Morphological and phylogenetic analyses reveal a new genus and two new species of Tubakiaceae from China

Zhaoxue Zhang¹, Taichang Mu¹, Shubin Liu¹, Rongyu Liu¹, Xiuguo Zhang¹, Jiwen Xia¹

¹ Shandong Provincial Key Laboratory for Biology of Vegetable Diseases and Insect Pests, College of Plant Protection, Shandong Agricultural University, Taian, 271018, China

Corresponding author: Jiwen Xia (xiajiwen1@126.com)

Academic editor: Pedro Crous | Received 4 September 2021 | Accepted 3 November 2021 | Published 22 November 2021

Citation: Zhang Z, Mu T, Liu S, Liu R, Zhang X, Xia J (2021) Morphological and phylogenetic analyses reveal a new genus and two new species of Tubakiaceae from China. MycoKeys 84: 185–201. https://doi.org/10.3897/mycokeys.84.73940

Abstract
Species of Tubakiaceae have often been reported as plant pathogens or endophytes, commonly isolated from a wide range of plant hosts. The isolated fungi were studied through a complete examination, based on multilocus phylogenies from combined datasets of ITS/LSU/rpb2 and ITS/tef1/tub2, in conjunction with morphological characteristics. Five strains isolated from Lithocarpus fohaiensis and Quercus palustris in China represented a new genus of Tubakiaceae, Obovoideisporodochium and three species, viz. Obovoideisporodochium lithocarpi sp. nov., Tubakia lushanensis sp. nov. and T. dryinoides.

Keywords
multigene phylogeny, new genus, new species, taxonomy, Tubakia

Introduction
Diaporthales represents an important order in Sordariomycetes containing taxa that are mainly isolated as endophytes, saprobes or plant pathogens on various hosts (Fan et al. 2018). Tubakiaceae is a family in Diaporthales, which has been
studied in recent years by Braun et al. (2018) by incorporating morphological and molecular data with appropriate genes to resolve species limitations in the family. Tubakiaceae currently comprises eight genera including Apiognomonioides U. Braun et al., Involutisuctellula U. Braun & C. Nakash., Oblongisporothyrium U. Braun & C. Nakash., Paratubakia U. Braun & C. Nakash., Racheliella Crous & U. Braun, Saprothyrium U. Braun et al., Sphaerosporithyrium U. Braun et al. and Tubakia B. Sutton (Braun et al. 2018).

Tubakia, the type genus of Tubakiaceae, was introduced by Sutton (1973). Species of Tubakia are endophytes in leaves and twigs of many tree species, but can also cause conspicuous leaf symptoms as plant pathogens (Harrington et al. 2012; Harrington & McNew 2016, 2018; Braun et al. 2018). The genus is characterised by unique pycnothyria, consisting of pigmented, radiating, seta-like cells (scutellum) on top of a columella, with small phialides on the underside of the scutellum producing ellipsoid, hyaline to brown conidia that are forced out from under the pycnothyrium for rain dispersal (Harrington & McNew 2018). Some species produce a second type of much smaller conidia (microconidia), either in “normal” pycnothyria or in separate, mostly smaller pycnothyria (Braun et al. 2018).

Saccardo (1913) introduced the genus Actinopelte for A. japonica, a scutellate fungus found in Japan on Castanea crenata (= C. pubinervis). Saccardo (1913) confused the large conidia of this species with asci, which was clarified and corrected by Theissen (1913) who provided a detailed discussion, description and illustration (Theissen 1913) of A. japonica. Von Höhnel (1925) revisited Actinopelte, added a new species, A. americana and introduced the new combination A. dryina, based on Leptothyrium dryinum. Yokoyama & Tubaki (1971) discussed the history of this genus in detail, published results of comprehensive examinations of Japanese collections in vivo and in vitro and described A. castanopsidis, A. rubra and A. subglobosa, based on Japanese collections. Since Saccardo’s Actinopelte turned out to be illegitimate (later homonym of Actinopelte Stitzenb. 1861), Sutton (1973) introduced the replacement name Tubakia and reallocated all species recognised and treated in Yokoyama & Tubaki (1971) to this genus. Twenty-one additional Tubakia species have subsequently been described including fifteen new Tubakia species and six combinations in Tubakia species (Yun & Rossman 2011; Harrington et al. 2012; Braun et al. 2014; Harrington & McNew 2018; Senanayake et al. 2017; Braun et al. 2018; Yun & Kim 2020).

