperatures.

(3) Only one of the 50 plant species tested at first, *Vaccinium uliginosum*, proved to be susceptible to *Bartheletia*. The leaf inoculated on the underside showed conidial sori after 6 d of incubation. Sorus formation declined after 9 d, and the last remnants of conidial sori disappeared after 15 d. Teliospores were not formed on this host. This result was verified by repeating the experiment with *V. uliginosum* and five other species of *Ericaceae*. None of these was susceptible either and again only *V. uliginosum* exhibited the same symptoms of colonization on seven of ten inoculated leaves. All control samples (inoculated *Ginkgo* leaves) showed heavy colonization.

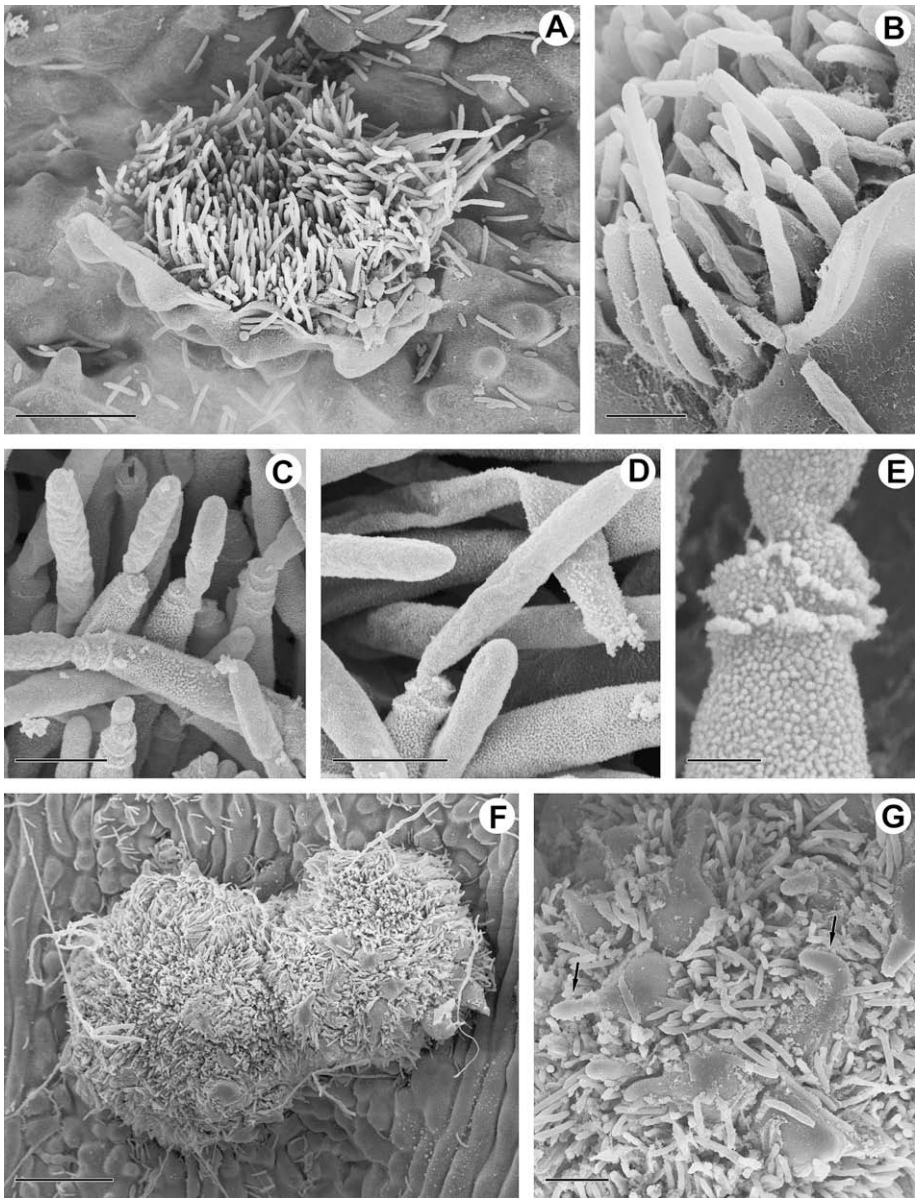

Fig 2 – *Bartheletia paradoxa*, anamorph. (A) Small conidial sorus breaking through the epidermis. Bar = 50 μm. (B) Tips of conidiophores at the edge of a small conidial sorus. Bar = 10 μm. (C–D) Tips of conidiogenous cells, mainly in side view. Bars = 5 μm. (E) Tip of a conidiogenous cell at higher magnification. Bar = 1 μm. The marginal frills of the secession scars suggest that both types of proliferation occur, percurrent (annelidic s. *lat.*) and sympodial. (F) Two large, slightly older conidial sori with copious slimy conidia and few young teliospores. Bar = 100 μm. (G) Surface of such a conidial sorus at higher magnification. The conidia are already mixed with young teliospores. Note the conspicuous cap-like apical wall thickenings of the teliospores (arrows). Bar = 20 μm.

Taxonomy

Bartheletiaceae R. Bauer, Scheuer, M. Lutz & Grube, **fam. nov.**
MycoBank no.: MB 511575

Familia basidiomycetum, teliosporis crassitunicatis et phragmobasidiis stipitatis, longitudinaliter septatis, statismosporis. Septa solum perforationibus plasmodesmatum similibus instructa, sine poro centrali.

Typus: *Bartheletia* G. Arnaud ex Scheuer, R. Bauer, M. Lutz, Stabentheiner, Melnik & Grube (see below)

Family of the basidiomycetes, with thick-walled teliospores and stipitate, longitudinally septate, statismosporic phragmobasidia. Septa only perforated by plasmodesma-like structures, without a central septal pore (Fig 1A–E).

