

Imaia, a new truffle genus to accommodate *Terfezia gigantea*

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Abstract: Originally described from Japan by Sanshi Imai in 1933, the hypogeous ascomycete *Terfezia gigantea* was subsequently discovered in the Appalachian Mountains of the USA. Morphological, electron microscopic, and phylogenetic studies of specimens collected in both regions revealed that, despite this huge geographic disjunction, (1) the Japanese and Appalachian specimens are remarkably similar both in morphology and the sampled rDNA sequences, (2) the species unambiguously falls into the *Morchellaceae* and is separated from the genus *Terfezia* in the *Pezizaceae*, (3) its spores are much larger than those of *Terfezia* spp. and are enclosed in a unique, electron-semitransparent, amorphous epispore that appears to be permeated with minute, meandering strands or canals. In addition to the molecular phylogenetic results, the numerous nuclei in ascospores, the dome shaped, striate ascus septal plugs and the long cylindrical Woronin bodies also strengthen the family assignment to the *Morchellaceae*. Moreover, the species occurs in moist, temperate forests as opposed to the xeric to arid habitats of other *Terfezia* spp. We propose the new, monotypic genus *Imaia* to accommodate the species.

Key words: Asa-Gray disjunction, *Ascomycota*, edible fungus, epispore, hypogeous, mycorrhizae, *Pezizomycetes*, phylogeny, sequestrate, ultrastructure

INTRODUCTION

The sequestrate, hypogeous *Terfezia gigantea* was described by Dr. Sanshi Imai in 1933 from forest soil at Nopporo, Hokkaido, Japan. Subsequently it was reported from the Appalachian region of the eastern United States (Gilkey 1947, Trappe and Sundberg 1977). The species was placed in *Terfezia* because its asci formed in the gleba in pockets separated by white veins, its peridium lacks pubescence or tomentum, and its globose spores seemed to be ornamented with spines (Imai 1933). These were defining features of the genus *Terfezia* at that time. The genus *Terfezia* belongs to the family *Pezizaceae* (Læsøe and Hansen 2007); its species are also termed “desert truffles,” and many are regularly collected mycorrhizal fungi in the Mediterranean area (Díez et al 2002). In addition to *T. gigantea*, two other *Terfezia* species – *T. longii* and *T. spinosa* – have been reported from the New World (Gilkey 1947).

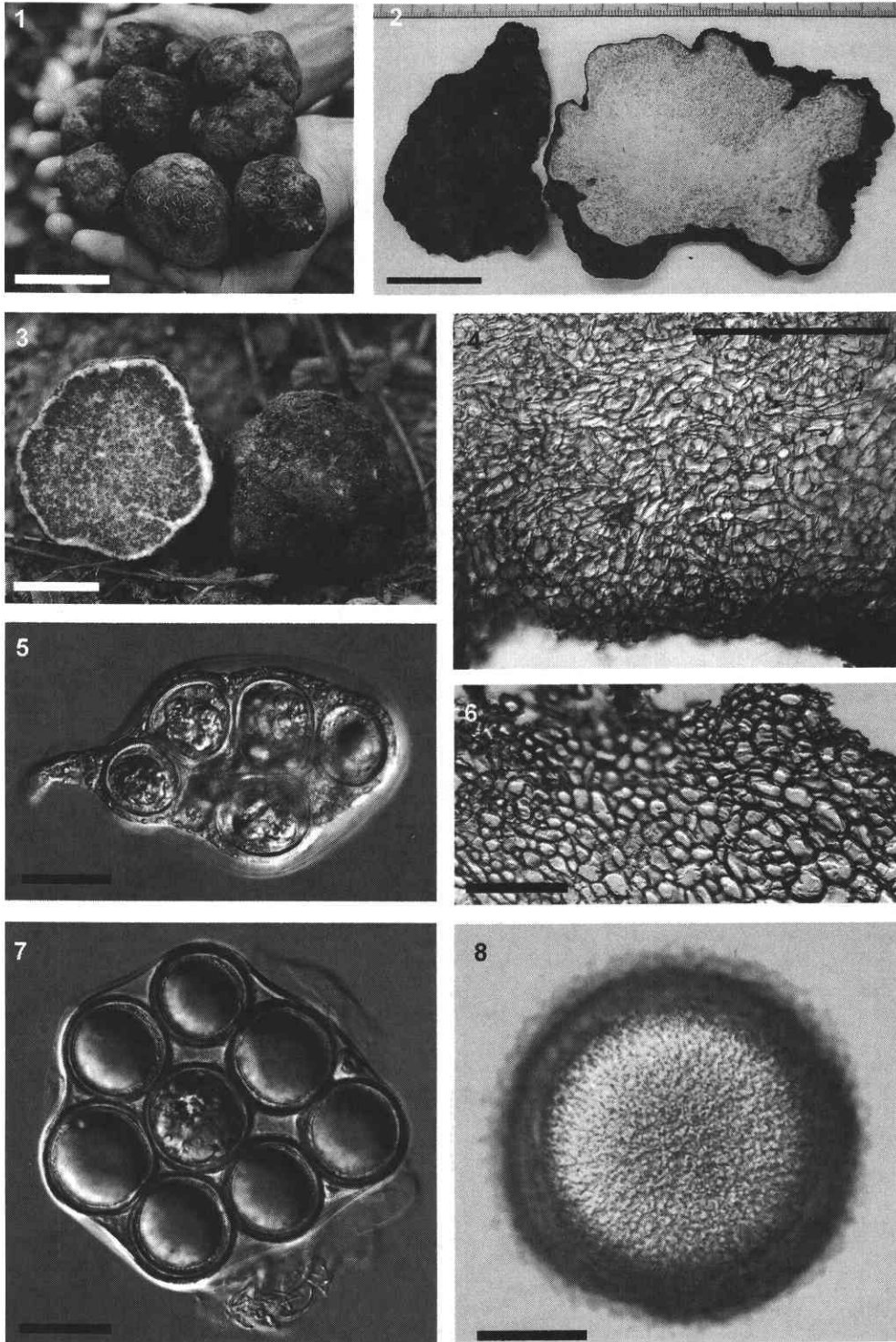
Some features of *T. gigantea* as conceived by the authors cited above have raised questions, however. It differs strikingly in several features from other members of *Terfezia*. Its thick-walled peridial cells, very large spores, and a spore ornamentation seemingly of minute spines embedded in mucilage were unique within the genus. Moreover, all other *Terfezia* spp. occur in xeric to arid habitats characterized by mild winters, whereas *T. gigantea* is known from mesic to wet forests that often experience cold winters.