During field trips to collect plant pathogens causing leaf spots symptoms in China, several specimens associated with typical diaporthalean symptoms were collected from various tree hosts, i.e. Betula dahurica (Betulaceae), Juglans regia (Juglandaceae), Prunus davidiana (Rosaceae), Lithocarpus fohaiensis, Quercus mongolica and Q. palustris (Fagaceae). Based on morphological analyses as well as phylogenetic data, this study presents a new genus of Tubakiaceae, Obvoideisporodochium and three species, viz. Obvoideisporodochium lithocarpi sp. nov., Tubakia lusbanensis sp. nov. and T. drynoides from diseased leaves of L. fohaiensis or Q. palustris.
A new genus and two new species of Tubakiaceae from China

Materials and methods

Isolation and morphological studies

The samples were collected from the Shandong and Yunnan Provinces, China. The strains were isolated from diseased leaves of Lithocarpus foehaiensis and Quercus palustris using tissue isolation methods. Tissue fragments (5 mm × 5 mm) were taken from the margin of leaf lesions and surface-sterilised by consecutively immersing in 75% ethanol solution for 1 min, 5% sodium hypochlorite solution for 30 s and then rinsing in sterile distilled water for 1 min. The pieces were dried with sterilised paper towels and placed on potato dextrose agar (PDA). All the PDA plates were incubated in a biochemical incubator at 25°C for 2–4 days. The colonies from the periphery were picked out and inoculated on to new PDA plates. Colony photos after 7 days and 15 days were taken by a digital camera (Canon Powershot G7X). Micromorphological characters were observed using an Olympus SZX10 stereomicroscope and Olympus BX53 microscope, all fitted with Olympus DP80 high definition colour digital cameras to photo-document fungal structures. All fungal strains were stored in 10% sterilised glycerine at 4°C for further studies. The holotype specimens are deposited in the Herbarium of Plant Pathology, Shandong Agricultural University (HSAUP). Ex-type cultures are deposited in the Shandong Agricultural University Culture Collection (SAUCC). Taxonomic information of the new taxa was submitted to MycoBank (http://www.mycobank.org).

DNA extraction and amplification

Genomic DNA was extracted from fungal mycelia grown on PDA, using a modified cetyltrimethylammonium bromide (CTAB) protocol as described in Guo et al. (2000). The internal transcribed spacer regions with intervening 5.8S nrRNA gene (ITS), the partial large subunit (LSU) nrRNA gene, part of the beta-tubulin gene region (tub2), partial translation elongation factor 1-alpha (tef1) and partial RNA polymerase II second largest subunit (rpb2) genes were amplified and sequenced by using the primer pairs ITS5/ITS4 (White et al. 1990), LR0R/LR5 (Rehner & Samuels 1994; Vilgalys & Hester 1990), Br2a/Br2b (Glass & Donaldson 1995), EF1-728F/EF-2 (O’Donnell et al. 1998; Carbone & Kohn 1999) and frpb2-5F/frpb2-7cR (Liu et al. 1999; Sung et al. 2007).

The PCR was performed using an Eppendorf Master Thermocycler (Hamburg, Germany). Amplification reactions were performed in a 25 μl reaction volume, which contained 12.5 μl Green Taq Mix (Vazyme, Nanjing, China), 1 μl of each forward and reverse primer (10 μM stock) (Biosune, Shanghai, China) and 1 μl template genomic DNA in amplifier, adjusted with distilled deionised water to a total volume of 25 μl. The PCR parameters were as follows: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at a suitable temperature for 50 s, extension at 72°C for 1 min and a final elongation step at 72°C for 10 min. The annealing temperatures for the genes were 55°C for ITS, 52°C for LSU, 53°C for tub2, 48°C for tef1
and 56°C for rpb2. The PCR products were separated with the 1% agarose gel, with added GelRed and UV light used to visualise the fragments. Sequencing was done bidirectionally, conducted by the Biosune Company Limited (Shanghai, China). Consensus sequences were obtained using MEGA v. 7.0 (Kumar et al. 2016). All sequences generated in this study were deposited in GenBank (Table 1).

**Phylogeny**

The generated consensus sequences for each gene were subjected to megablast searches to identify closely-related sequences in the NCBI’s GenBank nucleotide database (Zhang et al. 2000). For the ITS-LSU-rpb2 and ITS-tef1-tub2 analyses, subsets of sequences from the alignments of Braun et al. (2018) were used as backbones. Newly-generated sequences in this study were aligned with additional related sequences downloaded from GenBank (Table 1) using MAFFT 7 online service with the Auto strategy (Katoh et al. 2019, http://mafft.cbrc.jp/alignment/server/). To establish the identity of the isolates at species level, phylogenetic analyses were conducted, first individually for each locus and then as combined analyses (ITS-LSU-rpb2 and ITS-tef1-tub2).

Phylogenetic analyses were based on Maximum Likelihood (ML) and Bayesian Inference (BI) for the multilocus analyses. For BI, the best evolutionary model for each partition was determined using MrModelTest v. 2.3 (Nylander 2004) and incorporated into the analyses. ML and BI were run on the CIPRES Science Gateway portal (https://www.phylo.org/) (Miller et al. 2012) using RAxML-HPC2 on XSEDE v. 8.2.12 (Stamatakis 2014) and MrBayes on XSEDE v. 3.2.7a (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003; Ronquist et al. 2012), respectively. For the ML analyses, the default parameters were used and BI was carried out using the rapid bootstrapping algorithm with the automatic halt option. Bayesian analyses included four parallel runs of 5,000,000 generations, with the stop rule option and a sampling frequency of 50 generations. The burn-in fraction was set to 0.25 and posterior probabilities (PP) were determined from the remaining trees. All resulting trees were plotted using FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and the layout of the trees was done in Adobe Illustrator CC 2019.