Bartheletia paradoxa G. Arnaud ex Scheuer, *et al.*, **gen. sp. nov.** (Figs 1–4, 7–10)
MycoBank no.: MB 511576 (*Bartheletia*)
MycoBank no.: MB 511577 (*Bartheletia paradoxa*)
Synonym: *Bartheletia paradoxa* G. Arnaud, *Bull. Trimestriel Soc. Mycol. France* **69**: 300 (1954); as descr. gen.-spec., nom. inval. (Art. 36)

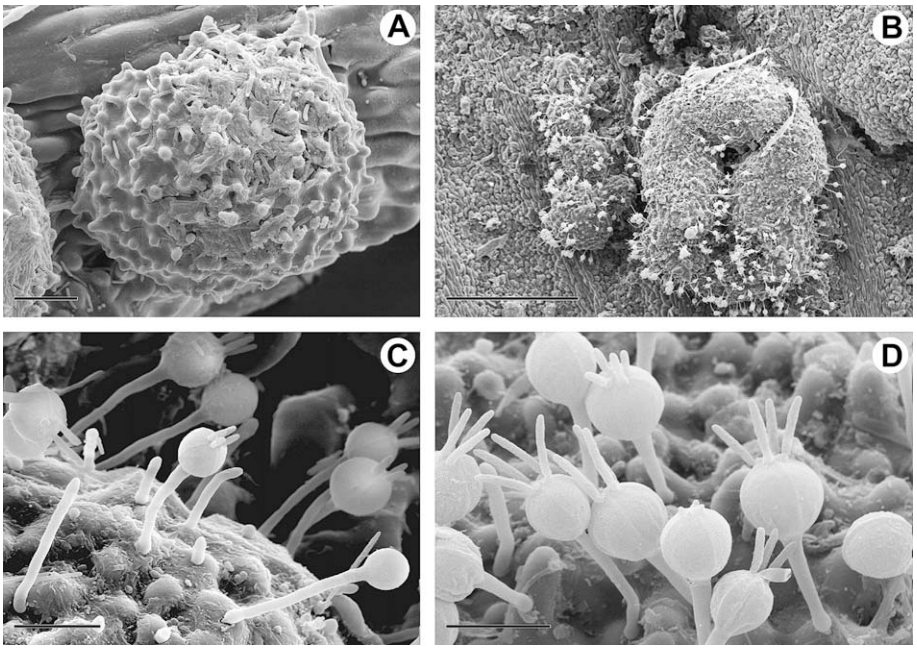

Fig 3 – *Bartheletia paradoxa*, telia and basidia. (A) Telium at the beginning of the dormant stage. Note the remnants of conidia on the surface and the protruding cap-like tips of teliospores. Bar = 50 μm. (B) Group of one large and a few small mature telia with basidia. Bar = 500 μm. (C) Young basidia on the surface of a telium. Bar = 20 μm. (D) Fully mature basidia producing basidiospores. Bar = 20 μm.

Fungus in foliis dejectis crescens. Sori conidiales in statu sicco *ca* 100–400 μm diam. Conidiophori ramosi. Cellulae conidiogenae graciles, holoblasticae, apicibus percurrentibus et/vel densissime distiche-sympodialiter proliferantibus. Conidia hyalina, unicellularia, bacilliformia, (15–)17–25(–28) × (2.5–)3(–3.5) μm, cicatricibus minutissimis. Teliosporae maturae crassitunicatae, (atro)fuscae, in teliis confertae vel intramatricales singularesque. Telia erumpentia, 150–850(–1200) μm diam. Basidia tenuistipitata, (20–)22–28(–30) μm diam., quadricellularia, longitudinaliter septata. Loci sporigeni basidiosporas aliquantum numerosas formant, primum complani, demum tumescentes, tandem paulum protuberantes. Basidiosporae ut conidia.

Typus: **Austria:** *Steiermark (Styria)*: Graz, Geidorf District, Botanical Garden (Institute of Botany/Plant Sciences, University of Graz), 47°04′52″N, 15°27′20″E, alt. 380 m, on rotting leaves of *Ginkgo biloba* (♀) of the previous growing season, 27 Oct 2003, *C. Scheuer* 4935 (GZU — holotypus; HAL, LE, TUB, UPS — isotypi). This material contains germinated teliospores with basidia.

Foliicolous fungus, apparently (mainly?) growing on fallen leaves. Mycelium in the leaf tissue of hyaline to pale brownish, sparsely septate hyphae, (2–)3–7(–8) μm wide and of rather irregular shape. Conidial sori and telia on petioles and leaf blades, mainly hypophyllous, single or in ± circular groups 2–5(–10) mm diam.

Anamorph (Figs 2, 8A–C)

Conidial sori similar to acervuli or sporodochia (but without a well-differentiated peridium or pseudoparenchymatic base), appearing in autumn on freshly fallen leaves of the current year, slimy, *ca* 100–400 μm in diam. when dry, single or in ± circular groups, more rarely evenly dispersed. At first, the sori produce copious conidia, which germinate readily to infect other newly fallen leaves. Conidiophores branched, hyaline, finely verruculose in the apical parts under SEM, originating from a ± compact base of irregularly interwoven, hyaline to pale brown intramatrical hyphae *ca* 3–8 μm wide. Conidiogenesis holoblastic. Conidiogenous cells mostly 15–35 μm long, terminal, or intercalary with one or two conidiogenous branches, unilocal with percurrent proliferation (annellidic *s. lat.*) and rather dense annellations and/or multilocal with distichous sympodial proliferation and densely stacked secession scars. Secession scars very inconspicuous in LM. Conidia hyaline, one-celled, straight, cylindrical-bacilliform or sometimes slightly broader below the middle, (15–)17–25(–28) × (2.5–)3(–3.5) μm, with a short attenuate base and minutely truncate scar ≥ 0.5 μm wide, uninucleate with the nuclear area in the middle, with minute guttules, finely verruculose under SEM. The conidiophores are soon replaced by teliospores (Fig 2F–G) originating from the same basal cushion of intramatrical hyphae.

Teleomorph (Figs 3, 4, 7, 8D–H, 9, 10)

Teliospores either single, immersed in the leaf tissues and ± evenly dispersed (most conspicuous in the epidermis), or conglutinated in compact, erumpent and finally ± superficial telia. Telia single or arranged in ± circular groups. Well-developed groups of telia usually with one or few large central telia, surrounded by smaller, often confluent telia, becoming smaller