Although there are fungal examples for the strange Eastern North American-Eastern Asian distribution—also known as the Asa Gray disjunction—the extreme disjunction of the known distribution of *T. gigantea* raised the question of conspecificity, although we could not discern morphological differences between specimens from the two geographical regions.

The main aim of the study presented here was (1) to test the generic placement of *T. gigantea* by molecular phylogenetic methods and (2) to study the peculiar microscopic characteristics of the species by both light and electron microscopy. Success of the latter procedure was facilitated by availability of fresh specimens fortuitously discovered by one of us (T.F.E.: FIGS. 1, 3).

MATERIALS AND METHODS

Collection, processing and examination of specimens.—Fresh specimens from North Carolina, often emergent and



FIGS. 1–8. Ascomata and light microscopic morphology of *I. gigantea*. 1. Fresh ascomata. Bar = 4 cm 2. Dried slices of a large ascoma (TMI 24279). Bar = 3 cm 3. Mature ascomata with the structure of the gleba. Bar = 2 cm. 4. Cross-section of the peridium (OSC 36074). Bar = 20 μ m. 5. A young ascus with thick ascus wall and ascospores with lipid droplets (TMI 24279). Bar = 40 μ m. 6. The peridium structure at a wart (OSC 36074). Bar = 20 μ m. 7. Eight medium matured ascospores within an ascus (TSH #Kasuya 05-1009). Bar = 40 μ m. 8. Micrograph of a mature ascospore with focus on the wall layer (OSC 130595). Bar = 20 μ m.

otherwise found by raking the forest floor, were digitally photographed in the field and the color of all tissues, aroma and taste recorded. Pieces were separated for scanning and transmission electron microscopy (SEM and TEM) and DNA extraction. Remaining specimens were dried in a portable, electric, forced-air, food dehydrator at 35 C. Other dried collections were borrowed from several herbaria, abbreviated hereafter according to Index Herbariorum (<http://sciweb.nybg.org/science2/IndexHerbariorum.asp>).

Razor-blade sections of fresh specimens were mounted in water and Melzer's reagent for light microscopy. Sections from dried specimens were rehydrated and mounted in 5% KOH. Maturity of specimens was judged by depth of spore color and thickness of the spore wall. A light microscope equipped with Nomarski interference contrast optics was used for the study. To study the number of nuclei the ascospores were stained in 6 µg/ml ethidium-bromide (Sigma Aldrich) and observed with a Nikon 80i microscope equipped with fluorescence optics.

Electron microscopy.—For SEM, spores of dry herbarium samples were fixed on double-sided tape, gold coated and studied in a SEM Hitachi 2360N. For TEM the samples were fixed in 2.5% glutaraldehyde in Sørensen buffer (0.1 M, pH 7.2). The samples were washed six times for 15 min in the same buffer and post fixed in osmium tetroxide (1% OsO₄ in Sørensen buffer, pH 7.2, 2 h). The samples were washed six times in distilled water, then dehydrated in an acetone series, and finally embedded in Spurr's (Spurr 1969) ERL. The infiltration was carried out in five steps, in ERL:acetone 1:2 one d, 1:1 one d, 2:1 one d, and one overnight and a half d in pure ERL. Ultra thin sections were stained with uranyl acetate (1% 4 min) and lead citrate (2% 4 min). The sections were studied with a TEM Hitachi 7100.

DNA extraction, PCR amplification and sequencing.—Small pieces (10–20 mg) of dried ascomata were cut after removing the specimen surfaces. Total DNA was extracted by two methods. The DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used according to manufacturer instructions. The samples were homogenized in Eppendorf-tubes with micropestles and sterilized sand by using the lysis buffer in the kit. In some cases the DNA extraction was carried out with CTAB buffer following the method described earlier (Jakucs et al 2005).

The primers NS1-NS8, ITS1F, ITS2, ITS3, ITS4, LR0R, LR3, LR3R and LR5 (White et al 1990, Gardes and Bruns 1993, Vilgalys and Hester 1990, Rehner and Samuels 1994, <http://www.lutzonilab.net/primers/page244.shtml>) were used for amplifying and sequencing the SSU (amplified with: NS1-4, NS3-8), the ITS region including the 5.8S gene (amplified with: ITS1F-ITS4), and the partial LSU of rDNA (amplified with: LR0R-LR5). A species-specific primer pair (tgigFOR: 5'-gtcactgtcggcaggactactgg-3' and tgigREV: 5'-gtgccgcgacgatgtgagta-3' targeting the ITS-1 and ITS-2 region, respectively) was designed to test the presence of extracted DNA of *T. gigantea* when the amplification by fungal specific primers was ambiguous due to contamination of the herbarium sample. High Fidelity PCR Enzyme Mix, Taq DNA Polymerase and dNTP mix (MBI Fermentas, Vilnius, Lithuania) were used in the PCRs. The amplifica-

tions were carried out in a T-Gradient 96 thermocycler (Biometra, Göttingen, Germany). To reduce the possibility of a polymerase mistake in the sequence of PCR products each amplification of a region was carried out in three parallel tubes which were mixed after the reaction and purified with PCR Clean up-M kit (Viogene, Hong-Kong, China). The possibility that the polymerase produce mistake at the same base position in three parallel tubes during the PCR is almost zero. For cycle sequencing of both strands, a BigDye[®] Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA) was used and the electrophoresis was carried out on ABI PRISM 3100 Genetic Analyser at the service laboratory of the Biology Research Center (Szeged, Hungary). The sequences were compiled from electrophoregrams using Pregap4 and Gap4 (Staden et al 2000).

Sequences of the above described regions of rDNA of 5 specimens from the USA (OSC 36074: ITS; OSC #Sundberg 2851: SSU, ITS, LSU; OSC Trappe #23199: SSU, ITS, LSU; C holotype of *Picoa pachyascus* Lange: ITS; OSC 130594: ITS) and two specimens from Japan (TMI 24279: SSU, ITS, LSU; TSH #Kasuya 05-1009: ITS) were determined (SUPPLEMENTARY TABLE). The sequences have been deposited in GenBank (EU327191–EU327203).