**Result**

**Phylogenetic analyses**

**ITS/LSU/rpb2 phylogeny**

The alignment contained 37 isolates representing *Tubakia* and allied taxa and a strain of *Greeneria uvicola* (FI12007) was used as outgroup. The final alignment contained a total of 2459 characters used for the phylogenetic analyses, including alignment gaps, viz. ITS: 1–676, LSU: 677–1545, rpb2: 1546–2459. Of these characters, 1858 were constant, 115 were variable and parsimony-uninformative and 486 were parsimony-
### Table 1. Species and GenBank accession numbers of DNA sequences used in this study. New sequences in bold.

| Species                  | Voucher1 | Host/Substrate | Country       | GenBank accession number | ITS       | LSU       | tef1       | tub2       | rpb2       |
|--------------------------|----------|----------------|---------------|--------------------------|-----------|-----------|------------|------------|------------|
| *Greeneria uvicola*      | FI12007  |                | Uruguay       | HG95609                  | QG87619   |           |            |            |            |
| *Involucrictetrella rubra* | CBS 192.71 | Quercus phyllyraeoides | Japan       | MG91899                  | MG591993  | MG592086  | MG592087   | MG592180   | MG976476   |
| *Mucuna altissima*       | MUC 2303 | Quercus phyllyraeoides | Japan       | MG91900                  | MG591994  | MG592087  | MG592181   | MG976477   |            |
| *ATCC 22473*             |          | Quercus phyllyraeoides | Japan       | MG91901                  | MG591995  | MG592088  | –           | –          | –          |
| *Oblongiporothryum castanopidus* | CBS 124732 | Catanopis cuspida | Japan       | MG91849                  | MG591942  | MG592087  | MG592131   | MG976453   |            |
| *CBS 189.71*             |          | Catanopis cuspida | Japan       | MG91850                  | MG591943  | MG592088  | MG592132   | MG976454   |            |
| *Obvoldiasporothryum lithocarpi* | SAUCC 0748* | Lithocarpus fohaiensis | China      | MW820279                 | MW821346  | MW996876  | MZ962157   | MZ962155   | MZ962156   |
| *SAUCC 0745*             |          | Lithocarpus fohaiensis | Japan      | MW820280                 | MW821347  | MW996877  | MZ962158   | MZ962156   | MZ962156   |
| *Protubakia subglobosa*  | CBS 124733 | Quercus glauca | Japan       | MG91913                  | MG592008  | MG592102  | MG592194   | MG976489   |            |
| *CBS 193.71*             |          | Quercus glauca | Japan       | MG91914                  | MG592009  | MG592103  | MG592195   | MG976490   |            |
| *Protubakia subglobosa*  | MUC 2293* | Quercus glauca | Japan       | MG91915                  | MG592010  | MG592104  | MG592196   | MG976491   |            |
| *Rachellula wingfieldiana* | CBS 143669 | Syzygium guineense | Africa      | MG91911                  | MG592006  | MG592100  | MG592192   | MG976487   |            |
| *Sphaeroporothryum mexicanum* | CPC 32258 | Quercus enunhi | Mexico      | MG91895                  | MG591989  | MG592082  | MG592176   | –          |            |
| *CPC 33021*              |          | Quercus enunhi | Mexico      | MG91896                  | MG591990  | MG592083  | MG592177   | MG976473   |            |
| *Tubakia americana*      | CBS 129014 | Quercus macrocarpa | USA         | MG91873                  | MG591966  | MG592058  | MG592152   | MG976449   |            |
| *Tubakia californica*    | CPC 31505* | Quercus kelloggi | USA         | MG91883                  | MG591928  | MG592023  | MG592117   | MG976451   |            |
| *Tubakia dypina*         | CBS 112097* | Quercus ruber | Italy        | MG91851                  | MG591944  | MG592039  | MG592133   | MG976455   |            |
| *Tubakia dypinaoides*    | SAUCC 1924 | Quercus palustris | China      | MW948482                 | MW94852   | MW324260  | MW324263   | MW324266   | MW324266   |
| *Tubakia hallii*         | CBS 329.75 | Quercus sp.    | France       | MG91874                  | MG591967  | MG592059  | MG592153   | MG976458   |            |
| *Tubakia hallii*         | MUCU 2290 | Catanopis creasta | Japan       | MG91876                  | MG591968  | MG592061  | MG592155   | MG976459   |            |
| *Tubakia hallii*         | MUCU 2291 | Catanopis creasta | Japan       | MG91877                  | MG591969  | MG592062  | MG592156   | MG976460   |            |
| *Tubakia hallii*         | MUCU 2292* | Quercus phyllyraeoides | Japan       | MG91878                  | MG591970  | MG592063  | MG592157   | MG976461   |            |
| *Tubakia houseiana*      | CBS 129013 | Quercus stellata | USA         | MG91880                  | MG591972  | MG592065  | MG592159   | MG976462   |            |
| *Tubakia iowensis*       | CBS 129012* | Quercus macrocarpa | USA         | MG91879                  | MG591971  | MG592064  | MG592158   | –          |            |
| *Tubakia japonica*       | ATCC 22472* | Catanopis creasta | Japan       | MG91886                  | MG591978  | MG592071  | MG592165   | MG976465   |            |
| *Tubakia koraiensis*     | KCTC46072 | Quercus mongolica | South Korea | KP866837                 | –         | –         | –          | –          | –          |
| *Tubakia liquidambaris*  | CBS 19744 | Liquidambar spiciflora | USA        | MG605068                 | MG605077  | MG603578  | –          | –          | –          |
| *Tubakia liquidambaris*  | SAUCC 1921 | Quercus palustris | China       | MW9484677                | MW985450  | MW9842262 | MW9842265  | MW9842266  | MW9842267  |
| *Tubakia meleagrina*     | CPC 32255* | Quercus cambei | Mexico       | MG91893                  | MG591987  | MG592080  | MG592174   | MG976472   |            |
| *Tubakia obovata*        | MUCU 2295* | Quercus serrata | Japan       | MG91897                  | MG591991  | MG592084  | MG592178   | MG976474   |            |
| *Tubakia panarazynoides* | MUCU 2294* | Quercus acutissima | South Korea | MG91898                  | MG591992  | MG592085  | MG592179   | MG976475   |            |
| *Tubakia sessiliflora*   | CBS 127490* | Quercus mongolica | South Korea | MG91907                  | KP260499  | MG592094  | MG592186   | –          |            |
| *Tubakia sierraflorana*  | CBS 127491 | Quercus mongolica | South Korea | HM997135                 | KP260490  | MG592095  | MG592187   | MG976484   |            |
| *Tubakia sierraflorana*  | CPC 33020 | Quercus enunhi | Mexico       | MG91910                  | MG592005  | MG592099  | MG592191   | MG976486   |            |
| *Tubakia sierraflorana*  | CBS 115011 | Quercus robur | Netherlands   | MG91912                  | MG592007  | MG592101  | MG592193   | MG976488   |            |
| *Tubakia sturtiana*      | CBS 639.93 | Quercus sp. | Italy        | MG91921                  | MG592016  | MG592110  | MG592202   | MG976493   |            |