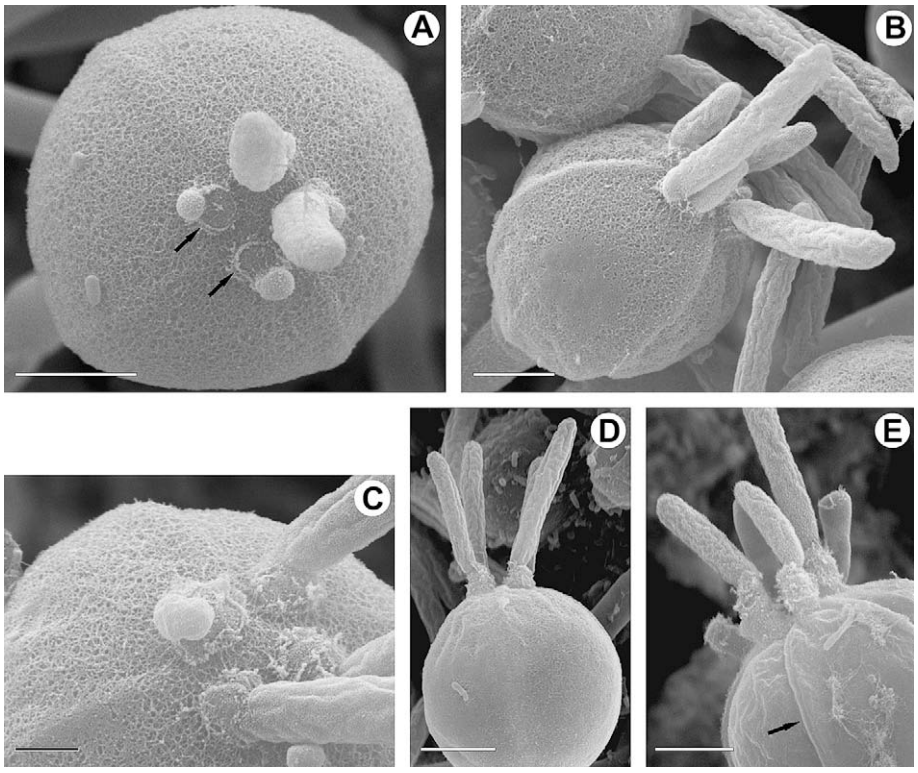

Fig 4 – *Bartheletia paradoxa*, basidia at successive stages of development. (A) Basidium seen from above with the four sporogenous loci. Note the two distinct secession scars (arrows). The septa appear as rounded longitudinal ridges. Bar = 5 μm. (B) Basidium producing basidiospores. Note that the sporogenous locus of the cell in the front is bearing two basidiospores of different size/age. Bar = 5 μm. (C) Slightly older basidium with the sporogenous loci already bulging. Bar = 2 μm. (D) Older basidium with protruding sporogenous loci. Bar = 5 μm. (E) Senescent basidium bearing distinctly protruding sporogenous loci with numerous marginal frills of secession scars. The septa between the strongly vacuolised, inflated cells now appear as furrows (arrow). Bar = 5 μm.

and sometimes passing into tiny fascicles and ± solitary superficial teliospores towards the periphery, nearly always surrounded by a halo of solitary intraepidermal teliospores (Figs 7C–D, 9A). In some collections remnants of an extramatrical mycelium bearing solitary teliospores were observed on the leaf surface.

Telia (Figs 3A–B, 7C–D, 9) similar to those of rust fungi, often developing from conidial sori, hemispherical or cushion-like to ± spherical, the largest ones often more irregular and with a conspicuous depression in the centre, single to gregarious to confluent, 150–850(–1200) μm in diam., consisting of a basal cushion of densely interwoven hyphae, the stalk hyphae of the teliospores, and a layer of densely packed and conglutinated brown-walled teliospores. The stalk hyphae and the basal cushion usually degenerate at some time during the dormant stage. In a vertical section through a younger telium (Fig 9B), the stalk hyphae of the teliospores appear ± fasciculate and conglutinated, *ca* (5–)6–9 μm wide, usually thick-walled; the stalk cell(s) immediately below the teliospore often with additional, somewhat irregular internal wall thickenings.

Solitary intramatrical teliospores (Figs 7E–F, 9A, 10A) in the epidermal cells or deeper in the leaf tissue, dispersed or somewhat agglomerated, spherical or broadly ellipsoidal to somewhat irregular in shape, brown, 25–40 μm in diam. Conglutinated teliospores in the telia (Figs 8F, 9, 10C) superficially similar to those of certain rust fungi (e.g. *Uromyces*), thick-walled, dark brown to blackish brown, (35–)50–125(–140) × 12–30 μm, often with a bifid base, and often with a protruding, conical to cylindrical wall thickening up to 25(–30) μm high at the apex, sometimes also with two of these thickenings. Loosely fascicular and solitary superficial teliospores more rounded, often pyriform (Fig 10B).

Teliospores in LM with only two sharply delimited wall layers (Figs 8E–F, 9C–D, 10), the outer one yellowish- to reddish-brown, (<1–)1.5–3 μm, the inner one conspicuously darker brown and rather evenly (1–)1.5–2(–3) μm thick. In solitary intramatrical teliospores, thickenings of the outer layer are usually almost absent or rather faint and irregular. In the ellipsoidal to strongly elongate, conglutinated teliospores in the telia, this outer layer usually shows two conspicuous thickenings, one in the cap-like apical part, the other towards the base, where it leaves only a narrow canal above the septum (Figs 8E–F, 10C). In the lateral part of such compressed and conglutinated teliospores, the outer layer is extremely thin and often hardly visible in LM. A thin outermost sublayer of the outer wall can often be observed in the apical wall thickening in LM (Fig 10C), but the layering appears to be much more complex under TEM (Fig 1F). In the very young teliospores (as they can be

