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RESEARCH ARTICLE

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Species limits and relationships within *Otidea* inferred from multiple gene phylogenies

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Key words

distribution
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gene conflict
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mapping morphological features
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Abstract The genus *Otidea* is one of the more conspicuous members of the *Pyronemataceae*, with high species diversity in hemiboreal and boreal forests. The genus is morphologically coherent and in previous higher-level multigene analyses it formed a highly supported monophyletic group. Species delimitation within *Otidea* is controversial and much confusion has prevailed in the naming of taxa. To provide a phylogenetic hypothesis of *Otidea*, elucidate species diversity and limits we compiled a four-gene dataset including the nuclear LSU rDNA and three nuclear protein-coding genes (RPB1, RPB2 and EF-1α) for 89 specimens (total 4 877 nucleotides). These were selected from a larger sample of material studied using morphology and 146 ITS (ITS1-5.8S-ITS2) and 168 LSU rDNA sequences to represent the full genetic diversity. Using genealogical concordance phylogenetic species recognition (GCPSR), Bayesian and maximum likelihood analyses of the individual datasets resolved 25 species of *Otidea*. An additional eight singletons are considered to be distinct species, because they were genetically divergent from their sisters. Sequences of multiple genes were included from 13 holotypes, one neotype and three epitypes. *Otidea angusta*, *O. myosotis* and *O. papillata* f. *pallidefurfuracea* are nested within *O. nannfeldtii*, *O. leporina* and *O. tuomikoskii*, respectively and are considered synonyms. *Otidea cantharella* var. *minor* is shown to be a distinct species. Five new species were discovered: *O. oregonensis* and *O. pseudoleporina* for North America; and *O. borealis*, *O. brunneoparva* and *O. subformicarum* for Europe. The analyses of the individual four gene datasets yielded phylogenies that were highly concordant topologically, except for the RPB1 that showed supported conflict for some nodes in Bayesian analysis. Excluding the RPB1 from the combined analyses produced an identical topology to the four-gene phylogeny, but with higher support for several basal nodes and lower support for several shallow nodes. We argue to use the three-gene dataset to retrieve the maximum support for the higher-level relationships in *Otidea*, but still utilise the signal from the RPB1 for the delimitation and relationships of closely related species. From the four gene regions utilised, EF-1α and RPB1 have the strongest species recognition power, and with higher amplification success EF-1α may serve as the best secondary barcoding locus for *Otidea* (with ITS being a primary). The phylogeny from the three- and four-gene datasets is fully resolved and strongly supported in all branches but one. Two major clades, as part of six inclusive clades A–F, are identified – and ten subclades within these: A) *O. platyspora* and *O. alutacea* subclades, and B) *O. papillata*, *O. leporina*, *O. tuomikoskii*, *O. cantharella*, *O. formicarum*, *O. unicisa*, *O. bufonia-onotica* and *O. concinna* subclades. Morphological features in *Otidea* appear to be fast evolving and prone to shifts, and are poor indicators of higher-level relationships. Nevertheless, a conspicuous spore ornament is a synapomorphy for the *O. unicisa* subclade (/*Otideopsis*); all other species in *Otidea* have smooth or verruculose (in SEM) spores. Exclusively pale to bright yellow apothecia and straight to curved, broadly clavate to distinctly capitate paraphyses are synapomorphies for a restricted *O. concinna* subclade (/*Flavoscypha*). The curved to hooked apices of the paraphyses is suggested to be a symplesiomorphic trait for the genus. The reaction of resinous exudates on the outermost excipular cells that coalesce into amber drops in Melzer's reagent is likely an ancestral state for clade B. We estimate that *Otidea* consists of 47 species worldwide, based on all available information (including morphology, ITS or LSU sequences, and literature descriptions). Three fifths of the species occur in Europe, with 20 species recognised as endemic. At least 14 species occur in North America and 17 in Asia, with eight and ten species considered endemic to each continent, respectively. Our knowledge about *Otidea* in Asia is still fragmentary and the diversity likely much higher.

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INTRODUCTION

Otidea is one of the more conspicuous members of the *Pyronemataceae* (*Pezizomycetes*), and contrary to most other members of the family, *Otidea* species generally fruit in non-disturbed habitats. They are restricted to the Northern Hemisphere and considered ectomycorrhizal. The diversity and abundance of *Otidea* is high in hemiboreal and boreal forests, both in *Picea*, *Pinus* and deciduous forests, on rich or calcareous as well as poor soil, on the ground or on plant debris. The genus is monophyletic and morphologically distinct (Hansen et al. 2013). The species produce large, 0.3–7.5 cm high apothecia, typically in rows or half rings. The apothecia are ear-shaped, i.e. split down to the base in one side, sometimes strongly elongated on the side opposite the split, narrowly ear-shaped or fan-shaped (Fig. 1a, b), cup-shaped (Fig. 1c–f), or as in a single species, closed and hypogeous (a truffle-like form). Within *Pezizomycetes*, ear-shaped apothecia are only otherwise present in the genera *Wynnella* (*Helvellaceae*) and *Wynnea* (*Sarcoscyphaceae*). Species limits within *Otidea* are highly problematic and no monograph exists. The delimitation of species has chiefly relied on apothecia shape, size, colour and appearance of the outer surface, along with characters of the spores and paraphyses (e.g. Cao et al. 1990, Dissing 2000). Accurate spore dimensions subjected to statistical methods have been proposed to discriminate species (Raitviir 1972). In addition, the colour

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Fig. 1 Diversity of apothecial shapes and colours in *Otidea*, showing exemplar character states reconstructed. a. Long, narrowly ear-shaped apothecia, *O. tuomikoskii* (Carbone & Vauras 26954F, TUR-A); b. broadly ear-shaped apothecia, *O. leporina* (KH.11.33, S); c–e. cup-shaped, split apothecia: c. *O. alutacea* clade 1 (KH.10.193, S); d. *O. mirabilis* (KH.09.188, S); e. *O. concinna* (KH.10.180, S); f. cup-shaped without a split, *O. propinquata* (KH.09.94, S).

of the basal tomentum has been considered an informative character (Harmaja 1976). Most recently high importance was given to the reaction of the excipular resinous exudates ('encrusted pigment') in Melzer's reagent to distinguish some species (Harmaja 2009). Despite the introduction of new characters and descriptions of several new species (e.g. Cao et al. 1990, Zhuang & Yang 2008, Harmaja 2009, Zhuang 2010) species diversity has been overlooked, and delimitations and identifications remained controversial. The variation of morphological features has not previously been adequately investigated using molecular characters. Peterson (1998) studied *Otidea* in the Pacific Northwest of North America using phylogenetic analyses of LSU and ITS rDNA sequences. He recognised eight species, but did not make comparative studies with European or Asian material. Liu & Zhuang (2006) studied the relationships among some species of *Otidea*, also using LSU rDNA sequences, but apart from a single Danish collection of *O. onotica*, they included only sequences from China and North America (from Peterson 1998). They concluded *Flavoscypha* and *Otideopsis*, taxa previously segregated from *Otidea*, are congeneric with *Otidea* (but see further on *Flavoscypha* under Discussion), and changed the rank of *Otideopsis* to subgenus, based on *O. yunnanensis*, and included also *O. unicisa* (as *O. grandis*). The study (Liu & Zhuang 2006) does not present any coherent species identification tools or descriptions. To provide a monograph of *Otidea* we have collected and studied fresh material, primarily in Sweden, and obtained material from other places in Europe, North America and Asia. The goals of this study were to:

1. resolve species limits within *Otidea* using genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al. 2000);
2. use the combined multi-gene dataset (LSU, RPB1, RPB2 and EF-1α) to provide a robust hypothesis for relationships within *Otidea*;
3. use comparative morphological studies to provide insight into evolutionary trends in morphological features and tree association; and
4. give insight into the geographical distribution of the species.

Our detailed species descriptions, illustrations and a key for identification are given in Olariaga et al. (2015).

MATERIALS AND METHODS

Taxon sampling

To obtain an estimate of *Otidea* genetic diversity we generated 112 ITS and LSU sequences using standard methods, and obtained 34 ITS and 57 LSU sequences from GenBank (total 146 ITS, 169 LSU), from a total of 171 *Otidea* collections. A larger number of collections was studied morphologically (450 collections). From these a subset of 89 collections was chosen, to represent the full range of phylogenetic diversity sampled, for a four-locus dataset comprising portions of the LSU rDNA, RPB1, RPB2 and EF-1α (Table 1). Of the 89 collections a large part included fresh or recent material to facilitate the amplification of the protein-coding genes, but also dried material (from 1948–2010). Two outgroup taxa, *Monascella botryosa* and *Warcupia terrestris*, were included for rooting purposes based on previous results, which support these as the closest sister group to *Otidea* (Hansen et al. 2013).

Molecular techniques

DNA was isolated from fresh (stored in 1 % SDS extraction buffer) or dried ascomata, and extracted as in Hansen et al. (1999), with the exception that fresh material was ground directly in an Eppendorf tube and dried material was shaken in a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK, USA) at 4 500 RPM for 20 s. The DNA was re-suspended in 35 µL water and dilutions 1 : 10 and/or 1 : 100 were used for PCR amplification. The following five gene regions were amplified: ITS1-5.8S-ITS2 and the 5' end of the nLSU rDNA, spanning domains D1 and D2, part of the nuclear genes that encode the two largest subunits of RNA polymerase II (RNA polymerase I (RPB1), A–C region, c. 700 bp (Matheny et al. 2002); and RNA polymerase II (RPB2), 6–11 region, c. 1 700 bp (Liu et al. 1999, Hansen et al. 2005)), and nearly the complete coding region of translation elongation factor 1-alpha (EF-1α, c. 1 000–1 500 bp; Rehner & Buckley 2005). PCR and sequencing primers

Table 1 Collections used in the molecular phylogenetic study, with voucher information and GenBank accession numbers. Numbers in parentheses following species names indicate multiple collections of a single species. Sequences generated in this study are in **bold**. For type specimens the original names are given here regardless of the synonymy shown in this study.

