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Evolutionary relationships of the cup-fungus genus *Peziza* and Pezizaceae inferred from multiple nuclear genes: RPB2, β-tubulin, and LSU rDNA

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Abstract

To provide a robust phylogeny of Pezizaceae, partial sequences from two nuclear protein-coding genes, RPB2 (encoding the second largest subunit of RNA polymerase II) and β-tubulin, were obtained from 69 and 72 specimens, respectively, to analyze with nuclear ribosomal large subunit RNA gene sequences (LSU). The three-gene data set includes 32 species of *Peziza*, and 27 species from nine additional epigeous and six hypogeous (truffle) pezizaceous genera. Analyses of the combined LSU, RPB2, and β-tubulin data set using parsimony, maximum likelihood, and Bayesian approaches identify 14 fine-scale lineages within Pezizaceae. Species of *Peziza* occur in eight of the lineages, spread among other genera of the family, confirming the non-monophyly of the genus. Although parsimony analyses of the three-gene data set produced a nearly completely resolved strict consensus tree, with increased confidence, relationships between the lineages are still resolved with mostly weak bootstrap support. Bayesian analyses of the three-gene data, however, show support for several more inclusive clades, mostly congruent with Bayesian analyses of RPB2. No strongly supported incongruence was found among phylogenies derived from the separate LSU, RPB2, and β-tubulin data sets. The RPB2 region appeared to be the most informative single gene region based on resolution and clade support, and accounts for the greatest number of potentially parsimony informative characters within the combined data set, followed by the LSU and the β-tubulin region. The results indicate that third codon positions in β-tubulin are saturated, especially for sites that provide information about the deeper relationships. Nevertheless, almost all phylogenetic signal in β-tubulin is due to third positions changes, with almost no signal in first and second codons, and contribute phylogenetic information at the "fine-scale" level within the Pezizaceae. The Pezizaceae is supported as monophyletic in analyses of the three-gene data set, but its sister-group relationships is not resolved with support. The results advocate the use of RPB2 as a marker for ascomycete phylogenetics at the inter-generic level, whereas the β-tubulin gene appears less useful.

Keywords: Fungi; Ascomycota; Pezizales; Protein coding genes; Third codon saturation; Combining data; Parsimony, Maximum likelihood and Bayesian inference

1. Introduction

The cup-fungus family Pezizaceae (Pezizales) are filamentous ascomycetes recognized macro-morphologically by the often fleshy, soft, and brittle, cup-shaped fruit bodies (ascomata) that range in size from a few mm to more than 10 cm in diameter. It includes epigeous, semi-hypogeous to hypogeous (truffle) taxa. Ten out of 22 currently accepted genera in the family (Eriksson et al., 2004) are exclusively truffle or truffle-like genera, but several truffle species have also been described in *Peziza* Fr.. A shared derived character, the operculate ascus, characterizes Pezizales. Molecular phylogenetic

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studies show the Pezizales (along with Orbiliaceae) to be the most basal lineage(s) within the Euascomycetes (e.g., Liu et al., 1999; Lumbsch et al., 2000; Platt and Spatafora, 2000), which makes work toward a robust phylogeny of this group of general importance in understanding ascomycete phylogeny and character evolution. The Pezizaceae is chiefly characterized by amyloid asci (asci that turn blue in iodine solutions) a feature only shared with the Ascobolaceae within the Pezizales. The ascospores are uninucleate, usually thin-walled, globose, ellipsoid, or fusiform, hyaline or pale brownish, smooth or with cyanophylic ornaments. The excipulum consists, at least partly, of large isodiametric cells. The trophic status of the species is little known, and although the majority is considered to be saprotrophs, many more are likely to be proven mycorrhizal. Ecologically it covers an extremely broad range of niches, fruiting on all types of soil, dung, and wood. The family is most diverse in temperate zones and arctic-alpine areas, but a few strictly tropical taxa are known. The family includes ca. 200 known species, with *Peziza* as the largest genus, estimated to comprise 84 species (Kirk et al., 2001). No intergeneric classification has ever been proposed for the Pezizaceae, and furthermore, *Peziza* has never been monographed and infrageneric relationships are poorly understood. In addition, species delimitations within *Peziza* continue to be problematic, although many keys (e.g., Dissing, 2000; Häffner, 1995; Hohmeyer, 1986) and descriptions (e.g., Donadini, 1978) of European *Peziza* species exist. Molecular phylogenetic studies (Hansen et al., 2001; Norman and Egger, 1996, 1999) have shown that *Peziza* is not monophyletic. Our previous results (Hansen et al., 2001), based on phylogenetic analyses of partial nuclear ribosomal large subunit RNA gene sequences (LSU), suggest that *Peziza* is composed of at least six distinct lineages, most of which include other genera of the Pezizaceae. Higher-level relationships within the Pezizaceae were however, not resolved or only with low support, using LSU rDNA sequences. To improve phylogenetic inference within Pezizaceae additional characters from alternative loci are needed. In this study, we investigate the utility of two potential phylogenetic markers, β-tubulin and RPB2 (the gene encoding the second largest subunit of RNA polymerase II) for intergeneric-level systematics within cup-forming fungi (Ascomycota).

RNA polymerase II is the key enzyme that transcribes pre-mRNA. The use of RPB2 was recently initiated as a suitable alternative to the commonly used nuclear ribosomal small subunit (SSU rDNA) region in ascomycete molecular systematics (Liu et al., 1999). RPB2 is a ca. 1000 amino acid protein, encoded in an approximately 3.0 kb gene. A favorable attribute of RPB2 is that only a single copy of the gene has (so far) been found in fungi. Different regions of RPB2 have different rates of evolutionary change, and are thus, useful for both species level and deep phylogenetic work, depending on the segment used. The region of RPB2 with the highest evolutionary rate is spanning conserved regions 6–7 (Liu et al., 1999). It has provided phylogenetic information in analyses of ascomycetes at the species level (within *Leotia*, Zhong and Pfister, 2004) using the nucleotides, and between families and orders (Diaporthales, Microascales, and Sordariales) using the amino acids (Zhang and Blackwell, 2001). Here, we evaluate the usefulness of RPB2 to infer intra- and intergeneric phylogenetic relationships within Pezizaceae, using the exonic nucleotides spanning conserved regions 6–11.

β-Tubulin is well characterized in Ascomycota, but has not been used in phylogenetics of cup-fungi. It is a 445–449 amino acid protein, encoded in an approximately 1.8 kb gene with 4–8 introns (May et al., 1987). At the amino acid level partial β-tubulin sequences have recently been used by Landvik et al. (2001) to infer higher-level phylogenetic relationships in the ascomycetes, but their results suggest it is less useful than other genes at this level. At the nucleotide level, β-tubulin has been informative at low taxonomic levels within the ascomycetes (e.g., Jong et al., 2001; O'Donnell et al., 1998; Schoch et al., 2001). β-Tubulin has been determined to be a single copy gene in some genera of the Ascomycota (e.g., Byrd et al., 1990; Neff et al., 1983; Orbach et al., 1986), but two highly divergent paralogs have been reported in others (e.g., May et al., 1987; Panaccione and Hanau, 1990). Within Pezizaceae we are using the nucleotide sequences from two large exons (6–7 in *Aspergillus nidulans* benA, May et al., 1987).

The goals of the present study were: (i) to reconstruct the evolutionary history of *Peziza* and the Pezizaceae, (ii) to explore and compare the utility, at the generic level in ascomycete phylogeny, of partial sequences from the three nuclear genes, LSU rDNA, RPB2, and β-tubulin, (iii) to explore, for the protein coding genes, if separate partitions of first and second vs. third codon positions contain the same phylogenetic information, and if different methods of phylogenetic inference affect the relationships reconstructed for the Pezizaceae.

2. Materials and methods

2.1. Specimens

A data matrix containing 69 unique species of Pezizales and *Neolecta vitellina* was constructed with sequences from LSU rDNA, RPB2, and β-tubulin genes (Table 1). For some species more than one collection was sequenced to verify the sequences of a particular gene and explore the intra-specific variation. Only a single accession was included in the phylogenetic analyses when all intra-specific sequences from different accessions were identical across all loci. Sixty-nine RPB2 and

Table 1
Species examined, sequenced, and used in the molecular phylogenetic study

Species	Collection number (Herbarium)[a] Geographic origin	LSU	β-Tubulin	RPB2
Amylascus tasmanicus (Rodway) Trappe	Trappe 18084 (C, dubl. OSC). Australia	AF335113	AY513297[b]	AY500465[b]
Ascobolus carbonarius P. Karst.	KH 00.008 (C). Denmark	AY500526[b]	AY513298[b]	AY500459[b]
Ascobolus crenulatus P. Karst.	KH.02.005 (C). USA	AY500527[b]	AY513299[b]	AY500462[b]
Ascobolus denudatus Fr.	KS-94-146 (C). Denmark	AY500528[b]	AY513300[b]	AY500460[b]
Boudiera dennisii Dissing & Sivertsen	Rana 81.113 (C). Norway	AY500529[b]	—	AY500508[b,c]
Boudiera tracheia (Gamundí) Dissing & T. Schumach.	Rana 79.049 (C). Norway	AY500530[b]	AY513301[b]	AY500507[b,c]
Byssonectria terrestris (Alb. & Schwein.: Fr.) Pfister	KS-94-4 (C). Denmark	AY500531[b]	AY513302[b]	AY500504[b,c]
Greletia reticulosperma Donadini, Riousset & G. Riousset	Part of isotype (herb. Roy Kristiansen). France	AY500532[b]	AY513303[b]	—
Hapsidomyces venezuelensis Krug. & Jeng.	Dumont et al. VE-4890z (Type TRTC). Venezuela	AY500533[b]	AY513304[b]	—
Hydnotryopsis sp.	Trappe 17231 (C, dubl. OSC). USA	AF335116	AY513305[b]	AY500472[b]
Iodophanus carneus (Pers.) Korf	JHP 00.027 (C). Denmark	AY500534[b]	AY513306[b]	AY500506[b,c]
Iodophanus hyperboreus T. Schumach.	Gr. 83.06 (C). Greenland	AY500535[b]	AY513307[b]	AY500458[b]
Iodowynnea auriformis (Le Gal) Medel, Guzmán & Chacón (1)	CUP-ME566 (CUP). Mexico	AF335117	AY513308[b]	—
Iodowynnea auriformis (2)	18510 PAN (FH). India	AF335118	AY513309[b]	AY500473[b]
Marcelleina persoonii (P. Crouan & H. Crouan) Brumm. (1)	KH 00.007 (C). Denmark	AY500536[b]	AY513310[b]	AY500463[b]
Marcelleina persoonii (2)	TL-5696 (C). Denmark	AY500537[b]	AY513311[b]	AY500464[b]
Marcelleina pseudoanthracina (Donadini) R. Kristiansen & J. Moravec	KH 02.15 (C). Norway	AY500538[b]	AY513312[b]	AY500509[b,c]
Melastiza contorta (Massee & Crossl.) Spooner & Y.J. Yao	KH.01.06 (C). Sweden	AY500539[b]	—	AY500505[b,c]
Morchella elata Fr.	—	U42667[d]	—	AF107810[d]
Neolecta vitellina (Bres.) Korf & J.K. Rogers	—	U42695[d]	AF170963[d]	AF107786[d]
Otidea onotica (Pers.: Fr.) Fuckel	KH-98-107 (C). Denmark	AF335121	AY513313[b]	—
Otidea umbrina (Pers.: Fr.) Bres.	KH.01.09 (C). Denmark	AY500540[b]	AY513314[b]	—
Pachyella adnata (Berk. & M.A. Curtis) Pfister	DHP-02.496 (FH). USA	AY500541[b]	AY513315[b]	AY500469[b]
Pachyella babingtonii (Berk. & Broome) Boud. (1)	KS-94-45 (C). Denmark	AF335122	AY513316[b]	AY500522[b,c]
Pachyella babingtonii (2)	KH-99-09 (C). USA	AF335123	AY513317[b]	AY500467[b]
Pachyella clypeata (Schwein.) Le Gal	FH No. 387 (FH). USA	AY500542[b]	AY513318[b]	—
Pachyella habrospora Pfister	A. de Meijer 1872 (Type FH). Brazil	AY500543[b]	AY513319[b]	—
Pachyella punctispora Pfister	KH-98-77 (C). Austria	AF335145	AY513320[b]	AY500468[b]
Pachyella violaceonigra (Rehm) Pfister	s.n. (FH). Switzerland	AF335125	AY513321[b]	AY500470[b]
Pachyphloeus citrinus Berk. & Broome	H. Saylor 2026 (FH, dubl. OSC). USA	AY500544[b]	AY513322[b]	AY500466[b]
Peziza ampelina Quél.	KH 00.011 (C). Denmark	AF335127	AY513323[b]	AY500492[b]
Peziza ampliata Pers.: Fr. (1)	JHC 92-386 (C). Denmark	AF335128	—	—
Peziza ampliata (2)	KH 00.027 (C). Denmark	—	—	AY500510[b,c]
Peziza apiculata Cooke	Winterhoff 86239 (herb. Winterhoff). Germany	AF335129	AY513324[b]	—
Peziza arvernensis Roze & Boud.	KH-98-12 (C). Denmark	AF335131	AY513325[b]	AY500497[b]
Peziza badiofusca (Boud.) Dennis	KH-98-113 (C). Sweden	AF335132	AY513326[b]	AY500475[b]
Peziza bananicola (Rehm) Sacc.	V. Demoulin 5529 (FH). New Guinea	AF335133	AY513327[b]	AY500483[b]
Peziza depressa Pers.	KH-98-28 (C). Denmark	AF335135	AY513328[b]	AY500474[b]
Peziza echinispora P. Karst.	Jukka Vauras 9110 F (TURA). Finland	AF335138	AY513329[b]	AY500496[b]
Peziza ellipsospora (Gilkey) Trappe	Trappe 13017 (C, dubl. OSC). USA	AF335139	AY513330[b]	AY500482[b]
Peziza emileia Cooke	Brummelen 1921 (L). The Netherlands	AF335140	AY513331[b]	—
Peziza gerardii Cooke (1)	KH-98-86 (C). Denmark	AF335142	—	AY500511[b,c]
Peziza gerardii (2)	KH-98-42 (C). Denmark	AF335144	—	AY500512[b,c]
Peziza gerardii (3)	TL-5693 (C). Denmark	AY500546[b]	AY513332[b]	AY500513[b,c]
Peziza gerardii (4)	DHP 02-495 (FH). Mexico	AY500547[b]	AY513333[b]	AY500471[b]
Peziza howsei Boud.	KH-97-98 (C). Denmark	AF335146	AY513334[b]	AY500493[b]
Peziza limnaea Maas Geest.	HFG-94.2 (C). Denmark	AF335147	AY513335[b]	AY500518[b,c]
Peziza lobulata (Velen.) Svrcek	KH 03.157 (FH). USA	AY500548[b]	AY513336[b]	AY500495[b]
Peziza michelii (Boud.) Dennis	TL-5692 (C). Denmark	AY500549[b]	AY513337[b]	AY500494[b]
Peziza natrophila A.Z.M. Khan (1)	Kew 59522 (K, Isotype). Bangladesh	AF335152	AY513338[b]	—
Peziza natrophila (2)	JHP 93.021 (C). Denmark	AF335153	AY513339[b]	AY500486[b]
Peziza obtusapiculata J. Moravec	TL-6474 (C). Denmark	AY500550[b]	AY513340[b]	AY500490[b,c]