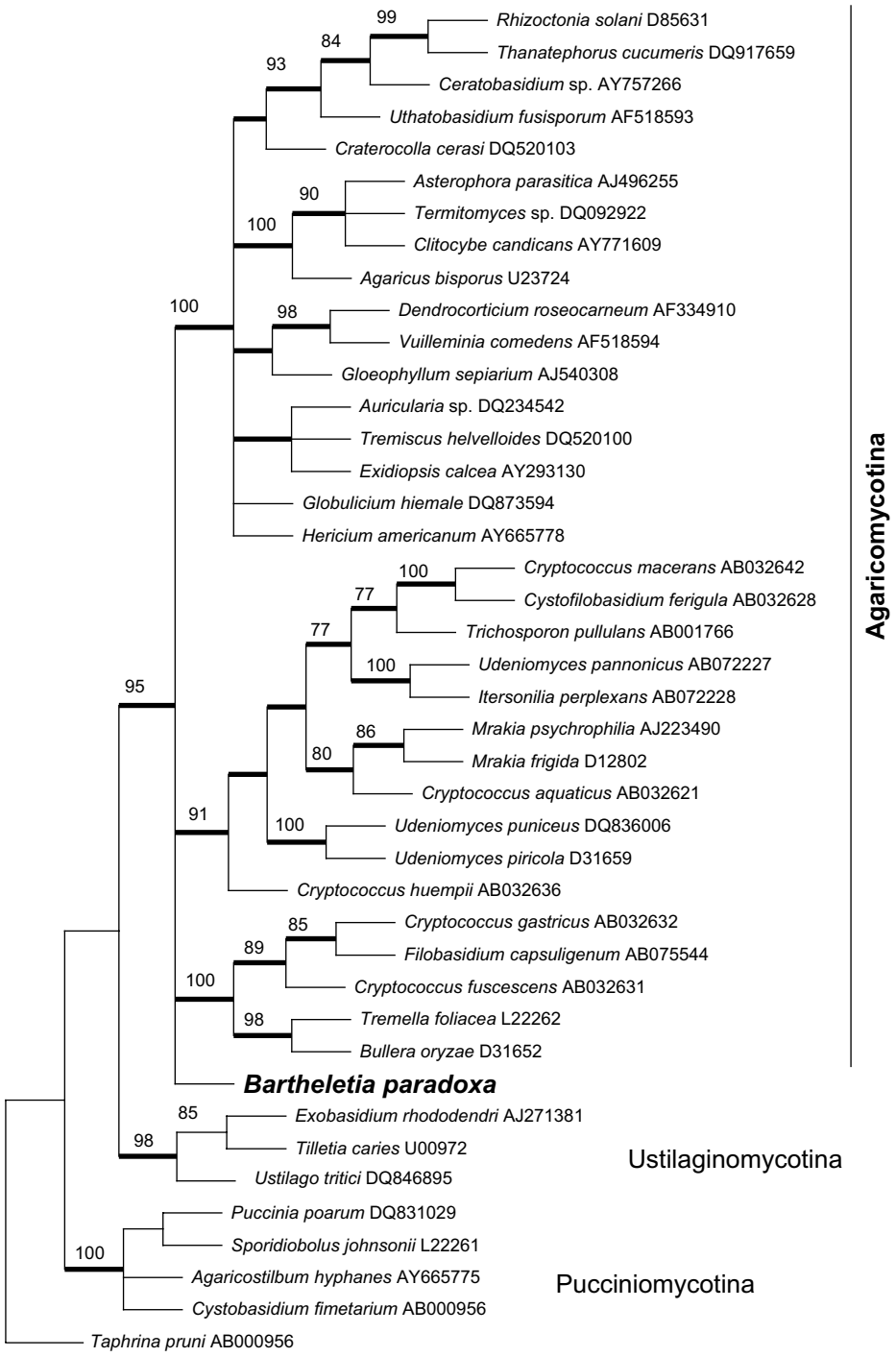

Fig 5 – Phylogenetic position of *Bartheletia paradoxa*, assessed by MP and Bayesian analysis of full-length nuSSU rDNA. BS values of 1 K replicates of a heuristic search are indicated by numbers above the respective branches. Branches supported by 95 % or greater PP in the Bayesian analysis are indicated by thicker lines. The most parsimonious phylogenetic tree had a length of 1691 steps, and the arithmetic mean of the estimated marginal likelihoods of the Bayesian analysis was -11001.79.

found in conidial sori, Fig 2G), the inner wall layer is still absent, and the outer one appears at first hyaline with glassy apical thickenings. The inner layer is subsequently formed during the maturation process, until the spore reaches the dormant stage. At first, this inner layer shows a basal canal leading towards the septum, but at maturity the canal is apparently closed (Fig 8D–F).

The teliospores germinate in autumn through a thin canal ≤ 1 μm wide, which perforates the (apical) wall of the teliospore (Figs 8F, 9C, 10C). Stalk cell of the basidium (10–)

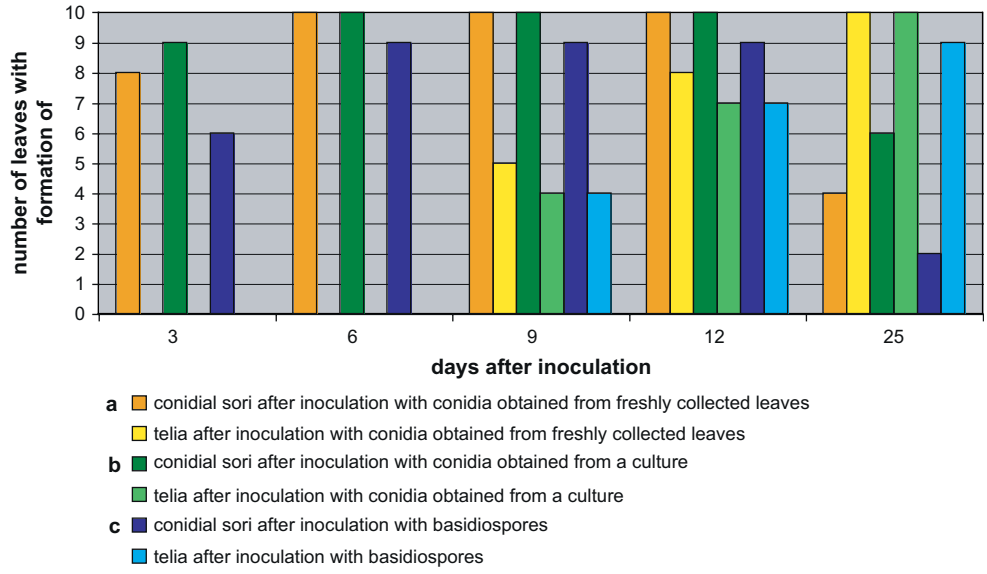

Fig 6 – *Bartheletia paradoxa*. Development of conidial sori and telia on *Ginkgo* leaves inoculated with suspensions of conidia and basidiospores. The control samples (*Ginkgo* leaves treated with tap water instead of a spore suspension) remained free of conidial sori and telia (not figured).

