Antonie van Leeuwenhoek (2018) 111:1003–1022
https://doi.org/10.1007/s10482-017-1001-9

ORIGINAL PAPER

A re-evaluation of *Tubakia*, including three new species on *Quercus* and six new combinations

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Received: 20 August 2017 / Accepted: 12 December 2017 / Published online: 18 December 2017

Abstract Morphological comparisons and phylogenetic analyses of *Tubakia* species from leaves of *Quercus* spp. in Iowa and other areas of eastern USA revealed three novel species: *Tubakia hallii*, *Tubakia macnabbii*, and *Tubakia tiffanyae*. These species, as well as *Tubakia dryina* and *Tubakia iowensis*, are common leaf endophytes and pathogens on *Quercus* and *Castanea* in eastern USA, as is *Tubakia americana* comb. nov, originally described from *Quercus* in New Jersey as *Actinopelte americana*. New combinations of species on leaves of other hosts in the eastern USA include *Tubakia gloeosporioides*, *Tubakia liquidambaris*, and *Tubakia nyssae*. Asian species of *Tubakia* are phylogenetically compared, and the new combination *Tubakia supraseptata* is made to accommodate a Japanese endophyte known only by its sexual state. The earlier description of *Dicarpella dryina* as the sexual state of *T. dryina* is questioned. The new combination *Tubakia stellata* is made to accommodate an unusual species from Brazil.

Keywords *Actinopelte* · *Actinothyrium* · Diaporthales · *Leptothyrella* · *Pirostoma*

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Introduction

Species of *Tubakia* B. Sutton are common leaf endophytes and pathogens on *Quercus* (oak) and other Fagaceae (Harrington and McNew 2016; Harrington et al. 2012). The genus is characterized by unique pycnothyria, consisting of pigmented, radiating, setae-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. The majority of the recognized species of *Tubakia* are found in eastern Asia (Kaneko and Kobayashi 1984; Kobayashi et al. 1979; Liu and Liu 2010; Yokoyama and Tubaki 1971; Yun and Rossman 2011). Traditionally, American species have been lumped into a single, broadly defined species, *Tubakia dryina* (Sacc.) Höhn. (Limber and Cash 1945), which has been reported most commonly from Europe (Boroń and Grad 2016; Harrington et al. 2012; Kowalski 2006). A comprehensive treatment of the genus is lacking, and the group is in need of phylogenetic analyses and taxonomic revision (Harrington and McNew 2016; Harrington et al. 2012).

Actinopelte was erected by Saccardo (1913) to accommodate fungi with fruiting bodies of radiating setae-like cells, though Saccardo (1913) mistook the large conidia of *Actinopelte japonica* Sacc. as asci. Höhnel (1925) transferred the European *Leptothyrium dryinum* Sacc. (Saccardo 1878) to *Actinopelte* and described *Actinopelte americana* Höhn. from material

collected by Ellis and Everhart in New Jersey. The genus name *Actinopelte* was previously occupied by a lichen taxon, so Sutton (1973) erected *Tubakia*, using *A. japonica* as the type species. Besides *Tubakia japonica* and *T. dryina*, Yokohama and Tubaki (1971) reported three new species in Japan: *Tubakia castanopsidis* (Yokohama and Tubaki) B. Sutton, *Tubakia rubra* (Yokohama and Tubaki) B. Sutton and *Tubakia subglobosa* (Yokohama and Tubaki) B. Sutton. More recently, *Tubakia seoraksanensis* Yun and Rossman was described from oak leaves in Korea (Yun and Rossman 2011), *Tubakia iowensis* T.C. Harr. and McNew was described from oak leaves in USA (Harrington et al. 2012), and *Tubakia chinensis* U. Braun, S. Bien and Hantsch was described from *Castanea* leaves in China (Braun et al. 2014).

Numerous described species of leaf fungi from North America that could be placed in *Tubakia*, including *A. americana* (Höhnel 1925), have been considered synonyms of *T. dryina* (Gilman and Archer 1929; Glawe and Crane 1987; Limber and Cash 1945). Aside from host species in the Fagaceae, Tehon (1924) described eastern USA species with typical Tubakia-type pycnothyria as *Actinothyrium gloeosporioides* Tehon from leafspots on sassafras (*Sassafras albidum*) and *Pirostoma nyssae* Tehon from leafspots on black tupelo (*Nyssa sylvatica*). Tehon and Stout (1929) described *Leptothyrella liquidambaris* Tehon and Stout from leaf spots on sweetgum (*Liquidambar styraciflua*). These three species were considered probable synonyms of the oak leafspot pathogen *A. dryina* by Limber and Cash (1945), and Tehon (1948) later agreed. Studies of a new leaf blight disease on bur oak (*Quercus macrocarpa*) led to a new species, *T. iowensis* T.C. Harrin and McNew, which was distinguished from a more narrowly defined *T. dryina* by morphology and DNA sequence analyses (Harrington et al. 2012). The association of *T. iowensis* with a serious leaf disease on *Q. macrocarpa* and observations of other undescribed species causing leaf diseases of other *Quercus* spp. (Harrington and McNew 2016) prompted a more thorough evaluation of the genus *Tubakia*.

This study provides a re-evaluation of the genus *Tubakia*, especially of the species common to eastern USA. Cultures and specimens were studied morphologically and phylogenetically and compared to the other known species of *Tubakia* from Eurasia. Three new species are described from eastern USA.

Materials and methods

Specimens and isolates

Either green and non-symptomatic leaves or leaves with necrotic spots or veins were collected primarily in Iowa and surrounding states. Cooperators throughout the eastern USA and California provided other fresh samples for study. For isolations from green or necrotic plant tissues without conidiomata, excised pieces were surfaced-sterilized with undiluted household bleach (5.25% sodium hypochlorite)for 3 min with stirring, followed by 30 s in 95% ethanol, and rinsed in sterile water before plating on malt extract agar (MEA; 1.5% Difco malt extract and 2% agar). If present, conidiomata could be easily dislodged from spots on leaf blades or along necrotic veins by scraping with a dissecting needle, and isolations were made by placing several conidiomata with conidia in a drop of water and streaking the drop across the surface of streptomycin malt extract agar (SMA, MEA with 100 mg/L streptomycin sulphate added after autoclaving). Crustose pycnothyria that formed on leaves, petioles or twigs were moistened with sterile water until swollen, the crustose covering was cut open with a scalpel, and the conidial contents streaked on SMA. Subcultures of developing colonies were made after 1–3 days onto malt yeast extract agar (MYEA, 2.0% Difco malt extract, 0.2% Difco yeast extract and 2.0% agar).

Culture numbers beginning with "A" are those of the Iowa State University collection (maintained by the senior author), and representative cultures were deposited in the Centraalbureau voor Schimmelcultures (CBS, now Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). Aside from our collections, cultures were obtained from CBS, and these cultures were also assigned "A" numbers (Table 1).

Specimen numbers beginning with RO and WO are those of the authors, and representative specimens were deposited in the Ada Hayden Herbarium (ISC) at Iowa State University. We examined herbarium specimens at ISC, the US National Fungus Collection (BPI), the Illinois Natural History Survey (ILLS), and the New York Botanical Garden (NY). Several of the exsiccati collections of Ellis and Everhart were examined, including a copy of Fungi Colombiani in the McClatchie Herbarium at NY.

Table 1 Collection numbers, substrate, location, and DNA sequences of representative cultures and specimens of *Tubakia* spp

Species	Specimen number(s)	Culture number(s)	Substrate	Location	LSU (28S) rDNA	ITS rDNA	*TEF*-1α
T. americana	NY E&E FC286 ex McClatchie, lectotype		Leaf spot on *Quercus coccinea*	Newfield, New Jersey		MG605063	
		A749, CBS 129014	Leaf spot on *Q. macrocarpa*	Ames, Iowa	JF704191	JF704202	
	ISC 453301	A1158, CBS 137353	Leaf spot on *Q. macrocarpa*	Ames, Iowa		MG605064	MG603571
		A682, CBS 335.86	*Q. pedunculata*	France		MG605065	MG603572
T. castinopsidis		A686, CBS 189.71, ex holotype	*Castanopsis cuspidata*	Japan	MG605076	MG605066	MG603573
T. hallii	ISC 453286, holotype	A666, CBS 129013, ex holotype	Leaf spot on *Q. stellata*	Kirbyville, Missouri	JF704192	JF704203	MG603574
	ISC 453288	A762, CBS 129015	Leaf spot on *Q. stellata*	Bella Vista, Arkansas	JF704193	JF704204	
T. dryina		A680, CBS 112097	*Q. robur*	Teviso, Italy	JF704187	JF704198	MG603575
		A681, CBS 114386	Leaf spot on *Q. robur*	Auckland, New Zealand	JF704188	JF704199	
	ISC 448611	A876, CBS 129016	Dead twig of *Q. alba*	Ames, Iowa	JF704189	JF704200	
	ISC 448612	A996, CBS 129018	Leaf spot on *Q. macrocarpa*	Muscatine Co., Iowa	JF704190	JF704201	
T. iowensis	BPI 881219, ISC 448599, holotype	A607, CBS 129012, ex holotype	Leaf of *Q. macrocarpa*	Ames, Iowa	JF704183	JF704194	
	ISC 448602	A1003, CBS 129019	Petiole of *Q. macrocarpa*	Ames, Iowa	JF704184	JF704195	MG603576
	BPI 881221, ISC 448604	A573, CBS 129019	Petiole of *Q. macrocarpa*	Ames, Iowa	JF704185	JF704196	
	ISC 448610	A995, CBS 129017	Leaf spot on *Q. macrocarpa*	Monroe, Wisconsin	JF704186	JF704197	
T. japonica		A872, CBS 191.71	*Quercus* sp.	Japan		MG605067	MG603577
T. liquidambaris	ISC 453303	A771, CBS 139744	Leaf spot on *Liquidambar styraciflua*	Bella Vista, Arkansas	MG605077	MG605068	MG603578
T. macnabbii	ISC 453290, holotype	A989, CBS 137349, ex holotype	Leaf spot on *Q. palustris*	Jackson Co., Missouri		MG605069	MG603579
	ISC 453292	A852, CBS 137347	Leaf spot on Castanea × Dunstan	Gainesville, Florida		MG605070	
	ISC 453291	A810, CBS 137346	Leaf vein of *Q. muehlenbergii*	Bella Vista, Arkansas		MG605071	
		A683, CBS 639.93	*Q. rubra*	Italy	MG605078	MG605072	

Table 1 continued

Species	Specimen number(s)	Culture number(s)	Substrate	Location	LSU (28S) rDNA	ITS rDNA	*TEF*-1α
T. rubra		A684, CBS 192.71, ex isotype	*Q. phillyraeoides*	Japan	MG605079	MG605073	
T. seoraksanensis		A786, ex-holotype	*Q. mongolica*	Korea		MG605074	MG603580
T. subglobosa		A685, CBS 193.71, ex holotype	*Q. glauca*	Japan	MG605080	MG605075	
T. supraseptata			*Q. glauca*	Japan	AF277127		
T. tiffanyae	ISC 453296, holotype	A803, CBS 137345 ex holotype	Leaf spot on *Q. rubra*	Ames, Iowa		MG605081	MG603581
	ISC 453299	A1042, CBS 137351	Leaf spot on *Q. rubra*	Iowa City, Iowa		MG605082	

Characterization of specimens and isolates

Isolates were grown on MYEA plates at room temperature (21 to 23 °C) or at various set temperatures (20, 25, 30, and 35 °C). For comparing growth rates, a plug of mycelium 4 mm in diam from the advancing margin of a 7-day-old colony was placed on a MYEA plate, and the radial growth rate (mm/d) was determined between 2 and 7 days. Three replicate plates were used for each isolate/temperature combination. For formal description, cultures were grown on MYEA at 25 °C in a dark incubator.