Species	Collection no. (Herb.) or Herb. / Culture coll. no.[1]	Putative host trees	Geographic origin, Year and Collector	GenBank accession no.[6]				
				ITS	LSU	EF-1α	RPB1	RPB2
Monascella botryosa	CBS 233.85; Type		Spain, 1985, J. Guarro	–	KC012688[2]	KC109256[2]	JX943733[2]	JX943831[2]
Otidea alutacea (1)	KH.09.133 (S)	*Corylus*, *Picea*	Norway, 2009, K. Hansen & I. Olariaga	**KM010071**	**KM823185**	**KM823253**	**KM823328**	**KM823381**
O. alutacea (2)	ARAN A3023204	*Tilia*	Spain, 2009, J.I. López Amiano	**KM010072**	**KM823186**	**KM823254**	**KM823329**	**KM823382**
O. alutacea (3)	KH.09.170 (S)	*Quercus robur*, *Picea abies*, *Corylus*, *Salix*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010059**	KC012691[2]	KC109261[2]	JX943732[2]	JX943830[2]
O. alutacea (4)	JS.08.81 (S)	*Quercus*	Sweden, 2008, J. Santos	**KM010062**	**KM823187**	**KM823255**	**KM823330**	**KM823383**
O. alutacea (5)	KH.10.193 (S)	*Corylus*, *Quercus*	Sweden, 2010, K. Hansen, K. Gillen & I. Olariaga	**KM010060**	**KM823188**	**KM823256**	**KM823331**	–
O. alutacea (6)	OSC 56747	*Pseudotsuga menziesii*, *Tsuga*, *Picea*, *Calocedrus*	USA, WA, 1996, E.T. Peterson	–	**KM823189**	**KM823257**	–	–
O. alutacea (7)	KH.09.135 (S)	*Corylus*, *Picea*	Norway, 2009, V. Kučera & I. Kautmanova	**KM010064**	**KM823190**	**KM823258**	**KM823332**	**KM823384**
O. alutacea (8)	KH.09.178 (S)	*Corylus*, *Populus*, *Picea*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010066**	**KM823191**	**KM823259**	**KM823333**	**KM823385**
O. alutacea (9)	S-F257085	*Quercus ilex*	Italy, 2010, M. Carbone	**KM010069**	**KM823192**	**KM823260**	**KM823334**	**KM823386**
O. alutacea (10)	OSC 56758	*Pseudotsuga menziesii*	USA, OR, 1996, E.T. Peterson	–	**KM823193**	**KM823261**	**KM823335**	–
O. alutacea (11)	Moorefun19 (OSC)	*Pseudotsuga menziesii*	USA, OR, 2010, J. Moore	**KM010070**	**KM823194**	**KM823262**	**KM823336**	**KM823387**
O. angusta	H6010804; holotype	Mixed woods with *Picea*, *Betula*, *Corylus* etc.	Finland, 1965, H. Harmaja	**KF717574**	**KM823195**	**KM823263**	**KM823337**	**KM823388**
O. apophysata	Herb. FK s.n., dupl. S-F257062	*Populus canadensis* and other hygrophilous trees	Germany, 1999, F. Kasparek	**KM010077**	**KM823196**	**KM823264**	**KM823338**	**KM823389**
O. borealis	S-F242694; holotype	*Picea abies*	Finland, 2010, M. Carbone	**KM010023**	**KM823197**	**KM823265**	**KM823339**	**KM823390**
O. brunneoparva (1)	KH.09.82 (S)	*Picea* forest	Sweden, 2009, K. Hansen & I. Olariaga	**KM010029**	**KM823198**	**KM823266**	**KM823340**	**KM823391**
O. brunneoparva (2)	S-F257086, dupl. TUR-A 198579	*Picea*, *Betula*	Finland, 2009, M. Carbone	**KM010025**	**KM823199**	**KM823267**	**KM823341**	**KM823392**
O. brunneoparva (3)	KH.08.107 (S); holotype	*Picea*, *Pinus*	Sweden, 2008, K. Hansen	**KM010026**	**KM823200**	**KM823268**	**KM823342**	**KM823393**
O. bufonia (1)	KH.09.248 (S)	*Quercus faginea*, *Q. rotundifolia*	Spain, 2009, J.L. Teres & P.M. Pasaban	JN942766[3]	JN941084[3]	**KM823269**	JQ012818[3]	**KM823394**
O. bufonia (2)	NV 2009.11.01 (S)	*Pinus*, *Cupressus*	France, 2009, G. Moyne	JN942765[3]	JN941085[3]	**KM823270**	JQ012817[3]	**KM823395**
O. bufonia (3)	KH.09.249 (S)	*Pinus pinaster*	France, 2009, J.L. Teres	**KM010079**	**KM823201**	**KM823271**	**KM823343**	**KM823396**
O. bufonia (4)	KH.09.172 (S)	*Quercus robur*, *Picea abies*, *Corylus*, *Salix*	Sweden, 2009, K. Hansen & I. Olariaga	JN942764[3]	JN941097[3]	**KM823272**	JQ012828[3]	**KM823397**
O. bufonia (5)	KH.07.37 (S)	*Fagus*	Denmark, 2007, K. Hansen & I. Olariaga	JN942767[3]	JN941098[3]	KC109262[1]	JQ012829[3]	JN993552[2]
O. caeruleopruinosa (1)	H6010805; holotype	Predominantly deciduous woods (*Quercus rubur*, *Corylus avellana* etc.)	Finland, 1978, H. Harmaja	**KF717575**	**KM823202**	**KM823273**	**KM823344**	**KM823398**
O. caeruleopruinosa (2)	MT 10082601 (dupl. S)	*Corylus avellana*, *Betula verrucosa*, *Buxus sempervirens*	Spain, 2010, M. Tabarés & S. Santamaría	**KM010030**	**KM823203**	–	**KM823345**	**KM823399**
O. cantharella (1)	NV 2008.09.16 (dupl. S)	*Picea abies*	France, 2008, J. Cavet	**KM010085**	**KM823204**	–	**KM823346**	**KM823400**
O. cantharella (2)	KH.09.125 (S); neotype	*Picea*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010084**	**KM823205**	**KM823274**	**KM823347**	**KM823401**
O. concinna (1)	KH.09.183 (S); epitype	*Quercus robur*, *Populus*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010032**	JN941089[3]	**KM823275**	JQ012832[3]	**KM823402**
O. concinna (2)	KH.09.250 (S)	*Quercus rotundifolia*, *Q. humilis*	Spain, 2009, F. Prieto & A. González	JN942775[3]	JN941095[3]	**KM823276**	JQ012825[3]	**KM823403**
O. daliensis	SEST-06081702	*Populus nigra*	Spain, 2003, J.L. Pérez Butrón	**KM010086**	**KM823206**	**KM823277**	**KM823348**	**KM823404**
O. flavidobrunneola (1)	KH.09.153 (S)	*Corylus*, *Picea*	Norway, 2009, K. Hansen & I. Olariaga	**KM010088**	**KM823207**	–	**KM823349**	**KM823405**
O. flavidobrunneola (2)	H6010830	*Quercus*	Finland, 1987, P. Askola	**KM010087**	**KM823208**	**KM823278**	–	**KM823406**
O. flavidobrunneola (3)	H6010806; holotype	Predominantly deciduous woods (*Quercus robur*, *Corylus avellana* etc.)	Finland, 1978, H. Harmaja	**KF717576**	**KM823209**	**KM823279**	**KM823350**	**KM823407**
O. formicarum (1)	S-F244372 (dupl. O)	*Picea*	Norway, 2009, J. Lorås	**KM010034**	**KM823210**	**KM823280**	**KM823351**	**KM823408**
O. formicarum (2)	H6003549; holotype	Spruce forest	Finland, 1970, L. Fagerström	**KF717577**	**KM823211**	**KM823281**	–	**KM823409**
O. formicarum (3)	JS.08.63 (S)	Spruce	Sweden, 2008, J. Santos	**KM010035**	**KM823212**	**KM823282**	–	–
O. kaushalii	T. Læssøe 6236 (C, dupl. BORH)	On rotten wood, *Fagaceae* (incl. *Castanopsis* and possible *Quercus*)	Malaysia, 1999, T. Læssøe	**KM010119**	AF335111[4]	**KM823326**	**KM823379**	**KM823455**
O. leporina (1)	KH.09.93 (S); epitype	*Picea abies*, *Pinus sylvestris*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010090**	**KM823213**	**KM823283**	**KM823352**	**KM823410**
O. leporina (2)	NV 2008.09.28 (dupl. S)	*Picea*	France, 2008, N. Van Vooren	**KM010092**	**KM823214**	**KM823284**	**KM823353**	**KM823411**
O. leporina (3)	OSC 56784	–	USA, OR, 1997, E.T. Peterson	–	**KM823215**	**KM823285**	–	**KM823412**
O. leporina (4)	OSC 56824	–	USA, CA, 1997, E.T. Peterson	–	**KM823216**	**KM823286**	–	**KM823413**
O. minor (1)	KH.98.84 (C)	Deciduous trees	Denmark, 1998, K. Hansen	**KM010041**	**KM823217**	**KM823287**	–	**KM823414**
O. minor (2)	KH.10.311 (S)	*Pinus sylvestris*	Sweden, 2010, K. Hansen, K. Gillen & I. Olariaga	**KM010042**	**KM823218**	**KM823288**	**KM823354**	**KM823415**
O. minor (3)	H6008618	*Acer*, *Betula*, *Populus tremula*, *Salix caprea*, *Sambucus*, *Sorbus*	Finland, 1992, R. Saarenoksa	**KM010039**	**KM823219**	**KM823289**	–	**KM823416**

O. minor (4)	CL 950914-01 (dupl. S)	*Quercus cerris*, *Pinus laricio* var. *calabrica*	Italy, 1995, C. Lavorato	**KM010044**	**KM823220**	–	**KM823355**	–
O. mirabilis (1)	KH.09.188 (S)	*Pinus sylvestris*, small *Quercus robur* plants, *Helianthemum* sp.	Sweden, 2009, E. Bohus-Jensen, K. Hansen & I. Olariaga	JN942770[3]	JN941086[3]	–	JQ012821[3]	**KM823417**
O. mirabilis (2)	KH.10.285 (S)	*Pinus sylvestris*	Sweden, 2010, K. Hansen, K. Gillen & I. Olariaga	**KM010094**	**KM823221**	–	**KM823356**	**KM823418**
O. mirabilis (3)	KH.01.09 (C)	*Picea*	Denmark, 2001, C. Lange	JN942769[3]	AY500540[5]	**KM823290**	JQ012820[3]	**KM823419**
O. mirabilis (4)	NV 2008.09.14 (dupl. S)	*Larix*	France, 2008, J. Cavet	JN942768[3]	JN941094[3]	**KM823291**	JQ012819[3]	**KM823420**
O. myosotis	H6003548; holotype	Mixed forest	Finland, 1970, L. Fagerström	**KF717578**	**KM823222**	**KM823292**	–	**KM823421**
O. nannfeldtii (1)	KH.10.302 (S)	*Picea* and *Pinus*	Sweden, 2010, K. Hansen, K. Gillen & I. Olariaga	**KM010101**	**KM823223**	**KM823293**	**KM823357**	**KM823422**
O. nannfeldtii (2)	JS.08.103 (S)	Spruce and deciduous trees	Sweden, 2008, J. Santos	**KM010045**	**KM823224**	**KM823294**	–	**KM823423**
O. nannfeldtii (3)	S-F249387 (Ex-H6017194) = *O. lohjaënsis* nom. prov. Harmaja	Spruce (in mixed forest)	Finland, 1978, H. Harmaja	**KM010093**	**KM823225**	–	–	–
O. nannfeldtii (4)	rh101310 (OSC)	Conifers	USA, OR, 2010, R. Helliwell	**KM010100**	**KM823226**	**KM823295**	**KM823358**	**KM823424**
O. nannfeldtii (5)	NV 2008.10.01 (dupl. S)	*Abies*, *Picea*, *Fagus*	France, 2008, N. Van Vooren	**KM010099**	**KM823227**	**KM823296**	**KM823359**	**KM823425**
O. nannfeldtii (6)	H6002902; holotype	Spruce forest	Finland, 1972, C.-A. Haeggström	**KF717581**	**KM823228**	**KM823297**	–	**KM823426**
O. onotica (1)	C-F-89691	Needle trees	Denmark, 2008, H. Knudsen	JN942773[3]	JN941090[3]	–	JQ012824[3]	**KM823427**
O. onotica (2)	KH.09.136 (S)	*Corylus* and *Picea*	Norway, 2009, K. Hansen & I. Olariaga	JN942772[3]	JN941096[3]	**KM823298**	JQ012830[3]	**KM823428**
O. onotica (3)	KH.10.284 (S); epitype	*Picea* mossy forest	Sweden, 2010, K. Hansen, K. Gillen & I. Olariaga	**KP006505**	**KM823229**	**KM823299**	**KM823360**	**KM823429**
O. onotica (4)	OSC 56759	–	USA, OR, 1996, E.T. Peterson	–	JN941088[3]	**KM823300**	JQ012831[3]	**KM823430**
O. oregonensis (1)	Moorefun 31 (S)	*Pseudotsuga menziesii*	USA, OR, 2010, J. Moore	**KM010047**	**KM823230**	**KM823301**	**KM823361**	**KM823431**
O. oregonensis (2)	Moorefun 58 (OSC holotype; S isotype)	*Pseudotsuga menziesii*, *Abies concolor*	USA, OR, 2010, J. Moore	**KM010048**	**KM823231**	**KM823302**	**KM823362**	**KM823432**
O. oregonensis (3)	OSC 56745	–	USA, ID, 1996, J. Trappe	AF072089	**KM823232**	**KM823303**	**KM823363**	**KM823433**
O. papillata (1)	TUR 102134	Needle forest	Finland, 1990, T. Lindholm	**KM010105**	**KM823233**	**KM823304**	**KM823364**	**KM823434**
O. papillata (2)	H6003547; holotype	Predominantly coniferous grass-herb forest (mainly in spruce needles)	Finland, 1971, H. Harmaja	**KF717582**	**KM823234**	**KM823305**	–	**KM823435**
O. papillata f. *pallidefurfuracea*	NV 2007.09.27 (S); isotype	*Picea abies*	France, 2007, N. Van Vooren	**KF717584**	**KM823235**	–	–	–
O. phlebophora	JV06-385 (C)	*Abies*	Denmark, 2006, L. Vesterholt & J. Vesterholt	**KM010049**	**KM823236**	**KM823306**	**KM823365**	**KM823436**
O. platyspora (1)	JV06-656 (C)	*Fagus*, *Quercus*	Denmark, 2006, J. Vesterholt	**KM010108**	**KM823237**	**KM823307**	**KM823366**	**KM823437**
O. platyspora (2)	KH.09.163 (S)	*Quercus robur*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010106**	**KM823238**	**KM823308**	**KM823367**	**KM823438**
O. propinquata (1)	KH.09.99 (S)	*Picea abies*	Sweden, 2009, K. Hansen & I. Olariaga	**KM010109**	**KM823239**	**KM823309**	**KM823368**	**KM823439**
O. propinquata (2)	NV 2008.09.15 (dupl. S)	*Picea*	France, 2008, J. Cavet	**KM010111**	**KM823240**	**KM823310**	**KM823369**	**KM823440**
O. pseudoleporina (1)	OSC 56809	–	USA, WA, 1997, J. Spatafora	AF072080	**KM823241**	**KM823311**	**KM823370**	**KM823441**
O. pseudoleporina (2)	Moorefun14 (S)	*Pseudotsuga menziesii*, *Abies concolor*, *Pinus lambertiana*	USA, OR, 2010, J. Moore	**KM010113**	**KM823242**	**KM823312**	**KM823371**	**KM823442**
O. pseudoleporina (3)	rh101910 (OSC); holotype	Conifers	USA, OR, 2010, R. Helliwell	**KM010112**	**KM823243**	**KM823313**	**KM823372**	**KM823443**
O. pseudoleporina (4)	OSC 56760	–	USA, WA, 1996, E.T. Peterson	AF072081	**KM823244**	**KM823314**	**KM823373**	**KM823444**
O. rainierensis	A.H. Smith 30553 (MICH); holotype	–	USA, WA, 1948, A.H. Smith	**KF717583**	**KM823245**	**KM823315**	–	**KM823445**
O. smithii (1)	ecv3345 (S)	*Betula* and *Cedrus*	USA, CA, 2005, E. Vellinga	JN942771[3]	JN941093[3]	**KM823316**	JQ012822[3]	**KM823446**
O. smithii (2)	OSC 56799	–	USA, WA, 1997, E.T. Peterson	AF072063	JN941087[3]	**KM823317**	JQ012823[3]	**KM823447**
O. subformicarum (1)	CMP 1179, RM 1095, dupl. S-F256980	*Pinus sylvestris*	Spain, 2009, C.M. Pérez del Amo & R. Gil	**KM010053**	**KM823246**	**KM823318**	**KM823374**	**KM823448**
O. subformicarum (2)	CL 050928-30, dupl. S-F256978	*Pseudotsuga menziesii*	Italy, 2005, C. Lavorato	**KM010052**	**KM823247**	**KM823319**	**KM823375**	**KM823449**
O. aff. *subformicarum* (1)	FH301036	*Pinus teocote*, *P. montezumae*, *Arbutus jalapensis*	Mexico, 2007, M.E. Smith	**KM010056**	**KM823248**	**KM823320**	**KM823376**	**KM823450**
O. aff. *subformicarum* (2)	FH301035	*Abies* forest	Mexico, 2007, M. Hernández	**KM010055**	**KM823249**	**KM823321**	**KM823377**	**KM823451**
O. tuomikoskii (1)	KH.09.130 (S)	*Picea*, *Pinus*	Norway, 2009, K. Hansen & I. Olariaga	JN942776[3]	JN941092[3]	**KM823322**	JQ012826[3]	**KM823452**
O. tuomikoskii (2)	NV 2008.09.08 (dupl. S)	*Picea abies*	France, 2008, N. Van Vooren	JN942777[3]	JN941091[3]	**KM823323**	JQ012827[3]	**KM823453**
O. tuomikoskii (3)	H6002901; holotype	*Picea abies*	Finland, 1972, R. Tuomikoski	**KF717585**	**KM823250**	**KM823324**	–	–
O. tuomikoskii (4)	OSC 56761	Conifers	USA, OR, 1996, E.T. Peterson	AF072085	**KM823251**	**KM823325**	**KM823378**	**KM823454**
O. unicisa	KH.06.06 (FH)	–	USA, MA, 2006, L. Millman	–	KC012693[2]	KC109264[2]	JX943731[2]	JX943829[2]
Otidea sp. 'b'	KH.09.79 (S)	*Picea* forest	Sweden, 2009, K. Hansen & I. Olariaga	**KM010120**	**KM823252**	**KM823327**	**KM823380**	**KM823456**
Warcupia terrestris	CBS 891.69		Canada, 1966, J.W. Paden	–	DQ220467[4]	KC109308[2]	JX943734[2]	JX943832[2]