15–90(–100) μm long, (2–)2.5–3.5 μm thick, only below the basidium up to 5 μm, with glassy walls (0.5–0.8 μm thick), often somewhat torulose, usually with a small but distinct swelling immediately above the exit of the germination canal. If the stalk cells originate from intramatrical teliospores, they grow through the leaf tissue (or the epidermal cell wall) to reach the surface where evenly dispersed basidia are formed. Basidia (Figs 3C–D, 4, 8G) globose to subglobose, with longitudinal septa (like the basidia of *Tremellales*), (20–)22–28(–30) μm diam. Each cell of the basidium produces a considerable number of basidiospores at its apex (Fig 4). The sporogenous loci are at first flat, with new basidiospores forming side by side, leaving a dense group of secession scars ≤ 1 μm in diam. Subsequently, the sporogenous loci bulge and finally protrude to form short proliferating warts. At the end of this stage, the vacuoles at the bases of the four cells enlarge, and the production of basidiospores stops. Basidiospores like the conidia, (15–)17–23(–25) x 2.5–3(–3.5) μm, rapidly infecting freshly fallen leaves. The basidiospores tend to conglutinate on the basidium, indicating that their surface is rather adhesive.

A teliospore is defined as a thick-walled probasidium (usually functioning as a resting spore), and a probasidium as a cell where karyogamy takes place (e.g. Kirk *et al.* 2001). Karyogamy has not yet been observed in the resting spores of *Bartheletia*, and the term teliospore is mainly used in the *Pucciniomycotina* and *Ustilaginomycotina*. However, Oberwinkler *et al.* (1983) adopted it for the resting spores of *Cystofilobasidium* (*Cystofilobasidiales*) in the *Agaricomycotina*. The teliospores and basidia of the (probably unrelated) genus *Cystofilobasidium* resemble those of *Bartheletia* to some degree, and we, therefore, decided to use the terms teliospore and telium here.

No original material studied by Arnaud (1954) is preserved (Nicot & Charpentié 1971; Carmichael *et al.* 1980), but identity of the collections we studied with this lost material (**France**: Jouy-en-Josas, Seine-et-Oise, 1932, *J. Barthelet*) was concluded from the illustrations he provided (Arnaud 1954: 289, Fig 10M–N).

Representative material (containing germinated teliospores with basidia): **Austria:** *Graz*, Botanical Garden of the university (type locality), 2 Oct 2002, *V. Mel'nik* (GZU: Scheuer 5067–5071; UPS F-128825); 9 Oct 2002, *V. Mel'nik* (GZU: Scheuer 5072–5075); 13 Oct 2002, *C. Scheuer* 5076 (GZU). — **Germany**: *Tübingen*, Old Botanical Garden, 17 Oct 2005, *R. Bauer* 3113 (TUB 015890). — **Korea:** *Suwon*, territory of the National Institute of Agricultural Science and Technology, 18 Oct 2003, *V. Mel'nik* (LE 214581).

Additional material (containing telia and/or conidial sori): **Austria** (GZU: Scheuer 3911, 3925, 3944, 4936, 4937, 5064–5066, 5077–5081, Jaklitsch WJ-2068). — **Denmark** (C-F-84030, 84031). — **Germany** (GZU: Scheuer 5085; HAL 2010-F; LE 230741, 230744, 230749; TUB 015888, 015889). — **The Netherlands** (GZU: Scheuer 5434). — **Russia** (GZU: Scheuer 4851; LE 212591, 230743). — **Sweden** (GZU: Scheuer 5082; LE 230738, 230742, 230748).

Discussion

This report shows a new type of septal architecture for *Basidiomycota* (Fig 1A–E). The multiple, but tiny, plasmodesma-like perforations should not be confused with multiperforate septa having pores essentially identical to central pores of related fungi. Such multiperforate septa are known, for example, from some lichen-forming ascomycetes and the basidiomycetous plant parasite *Kriegeria eriophori* (Wetmore 1973; Doublés & McLaughlin 1991).

The combination of percurrent and sympodial proliferation at the tips of the conidiogenous cells observed under SEM

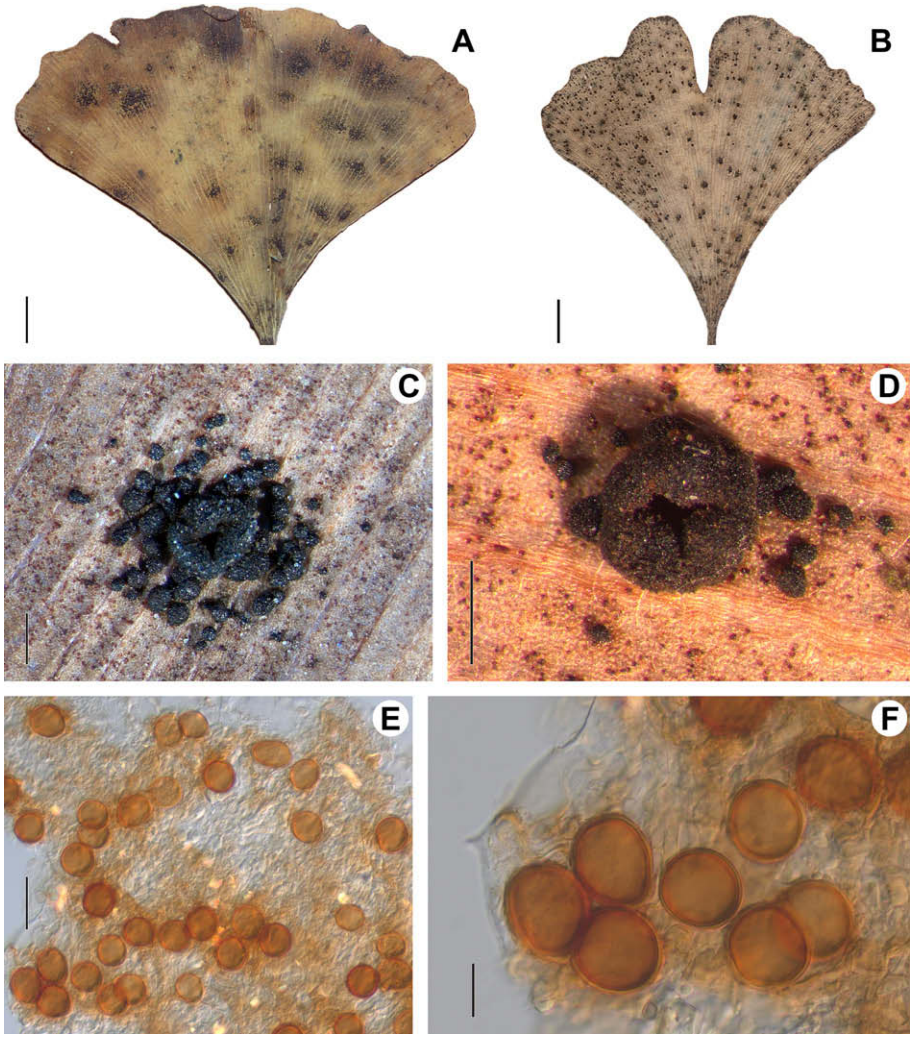

Fig 7 – *Bartheletia paradoxa*. (A–B) *Ginkgo biloba* leaves with groups of telia. Note the dull grey or brown halo around the blackish groups of telia, where solitary teliospores are immersed in the leaf tissue. Bars = 1 cm. (C–D) Two groups of telia with the peripheral halo of solitary intramatrical teliospores. Bars = 200 μm. (E–F) Solitary teliospores in the leaf tissue. Bars = (E) 50 μm, (F) 20 μm.