Material was mounted in 20% lactic acid or in lactophenol with cotton blue for microscopic examination. Digital photographs and measurements were taken on an Olympus compound microscope (Olympus BH-2, Nomarski DIC optics) fitted with a digital camera (DFC295) and software from Leica (Bannockburn, Illinois).

DNA sequencing

In most cases, DNA was extracted from specimens or cultures using PrepMan^{TM} Ultra (Applied Biosystems, Foster City, California) following the manufacturer's instructions. Isolates were grown on MYEA for 7–14 days at room temperature, and surface growth on the MYEA plates was used for DNA extraction. Attempts were also made to extract DNA from conidiomata on leaf surfaces of fresh field material or dried herbarium material.

The large subunit (LSU, 28S) rDNA region was amplified with primers LROR and LR5, and the PCR products were sequenced with primers LR0R and LR3 (published online: https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi/) as described in Harrington et al. (2010). Sequencing of portions of internal transcribed spacer region of rDNA (ITS) were attempted from DNA extracted from all cultures and from some conidiomata scraped from leaf surfaces. The primers ITS1-F and ITS4 (Gardes and Bruns 1993, White et al. 1990) and cycling conditions were as described earlier (Harrington et al. 2014). For conidiomata scraped from herbarium specimens, DNA was similarly extracted, but PCR and DNA sequencing utilized the fungal specific primer ITS1-F with the *Tubakia*-specific reverse primer TubITS4Rb (5′-ATC CGA GGT CAA CCA GTA AA). A portion of the translocation elongation factor 1-alpha (*TEF*-1α) was

amplified with primers that we developed for *Ceratocystis fimbriata*, namely, EFCF1 (5′-AGT GCG GTG GTA TCG ACA AG) and EFCF6 (5′-CAT GTC ACG GAC GGC GAA AC) and sequenced with the same primers and EFCF2 (5′-TGC TCA CGG GTC TGG CCA T) and EFCF3 (5′-ATG GCC AGA CCC GTG AGC A), using the cycling conditions and recipe of Oliveira et al. (2015).

All sequencing was conducted at the Iowa State University DNA Sequencing and Synthesis Facility. Complementary sequences were compared with Sequence Navigator v 1.0.1 or Auto Assembler v 1.3.0 (Perkin Elmer Applied Biosystems, Foster City, California). Sequences of LSU-rDNA were generated for 33 *Tubakia* isolates, sequences of ITS-rDNA for nearly 500 isolates and specimens, and sequences of *TEF*-1α sequences for 56 *Tubakia* isolates. Sequences of isolates representing putative taxonomic units were used in phylogenetic analyses, and a select number of representative sequences were deposited in GenBank (Table 1). Generated sequences were compared to those of other fungi using BLAST searches (v. 2.2.24, National Centre for Biotechnology Information, National Institute of Health, Bethesda, MD), and the closest matches were downloaded and included in the datasets for phylogenetic analyses.

Phylogenetic analyses

The sequences in each of the three datasets were manually aligned, with little ambiguity among the ingroup taxa (*Tubakia* spp.), except that there was some ambiguity in the alignment of the ITS sequences due to short indels in ITS1. The LSU dataset had 41 sequences, including those of *Tubakia* spp., representative Diaporthales that had sequences most similar to those of the *Tubakia*, and *Chaetomium brasiliense* (Sordariales). Of the 527 aligned LSU characters, 424 were constant and 52 were parsimony informative. The ITS dataset had 59 sequences representing the diversity of ITS sequences of *Tubakia* spp., along with the sequences of *Gnomonia setacea* and *Greeneria uvicola*, whose sequences were most easily aligned with those of *Tubakia*. The ITS alignment had 581 characters, including the 5.8 S gene, with 346 characters constant and 128 characters that were parsimony informative. The *TEF*-1α dataset had 53 sequences that we generated, as well as sequences of *C. globosum* and *Metarhizium guizhouense* (Hypocreales) as outgroup taxa, which were selected because their sequences showed the greatest similarity to those of *Tubakia* in BLAST searches. The 1261 aligned *TEF*-1α characters included the entire second exon and most of the third exon, as well as the intervening intron of about 170 aligned bp, which was unambiguously aligned for the *Tubakia* sequences. Of the 1261 characters, 957 were constant and 133 were parsimony informative.

The aligned sequences were analysed for maximum parsimony (MP) using PAUP v.4.0b10 (Swofford 2002). Gaps were treated as a fifth base, all characters had equal weight, and the heuristic searches utilized simple stepwise addition and tree-bisection-reconnection. Bootstrap analyses also were conducted in PAUP with 1000 replications, but the maximum number of trees in each bootstrap replication in the ITS analysis was set to 100 per run. Posterior probability (Bayesian) estimates for branches were determined with Mr. Bayes 3.2.1 (Ronquist and Huelsenbeck 2003), in which gaps are treated as missing data. The settings included a general time-reversible (GTR) model and gamma distribution. There were 1,000,000 (ITS) or 2,000,000 (LSU and *TEF*-1α) generations, a diagnosis frequency of 5000, and a sample frequency of 1000. The first 25% of samples were discarded (burninfrac = 0.25), and Bayesian posterior probability estimates were calculated by majority rule consensus of the trees after burn-in.

Results

Morphological comparisons

The characteristic scutella of radiating, setae-like hyphae were found on symptomatic leaves of *Quercus* spp., *Castanea dentata* (chestnut), *Lithocarpus densiflorus* (tanoak), and *Liquidambar styraciflua* in eastern USA and California (Fig. 1). A range of leaf symptoms was seen (Fig. 1a, d, h), but there was only limited variation in the diameter of scutella and the size of conidia, which ranged from hyaline to brown, often with a finely punctate, thick outer wall (Fig. 1j). Naked sporodochia were also seen in some samples, especially on necrotic tissue along the midveins on the underside of *Quercus* leaves, and the conidia from these sporodochia were similar in size and coloration to those formed under the radiate scutella. A second

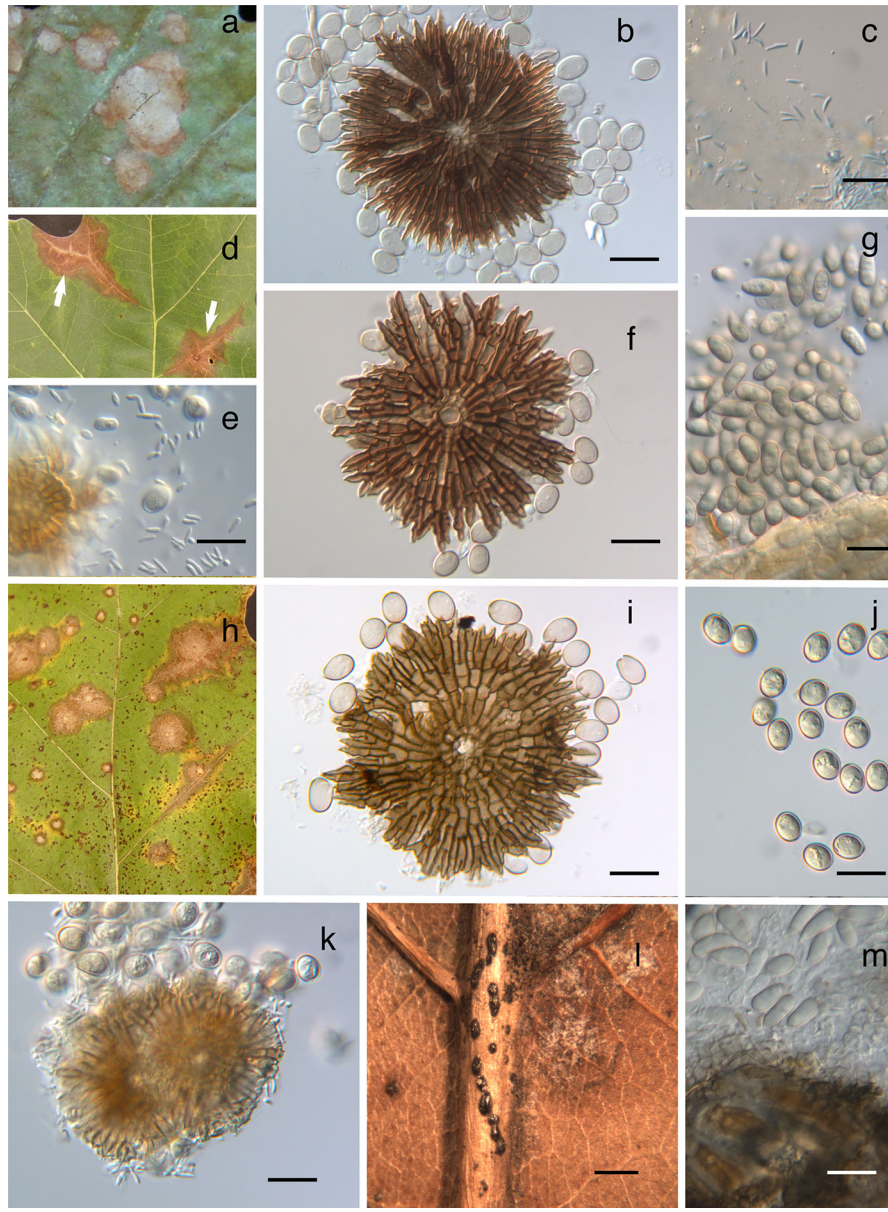

Fig. 1 Leaf symptoms, conidiomata and spores of three new *Tubakia* species. **a–c** *T. hallii*. **a** Irregular leaf spots on *Quercus stellata*. **b** Pycnothyrium with conidia on leaf of *Q. stellata*, from the holotype. **c** Microconidia from a culture (CBS 129015 = A762) on media with sterile leaf pieces. **d–g** *T. macnabbii*. **d** Veinal necrosis (arrows) on leaf of *Q. rubra*. **e, f** Poorly developed pycnothyrium with conidia and microconidia and mature pycnothyrium with conidia, from the holotype. **g** Conidia from a squashed crustose-type pycnothyrium (from ISC 453295, strain A1258). **h–m** *T. tiffanyae*. **h** Necrotic flecks, small circular leafspots, and larger irregular leaf spots on a leaf of *Q. rubra*. **i–k** Pycnothyrium, conidia, and poorly developed pycnothyrium with conidia and microconidia, from the holotype. **l, m** Crustose-type pycnothyrium on leaf vein of *Q. rubra* and conidia from a crushed pycnothyrium (ISC 453298). Bars in all photos = 20 µm, except **l**, where the bar = 1 mm