Phylogenetic analyses.—Several pilot analyses of different datasets of SSU, ITS and partial LSU nrDNA sequences were carried out with MEGA3.1 (Kumar et al 2004), but none of them gave more information regarding the position of *T. gigantea* within the *Pezizales* than use of the data sets published by O'Donnell et al (1997). We analyzed a combined dataset of SSU and LSU sequences published by O'Donnell et al (1997), but as it could not be complemented with the sequences of any real *Terfezia* species we had to analyze separate LSU and SSU datasets complemented with other sequences (FIG 13). We present only the analyses of the SSU dataset in detail, the branching of sequences of *Imaia* and the topology in the *Morchellaceae-Discinaceae* being the same as when the combined dataset was analyzed; however the latter gave generally bigger distances between the taxa. The sequences were aligned by use of Multalin (Corpet 1988) running on the INRA server (<http://prodes.toulouse.inra.fr/multalin/multalin.html>). The alignments were checked and adjusted manually with ProSeq 2.9 (Filatov 2002). The best fit nucleotide substitution model was selected with the program Modeltest 3.06 (Posada and Crandall 1998) considering the selection of Akaike Information Criterion (AIC).

ML phylogenetic analyses were carried out with the program PHYML (Guindon and Gascuel 2003). The GTR nucleotide substitution model was used with ML estimation of base frequencies. The proportion of the invariable sites was estimated and optimized. Four substitution rate categories were set and the gamma distribution parameter was estimated and optimized. Bootstrap analysis with 1000 replicates was used to test the support of the branches. The same substitution model was used in Bayesian analyses performed with program MrBayes 3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). The Markov chain was run over 2 000 000 generations, sampling

every 100th step, and with a burn in at 7500 sampled trees. The alignment used in the analyses have been deposited in TreeBase (S2101, M3958). The phylogenetic trees were visualized and edited by the Tree Explorer of the MEGA 3.1 program (Kumar et al 2004).

RESULTS

LM, SEM and TEM.—The characteristic morphology and anatomy of *T. gigantea* were detected by the study of both the herbarium materials and the fresh ascospores (FIGS. 1–12, SUPPLEMENTARY TABLE). The SEM micrographs showed remnants of asci attached to the mostly globose ascospores or sometimes whole asci with eight ascospores (FIG. 9). The ascospore ornamentation, interpreted by past workers (Imai 1933, Trappe and Sundberg 1977) by light microscopy as minute spines (FIG. 8), was not evident by SEM (FIG. 9). Instead, the spore surface appears to be amorphous, suggested by Trappe and Sundberg (1977) to consist of “mucilagenous material” in which the spines were embedded. This amorphous material as viewed by SEM is verruculose with a scattering of minute pores, but this could be an artifact of shrinking of a mucilagenous layer, see below.

Several nuclei (more than 4) in one ascospore could be detected by fluorescence microscopy.

TEM of cross sections of the ascospores revealed no spines or other, regular ornamentation (FIG. 10). Rather, an amorphous epispore 3–5 µm thick covers the ascospores (FIG. 10). The verruculose surface of the epispore shown in SEM is not evident by light microscopy and is likely an artifact resulting from shrinkage of the amorphous material during the drying of the specimens. In some spores a thin dark, electron dense layer was detected below the amorphous layer. The TEM also showed myriad, minute, sinuous strands or canals permeating the epispore but no sign of spines (FIG. 10).

Although the ascoma from which material for TEM could be fixed was overmature, other ultrastructural features were also evident in the TEM micrographs. The Woronin bodies are generally long cylindrical (FIG. 11) or rarely angular or globose. Ascus septal plugs are dome shaped and striate (FIG. 12).

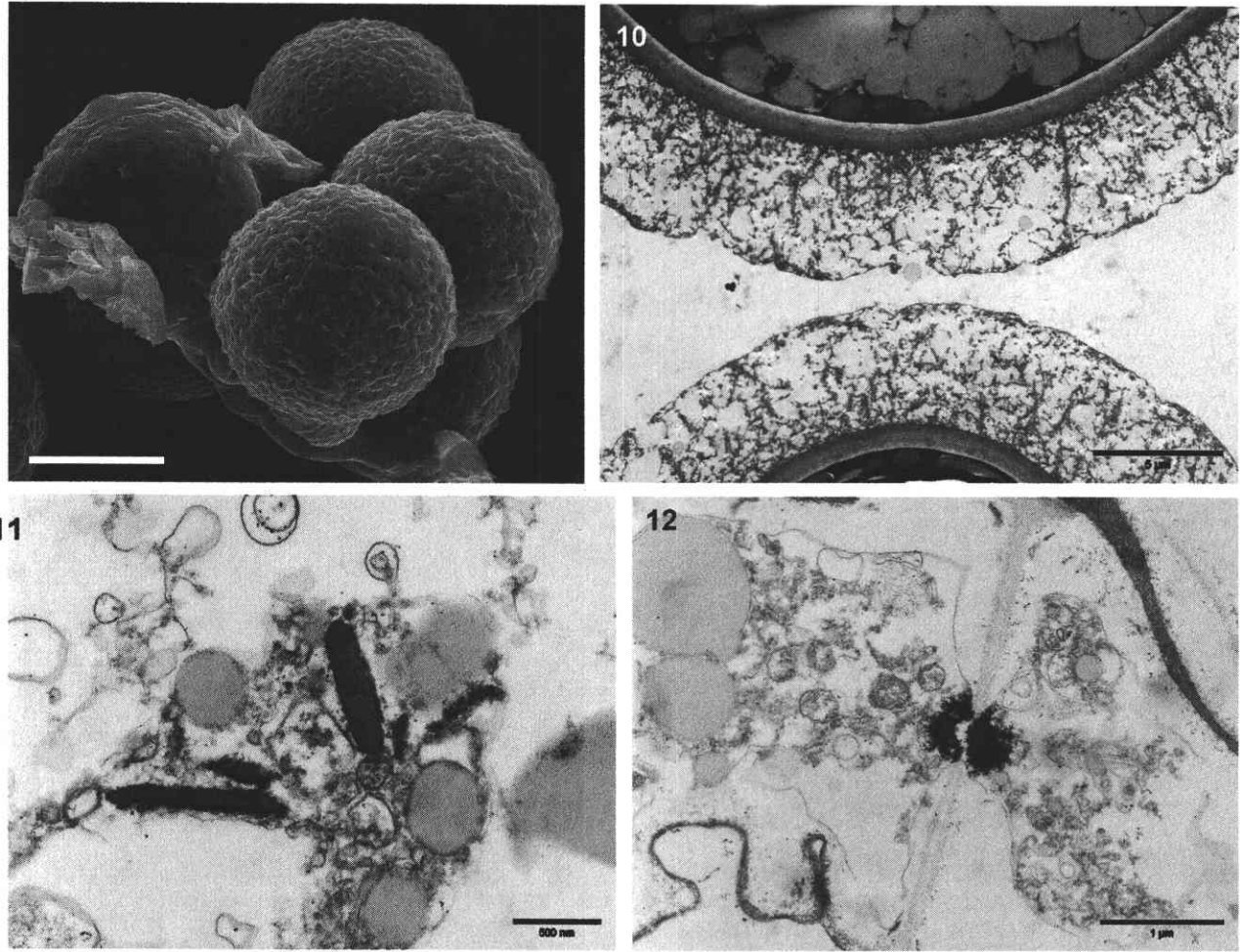
Molecular analysis.—The ITS regions of five North American and two Japanese samples were amplified and sequenced (SUPPLEMENTARY TABLE). The relatively long (approximately 900 bp) amplicons of the ITS1F-ITS4 region of rDNA initially showed that the samples differed from species of *Terfezia s. str.*, the ITS of which is approximately 600 bp. The ITS sequences of *T. gigantea* could not be unambiguously aligned with the ITS of *Terfezia s. str.* (Díez et al 2002), and gave