(Fig. 2C–E) needs further investigation by TEM. The similarity to more typical polyblastic conidiogenous cells with sympodial proliferation (e.g. Cole & Samson 1979; Kirk *et al.* 2001) might be obscured by the extremely broad and densely arranged secession scars of *Bartheletia*. Such a combination of sympodial and percurrent proliferation would not be uncommon, especially in connection with holoblastic conidiogenesis (e.g. Wang 1990; Uwe Braun, pers. comm.). However, this mode of conidiogenesis appears somewhat divergent when compared with the predominantly thallic mode in other *Agaricomycotina* (e.g. Walther *et al.* 2005; Walther & Weiß 2006).

The culture and inoculation experiments confirm our observations in the natural environment, i.e. rapid colonization of freshly fallen leaves, and the prompt formation of conidia for mass reproduction and dispersal, and of teliospores for dormancy (Fig 6). The results also suggest that *Bartheletia* is not necessarily an endotrophic fungus waiting for the senescence and the fall of leaves before it becomes apparent, because all control samples of freshly fallen *Ginkgo* leaves treated only with tap water instead of a spore suspension remained free of conidial sori and telia. It would be easier to explain the widespread occurrence of *Bartheletia* in temperate areas if an endotrophic lifestyle could be demonstrated, especially as *Ginkgo* trees tend to be solitary or widely scattered. Further isolation experiments are required to trace *Bartheletia* in symptomless *Ginkgo* leaves or twigs with standard techniques for endotrophic fungi. Attempts to amplify *Bartheletia* DNA with fungal-specific primers directly from living and senescing *Ginkgo* leaves and twigs failed, but in spite of a (mainly?) saprotrophic life style, *Bartheletia* appears to be host-specific, and the formation of teliospores seems to be restricted to *G. biloba* leaves. Whether accidental occurrence on other hosts, such as the conidial sori on *Vaccinium uliginosum* leaves in our inoculation experiment, could play a role in nature is uncertain.

We tried to find the germination product of the teliospores at all times of the year, but have only encountered basidia and basidiospores in the autumn (mainly October). We assume that