[1] Herbaria are cited according to acronyms in Index Herbariorum (http://sweetgum.nybg.org/ih/), except for SEST: Sociedad de Ciencias Naturales de Sestao; and the private herbaria of CMP: C.M. Pérez del Amo; RM: R. Gil; FK: F. Kasparek; CL: C. Lavorato; MT: M. Tabarés; NV: N. Van Vooren.

Published sequences generated by us: [2] Hansen et al. (2013), [3] Schoch et al. (2012), [4] Perry et al. (2007), [5] Hansen et al. (2005).

[6] ITS: Internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene of the nrDNA; LSU: 28S large subunit of the nrRNA gene; EF-1α: Translation elongation factor 1-alpha; RPB1: RNA polymerase II largest subunit; RPB2: RNA polymerase II second largest subunit.

Table 2 Newly designed *Otidea* specific primers, or previously published primers successfully used for *Otidea* in this study, for RPB1, RPB2 and EF-1α (5'–3')[1].

Locus	Primer	Reference	Sequence	PCR	Sequencing
RPB1	RPB1-Otidea-A	This study	GAGTGTCCGGGGCATTTYGG	×	
	RPB1-PyrC rev	Hansen et al. (2013)	TTCGCRCGRATRATRTCTCC	×	
	RPB1-Otidea-A2	This study	ATTGGAYGAAGTGAGTGCCAC	×	×
	RPB1-Otidea-C2	This study	GMAGTACDGTGATGAYCATCC		×
RPB2	RPB2-Otidea6F	This study	TGGGGHCTTGTTTGYCCTGC	×	×
	RPB2-Otidea7R	This study	CCCATRGCTTGCTTGCCCAT	×	×
	RPB2-Otidea-b7F	This study	TGYGARATTCACCCTAGCATGA		×
	RPB2-Otidea7F	This study	ATGGGCAAGCAAGCYATGGG	×	×
	fRPB2-11aR	Liu et al. (1999)	GCRTGGATCTTRTCRTCSACC	×	×
EF-1α	526F	S. Rehner unpubl.[2]	GTCGTYGTYATYGGHCAYGT	×	×
	EF-df	S. Rehner unpubl.[2]	AAGGATGGHCAGACYCGYGARCAYGC		×
	1567R	S. Rehner unpubl.[2]	ACHGTRCCRATACCACCRATCTT		×
	2218R	Rehner & Buckley (2005)	ATGACACCRACRGCRACRGTYTG	×	×
	1577F	Rehner & Buckley (2005)	CARGAYGTBTACAAGATYGGTGG	×	×
	EF-2F	S. Rehner unpubl.[2]	AACATGATSACTGGTACYTCC	×	
	Otidea-EF1 1567R	This study	ACTGTTCCAATACCACCRATCT	×	×
	Otidea-EF1 2F	This study	CCGTGACTTCATCAAGAACATGA	×	×
	Otidea-EF1-df	This study	AAGGAYGGYCAGACYCGTGARCAC	×	×
	Otidea-EF1-ir	This study	GCGTGYTCACGRGTCTGRCCRTC	×	

Primers designed in this study for RPB1, RPB2 and EF-1α are modified for *Otidea*; for location of most of these see Matheny et al. (2002) for RPB1, Liu et al. (1999) for RPB2 and S. Rehner unpubl.[2] for EF1-α.

[1] 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.

[2] See http://www.aftol.org/pdfs/EF1primer.pdf

for the protein-coding genes were previously published and/or newly designed *Otidea* specific primers, with the optimal primers listed in Table 2. Initially the following primers were in addition used: gRPB1-A and fRPB1-C rev (Matheny et al. 2002); and fRPB2-5F (Liu et al. 1999), RPB2-P7Fa, RPB2-P7Ra (Hansen et al. 2005), RPB2-Pyr6Fb, RPB2-Pyr7R, RPB2-Pyr7F (Hansen et al. 2013). For RPB1, *Otidea* specific internal sequencing primers were designed and these were successfully used for PCR products that showed very weak or multiple PCR bands (without gel purification) (Table 2). The sequence spanning RPB2 regions 6–11 was amplified as one piece, or two pieces when required. When amplified in one piece, the primer RPB2-Otidea-b7F was used to sequence a short part missing between regions 6–7 and 7–11 in some cases. Initially, to sequence across the RPB2 6F primer site, to be able to make a specific 6F *Otidea* primer, and in a few instances where the region 6–7 did not successfully amplify, the regions 5–7 were amplified. The EF-1α region was PCR amplified in one piece for all recent collections, or more pieces for older material using different primer combinations (Table 2). The ITS and LSU regions were amplified in one piece for DNA extracted from fresh material using the primers ITS1 or ITS5 and LR5, and otherwise as separate pieces: ITS using the ITS5 and ITS4, and in a few instances ITS1 and ITS4, ITS5 and 5.8S, or ITS3 and ITS4 (Hibbett et al. 1995, White et al. 1990); and LSU using LR0R and LR5 (or LR3) (Moncalvo et al. 2000). The same primers were used for sequencing the LSU region. The ITS was sequenced using the primers ITS1 and ITS4 and/or in a few instances ITS5, 5.8S and ITS3. PCR amplifications were performed using Illustra™ Hot Start Mix RTG PCR beads (GE Healthcare, UK) in a 25 µL volume following the manufacturer's instructions. PCRs were conducted in an Applied Biosystems GeneAmp® PCR System 9700, and 2720 Thermal Cycler. PCR amplification conditions follow Hansen et al. (2013), except a hot start of 94 °C for 4 min was added to the program for LSU and ITS, and an additional program was used for RPB1: 94 °C for 90 s, 40 cycles of 94 °C for 30 s, 55 °C for 90 s, and 68 °C for 3 min, followed by 68 °C for 5 min and a 12 °C soak. The amplified products were either directly purified using an enzymatic method with 1× Exonuclease I (Exo I) 20 u/µL and 4× FastAP™ Thermosensitive Alkaline Phosphatase 1 u/µL (Fermentas Life Sciences), or when multiple bands were amplified, products were size-fractionated on a 1 % agarose gel run in TBE buffer, stained with GelRed™ (Biotium Inc.), visualized over a UV trans-illuminator, excised and purified using QIAquick spin columns (Qiagen). Cycle sequencing reactions were conducted in a 20 µL volume (containing 1–2 µL of ABI BigDye v3.1 terminator reactions mix), and sequencing reactions were purified using the DyeEx 96 Kit (Qiagen). Electrophoresis and data collecting were done on an ABI PRISM 3100 Genetic Analyzer (ABI, Foster City, CA).

Sequence alignment and phylogenetic analyses

Sequences were edited and assembled using Sequencher v. 4.10.1 (Gene Codes Corp., Ann Arbor, MI) and deposited in GenBank (Table 1). Nucleotide sequences were aligned manually using Se-Al, v. 2.0a11 (Rambaut 2002). Each alignment of the protein-coding genes was translated to amino acids in MacClade v. 4.05 (Maddison & Maddison 2000) to determine intron positions, and for examination and refinement of the nucleotide alignment. The introns in the protein-coding genes were highly variable between the ingroup and outgroup, and could not be unambiguously aligned. Therefore the introns in *Monascella terrestris* and *Warcupia botryosa* were excluded from all analyses. The full alignment containing all four loci (LSU, RPB1, RPB2, EF-1α) is available from TreeBASE under accession no. 16681. Individual and combined analyses of the LSU, RPB1, RPB2 and EF-1α data were performed using Metropolis-coupled Markov chain Monte Carlo (MCMCMC) as implemented in MrBayes v. 3.2.1 (Ronquist & Huelsenbeck 2003, Altekar et al. 2004, Ronquist et al. 2012) and maximum likelihood-based inference (ML) as implemented in RAxML v. 7.2.6 as mpi (Stamatakis 2006). MrBayes v. 3.2.1 was run in parallel using 8 processors on a MacPro 3.1 (Quad-Core Intel Xeon). The RAxML analyses were run on the freely available Bioportal, University of Oslo (Kumar et al. 2009).

All gene regions were analysed using the nucleotide data. Each of the four gene regions (LSU, RPB1, RPB2 and EF-1α) were specified as distinct partitions, and each of the three protein-coding genes were further partitioned as:

1. first and second codon positions;
2. third codon position; and
3. introns.

Thus, each protein-coding gene was analysed with three partitions, and the concatenated three-gene (excluding RPB1 due to supported conflict among the loci) and four-gene datasets were analysed with seven and ten partitions, respectively.

The Bayesian analyses were run in parallel using model jumping (/mixed models), and with all parameter values, except branch lengths and tree topologies, unlinked. Site-specific rates were allowed to vary across partitions. Rather than selecting a substitution model using a priori model selection procedure, MrBayes v. 3.2 can (with a four-by-four nucleotide model as a component) sample across 203 possible time-reversible rate matrices according to their posterior probability, using model jumping during the MCMC simulation to integrate out the uncertainty concerning the correct substitution model (Ronquist et al. 2012). The analyses consisted of four parallel searches, each with four chains, run for 3 M generations, and initiated with random starting trees. The chains were sampled every 1 K generations from the posterior distribution. A majority rule consensus tree was assembled and the posterior probabilities (PP) were calculated from the last 75 % of the posterior tree sample (9 000 K trees). The incremental heating scheme for the analyses used the default settings in MrBayes (i.e., three heated chains and one cold chain). The default settings were also used to set unconstrained branch length and uninformative topology (uniform) priors.

For the ML analyses a GTRCAT model with 25 per site rate categories was assigned and all free model parameters estimated by the program. An ML bootstrap analysis (ML-BP) using 1 000 rapid bootstrapping replicates from random starting trees was performed, followed by a subsequent ML search similarly using 1 000 replicates. The likelihood of the final tree was evaluated and optimized under GAMMA. Identical sequences were excluded under the ML analyses of the individual gene datasets.

Morphological characters state coding and mapping

A species phylogeny (34 taxa) using the three-gene dataset (RPB2, EF-1α and LSU), with only one representative collection from each species of *Otidea,* was constructed for summarizing trends in morphological and ecological features. Bayesian and ML analyses were conducted as specified above. Four morphological features were mapped along the side of the species phylogeny, apothecium shape and colour (traits 1–2), shape of the apices of the paraphyses (trait 3) and spore size (trait 4), which have been used previously to delimit species of *Otidea*. In addition we mapped two newly discovered features, reactions of resinous exudates on the surface of the outermost ectal excipulum cells in Melzer's reagent (MLZ) (trait 5), and presence of resinous exudates on the mycelium at the base of the apothecia (trait 6).

The basic apothecial shape in *Otidea* is ear-shaped, i.e. a cup with a split in one side to the base, often more elongated on the side opposite the split. The apothecia are nearly always narrowly to broadly ear-shaped initially, but as they grow they can expand in various ways. The coding here refers to the last stage of the apothecial development.

Apothecial shape is treated using four states:
(0) long, narrowly ear-shaped;
(1) broadly ear-shaped / fan-shaped, i.e. with a broadly rounded margin;
(2) shallow to deeply cup-shaped, i.e. with a horizontal upper margin, split; and
(3) cup-shaped without a split (Fig. 1).

Apothecial colours are treated as:
(0) medium brown / greyish brown / yellowish brown;
(1) dark brown with ± reddish, purplish, or olivaceous tones;
(2) light orange / ochraceous yellow / ochre orange; and
(3) pale to bright yellow / citrine yellow (in hymenium or outer surface) (Fig. 1).

The shape of the apices of the paraphyses is coded as:
(0) curved to hooked, predominantly of the same width as the lower part or slightly enlarged, occasionally with a few slightly swollen areas or notches / short irregular proliferations, especially on the concave side (Fig. 2a–c);
(1) strongly inrolled with pronounced notches (Fig. 2d); or
(2) straight to bent, or bent to curved, broadly clavate to distinctly capitate, i.e. abruptly enlarged (Fig. 2e, f).