the highest scores with ITS sequences of species from the *Morchellaceae* during the Blast search in GenBank. Although an ITS sequence and a partial LSU sequence of *T. gigantea* were deposited in GenBank prior to our study, based on the analysis of those sequences (data not shown) we conclude that those sequences were obtained from another fungus.

Only one nucleotide differed among the ITS sequences of specimens from both Japan and the USA. However, the gene regions differed between the North American and Japanese specimens: the differences (p-distance) were 0.23% on the SSU, 4.11% on the ITS-1, 1.94% on the 5.8S gene, 6.25% on the ITS-2 and 0.78% on the LSU. Sequences of one specimen from North America and one from Japan were inserted into the phylogenetic analyses. The delimitation and relationships among the main groups of the *Pezizales* were in accordance with previous studies (O'Donnell et al 1997, Percudani et al 1999, Tedersoo et al 2005, Hansen and Pfister 2006, Læsøe and Hansen 2007). *Terfezia gigantea* separated unambiguously from the species of *Terfezia sensu stricto*. The *Terfezia* species belong to the *Pezizaceae*, whereas *T. gigantea* clustered with the *Morchellaceae* during the different phylogenetic analyses of the SSU and partial LSU datasets (FIG. 13). Most branches of the LSU-sequence-based phylogenetic trees received lower bootstrap support than those inferred from SSU dataset, as was the case in an earlier study (O'Donnell et al 1997). *Terfezia gigantea* formed a highly supported, monophyletic group with *Leucangium carthusianum* (Tul. & C. Tul.) Paol. in all analyses of both the combined LSU and SSU and the SSU data sets (ML > 90%, PP 100%). Although the branching order of the lineages within the *Morchellaceae* was not resolved, the members of the family itself, including *T. gigantea* and *L. carthusianum*, always formed a monophyletic group with strong support. The family *Discinaceae* was always the sister group of the *Morchellaceae* and the *Morchellaceae-Discinaceae* group received strong bootstrap support in all analyses of either SSU or LSU datasets. *Terfezia gigantea* clearly separated from the other genera. The genetic distances (calculated with the best-fit model) of the SSU and LSU sequences of *T. gigantea* and *L. carthusianum* were higher than several intergeneric distances of the taxa analyzed (e.g. *Disciotis-Verpa*, *Disciotis-Morchella*, *Labyrinthomyces-Redellomyces-Dingleya*).

TAXONOMY

Results of the TEM and molecular phylogenetic analyses call for a new genus to accommodate *Terfezia*



FIGS. 9–12. Ultrastructural characteristics of *I. gigantea*. 9. SEM micrograph of ascospores with remnants of the ascus (CBM FA-35455). Bar = 20 μ m. 10. TEM micrographs of the ascospore wall (OSC 130595). Bar = 5 μ m. 11. TEM micrographs of cylindrical, elongated Woronin bodies in a degrading cell (OSC 130595). Bar = 500 nm. 12. Dome-shaped septal pore plug with V-shaped striations (OSC 130595). Bar = 1 μ m.

gigantea. We also redescribe it with special attention devoted to spore characters.

Imaia Trappe and Kovács, gen. nov.

Mycobank number: MB512081

A *Terfeziis* sporis grandissimis (35–) 42–62 (–69) μ m episporam includentibus, epispora amorphia 2–5 μ m crassa, canales minutos, sinuosos continenti, et ordinibus DNA divergentibus differt. Species typica: *Terfezia gigantea* Imai.

Ascomata globose to ellipsoid or irregular, brown, often cracked at maturity. *Peridium* of angular cells with thick walls. *Gleba* composed of brown pockets of asci separated by white veins. *Spores* globose to subglobose, up to 70 μ m long, with a thick, amorphous episporum. *DNA sequences* divergent from those of *Terfezia*.

Etymology: in honor of Dr. Sanshi Imai, discoverer of the type species.

Imaia gigantea (Imai) Trappe & Kovács, comb. nov.

FIGS. 1–12

Basionym: *Terfezia gigantea* Imai, Proc. Imp. Acad. Japan 9: 184. 1933

= *Picoa pachyascus* M. Lange, Mycologia 48: 877. 1956.