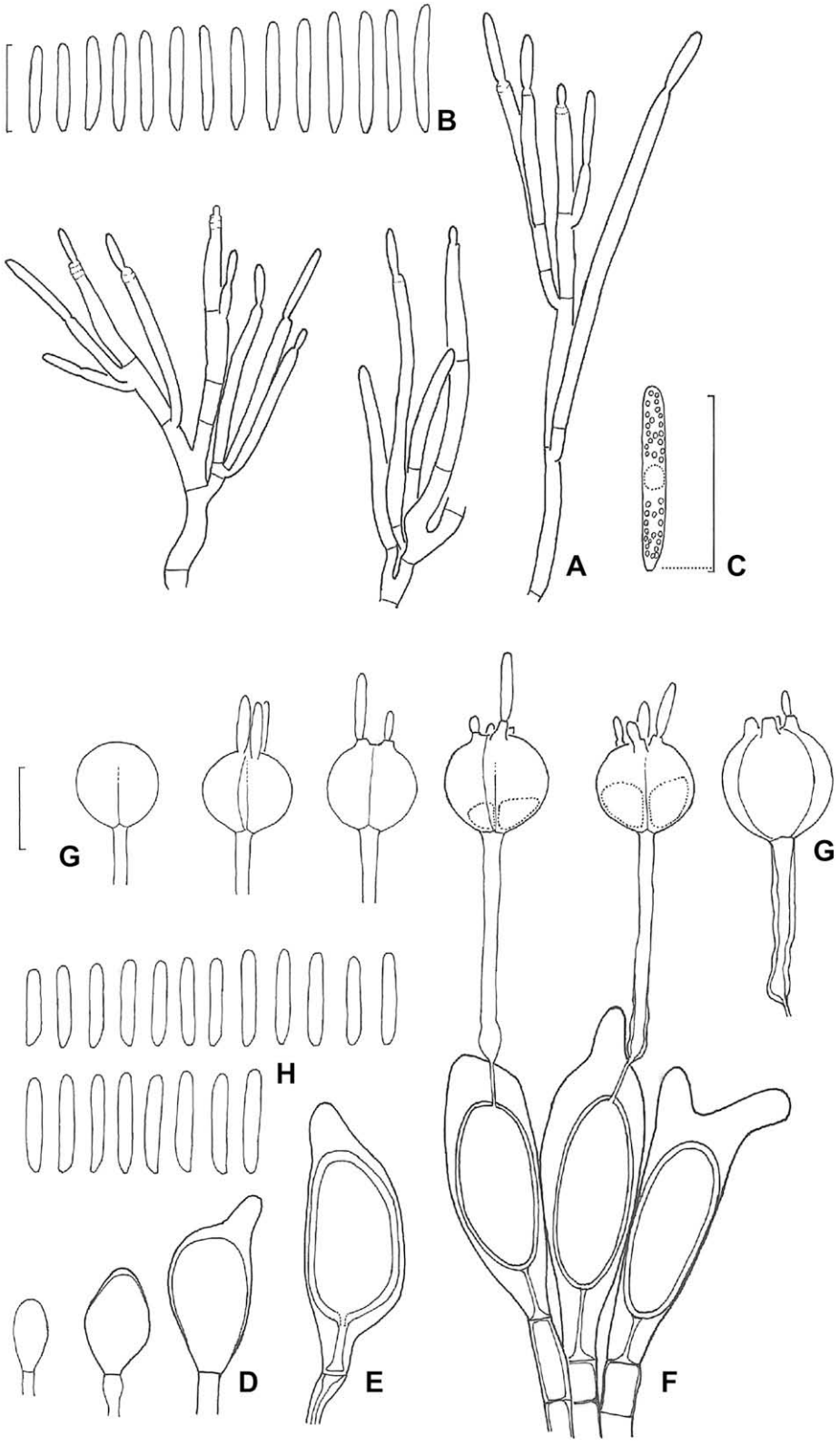

Fig 8 – *Bartheletia paradoxa*. (A) Conidiophores from a large conidial sorus. (B) Conidia. (C) Conidium drawn from living material. Note the orbicular nuclear area and the minute guttules. (D) Three young teliospores at successive developmental stages, as they are formed between the bases of the conidiophores of an older conidial sorus. Note that the inner wall layer is apparently still absent. (E) Nearly mature teliospore, apparently shortly before dormancy. Note that the inner wall layer is apparently not yet closed at the basal point. (F) Mature teliospores, two of them with basidia. (G) Basidia at successive stages of development. (H) Basidiospores. Bars = 20 μm.

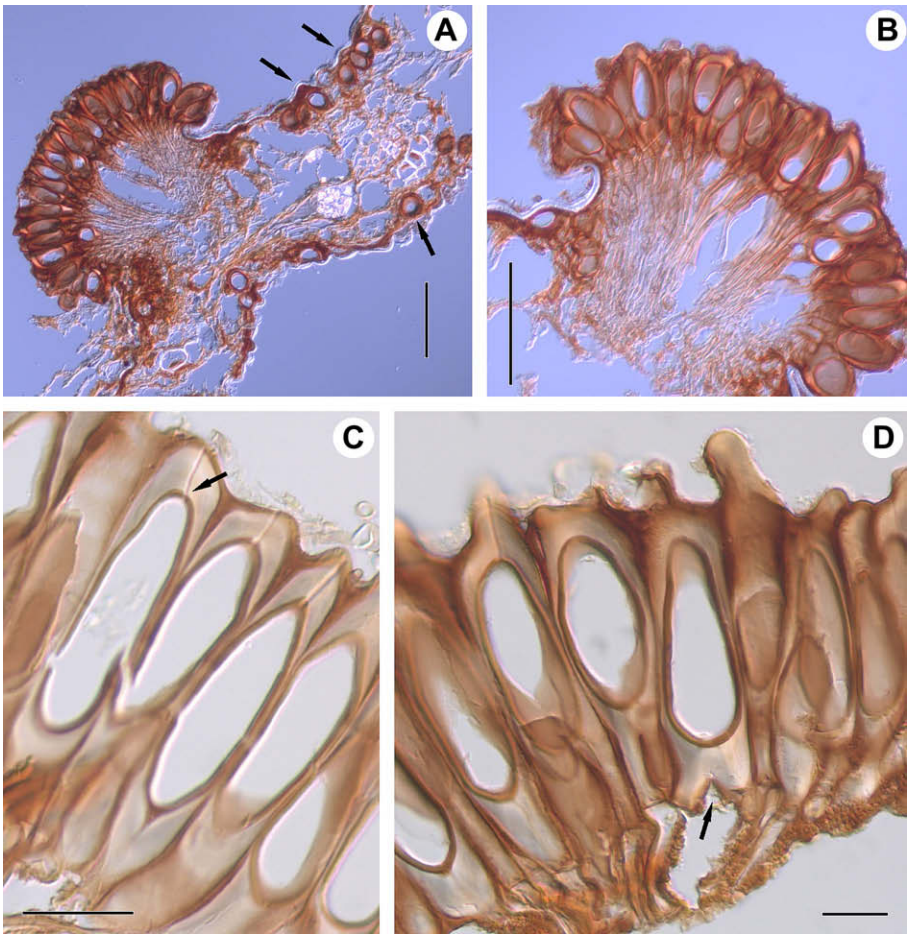

Fig 9 – *Bartheletia paradoxa*, telium and teliospores in longitudinal section. (A–B) Small telium with the fascicular stalk hyphae still present. Bars = 100 μm. (A) Note the solitary teliospores in the surrounding leaf tissue (arrows). (B) The same telium at higher magnification. (C–D) Elongate teliospores from large telia. Note that the darker inner wall layer is of ± even thickness, while the lighter outer layer is extremely thickened at the apex and the base, but very thin in the lateral part. Bars = 20 μm. (C) Three germinated teliospores with the germination canal (arrow) well visible. (D) Note the teliospore with the distinctly bifid base (arrow), and the degenerated stalk hyphae with their walls encrusted by reddish brown amorphous matter.

they are already present in sufficient quantity on rotting *Ginkgo* leaves of the preceding year on the ground before the tree sheds the current year's leaves. The colonization of freshly fallen leaves, and the subsequent formation of conidia and teliospores, is apparently triggered by the adhesive basidiospores, which could be transported to recently fallen leaves by animals on the ground (e.g. arthropods, snails). *Ginkgo* leaves are substantial and fall in a single rush, all being lost within 1–2 d. Therefore, contact between basidia with basidiospores and newly fallen leaves might, alternatively, be established by the pressure of the thick layer of new leaf litter, which is sometimes weighed down by early snow falls. Although in principle insects, birds or squirrels might also carry basidiospores from the ground to still attached senescing leaves, no conidial sori were found on the tree.

The basidiospores infect the new substratum, and the mycelium colonizes the leaf tissue rapidly. However, efficient colonization of newly fallen leaves, as well as mass reproduction and dispersal, apparently depend on the slimy conidial masses of the anamorph. Although the basidiospores are easily detached from the basidia (see above), the shape and structure of both does not suggest that these are ballistospores. Dispersal over greater distances would, therefore, mainly rely on conidia adhering to insects and other animals. However, as the surface of the basidiospores is somewhat adhesive, long-distance dispersal by the same vectors cannot be excluded. Rain-splash certainly dilutes the slimy conidial masses and spreads the conidia over other leaves, accelerating substrate colonization and mass reproduction in a locality. A fast-growing, whitish, extramatrical mycelium occurred in thick layers of freshly fallen leaves on the ground, but was not tested for conspecificity. However, solitary teliospores were found in remnants of this mycelium on dried leaves in some herbarium specimens.