(*continued on next page*)

Table 1 (*continued*)

Species	Collection number (Herbarium)[a]. Geographic origin	LSU	β-Tubulin	RPB2
Peziza phyllogena Cooke (1)	KH-99-03 (C). USA	AF335155	AY513341[b]	AY500480[b]
Peziza phyllogena (2)	s.n. (TUR, Type of *Peziza kallioi*). Finland	AF335156	AY513342[b]	AY500481[b]
Peziza polaripapulata (J. Moravec) K. Hansen (1)	KS-95-10A/B (C). Denmark	AF335157	AY513343[b]	—
Peziza polaripapulata (2)	KH-96-11 (C). Denmark	AY500551[b]	AY513344[b]	AY500515[b,c]
Peziza polaripapulata (3)	KH-96-12 (C). Denmark	—	—	AY500514[b]
Peziza quelepidotia Korf & O'Donnell	—	U42693[d]	—	AF107809[d]
Peziza retrocurvata K. Hansen & Sandal	KS-94-182 (C). Denmark	AF335159	—	AY500516[b]
Peziza saniosa Schrad.: Fr.	KH-97-137 (C). Denmark	AF335160	AY513345[b]	AY500476[b]
Peziza subcitrina (Bres.) Korf	KH-97-133 (C). Denmark	AF335162	AY513346[b]	AY500520[b,c]
Peziza subisabellina (Le Gal) Blank, Häffner & Hohmeyer (1)	RK 96.54 (herb. Roy Kristiansen). Norway	AF335163	AY513347[b]	AY500484[b]
Peziza subisabellina (2)	Winterhoff 8844 (herb. Winterhoff). Germany	AF335164	AY513348[b]	AY500485[b]
Peziza subviolacea Svrcek	KH-98-29 (C). Denmark	AF335165	AY513349[b]	AY863000[b]
Peziza succosa Berk.	KH-98-07 (C). Denmark	AF335166	AY513350[b]	AY500487[b]
Peziza succosella (Le Gal & Romagn.) Aviz.-Hersh. & Nemlich	KH-97-139 (C). Denmark	AF335167	AY513351[b]	AY500517[b]
Peziza varia (Hedw.: Fr.) Fr. (1)	KH-97-54 (C). Denmark	AF335134	AY513352[b]	AY500519[b,c]
Peziza varia (2)	KH-99-04 (C). USA	AF335151	AY513353[b]	AY500499[b]
Peziza varia (3)	KH-97-107 (C). Denmark	AF335150	AY513354[b]	AY500498[b]
Peziza varia (4)	KH 00.033 (C). Denmark	—	—	AY500500[b]
Peziza vesiculosa Bull.	JV 95-652 (C). Denmark	AY500552[b]	AY513355[b]	AY500489[b]
Peziza whitei (Gilkey) Trappe	Trappe 17049 (C, dubl. OSC). Australia	AF335168	AY513356[b]	AY500491[b]
Peziza sp. 3	PM-120-97 (Herb. Roy Kristiansen). Norway	AF335171	AY513357[b]	AY500488[b]
Peziza sp. 4	KH-97-85 (C). Denmark	AF335172	AY513358[b]	AY500521[b,c]
Plicaria carbonaria (Fuckel) Fuckel	DHP 9215 (FH). USA	AY500553[b]	AY513359[b]	AY500479[b]
Plicaria trachycarpa (Curr.) Boud.	KH-97-93 (C). Denmark	AY500554[b]	AY513360[b]	AY500478[b]
Ruhlandiella berolinensis Henn.	Isoneotype, 1230 (C). Canary Islands	AF335175	AY513361[b]	AY500477[b]
Sarcosphaera coronaria (Jacq.) Boud. (1)	TL-5450 (C). Denmark	—	AY513362[b]	AY500524[b,c]
Sarcosphaera coronaria (2)	KS-94-19 (C). Denmark	—	—	AY500523[b,c]
Sarcosphaera coronaria (3)	KS-94-24A (C). Denmark	AY500555[b]	—	AY863001[b,c]
Scabropezia flavovirens (Fuckel) Dissing & Pfister	KH-97-68 (C). Denmark	AY500556[b]	AY513363[b]	AY500461[b]
Smardaea amethystina (W. Phillips) Svrcek	KH-97-132 (C). Denmark	AF335176	AY513364[b]	—
Terfezia arenaria (Moris) Trappe	Trappe 11093 (FH, dubl. OSC). Spain	—	AY513365[b]	—
Terfezia boudieri Chatin	Trappe 4916 (FH, dubl. OSC). Libya	AY500557[b]	AY513366[b]	—
Terfezia claveryi Chatin	Trappe 3195 (FH, dubl. OSC). Kuwait	AY500558[b]	—	AY500503[b]
Tirmania nivea (Desf.: Fr.) Trappe	Trappe 23190 (C, dubl. OSC). Israel	AF335177	AY513367[b]	AY500525[b,c]
Tirmania pinoyi (Maire) Malençon	Trappe 13587 (C, dubl. OSC). Saudi Arabia	AF335178	AY513368[b]	AY500502[b]

Corresponding voucher information and GenBank accession numbers included. Numbers in parentheses following species names are used to indicate multiple collections of a single taxon.

[a] Herbaria are cited according to acronyms in Index Herbariorum (http://www.nybg.org/bsci/ih/ih.html), except for the two private herbaria of Wulfard Winterhoff and Roy Kristiansen.

[b] New sequences.

[c] Only 6–7 region of RPB2 sequenced.

[d] Sequences from other authors obtained from GenBank.

72 β-tubulin sequences are newly determined in this study. In addition, 32 LSU rDNA sequences are new, and analyzed together with 46 LSU rDNA sequences from our previous study (Hansen et al., 2001). LSU and RPB2 sequences of *Peziza ampliata* are from different collections (Table 1). All seven clades previously identified within the Pezizaceae using LSU rDNA sequences (Hansen et al., 2001) were represented in the analyses. Sixteen of the 22 currently accepted genera in the Pezizaceae (Eriksson et al., 2004) were included, with 44 collections of *Peziza* representing 32 *Peziza* species. To confirm the monophyly of the Pezizaceae, species belonging to the Ascobolaceae (*Ascobolus* Pers.: Fr.), Morchellaceae (*Morchella* Pers.: Fr.), and Pyronemataceae (*Byssonectria* P. Karst., *Melastiza* Boud., and *Otidea* (Pers.) Bonord.) were also included. *N. vitellina* was used to root the tree, because phylogenetic analyses at more inclusive levels place *Neolecta* Speg. basal to the rest of the fruitbody producing ascomycetes and the budding yeasts (Landvik, 1996; Landvik et al., 2001).

2.2. Molecular techniques

DNA was isolated from dried or fresh ascomata (stored in extraction buffer) and was extracted as in Hansen et al. (1999), except the dried material was not ground in liquid nitrogen, but shaken in a Fastprep FP120 Cell Disruptor (BIO 101, CA). Serial dilutions of DNA (1:10, 1:100, and 1:1000) were used as template for the polymerase chain reaction (PCR). For RPB2 and β-tubulin only dilutions 1:10 and 1:100 were used. The 5′ end of the LSU-rDNA, spanning domains D1 and D2, was amplified using the primers LROR and LR5 (for a few taxa LR5 was replaced with LR3 or LR7) (Moncalvo et al., 2000). In addition to the primers used for PCR, internal primers LR3 and LR3R were used for sequencing. Degenerate primers designed by Liu et al. (1999) to obtain sequences spanning conserved regions 6–11 in RPB2 (Denton et al., 1998; James et al., 1991) were modified for Pezizales (Table 2) based on RPB2 sequences of *Peziza quelepidotia* and *Morchella elata*. After generating additional Pezizaceae RPB2 sequences some of the primers were improved further for members of the family (P6Fa, P7Ra, and P7Fa). The sequence spanning regions 6–11 was amplified as one piece, or two pieces when required. The primer Pb7F was designed to amplify and sequence the overlapping piece between regions 6–7 and 7–11 (Denton et al., 1998; James et al., 1991). In a few instances, where the regions 6–7 did not successfully amplify, the regions 5–7 was amplified instead (Denton et al., 1998; James et al., 1991). Degenerate primers to amplify and sequence a ca. 860 bp region of the β-tubulin gene, spanning two large exons (exons 6 and 7 in *A. nidulans* benA) and one small intron (May et al., 1987), were designed through comparison of known ascomycete β-tubulin sequences from GenBank in a multiple alignment (odd numbers are forward primers, even numbers are reverse) (Table 2). The β-tubulin gene region was amplified and sequenced using the two most external primers, PB1/PB1a and B42F, or with a combination of primer pairs, PB3/PB3a-B42F, PB1a-PB2/PB4, and PB1–PB6, to obtain overlapping PCR products from genomic DNA preparation.

The LSU rDNA and β-tubulin gene were amplified using *Taq* DNA polymerase (Gibco-BRL, Life Technologies, Carlsbad, California, USA), whereas RPB2 was amplified using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Life Technologies, Carlsbad, California, USA). For some problematic taxa β-tubulin was likewise amplified using the Platinum *Taq* DNA Polymerase High Fidelity. The PCR conditions for LSU rDNA, and some β-tubulin reactions, included: 35–40 cycles at 94° for 30 s, 60° for 30 s, and 72° for 1:30 min, followed by a 4° soak. In addition, the following programs were used to amplify β-tubulin: (1) 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 7 min and a 4 °C soak, (2) a hot start at 94 °C for 5 min, then two cycles at 94 °C for 1 min, and 60 °C for 2 min, and 72 °C for 1 min, and then decreasing the annealing temperature 3 °C every second cycle to 50 °C is reached, with 24 cycles at 50 °C annealing, followed by a 72 °C for 30 min and a 4 °C soak. PCR conditions for RPB2 included: hot start at 95 °C for 5 min, then 35–40 cycles of 95 °C for 1 min, 60 °C for 2 min, an increase of 0.2 °C/1 s to 72 °C, and 72 °C for 2 min, and a final extension of 72 °C for 10 min with a 4 °C soak. These conditions were also used to amplify β-tubulin when using the Platinum *Taq* DNA Polymerase High Fidelity.

PCR products were purified either directly, or following agarose gel electrophoresis and band excision, using QIAquick spin columns (Qiagen, 1997). Cycle sequencing, using BigDye terminator (Applied Biosystems, Foster City, CA), was done in a Peltier Thermal Cycler PTC-200 (MJ Research, Watertown, MA) using the following program: 96° for 3 min, then 25 cycles of 96° for 10 s, ramping 1.0°/s to 60°, 60° for 4 min, followed by a 4° soak. Cycle sequencing reaction volumes were 8 μl. Sequencing reactions were purified using ethanol precipitation. Seventy-four microliters of a mixture of 1 μl of 0.5 M MgCl and 1000 μl of 100% ethanol was added to the cycle-sequencing product, vortexed and left in the

Table 2
RPB2 and β-tubulin degenerate primers (5′–3′)[a]

Primer	Sequence	Location
RPB2-P5F	GAYGACAGAGATCACTTYGG	—
RPB2-P6F	TGGGGWYTSGTMTGYCCTGC	—
RPB2-P6Fa[b]	TGGGGRYTKGTBTGYCCKGCHGA	—
RPB2-P7R	CCCATSGCYTGYTTACCCAT	—
RPB2-P7Ra[b]	CCCATNGCYTGYTTRCCCAT	—
RPB2-P7F	ATGGGTAARCARGCSATGGG	—
RPB2-P7Fa[b]	ATGGGYAARCARGCNATGGG	—
RPB2-P11aR	GCGTGGATYTTGTCRTCSACC	—
RPB2-Pb7F[c]	TGYGARATYCAYCCNAGTATGA	—
β-tub-PB1	TCCTCGTYGATCTBGAGCCYGGTAC	1757
β-tub-PB1a[b]	GATCTGGAGCCTGGTACCATGGA	1765
β-tub-PB42F	ACCTCCTTCATGGMGACCTTDCC	2620
β-tub-PB42Fa[b]	CCTTCATGGAGACCTTGCCAC	2618
β-tub-PB3	ACYCTCAAGCTCTCCAASCCSTC	2254
β-tub-PB3a[b]	TGCATGAGGACYCTBAAGCTCTC	2245
β-tub-PB2	CCRGACATGACRGCRGAGAC	2300
β-tub-PB4[d]	GAACCATGTTSACRGCCAACTTCC	2366
β-tub-PB6	GCCATCATGTTCTTRGGGTCGTACA	2491
β-tub-PB5	AGGAAGTTGGCYGTSAACATGGT	2365
β-tub-PB8	GAGAGCTTVAGRGTCCTCATGCA	2245
β-tub-PB7	GTCGACCAGGTCCTCGACGT	1903

RPB2 primers from Liu et al. (1999) modified for the Pezizales; for location see Liu et al. (1999). β-Tubulin primer location relative to *A. nidulans* benA β-tubulin gene sequence (GenBank M17519).