Spore size is here divided into four states, based on spore length:
(0) < 12 µm;
(1) 12–16.5 µm;
(2) 16.5–18 µm; or
(3) > 18 µm.

Small, pigmented, resinous exudates (lumps or drops) are present on the outermost ectal excipulum cells of most *Otidea* species (Fig. 2k). The reaction of the exudates in MLZ can be:
(0) absent;
(1) the exudates dissolve;
(2) coalesce into spheroid drops, referred to as amber drops (Fig. 2m); or
(3) partly convert into small reddish particles (Fig. 2n).
The reactions were observed by adding MLZ to a water mount (if mounted directly in MLZ the drops coalesce instantly and can be washed away).

Resinous exudates on the mycelium, at the base of the apothecia and spreading out in the substrate, were coded as:
(0) absent or inconspicuous (i.e. only a few refractive drops or scattered minute exudates); or
(1) abundantly present (Fig. 2o, p).

Morphological characters for coding individual species are based on our own observations (including 142 living collections).

Species of *Otidea* are considered to be ectomycorrhizal and tree association (trait 7) was coded as:
(0) broadleaved;
(1) coniferous; or
(2) mixed broadleaved and coniferous trees.
The tree association is based on our own field observations (inferred from the tree(s) growing by the apothecia) or notes given with the herbarium collections. Mixed trees refer to cases where different collections of a species were found associated with either broadleaved or coniferous trees, or collections were from mixed forest stands where no decisive association could be inferred.

Phylogenetic species recognition by genealogical concordance

Genealogical concordance phylogenetic species recognition (GCPSR: Taylor et al. 2000) was used to investigate species limits. Similar to the criteria proposed by Dettman et al. (2003) and O'Donnell et al. (2011), a clade was recognised as an independent evolutionary lineage if it was well supported as monophyletic in at least one single-locus Bayesian and ML genealogy, as judged by both Bayesian posterior probabilities (PP ≥ 95 %) and ML bootstrap proportions (ML-BP ≥ 70 %), and its genealogical exclusivity was not contradicted in any other single-locus genealogy at the same level of support. In Fig. 3, bold green branches indicate the clades that satisfied this criterion and therefore were identified as independent evolutionary lineages. For deciding which independent evolutionary lineages represented phylogenetic species, characteristics of the lineages in combined data analyses were also considered. Two ranking criteria were applied following Dettman et al. (2003): "(1) genetic differentiation: to prevent minor tip clades from being recognised, phylogenetic species had to be relatively

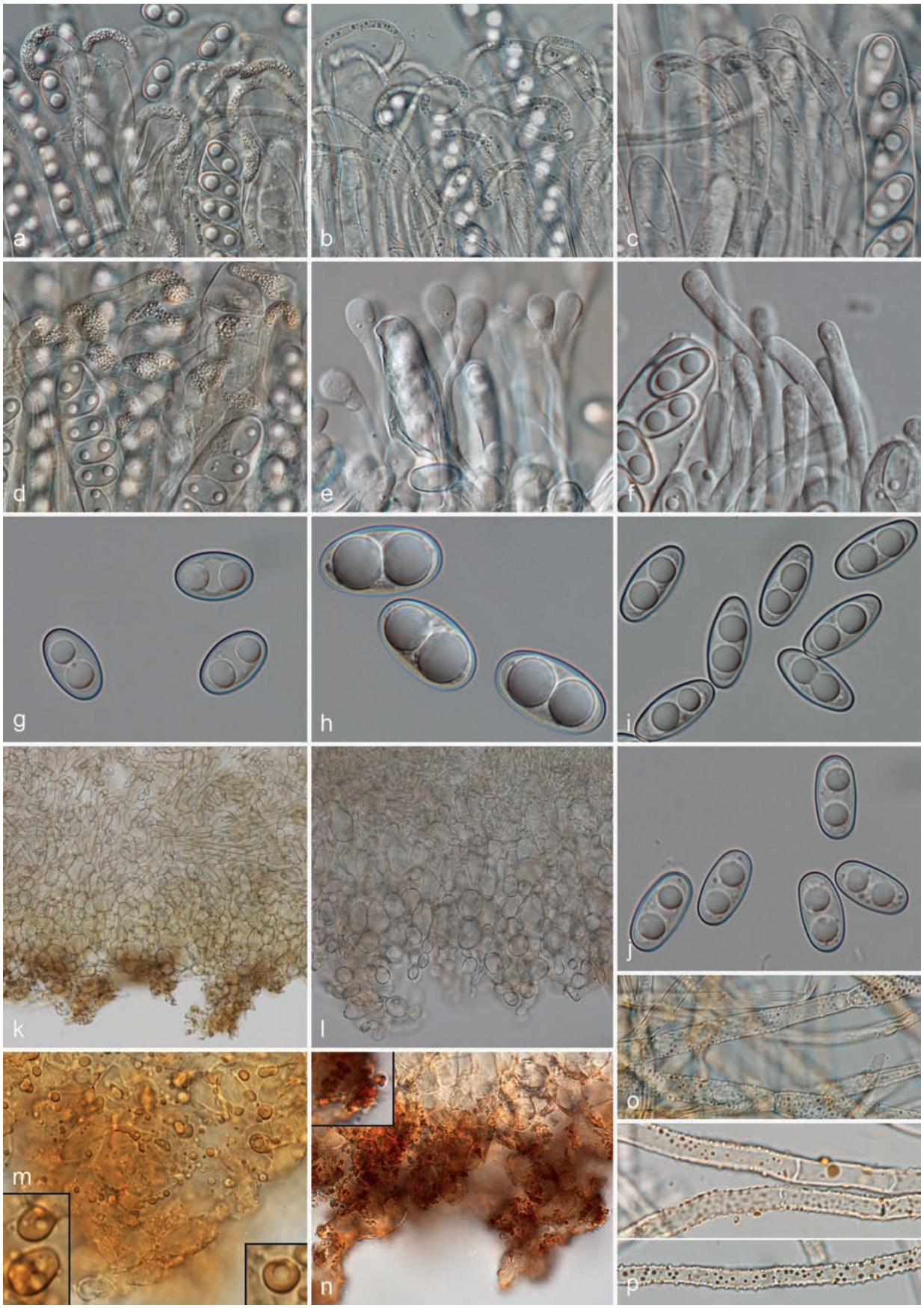

Fig. 2 Microscopic characters in *Otidea*, showing states reconstructed. a–f. Apices of paraphyses: a–c. curved to hooked, equal width throughout or slightly enlarged; a. *O. leporina* (KH.08.98, S) with few low, swollen areas or notches; b. *O. cantharella* (KH.09.155, S), equal width; c. '*O. alutacea* clade 2' (KH.13.50, S), hooked at extreme apices; d. strongly inrolled with pronounced notches, *O. brunneoparva* (holotype, S); e, f. straight to bent: e. *O. rainierensis* (holotype, MICH), capitate; f. *O. minor* (epitype, S), subclaviform; g–j. spores, shown to the same scale: g. *O. brunneoparva* (KH.08.107, S); h. *O. platyspora* (KH.09.163, S); i. *O. mirabilis* (KH.10.308, S), narrowly fusoid; j. *O. alutacea* (KH.13.50, S), oblong; k. medullary and outer excipulum, showing warts with resinous exudates, *O. cantharella* (KH.12.99, S); l. outer excipulum without resinous exudates in *O. alutacea* (KH.13.50, S); m, n. resinous exudate reaction in Melzer's reagent: m. coalesce into amber drops, *O. onotica* (epitype, S), insert showing close-up of amber drops; n. turn into small reddish particles, *O. mirabilis* (KH.10.308, S), insert showing close-up of red particles; o, p. resinous exudates on mycelium at apothecial base and substrate: o. *O. propinquata* (KH.09.99, S); p. *O. tuomikoskii* (holotype, H).

distinct and well differentiated from other species. (2) Exhaustive subdivision: all individuals had to be placed within a phylogenetic species." If an individual was not included in one of the independent evolutionary lineages (green branches in Fig. 3), we traced down the nodes of the tree from that individual, collapsing clades not subtended by green branches, until all individuals were included in a clade subtended by a green branch and recognised such clades as phylogenetic species (indicated with green circles in Fig. 3).

RESULTS

Nucleotide sequences and introns

We generated sequences from the ITS, LSU, RPB1, RPB2 and EF-1α to access species limits within *Otidea*. A total of 446 sequences were obtained, with 335 new sequences reported here: 3 939 bp from the protein-coding genes (53 RPB1, 76 RPB2, 75 EF-1α) and 938 bp from 68 LSU from 84 collections of *Otidea*. Four datasets were produced of LSU, RPB1, RPB2 and EF-1α from 89 collections. Sixty-tree new ITS rDNA sequences are provided, varying in length from 539–752 bp for complete ITS1 to ITS2 (excluding gaps and tandem repeats in *O. subformicarum* and *O.* aff. *subformicarum*) (Table 1). The ITS sequences were too divergent to reliably align across the breadth of *Otidea*, due to the number and complexity of indels, and were therefore not used in analyses of the entire genus. The ITS region showed overall low intraspecific variation. The additional new ITS and LSU sequences are given in Olariaga et al. (2015). Of the 89 collections included in the combined dataset, 16 collections lack RPB1, 8 RPB2 and 9 EF-1α (Table 1). In the combined dataset, sequences of at least three different markers were successfully obtained for 93 % of the collections, and all four markers for 72 %. Only two collections with a single marker were included. The nearly complete coding region of EF-1α was obtained for most collections, but for two collections only the first 815–866 bp were obtained, and for *O. mirabilis* (KH.01.09) only the last 946 bp. Complete sequences spanning regions 6–11 were obtained for a little more than half of the collections (58 %, representing all species except two), but for 33 collections only the 6–7 region was obtained (c. 800 bp). For *O. flavidobrunneola* (KH.09.153) only the 7–11 region (944 bp) was obtained. See Table 3 for proportions of variable and parsimony informative characters for the individual and combined data partitions.

Spliceosomal intron positions in the protein-coding genes were recognised by sequence comparisons and the conserved dinucleotide sequences at the intron ends (GT at start and AG at end). The A–C region of RPB1 contains two closely spaced spliceosomal introns at the 5' end of the gene, whose combined length is 128 bp (for *Otidea* only). The first intron occupies a phase 1 insertion with respect to the reading frame, while the second intron has a phase 0 insertion. The RPB1 exon regions include a 3 bp indel shared by clade A, *O. papillata* and the outgroup. The 6–11 region of RPB2 contains two spliceosomal introns whose combined length is 144 bp. The first intron is located between 6–7 and 7–11 regions, and the second intron towards the 3' end of the 7–11 region. Both introns have phase 0 insertions with respect to the reading frame. The EF-1α contains four spliceosomal introns, placed throughout the region. Their combined length is 230 bp. The first intron occupies a phase 0 insertion, and the last three phase 1 insertions.

Phylogenetic species recognition

Based on the grouping and ranking criteria we recognised 25 *Otidea* species. All of these, except for '*O. alutacea* clade 3', were strongly supported as monophyletic by Bayesian PP (≥ 95 %) and ML-BP (≥ 80 %) in at least two of the individual gene trees, and 13 were strongly supported by all four genealogies (Table 4). '*Otidea alutacea* clade 3', *Otidea leporina*, *O.* aff. *subformicarum* and *O. flavidobrunneola* were not resolved as monophyletic in one or two of the individual gene trees (Table 4), but their monophyly was not strongly contradicted in any of these trees. In the combined analyses of the four-gene dataset all species were supported as monophyletic by 100 % Bayesian PP and ML-BP. Although the monophyly of eight putative species (*O. apophysata*, *O. borealis*, *O. daliensis*, *O. kaushalii*, *O. phlebophora*, *O. rainierensis*, *O. unicisa* and *Otidea* sp. 'b'), represented by single collections, could not be tested, they were considered to be distinct because they were all genetically divergent from their sisters. For *O. daliensis* and *O. unicisa*, LSU and ITS sequences from one or two additional collections were available from GenBank and our LSU analyses support these as monophyletic groups (Olariaga et al. 2015). The 33 species recognised here, by genealogical concordance or genetic divergence, can all be recognised by a combination of morphological features (excluding the three putative species in the *O. alutacea* complex). Three of the species recognised by GCPSR had internal phylogenetic structure, i.e. included several independent evolutionary lineages, indicated by green branches in Fig. 3 (for ranking criteria see Methods). Within *Otidea nannfeldtii* there were two strongly supported subgroups of four collections from Sweden and Finland (including the holotype of *O. angusta*), and two collections from Finland and France (including the holotype of *O. nannfeldtii*) – and a single unresolved collection from western North America. We collapsed these subgroups into a single species, as the branches were short and we believe their reciprocal monophyly may be

Table 3 Data partitions, including number of nucleotides, variable uninformative characters (VC), parsimony informative characters (PIC) and percent PIC.

Datasets	No. of sequences	Total characters	VC	PIC	Percent PIC[1, 2] (%)
LSU rDNA	89	938	40	239	25.48
RPB1, all sites	73	724	64	210	29.01
RPB1, 1 and 2 codons	73	398	20	46	21.90
RPB1, 3 codons	73	198	32	134	63.81
RPB1 introns	73	128	12	30	14.29
RPB2, all sites	81	1820	106	506	27.80
RPB2, 1 and 2 codons	81	1118	21	43	8.50
RPB2, 3 codons	81	558	69	401	79.25
RPB2 introns	81	144	16	62	12.25
EF-1α, all sites	80	1395	61	412	29.53
EF-1α, 1 and 2 codons	80	777	10	34	8.25
EF-1α, 3 codons	80	388	36	231	56.07
EF-1α, introns	80	230	15	147	35.68
Combined 4 genes	89	4877	271	1367	28.03

[1] For datasets including all sites: percent PIC out of total number of characters in individual datasets.
[2] For datasets per codon positions and introns: percent PIC out of total number of PIC in individual datasets including all sites.