1 Isolates marked with "*" are ex-type or ex-epitype strains.
informative. MrModelTest recommended that the Bayesian analysis should use Dirichlet base frequencies for the ITS, LSU and rpb2. The GTR+I+G model was proposed for ITS, LSU and rpb2. The MCMC analysis of the three concatenated genes, run for 700,000 generations, resulted in 14,001 trees. The initial 3500 trees, representative of the analysis burn-in phase, were discarded, while the remaining trees were used to calculate posterior probabilities in the majority rule consensus trees (Fig. 1; first value: PP > 0.74 shown). The alignment contained a total of 744 unique site patterns (ITS: 266, LSU: 128, rpb2: 350). The topology of the ML tree confirmed the tree topology obtained from the Bayes analyses and, therefore, only the ML tree is presented (Fig. 1). Bayesian posterior probability (> 0.74) and ML bootstrap support values (> 74%) are shown as first and second position above nodes, respectively. The 37 strains were assigned to 25 species clades, based on the three-gene phylogeny (Fig. 1).

ITS/tef1/tub2 phylogeny

The alignment contained 37 isolates representing Tubakia and allied taxa and a strain of Greeneria uvicola (FI12007) was used as outgroup. The final alignment contained a total of 1939 characters used for the phylogenetic analyses, including alignment gaps, viz. ITS: 1–676, tef1: 677–1358, tub2: 1359–1939. Of these characters, 1077 were constant, 136 were variable and parsimony-uninformative and 726 were parsimony-informative. MrModelTest recommended that the Bayesian analysis should use Dirichlet base frequencies for the ITS, tef1 and tub2 data partitions. The GTR+I+G model was proposed for ITS and HKY+I+G for tef1 and tub2. The MCMC analysis of the three concatenated genes, run for 170,000 generations resulted in 3401 trees. The initial 850 trees, representative of the analysis burn-in phase, were discarded, while the remaining trees were used to calculate posterior probabilities in the majority rule consensus trees (Fig. 2; first value: PP > 0.74 shown). The alignment contained a total of 997 unique site patterns (ITS: 266, tef1: 416, tub2: 315). The topology of the ML tree confirmed the tree topology obtained from the Bayes analyses and, therefore, only the ML tree is presented (Fig. 2). Bayesian posterior probability (> 0.74) and ML bootstrap support values (> 74%) are shown as first and second position above nodes, respectively. The 37 strains were assigned to 25 species clades, based on the three-gene phylogeny (Fig. 2).

Based on phylogenetic data (Figs. 1 and 2) and morphological analyses, the present study revealed a new genus of Tubakiaceae, Obovoideisporodochium and three species, viz. Obovoideisporodochium lithocarpi sp. nov., Tubakia lushanensis sp. nov. and T. dryinoides.