[a] Follow the international nomenclature for degenerate positions: R = G or A, K = G or T, S = G or C, W = A or T, M = A or C, Y = T or C, B = G, T or C, H = A, T or C, N = G, A, T or C.

[b] Primers improved further for Pezizaceae.

[c] Forward primer positioned just before RPB2-P7F, designed to amplify overlapping piece between region 6–7 and 7–11.

[d] Alternative for β-tub-PB2.

dark for 20 min, and centrifuged at 5300 rpm for 30 min. The supernatant was draw off and the samples left upside down to air-dry. Electrophoresis and data collecting were done on an ABI PRISM 3100 automated DNA sequencer (Perkin-Elmer/ABI).

2.3. Sequence alignment

Sequences were edited and contigs assembled using Sequencher 3.0 (GeneCodes, Ann Arbor, Michigan). Sequences are deposited in GenBank (Table 1). Nucleotide sequences were aligned by hand using the software Se-Al v 2.0a8 (Rambaut, 1996). Alignments are available from TreeBASE (http://www.treebase.org/treebase/) as Accession Nos. M2134 (LSU), M2132 (RPB2), M2131 (β-tubulin), M2133 (LSU, RPB2, and β-tubulin combined data set). The LSU rDNA was aligned with gaps inserted to optimize aligned sites, and gapped positions were included and treated as missing data in analyses. Only the exons of RPB2 and β-tubulin were included in analyses and these were easily aligned; no indels were present in β-tubulin and only a few were found in RPB2. Introns differed in length and were too variable to align among species. The aligned LSU, RPB2, and β-tubulin sequence matrices were first analyzed separately, and then combined.

2.4. Search strategies

Individual and combined analyses of the LSU rDNA, RPB2, and β-tubulin were performed using PAUP* 4.0b 10 (Swofford, 2000) and MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) on an iMac 600 MHz, a G4, and G5 Mac computer. Maximum parsimony (MP) analyses with heuristic searches consisted of 1000 random sequence addition replicates with tree bisection–reconnection (TBR) branch swapping, MULPARS in effect, and saving all equally most parsimonious trees (MPTs). All characters were equally weighted and unordered. RPB2 and β-tubulin were also analyzed by codon position using parsimony. In analyses of 1 and 2 codon positions a two-step search was performed (due to the exceedingly large number of trees generated): first, 1000 heuristic searches were performed with random sequence addition and TBR branch swapping, with MAXTREES unrestricted, keeping only up to 15 trees per replicate, then exhaustive swapping was performed on all of the MPTs discovered with MAXTREES set to 15,000. Robustness of individual branches was estimated by maximum parsimony bootstrap proportions (BP), using 500 bootstrap replicates, each consisting of a heuristic search with 100 random addition sequence replicates, TBR branch swapping, and MAXTREES set at 100. Transition and transversion base substitutions in the sequence data were assessed using MacClade 4.0 (Maddison and Maddison, 2000) and PAUP files consisting of the MPTs.

To determine which model of nucleotide substitution with the least number of parameters best fit each data set, hierarchical likelihood ratio tests were performed as implemented in the program Modeltest v3.06 (Posada and Crandall, 1998). A Tamura–Nei model (Tamura and Nei, 1993) with unequal base frequencies, gamma distributed among site rate variation, and a proportion of invariable sites (TrN + I + G) was selected for all dataset

Table 3
Maximum likelihood best-fit evolutionary models and parameters for each data set selected by hierarchical likelihood ratio tests

	LSU rDNA	RPB2	β-Tubulin	Combined
Number of sites	974	1707	773	3454
Model	TrN + I + G[a]	TrN + I + G[a]	TrN + I + G[a]	TRN + I + G[a]
−L ln	10111.0727	31839.7584	10833.3334	53820.9918
Base frequencies				
A	0.2885	0.2747	0.2408	0.2697
C	0.1597	0.2545	0.2762	0.2460
G	0.2583	0.2132	0.1806	0.2104
T	0.2934	0.2576	0.3024	0.2740
Substitution model: rate matrix				
[A–C]	1.0000	1.0000	1.0000	1.0000
[A–G]	3.6466	4.7759	4.6152	4.5128
[A–T]	1.0000	1.0000	1.0000	1.0000
[C–G]	1.0000	1.0000	1.0000	1.0000
[C–T]	7.1249	6.0905	7.5017	6.3063
[G–T]	1.0000	1.0000	1.0000	1.0000
Among-site rate variation				
I[b]	0.4429	0.3717	0.5282	0.4478
G[c]	0.6546	0.6597	0.8268	0.7103

[a] Tamura–Nei model (Tamura and Nei, 1993) with unequal base frequencies, gamma distributed among site rate variation and a proportion of invariable sites.
[b] Proportion of invariable sites.
[c] Variable sites gamma distribution parameter.

(Table 3). Maximum likelihood (ML) analyses consisted of heuristic searches with TBR branch swapping, using one of the MPTs for each data set as a starting tree. Estimated model parameter values resulting from the Modeltest run (Table 3) were entered manually into PAUP.

Bayesian analyses used uniform prior probabilities, the general time-reversible model of molecular evolution (Rodrígez et al., 1990), in which a proportion of the sites were assumed to be invariable, while the rate for the remaining sites was approximated from a gamma distribution with four categories (GTR + I + G), and a random starting tree. For the combined three-gene analysis, each data set was specified as distinct partitions. Four simultaneous chains of Markov Chain Monte Carlo were run starting from random trees for 1,000,000 generations, sampling every 100 generations. For the LSU data set stationarity was reached at approximately generation 67100; thus the first 671 trees were discarded (the "burn in" of the chain), and phylogenetic inferences are based on the last 9330 trees sampled. For the RPB2 data stationarity was reached at ca. generation 75,000, for β-tubulin at ca. generation 70,000, and for combined data at ca. generation 100,000. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the trees kept. If $\geqslant$95% of the sampled trees contained a given clade we considered it to be significantly supported by our data.

Prior to combined analyses the combinability of the data were explored. Several studies have suggested that some of the most readily available methods (e.g., the ILD test) for detecting conflicts among data partitions are poor predictors as to whether or not combining two data partitions is likely to lower phylogenetic accuracy (e.g., Barker and Lutzoni, 2002; Cunningham, 1997; Dolphin et al., 2000; Dowton and Austin, 2002; Hibbett and Donoghue, 2001; Yoder et al., 2001). Therefore, congruence of the separate data sets was assessed by visual inspection of the individual bootstrap values. We considered the phylogenies to be incongruent only if they displayed strongly bootstrap supported incongruence, rather than weakly supported incongruence (e.g., Mason-Gamer and Kellogg, 1996; Wiens, 1998). We used the following bootstrap categories: unsupported, <50%; weak, 50–74%; moderate, 75–84%; strong, 85–100% (from Whitten et al., 2000). Incongruence is then considered conflict of clades with BP $\geqslant$85%; clades that are strongly supported in one analyses that conflict with different and strongly supported clades in the others.

3. Results

3.1. Nucleotide sequences

The RPB2 alignment consisted of 1707 nucleotides (excluding introns) corresponding to positions 1592–3304 in *Saccharomyces cerevisiae* RPB2 gene (GenBank M15693). Complete sequences spanning regions 6–11 were obtained for 48 specimens. For 21 specimens only the 6–7 region was obtained (ca. 720 bp) (see Table 1) and for *Peziza subviolacea* only the 7–11 region. A 900 bp region of LSU rDNA was sequenced for most specimens. The β-tubulin alignment consisted of 773 nucleotides (excluding introns) corresponding to positions 1757–2619 in *A. nidulans* benA (May et al., 1987). There was no evidence of gene duplication in the Pezizaceae β-tubulin sequences.

RPB2 contained 1 deletion site (ranging from two amino acids in *Neolecta*, to 3–8 in Pezizales) at positions 2008–2031 compared to *S. cerevisiae* RPB2 gene (GenBank M15693) and two insertions sites (a single synapomorphic indel in *Boudiera* Cooke and *Pachyella* Boud. species at position 2176–2178, and a 3–7 amino acid indel at position 2206–2226). Intron positions in the protein coding nuclear genes were recognized by sequence comparisons and the conserved dinucleotide sequences at the ends of introns (GT at start and AG at end). Four introns were recognized within the RPB2 region using the *S. cerevisiae* RPB2 gene sequence for position identification. The first intron is present in all in-group taxa except for *Morchella* and *Marcelleina persoonii*, inserted between positions 2313 and 2314, and ranges in length from 47 to 73 nucleotides. The second intron is only present in *Neolecta*, *Ascobolus carbonarius*, *A. denudatus*, *Iodophanus carneus*, *Pachyella adnata*, *Pachyella punctispora*, *Pachyella violaceonigra*, *Hydnotryopsis* sp., and *Peziza lobulata*, inserted between positions 2985 and 2986, and ranges in length from 34 to 65 nucleotides. The third intron is present in all in-group taxa sequenced except for *Morchella*, *Pachyella babingtonii*, and *Peziza howsei*, inserted between position 3055 and 3056, and ranges in length from 46 to 99 nucleotides. The fourth intron is present in *Neolecta*, *M. persoonii*, *Iodowynnea auriformis*, *Hydnotryopsis* sp., *Peziza gerardii*, *P. michelii*, *P. succosa*, *P. ampelina*, *P. subviolacea*, *P. vesiculosa*, *P. quelepidotia*, *Peziza natrophila*, and *P. obtusapiculata*, inserted between positions 3167 and 3168, and ranges in length from 38 to 70 nucleotides. Intron 1 and 2 occupies a phase O insertion with respect to the reading frame, while the third intron has a phase 1 insertion and the fourth intron a phase 2 insertion.

Four introns were recognized within the β-tubulin region compared to *S. cerevisiae* β-tubulin gene (GenBank V01296). The first and second introns are only present in *Neolecta*, between positions 705 and 706, and positions 961 and 962, respectively. The third intron is unique to the in-group taxa (Pezizales), inserted between positions 968 and 969, and ranges in length from 50 to 67 nucleotides. The fourth intron is present in all Pezizaceae and Ascobolaceae (*Ascobolus*) taxa sequenced, but not in *Byssonectria* (sequences of other representatives

of Pyronemataceae ended before the intron site). This intron is inserted between positions 1341 and 1342, and ranges in length from 52 to 78 nucleotides. Intron 1 and 4 occupies a phase 2 insertion with respect to the reading frame, while the intron 2 have a phase O insertion and intron 3 a phase 1.

The base composition of all three gene-regions was with approximately equal AT/CG ratios; RPB2 and LSU being identical with a slightly lower CG content (48%), and β-tubulin with a slightly lower AT content (47%) (Table 4). However, the overall AT/CG ratios were biased per codon position in the protein coding regions. RPB2 had more As and Ts relative to Cs and Gs at second codon positions (62%), while first and third positions were more balanced (with 45 and 49%, respectively). β-Tubulin also had more As and Ts at second codon positions (57%), but showed a higher average CG content at first and third positions (57 and 60%, respectively). All gene regions had more transitions than transversions (Table 4). The transition/transversion ratio in the β-tubulin region was high compared to the RPB2 and LSU rDNA regions (Table 4), with a strong bias toward C–T transitions (53.4% of the total number of transitions). Substitutions within the β-tubulin exons were strongly biased towards third codon position; of the 325 variable sites within exons, 63 (19.4%) were in the first position, 23 (7.1%) in the second, and 239 (73.5%) in the third position. Although less prominent, substitutions within RPB2 were also biased toward third codon; of the 989 variable sites, 268 (27%) were in first position, 171 (17%) in the second, and 550 (56%) in the third position.

Table 4
Number of transition and transversion base substitutions and composition in the LSU, RPB2, and β-tubulin regions

Substitution type and sequence composition	LSU	RPB2	β-Tubulin
Transition			
A–G	105	431	76
G–A	235	592	123
T–C	234	771	371
C–T	262	681	652
Transversion			
A–C	29	235	41
C–A	26	208	90
A–T	54	175	26
T–A	90	224	95
G–C	25	144	15
C–G	19	144	72
G–T	74	132	16
T–G	73	147	45
Transition/transversion ratio	2.14	1.76	3.06
Average A + T frequency	0.52	0.52	0.47
Average C + G frequency	0.48	0.48	0.53
Average A + T/C + G ratios	1.08	1.08	0.89

3.2. Lineages in Pezizaceae

Fourteen fine-scale lineages are identified within Pezizaceae with moderate to strong bootstrap support in combined analysis of LSU, RPB2, and β-tubulin (BP 78–100%) or with Bayesian PP of 100%, and are resolved by ML analysis. To facilitate results and discussion, we have labeled these lineages as indicated in Table 5. Species of *Peziza* occur in eight of the lineages, spread among other genera of the family. *Peziza retrocurvata* and *Peziza* sp. 3, represented by single specimens, constitutes separate lineages with uncertain placement. The supported fine-scale topology is identical to those obtained in previous analyses of LSU rDNA sequences including a larger sample of taxa (Hansen et al., 2001). Relationships among the lineages are still not resolved with confidence as measured by bootstrapping, but Bayesian analyses of RPB2 and the tree-gene data set did resolve several more inclusive clades with PP >95% (labeled A–F, Sections 3.4 and 3.6).

3.3. LSU phylogeny

Parsimony analysis of the LSU rDNA data set yielded 83 equally MPTs (see Table 6). The Pezizaceae is highly supported as monophyletic (BP 100%) with Ascobolaceae as the sister group (BP 98%) (Fig. 1). The strict consensus tree of all MPTs is highly resolved, but the deep level relationships are not well supported. Thirteen of the 14 fine-scale lineages are resolved, and twelve of these supported with BP $\geqslant$75% (Table 5 and Fig. 1). The *Peziza* s. str. lineage (a and b) is not monophyletic in the strict consensus tree. The *Peziza* s. str.-a sub-lineage is monophyletic and strongly supported (BP 89%), and the *Peziza* s. str.-b sub-lineage, excluding *Peziza subcitrina*, is resolved but with low support (BP 53%). The *P. depressa–Ruhlandiella* lineage is only weakly supported (BP 67%). Overall, MP yields 28 clades with BP $\geqslant$75% (Table 6 and Fig. 1).