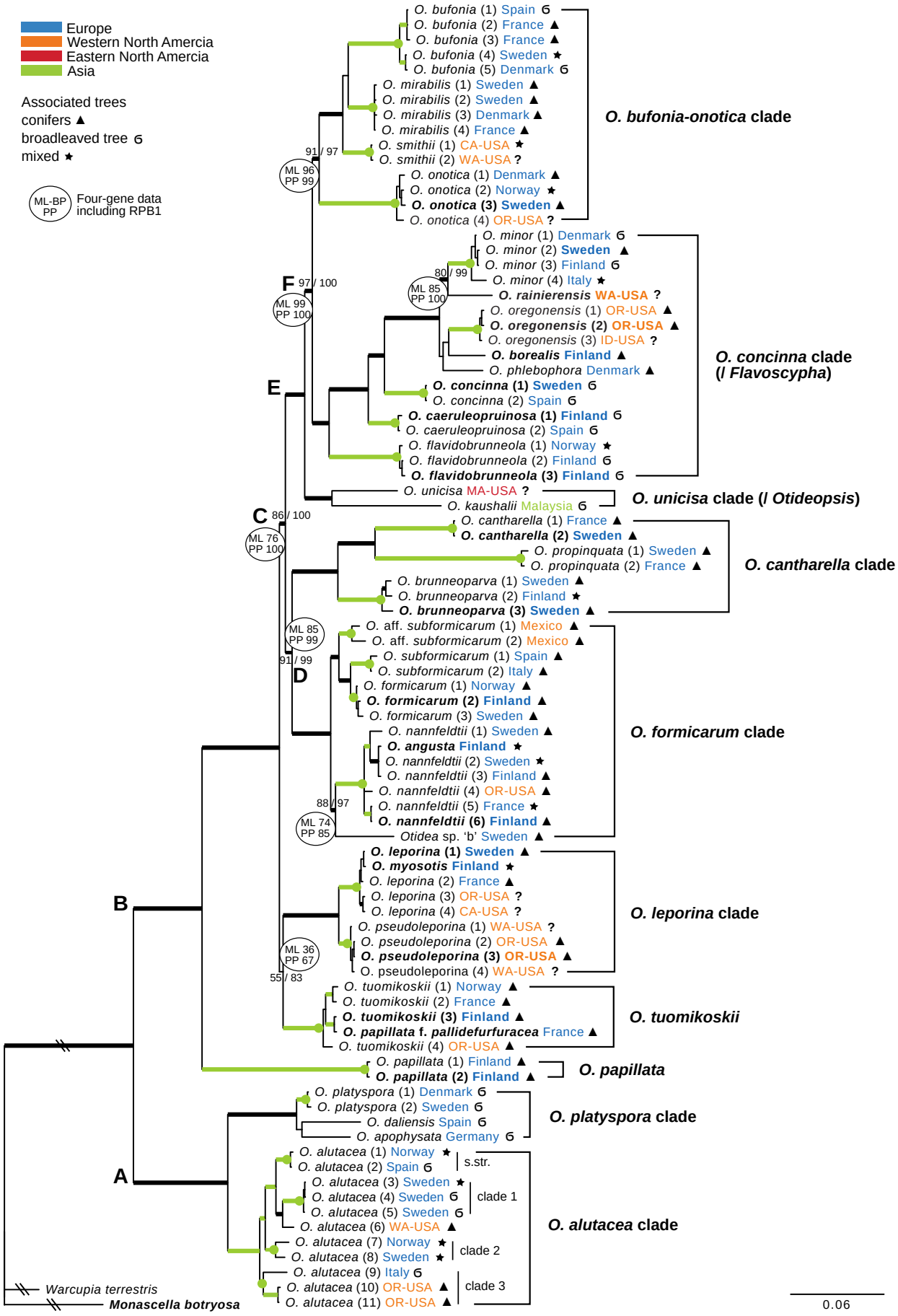

Fig. 3 Phylogeny of *Otidea* produced from Bayesian analysis of the combined LSU, RPB2 and EF-1α loci. Sequences of *Monascella botryosa* and *Warcupia terrestris* were used to root the phylogeny. Thick branches received high support in the analyses (Bayesian posterior probabilities ≥ 95 %, maximum likelihood bootstrap ≥ 75 %). Support values from analyses of four loci combined (i.e. LSU, RPB1, RPB2 and EF-1α) are given for some nodes in circles, to show the influence of RPB1 on the support values. Green branches were concordantly supported by the majority of the four loci (including RPB1), or were well supported by at least one locus but not contradicted by any other locus. Green circles at nodes indicate that all taxa united by it belong to the same phylogenetic species (see text for details). **Bold** taxon names indicate type material (holo-, iso-, neo- or epitypes). Six nodes (A–F) and 8 subclades are labelled for discussion.

Table 4 Support values for *Otidea* species recognized by genealogical concordance in analyses of individual gene partitions and in the combined four-gene dataset. Percent Bayesian posterior probabilities (PP) / RAxML bootstrap (ML-BP). NA, not applicable because only a single sequence of the particular gene and species was obtained.

Species[1]	LSU PP / ML-BP[2]	EF-1α PP / ML-BP	RPB1 PP / ML-BP	RPB2 PP / ML-BP	Combined four-gene data[3]
O. alutacea s.str.	94 / 97	100 / 100	100 / 100	100 / 100	100 / 100
O. alutacea clade 1	100 / 100	100 / 99	100 / 100	100 / 98	100 / 100
O. alutacea clade 2	93 / 64	100 / 96	100 / 86	73 / 76	100 / 100
O. alutacea clade 3	– / –	94 / 76	100 / 89	– / –	100 / 97
O. brunneoparva	100 / 100	100 / 99	100 / 100	100 / 100	100 / 100
O. bufonia	100 / 100	100 / 98	100 / 100	100 / 98	100 / 100
O. caeruleopruinosa	100 / 100	NA	100 / 100	99 / 100	100 / 100
O. cantharella	100 / 100	NA	100 / 100	100 / 100	100 / 100
O. concinna	100 / 100	100 / 100	100 / 100	100 / 100	100 / 100
O. flavidobrunneola	100 / 100	100 / 100	100 / 100	54 / –	100 / 100
O. formicarum	100 / 92	99 / 100	NA	87 / 66	100 / 100
O. leporina	– / –	100 / 99	– / –	100 / 100	100 / 100
O. minor	100 / 97	100 / 100	100 / 100	100 / 100	100 / 100
O. mirabilis	100 / 100	100 / 99	100 /98	100 / 100	100 / 100
O. nannfeldtii	100 / 99	100 / 100	100 / 98	100 / 100	100 / 100
O. onotica	100 / 99	100 / 100	100 /100	100 / 100	100 / 100
O. oregonensis	100 / 100	100 / 100	100 / 98	100 / 92	100 / 100
O. papillata	100 / 100	100 / 100	NA	100 / 100	100 / 100
O. platyspora	97 / 69	100 / 100	100 / 97	83 / 61	100 / 100
O. propinquata	100 / 100	100 / 100	74 / 99	100 / 100	100 / 100
O. pseudoleporina	100 /100	99 / 83	100 / 93	98 / 97	100 / 100
O. smithii	100 / 100	100 / 100	95 / 87	99 / 100	100 / 100
O. subformicarum	100 / 99	100 / 99	100 / 100	100 / 95	100 / 100
O. aff. *subformicarum*	– / –	100 / 100	100 / 100	70 / 55	100 / 100
O. tuomikoskii	100 / 93	100 / 100	100 / 98	100 / 99	100 / 100

[1] Support values not applicable for the following eight species represented by single collections, which are therefore not included in the table: *O. apophysata*, *O. borealis*, *O. daliensis*, *O. kaushalii*, *O. phlebophora*, *O. rainierensis*, *Otidea* sp. 'b', *O. unicisa.*

[2] –, clade not resolved as monophyletic.

[3] PP and ML-BP values for the combined dataset.

compromised with the addition of further collections from other geographic areas. Also the minor morphological features used to differentiate *O. angusta* from *O. nannfeldtii* did not correlate with the groupings, and we placed *O. angusta* in synonymy with *O. nannfeldtii* (Olariaga et al. 2015). *Otidea bufonia* contained two subgroups that reflected the geographical origins of the collections. The one subgroup was composed of two collections from Scandinavia, and the other of three collections from Central / Southern Europe. These lacked significant genetic differentiation and were collapsed into a single species. *Otidea tuomikoskii* likewise showed some phylogenetic structure, with two subgroups of four collections from Europe (northern and central European collections mixed) and sister to these a single collection from western North America. Due to missing data for several of the *O. tuomikoskii* collections these cannot be fully evaluated, but all branches were very short suggesting these represent a single species. We were not able to obtain multiple genes for the isotype of *O. papillata* f. *pallidefurfuracea*, but ITS and LSU sequences were identical to the holotype of *O. tuomikoskii*.

Sequences of multiple genes were generated from 13 holotypes (Table 1) and are marked in **bold** in Fig. 3. The holotype of *O. myosotis* was deeply nested within *O. leporina* and based on GCPSR synonymous. Overall *O. leporina* showed very little genetic divergence. The LSU, EF-1 and RPB2 sequences of the holotype of *O. myosotis* and the two European *O. leporina* collections were either identical or differed 1–3 bp from each other. The ITS of the *O. myosotis* holotype and the French collection (NV 2008.09.28) were identical and the epitype ITS sequence of *O. leporina* differed only 2 bp from those. Five new species were identified: *O. borealis*, *O. brunneoparva*, *O. oregonensis*, *O. pseudoleporina* and *O. subformicarum*; they are described in Olariaga et al. (2015). *Otidea cantharella* var. *minor* was supported as a distinct species. New collections, with photographs and multiple genes provided, have been selected as a neotype for *O. cantharella* and epitypes for *O. concinna*, *O. leporina* and *O. onotica* (Olariaga et al. 2015) and are marked in Fig. 3. The type species of *Otidea*, *O. onotica*, is deeply nested within *Otidea* (in clade E, see below).

Gene-conflict in relationships of Otidea and phylogenetic signal in data partitions

No supported conflicts were detected between the individual gene phylogenies in terms of relationships among the 33 species recognised, except for the RPB1. In Bayesian analyses of RPB1 alone, *O. propinquata*, *O. cantharella* and *O. brunneoparva* were supported as successive sister species to the rest of *Otidea* (all branches PP 95 %) (Fig. 4). In all other single gene analyses these three species form a strongly supported monophyletic group (all PP / ML 100 %, except LSU ML 89 %), deeply nested within *Otidea*. Concurrently, *O. papillata* formed a monophyletic group with the inclusive clade A (PP 99 %) in analyses of the RPB1, as opposed to a monophyletic group with clade B in the other genes (PP RPB2 92 %, EF-1α 100 %, LSU 98 %). To explore the influence of these conflicts on the analyses of the combined loci, analyses were conducted on a three-gene dataset (excluding RPB1) and a four-gene dataset (all loci). In the Bayesian analyses of the three- and four-gene datasets, respectively, an average standard deviation of split frequencies between runs (diagnosed from the last 75 % of the tree sample) reached 0.0044 and 0.0049, and the Potential Scale Reduction Factor 1.000, and the tree samples were considered to be stationary. In the searches with RAxML the three- and four-gene alignments had 1 908 and 2 296 distinct patterns with a proportion of gaps and undetermined characters of 23.23 % and 23.02 %, respectively. The partitioned ML analyses recovered a single best scoring tree of $-\ln L$ = 22,559.87 and $-\ln L$ = 26,706.20 for the three- and four-gene datasets, respectively. Bayesian and ML analyses of the three-gene dataset produced an identical topology to the four-gene phylogeny, but with higher ML-BP support for two deeper nodes (C and D) surrounding the *O. cantharella* clade (Fig. 3). At the