Taxonomy

Obovoideisporodochium Z. X. Zhang, J. W. Xia & X. G. Zhang, gen. nov.
MycoBank No: 841103

Type species. Obovoideisporodochium lithocarpi Z. X. Zhang, J. W. Xia & X. G. Zhang
A new genus and two new species of Tubakiaceae from China

Figure 1. Phylogram of Tubakiaceae, based on the concatenated ITS, LSU and rp2 sequence alignment. The BI and ML bootstrap support values above 0.74 and 74% are shown at the first and second position, respectively. The tree is rooted to Greeneria uvicola (culture FI12007) and ex-type cultures are indicated in bold face. Strains from the current study are in red. Some branches were shortened for layout purposes – these are indicated by two diagonal lines with the number of times a branch was shortened indicated next to the lines.

Etymology. Composed of “obovoideisporo-” (obovoid spores) and “-dochium” (referring to the conidioma, i.e. sporodochium).
Figure 2. Phylogram of Tubakiaceae, based on the concatenated ITS, \textit{tef1} and \textit{tub2} sequence alignment. The BI and ML bootstrap support values above 0.74 and 74% are shown at the first and second position, respectively. The tree is rooted to \textit{Greeneria uvicola} (culture Fi12007) and ex-type cultures are indicated in bold face. Strains from the current study are in red. Some branches were shortened for layout purposes – these are indicated by two diagonal lines with the number of times a branch was shortened indicated next to the lines.
Description. Genus of Tubakiaceae. Living as endophyte in leaves and causing leaf spots. Asexual morph: mycelium consisting of septate, smooth and hyaline hyphae, thin-walled. Conidiomata sporodochial, appeared within 20 days or longer, formed on agar surface, slimy, pale bluish-green, semi-submerged. Sporodochial conidiophores densely and irregularly branched, bearing apical whorls of 2–3 phialides; sporodochial phialides monophialidic, subulate to subcylindrical, smooth, thin-walled, tapering towards apex, swelling at base. Conidia formed singly, obovoid to ellipsoid, smooth, thin walled, apex obtuse, base with inconspicuous to conspicuous hilum. Sexual morph: unknown.

Notes. In the two phylogenetic trees (Figs.1 and 2), Obovoideisporodochium is allied to Racheliella, Oblongisporothyrium and Paratubakia, but forms a separate lineage with full support (PP = 1, ML-BS = 100%), suggesting a genus of its own.

Obovoideisporodochium lithocarpi Z. X. Zhang, J. W. Xia & X. G. Zhang, sp. nov.
MycoBank No: 841104
Fig. 3

Type. China, Yunnan Province: Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, on diseased leaves of Lithocarpus fohaiensis (Fagaceae), 11 Sep 2020, Z. X. Zhang, (holotype HSAUP0748, ex-type living culture SAUCC 0748).

Etymology. Name refers to the genus of the host plant Lithocarpus fohaiensis.

Description. Asexual morph: mycelium consisting of septate, smooth and hyaline hyphae, thin-walled, 1.0–2.0 μm. Colonies on PDA incubated at 25°C in the dark with an average radial growth rate of 5–6 mm/d and reaching 75–80 mm diam. in 14 d, formed some conspicuous concentric circles, aerial mycelium cottony, white initially, then becoming greyish-sepia. Conidiomata sporodochial, appeared within 20 days or longer, formed on agar surface, slimy, pale bluish-green, semi-submerged. Sporodochial conidiophores densely and irregularly branched, 12.0–26.5 × 1.5–3.0 μm, bearing apical whorls of 2–3 phialides; sporodochial phialides monophialidic, subulate to subcylindrical, 9.5–20.0 × 1.5–3.0 μm, smooth, thin-walled, tapering towards apex, swelling at base. Conidia formed singly, obovoid to ellipsoid, 5.5–8.0 × 2.5–4.0 μm, length/width ratio 1.7–3.1, hyaline, smooth, thin walled, apex obtuse, base with inconspicuous to conspicuous hilum, 0.4–0.9 μm diam. Sexual morph: unknown.

Culture characteristics. Cultures incubated on MEA at 25°C in darkness, attaining 52.0–58.0 mm diam. after 14 d (growth rate 3.5–4.0 mm diam./d), grey-white to creamy white with irregular margin, spread like petals from the inside and outside, reverse dark to light brown, distributed in an irregular circle. Conidial formation not observed.

Additional specimen examined. China, Yunnan Province: Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, on diseased leaves of Lithocarpus fohaiensis (Fagaceae), 11 Sep 2020, Z. X. Zhang, HSAUP0745; living culture SAUCC 0745.
Notes. In the two phylogenetic trees (Figs. 1 and 2), *Obovoideisporodochium lithocarpi* is related to *Racheliella wingfieldiana*, *Oblongisporothyrium castanopsidis*, *Paratubakia subglobosa* and *P. subglobosoides*, but forms a separate single species lineage with full support (PP = 1, ML-BS = 100%). Furthermore, the conidia of *O. lithocarpi* (5.5–8.0 μm × 2.5–4.0 μm) are smaller than those of *R. wingfieldiana* (11.0–15.0 μm × 6.5–7.5 μm), *Ob. castanopsidis* (14.0–17.0 μm × 7.0–9.5 μm), *P. subglobosa* (10.0–13.0 μm × 8.0–11.0 μm) and *P. subglobosoides* (10.0–12.5 μm × 5.5–10.0 μm) and *Racheliella, Oblongisporothyrium* and *Paratubakia* spp. form crustose conidiomata and true pycnothyria.
A new genus and two new species of Tubakiaceae from China

_Tubakia lushanensis_ Z. X. Zhang, J. W. Xia & X. G. Zhang, sp. nov.