The optimal maximum likelihood tree (MLT) was found with a log likelihood score of −10111.0727 (Table 3). The topology of the strict consensus tree identified by MP analysis (see Fig. 1) was found by ML analysis, except for minor rearrangements within the fine-scale lineages (not shown). Bayesian analysis identified 12 of the 14 lineages (PP $\geqslant$99%) (Table 5 and Fig. 1). The *Plicaria–Hapsidomyces* lineage is not resolved. As in MP analysis, *Peziza* s. str. do not form a monophyletic group, but sub-lineage-a is resolved with only PP of 84% and sub-lineage-b, excluding *P. subcitrina*, is supported with PP of 97%. Contrary to MP analysis, Bayesian analyses recovered the *P. depressa–Ruhlandiella* lineage with confidence (100% PP). The sister-group relationship of the *Marcelleina–P. gerardii* lineage and the rest of the Pezizaceae are also strongly indicated (100% PP). Overall, Bayesian analysis records 31 clades with PP $\geqslant$95% (Table 6).

Table 5
Comparative parsimony bootstrap proportions (BP), Bayesian posterior probabilities (PP), and presence or absence (+/−) in maximum likelihood analyses (ML) of selected lineages within Pezizaceae, obtained from separate and combined data partitions of LSU rDNA, RPB2, and β-tubulin sequence data

Lineages	LSU rDNA			RPB2					β-Tubulin					LSU, RPB2, β-tubulin		
	BP	PP	ML	All sites			1, 2[a]	3[b]	All sites			1, 2[a]	3[b]	BP	PP	ML
				BP	PP	ML	BP	BP	BP	PP	ML	BP	BP			
Marcelleina–P. gerardii	78	100	+	86	100	+	84	–	–	100	+	59	–	89	100	+
Boudiera–Pachyella	75	100	+	–	68	+	–	–	–	96	+	<50	–	78	100	+
Sarcosphaera–Hydnotryopsis	100	100	+	100	100	+	97	100	100	100	+	–	100	100	100	+
Peziza s. str. (a and b)	–	–	–	–	93	+	53	–	–	–	+	–	–	<50	100	+
Peziza s. str.-a	89	84	+	67	100	+	–	–	99	100	+	–	96	100	100	+
Peziza s. str.-b	–	–	–	<50	100	+	–	–	–	–	–	–	–	<50	97	+
P. natrophila–quelepidotia	100	100	+	100	100	+	100	100	–[e]	–[e]	–[e]	–[e]	–[e]	100	100	+
P. subisabellina–bananicola	99	100	+	100	100	+	100	100	100	100	+	–	99	100	100	+
P. phyllogena	99	100	+	100	100	+	100	100	100	100	+	–	100	100	100	+
Plicaria–Hapsidomyces	88	–	+	100[c]	100[c]	+[c]	99[c]	100[c]	91	100	+	–	94	98	100	+
P. depressa–Ruhlandiella	67	100	+	71	100	+	68	57	<50	90	+	–	–	89	100	+
P. succosa–michelii	82	99	+	100	100	+	79	99	95	100	+	–	94	100	100	+
Iodowynnea	100	100	+	–[d]	–[d]	–[d]	–[d]	–[d]	100	100	+	55	100	100	100	+
P. polaripapulata-sp. 4	100	100	+	100	100	+	68	100	100	–	–	–	99	100	100	+
Scabropezia–Amylascus	100	100	+	100	100	+	100	100	98	100	+	–	96	100	100	+
Iodophanus	100	100	+	100	100	+	100	–	99	100	+	75	90	100	100	+

[a] First and second codon positions included.
[b] Third codon positions included.
[c] The RPB2 region of *Hapsidomyces venezuelensis* was not sequenced, and values refer to the grouping of two specimens of *Plicaria*.
[d] The RPB2 was sequenced from only a single specimen of *Iodowynnea auriformis*.
[e] The β-tubulin region of *P. quelepidotia* was not sequenced.

Table 6
Phylogenetic information of LSU, RPB2, and β-tubulin individual and combined data sets, based on parsimony analyses

Data sets	No. of taxa	Total characters included	Constant characters	Parsimony informative characters	Percent informative characters[a] (%)	No. MP trees	Tree length	CI	RI	No. of pezizaceous clades ⩾75% boostrap support	No. of pezizaceous clades ⩾95% posterior probabilities
LSU rDNA	78	974	567	338[a]	34.70	83	1854	0.365	0.698	28	31
RPB2, all sites	68	1707	718	883[a]	51.73	2	7889	0.238	0.561	29	41
RPB2, 1 codon	68	569	301	214[b]	24.24						
RPB2, 2 codon	68	569	398	122[b]	13.82						
RPB2, 3 codons	68	569	19	547[b]	61.95	11	6347	0.193	0.519	27	
RPB2, 1 and 2 codons	68	1138	699	336[b]	38.05	>15,000	1462	0.449	0.773	24	
β-tub., all sites	70	773	448	277[a]	35.83	5	2393	0.232	0.562	25	30
β-tub., 1 codon	70	258	195	41[b]	14.80						
β-tub., 2 codon	70	257	234	8[b]	2.89						
β-tub., 3 codon	70	258	19	228[b]	82.31	99	2200	0.209	0.548	24	
β-tub., 1 and 2 codons	70	516	429	49[b]	17.69	>15,000	165	0.576	0.795	4	
LSU, RPB2, β-tub.	79	3454	1733	1498[a]	43.37	6	12289	0.253	0.579	42	52

[a] For data sets including all sites: percent informative characters out of total number of characters for individual data sets.
[b] For data sets per codon positions: percent informative characters out of total number of informative characters in individual data sets including all sites.

3.4. RPB2 phylogeny

Parsimony searches of all RPB2 nucleotide sites identified two MPTs (see Table 6). The strict consensus tree is almost completely resolved, except for a trichotomy of the *P. phyllogena*, *Plicaria–Hapsidomyces*, and *P. subisabellina–bananicola* lineages (see Fig. 2). Most of the deeper branches are however, with no bootstrap support (50%).

The placement of the root is uncertain, and differs when the RPB2 data are assessed by different methods of phylogenetic inference (MP, ML, and Bayesian analyses). The basal branches of the MPTs are resolved, but without support (BP <50%). The Pezizaceae, as defined by Hansen et al. (2001) includes *Marcelleina* Brumm., Korf & Rifai, previously Pyronemataceae. However, in the RPB2 MPTs *Marcelleina* and *P. gerardii* forms a strongly supported clade outside of the Pezizaceae, as a

sister group to all of the Pezizales sampled (including Ascobolaceae, Pyronemataceae, and Morchellaceae, which form a clade as successive sisters to Pezizaceae) (Fig. 2). In the MLT *Marcelleina* and *P. gerardii* form a sister group to the Ascobolaceae/Pyronemataceae/Morchellaceae clade, as sister to Pezizaceae (tree not shown). Using Bayesian analyses Pezizaceae sensu Hansen et al. (2001) is monophyletic, but only with PP of 85% (tree not shown). The uncertainty in the placement of the root is reflected in MP analyses of RPB2 by codon position. In analysis of first and second codon positions Pezizaceae is weakly supported as monophyletic (BP 63%), but the basal node of the ingroup topology is unresolved in the strict consensus tree, with Ascobolaceae, Pyronemataceae/Morchellaceae, and Pezizaceae forming a trichotomy (tree not shown). Although third codon positions possess 62% of the phylogenetic informative sites in the RPB2 data (547 sites, Table 6), deeper nodes are not resolved with any confidence using third codon positions only. In the strict consensus of the MPTs of third codon positions Pyronemataceae, Morchellaceae, and Ascobolaceae are erroneously highly nested within the Pezizaceae, but with no support (tree not shown). MP analysis of first and second codon positions yielded >15,000 trees whereas third codon positions yielded only 11 trees (Table 6).

Ten of the 14 fine-scale lineages identified within the Pezizaceae are supported with BP $\geqslant$86% (Table 5 and Fig. 2). The *P. depressa–Ruhlandiella* lineage is weakly supported (BP 71%). Contrary to all LSU analyses, the *Boudiera–Pachyella* lineage is not monophyletic; *Boudiera* form a monophyletic group with *Iodophanus* Korf, but without support (BP <50%). As in analyses of LSU the *Peziza* s. str. lineage is not monophyletic, but both of the sub-lineages -a and -b are resolved.

The relationships among the lineages (Fig. 2) differ largely from the relationships in the LSU MP strict consensus tree (Fig. 1), but are mostly with <50% bootstrap support in both genes and thus will not be discussed in detail. RPB2 did identify a more inclusive clade A with moderate support (BP 78%) of the *P. subisabellina–bananicola*, *P. phyllogena*, *Plicaria*, and *P. depressa–Ruhlandiella* lineages (Fig. 2). The most distinct difference in clade support between LSU and RPB2 phylograms is the support for the *Peziza* s. str.-a lineage, with 89% BP in LSU MPTs and only 67% in RPB2 MPTs (Table 5). Support for the *P. depressa–Ruhlandiella* lineage, raised from 67% BP in LSU to 71% BP in RPB2. The *P. depressa–Ruhlandiella* lineage, excluding *Ruhlandiella* Henn. and *Peziza whitei*, is strongly supported in the RPB2 MPT (BP 100%, Fig. 2). Overall, MP of all RPB2 sites records 29 clades with BP $\geqslant$75% compared to 28 for the LSU data (Table 6).

The optimal MLT was found with a log likelihood score of −31839.7584 (Table 3). In ML and Bayesian analyses of RPB2 all 14 fine-scale lineages are resolved as monophyletic (Table 5). Notable the *Peziza* s. str. sub-lineages -a and -b are each with PP of 100%. The Bayesian tree agrees with the ML tree (trees not shown) in the branching order of taxa within the Pezizaceae, with only minor changes. The more inclusive clades, A–F, identified by Bayesian analysis with significant support (PP 96–100%, see Fig. 2 except for clade B) were also present in the ML tree. *Marcelleina* and *P. gerardii* are weakly resolved as a sister group to the rest of the Pezizaceae, which form a highly supported clade F (PP 100%). Clade F members, excluding *Iodophanus*, *Boudiera*, and *Pachyella* form a clade E (PP 100%). *Peziza* s. str. (sub-lineages-a and -b) are sister group(s) to the D_1 clade with PP of 96%. In contrast to MP, but in agreement with ML, the *Scabropezia–Amylascus* lineage form a monophyletic group with clade A (forming clade B, PP 100%). Clade B and *P. retrocurvata* are suggested as a sister group to the *Iodowynnea*, *P. polaripapulata*-sp. 4, and *P. succosa–michelii* lineages (forming clade C, PP 100%). Overall, Bayesian analysis identified 41 clades with PP $\geqslant$95% (Table 6).

3.5. β-Tubulin phylogeny

Parsimony searches of all β-tubulin nucleotide sites identified five MPTs (see Table 6). None of the analyses of β-tubulin, MP, ML, or Bayesian, resolved the Pezizaceae as monophyletic; a clade of Ascobolaceae and Pyronemataceae are highly nested within the Pezizaceae in all trees (e.g., Fig. 3) (no β-tubulin sequences of Morchellaceae taxa were included). This structure of the β-tubulin phylograms is similar to RPB2 third codon positions phylograms. The few resolved deep branches are with weak or no support (Fig. 3). In analyses of first and second codon positions, the basal branches of the strict consensus tree (of >15,000 MPTs) are congruent with results from LSU data, placing Ascobolaceae as the closest relative to a monophyletic Pezizaceae, and Pyronemataceae as a sister to the rest of the ingroup (tree not shown). These branches are however, with <50% bootstrap support. Nearly the entire phylogenetic signal in the β-tubulin data set stems from third codon positions; MP of third codon positions identified 24 clades with BP $\geqslant$75% out of the 25 recorded using all sites (Table 6). MP of first and second codon positions identified only four clades with BP $\geqslant$75%. Third codon positions possess 82% of the phylogenetic informative sites in the β-tubulin data (228 sites) (Table 6).

The strict consensus tree of all MPTs of all sites shows a large polytomy (see Fig. 3). Nevertheless, nine of the 14 fine-scale lineages are supported by $\geqslant$91% bootstrap support (Fig. 3 and Table 5). The *Marcelleina–P. gerardii*, *Boudiera–Pachyella*, and *Peziza* s. str. lineages are broken up into smaller lineages. A β-tubulin sequence of *P. quelepidotia* was not available and thus, *P. natrophila* constitutes a separate lineage. The *Peziza* s.