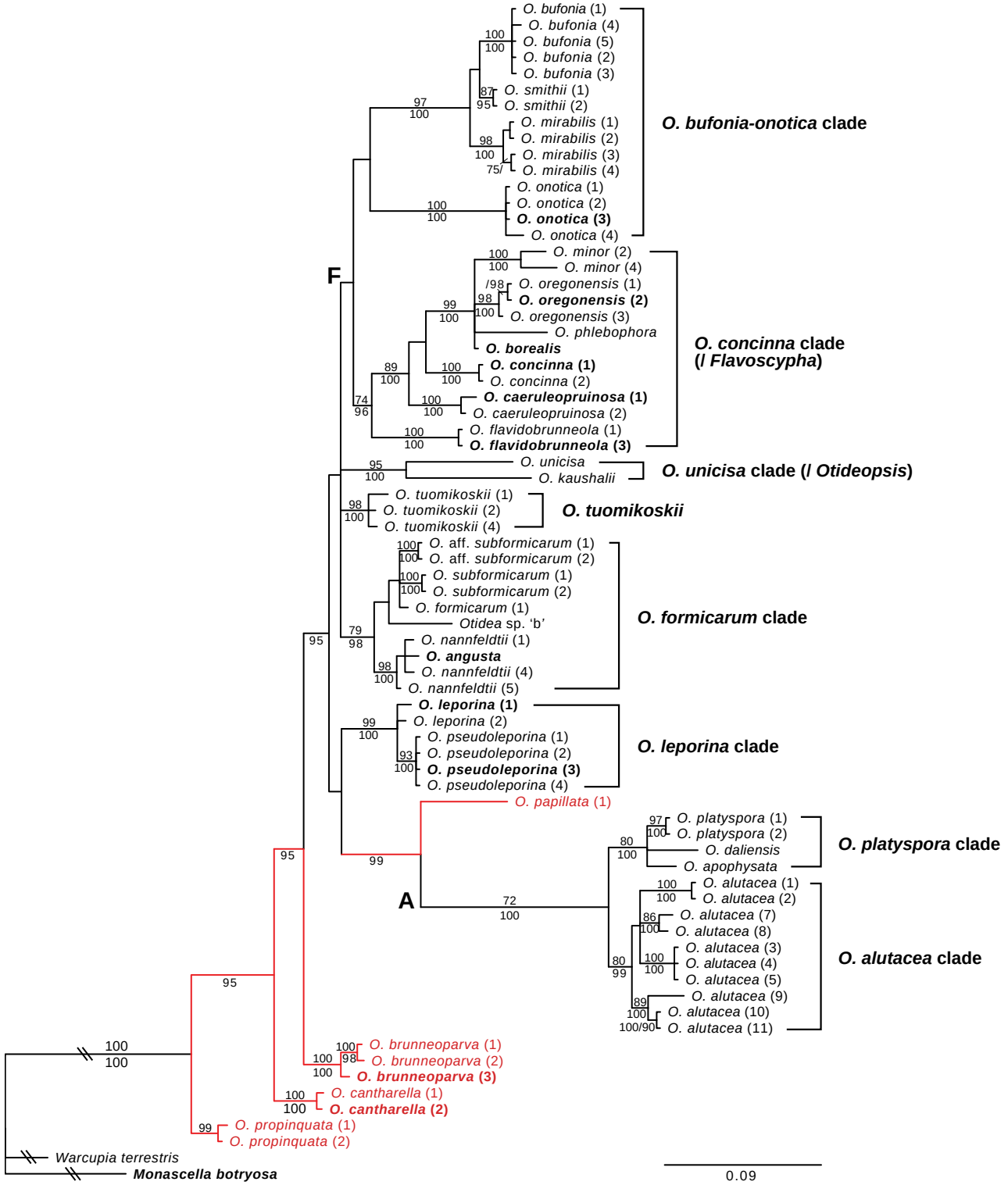

Fig. 4 Phylogeny of *Otidea* produced from Bayesian analysis of the RPB1 alone. Maximum likelihood bootstrap ≥ 70 % and Bayesian posterior probabilities ≥ 95 % are shown above and below the branches, respectively. Branches showing supported conflict with the LSU, RPB2 and EF-1α single gene phylogenies are highlighted in red. **Bold** taxon names indicate type material. A and F refer to two of six nodes supported in Fig. 3.

same time the support values for several shallow nodes in the tree were lowered. Excluding the RPB1 did not markedly change the Bayesian PP values (to raise above 95 %), except for a single node (monophyly of *O. nannfeldtii* and *Otidea* sp. 'b') that rose from 85 % to 97 % PP. For this node ML-BP also increased from 74 % to 88 % when RPB1 was excluded. Overall the localised conflicts in the RPB1 affected support values for several deeper nodes in combined analyses, but the LSU, RPB2 and EF-1α gene partitions contributed significantly strong support for the *O. cantharella* clade and surrounding nodes in combined analyses. We suggest the topology that is identical and with support in both the three- and four-gene phylogenies, represents the best hypothesis for the higher-level relationships (Fig. 3). The RPB1 still adds valuable information for the delimitation and relationships of closely related species, and we present the support values from the combined analyses including the RPB1 for selected nodes (Fig. 3). The RPB2 and EF-1α regions account for the greatest number of putative parsimony informative characters (PIC) within the combined dataset (37.02 % and 30.14 %), whereas LSU and RPB1 account for much less (17.48 % and 15.36 %). Nevertheless, RPB1 exhibits a similar level of phylogenetic signal per

sequenced base pair (based on number of PIC) as EF-1α, and slightly more than RPB2 (Table 3). The third codon positions of each of the protein-coding genes provided the most PIC, with the most pronounced percentage in the RPB2 region (79 %) compared to RPB1 and EF-1α (64 % and 56 %, respectively). The combined four-gene dataset included 1 367 PIC, with 766 (56 %) provided by third codon positions. The introns provided 239 PIC (17 % of the total PIC), with the largest amount (147 PIC) from the four introns in the EF-1α.

Relationships among species of Otidea and geographical distribution

The three-gene phylogeny of *Otidea* is fully resolved and highly supported in all deeper branches as inferred by both Bayesian PP and ML-BP, except for the single node joining the *O. leporina* clade and *O. tuomikoskii*, which has no support. Six inclusive clades are identified and labelled A–F (PP 100 % / ML-BP 86–100 %). Ten subclades within the more inclusive clades are highly supported and recognised (PP and ML-BP 100 %, except the *O. onotica-bufonia* clade PP 97 %, 99 % / ML-BP 91 %, 96 %; from three-gene, and four-gene analyses). To facilitate results and discussion, we have named these as indicated on Fig. 3. Two major sister clades, A and B, are highly supported. Clade A is composed of the *O. platyspora* clade, namely *O. platyspora*, *O. daliensis* and *O. apophysata*, and the *O. alutacea* species complex. *Otidea platyspora* and *O. apophysata* are known only from a few countries in Europe, and are here represented by two collections from Scandinavia and one from Germany, respectively. *Otidea daliensis* is described from China, but here suggested to occur in Europe (Spain; our LSU sequence is identical to a GenBank LSU sequence of the holotype DQ443445). ML analyses support *O. daliensis* and *O. apophysata* as sister species (ML-BP 74 % 3 genes, 77 % 4 genes). Our molecular and morphological data suggests the *O. alutacea* clade is a complex of at least four, recently radiated species. Additional sampling of fresh material from each of these is needed to accurately assess their species boundaries using multiple genealogies.

Clade B comprises the majority of *Otidea* species. The only two known collections of *O. papillata* (from Finland) constitute a distinct, separate lineage, strongly supported as a sister group to the rest of clade B (PP / ML-BP 100 %).

The relationship of the *O. leporina* clade and *O. tuomikoskii* is without support, but both are strongly supported as sister groups to clade C. The *O. leporina* clade is composed of the closely related species, *O. leporina* and *O. pseudoleporina*. *Otidea leporina* includes three collections from Europe and two from western North America. Based on two LSU sequences in GenBank that were published under the name *O. crassa* (HMAS 83570: DQ443443; and HMAS 583571 (holotype of *O. crassa*): DQ443444) (Liu & Zhuang 2006), it also occurs in China. *Otidea tuomikoskii* (as delimited here) likewise includes collections from both Europe and western North America, and based on a morphological description by Cao et al. (1990) it may also be present in Asia. *Otidea pseudoleporina* includes four collections from Oregon and Washington, and is only known from western North America.

Clade D is composed of the *O. cantharella* and *O. formicarum* subclades. The *O. cantharella* subclade constitutes three species: *O. brunneoparva*, *O. cantharella* and *O. propinquata*. The branch leading to *O. propinquata* is, apart from the branch leading to *O. papillata*, the longest in the tree (Fig. 3). These three distinct species are represented by sequences only from Europe, i.e. Scandinavia and France. The *O. formicarum* subclade is composed of two strongly supported monophyletic groups: 1) *O. formicarum*, *O. subformicarum* and *O.* aff. *subformicarum*; and 2) *O. nannfeldtii* and *Otidea* sp. 'b'. *Otidea formicarum* and *O. subformicarum* form a strongly supported clade (PP 100 %, 100 % / ML-BP 99 %, 100 %). These two species are restricted to Scandinavia and Southern Europe, respectively, whereas *O.* aff. *subformicarum* includes two collections from Mexico. *Otidea nannfeldtii* is suggested to be transcontinental (Europe – North America), whereas *Otidea* sp. 'b' so far is only known from a single Swedish collection.

The *O. unicisa* clade is placed within clade E, as a strongly supported sister group to clade F. It includes the eastern North American *O. unicisa* and the Asian *O. kaushalii*.

Clade F is composed of two larger subclades: *O. bufonia-onotica* and *O. concinna*. The *O. bufonia-onotica* subclade includes two strongly supported groups: 1) *O. bufonia*, *O. mirabilis* and *O. smithii* (all 100 %); and 2) *O. onotica*. *Otidea bufonia* and *O. mirabilis*, including collections from Europe, are supported as sister species by ML-BP (81 %, 84 %), and the western North American *O. smithii* a sister species to those. Based on subsequent morphological study of four North American collections (RH1218 and RH1393 (MIN), UPS F-629510 and F-629511) we suggest *O. bufonia* may also be present in North America. An LSU sequence from a mycorrhizal root tip (see Mycorrhizal status and putative tree associations under Discussion), and two LSU sequences in GenBank (HMAS 83579: DQ443448; and HMAS 83568: DQ443449, published as *O. leporina*; Liu & Zhuang 2006), suggest *O. bufonia* and *O. mirabilis*, respectively, are also present in Asia. *Otidea onotica* includes collections from Scandinavia and North America, and based on a morphological description by Cao et al. (1990) it may also be present in Asia. The *O. concinna* subclade includes five European species and two North American. Deeply nested is a strongly supported monophyletic group consisting of two sister clades: 1) *O. minor* and *O. rainierensis* (PP 99 %, 100 % / ML-BP 80 %, 85 %); and 2) *O. oregonensis* and *O. borealis* (PP 95 %, 98 % / ML-BP 70 %, 77 %) and *O. phlebophora* (placed without support). As successive sister species (all PP and ML-BP 100 %) to this deeply nested clade are *O. concinna*, *O. caeruleopruinosa* and *O. flavidobrunneola.* The placement of *O. phlebophora*, the type species of the genus *Flavoscypa*, shows *Flavoscypha* belongs to *Otidea*. The other species Harmaja (1974) intended to include in *Flavoscypha*, *O. concinna* (*F. cantharella*, misapplied by Harmaja 1974; see Harmaja 2009), and the later combined *F. cantharella* var. *minor* (Häffner 1994) (= *O. minor*) are both shown to belong to the *O. concinna* clade. The current knowledge on the continental distribution of *Otidea* species is summarised in Fig. 5.

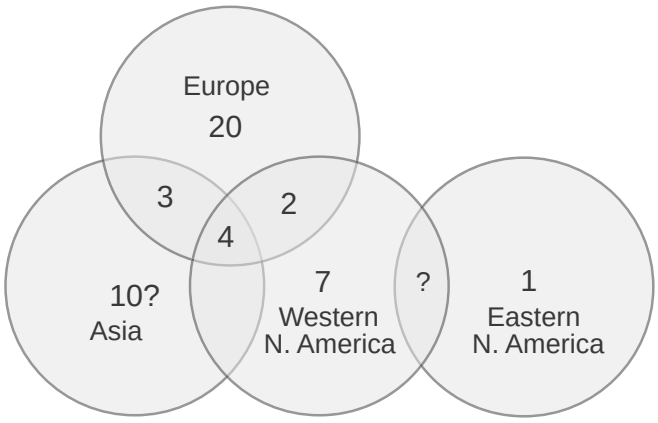

Fig. 5 Venn diagram summarizing the high level of continental endemism of *Otidea* in the Northern Hemisphere. Our knowledge on species occurring in Asia and mid-region to eastern North America is still fragmentary and the number of species in those areas is likely higher. The four lineages in the *O. alutacea* complex are preliminarily included as distinct species.