type of pycnothyria with crustose coverings (Harrington et al. 2012) was occasionally found on living and dead leaves, petioles, twigs, or acorns of *Quercus* spp. (Fig. 1l). Conidia from crustose pycnothyria (Fig. 1g, m) were similar in appearance to those under radiate scutella, though the sizes of conidia from the two types of pycnothyria typically differed. Herbarium samples from Illinois (ILLS), Iowa State University (ISC) and

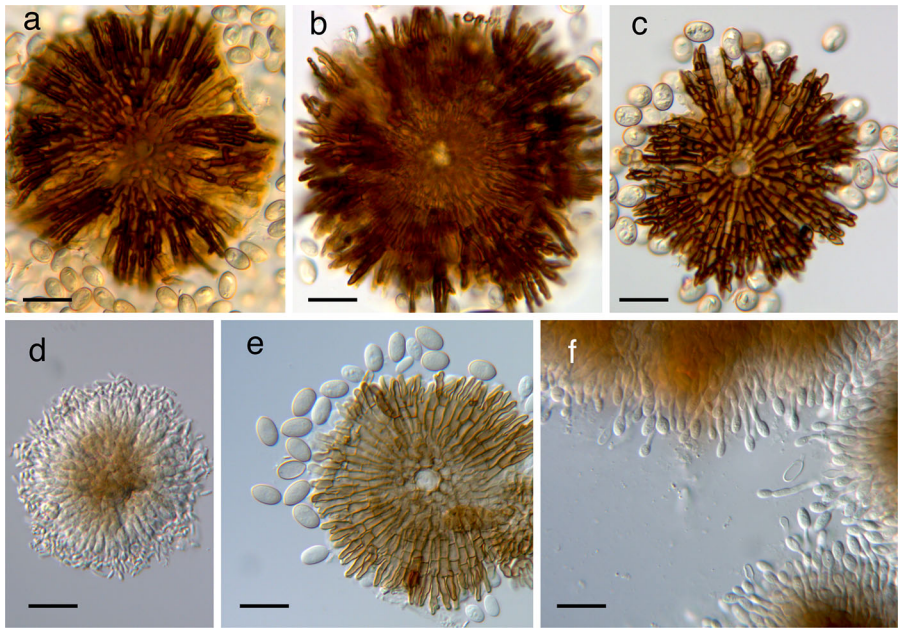

Fig. 2 *Tubakia americana* and a second *Tubakia* sp. on lectotype material. **a**, **b** Pycnothyria and conidia of *T. americana* from a leafspot on the lectotype. **c** Pycnothyrium and conidia of a second *Tubakia* sp. on a separate leafspot on the lectotype material. **d**–**f** *T. americana* on *Quercus macrocarpa* leaves and twigs in Iowa. **d, e** Underdeveloped pycnothyrium with microconidia and fully-developed pycnothyrium with conidia from a leaf of *Q. macrocarpa* (ISC 453301, strain A1158). **f** Conidiophores and conidia from a squashed, crustose pycnothyrium taken from a twig of *Q. rubra* (ISC 453304, strain A1007). Bars in all photos = 20 µm

the New York Botanical Garden (NY) also showed *Tubakia* spp. on leaves of oaks and numerous other hosts that were similar to those on fresh leaves. Some herbarium samples showed fruiting structures of two *Tubakia* species that differed by the size and shape of their conidia on different leaf spots of the same leaf (Fig. 2).

Pure cultures were obtained from over 500 collections of *Tubakia* spp., mostly by isolating from surfaced sterilized leaves or twigs of *Q. macrocarpa* in Iowa. In contrast to pycnothyria and conidia on leaves collected in the eastern USA, which showed limited variation, cultures of *Tubakia* spp. showed a wide range in growth rate and pigmentation of mycelium on MYEA. Most cultures were fast growing and had scalloped margins, initially white and later darkening grey to black (Harrington et al. 2012). Sporodochia with conidia rarely or commonly formed on MYEA, depending on the putative species, and aerial mycelium often obscured the sporodochia. The CBS isolates of *Tubakia* spp. from Japan showed a wide range in pigmentation and growth rate, but isolates from other countries appeared similar to the USA isolates.

Phylogenetic analyses

The LSU-rDNA sequences showed limited variation among the *Tubakia* collections from the USA, although the Japanese sequences were more variable (Fig. 3). There were 18 most parsimonious trees of 157 steps, with the homoplasy index (HI) = 0.2229 (= 0.3398 excluding uninformative characters), retention index (RI) = 0.7697, and rescaled consistency index (RC) = 0.5981. In the Bayesian analysis, the level of convergence from two parallel runs after 2,000,000 generations had a mean standard deviation of split frequencies of 0.005440. The MP trees (Fig. 3) grouped the putative *Tubakia* spp. in a single clade with moderate support (88/.99 bootstrap/posterior probability support). There were only two LSU sequences among the *Tubakia* spp. found in the eastern USA, though a GenBank sequence (HM122939) of an unidentified isolate recovered from a dead oak leaf in Arizona differed by 1 or 2 bp,

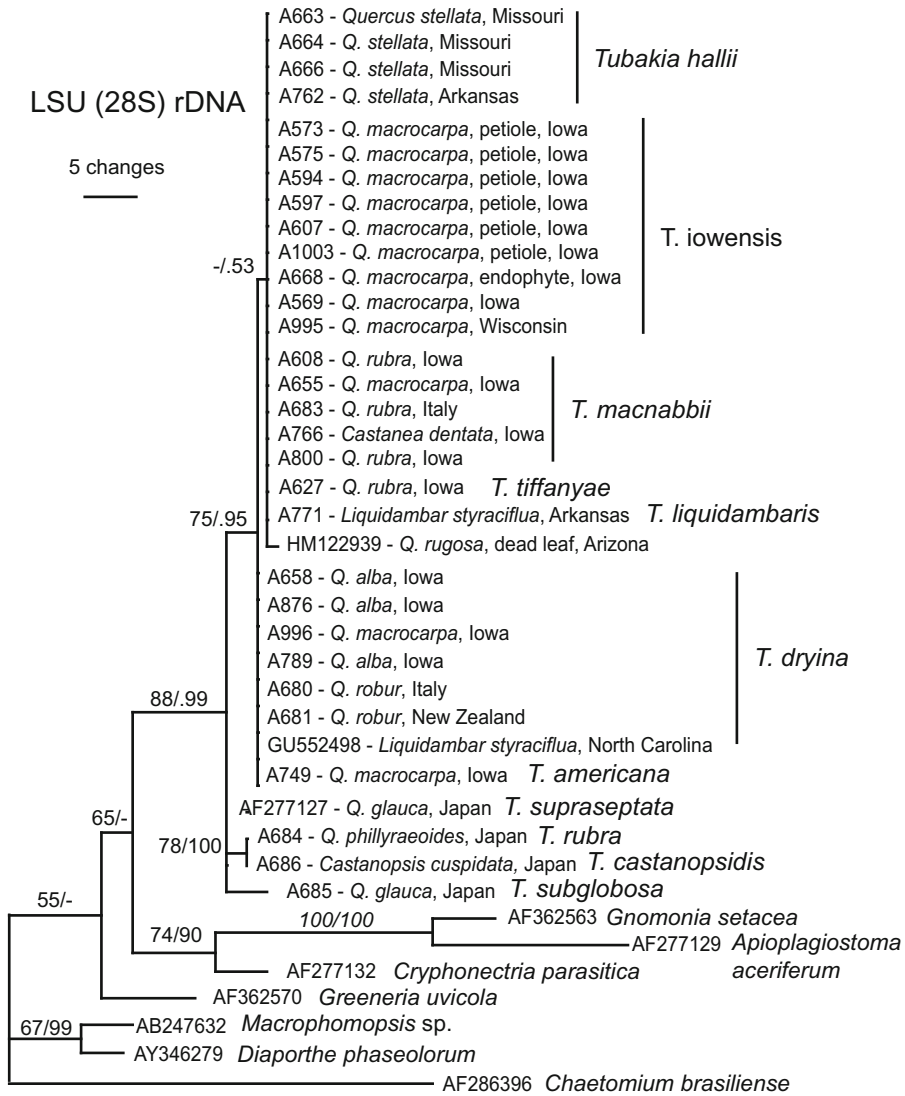

Fig. 3 One of 18 most parsimonious trees of *Tubakia* spp. and representative Diaporthales using partial sequences of the large subunit (28S) rDNA gene. The tree is rooted to *Chaetomium brasiliense*. Bootstrap values (greater than 50%, 1000 replications)/posterior probability estimates are given above the branches

respectively, from the LSU sequences of the other American species. The base of the *Tubakia* clade comprised four Japanese species and the deposited sequence (AF277127) of a sexual species, originally described as *Apiognomonia supraseptata* S. Kaneko and Ts. Kobay. With the tree rooted to *C. brasiliense* at an internal node with basal polytomy, the LSU sequences of the *Tubakia* clade grouped most strongly with Diaporthales with 2-celled ascospores, including representatives of *Apioplagiostoma*, *Cryphonectria*, and *Gnomonia*, as well as the asexual *G. uvicola* (Fig. 3). The LSU sequence of an Italian isolate (A683 = CBS 639.93) deposited as *Diacarpella dryina* Belisario and Barr was placed among the USA isolates of *Tubakia* spp., but *Diacarpella* has single-celled ascospores, unlike *A. supraseptata* and the sexual relatives of *Tubakia* spp.