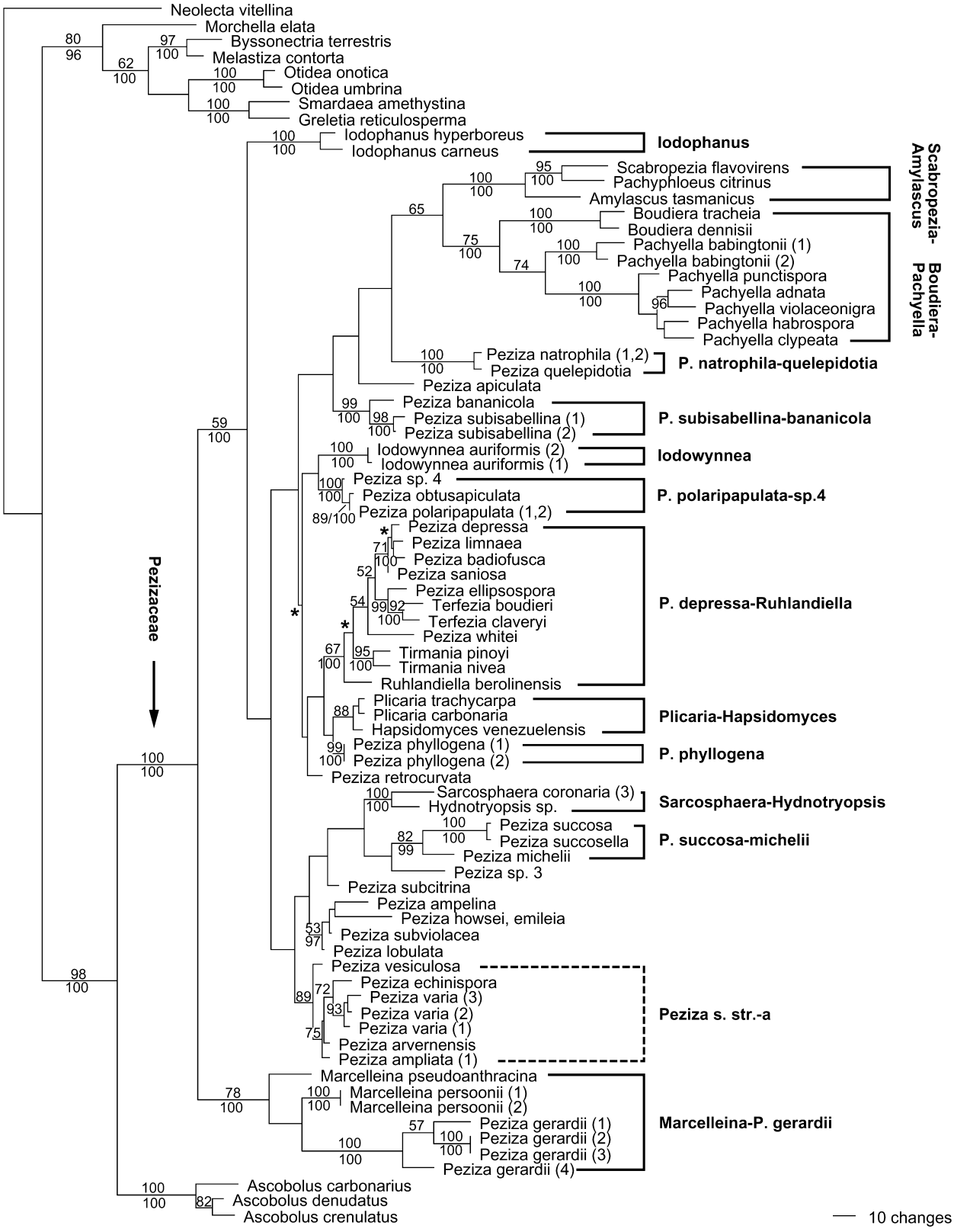

Fig. 1. Phylogeny of the Pezizaceae inferred from LSU rDNA sequences. One of 83 equally most parsimonious trees. Terminal taxa represent individual specimens (Table 1). Branches with asterisks collapse in the strict consensus of all most parsimonious trees. Numbers above branches are bootstrap frequencies (>50%); those below are posterior probabilities (⩾95%). Branch lengths are proportional to the number of steps (character changes) along the branch. Thirteen resolved fine-scale lineages (of 14 identified in the combined trees, Figs. 4 and 5 and Table 5) are indicated for discussion in the text. Dashed bracket indicate resolved sub-lineage-a of the *Peziza* s. str. lineage.

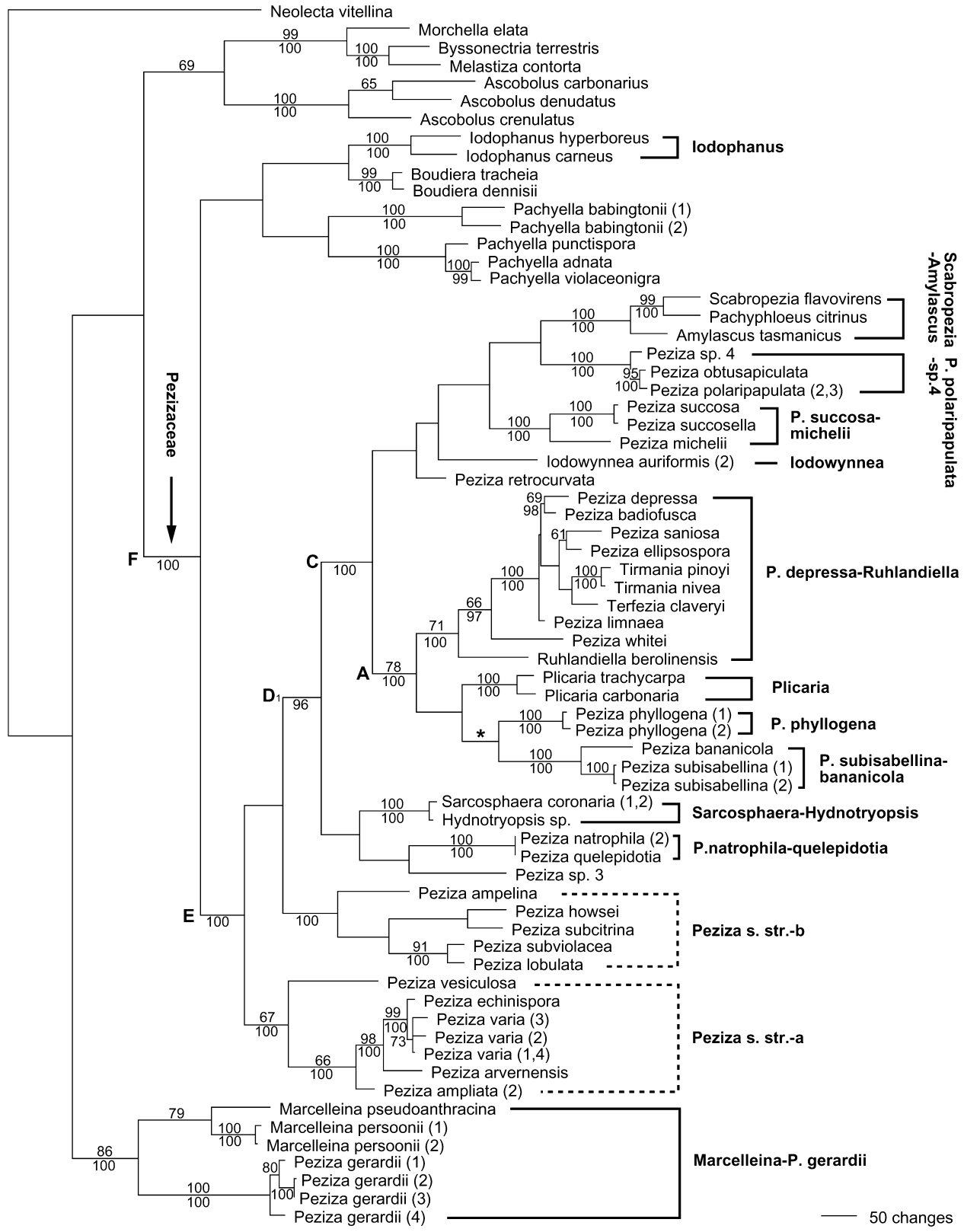

Fig. 2. Phylogeny of the Pezizaceae inferred from RPB2 sequences, showing one of two equally most parsimonious trees. The asterisk indicates a branch that collapses in the strict consensus of the two most parsimonious trees. Numbers above branches are boostrap frequencies (>50%); those below are posterior probabilities ($\geqslant$95%). Branch lengths are proportional to the number of steps along the branch. Twelve resolved fine-scale lineages within Pezizaceae (of 14 identified in the combined trees, Figs. 4 and 5 and Table 5), and five supported more inclusive clades A, C, D_1, E, and F, are indicated for discussion in the text. Dashed brackets indicate resolved sub-lineages -a and -b of the *Peziza* s. str. lineage.

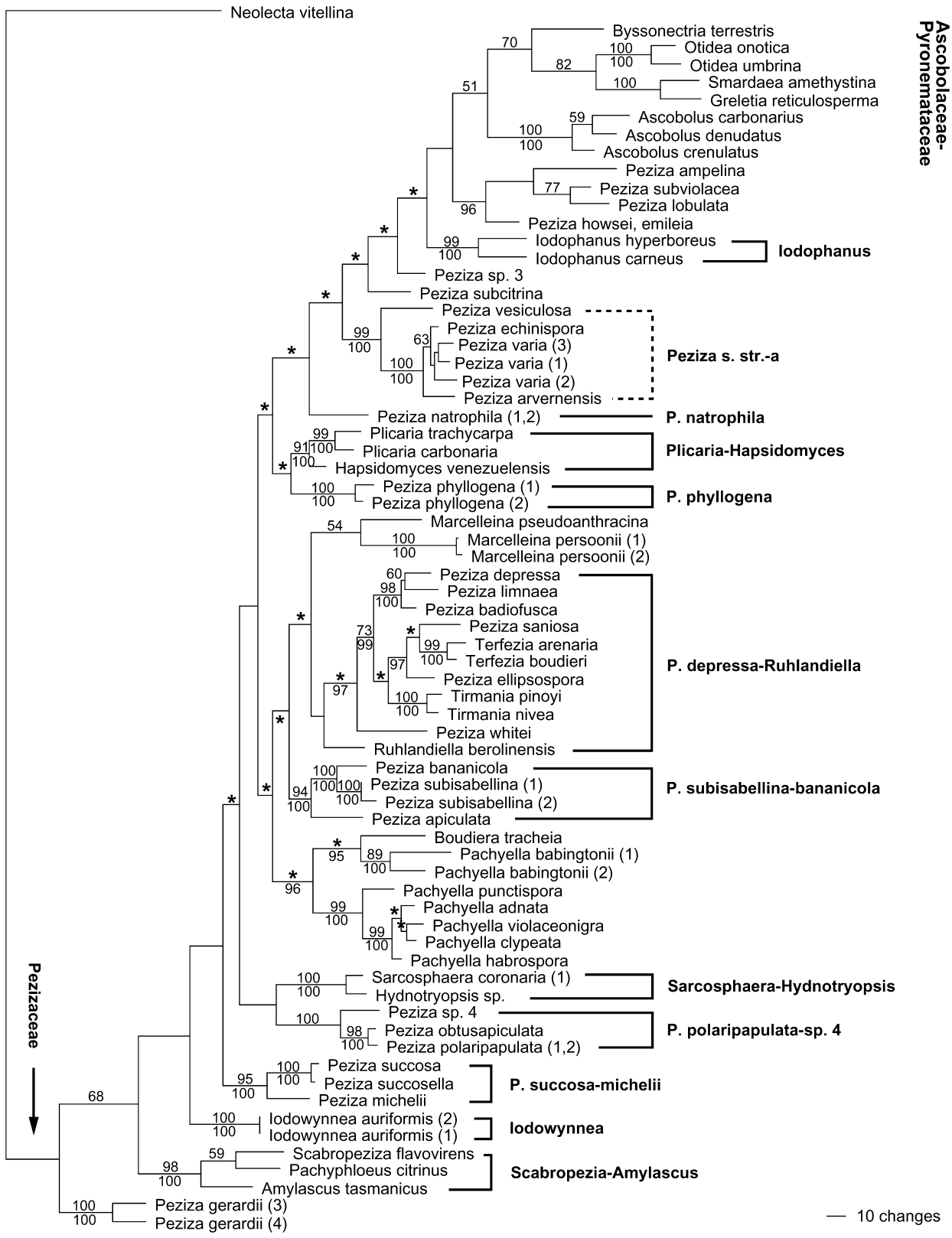

Fig. 3. Phylogeny of the Pezizaceae inferred from β-tubulin sequences, showing one of five equally most parsimonious trees. Branches with asterisks collapse in the strict consensus tree of the five most parsimonious trees. Numbers above branches are boostrap frequencies (>50%); those below are posterior probabilities ($\geqslant$95%). Branch lengths are proportional to the number of steps along the branch. Eleven resolved fine-scale lineages within Pezizaceae (of 14 identified in the combined trees, Figs. 4 and 5, Table 5) are indicated for discussion in the text. Dashed bracket indicate resolved sub-lineage-a of the *Peziza* s. str. lineage.

str.-a sub-lineage is highly supported (BP 99%), congruent with LSU data. The *Peziza* s. str.-b sub-lineage is resolved, excluding *P. subcitrina*, but with BP <50%.

Two optimal MLTs were found with a log likelihood score of −10833.3334 (Table 3), differing only in the length of the branches leading to *M. persoonii* and *P. gerardii*, respectively. The MLT recovered all 14 lineages, except for the *P. polaripapulata*-sp. 4 lineage (Table 5, tree not shown). Bayesian analyses shows, as MP analysis, a large polytomy, but identified 12 of the 14 lineages with PP ⩾96% (the *P. depressa–Ruhlandiella* lineage, excluding *Ruhlandiella*) (Table 5, tree not shown). *Peziza* s. str. did not form a monophyletic group, but the sub-lineage-a was identified with PP of 100% and the sub-lineage-b, excluding *P. subcitrina*, was identified with PP of 96%. Contrary to MP analyses, ML, and Bayesian analyses identified *P. gerardii* and *Marcelleina*, and *Boudiera* and *Pachyella*, respectively, as monophyletic (PP 100 and 96%). Overall, Bayesian analysis records 30 clades with PP ⩾95% (Table 6).

3.6. Combined LSU, RPB2, and β-tubulin phylogeny

The phylogenies derived from the separate LSU, RPB2, and β-tubulin data sets did not possess any "hard" incongruence and were therefore combined. The RPB2 region accounts for the greatest number of potentially parsimony informative characters within the combined dataset (58.95%), followed by the 5′ portion of the LSU rDNA (22.56%) and the β-tubulin region (18.49%). RPB2 sequences possess ca. 1.48 times more phylogenetic signal than β-tubulin and LSU sequences (Table 6). The performance of the MPTs, with regard to the number of pezizaceous clades with BP ⩾75%, was increased when the data were combined (42 clades vs. 28, 29, and 25; Table 6). A small number of these clades (potentially four or five), compared to the number of clades in the RPB2 and β-tubulin trees, may be resolved due to the larger number of pezizaceous sequences included from the LSU data set (68 pezizaceous sequences in the combined data set vs. 61 in the RPB2 and 60 in the β-tubulin data sets). The numbers of sequences included in the LSU and combined data set differed only by one taxon (Table 6). Also the number of pezizaceous clades with Bayesian PP ⩾95% was increased when the data were combined (52 clades compared to 41, 31, and 30 for separate analyses of RPB2, LSU, and β-tubulin; Table 6).