Mycobank No: 841105

Fig. 4

**Type.** China, Shandong Province: Zibo Lushan National Forest Park, on diseased leaves of _Quercus palustris_ Münchh (Fagaceae), 20 Sep 2020, Z. X. Zhang, (holotype HSAUP1923, ex-type living culture SAUCC 1923).

**Etymology.** Named after the type locality, Lushan National Forest Park.

**Description.** Asexual morph: Leaf spots irregular, occurring on leaf veins and at leaf edges. Colonies on PDA incubated at 25°C in the dark with an average radial growth rate of 5–7 mm/d and occupying an entire 90 mm Petri dish in 14 d, forming some conspicuous concentric circles, aerial mycelium cottony, white initially, then becoming greyish-sepia. Conidiomata pycnidial, usually globose or subglobose when viewed from above, formed on agar surface, black, semi-submerged, up to 200 μm diam. Pycnidial wall composed of an outer layer of yellow-brown, thick-walled textura angularis and an inner layer with hyaline, thin-walled cells. Conidiophores reduced to conidiogenous cells lining the inner cavity, ampulliform or flask-shaped, smooth, hyaline, 9.0–15.0 μm × 2.0–4.0 μm. Conidia solitary, globose to irregular globose, ellipsoid to broad ellipsoid, 10.0–18.0 μm × 7.5–16.0 μm, length/width ratio 1.0–1.7, slightly lighter and wall thin when immature, slightly darker and wall thickened when ripening, smooth, apex rounded, base with peg-like hila, 1.3–2.3 μm diam. Microconidia not observed. Sexual morph not observed.

**Culture characteristics.** Cultures incubated on MEA at 25°C in darkness, attaining 52.0–56.0 mm diam. after 14 d (growth rate 3.7–4.0 mm diam./d), creamy white to pale brown with regular margin, grey near the centre and hyphae clusters, reverse brown to dark brown rings, heterogeneous colour, with creamy-white edge. Conidial formation not observed.

**Additional specimen examined.** China, Shandong Province: Zibo Lushan National Forest Park, on diseased leaves of _Quercus palustris_ Münchh. (Fagaceae), 20 Sep 2020, Z. X. Zhang, HSAUP1921; living culture SAUCC 1921.

**Notes.** The phylogenetic analysis of a combined three-gene alignment (ITS, _tef1_ and _tub2_) showed that _T. lushanensis_ formed an independent clade and is phylogenetically distinct from its closest sister species _T. seoraksanensis_. This species can be distinguished from _T. seoraksanensis_ by 65 different nucleotides in the concatenated alignment (21/628 in the ITS, 31/581 in the _tef1_ and 13/521 in the _tub2_). Morphologically, _T. lushanensis_ differs from _T. seoraksanensis_ in having smaller conidia (10.0–18.0 μm × 7.5–16.0 μm vs. 13.0–25.0 μm × 10.0–15.0 μm) (Yun & Rossman 2011). Furthermore, the MEA's colony colour of _T. lushanensis_ is different from _T. seoraksanensis_ (surface: creamy white, pale brown to grey vs. whitish to pale yellow; reverse: creamy white, brown to dark brown vs. olive brown, light olive brown to yellow; Yun & Rossman 2011). Therefore, we describe this fungus as a novel species.
**Tubakia dryinoides** C. Nakash., *Fungal Systematics and Evolution* 1: 80 (2018)  
Fig. 5

**Description.** Asexual morph: Living as endophyte in leaves, forming distinct leaf lesions, shape and size variable, subcircular to angular-irregular, pale brown to brown. Colonies on PDA incubated at 25°C in the dark with an average radial growth rate of 5–7 mm/d and occupying an entire 90 mm Petri dish in 14 d, forming some conspicuous concentric circles, aerial mycelium cottony, white initially, then becoming greyish-sepia. Conidiomata sporodochial, appeared within 14 days or longer, formed on agar surface, slimy, black, semi-submerged. Sporodochial conidiophores densely
A new genus and two new species of *Tubakiaceae* from China

**Figure 5.** *Tubakia drynoides* (SAUCC 1924). **a** diseased leaf of *Quercus palustris*; **b** surface of colony after 15 days on MEA; **c** reverse of colony after 15 days on MEA; **d** conidiomata; **e–g** conidiophores, conidiogenous cells with conidia; **h–i** conidia. Scale bars: 10 μm (**e–i**).

and irregularly branched, 11.0–24.0 μm × 1.5–5.0 μm, bearing apical whorls of 2–3 phialides; sporodochial phialides monophialidic, subulate to subcylindrical, 9.0–16.0 μm × 1.5–5.0 μm, smooth, thin-walled, apex obtuse to truncate, sometimes forming indistinct periclinal thickenings. Conidia solitary, ellipsoid to obovoid, 6.5–14.0 μm × 4.0–6.0 μm, wall thin, up to 1.0 μm, hyaline to subhyaline, smooth, apex and base broadly rounded, with inconspicuous to conspicuous basal hilum (frill), occasionally somewhat peg-like and truncate when conspicuous. Microconidia not observed. Sexual morph not observed.