Ascomata hypogeous to emergent, 3.5–5.5 (–15) cm broad, globose to ellipsoid or irregular, with a basal mycelial tuft or occasionally a sterile basal projection. *Peridium* in youth obscurely verrucose and pale yellow to brownish orange, at maturity brown or orange brown with prominent, rounded to subpolygonal warts, minutely scurfy, pubescent or smooth between the warts, often cracked in age or when exposed, 0.4–0.5 mm thick, the thickest part near the ascoma base, with a thin yellow to brown pellis and a thicker, white to pale brown subpellis. *Gleba* solid, fleshy, white and gelatinous-moist in youth, maturing to dark brown pockets of fertile tissue separated by sterile veins

concolorous with the peridial subpellis, moist but not gelatinous. *Odor* pronounced, variously described by collectors as "spicy-sweet," "rich, fragrant and fruity," "potato-like" or "somewhat disagreeable". *Taste* mild.

Spores globose to occasionally subglobose or subpolygonal (from mutual pressure in asci), (30–)35–40 μm broad when globose and (36–) 40–47 \times 30–36 μm when subglobose excluding the epispore, (35–) 42–62 (–69) μm including the epispore, hyaline and smooth in youth, when mature with brownish-yellow to deep orange yellow walls 0.9–2.5 μm thick, randomly arranged in asci. *Epispore* 2–5 μm thick, hyaline, electron-semi-transparent, amorphous, appearing by light microscopy to embed minute spines, but such spines not evident in TEM. *Chemical reactions*: in Melzer's reagent spores dark brown to reddish brown, orange or golden brown; in cotton blue lightly cyanophilic.

Asci randomly arranged within fertile pockets in the gleba, 8-spored, hyaline, globose to subglobose, subpyriform, obovoid, ellipsoid or irregular, in youth with a stipe up to 28 μm long and walls up to 10 μm thick, at maturity generally astipitate, (100–)130–200 \times (80–)95–155 μm , the walls 2–3 μm thick. *Chemical reactions*: in Melzer's reagent orange brown, in cotton blue light blue.

Peridial pellis 300–720 μm thick, pseudoparenchymatous, of subglobose, polygonal, subpolygonal, or irregular cells 5–15 μm broad at the septa but inflated up to 35–80 μm , the walls yellow to orange brown and ± 2 μm thick. *Subpellis* 75–150 μm thick, differentiated from the pellis as smaller, prosenchymatous cells 5–10 μm broad at the septa but inflated up to 8–18 μm , the walls hyaline to pale yellow and ± 1 μm thick, grading to interwoven hyphae confluent with the gleba. *Gleba* of hyaline, interwoven hyphae, 4–20 μm broad at septa but some inflated to 18(–50) μm , the walls ± 1 μm thick; hyphae of sterile veins similar to those of fertile pockets. Paraphyses lacking in all developmental stages.

Distribution, habit, habitat and season. In Japan from Hokkaido to Hiroshima Pref. on Honshu, and the Appalachian Mountains of the U.S.A., from Pennsylvania south to Tennessee and North Carolina, occurring in groups in moss or in the upper 10–15 cm of humus of broad-leaf or mixed broad-leaf-conifer forests; September to November and December (Japan). Probably mycorrhizal but specific hosts unknown.

Etymology. Latin, *gigantea* (gigantic): the type collection from Japan included a specimen 10 \times 15 cm.

Collections examined. JAPAN. HOKKAIDO PREF.: Noporo, Ishikari, Oct. S. Imai (HOLOTYPE, TNS) and Gilkey #781 (ISOTYPE, OSC). GUNMA PREF.: Kusatsu-machi,

Tonozuka, 24 Sep 1977, T. Yamada (TNS). Agatsuma-gun, Kusatsu-machi, 13 Oct 1984 and 1984? (TNS). Agatsuma-gun, Kusatsu-machi, Denzuka, 5 Nov 1978, Y. Otani (TNS). Agatsuma-gun, Iriyama, Kuni-mura, 17 Dec 2004, H. Nakamura (CBM). IWATE PREF.: Shimo-hei-gun, Iwazumi-cho, Udouge-zawa, Hayasaka plateau, 6 Oct 1998, T. Fukiharu (CBM), NAGANO PREF.: 3 Nov 2004, A. Yamada, Kasuya 05-1009 (TSH). TOTTORI PREF.: Saihaku-gun, Nakayama-cho, Hatai, 8 Nov 1997, M. Takami (TMI). HIROSHIMA PREF.: Hiba-gun, Saijyo-cho, Inisaka, S. Ushijima (TMI). Unknown location: Oct 1986 (TNS). USA. NORTH CAROLINA, McDOWELL CO.: Armstrong Creek, 12 Sep 1998, Trappe 23199 (OSC). TRANSYLVANIA CO.: Pisgah National Forest, South Fork of Mills River, 1 Sept 2006, Todd F. Elliott, Trappe 31914 (OSC 130594) and 11 Nov 2006, Trappe 31915 (OSC 130595). MACON CO.: Near Highlands. Sep 1947, M. Lange 1917 (HOLOTYPE of *Picoa pachyascus* Lange, C); Highlands Biological Station 15 Oct 1960, C. T. Rogerson & R. Peterson, Rogerson #3951 (CUP, NY, OSC); WATAUTAGA CO.: Boone. 23 Oct 1976, D. R. Fravel (NCSC, OSC). PENNSYLVANIA, CENTER CO.: Reitz Gap. 7 Oct 1939, L. O. Overholts 22168 (OSC, PAC). TENNESSEE, SEVIER CO.: Great Smoky Mountains National Park, 15 Sep 1974, W. J. Sundberg 2851 (OSC, SFSU, SIU).

Illustrations. Imai (1933), FIGS. 8–10, p. 185; Gilkey (1947), FIGS. 15 and 16, p. 443; Lange (1956), FIG. 1, p. 877; Trappe and Sundberg (1977), FIGS. 1–4, pp. 434–435.