Analysis of the ITS rDNA sequences gave 49 MP trees of 441 steps, HI = 0.2404 (= 0.3365 excluding uninformative characters), RI = 0.9011, and RC = 0.6845. In the Bayesian analysis, the level of convergence from two parallel runs after 1,000,000

generations had a mean standard deviation of split frequencies of 0.008518. Strong support (99/.97) for the branch comprising 13 *Tubakia* spp. was seen (Fig. 4), with three Japanese species (*T. rubra*, *T. subglobosa*, and *T. castanopsidis*) in a group sister to the other *Tubakia* spp. Aside from these three Asian species, the *Tubakia* sequences formed two sister clades. One of these clades comprised primarily

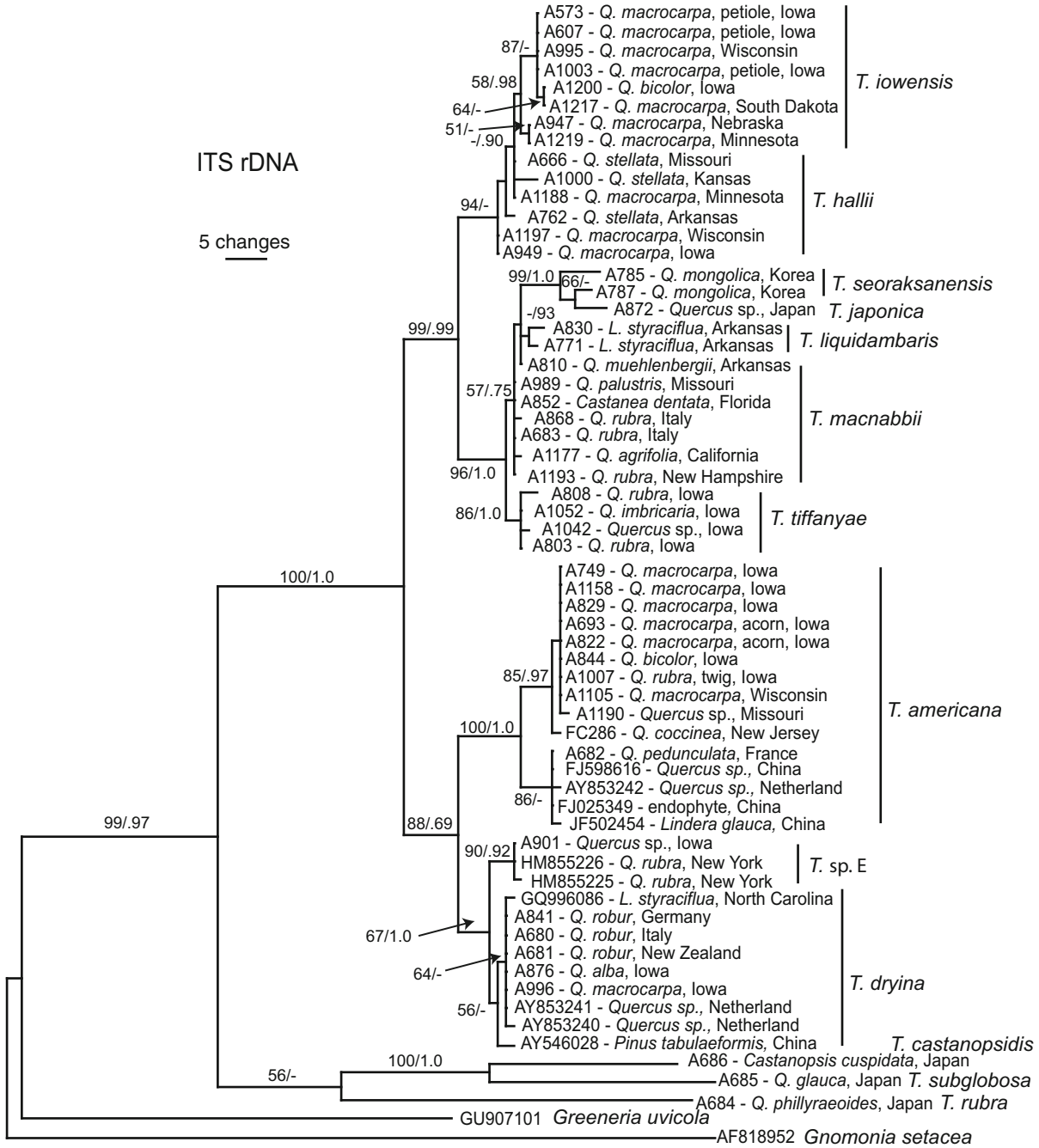

Fig. 4 One of 49 most parsimonious trees of *Tubakia* spp. and two representative Diaporthales (*Gnomonia setacea* and *Greeneria uvicola*) using partial sequences of the internal transcribed spacer regions (ITS) of rDNA. The tree is unrooted. Bootstrap values (greater than 50%, 1000 replications)/posterior probability estimates are given above the branches

collections from the USA and Europe, mostly from *Quercus* sect. *Quercus*, including collections considered to be *T. dryina* sensu stricto (Harrington et al. 2012). This clade was further separated into the *T. dryina* group (*T. dryina* sensu stricto along with an isolate from an oak tree in Iowa and two sequences deposited in GenBank from *Q. rubra* in New York, designated as *Tubakia* sp. E) and a second group that included the lectotype (FC 286) of *A. americana* from *Q. coccinea* in New Jersey, USA. The latter sequence differed by only one or 2 bp from those of isolates from sect. *Quercus* in and around Iowa, and it was also similar to sequences of some Eurasian collections (Fig. 4).

The other major clade of *Tubakia* spp. included two subclades (Fig. 4). One of these clades was primarily on the white oak group (sect. *Quercus*) and included the recently described *T. iowensis* (Harrington et al. 2012). The second subclade included most of the USA collections, which primarily were isolated from members of the red oak group (*Quercus* sect. *Lobate*). Two isolates from sweetgum (considered to represent *L. liquidambaris* Tehon and Stout), a USA isolate from chestnut, and the Asian species *T. japonica* (the type species for the genus) and *T. seoraksanensis* were within the USA red oak group (Fig. 4).

The *TEF*-1α analyses resulted in trees with topologies similar to those of the ITS trees. There were 23 MP trees of 488 steps in the *TEF*-1α analysis, with HI = 0.2193 (= 0.3262 excluding uninformative characters), RI = 0.8856, and RC = 0.6914. In the Bayesian analysis, the level of convergence from two parallel runs after 2,000,000 generations had a mean standard deviation of split frequencies of 0.009960. The sequence of the Japanese species *T. castanopsidis* was outside of the other *Tubakia* spp. in the tree (Fig. 5). The sequences of the other collections grouped similarly to the groupings found with the ITS rDNA sequences. Three broad groups were recognized among the American isolates. One included *T. dryina*, a second included *T. iowensis,* and a third comprised most of the collections from *Quercus* sect. *Lobatae*, a collection from chestnut, *T. japonica*, and the Korean species *T. seoraksanensis*. Two isolates from sweetgum grouped outside of the red oak group in the *TEF*-1α analysis (Fig. 5), but they were within the red oak group in the ITS analysis (Fig. 4).

Adding the respective ITS sequences to the *TEF*-1α dataset yielded maximum parsimony trees that were nearly identical to the *TEF*-1α tree, including placement of the sweetgum isolates outside the American red oak group. The Asian species *T. seoraksanensis* and *T. japonica* were still placed inside the American red oak group in the combined trees, but *T. iowensis* and its most closely related species were more clearly separated as sister groups (not shown).

Each group of collections with similar ITS and *TEF-1*α sequences were morphologically examined as potential species. Those USA groups with unique phenotypes (morphology, host range or symptom type) were considered to be species, a number of which could be accommodated within previously described species. Three new species are described, and six other species are transferred to *Tubakia.*

Taxonomy

Three new American species of *Tubakia*

Tubakia hallii T.C. Harr. and McNew, sp. nov. (Fig. 1a–c), MycoBank No. MB 822391

Etymology: named for Richard B. Hall, deceased forest geneticist at Iowa State University.

Conidiomata (pycnothyria) superficial, hypophyllous or epiphyllous on necrotic interveinal spots and along necrotic mid and lateral leaf veins, radiate scutella 45–155 µm diam, composed of a series of dark-brown, thick-walled cells originating from a central cell, ending in a blunt to acute point. *Sporodochia* superficial with only a few radiating dark hyphae, mainly hypophyllous, on necrotic interveinal tissues and along leaf veins. *Conidiophores* on underside of scutella or on top of sporodochia. *Conidia* hyaline, turning light brown with age, smooth to slightly varicose, aseptate, obovoid to ovoid, 9.5–14.5 (16) × 7.5–10 (11) µm (mean 12.4 × 8.7 µm). Microconidia not seen on leaves.