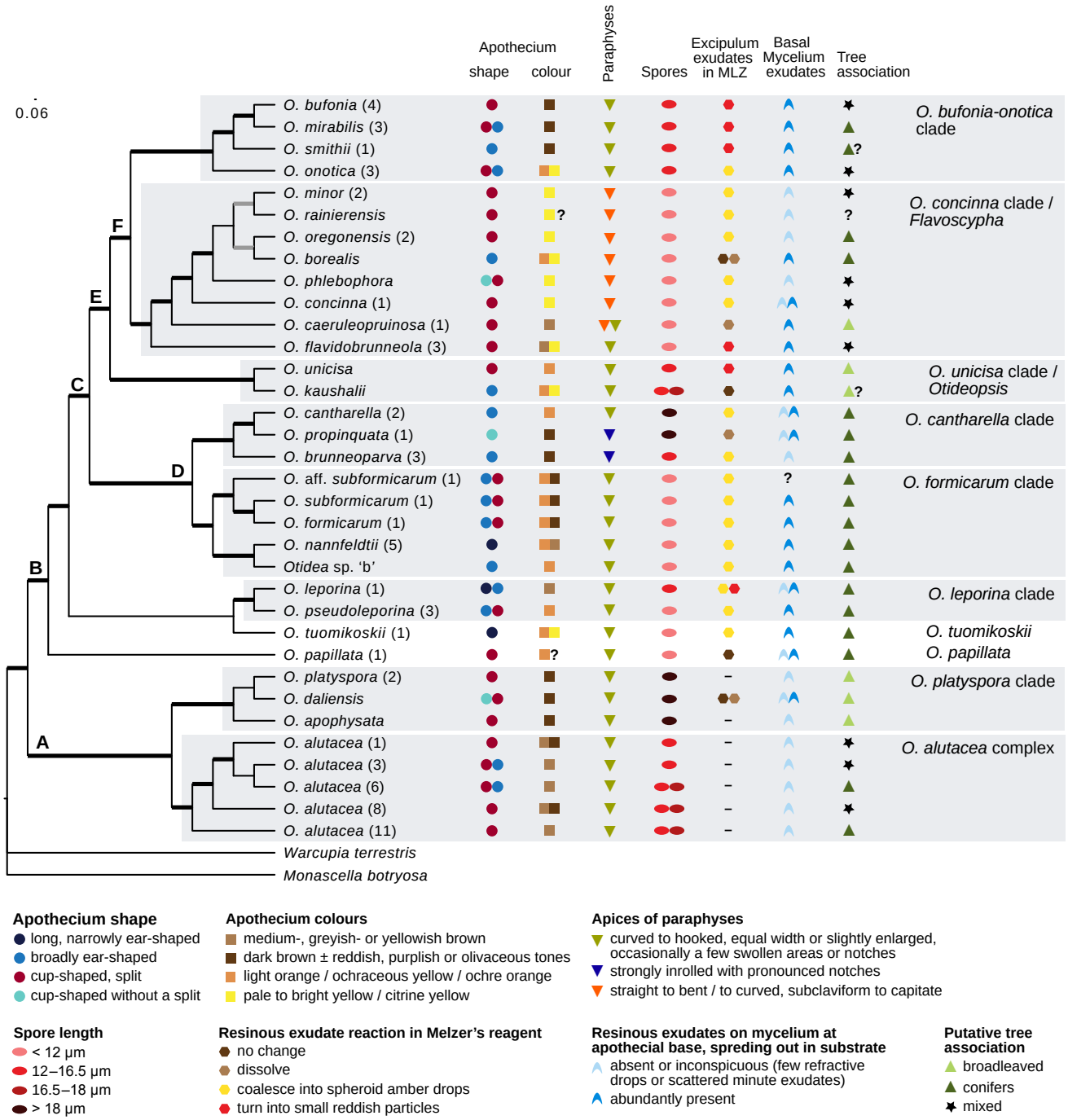

Fig. 6 Selected morphological character states and putative tree association in *Otidea*, mapped on a Bayesian consensus tree from combined LSU, RPB2 and EF-1α analysis, including one representative collection from each species (as inferred from Fig. 3). Sequences of *Monascella botryosa* and *Warcupia terrestris* were used to root the phylogeny. Thick black branches received high support in the analyses (Bayesian posterior probabilities ≥ 95 %, maximum likelihood bootstrap ≥ 75 %); thick grey branches received high support only in Bayesian analyses. Six nodes (A–F) and 8 subclades are labelled for discussion. Traits and states are given in detail under Materials and Methods. Uncertain state for a taxon is given as '?' Not applicable is given as a '–'.

Evolutionary trends in morphological features and tree association

Morphological features and putative tree association of *Otidea* species are depicted on the 50 % majority rule consensus species tree from the Bayesian analysis in Fig. 6. No unique morphological or ecological features appear to support any of the inclusive clades A–F. Some subclades however, show distinct features and trends.

The apothecium with a split to the base in one side is a synapomorphy for *Otidea* within *Pezizomycetes*, but apothecia without a split are often produced by *O. daliensis* and *O. phlebophora* (in clades A and F), and the split has been completely lost at least once in *Otidea*, in *O. propinquata* (Fig. 1f) (in clade D). *Otidea* apothecia are nearly always narrowly to broadly ear-shaped initially, but as they grow they can expand and flatten, and become broadly ear-shaped/fan-shaped or cup-shaped, i.e. with a rounded upper margin or a horizontal upper margin, respectively (Fig. 1b–f). Only in two species, *O. tuomikoskii* (Fig. 1a) and *O. nannfeldtii* (*O. formicarum* clade), and sometimes in *O. leporina*, the apothecia retain the narrow ear-shape in later stages. In the early diverging clade A and *O. papillata*, and in the deeply nested clade F all species but two, become cup-shaped. Narrow to broadly ear-shaped apothecia prevail in clade D and in the *O. leporina* clade. The hypogeous *O. subterranea* (not sampled in our multi-gene phylogeny, but nested in the *O. platyspora* clade based on LSU sequences) shows, like other pezizalean truffles or truffle-like forms, a completely

different apothecium type (a ptychothecium), being closed and with a solid or partly solid gleba.

The apothecium colours in *Otidea* are various tones of brown, orange and yellow. Exclusively pale to bright yellow, or citrine yellow is a synapomorphy for the *O. concinna* clade (Fig. 1e). Yellow tones are also found in the hymenium of *O. onotica* and *O. tuomikoskii* (Fig. 1a). Exclusively dark brown colours are present in three clades, the *O. platyspora*, *O. cantharella* and *O. bufonia-smithii* clades (Fig. 1d, f), but *O. cantharella* itself is with orange to ochraceous yellow tones. Medium to greyish brown apothecia characterise the *O. alutacea* clade (Fig. 1c), but are also present in *O. caeruleopruinosa*, *O. flavidobrunneola* (*O. concinna* clade), *O. nannfeldtii* (*O. formicarum* clade) and *O. leporina* (*O. leporina* clade). Pink tinges or spots (not mapped) can be seen in a number of species (*O. nannfeldtii*, *O. onotica*, *O. pseudoleporina* and *O. unicisa*), but their presence varies considerably. The tinges are most common and pronounced in *O. onotica*.

The curved to hooked apices of the paraphyses, predominantly of the same width as the lower part or slightly enlarged, occasionally with a few slightly swollen areas or notches (Fig. 2a–c), are present across *Otidea* and are suggested to be a symplesiomorphic trait for the genus. Two different types of paraphyses have evolved within *Otidea*. Strongly inrolled paraphyses with pronounced notches are found only in the *O. cantharella* clade (in *O. brunneoparva* (Fig. 2d) and *O. propinquata*), and straight to bent or bent to curved paraphyses with broadly clavate to distinctly capitate apices are unique to a restricted *O. concinna* clade (Fig. 2e, f) (excluding the early diverging *O. flavidobrunneola*).

All epigeous species of *Otidea* have smooth spores, except for the species in the *O. unicisa* clade (not mapped in Fig. 6). Spores in *O. unicisa* have low, delicate warts and short, irregular ridges, and in *O. kaushalii* spines up to 1 µm high. The hypogeous *O. subterranea* has finely verruculose spores in SEM (Smith & Healy 2009). The basic spore shape in *Otidea* is ellipsoid, but fusoid spores are typical in the *O. bufonia-mirabilis* lineage and oblong spores in the *O. alutacea* complex (Fig. 2g–j). The spore size, here based on spore length divided in four categories, shows some pattern across *Otidea* (Fig. 6). Small spores (< 12 µm) are dominant in clade B, and absent from clade A. Medium-sized spores are found in several clades: 12–16.5 µm in the *O. alutacea*, *O. leporina*, *O. cantharella*, *O. unicisa* and *O. bufonia-onotica* clades; and 16.5–18 µm partly in the *O. alutacea* clade. Large spores (> 18 µm) have evolved in two separate clades, the *O. platyspora* and *O. cantharella* clades (within clade A and B).

Resinous, pigmented exudates on the outermost excipular cells were found to be present in all species in clade B (Fig. 2k), but absent from all but *O. daliensis* in clade A (Fig. 2l). Resinous exudates that coalesce into spheroid drops that contain hyaline bubbles (amber drops) are the most common reaction in MLZ and are likely the ancestral state for clade B (Fig. 2m). In the *O. bufonia-smithii* clade, in *O. flavidobrunneola* and *O. unicisa* (all in clade E), the exudates instead convert partly into small reddish particles / turn reddish in MLZ (Fig. 2n). In four species the resinous exudates do not react or simply dissolve in MLZ (Fig. 6).

All *Otidea* species have a conspicuous basal tomentum covering the base of the apothecia and spreading out in the substrate. Exudates, turning into differently shaped, pigmented deposits were found to be abundantly present on the surface of the hyphae in species across *Otidea* (Fig. 2o, p, 6), but absent or inconspicuous in the *O. alutacea* complex and in a restricted *O. concinna-minor* clade (except for *O. borealis*). In three clades (the *O. formicarum*, *O. unicisa*, *O. bufonia-onotica*), resinous exudates are abundantly present in all species. The exudates can appear like ornamentation, but do dissolve and disappear in MLZ.

Most species of *Otidea* appear to be restrictedly associated with broadleaved or coniferous trees (Fig. 3, 6), with the clear exceptions of *O. bufonia*, *O. minor* and *O. onotica*. The majority of the species are associated with coniferous trees, but species in the *O. platyspora* and *O. unicisa* clades are found exclusively with broadleaved trees (within clade A and B, respectively). The earliest diverging groups within clade B, *O. papillata*, *O. tuomikoskii*, the *O. leporina* clade and clade D are associated with coniferous trees, suggesting this might be the ancestral state for clade B. The deeply nested clade F is composed of species associated with either coniferous or broadleaved trees, or both (several with uncertain state).

DISCUSSION

Conflicts among data partitions and signal for species delimitation

The RPB1 data showed supported conflict in the Bayesian analysis with regard to the placement of *O. brunneoparva*, *O. cantharella* and *O. propinquata* (the *O. cantharella* clade in Fig. 3) and *O. papillata* (Fig. 4). Nevertheless, the phylogenetic signal in the individual LSU rDNA, RPB2 and EF-1α datasets is so strong that the incongruence from RPB1 is wiped out in the four-gene analyses. Also the RPB1 represented the smallest dataset (73 taxa out of 89 in total, 724 bp out of 4 877 bp in total; Table 3), although with taxa represented from all clades, and thus had the lowest impact in combined analyses. Interestingly, even though the conflicting branches in RPB1 were resolved with high support in Bayesian (and without support in ML) analyses, ML-BP was more sensitive than Bayesian PP to excluding the RPB1 data in combined analyses. Insensitivity in Bayesian PP to conflicts among gene partitions has been noted in other studies (Sung et al. 2007), especially with regard to short internodes, such as nodes C, D and F in our phylogeny (Fig. 3). Incongruence between phylogenies obtained using individual genes is a challenge in molecular phylogenetics at all taxonomic levels. Supporters of a conditional combinability approach might claim that the RPB1 should be excluded because of the incongruence, whereas others might argue that the RPB1 should be included in the combined dataset for total evidence (e.g. reviewed by Huelsenbeck et al. 1996). As suggested by others (e.g. Sung et al. 2007), our results indicate it is advantageous to explore the impact of a localised conflict and the potential loss of signal for other nodes as well, rather than simply excluding the gene partition from the combined analyses. A possible explanation for the conflict is that we could be dealing with paralogous copies of RPB1 for *O. brunneoparva*, *O. cantharella* and *O. propinquata*. Two paralogs of the RPB1 gene have been found in some plants (Luo et al. 2006). Analytical factors are another explanation, although complex evolutionary models with independent parameters for each partition, taking into account heterogeneity among each gene partitions (e.g. rate variation, codon saturation) were employed. We utilised both the three- and four-gene datasets, retrieving the maximum support for the higher-level relationships within *Otidea* from the three-gene dataset (excluding the localised incongruence from RPB1), and for the species delimitation and species groups using the four-gene dataset. From the four gene regions utilized here, EF-1α and RPB1 had the strongest species recognition power, resolving all but one and two species, respectively, with high support (PP ≥ 95 %, ML-BP ≥ 70 %) (Table 4). The LSU and RPB2 however, failed independently to resolve or highly support six of the species and are thus not alone reliable as species delimitation genes for *Otidea*. Since

the amplification success of EF-1α was higher than for RPB1 (80 vs 73 collections) the EF-1α may serve as the best secondary barcoding locus for *Otidea*, with ITS being the primary locus. It is noteworthy that the EF-1α had the lowest amount of PIC from third codon positions compared to RPB2 and RPB1, and a large amount of the PIC from the four intron positions (36 %).

Species delimitation, diversity and distribution

One of the primary objectives of the present study was to clarify species limits within the genus *Otidea* using GCPSR (Taylor et al. 2000). Of the 33 species recognised within *Otidea*, all 25 lineages represented by two or more collections fulfilled the GCPSR criteria of Dettman et al. (2003) (see Materials and Methods). Our multi-gene phylogeny includes only a single collection from Asia, *O. kaushalii*, and the Asian species *O. daliensis* represented by material from Spain. Based on LSU sequences available in GenBank (Liu & Zhuang 2006) and/or morphology we accept five additional Asian taxa, *O. brevispora*, *O. lactea*, *O. purpurea*, *O. sinensis* and *O. yunnanensis*. We estimate that at least another four species endemic to Asia exist, i.e. *O. bicolor*, *O. olivaceobrunnea*, *O. subpurpurea* and *O. tianshuiensis* (Cao et al. 1990, Zhuang & Yang 2008, Zhuang 2010). However, the genetic exclusivity of these Asian species still needs to be tested. We suggest the Asian *O. kunmingensis* (Zhuang & Yang 2008) belongs to the *O. alutacea* complex (clade 1; Fig. 3). *Otidea subterranea*, the only known hypogeous member of *Otidea*, was recently described from Midwestern USA, Iowa (Smith & Healy 2009). Based on our analyses of the LSU it is nested within the *O. platyspora* clade (PP 100 %, ML-BP 99 %). Based on our morphological studies of a collection (MINN 933306) and an ITS sequence (unpublished by R. Healy), one additional undescribed *Otidea* species is present in Midwestern USA, Minnesota. Including four putative species from Europe, *Otidea* sp. 'a', *Otidea* sp. 'b', '*O. fusconigra*' (a provisional name; Jamoni 2004) and *O. integra* based on analyses of the ITS-LSU regions (not shown), we suggest *Otidea* comprises 47 species worldwide (Fig. 5). Noteworthy, species occurring in Europe constitute three fifths (i.e. 29/47) of the lineages within *Otidea*, with 20 species recognised as endemic to Europe. At least 14 *Otidea* species occur in North America and 17 in Asia, with respectively eight and ten species considered endemic to each continent. Only *O. unicisa* was found to be restricted to eastern North America, where it is common in broad-leaved forests, but surely the diversity of *Otidea* on the East coast is higher as it is still poorly explored. Similarly a much higher diversity is expected in Asia where our knowledge is still fragmentary.