Parsimony analyses of all three genes yielded six MPTs (see Table 6). The strict consensus tree is nearly completely resolved; the six MPTs differ only in minor arrangements between closely related species of *Terfezia* (Tul. & C. Tul.) Tul. & C. Tul. (*T. arenaria*, *T. boudiera*, and *T. claveryi*) and between three closely related species of *Peziza* (*P. badiofusca*, *P. depressa*, and *P. limnaea*) within the *P. depressa–Ruhlandiella* lineage (Fig. 4). The Pezizaceae are supported as monophyletic with moderate support (BP 81% and PP 100%), as suggested by the LSU data (BP 100% and PP 100%). The placement of the root is resolved, but without support (BP <50% and PP 76%). In all analyses of the combined data Ascobolaceae are placed most basally, followed by a Pyronemataceae/Morchellaceae clade, as successive sisters to Pezizaceae (Figs. 4 and 5).

The 14 fine-scale lineages are resolved with BP of 78–100%, except for the *Peziza* s. str. lineage that has no support (BP <50%) (Table 5 and Fig. 4). The *Peziza* s. str.-a sub-lineage is highly supported (BP 100%) and the -b sub-lineage, excluding *P. subcitrina*, is with moderate support (BP 84%). None of the separate data sets resolve *Peziza* s. str. as monophyletic using MP. Bootstrap support for the 14 lineages in the three-gene tree is in all cases identical or higher than obtained in the separate analyses. The bootstrap support for the *P. depressa–Ruhlandiella* lineage was notable raised from weak (67, 71, and <50%) in analyses of the individual data sets, to strong support in the combined analyses (BP 89%) (Table 5). Parsimony analyses of RPB2 and β-tubulin separately do not identify the *Boudiera-Pachyella* lineage as monophyletic, but both ML and Bayesian analyses do (β-tubulin PP 96% and RPB2 PP 68%). The combined data resolve the *Boudiera–Pachyella* lineage with similar support as the LSU data (BP 75 and 78%). The combined data shows moderate support (BP 84%) for a close relationship between *P. apiculata* and *P. subisabellina–bananicola*, whereas β-tubulin shows high support (BP 94%). This relationship was not resolved in MP analyses of LSU, but analyses using ML and a Bayesian approach supports this (100% PP). No RPB2 sequence was available for *P. apiculata*.

One optimal MLT was found with a log likelihood score of −53820.9918 (Table 3). ML and Bayesian analyses of the combined data identified all 14 fine-scale lineages with PP of 100% (Table 5 and Fig. 5). The *Peziza* s. str. sub-lineages -a and -b are both with significant support (PP 100 and 97%).

The relationships between the lineages are resolved with mostly weak or no MP bootstrap support. The more inclusive clade A, supported by MP analyses of RPB2 (BP 78%), is likewise supported by the three-gene data set with moderate bootstrap support (76%, Fig. 4). Bayesian analysis supports some deeper branches, congruent with the strict consensus tree of all MPTs and the ML tree (C, E, and F, PP 100 and 99%, Fig. 5). The topology within Pezizaceae, derived from ML and Bayesian analyses of the combined data, is mostly congruent with Bayesian analyses of RPB2. The placement of the *Boudiera–Pachyella* lineage differs by grouping with *Iodophanus* in separate analyses of RPB2. The more inclusive clades A–F supported by Bayesian analyses of RPB2 (Fig. 2) are likewise supported by Bayesian analyses of the three-gene data (Fig. 5), except for D_1. The D_1 node collapses in the combined tree, with *Peziza* s. str.,

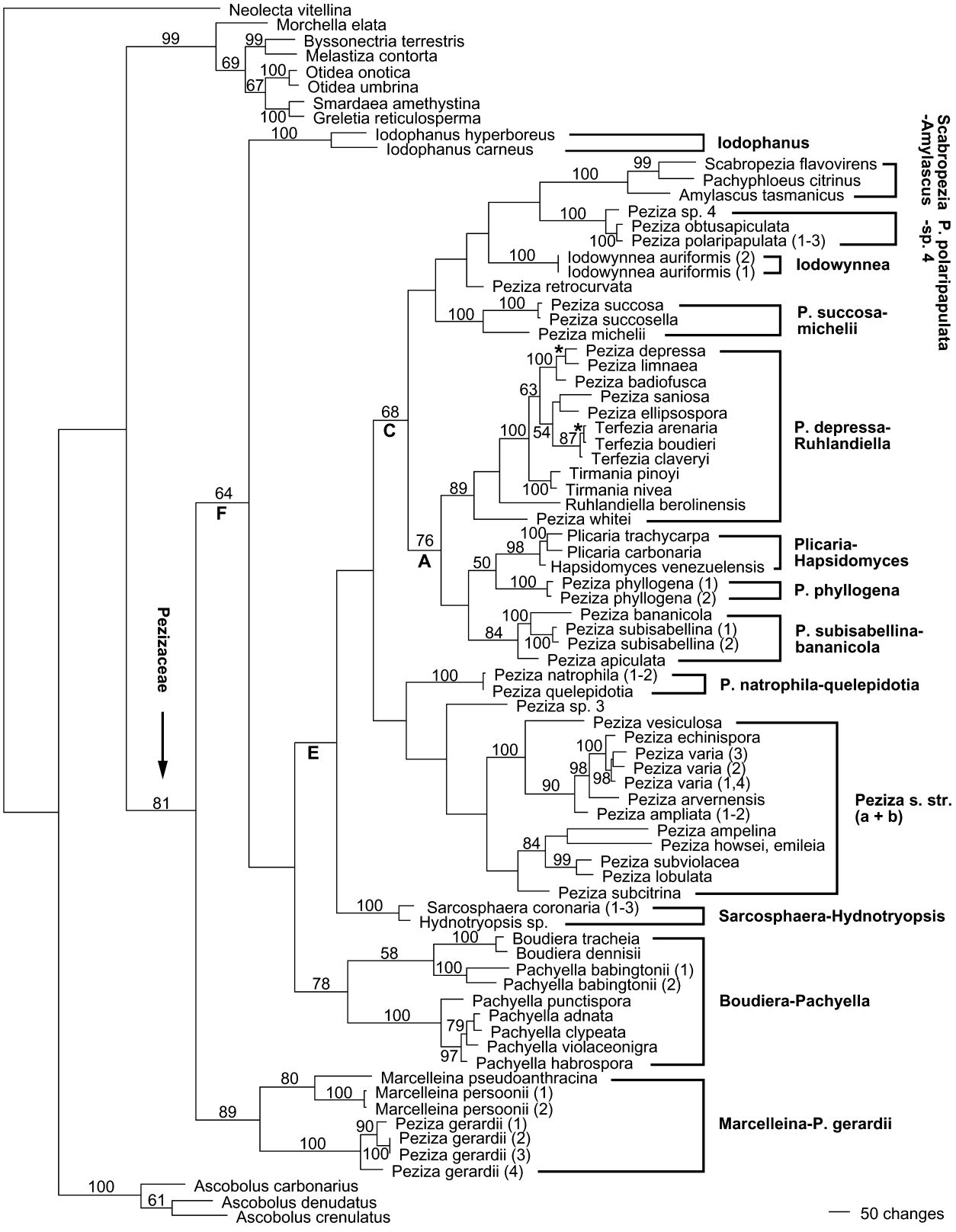

Fig. 4. Phylogeny of the Pezizaceae inferred from combined analysis of LSU rDNA, RPB2, and β-tubulin sequences, showing one of six equally most parsimonious trees. The asterisks indicate the two branches that collapse in the strict consensus tree. Numbers at branches are boostrap frequencies (>50%). Branch lengths are proportional to the number of steps along the branch. Fourteen fine-scale lineages identified within Pezizaceae, and four resolved more inclusive clades, A, C, E, and F (corresponding to the clades in Figs. 2 and 5), are indicated for discussion in the text.

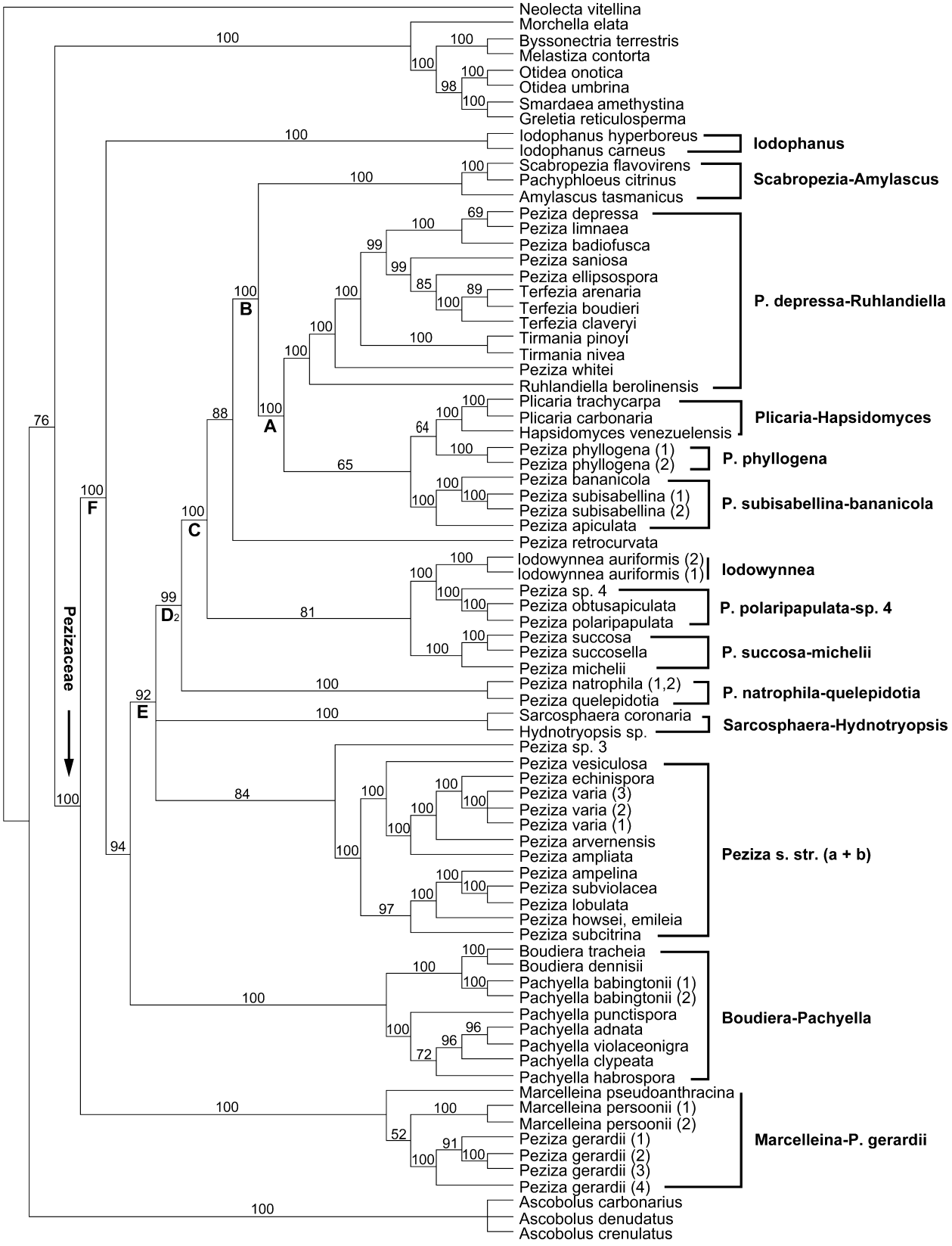

Fig. 5. Fifty percent majority consensus tree of the Pezizaceae, based on 9000 trees sampled from a Bayesian MCMC analysis of the combined LSU rDNA, RPB2, and β-tubulin data set (with each data set specified as distinct partitions). Numbers at branches are resulting posterior probabilities. Fourteen fine-scale lineages identified within Pezizaceae, and six more inclusive clades, A, B, C, D_2, E, and F (corresponding to the clades in Fig. 2), are indicated for discussion in the text.

the *Sarcosphaera–Hydnotryopsis* lineage, and clade D_2 forming a trichotomy (Fig. 5).

4. Discussion

4.1. Usefulness of RPB2, β-tubulin, and LSU rDNA

The RPB2 exons exhibit a higher percentage of potentially parsimony-informative characters than LSU and β-tubulin exons, which exhibit similar amounts (Table 6). Analyses of RPB2 resulted in a slightly more resolved MP strict consensus tree than LSU with a few more pezizaceous clades with higher bootstrap support. The LSU data set includes six pezizaceous sequences that are not available in the RPB2 data set, and these sequences result in two clades with BP $\geqslant$75% in all MPTs of the LSU data (the *Iodowynnea* lineage and a *Terfezia* clade). Taking this into account, MP of RPB2 records three more clades with BP $\geqslant$75% than the LSU data (29 compared to 26). At deeper level relationships RPB2 did not perform notable better than LSU data using MP; only one more inclusive clade received moderate support (clade A; BP 78%). Using a Bayesian approach however, RPB2 recovered 10 more clades with PP $\geqslant$95% than did LSU, and resolved several more inclusive clades with confidence (A–F in Fig. 2).