Colonies on MYEA with optimal growth between 25–30 °C, diam 50–70 mm after 7 days, initially white with dense aerial mycelium, smooth to scalloped at edge, turning cream to light grey at 10 days, developing concentric rings of dense mycelium, underside yellow, becoming golden yellow to brown

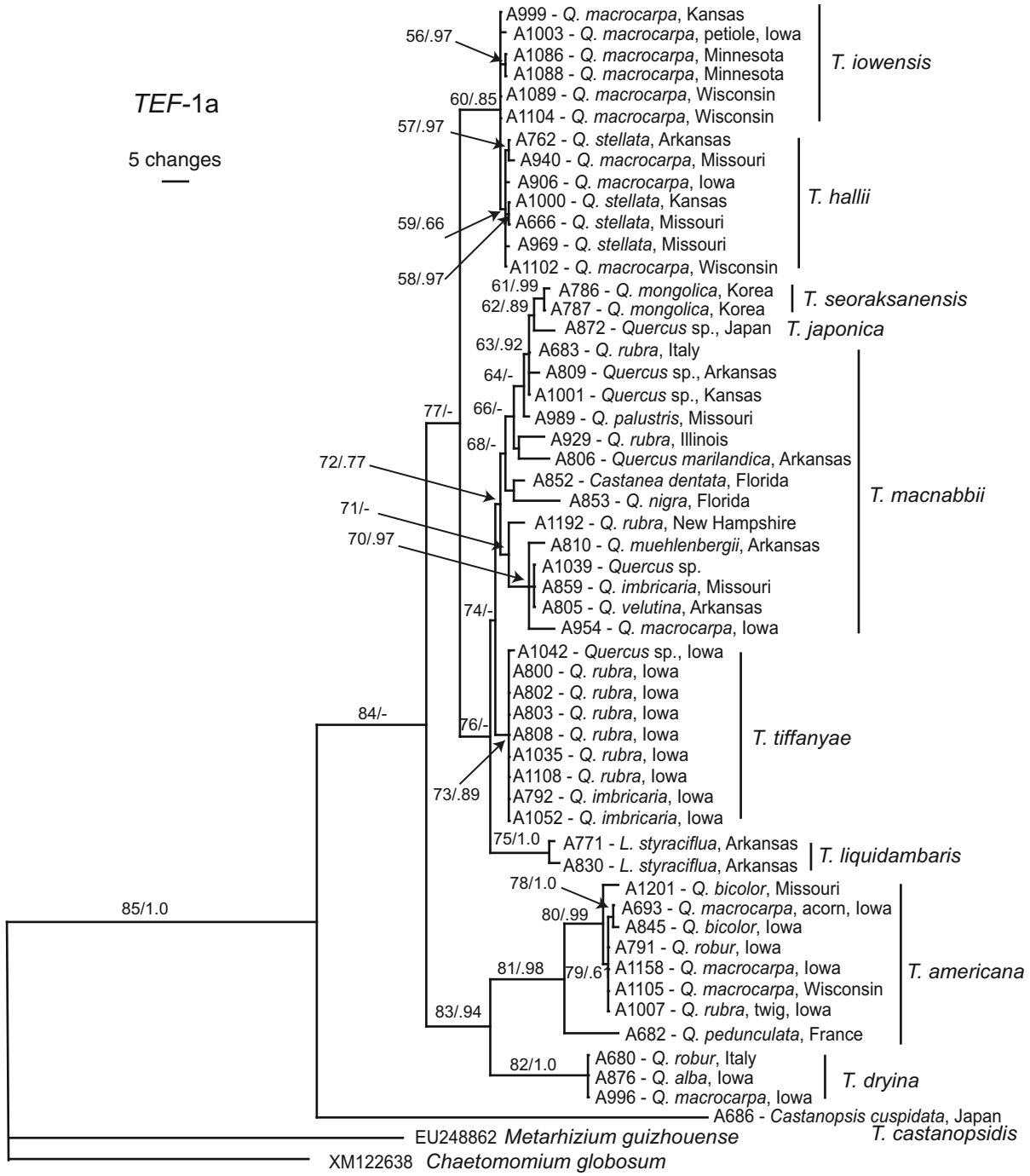

Fig. 5 One of 23 most parsimonious trees of *Tubakia* spp. using partial sequences of the translocation elongation factor 1α (*TEF*-1α). The tree is unrooted, but *Chaetomium globosum* and *Metarhizium guizhouense* are not in the Diaporthales. Bootstrap values (greater than 50%, 1000 replications)/posterior probability estimates are given above the branches

at 10 days, sometimes with dark cell masses on surface or subsurface. *Conidiophores* rare to abundant, short, hyaline, aggregated (sporodochia) on agar surface, producing conidia in dark brown to black, wet masses. *Conidia* hyaline, becoming light brown, thick-walled, aseptate, ellipsoidal to obovate, 12.5–16.5

(17) × 5.3–7.5 µm (mean 14.5 × 6.2 µm). *Microconidia* not seen on agar media but produced from scutella developing on autoclaved pieces of leaves of *Q. macrocarpa* placed on MEA, hyaline, aseptate, fusiform, 3.5–7.5 × 1.0–2.5 µm.

Holotype: USA, Missouri, Kirbyville, on leaf of *Quercus stellata*, 2 Sep 2008, D. Brandt (Holotype, *ISC 453286*; ex-type strain, *CBS 129013 = A666*).

Other material examined: USA, Arkansas, Bella Vista, leaf of *Q. stellata*, 27 Aug 2009, *D. McNew*, N36°46′43″W94°29′39″, (*ISC 453288*; strain *CBS 129015 = A762*); Iowa, Marion, on leaf of *Q. macrocarpa*, 25 Aug 2010, *D. McNew* (*ISC 453287*; strain *CBS 137348 = A949*); Wisconsin, Lafayette County, leaves of *Q. macrocarpa*, 23 Sep 2011, *M. Guthmiller*, N42°78′06″W90°00′01″, (*ISC 453289*; strain *A1102*).

Comments: This species was called *Tubakia* sp. B in earlier publications (Harrington and McNew 2016; Harrington et al. 2012). It is similar in DNA sequences and morphology to *T. iowensis*. However, the two species differ in both ITS and *TEF-1α* sequences; in analysis of the combined dataset, the two species form monophyletic sister groups and appear to have recently diverged. In contrast to *T. iowensis*, *T. hallii* has not been associated with bur oak blight (Harrington et al. 2012) and appears to have broader host and geographic ranges. In contrast to *T. iowensis*, no crustose pycnothyria of *T. hallii* have been seen on the petioles of overwintering leaves. Radiate scutella tend to be larger in *T. hallii* (up to 155 µm) than in *T. iowensis* (up to 110 µm). In culture on MYEA *T. hallii* tends to produce conidia, but *T. iowensis* does so rarely. Whereas *T. iowensis* is mostly restricted to *Q. macrocarpa* and causes necrosis of leaf veins, *T. hallii* has been associated with both leaf spots and necrotic leaf veins of numerous members of *Quercus* sect. *Quercus*: *Q. alba, Q. bicolor*, *Q. macrocarpa*, *Q. muehlenbergii*, and *Q. stellata* in Wisconsin, Illinois, Iowa, Missouri, Kansas and Arkansas.

Tubakia macnabbii T.C. Harr. and McNew, sp. nov., Fig. 1d–g, MycoBank No. MB 822392

Etymology: named for H. Sande McNabb, deceased forest pathologist at Iowa State University.

Conidiomata (pycnothyria) superficial, hypophyllous or epiphyllous, on necrotic spots and along mid and lateral leaf veins, radiate scutella (45) 65–135 (178) µm diam, composed of a series of dark brown, thick-walled cells radiating from a central cell, ending in a blunt to acute point. *Sporodochia* with no scutella or poorly developed scutella, epiphyllous or hypophyllous on necrotic veins and tissue, light to dark brown due to mass of conidia. *Conidiophores* on underside of scutella or on sporodochia. *Conidia* from radiate pycnothyria and sporodochia hyaline, turning light brown with age, smooth to slightly varicose, aseptate, obovoid to ellipsoidal, 9.5–14.5 × (6) 7–9 (10) µm (mean 11.8 × 8.2 µm). Microconidia sterile, hyaline, aseptate, fusiform, 3.5–9 (11.5) × 1–3 µm, from small, radiate pycnothyria, alone or along with macroconidia. *Crustose conidiomata* (pycnothyria) hypophyllous or epiphyllous on necrotic mid and lateral veins of late–season, current years leaves and overwintering leaves still hanging from tree, black, pulvinate, irregularly shaped, 0.7–1.5 mm diam, single or grouped, covered with thick-walled cells, breaking open with swelling when wet. *Conidia* hyaline to light brown, aseptate, ellipsoidal to obovate to irregular in shape, (8) 10–15 (19) × (6) 6.5–8 (9) µm (mean 13 × 7.1).

Colonies with optimal growth on MYEA at 25 °C, diameter 50–65 mm after 7 days, creamy-white aerial mycelium, smooth to scalloped at edge, may develop concentric rings of dense mycelium, underside golden yellow to slightly darker with age, sometimes with dark cell masses on surface or subsurface. *Conidiophores* rare to abundant, short, hyaline, sometimes aggregated (sporodochia) on agar surface. *Conidia* hyaline to dark brown, thick-walled, smooth to slightly varicose, aseptate, obovoid to ellipsoidal, 8.5–15 × 5.5–10 µm.

Holotype: USA, Missouri, Jackson County, leaves of *Quercus palustris*, Sep 2010, *D. Brandt,* (*ISC 453290*; ex-type strain *CBS 137349 = A989*).

Other material examined: USA, Arkansas, Bella Vista, leaf of *Q. muehlenbergii*, 27 Aug 2009, *D. McNew* (*ISC 453291*, strain *CBS 137346 = A810*); California, Los Osos, twig of *Q. agrifolia*, 27 April 2012, *S. Latham*, (strain *A1177*); Florida, Alachua County, leaf of *Castanea* 'Dunstan,' Nov 2009, *T. Schubert* (*ISC 453292*, strain *CBS 137347 = A852*);

Illinois, Nauvoo, leaf of *Q. rubra*, 28 Jul 2010, *D. McNew* (*ISC 453293*, strain *A929*); Iowa, Montgomery County, leaf of *Q. macrocarpa*, 27 Aug 2010, *T. Harrington* (*ISC 453294* strain *A954*); Ohio, Dublin, leaf of *Q. rubra*, Mar 2014, *T. Hollowell* (*ISC 453295*, strain *A1258*); ITALY, near Rome, isolated from overwintered leaf of *Q. rubra,* Dec 1993, *A. Belisario* (strain *CBS 639.93* = *A683*); ITALY, Unknown, dead leaf, Jul 1989, *H. A. van der Aa* (strain *CBS 387.89* = *A868*).

Comments: This was the most commonly encountered *Tubakia* species in the eastern USA, where it appears to be indigenous and widespread. It was reported as *Tubakia* sp. D in earlier work (Harrington and McNew 2016) and has been found primarily on *Quercus* sect. *Lobatae*. We have examined material or isolates from *Quercus* in Kansas, Missouri, Oklahoma, Arkansas, Louisiana, Florida, Maryland, New Hampshire, Ohio, Illinois, Wisconsin, Minnesota, Iowa and California. Oak hosts in eastern USA include *Q. alba*, *Q. hemisphaerica*, *Q. imbricaria*, *Q kelloggii*, *Q. laurifolia*, *Q. macrocarpa*, *Q. marilandica*, *Q. muehlenbergii*, *Q. nigra*, *Q. palustris*, *Q. rubra*, *Q. stellata, Q. velutina*, and *Q virginiana*. We have also examined material from *Castanea* spp. in Florida and Iowa. Although no species of *Tubakia* had been identified west of the Great Plains, California cultures and specimens of *T. macnabbii* from leaves and twigs of *Q. agrifolia* (San Luis Obispo County), *Q. wislizenii* and *Q. kelloggii* (Richland and Shasta Counties), and *Lithocarpus densiflorus* (tanoak) from Santa Clara County were sent to us by S. Latham.