Much confusion has prevailed in the naming of *Otidea* species, due to different interpretations of European names, because original material has not been studied or does not exist, and neither comparative morphological nor molecular studies across different geographical areas have been conducted. As has been shown within other groups of fungi (e.g. Nuytinck et al. 2007, O'Donnell et al. 2011), our study shows that the majority of *Otidea* species in North America are distinct from European species. Forty-four names including seven varieties or forms, currently accepted in *Otidea*, have been described from Europe, representing 19 distinct species. On the contrary, only six names currently recognised in *Otidea*, have been described from North America, *O. alutacea* var. *microspora* (a doubtful name), *O. kauffmanii* (a synonym of *O. rainierensis*), *O. rainierensis*, *O. smithii*, *O. subterranea* and *O. unicisa* (Peck 1874, Kanouse 1949, Smith & Healy 2009), and European names have until now, often erroneously, been applied to American taxa (e.g. Kanouse 1949, Peterson 1998). Peterson (1998) recognised eight species in the Pacific Northwest, and we included representatives of all of these species. We have studied the material morphologically and sequenced additional loci, and confirm or correct names for the sequences in GenBank. Sequences under the name *O. umbrina* (OSC 56758: ITS AF072074 and LSU AF086581; OSC 56813: LSU AF086584; and OSC 56782: LSU AF086586) belong to the *O. alutacea* complex ('*O. alutacea* clade 3', Fig. 3). Two new species endemic to North America were discovered, *O. pseudoleporina* and *O. oregonensis*, for taxa previously recognised under the European names *O. cantharella* var. *minor* or *O. concinna*, and *O. rainierensis*, respectively (Kanouse 1949, Peterson 1998) (see Table 1 for GenBank accession numbers, and Olariaga et al. 2015).

Our results suggest that four species, *O. bufonia*, *O. leporina*, *O. onotica* and *O. tuomikoskii*, have a western North American-Eurasian distribution (Fig. 5). The presence of these in Asia needs to be confirmed using multiple gene sequences. Also, from North America only one sequence each represents *O. onotica* and *O. tuomikoskii* in our multi-gene phylogeny and although the branches are short, further sampling could reveal genetic differentiation between North American and European collections. Species with a disjunct Eurasian-North American or European-North American distribution have been documented in other *Pezizomycetes*: in *Geopyxis* (Wang & Hansen unpubl.), in *Phillipsia* (Hansen et al. 1999), in *Morchella* (Du et al. 2012). The two *Otidea* species with a putative European-North American distribution (Fig. 5), *O. nannfeldtii* and '*O. alutacea* clade 3', however, need to be further tested including more samples and multiple gene sequences. In other ectomycorrhizal fungi such wide distributions are absent or rare (Nuytinck et al. 2007) or best explained by recent introductions with host trees (Pringle et al. 2009). Likewise, emerging population genetic studies of Northern Hemisphere, ectomycorrhizal morphospecies have shown that these display intercontinental divergence, and almost no intracontinental phylogeographic structure, providing strong evidence for a lack of ongoing gene flow between European and North American populations. These may be considered phylogenetic or cryptic species using GCPSR (Grubisha et al. 2012, Vincenot et al. 2012).

No species were found with an exclusively disjunct Asian-North American distribution. Three species are suggested to be Eurasian, based on ITS and LSU sequences: *O. alutacea* s.str., *O. daliensis* and *O. mirabilis* (Olariaga et al. 2015).

Phylogenetic relationships and morphological features

Our multi-gene analyses provide the first robust hypothesis of the evolutionary relationships within *Otidea* (Fig. 3). Morphological features in *Otidea* appear in general to be fast evolving and prone to shifts and were found to be poor indicators of higher level relationships; no unique morphological or ecological features seem to support the inclusive clades A–F (Fig. 6). Several characters appear plastic (difficult or impossible to score as a single state for a species), e.g. colour and shape of the apothecia, shape of paraphyses, and non-mapped characters such as apothecial size, and presence or absence of a stipe. This might explain the difficulties and confusions that have prevailed in *Otidea* taxonomy. Two exceptions are the presence of a conspicuous ornamentation on the spores that is a synapomorphy for the *O. unicisa* clade; and the straight to bent or curved, broadly clavate to distinctly capitate paraphyses, a synapomorphy for a restricted *O. concinna* clade (excluding the early diverging *O. caeruleopruinosa* and *O. flavidobrunneola*) (Fig. 2e, f, 6). The resinous, pigmented exudates on the outermost excipulum cells characterise clade B (Fig. 2k), but are also present in *O. daliensis* in clade A. Most species and several clades of *Otidea* show distinct combinations of morphological and ecological features. These are described and discussed in the Taxonomy section in Olariaga et al. (2015).

No obvious morphological features unite all three species in the *O. cantharella* clade and some of the longest branches in the tree are found in this clade (see also gene-conflict above). The grouping could be a result of 'long-branch attraction' (Felsenstein 1978, Bergsten 2005), or if showing the true history suggests these taxa have been separated for a long time or still exhibit rate heterogeneity in these gene regions. Two of the species share large spores (*O. cantharella*: 18–21 µm long; *O. propinquata*: 19–21 µm long), otherwise only present in the *O. platyspora* clade, and *O. propinquata* and *O. brunneoparva* share strongly notched paraphyses, not present in other species of *Otidea* (Fig. 2d, h, 6). The three species appear to be associated with *Picea*, and have clearly stipitate apothecia, typically produced in abundant needle litter. Some of the shortest branches in the tree are present in the *O. formicarum* clade, suggesting these diversified more recently. Morphologically this clade is likewise uniform, with all species having small spores (9.5–12 µm long), curved to hooked paraphyses of equal width throughout, occasionally with a few notches, resinous exudates in the outermost excipular cells coalesce into amber drops in MLZ and the mycelium at the base of the apothecia is with abundant yellow resinous exudates. The apothecia vary somewhat in shape and colours within the clade (Fig. 6).

Our multi-gene analyses confirm that the genus *Otideopsis* is deeply nested in *Otidea* (Liu & Zhuang 2006). *Otidea kaushalii* and *O. unicisa* form the *O. unicisa* clade, strongly placed as a sister group to the *O. bufonia-onotica-O. concinna* clades (Fig. 3). An LSU sequence from GenBank (DQ443452) of *O. yunnanensis*, the type species of *Otideopsis*, differs only 8 bp from our LSU sequence of *O. kaushalii*. *Otideopsis* was erected based on the ornamented spores (Liu & Cao 1987), but a conspicuous spore ornament has evolved at least once within *Otidea*, in the *O. unicisa* clade. The generic placement of *O. kaushalii* and *O. unicisa* has been debatable. Both have been considered species of *Sowerbyella*, again based especially on the ornamented spores (Moravec 1986, 1994). Later *O. kaushalii* was placed in *Aleurina* (Zhuang & Korf 1987) and in *Otideopsis* (Moravec 1988). Harmaja (1986) however, considered *O. unicisa* a species of *Otidea*. The split apothecia, with a densely pustulate outer surface, curved to hooked paraphyses, and abundant resinous exudates on the outermost ectal excipulum cells and on the basal mycelium in *O. kaushalii*, *O. unicisa*, and *O. yunnanensis* are typical for *Otidea*.

The placement of the type species of *Otidea*, *O. onotica*, as a sister species to the coherent *O. bufonia-smithii* clade is surprising. The species share large, cup-shaped, split to broadly ear-shaped apothecia, medium-sized spores and curved paraphyses of the same width throughout, occasionally with a few swollen areas or notches (Fig. 6). But the ochraceous yellow apothecia, often with pink tinges or spots in *O. onotica* are strikingly different from the dark purple or olivaceous brown apothecia in *O. bufonia*, *O. mirabilis* and *O. smithii*. Also the reaction of the resinous exudates in the outermost excipulum in MLZ differs, coalescing into amber drops in *O. onotica* (Fig. 2m) and turning into small reddish particles in the *O. bufonia-O. smithii* clade (Fig. 2n).

We show here for the first time that the genus *Flavoscypha* (/ the *O. concinna* clade) is deeply nested within *Otidea*. Liu & Zhuang (2006) concluded *Flavoscypha* is congeneric with *Otidea*, but in fact their study did not include the European *F. phlebophora* or *F. cantharella* sensu Harmaja (= *O. concinna*); the material they included as *F. cantharella* is the new North American species, *O. pseudoleporina* (= *O. concinna* sensu Peterson 1998; nested in the *O. leporina* clade). Considering *Flavoscypha* is primarily based on an outer excipulum structure of small-celled *textura prismatica* with clavate terminal cells (Harmaja 1974), the phylogenetic placement is not surprising. Our results suggest that all species in the *O. concinna* clade have a distinct excipulum structure in *Otidea*, of a *textura prismatica* to *angularis* (as compared to *textura angularis* in the rest of *Otidea*, except for *O. papillata* that has a *textura prismatica* to *intricata*). Also the bright yellow apothecial colours of *Flavoscypha* are unique to a restricted *O. concinna* clade, i.e. the node of *O. concinna-O. minor*. Unique to this restricted clade is also the straight to bent paraphyses with broadly clavate to distinctly capitate apices, lacking notches (Fig. 6). Other features, such as branching and anastomosing veins or ribs at the base of the apothecia are only found in *O. phlebophora*, *O. minor* and *O. oregonensis*, being most pronounced in *O. phlebophora*.

Mycorrhizal status and putative tree associations

Species of *Otidea* are considered to be ectomycorrhizal, although still only a limited number of molecular ectomycorrhizal community studies have documented *Otidea* from root samples (reviewed in Tedersoo & Smith 2013) and direct evidence is lacking for most species. Nevertheless, ectomycorrhizal fungal communities are typically species rich and consist of a small number of common species, and a large number of rare species (e.g. Horton & Bruns 2001). *Otidea*, along with several other *Pyronemataceae* species appears to belong to the rare group or sampling could be an issue, the occurrence of infected roots being too scattered for detecting. Specifically using ITS sequences from root tips, *O. alutacea* (nearly identical or identical to *O. alutacea* (10)(11); Table 1 and Fig. 3) has been identified from *Quercus douglasii* in dry forest in California (DQ974738; from a single soil core; Smith et al. 2007 as *O. umbrina*) and from *Quercus garryana* in south-western Oregon (EU018574; including morphotyping of the ectomycorrhizae; Moser et al. 2009 as *Otidea* sp.); *O. tuomikoskii* has been identified with *Pseudotsuga* in California (AY310846; Kennedy et al. 2003 as *Otidea*) and in boreal forest in northern Sweden (AY839228; Toljander et al. 2006); and *O. bufonia* on *Pinus thunbergii* in Korea (AB587756; Obase et al. 2011 as *Otidea* sp.). *Otidea* species always produce apothecia alongside ectomycorrhizal plants. Based on our observations, and field notes with herbarium collections, the majority of species occur with either *Pinaceae* or *Fagaceae*, but many species can also be found with *Corylus*, *Populus* and *Salix* (but *Populus* and *Salix* often occur in mixed stands). The associated trees (as inferred from the tree(s) growing alongside the collections) plotted on our multi-gene species phylogeny (Fig. 3, 6) indicate that most clades are either associated with conifers or broadleaved trees. The exceptions are some species in the *O. alutacea* complex (in clade A) and in the deeply nested clade F that might associate with both conifers and broadleaved trees. The growing knowledge about *Otidea* host(s) and host specificity will be important for understanding speciation and species distribution. The novel species, *O. oregonensis* and *O. pseudoleporina*, apparently endemic to western North America, appear to be strictly associated with native western North American trees, most likely *Pseudotsuga menziesii*, although some collections are in addition noted to occur with e.g. *Abies concolor*, *Pinus lambertiana* and *Quercus chrysolepis*. We suggest these two species originated by shifting associations and spreading to geographically novel and unexploited host(s), native to western North America. The closest sister species of *O. pseudoleporina*, the European-North American *O. leporina*, is conversely able to associate with both native European and western North American trees. The closest sister species to *O. oregonensis* is the European endemic *O. borealis*.

CONCLUSIONS AND FUTURE DIRECTIONS

The phylogenetic analyses presented here provide a robust hypothesis for relationships within *Otidea*. Identifying the conflict in the RPB1 gene partition improves the support for the phylogenetic relationships, but complete exclusion of this gene partition is not without cost for other nodes. Even so, the combined three- and four-gene analyses converge on the same topology. Overall our study shows that morphological features within *Otidea* are homoplasious and of limited value for higher-level relationships. Also several features are plastic, which may explain some of the difficulties that have prevailed in species delimitation in *Otidea*. Nevertheless, some subclades and all species identified so far (apart from putative species in the *O. alutacea* complex) can be recognised by a combination of morphological and ecological features. We recognise 33 species using GCPSR and genetic distinctiveness, and estimate a total of 47 *Otidea* species worldwide. With most of the currently described species and names clarified, analyses incorporating a more intense sampling of collections from Asia and further sampling especially from mid-region and eastern North America can now be undertaken. Such analyses coupled with morphological studies, including the newly discovered features (e.g. reactions of excipular resinous exudates in MLZ and KOH, and tomentum colours and exudates), will be able to improve our understanding of the biogeography and diversification of *Otidea* species. We hypothesise that nine species have a transcontinental distribution (four species western North American-Eurasian, two European-North American, and three Eurasia), which should be explored further. Although most *Otidea* species appear to have a rather broad host range, our results indicate that most clades are exclusively associated with coniferous or broadleaved trees. As molecular ectomycorrhizal community studies continue to progress so will hopefully our knowledge about the host specificity and distribution of *Otidea* species.

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