DISCUSSION

Imai (1933) thought *Imaia gigantea* resembled *T. boudieri* but with larger spores. However, *T. boudieri* differs in peridium structure, gleba color, spore size, spore ornamentation, and in usually having less than 8 spores per ascus. Trappe and Sundberg (1977) cited the larger, more obvious spines of *Terfezia pfeilii* [now *Kaliharituber pfeilii* (Henn.) Trappe & Kagan-Zur] spores as a major difference from *I. gigantea*. The amorphous epispore of *I. gigantea* contains numerous, sinuous strands or canals; where these terminate at the spore surface, they may produce the ornamentation seen by SEM as minute pores. That could produce an illusion of spines when spores are viewed in face view by light microscopy. Gilkey (1947) could not find these spines but emphasized the difficulty of interpreting the spore surface as "such minute sculpturing ... may be modified by various factors such as degree of spore maturity." This accords with our findings, as the minute pore-like structures seen in SEM were generally found on mature spores, whereas few or no "pores" were detected on the lighter spores in younger ascomata. Although those strands/canals and the lack of spines in TEM cannot be interpreted with confidence from our TEM micrographs, it is clear that a spinose ornamentation

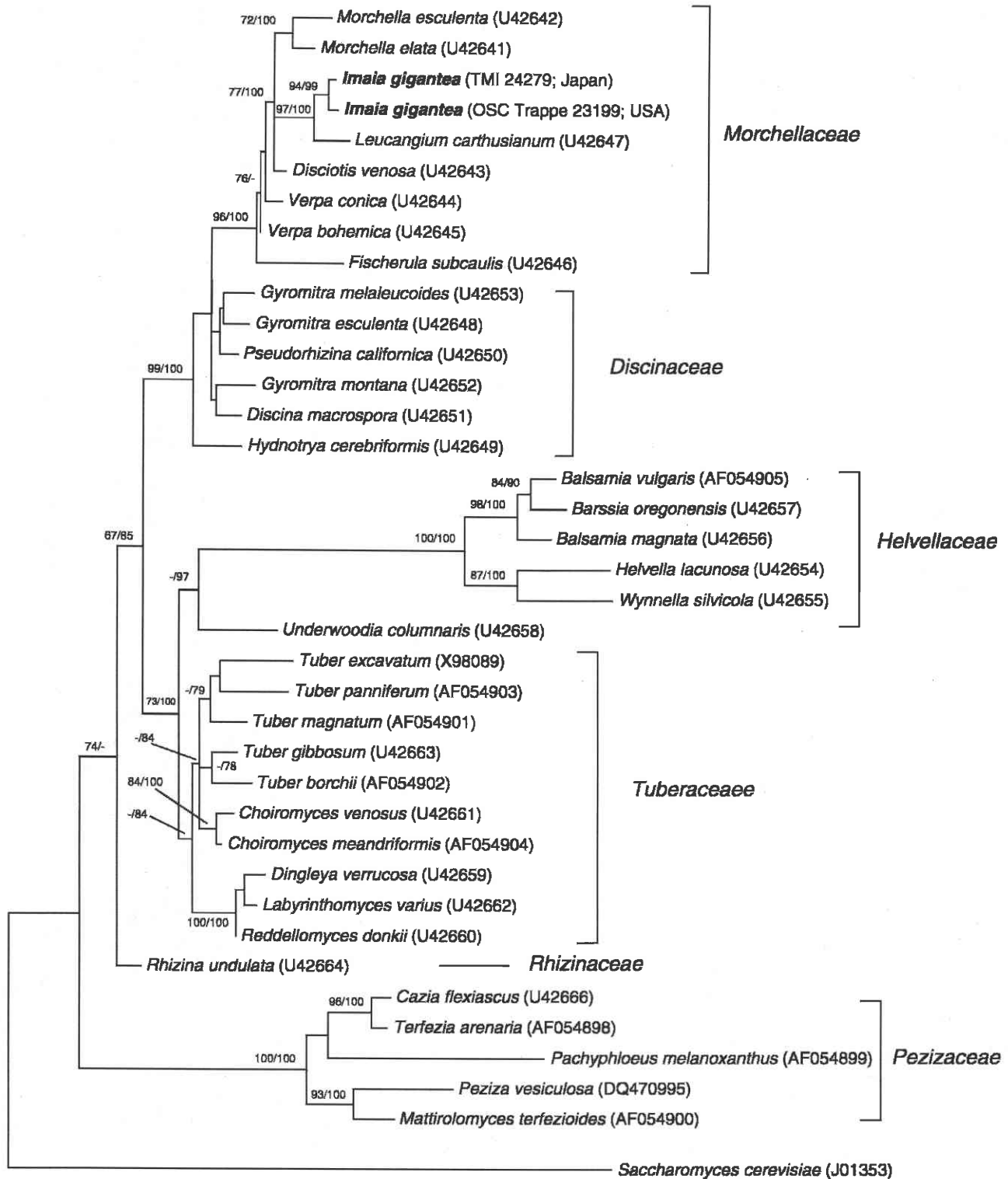


FIG. 13. The maximum likelihood tree showing the phylogenetic position of *I. gigantea* within the Pezizales. The ML tree inferred from rDNA SSU sequences of 37 pezizalean taxa and *Saccharomyces cerevisiae* as outgroup with PhyML program package (Guindon and Gascuel 2003). The *I. gigantea* sequences are shown in bold, their herbarium number and country in parentheses. GenBank accession numbers of the sequences from earlier studies (most of them are from O'Donnell et al 1997) are shown in parentheses. The analysis was based on an alignment 1548 characters long. The first value at the branches shows

formed by a secondary wall, as in *T. leptoderma* Tul. (Janex-Favre et al 1988), is absent in the case of *I. gigantea*.

Lange (1956) described *Picoa pachyascus* Lange as a new species but later his description proved to be based on a young ascoma of *I. gigantea* (Trappe and Sundberg 1977), and in the present study the ITS sequences obtained from the holotype of *P. pachyascus* were identical with ITS sequences of *I. gigantea* collected in the USA.

Our molecular phylogenetic data clearly show that *I. gigantea* is in a different clade from desert truffles in the genus *Terfezia sensu stricto* and is separated by considerable genetic distance from all the other genera included into the analyses. Moreover, the sequences of all the rDNA regions studied placed *I. gigantea* into the *Morchellaceae*, while *Terfezia sensu stricto* belongs to *Pezizaceae*. The *I. gigantea* sequences always clustered unambiguously with *Leucangium carthusianum*, which in turn grouped with *Fischerula subcaulis* in previous studies (O'Donnell et al 1997, Hansen and Pfister 2006, Læsøe and Hansen 2007). The family position of these two species was categorized as either *incertae sedis* (O'Donnell et al 1997) or placed into the *Morchellaceae* (Hansen and Pfister 2006, Læsøe and Hansen 2007). However those authors emphasized the effect of long-branch attraction on the grouping of these taxa. The ultrastructural characteristics also strongly support the grouping of *I. gigantea* with *L. carthusianum* and their family position. The septal plug structure and the features of the Woronin bodies of *I. gigantea* resemble those of *L. carthusianum* (Li 1997) and are characteristic of the *Morchellaceae* (Kimbrough 1994), as are the numerous nuclei in single ascospores as also detected in *I. gigantea*.