Two CBS cultures from *Q. rubra* (sect. *Lobatae*) leaves collected in a nursery near Rome, Italy were also identified as *T. macnabbii*, but it is unlikely that the fungus is native to Europe because members of sect. *Lobatae* are not native there, and this is the only report of a *Tubakia* sp. on sect. *Lobatae* outside of North America (Harrington et al. 2012). The fungus may have been transported to the nursery on acorns collected from eastern USA. A sexual state, *Dicarpella dryina*, was reported in a leaf of *Q. rubra* collected from the same nursey (Belisario 1991), but as discussed later, it is not thought that the perithecium was actually that of a *Tubakia* sp.

Tubakia macnabbii is distinguished from *T. dryina* by the production of crustose pycnothyria on vein and leaf tissue, rather than twigs (Harrington et al. 2012). The conidia from crustose pycnothyria of *T. macnabbii* are larger, and cultures lack the concentric rings typical of *T. dryina*. The conidia of *T. macnabbii* from radiate scutella are slightly smaller than those of *T. hallii* and *T. iowensis*, which are found on species in *Quercus* sect. *Quercus*. Unlike *T. iowensis*, *T. macnabbii* readily produces conidia in culture.

Sequence analyses suggest that there is substantial genetic variation in *T. macnabbii*, and there may be other cryptic species in the group. Surprisingly, two Asian species, *T. japonica* and *T. seoraksanensis*, form a monophyletic group within *T. macnabbii* according to ITS and *TEF-1α* analyses (Figs. 4, 5), but the Asian species are readily distinguished from the American *T. macnabbii* by their larger conidia. Further phylogenetic analyses are needed to resolve this anomaly. With the Asian species excluded and the ITS and *TEF-1α* sequences combined, isolates from the red oak group form two sister clades: *T. macnabbii* and the following new species. Two isolates of *Tubakia* from sweetgum in Arkansas, which appear to represent *Leptothyrella liquidambaris* (Tehon and Stout 1929), are sister to the red oak species (Fig. 5), and the sweet gum isolates differ from those of *T. macnabbii* in culture morphology. Two other species with similar pycnothyria and conidia were described on other host families, but no cultures or DNA were available, and these will be presented as new combinations in *Tubakia*. A second species on red oaks has slightly larger conidia and a more limited geographic range, and it is described below.

Tubakia tiffanyae T.C. Harr. and McNew, sp. nov., Fig. 1h–m, MycoBank No. MB 822393

Etymology: named for Lois H. Tiffany, deceased mycologist at Iowa State University.

Conidiomata (pycnothyria) superficial, hypophyllous or epiphyllous, on circular leaf spots or along necrotic leaf veins, radiate scutella (40) 60–150 µm diam, composed of a series of dark brown, thick-walled cells originating from a central cell, ending in a blunt to acute point. *Sporodochia* with no scutella or poorly developed scutella epiphyllous or hypophyllous on necrotic vein tissue, light to dark brown due to masses of conidia. *Conidiophores* on underside of scutella or on sporodochia. *Conidia* hyaline, turning light brown with age, smooth to slightly varicose,

aseptate, obovoid to ovoid (9) 10–15.5 × (8) 8.5–11.5 (12) µm (mean 12.9 × 9.6 µm). *Microconidia* sterile, hyaline, aseptate, fusiform 3–8 × 1–2.5 µm may develop from small, pycnothyria, alone or along with macroconidia.

Crustose conidiomata (pycnothyria) developing in late summer to fall on underside of necrotic veins and found on overwintering leaves still hanging from twigs, erumpent, brown to black, irregularly shaped, 80–580 µm diam, single to grouped, covered with dark, thick-walled cells that break open in fissures due to swelling when wet. *Conidia* hyaline to brown, ellipsoidal to obovate to irregularly shaped, aseptate, (11) 13–18 (20) × (6) 7–9.5 µm (mean 15.2 × 7.9 µm).

Colonies with optimal growth on MYEA at 25 °C, 40–58 mm diam after 7 days, creamy white aerial mycelium, smooth to scalloped at edge, developing concentric rings of dense mycelium, underside golden yellow to slightly darker with age, sometimes with dark cell masses on surface or subsurface. *Conidiophores* rare to abundant, short, hyaline, sometimes aggregated (sporodochia) on agar surface. *Conidia* hyaline to dark brown, thick-walled, smooth to slightly varicose, aseptate, obovoid to ellipsoidal, 10–16 × 7–11 µm.

Holotype: USA, Iowa, Ames, leaf of *Quercus rubra*, 5 Sep 2009, *T. Harrington*, N42°04′47″W93°65′22″, (*ISC 453296*, ex-holotype strain *CBS 137345 = A803*).

Other material examined: USA, Iowa, Ames, leaf of *Q. imbricaria*, 27 Jul 2011, *D. McNew*, N42°29′99″W93°64′45″, (*ISC 453297*, strain *CBS 137352 = A1052*); leaf of *Q. rubra*, Mar 2014, *D. McNew*, N42°02′87″W93°63′21″, (*ISC 453298*); Iowa County, leaf of *Q. rubra*, 29 Jul 2011 (*ISC 453299*, strain *CBS 137351 = A1042*); Hardin County, Steamboat Rock, leaf of *Q. ellipsoidalis*, 9 Sep 1913, *J. P. Anderson* (*ISC 319897*); Minnesota, Washington County, leaf of *Q. rubra*, Sep 2013, *J. Pokorny* (*ISC 453300*, strain *A1252*).

Comments: This species is closely related to *T. macnabbii* but has wider conidia than any of the other American *Tubakia* spp. It was referred to as *Tubakia* sp. C by Harrington and McNew (2016), where it was noted that affected leaves usually show characteristic circular spots with light-coloured centres (Fig. 1h) in addition to the veinal necrosis more typical of symptoms caused by the other *Tubakia* spp. It has been identified in Iowa and Minnesota on leaves of *Q. ellipsoidalis, Q. imbricaria*, and *Q. rubra.*

New Combinations in *Tubakia*

Tubakia americana (Höhn.) T.C. Harr. and McNew, comb. nov., Fig. 2, MycoBank No. MB 822395

≡ *Actinopelte americana* Höhn., Mitt. Bot. Inst. Tech. Hoechst. Wein 2:68. 1925, MB 256634

Conidiomata (pycnothyria) with radiate scutella epiphyllous or hypophyllous on leaf spots or along necrotic leaf veins of *Quercus*. *Scutella* convex, 40–110 µm diam, of brown, thick-walled cells radiating from a central point, ending in a 2–3 µm blunt tip. *Sporodochia* hypophyllous under leaf spots or along necrotic veins, irregular in shape, sometimes with small, poorly developed scutella. *Conidiophores* short, forming under developing scutellum or on sporodochia. *Conidia* of pycnothyria and sporodochia thick-walled, finely varicose to smooth, initially hyaline, later light brown, obovoid to ellipsoidal, aseptate, 9–13 (14) × 6–8 (8.5) µm (mean 11.1 × 6.9 µm). *Microconidia* sometimes produced from same pycnothyrium as macroconidia or from smaller pycnothyria, hyaline, fusiform, curved, aseptate, (3.5) 4–7 × 2–3 µm. *Crustose conidiomata* (pycnothyria) forming in winter or spring, erumpent on twigs or inside of acorn caps, black, irregular in shape, 150–500 µm diam. *Scutella* of thick-walled cells, *textura angularis*, dehiscing marginally or by irregular fissures. *Conidiophores* lining inside of crustose pycnothyrium. *Conidia* thick walled, hyaline to light brown, obovoid to ellipsoidal, aseptate, 9.5–14 × 5.5–7.5 µm.

Colonies with optimal growth at 25 °C on MYEA, attaining a diameter of 56–64 mm after 7 days, initially with a distinct ring of dense aerial mycelium, later developing concentric rings of fluffy white to grey aerial mycelium with wet conidial masses that are initially hyaline, becoming olive green then black, coalescing into large areas. *Conidia* in culture abundant, thick walled, smooth to finely varicose, hyaline to dark brown, obovoid to ellipsoidal, aseptate, 9–15 (16.5) × (4) 4.5–7.5 (8) µm.

Lectotype: USA, New Jersey, Newfield, leaf of *Quercus coccinea*, Aug 1883, Ellis and Everhart (designated here as *FC 286* NY ex McClatchie Herbarium, MBT 379548; *Isolectotypes*: Ellis and Everhart FC 286 = *ISC 453285*; Ellis and Everhart FC 286, NY ex Mr. F. S. Earle Herbarium; Ellis and Everhart FC 286, NY Ellis collection).

Other material examined: USA, Iowa, Ames, Brookside Park, leaves *of Q. macrocarpa*, 12 Oct 2011, *D. McNew* N42°03′10″W93°63′24″, (*ISC 453301*, strain *CBS 137353* = *A1158*); from leaf spot on *Q. macrocarpa*, Jun 2009, *D. McNew* (strain *CBS 129014* = *A749*); acorn cup of *Q. macrocarpa,* 19 May 2009, *D. McNew,* N42°02′98″W93°63′21″, (*ISC 453302*, strain *A693*); twig of *Q. rubra,* 6 Apr 2011, *D. McNew,* N42°02′95″W93°63′22″, (*ISC 453304*, strain *CBS 137350* = *A1007*); Missouri, Lewis County, from leaf of *Q. bicolor,* 5 Aug 2012, *D. McNew* (*ISC 453305*, strain *A1201*); Wisconsin, Green County, from leaf of *Q. macrocarpa*, 23 Sep 2011, *M. Guthmiller*, N42°78′69″W89°63′32″, (*ISC453306*, strain *A1105*); Racine, from leaf of *Q. rubra*, 14 Jul 1887, *J. J. Davis* (Ellis collection NY ex Herb. J. J. Davis). FRANCE, Seignosse le Penon, from *Cynips kollari* gall on *Q. pedunculata* (= *Q. robur*) leaf, *H.A. van der Aa* (strain *CBS 335.86* = *A682*).