**Culture characteristics.** Cultures incubated on MEA at 25°C in darkness, attaining 38.0–42.0 mm diam. after 14 d (growth rate 2.7–3.0 mm diam./d), margin scal-
loped, at first creamy white, grey near the centre, reverse light brown to dark, with olivaceous edge. Conidial formation not observed.

**Specimen examined.** China, Shandong Province: Zibo Lushan National Forest Park, on diseased leaves of *Quercus palustris* (Fagaceae), 20 Sep 2020, Z. X. Zhang, HSAUP1924, living culture SAUCC 1924.

**Notes.** Braun et al. (2018) described *Tubakia dryinoides*, based on morphological and molecular data. The holotype of *T. dryinoides* (NBRC H-11618) was collected from *Quercus phillyraeoides* A. Gray (Braun et al. 2018). In our current research, isolate (SAUCC 1924) collected from diseased leaves of *Quercus palustris* clustered in the *Tubakia dryinoides* clade by strong support (Figs. 1 and 2). We, therefore, consider the isolated strain (SAUCC 1924) as *T. dryinoides*. The conidiomata of *T. dryinoides* is only known from true pycnothyria and the sporodochial conidiomata of the isolated strain (SAUCC 1924) is new for *T. dryinoides* (Braun et al. 2018). Additionally, the conidia of our isolate (SAUCC 1924) is narrower than the original description of *T. dryinoides* (4.0–6.0 μm vs. 5.5–10.0 μm; Braun et al. 2018).

**Discussion**

In the study of the phylogenetic affinity and position of *Tubakia* in the Ascomycota hierarchical system, Senanayake et al. (2017) placed this genus in the newly-introduced family Melanconiellaceae. However, the recently-published phylogenetic analyses, including sequence data of the type species of *Tubakia*, confirmed that *Tubakia* warranted a family of its own, Tubakiaceae (Braun et al. 2018) and the description of eight genera including *Apiognomonioides* U. Braun et al., *Involutiscutellula* U. Braun & C. Nakash., *Oblongisporothyrium* U. Braun & C. Nakash., *Paratubakia* U. Braun & C. Nakash., *Racheliella* Crous & U. Braun, *Saprothyrium* U. Braun et al., *Sphaerosporithyrium* U. Braun et al. and *Tubakia* B. Sutton (Braun et al. 2018). The family comprises genera and species with sporodochia, crustose to pustulate pycnothyrioid stromatic conidiomata and superficial scutellate pycnothyria, monophialidic, colourless, conidiogenous cells, often with collarettes and conidia formed singly, mostly globose to broad ellipsoid-obovoid, aseptate, hyaline to pigmented, often with basal frill or truncate peg-like hilum.

The present study found two new species, one of which represents a novel genus in Tubakiaceae. In order to support the validity of the new species, we followed the guidelines of Braun et al. (2018). Based on ITS/LSU/rpb2 and ITS/tef1/tub2 molecular data, phylogenetic analyses revealed that two of the obtained isolates (SAUCC 0745 and SAUCC 0748) cluster in a separate lineage, fully supported at genus-level and related to the genera *Racheliella, Oblongisporothyrium* and *Paratubakia*. The new genus is named *Obovoideisporodochium* gen. nov. (type species: *Obovoideisporodochium lithocarpi* sp. nov.). The phylogenetic analyses also revealed that three isolates (SAUCC 1921, SAUCC 1923 and SAUCC 1924) pertain to the genus *Tubakia*. Owing to different nucleotides in the concatenated alignment and morphology, two isolates (SAUCC
A new genus and two new species of Tubakiaceae from China

1921 and SAUCC 1923) of Tubakia were identified as a new species, namely *T. lushanensis* sp. nov, whereas the third isolate (SAUCC 1924) was identified as *T. dryinoides*.

The centre of genetic diversity of Tubakia appears to be in East Asia, where Quercus and other genera of Fagaceae are the most common hosts (Harrington & McNew 2018). Our study supports this phenomenon well. *Tubakia lushanensis* (SAUCC 1921 and SAUCC 1923) and *T. dryinoides* (SAUCC 1924) were isolated from *Quercus palustris* (Fagaceae), thereby increasing the genetic diversity of Tubakia in East Asia.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 31900014, 31750001 and 31770016).

References

Braun U, Bien S, Hantsch L, Heuchert B (2014) *Tubakia chinensis* sp. nov. and a key to the species of the genus Tubakia. Schlechtendalia 28: 23–28.