Bartheletia is not an extremely inconspicuous fungus, so the 1932 collection indicates that it did not spread only recently, but may be uncommon due to 'tidy' horticultural practice. The presence or absence of *Bartheletia* seems to depend mainly

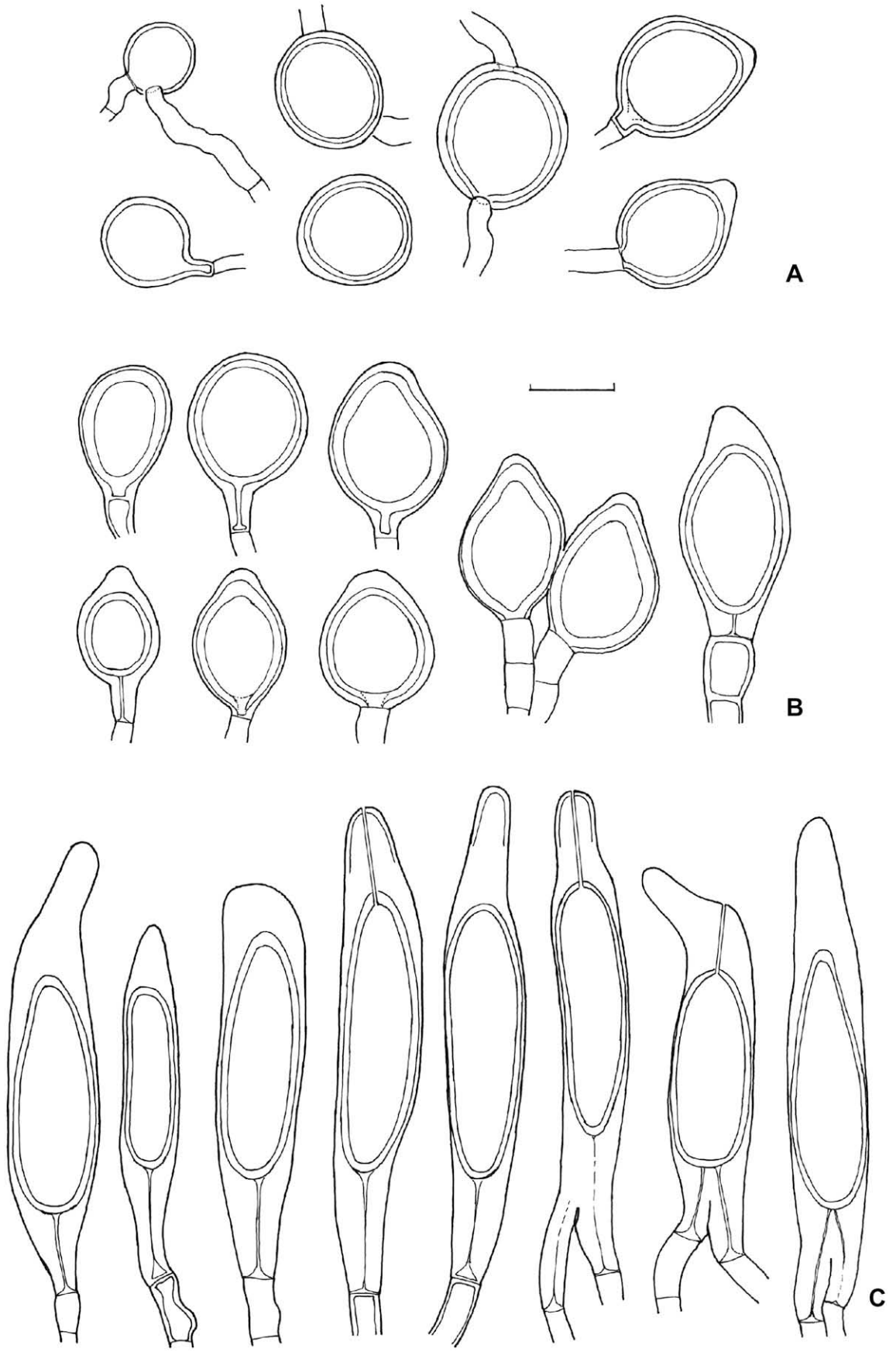

Fig 10 – *Bartheletia paradoxa*, teliospores. (A) Intramatrical, solitary teliospores, with two young teliospores on the left. (B) Teliospores from superficial (not intramatrical) mycelium and from small fascicular telia with non-conglutinated teliospores. (C) Elongate teliospores from a large telium (these teliospores are drawn singly, but of course they are firmly conglutinated in the telium). Note the distinctly bifid base of the three teliospores on the right. Bar = 20 μm.

on the availability of infected rotting leaves of the preceding growing season on the ground.

Considering the climate in autumn (October) in temperate areas of the Northern Hemisphere, rapid growth and effective mass reproduction are advantageous to this host-specific fungus, as it evidently needs only two to four weeks to complete its life cycle: (1) karyogamy (in the germinating teliospore?); (2) meiosis (in the young, non-septate basidium?); (3) formation and dispersal of basidiospores; (4) colonization and exploitation of new substratum; and (5) rapid formation of thick-walled teliospores for hibernation and also aestivation. A distinct dikaryotic stage, such as occurs in most *Dikarya* has not been observed, and the conidia are uninucleate. More details of sexual reproduction require further studies.

As we cannot exclude with certainty the possibility that *B. paradoxa* belongs to an already described order, we suggest only a new family to accommodate this fungus here. Our molecular analyses demonstrate that *Bartheletia* has an unresolved position at the basal branching of the *Agaricomycotina* (Fig 5). This phylogenetic hypothesis is consistent with the morphological and ultrastructural data. The formation of teliospores (except for *Xanthophyllomyces*) and dolipores without parenthesomes are unique features in *Agaricomycotina* and characterize *Cystofilobasidiales* (Oberwinkler *et al.* 1983; Suh *et al.* 1993; Sampaio *et al.* 2001). That order is one of the most basal agaricomycotinous lineages (Fell *et al.* 2000, 2001), but teliospores of *Cystofilobasidium* germinate with holobasidia, whereas *Bartheletia* has longitudinally septate phragmobasidia and further multiple plasmodesma-like perforations in the septa.

Our results suggest that *Bartheletia,* like *G. biloba*, is a living fossil, which apparently used *G.biloba* as its Noah's Ark. With *B. paradoxa* we have a unique possibility for further molecular investigations of a fungus at the base of the *Agaricomycotina*.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mycres.2008.06.008.

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