Comments: Höhnel (1925) described *Actinopelte americana* from specimen FC 286 in Ellis and Everhart's Fungi Columbiani, distinguishing this species from the European *A. dryina* Sacc. by its smaller conidia. The particular FC 286 specimen that Höhnel examined appears to be lost (personal communication, Farlow Herbarium). A copy of Fungi Columbiani FC 286 from the McClatchie Herbarium (NY), which we designate as the lectotype, shows pycnothyria of two *Tubakia* spp. associated with different necrotic spots. One of these *Tubakia* species has relatively small conidia, as described by Höhnel (1925) for *A. americana*, and the tips of the setae of the scutella are blunt (Fig. 2a, b). The second type of pycnothyria on the lectotype material has scutella with acute tips and larger conidia (Fig. 2c), matching our concept of *T. macnabbii*. The FC 286 material from four other sets of Fungi Columbiani (in NY and ISC) each contained leaves with shields of the two *Tubakia* species.

Pycnothyria of the small-spored species were scraped from a leaf spot of the lectotype and used for DNA extraction and PCR using a ITS rDNA primer pair specific to *Tubakia* spp. The PCR product was directly sequenced with the same primers, and the sequence matched that of *Tubakia* sp. A (Harrington and McNew 2016; Harrington et al. 2012), which has been commonly isolated as a twig and leaf endophyte in *Q. macrocarpa* and other *Quercus* spp. in Iowa (Fig. 4). *Tubakia americana* also is associated with leaf spots and necrotic veins on *Q. macrocarpa* in Iowa.

The *T. americana* conidia from pycnothyria with radiate scutella are the narrowest of all the species of *Tubakia* in the USA. Phylogenetic analyses suggest that *T. americana* is closely related to *T. dryina* (Figs. 3, 4, 5), and both species produce crustose pycnothyria on twigs (Harrington et al. 2012). In culture, the mycelium of *T. americana* is much lighter in colour than the dark mycelium of *T. dryina* (Harrington et al. 2012). Species of *Quercus* sect. *Quercus* (*Q. bicolor, Q. macrocarpa* and *Q. robur*) are the primary hosts of *T. americana* in Iowa. However, the fungus also has been found on *Q. rubra*, and the lectotype material was from *Q. coccinea*, also a member of *Quercus* sect. *Lobatae*. Specimens and cultures have been examined from Wisconsin, Illinois, Iowa, Missouri, and New Jersey. Phylogenetic analyses suggest that the Eurasian population of *T. americana* is distinct (Figs. 4, 5), but strain CBS 335.86 from France appears similar to the USA strains of *T. americana*. Boroń and Grad (2016) reported two distinct ITS rDNA sequences from *T. dryina* collections in Poland, and their Hp2 sequence appears to be that of the European *T. americana*.

Tubakia gloeosporioides (Tehon) T.C. Harr. and McNew, comb. nov., MycoBank No. MB 822396

≡ *Actinothyrium gloeosporioides* Tehon, Mycologia 16:136. 1924, MB 221305

Specimens on *Sassafras albidum* from Illinois (holotype ILLS 3311, as well as ILLS 2972, ILLS 3671, ILLS 29748, and ILLS 17547) and New Jersey (as *Leptothyrium dryinum* f. *sassafras*, NY E&E Fungi of New Jersey) have pycnothyria 45–132 µm diam (reported as 50–95 µm diam by Tehon 1924) and conidia 9.5–12.5 × 7–8.5 µm (reported as 11–12 × 6–7.5 µm by Tehon 1924), which are similar

to pycnothyria and conidia of *T. macnabbii*. Without cultures or DNA, it is not possible to distinguish *T. gloeosporioides* from *T. macnabbii*, but the former is probably distinct based on its unique host, which is in the family Lauraceae. Glawe and Crane (1987) reported the holotype ILLS 3311 as being lost and unnecessarily designated a lectotype, which is no longer valid.

Tubakia liquidambaris (Tehon and Stout) T.C. Harr. and McNew, comb. nov., MycoBank No. MB 822396

≡ *Leptothyrella liquidambaris* Tehon and Stout, Mycologia 21:192. 1929, MB 188944

Herbarium specimens on *Liquidambar styraciflua* from Arkansas (ISC 453303), Illinois (holotype ILLS 1445 and ILLS 29743), Maryland (BPI 391888), and Mississippi (BPI 840865) have pycnothyria 60–138 µm diam (91–112 µm diam by Tehon and Stout 1929) and conidia 9–14 × 6.5–9 µm (8.4–10.2 × 6–6.8 µm by Tehon and Stout 1929), similar to those of *T. macnabbii*. Cultures from necrotic spots on leaves of *L. styraciflua* in Bella Vista, Arkansas (CBS 139744 = A771 and CBS 139745 = A830, both from ISC 453303) are distinct from cultures of *T. macnabbii* in having slower growth, a flat surface, and abundant production of conidia formed from sporodochia in concentric rings. In addition to the different host, differences in DNA sequences (Figs. 4, 5) of the two Arkansas strains also support recognition of this species as distinct from *T. macnabbii*.

Tubakia nyssae (Tehon) T.C. Harr. and McNew, comb. nov., MycoBank No. MB 822398

≡ *Pirostoma nyssae* Tehon, Mycologia 16:137. 1924, MB 196764

Specimens from Illinois (holotype ILLS 2940 and ILLS 8871) and Virginia (BPI 391890 = ILLS 41874 and BPI 391891) are from *Nyssa sylvatica*, which is outside of the Fagaceae, suggesting that this is a distinct species of *Tubakia*. Morphologically *T. nyssae* is similar to *T. macnabbii*, with pycnothyria in discreet leaf spots, the scutella 70–116 µm diam (60–95 µm diam according to Tehon 1924) with acute tips. Conidia are hyaline to light brown, ellipsoid to subglobose, 10–14 × 7–9 µm (12 × 7 µm according to Tehon 1924). The designation of a lectotype by Glawe and Crane (1987) was unnecessary as the holotype (ILLS 2940) is still available.

Tubakia stellata (Farr) T.C. Harr. and McNew, comb. nov., MycoBank No. MB 822399

≡ *Actinopelte stellata* Farr, Mycopathologia 31:63. 1967, MB 325826

Known only from the holotype (BPI 391941) on leaves of *Byrsonima coriacea* (= *B. spicata*) in Pará, Brazil (Farr 1967), *T. stellata* is the only species of *Tubakia*, other than *T. dryina*, from the Southern Hemisphere. This is also the only record of a *Tubakia* from the tropics and the tropical host family (Malpighiaceae). The scutella of the holotype are made up of unbranching cells that expand from the centre and then abruptly narrow to an acute tip. The scutella are reddish brown, 47–70 µm diam, with conidia formed underneath, hyaline, aseptate, globose to irregularly globose, 6.5–8 µm diam. No microconidia were seen. The shape of the scutella and conidial development sufficiently resemble other *Tubakia* species to warrant the new combination. No cultures or DNA sequences are available.

Tubakia supraseptata T.C. Harr. and McNew, comb nov., MycoBank No. MB 822400

≡ *Apiognomonia supraseptata* Kaneko and Kobayashi, Trans. Mycol. Soc. Japan 25:11. 1984, MB 105925

Kaneko and Kobayashi (1984) isolated an endophyte from *Quercus* that formed perithecia but no conidia in culture. Aside from the production of perithecia, the illustrated culture had a mycelial morphology typical of *Tubakia*. The deposited LSU rDNA sequence from a culture taken from the holotype (strain CBS 632.92) clearly places this fungus within the genus *Tubakia* as defined herein (Fig. 3). As pointed out by Kaneko and Kobayashi (1984), *Apiognomonia* Höhn. has two celled ascospores in which the top cell is larger than the bottom cell, but in *A. supraseptata* the top cell is smaller. Also, *Apiognomonia* spp. have Discula-like asexual states (Sogonov et al. 2007).

Based on Kaneko and Kobayashi's (1984) LSU sequence obtained from the ex-holotype strain, *T. supraseptata* is the only *Tubakia* sp. with a clearly

demonstrated sexual state. Although many species of *Tubakia* produce small conidia that do not germinate and appear to be spermatia, no clear connection between a *Tubakia* asexual state and a perithecial state had been made. As discussed below under doubtful and excluded species, the connection of *Dicarpella dryina* (with one-celled ascospores) to *Tubakia dryina* (Belisario 1991) is questioned.

Previously recognized species of *Tubakia*

Tubakia castinopsidis (Yokoyama and Tubaki) B. Sutton, Trans Brit. Mycol. Soc. 60:165. 1973, MB 530653

≡ *Actinopelte castanopsidis* Yokoyama andTubaki, IFO Res. Comm. 5:50. 1971, MB 308270

Yokoyama and Tubaki (1971) described *T. castinopsidis* from leaves of *Castanopsis cuspidata* in Japan, scutellum 100–150 µm diam, conidia hyaline, oblong to cylindrical, 12–13 × 7–8 µm. The culture from the holotype (CBS 189.71 = IFO-9263) does not produce conidia or pycnothyria, but DNA sequences generated from this culture are clearly placed in *Tubakia* (Figs. 3, 4, 5).

Tubakia chinensis U. Braun, S. Bien and Hantsch, Schlechtendalia 28:23. 2014, MB 809784

This species was described from leafspots on *Castanea henryi* in China as having scutella up to 200 µm diam and conidia 25–40 × 20–30 µm (Braun et al. 2014). The measurements are similar to those of *T. japonica*, which is found in Japan on *Castanea*. No culture or DNA sequence was available at the time of this study.

Tubakia dryina (Sacc.) B. Sutton, Trans Brit. Mycol. Soc. 60:165. 1973, MB 325072

≡ *Leptothyrium dryinum* Sacc., Michelia 1:201. 1878, MB 24303

≡ *Actinopelte dryina* (Sacc.) Höhn., Mit. Bot. Inst. Tech. Hochsch. Wein. 2:69. 1925, MB 263534

As redefined by Harrington et al. (2012), *T. dryina* has radiate scutella, 60–125 µm diam, producing hyaline to light-brown, thick-walled conidia, 10–15 × 7–10.5 µm. It can apparently kill small twigs of *Quercus* spp., on which it produces crustose pycnothyria (Harrington et al. 2012; Holdenreider and Kowalski 1989). Conidia from the crustose pycnothyria are 10–16 × 6–7.5 µm, which are narrower than those from radiate pycnothyria. The DNA sequences of *T. dryina* are unique but similar to those of *T. americana* and an unidentified species (*Tubakia* sp. E) from eastern USA (Fig. 4). *Tubakia dryina* appears to be primarily a European species and found east to Iran (Zahedi et al. 2011), and it commonly causes a leaf spot on *Q. alba* in the eastern USA (Harrington and McNew 2016; Harrington et al. 2012). It has been found on the European species *Q. robur* in various locations around the world, including New Zealand, which is the only report of *Tubakia* in the Southern hemisphere besides *Tubakia stellata* in Brazil.