Although *Imaia* and *Leucangium* share these characters in common, the spores of the two genera differ drastically. *Imaia* spores are subglobose to globose, thick-walled, and enclosed in a thick, amorphous epispore. The only species of *Leucangium* described up to now, *L. carthusianum*, has fusoid-apiculate, smooth, and thin-walled ascospores.

Imaia gigantea together with *L. carthusianum* represent a hypogeous lineage within the *Morchellaceae*. The hypogeous character is usually connected with the mycorrhizal strategy (Urbanelli et al 1998, Izzo et al 2005, Trappe and Claridge 2005, Frank et al 2006, Smith et al 2006). Moreover the ectomycorrhiza

formed by *L. carthusianum* with *Pseudotsuga menziesii* was described (Palfner and Agerer 1998), and isotopic analyses also showed mycorrhizal characteristics of that species (Hobbie et al 2001). Accordingly, we hypothesize that *I. gigantea* is mycorrhizal, and it will be tested by the methods of stable nitrogen and carbon isotope measurements (Hobbie et al 2001, 2002, 2005).

Imaia gigantea has been collected only from Japan and eastern North America. Although, the Japanese and North American nrDNA sequences differ somewhat, there is no compelling reason to separate them into different species, especially in light of their identical morphologies. Similar results were found when the Japanese and North American specimens of *Choirioactis geaster* (Pezizales, Ascomycota) were compared, although the samples from the two distant localities formed separated clades in molecular phylogenetic analyses, no clear morphological difference could be detected between them (Peterson et al 2004, Pfister and Kurogi 2004).

Eastern Asia has close floristic relationships with eastern North America (Gray 1840, Li 1952, Wen 1999 and references therein), but molecular analyses have proven that several plant taxa showing this disjunction are not monophyletic (e.g. Wen 1999). Imai (1961), Hongo (1978), Hongo and Yokoyama (1978), Mao et al (1986), Zang (1986) and Wu and Mueller (1997) reported disjunct distributions of several fungal species between eastern Asia and eastern North America. Later molecular biogeographic studies either supported (Wu et al 2000, Mueller et al 2001) or queried (Mueller et al 2001) the monophyly of disjunct fungal taxa. According to our data from Japanese and American *I. gigantea* samples, this species represents a truly disjunct taxon.

According to Li (1952) the two regions are similar in geography and geological history, having largely remained undisturbed by major changes since the Paleozoic and seemingly not submerged since the close of the Cretaceous. Li (1952) hypothesizes that the present isolated and disjunct floras of eastern Asia and eastern North America "appear to be the remnants of a great mesophytic forest that extended over all the northern hemisphere and reached the arctic regions in the Tertiary. Geological changes, including mountain elevation, submergence, climatic variations, glaciations, etc., have destroyed and changed the floras of many lands so that this

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the bootstrap values (1000 replicates) as percentages, whereas the values after the slash are the posterior probabilities calculated by Bayesian analysis as percentages. The values below 70% are not shown. Bar = 2 changes / 100 characters.

mesophytic forest of the Tertiary in the northern hemisphere survives mainly in eastern Asia and eastern North America, with only scattered relics in southeastern Europe, western Asia, and western North America." Nevertheless, the data on phylogeography and biogeography of fungi are still sporadic (Lumsch et al 2008). This is especially true for the hypogeous fungi: only recently have a few groups been studied intensively (Jeandroz et al 2008, Hosaka et al 2008).

Imaia gigantea is edible (Trappe et al 2007) and is being commercially harvested on a small scale (Elliott TF, unpublished data). This development may spur interest in assembling more data on biogeography and mycorrhizal associations of this fungus.

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LITERATURE CITED

- Corpet F. 1988. Multiple sequence alignment with hierarchical clustering. *Nuc Acids Res* 16:10881–10890.
- Díez J, Manjón JL, Martín F. 2002. Molecular phylogeny of the mycorrhizal desert truffles (*Terfezia* and *Tirmania*), host specificity and edaphic tolerance. *Mycologia* 94: 247–259.
- Filatov DA. 2002. ProSeq: A software for preparation and evolutionary analysis of DNA sequence data sets. *Mol Ecol Notes* 2:621–624.
- Frank JL, Southworth D, Trappe JM. 2006. NATS truffle and truffle-like fungi 13: *Tuber quercicola* and *T. whetstoneense*, new species from Oregon, and *T. candidum* redescribed. *Mycotaxon* 95:229–240.
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol Ecol* 2: 113–118.
- Gilkey H. 1947. New or otherwise noteworthy species of Tuberales. *Mycologia* 36:441–452.
- Gray A. 1840. Dr. Siebold, Flora Japonica, sectio prima, Plantas ornatui vel usui inservientes; digessit Dr. J. G. Zuccarini: fasc. 1–10, fol. (A review). *Amer J Sci Arts* 39: 175–176.
- Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704.
- Hansen K, Pfister DH. 2006. Systematics of the Pezizomycetes – the operculate discomycetes. *Mycologia* 98: 1029–1040.
- Hobbie EA, Weber NS, Trappe JM. 2001. Mycorrhizal vs saprotrophic status of fungi: the isotopic evidence. *New Phytol* 150:601–610.
- , ———, ———, van Klinken CJ. 2002. Using radiocarbon to determine mycorrhizal status of fungi. *New Phytol* 156:129–136.
- , Jumpponen A, Trappe JM. 2005. Foliar and fungal ¹⁵N:¹⁴N ratios reflect development of mycorrhizae and nitrogen supply during primary succession: testing analytical methods. *Oecologia* 146:258–268.
- Hongo T. 1978. Biogeographical observations on the Agaricales of Japan. *Trans Mycol Soc Japan* 19:319–323.
- , Yokoyama K. 1978. Mycofloristic ties of Japan to the continents. *Mem Fac Educ Shiga Univ, Nat Sci* 28:76–80.
- Hosaka, Castellano M, Spatafora J. 2008. Biogeography of *Hysterangiales* (*Phallomycetidae*, *Basidiomycota*). *Mycol Res* 112:448–462.
- Huelsensbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17:754–755.
- Imai S. 1933. On two new species of Tuberales. *Proc Imp Acad Japan* 9:182–184.
- . 1961. Basidiomycetes of Japan. *Rec Advanc Bot* 2: 278–281.
- Izzo A, Meyer M, Trappe JM, North M, Bruns T. 2005. Hypogeous ectomycorrhizal fungal species on roots and in small mammal diet in a mixed-conifer forest. *For Sci* 51:243–254.
- Jakucs E, Kovács GM, Agerer R, Romsics C, Erős-Honti Z. 2005. Morphological–anatomical characterization and molecular identification of *Tomentella stuposa* ectomycorrhizae and related anatomotypes. *Mycorrhiza* 15: 247–258.
- Janex-Favre MC, Parguey-Leduc A, Rioussat L. 1988. L'ascocarpe hypogée d'une terfez Française (*Terfezia leptoderma* Tul., *Tuberales*, *Discomycetes*). *Bull Soc Myc Fr* 104:145–178.
- Jeandroz S, Murat C, Wang Y, Bongante P, Le Tacon F. 2008. Molecular phylogeny and historical biogeography of the genus *Tuber*, the "true truffles." *J Biogeography* 35:815–829.
- Kimbrough JW. 1994. Septal ultrastructure and ascomycete systematics. In: Hawksworth DL, ed. *Ascomycete Systematics: Problems and Perspectives in the Nineties*. New York: Plenum Press. p 127–141.
- Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5:150–163.