Braun U, Nakashima C, Crous PW, Groenewald JZ, Moreno-Rico O, Rooney-Latham S, Blomquist CL, Haas J, Marmolejo J (2018) Phylogeny and taxonomy of the genus Tubakia s. lat. Fungal Systematics and Evolution 1: 41–99. https://doi.org/10.3114/fuse.2018.01.04

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91(3): 553–556. https://doi.org/10.1080/00275514.1999.12061051

Fan XL, Bezerra JDP, Tian CM, Crous PW (2018) Families and genera of diaporthalean fungi associated with canker and dieback of tree hosts. Persoonia 40: 119–134. https://doi.org/10.3767/persoonia.2018.40.05

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61(4): 1323–1330. https://doi.org/10.1128/AEM.61.4.1323-1330.1995

Guo LD, Hyde KD, Liew ECY (2000) Identification of endophytic fungi from Livistona chinensis based on morphology and rDNA sequences. New Phytologist 147(3): 617–630. https://doi.org/10.1046/j.1469-8137.2000.00716.x

Harrington TC, McNew DL (2016) Distribution and intensification of bur oak blight in Iowa and the Midwest. In: Porter KM and Conkling BL (eds): Forest health monitoring: national status, trends, and analysis 2015. Forest Service & Research Station, Southern Research Station, General Technical Report SRS-213: 105–110.

Harrington TC, McNew DL (2018) A re-evaluation of Tubakia, including three new species on Quercus and six new combinations. Antonie van Leeuwenhoek 111(7): 1003–1022. https://doi.org/10.1007/s10482-017-1001-9
Harrington TC, McNew DL, Yun HY (2012) Bur oak blight, a new disease on *Quercus macrocarpa* caused by *Tubakia iowensis* sp. nov.. Mycologia 104: 79–92. https://doi.org/10.3852/11-112

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogeny. Bioinformatics 17(17): 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20: 1160–1166. https://doi.org/10.1093/bib/bbx108

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Kumar S, Tamura K, Nei M (2004) MEGA3: Molecular Evolutionary Genetics Analysis Version 3.1. Bioinformatics 20(2): 2067–2069. https://doi.org/10.1093/bioinformatics/bth329

Liu YJ, Whelen S, Hall BD (1999) Phylogenetic Relationships among Ascomycetes: evidence from an RNA polymerase II subunit. Molecular Biology and Evolution 16(12): 1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092

Miller MA, Pfeiffer W, Schwartz T (2012) The CIPRES Science Gateway: enabling high-impact science for phylogenetics researchers with limited resources. In: Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment. Bridging from the extreme to the campus and beyond. Association for Computing Machinery, USA, 1–8. https://doi.org/10.1145/2335755.2335836

Nylander JAA (2004) MrModelTest v. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

O’Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama Disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences of the United States of America 95(5): 2044–2049. https://doi.org/10.1073/pnas.95.5.2044

Rehner SA, Samuels GJ (1994) Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycological Research 98(6): 625–634. https://doi.org/10.1016/S0953-7562(09)80409-7

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian Phylogenetic Inference under Mixed Models. Bioinformatics 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Saccardo PA (1913) Notae mycologicae. Series XVI. Annales Mycologici 11: 493–511.

Senanayake IC, Crous PW, Groenewald JZ, Maharachchikumbura SSN, Jeewon R, Phillips AJL, Bhat JD, Perera RH, Li QR, Li WJ, Tangthirasunun N, Norphanphoun C, Karunarathna SC, Camporesi E, Manawasighe IS, Al-Sadi AM, Hyde KD (2017) Families of Diaporthales based on morphological and phylogenetic evidence. Studies in Mycology 86: 217–296. https://doi.org/10.1016/j.simyco.2017.07.003
Stamatakis A (2014) RAxML Version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Sutton BC (1973) Tubakia nom. nov. Transactions of the British Mycological Society 60: 164–165. https://doi.org/10.1016/S0007-1536(73)80077-4

Sung GH, Sung JM, Hywel-Jones NL, Spatafora JW (2007) A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): identification of localized incongruence using a combinatorial bootstrap approach. Molecular Phylogenetics and Evolution 44(3): 1204–1223. https://doi.org/10.1016/J.YMPEV.2007.03.011

Theissen F (1913) Ueber einige Mikrothyriaceen. Annales Mycologici 11: 493–511.

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. Journal of Bacteriology 172(8): 4238–4246. https://doi.org/10.1128/JB.172.8.4238-4246.1990

Von Höhnel F (1925) Neue Fungi imperfecti. 5. Mitteilung. Mitteilungen des Botanischen Instituts der Technischen Hochschule Wien 2: 65–73.

White T, Burns T, Lee S, Taylor J (1990) Amplification and direct sequencing of ribosomal RNA genes for phylogenetics. In: Innis MA (Ed.) PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Yokoyama T, Tubaki K (1971) Cultural and taxonomical studies on the genus Actinopelte. Institute of Fermentation, Osaka, Research Communication 5: 43–77. https://doi.org/10.1007/BF02051502

Yun HY, Kim YH (2020) Tubakia koreana sp. nov. causing Quercus leaf blight. Mycotaxon 135(1): 223–229. https://doi.org/10.5248/135.223

Yun HY, Rossman AY (2011) Tubakia seoraksanensis, a new species from Korea. Mycotaxon 115: 369–373. https://doi.org/10.5248/115.369

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7: 203–214. https://doi.org/10.1089/10665270050081478