Belisario (1991) reported perithecia of a new species, *Dicarpella dryina*, as the sexual stage of *T. dryina.* The perithecia were in leaves of *Q. rubra* collected in a nursery near Rome, Italy affected by an introduced strain of *T. macnabbii*. However, the connection of the *Dicarpella* to either *T. dryina* or *T. macnabbii* is questioned.

Tubakia iowensis T.C. Harr. and McNew, Mycologia 104:86. 2012, MB 561043

Compared to *T. hallii*, *T. iowensis* has a narrower geographic range and a much narrower host range (Harrington and McNew 2016; Harrington et al. 2012), but the two species are difficult to distinguish morphologically and phylogenetically (Figs. 3, 4, 5). Pycnothyria of *T. iowensis* with radiate scutella are somewhat smaller (40–110 µm diam) than those of *T. hallii* (45–155 µm diam). The crustose pycnothyria of *T. iowensis* on the petioles of leaves overwintering on twigs of bur oak trees is considered the most critical feature of bur oak blight, which occurs only on *Q. macrocarpa* var. *oliviformis*. Veinal necrosis caused by *T. iowensis* on *Q. bicolor* leaves in proximity of bur oak trees with bur oak blight has been seen, but it is not common. *T. iowensis* has been identified in Iowa and each of the states surrounding Iowa, and in northeastern Kansas (Harrington and McNew 2016).

Tubakia japonica (Sacc) B. Sutton, Trans Brit. Mycol. Soc. 60:165. 1973, MB 325073

≡ *Actinopelte japonica* Sacc., Annales Mycologici 11:315. 1913, MB 247526

This is the type species for the genus and has exceptionally large pycnothyria and conidia. Only *T. chinensis* is reported to have such large conidia (Braun et al. 2014). Specimens of *T. japonica* (on leaf spots on *Castanea* spp. in Japan, *holotype*: BPI 391948 = Sydow's Fungi Exotica Exsiccate no. 526; and BPI 391940) have large scutella (75–210 µm diam) and very large conidia (30–48 × 28–38 µm). These measurements fall within Saccardo's (1913) original description of *A. japonica* as having large asci (conidia), 35–42 × 28–33 µm and superficial perithecia (pycnothyria) 220–240 µm diam. Yokoyama and Tubaki (1971) reported microconidia 5–10 × 1–2 µm. In 2014, Schwarze deposited specimens of *A. japonica* on *Quercus* spp. from New Jersey, USA, but his collections (BPI 391916, BPI 391923 and BPI 391899) have conidia 10–15 × 5–7.5 µm, similar to those of *T. americana*. Another collection (BPI 391900) of *A. japonica* from West Virginia, USA has conidia 10–14 × 7.5–10 µm, similar to those of *T. macnabbii*.

The DNA sequences of a culture of *T. japonica* from Japan (CBS 191.71 = A872) are similar to those of *T. seoraksanensis* from Korea and *T. macnabbii* of the USA (Figs. 4, 5), but these species are morphologically distinct.

Tubakia rubra (Yokoyama and Tubaki) B. Sutton, Trans Brit. Mycol. Soc. 60:165. 1973, MB 325074

≡ *Actinopelte rubra* Yokoyama and Tubaki, IFO Res. Comm. 5:47. 1971, MB 308271

The ex-holotype culture (CBS 192.71 = IFO-9271) from *Q. phillyraeoides* in Japan has a distinctive red pigmentation, as described by Yokoyama and Tubaki (1971), but the culture no longer sporulates. Yokoyama and Tubaki (1971) reported that the fungus does not produce symptoms on leaves, but when green or senescent leaves are incubated in a moist chamber they readily produce red pycnothyria, 60–110 µm diam, with conidia hyaline to yellow orange, ellipsoidal to ovate, 12–15 × 10–13 µm. Microconidia are hyaline 8–10 × 1 µm. *Tubakia rubra*, *T. subglobosa* and *T. castanopsidis* have ITS rDNA sequences that differ significantly from the other *Tubakia* spp. (Figs. 3, 4).

Tubakia seoraksanensis H.Y. Yun, Mycotaxon 115:371. 2011, MB 519212

This species causes leaf spots and veinal necrosis on leaves of *Q. mongolica* in Korea (Yun and Rossman 2011). Specimens examined include the holotype (BPI 880799, ex holotype strain CBS 127490 = A785) and the isotype (BPI 880798, ex isotype strain CBS 127492 = A787). The scutella are 90–128 µm diam with hyaline to light brown, with thick-walled conidia 12–19 × 9–14 µm (scutella 90–160 µm diam and conidia 13–25 × 10–15 µm according to Yun and Rossman, 2011). The conidia are larger than all other described *Tubakia* spp. except *T. japonica* and *T. chinensis*, and the DNA sequences of *T. seoraksanensis* and *T. japonica* are similar (Figs. 4, 5).

Tubakia subglobosa (Yokoyama and Tubaki) B. Sutton, Trans Brit. Mycol. Soc. 60:165. 1973, MB 325075

≡ *Actinopelte subglobosa* Yokoyama and Tubaki, IFO Res. Comm. 5:49. 1971, MB 308272

This species was originally described as having subglobose to globose conidia 10–13 × 9–11 µm and cultures with a distinctive cinnamon-red colour (Yokoyama and Tubaki 1971). A culture from the holotype (CBS 193.71 = IFO-8931 = A685) from leaves of *Q. glauca* in Japan no longer produces conidia or the distinctive colour, but its ITS rDNA sequence is unique and groups *T. subglobosa* with two other Japanese species, *T. castanopsidis* and *T. rubra* (Figs. 3, 4). *Tubakia subglobosa* was recently reported from Korea (Yun and Kim 2017).

Doubtful and excluded species

Leptothyrium castaneicola Ellis and Ev., Jour. Myc. 4:103. 1888. nomen dubium, MB 187659

This species was described from leaves of *Castanea* as having scutella-like perithecia 115–150 µm diam on the green parts of the leaf (Ellis and Everhart 1888). No spore measurements were given. Glawe and Crane (1987) found no fruiting structures in the probable holotype of *L. castaneicola* (NY specimen 009278112 from the Ellis collection), and we also could find no *Tubakia*-like fruiting structures in that specimen.

Dicarpella dryina Belisario and Barr, Mycotaxon 41:154. 1991, MB 128110

Belisario (1991) found perithecia and ascospores of a fungus on overwintering leaves of *Quercus rubra* in

a nursery in central Italy, and a single-ascospore culture from one of the leaves was thought to be conspecific with *T. dryina*. However, the *Tubakia* associated with leaf spots on *Q. rubra* in the same nursery (CBS 387.89) and Belisario's deposit (CBS 639.93) are *T. macnabbii*, not *T. dryina* (Figs. 3, 4, 5). *T. macnabbii* will produce conidia in abundance on overwintered leaves of *Q. rubra*, so it is possible that conidia of a *Tubakia* contaminated the putative single-ascospore isolation deposited as *D. dryina*.

Dicarpella Syd. and P. Syd. has one-celled ascospores (Reid and Dowsett 1990) instead of the two-celled ascospores found in *T. supraseptata*, *Apiognomonia* (Sogonov et al. 2007), and other sexual relatives of *Tubakia* spp. based on LSU sequences (Fig. 3). If the perithecia described by Belisario (1991) were those of a *Tubakia* sp., *Dicarpella* Syd. and P. Syd. (1921) is an illegitimate homonym of *Dicarpella* Bory (1824), and thus, *D. dryina* is an illegitimate combination. Also, the basionym of *D. dryina* could not be transferred to *Tubakia* as that combination is occupied by *Tubakia dryina* (Sacc.) B. Sutton.

Discussion

The genus *Tubakia* is well defined by phylogenetic analyses and its unique pycnothyria with radiate scutella. The studied species in the USA also form a second type of pycnothyrium, with a crustose covering rather than a scutellum of radiating setae. The two fruiting structures appear to be evolving in parallel, as the conidia size and shape differ slightly in each of the studied species. Microconidia that do not germinate also may be produced from under the radiate scutella, and these spermatia-like spores suggest that most *Tubakia* species form ascomata. However, only *T. supreseptata* is known to produce ascomata, and the cultures of this species that produced the ascomata did not produce pycnothyria or another conidial state (Kaneko and Kobayashi 1984). The ascospores of *T. supreseptata* are two-celled, as are ascospores of the relatives of *Tubakia* based on LSU rDNA sequence analysis. If *D. dryina* Belisario and Barr is the sexual state of a *Tubakia*, as suggested (Belisario 1991), it would be the sexual state of *T. macnabbii*, not *T. dryina*.

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. Many *Tubakia* spp. are readily isolated from green leaves of Fagaceae, and all of the species may have an endophytic phase of growth. Pycnothyria may appear on leaf or twig tissue killed by other agents, but most of the species appear capable of causing necrotic leaf spots or necrosis along leaf veins (Harrington and McNew 2016). At least one species, *T. iowensis*, is capable of causing a serious leaf disease, bur oak blight (Harrington et al. 2012). *T. macnabbii* and *T. hallii* also have been associated with significant defoliation of *Quercus* spp. (Harrington and McNew 2016), and *T. dryina* may cause twig death of *Quercus* in Europe (Holdenreider and Kowalski 1989). As endophytes, it appears that some of the species can have broad host ranges.

Most of the recognized species of *Tubakia* in the USA differ only slightly from other species in sizes of conidia and pycnothyria. However, differences in leaf symptoms and relatively narrow host ranges distinguish some species. Three of the new combinations that we made were based on species described from hosts outside the Fagaceae, but the pycnothyria and conidia on leaves fit our concept of *T. macnabbii*. Cultures of *T. liquidambaris* were clearly distinct from those of *T. macnabbii*, but *T. gloeosporioides* and *T. nyssae* warrant further study. On the other hand, it appears likely that there are other cryptic species of *Tubakia* awaiting description.

Acknowledgements The authors are grateful to the numerous individuals who provided specimens and cultures for this study. The provision of materials by Suzanne Latham (California Department of Food and Agriculture) is especially appreciated.

Funding The work upon which this publication is based was funded through grants awarded by the Northeastern Area State and Private Forestry, U. S. Forest Service.

Conflict of interest Thomas C. Harrington and Douglas L. McNew declare that they have no conflict of interests.

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