- Læsøe T, Hansen K. 2007. Truffle trouble: what happened to the Tuberales? *Mycol Res* 111:1075–1099.
- Lange M. 1956. A new species of *Picoa*. *Mycologia* 6:877–878.
- Li H-L. 1952. Floristic relationships between eastern Asia and eastern North America. *Trans Amer Phil Soc* 42(pt. 2):371–409.
- Li L-T. 1997. Ultrastructural studies of *Leucangium carthusianum* (hypogeous Pezizales). *Int J Plant Sci* 158:189–197.
- Lumbsch HT, Buchanan PK, May TW, Mueller GM. 2008. Phylogeography and biogeography of fungi. *Mycol Res* 112:423–424.
- Mao X, Zang Y, Yao B. 1986. The distributional features of macrofungi in Mt. Mang, Hunan. *Acta Mycol Sin Suppl* 1:397–406.
- Mueller GM, Wu X-N, Huang Y-Q, Guo S-Y, Aldana-Gomez R, Vilgalys R. 2001. Assessing biogeographic relationships between North American and Chinese macrofungi. *J Biogeography* 28:271–281.
- O'Donnell KO, Cigelnik E, Weber NS, Trappe JM. 1997. Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. *Mycologia* 89:48–65.
- Palfner G, Agerer R. 1998. *Leucangium carthusianum* (Tul.) Paol. (= *Picoa carthusiana* Tul. & Tul.) + *Pseudotsuga menziesii* (Mirb.) Franco. *Descr Ectomyc* 3:37–42.
- Percudani R, Trevisi A, Zambonelli A, Ottonello S. 1999. Molecular phylogeny of truffles (Pezizales: Terfeziaceae, Tuberales) derived from nuclear rDNA sequence analysis. *Mol Phyl Evol* 13:169–180.
- Peterson KR, Bell CD, Kurogi S, Pfister DH. 2004. Phylogeny and biogeography of *Chorioactis geaster* (Pezizales, Ascomycota) inferred from nuclear ribosomal DNA sequences. *Harvard Papers Bot* 8:141–152.
- Pfister DH, Kurogi S. 2004. A note on some morphological features of *Chorioactis geaster* (Pezizales, Ascomycota). *Mycotaxon* 89:277–281.
- Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Redhead SA. 1989. A biogeographical overview of the Canadian mushroom flora. *Can J Bot* 67:3003–3062.
- Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycol Res* 98:625–634.
- Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
- Smith ME, Trappe JM, Rizzo DM. 2006. *Genea*, *Genabea* and *Gilkeya* gen nov.: ascomata and ectomycorrhiza formation in a *Quercus* woodland. *Mycologia* 98:699–716.
- Spurr AR. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26:31–43.
- Staden R, Beal KF, Bonfield JK. 2000. The Staden package, 1998. *Methods Mol Biol* 132:115–130.
- Tedersoo L, Hansen K, Perry BA, Kjøller R. 2006. Molecular and morphological diversity of pezizalean ectomycorrhiza. *New Phytol* 170:581–596.
- Trappe JM, Claridge AW. 2005. Hypogeous fungi: evolution of reproductive and dispersal strategies through interactions with animals and mycorrhizal plants. In: Dighton J, White JF, Oudemans P, eds. *The Fungal Community—Its Organization and Role in the Ecosystem*. Boca Raton: Taylor & Francis. p 613–623.
- , Sundberg WJ. 1977. *Terfezia gigantea* (Tuberales) in North America. *Mycologia* 69:433–437.
- Trappe M, Evans F, Trappe JM. 2007. *Field Guide to North American Truffles*. Berkeley: Ten Speed Press. 136 p.
- Urbanelli S, Sallicandro P, De Vito E, Bullini L, Palenzona M, Ferrara AM. 1998. Identification of *Tuber mycorrhizae* using multilocus electrophoresis. *Mycologia* 90:389–395.
- Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* 172:4238–4246.
- Wen J. 1999. Evolution of eastern Asian and eastern North American Disjunct distributions in flowering plants. *Ann Rev Ecol Syst* 30:421–455.
- White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: a Guide to Methods and Applications*. New York: Academic Press. p 315–322.
- Wu X-N, Mueller GM. 1997. Biogeographic relationships between the macrofungi of temperate eastern Asia and eastern North America. *Can J Bot* 75:2108–2116.
- , ——, Lutzoni FM, Huang Y-Q, Guo S-Y. 2000. Phylogenetic and biogeographic relationships of Eastern Asian and Eastern North American disjunct *Suillus* species (Fungi) as inferred from nuclear ribosomal RNA ITS sequences. *Mol Phyl Evol* 17:37–47.
- Zang M. 1986. The mycogeography of tropical fungi from Yunan, Tibet. *Acta Mycol Sin Suppl* 1:407–418.