The β-tubulin region had lower resolving power than LSU and RPB2, but was also the shortest of the three regions (Table 6). Thus, fewer potentially parsimony-informative sites were obtained in the β-tubulin data set, despite the fact that the level of phylogenetic signal in the β-tubulin data is comparable to the level found in the LSU data (36 and 35% informative characters; Table 6). Nevertheless, the inability of the β-tubulin data to resolve the higher level relationships and the erroneous placement of the Ascobolaceae and Pyronemataceae within the Pezizaceae (Fig. 3) is most likely due to saturation of third codon position in β-tubulin among the most divergent taxa (based on the assumption that silent substitutions in third codon positions in protein coding genes occur so rapidly that they essentially become randomized, i.e., saturated, especially for relatively deep divergences, compared to slower-evolving first and second codon positions). Therefore, it could be argued that the β-tubulin third codon positions should be excluded or downweighted. Since, however, almost all phylogenetic signals, as measured by resolution and levels of support, are due to changes in third positions, with almost no signal in first and second codons, we will argue that third codon positions should not be excluded or downweighted. A similar pattern of phylogenetic signal and saturation, in third vs. first and second codon positions in β-tubulin, was found at the inter-generic level of lichen-forming Porpidiaceae (Buschbom and Mueller, 2004). In addition, studies of other protein coding genes have shown that rapidly evolving and putatively saturated or homoplastic third codon positions may sometimes contain most of the phylogenetic structure in a data set (e.g., Björklund, 1999; Källersjö et al., 1999; Simmons et al., 2002; Wenzel and Siddall, 1999). Although third positions appear to be misleading in the placement of the root in our analyses, none of those branches are with support, and third positions contribute phylogenetic information at the "fine-scale" level within the Pezizaceae. Judging from the CI and RI values the third positions do have more homoplasy than first and second positions; the CI and RI are highest for first and second codon positions for both β-tubulin and RPB2, and are overall similar for the two genes (Table 6). The CI and RI are slightly higher for LSU data compared to the coding genes with all sites included.

The transition/transversion ratio in the β-tubulin region is high (3.06) compared to LSU and RPB2, with a very strong bias toward C–T transitions (53% of the transitions). An even higher transition/transversion ratio in β-tubulin exons was reported within *Fusarium* (7.34 for the *G. fujikuroi* species complex, O'Donnell et al., 1998). A lower AT base composition, as in our data set, was also reported in the β-tubulin exons within *Fusarium* (44% compared to 47% in the Pezizales sequences). RPB2 and LSU rDNA have identical AT/CG composition, with a lower CG content. The transition/transversion ratio in RPB2 was lower than in LSU rDNA (1.76 compared to 2.14). Differential weighting schemes in parsimony analyses based on the ratio of transition and transversion frequencies have been advocated (e.g., Hillis et al., 1994), so that potentially homoplastic transitions will have less influence on the topologies recovered. Nonetheless, we did not apply a transition–transversion weighting scheme in MP analyses of the β-tubulin region, because recent work has shown that transitions, although more homoplastic, can provide more phylogenetic information than transversions (Broughton et al., 2000).

Four introns are present in the RPB2 data set, but presence or absence of these introns seems random across the Pezizales. This confirms the results by Liu et al. (1999), that introns in RPB2 may have been gained and lost frequently over the course of fungal evolution. On the contrary, Landvik et al. (2001) used gains and losses of introns in the β-tubulin region as phylogenetic characters within the Ascomycota. The introns within the β-tubulin region we sequenced, also seem to be phylogenetically informative at higher levels; the third intron is unique to the in-group taxa (Pezizales), and the fourth intron is present in all Pezizaceae and Ascobolaceae taxa sequenced, but not in *Byssonectria* (sequences of other representatives of Pyronemataceae ended before the fourth intron site).

The first and second introns are present in *Neolecta* and not in Pezizales.

Overall the RPB2 appeared to be the most informative single gene region based on resolution and clade support (Tables 5 and 6). Not surprisingly, the RPB2 trees were also the most congruent with the combined trees. The LSU rDNA sequences were almost as useful as the RPB2 region, but had fewer potentially parsimony-informative characters and therefore resulted in a less resolved strict consensus tree. Within the Pezizales the LSU region is however, much easier to amplify than the RPB2 region when using DNA from (dried) fungal fruitbodies. The phylogenetic resolution within our set of β-tubulin data is low and other alternative protein coding genes, such as RPB1 or EF-1, may prove to be more useful for phylogenetic studies at inter-generic levels in ascomycetes. The three-gene data set performed best overall, with more resolved clades, higher bootstrap support, and higher Bayesian posterior probabilities (Tables 5 and 6).

4.2. Limits and relationships of Pezizaceae

Based on our LSU results (Fig. 1), a higher-level phylogenetic study of Pezizales using SSU rDNA sequences (Landvik et al., 1997), and the synapomorphic amyloid reaction of the ascus, we consider Ascobolaceae to be the closest sister group of Pezizaceae. Pezizaceae sensu Hansen et al. (2001) is supported as monophyletic in analyses of the three-gene and LSU rDNA data sets, contrary to β-tubulin and RPB2. The relationships and limits of Pezizaceae resolved by β-tubulin and RPB2 are likely artifacts; due to the limited sampling of members of Ascobolaceae and other Pezizalean families, and a too distant outgroup. Some of the longest branches in the MPTs of RPB2 lead to the *Ascobolus* clade, Pyronemataceae–Morchellaceae, *Marcelleina*, and *P. gerardii* (Fig. 2). Ascobolaceae is only represented by species of *Ascobolus*, and the inclusion of species of *Thecotheus* Boud. and *Saccobolus* Boud. could "break up" long branches and possible change the branching pattern deep in the tree. *N. vitellina* may be a too distantly related out-group when using the protein coding genes RPB2 and β-tubulin (which are prone to third codon saturation). The branch leading to *Neolecta* in the MPTs of both RPB2 and β-tubulin are long compared to the ingroup branches (448 and 141 bp, respectively; Figs. 2 and 3), reflecting the highly divergent relationship of this taxon to the ingroup. As an alternative, a more closely related taxon, *Orbilia* Fr., which has been suggested as the sister group to the Pezizales (Harrington et al., 1999; Platt and Spatafora, 2000) could be used as an outgroup. That choice, however, would be controversial, since a recent phylogenetic study of 1551 SSU rDNA sequences from all main groups of fungi found Orbiliales to be nested within the Pezizales (Tehler et al., 2003).

4.3. Evolutionary relationships within Peziza and the Pezizaceae

Within Pezizaceae the combined tree topologies is taken as the best estimates of the organismal phylogeny, based on the strongest statement of all available molecular evidence that is reflected in higher support for the monophyly of more clades. Fourteen fine-scale lineages are recognized within Pezizaceae, in overall agreement with our previous analyses of LSU rDNA sequences (Hansen et al., 2001). In Hansen et al. (2001) we discussed seven, in some cases, more inclusive groups based on the strict consensus of the MPTs and the MLT; some of these did not receive any bootstrap support (<50%). Several of those more inclusive clades are not identified in this study. The discussion here will focus on differences and areas of improved resolution and support as compared to our previous phylogenetic study (Hansen et al., 2001). For a detailed discussion of morphological characters supporting the lineages and previous taxonomic placements of the taxa see Hansen et al. (2001).

Confirming our previous results, *Marcelleina* and the morphologically distinct *P. gerardii* form a strongly supported lineage (Figs. 4 and 5 and Table 5). *Marcelleina* does not have blueing asci in iodine solutions, as is characteristic for the Pezizaceae, but apothecial features, such as the excipulum structure and pigmentation, corroborate this grouping. The *Marcelleina–P. gerardii* lineage is suggested to be a sister group to the rest of the Pezizaceae in all analyses of the three-gene data set.

A close relationship between *Boudiera* and *Pachyella* is indicated by all data, except MP analyses of RPB2 and β-tubulin separately (Table 5). The combined data resolve the *Boudiera–Pachyella* lineage with moderate support (BP 78% and PP 100%). Species of *Boudiera* lack gelatinous excipular tissue and hyphoid hairs embedded in a gel, typical of the genus *Pachyella* (Pfister, 1973). Nevertheless, the lineage is united by asci evenly amyloid over the entire length, the overall anatomical structure of the apothecia, and the fruiting habitat, being water-soaked, regularly inundated substrates (*Pachyella* species most commonly occur on wood and *Boudiera* on sand and silt). *Pachyella* is resolved as monophyletic in all analyses of LSU (BP 74% and PP 85%) and in MP analyses of RPB2 (BP <50%). In all other analyses *P. babingtonii* forms a monophyletic group with *Boudiera* (in combined analyses BP 58% and PP 99%), as sister to a highly supported clade comprised of the rest of the *Pachyella* species. Confirming morphological observation by Pfister (1973, 1995), *Pachyella violaceonigra*, *P. clypeata*, *P. adnata*, and *P. habrospora* form a group of closely allied species in all MP analyses (Figs. 1–4). These species (along with *Pachyella megalosperma* (Le Gal) Pfister, *P. peltata* Pfister & Cand., and *P. pseudosuccosa* (Le Gal) Pfister) are united by almost identical apothecial anatomy, distinguished from one another mainly

in differences in their spore ornamentations, while *P. babingtonii* and *P. punctispora* each have distinctive excipular structures (Pfister, 1973, 1995). Pfister (1973) noted that *P. punctispora* seems to be transitional between relatively simple *P. babingtonii* and the other larger more complex species of the genus. Häffner (1992) proposed to divide *Pachyella* in three sections, with *Pachyella* section *Babingtoniae* Häffner for *P. babingtonii*. A segregation of *P. babingtonii* from *Pachyella* is suggested by our analyses. To try to obviate long internode lengths in the *Boudiera–Pachyella* lineage, additional species of *Pachyella* (now including 6 of the 10 known species) and *Boudiera* (including 2 of 10 known species) were added, since our previous study (Hansen et al., 2001). Nevertheless, some of the longest branches within Pezizaceae in the LSU and RPB2 MPTs are still found in this lineage.

The two species of *Iodophanus*, *I. carneus* and *I. hyperboreus*, form a separate lineage (BP 99-100%, PP 100%; Table 5), as a sister group to the rest of the clade F members in the three-gene analyses. This position, however, is only weakly supported (Figs. 4 and 5). *Iodophanus* is quite distinct from the rest of the ingroup; the branch uniting the two species of *Iodophanus* is 215 steps. In RPB2 ML and Bayesian analyses *Iodophanus* forms a sister group to the *Boudiera–Pachyella* lineage (not shown), and in RPB2 MP analysis *Iodophanus* forms a monophyletic group with *Boudiera* (Fig. 2), but without support (BP <50%, PP 68%). In MP analyses of first and second codon positions of RPB2, however, *Iodophanus* is placed separate as a sister group to the rest of clade F, congruent with the three-gene analyses. *Iodophanus* has been considered closely related to *Boudiera* (Korf, 1972), when placed in the tribe Iodophaneae in Ascobolaceae. Korf (1972) considered the Iodophaneae to represent the link between Pezizaceae and Ascobolaceae. *Iodophanus* species are distinct by having carotenoid pigments, thick-walled spores in early stages of development, and diffusely amyloid asci, which protrude prominently at maturity.

The majority of *Peziza* species occur in two major lineages, the *Peziza* s. str. and the *P. depressa–Ruhlandiella* lineages (Figs. 4 and 5). In addition to those sampled, many more species belong to the two lineages based on LSU rDNA sequences and morphology (see Hansen et al., 2001). *Peziza* s. str. is composed of two sub-lineages (a and b). The type species of *Peziza*, *P. vesiculosa*, is in the sub-lineage-a, and thus this lineage will have to serve as a core group for a future circumscription of the genus. *Peziza* s. str.-a was recovered, with mostly strong support, across all data sets, whether examined individually or combined using MP, ML, or Bayesian analyses (Figs. 1–5 and Table 5). *Peziza* s. str.-b was only resolved in analyses of RPB2 and the combined data, and with low bootstrap support (<50%), but with Bayesian PP of 97–100%. If *P. subcitrina* is not considered a member, *Peziza* s. str.-b is resolved in all individual data sets, although with weak or no bootstrap support. *P. subcitrina* deviates from other lineage-b members in its golden yellow apothecia and eguttulate spores. *Peziza* s. str.-b members mostly exhibit violaceous to vinaceous pigments in the apothecia and two small guttules in the spores. *Peziza* s. str.-a contains common *Peziza* species, characterized by mostly yellowish brown disc- to cup-shaped apothecia, and simple, hyaline or pale yellowish paraphyses. For a detailed morphological and molecular phylogenetic study of *Peziza* s. str.-a see Hansen et al. (2002). The two sub-lineages recall, to various degrees, previous classifications of Boudier (1907) and Le Gal (1953) at the genus and subgenus level, based mainly on presence or absence of spore guttules (see Hansen et al., 2001). Species in sub-lineage-a were placed in the genus *Aleuria* emend. Boud. non-Fuckel (= Le Gal's section "Eguttulisporæ" 1953), whereas species in sub-lineage-b were treated in both *Aleuria* and *Galactinia* (Cooke) Boud. (Boudier, 1907; Le Gal, 1953). Although none of the separate data sets resolve *Peziza* s. str. as monophyletic using MP, and the combined data resolve the lineage with no bootstrap support (<50%), ML and Bayesian analyses of the three-gene data support the lineage with PP of 100% (Table 5). In Hansen et al. (2001), including a larger sample of *Peziza* species, the lineage was resolved in individual MP analyses of LSU (but BP <50%). A distinct amyloid ring zone at the apex of the asci, a synapomorphy for *Peziza* s. str. (a + b), adds, however, strong support for the recognition of this lineage. In addition, the species are distinguished by producing mostly smooth to finely warted spores, that are eguttulate or have two small guttules, and, where known, an *Oedocephalum* anamorph state.

The *P. depressa–Ruhlandiella* lineage is supported by asci that are intensely and unrestrictedly amyloid over the apex (an ascus blueing type only shared with species in the *P. succosa–michelii* lineage); mostly highly warted, ridged to reticulate spores, with two large guttules; and anamorphs referable to *Chromelosporium*.

The *P. depressa–Ruhlandiella* lineage gained strong support in MP analyses of the three-gene data set (BP 89%) as compared to the individual data sets (Fig. 4). The lineage is also present in ML analyses of all data sets and with PP of 100% in Bayesian analyses of LSU, RPB2, and the three-gene data sets (Table 5). The *P. depressa–Ruhlandiella* lineage includes epigeous species of *Peziza* (Boudier's (1907) genus *Galactinia* in part) and several hypogeous taxa without active spore discharge. The epigeous *Peziza* species are, in addition to the above-mentioned characters, united morphologically by mostly dark brownish, disc- to cup-shaped apothecia, often with an olivaceous or purple tinge, and dark pigmented paraphyses. Corroborating previous molecular phylogenetic results (Hansen et al., 2001; Norman and Egger, 1999) the genera *Terfezia* and *Tirmania* Chatin,

so-called desert truffles (Montecchi and Sarasini, 2000), are nested within the *P. depressa–Ruhlandiella* lineage (BP 100%, PP 100% in the three-gene analyses). *Tirmania* species highly resemble *Terfezia* species in excipulum structure and in habitat, which is mostly arid and semi-arid ecosystems in the Mediterranean region (Montecchi and Sarasini, 2000), and in forming mycorrhizal symbiosis with various *Helianthemum* species (Cistaceae) (Dexheimer et al., 1985; Fortas and Chevalier, 1992). *Tirmania* species are mainly distinguished from *Terfezia* in the amyloid asci, smooth spores, and by the discoloration of the outer layer of the ascomata when handled; *Terfezia* species have non-amyloid asci and ornamented spores. As suggested in Hansen et al. (2001) the amyloid reaction of the asci might be lost in some lineages within the *Pezizaceae*, such as in the branch leading to *Terfezia* and other truffles (e.g., some species of *Pachyphloeus* Tul. & C. Tul., for example *Pachyphloeus citrinus*, and *Cazia* Trappe, the latter included in Hansen et al. (2001)). Congruent with results by Díez et al. (2002) based on ITS rDNA sequences from a larger sample of specimens, *Terfezia* and *Tirmania* are supported as monophyletic and closely related genera. Contrary to Percudani et al. (1999) and Díez et al. (2002), which indicated that *Peziza badia* Pers. is not a sister species of *Terfezia*, all of our analyses support a close relationship with species of *Peziza* in the *P. depressa–Ruhlandiella* lineage (which includes *P. badia* in Hansen et al. (2001)). Other truffle or truffle-like members of the *P. depressa–Ruhlandiella* lineage are *Peziza ellipsospora*, *P. whitei*, and *Cazia*. The hypogeous or semi-hypogeous members of the *P. depressa–Ruhlandiella* lineage show diverse ascomatal forms: ptychothecia, stereothecia, and exothecia (see Hansen et al., 2001). *Ruhlandiella* is semi-hypogeous and develops solid ascomata in which the hymenium covers the outer surface, permanently exposed (exothecia). *Ruhlandiella* and *Peziza whitei* seems to occur only with ectomycorrhizal members of the Myrtaceae (Castellano et al., 1989) and have been shown to be able to form ectomycorrhizae (Warcup, 1990; Warcup and Talbot, 1989 with *Ruhlandiella* as *Muciturbo*). The *P. depressa–Ruhlandiella* lineage could be a mycorrhizal lineage; most of the epigeous *Peziza* species occur on naked, often disturbed soil, under ectomycorrhizal forming trees.

There has been disagreement as to whether *Plicaria*, with globose spores, should be recognized as a genus separate from *Peziza*, with ellipsoid spores. Supporting previous molecular phylogenetic analyses (Hansen et al., 2001; Norman and Egger, 1996, 1999) and hypotheses based on morphology (Egger, 1987; Moravec and Spooner, 1988), a close relationship of *Plicaria* to species of *Peziza* in the *P. depressa–Ruhlandiella* lineage is moderately supported in MP and Bayesian analyses of RPB2 and the three-gene data set (in clade A; BP 78 and 76%, PP 100%, Figs. 2, 4, and 5). In both *Plicaria* and some of the *Peziza* species in the *P. depressa–Ruhlandiella* lineage spores become pale brownish at maturity, the paraphyses are encrusted with amorphous matter, and dark ascomatal pigments are present, *Chromelosporium* anamorphs (e.g., Moravec and Spooner, 1988) are found, and there is a positive tyrosine reaction (Egger, 1987). The two species of *Plicaria* in this analyses form a strongly supported monophyletic group with *Hapsidomyces venezuelensis* in MP analyses of LSU, β-tubulin, and the combined data (BP 88, 91, and 98%, respectively) (no RPB2 sequence of *Hapsidomyces* J.C. Krug & Jeng was obtained). The monotypic *Hapsidomyces* was, in contrast to our molecular results, considered closely related to *Boudiera* when described (Krug and Jeng, 1984), based on excipulum structure and asci that project beyond the hymenium at maturity. In contrast to the epigeous *Peziza* species in the *P. depressa–Ruhlandiella* lineage, *Plicaria*, and *Hapsidomyces* have asci that are weakly amyloid over their entire length and globose spores. A larger sample of *Plicaria* species is needed to explore further the relationships to *Hapsidomyces*. Of particular note is that to date, *Hapsidomyces* is the only pezizaceous coprophilous taxon outside the *Peziza* s. str.-a lineage and *Iodophanus*. The genus *Plicaria* is pyrophilous.

Peziza phyllogena constitutes a separate lineage from the epigeous *Peziza* species in the *P. depressa–Ruhlandiella* lineage (Figs. 4 and 5), despite its many shared characters. It differs by having many small ascospore guttules aggregated toward the poles, rather than two large guttules, and by fruiting on decaying wood or woody debris in wet habitats in the spring (e.g., Elliott and Kaufert, 1974 as *P. badioconfusa*).

Peziza subisabellina and *Peziza bananicola*, which form a strongly supported lineage in all analyses (BP 99–100%, PP 100%; Table 5), are two unusual species of *Peziza*. Both possess diffusely amyloid asci, a very thick and dense excipulum, nearly fusiform spores with many small guttules (*P. subisabellina*) or narrowly ellipsoid eguttulate spores (*P. bananicola*), and apothecia colors in the range of white, yellowish, rose, reddish to purplish brown. *Peziza luteorosella* belong to this lineage based on LSU rDNA sequences (unpublished data by K. Hansen). *P. bananicola* and *P. luteorosella* (Le Gal) Pfister are among the very few strictly tropical *Peziza* species known. The RPB2 and three-gene data suggest that the *P. subisabellina–bananicola* lineage is closely related to the *P. depressa–Ruhlandiella*, *P. phyllogena*, and *Plicaria–Hapsidomyces* lineages (clade A. Figs. 2, 4, and 5).

Peziza apiculata is strongly supported as a sister taxon to the *P. subisabellina–bananicola* lineage by all analyses of β-tubulin and the three-gene data set (BP 94 and 84%, respectively; PP 100%; Figs. 3–5), and by ML and Bayesian analyses of LSU (PP 100%). No RPB2 sequence was obtained from *P. apiculata*. As in our previous study (Hansen et al., 2001), MP analyses of LSU did not resolve the

position of *P. apiculata* with any confidence. *Peziza apiculata* and *P. obtusapiculata* (here supported in the *P. polaripapulata*-sp. 4 lineage), represent just two of several *Peziza* species with apiculate spores (e.g., Häffner, 1995; Moravec, 1985), placed in subgenus *Phaeopezia* Sacc., along with "pseudoapiculate spored Pezizas" such as *P. phyllogena* and *P. polaripapulata* (Häffner, 1995). A close relationship among these taxa is not supported in our analyses.

The *P. succosa–michelii* lineage has moderate to strong support (BP 82–100%, PP 99–100%; Table 5). It includes common species of *Peziza* which share features with the epigeous *Peziza* species in the *P. depressa–Ruhlandiella* lineage, such as spores with irregular elongated warts and two large guttules, asci strongly amyloid over the apex, and brownish colors in the hymenium. Nevertheless, *Peziza succosa*, *P. succosella*, and *P. michelii* possess lactiferous hyphae in the excipulum, which yield a yellow juice when cut or bruised. Some *Peziza* species in the *P. depressa–Ruhlandiella* lineage also possess lactiferous hyphae, but the juice is bluish or watery. Other species with yellowish juice, such as *P. infuscata* Quél., *P. berthetiana* Donadini, and *P. sesiana* Garofoli & Baiano (Garofoli and Baiano, 1996), may belong in this clade.

A close relationship between *Sarcosphaera* Auersw. and the truffle *Hydnotryopsis* Gilkey was suggested for the first time in our previous study (Hansen et al., 2001) and is here confirmed with strong support in both separate and combined analyses (BP 100%, PP 100%, Table 5). Likewise, a close relationship between *Scabropezia* Dissing & Pfister, and the truffles *Pachyphloeus* and *Amylascus* Trappe is strongly indicated (BP 98–100%, PP 100%). The *Scabropezia–Amylascus* lineage is supported as a sister group to clade A in Bayesian analyses of RPB2 and the three-gene data set (PP 100%, Fig. 5). The position of the *Sarcosphaera–Hydnotryopsis* lineage is uncertain. It forms a trichotomy with *Peziza* s. str. and the D_2 clade in Bayesian analyses of the three-gene data set. These results confirm that transitions to truffle and truffle-like forms evolved at least three times within the Pezizaceae (in the lineages: *P. depressa–Ruhlandiella*, *Scabropezia–Amylascus*, and *Sarcosphaera–Hydnotryopsis*).

Our results support the strictly tropical or subtropical *Iodowynnea* as distinct from the main groups of *Peziza*. It was segregated from *Peziza* based on its large, convoluted to highly folded apothecia, arising from a buried stipe, and longitudinally verrucose-striate spores (Medel et al., 1996). The ascus blueing type in *Iodowynnea* also differs from most *Peziza* species, being evenly and strongly amyloid over the entire length.

Peziza natrophila and *P. qulepidotia* form a distinct lineage. This confirms previous molecular phylogenetic results (Hansen et al., 2001; Norman and Egger, 1999), and supports the recognition of the genus *Lepidotia* Boud., for *Lepidotia hispida* (Quél.) Boud. (= *P. quelepidotia*) (Boudier, 1885). *Lepidotia* was distinguished by ellipsoid, eguttulate spores, and distinctly stipitate or obconical apothecia with triangular, submembranaceous scales. The ascus blueing type, which is weak over the entire length, supports the segregation from the main groups of *Peziza*. Bayesian analyses of the three-gene data set places the *P. natrophila–quelepidotia* lineage as a sister group to clade C (Fig. 5). As pointed out previously (Hansen et al., 2001), until further comparative studies have been carried out we cannot exclude the possibility that *P. natrophila* and *P. qulepidotia* are con-specific.

5. Concluding remarks and outlook

Analyses of the three-gene data set improve understanding of the relationships within *Peziza* and the Pezizaceae. Nevertheless, even with 3.5 kb of sequence per taxon the backbone of the Pezizaceae phylogeny remains poorly supported using MP. The superior levels of resolution and support in the Bayesian combined analyses within the Pezizaceae, congruent with ML analyses, will however, allow us to draw conclusions from the phylogenies with reasonable confidence. Most importantly it is clear that the traditional circumscription of *Peziza* must be abandoned. *Marcelleina* and *P. gerardii* are confirmed to be a sister group to the rest of the Pezizaceae. Fourteen, most highly supported, fine-scale lineages are recognized and eight of these include species of *Peziza*. Two lineages comprised of most of the *Peziza* species are supported, the *Peziza* s. str. and the *P. depressa–Ruhlandiella* lineages, the latter including several genera of truffles (e.g., *Terfezia* and *Tirmania*). The *P. depressa–Ruhlandiella* lineage is, for the first time, strongly supported by bootstrap. *Peziza* s. str. is recognized to include sub-lineage-a and -b, based on analyses of the three-gene data and the synapomorphic ascus blueing type. The analyses support several scenarios for a revised classification of *Peziza* and Pezizaceae: e.g., (1) a very wide concept of the genus *Peziza* in which all genera of the Pezizaceae, or all genera of clade E (Fig. 5), are included, or (2) a restructuring of *Peziza* resulting in the recognition of several genera. We advocate the latter and suggest the circumscription of *Peziza* be restricted to the *Peziza* s. str. lineage. Under this scenario the analyses support the continued segregation, perhaps with emendation, of several morphologically distinct smaller genera viz. *Iodophanus*, *Boudiera*, *Pachyella* (possible excluding *P. babingtonii*), *Iodowynnea*, *Sarcosphaera*/*Hydnotryopsis*, and *Scabropezia*/*Amylascus*/*Pachyphloeus*. A close relationship between *Plicaria* and the monotypic *Hapsidomyces* is here suggested for the first time. The reinstatement of the genus *Lepidotia*, for the *P. natrophila–qulepidotia* lineage is supported. *Iodophanus* is suggested to be a sister group to the rest of the Pezizaceae (exclusive of *Marcelleina* and *P. gerardii*). *Pachyella* and *Boudiera* are recognized as closely related.

The *P. subisabellina–bananicola* lineage forms, for the first time, a clade with the *P. depressa–Ruhlandiella*, *P. phyllogena*, and *Plicaria–Hapsidomyces* lineages. *Scabropezia/Amylascus/Pachyphloeus* is suggested to be a sister group to the clade of the *P. depressa–Ruhlandiella*, *P. phyllogena*, *Plicaria–Hapsidomyces* and *P. subisabellina–bananicola* lineages. In summary, this study provides a framework for a revision of *Peziza* and Pezizaceae, in which the taxonomic ranks for the various lineages will be determined based on both molecular and morphological characters. Such a revision of the Pezizaceae is under way (Hansen and Pfister, in progress).

The Pezizaceae phylogeny in this study, inferred from three unlinked loci, offers an opportunity to further study and evaluate traditionally employed morphological characters, anamorph types, and trophic states within the group. Importantly it is now possible to focus studies on monophyletic groups or subgroups. Further studies of relationships within some of the more inclusive clades, e.g., the A, B, and C clades, with a broader sampling of taxa are still needed to resolve certain generic or sub-generic boundaries.

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