Mycological Progress (2018) 17:77–88
https://doi.org/10.1007/s11557-017-1373-2

ORIGINAL ARTICLE

DGfM

Otidea species from China, three new species with comments on some previously described species

Feng Xu[1] · Zai-Wei Ge[2] · Katherine F. LoBuglio[1] · Donald H. Pfister[1]

Received: 5 August 2017 / Revised: 16 October 2017 / Accepted: 20 December 2017 / Published online: 8 January 2018

Abstract

The genus *Otidea* was recently monographed and studied phylogenetically, but knowledge of the diversity and distribution of *Otidea* species in China is fragmentary. In this study, collections from China were examined morphologically and included in phylogenetic analyses. Using LSU, *TEF1-α*, and *RPB2* new species were placed within previously recognized clades in the genus. The results agree with both Genealogical Concordance Phylogenetic Species Recognition (GCPSR) and genetic divergence as previously reported. Three new species, *Otidea hanseniae*, *Otidea korfii* and *Otidea purpureogrisea* are recognized based on phylogenetic reconstruction using ITS, LSU, *TEF1- α* and *RPB2*. Comments on some incompletely known species are added. With the discovery of these three new species, the genus *Otidea* in China proves to be more diverse than previously recognized.

Keywords Morphology · *Otidea hanseniae* sp. nov. · *Otidea purpureogrisea* sp. nov. · *Otidea korfii* sp. nov. · *Pyronemataceae*

Introduction

Recent studies of *Otidea* (Pers.) Bonord. (*Pezizales*, *Pyronemataceae*) show species of the genus to be widely distributed in the Northern Hemisphere where they are ectomycorrhizal (Hansen and Olariaga 2015). Their relatively large epigeous apothecia are cupulate, split or ear-shaped. A hypogeous species is known, *O. subterranean* Healy & M.E. Sm. (Smith and Healy 2009, 2016). The genus has been demonstrated to be monophyletic and morphologically distinct (Liu and Zhuang 2006; Hansen et al. 2013). There is a recent worldwide treatment of the genus and phylogenetic relationships among the species have been examined, but knowledge of the diversity and distribution of *Otidea* species in China is incomplete (Hansen and Olariaga 2015; Olariaga et al. 2015). Zhuang (2014) reviewed the genus and provided a key to 18 species from China 11 of which were known only from China. Hansen and Olariaga (2015) mentioned at least 17 species from Asia; 10 of these were considered endemic. Although phylogenetic relationships of some of the Chinese *Otidea* species were studied using the LSU gene sequences (Liu and Zhuang, 2006), molecular data for other species was lacking. Those lacking molecular information were not included in the four-gene phylogenetic study by Hansen and Olariaga (2015). Hansen and Olariaga (2015) introduced new morphological characters but these characters were not studied in the Chinese collections. Through new collection efforts and access to material that was unavailable to Hansen and Olariaga (2015) we have been able to further document these fungi in China.

This study began when we undertook identification of a single collection (XF 007) from Gansu Province, China that seemed closely allied to collections named *O. cochleata* (L.) Fuckel and *O. propinquata* (P. Karst) Harmaja. Upon morphological and molecular comparison with several specimens we found not one but three previously unrecognized

Section Editor:Teresa Iturriaga and Marc Stadler

This article is part of the "Special Issue on ascomycete systematics in honor of Richard P. Korf who died in August 2016"

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11557-017-1373-2) contains supplementary material, which is available to authorized users.

✉ Donald H. Pfister
dpfister@oeb.harvard.edu

[1] Harvard University Herbaria and Department of Organismic and Evolutionary Biology, Harvard University, 22 Divinity Avenue, Cambridge, MA 02138, USA

[2] Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

species. This suggests that the number of *Otidea* species in China is higher than expected and warrants further work.

Material and methods

Sampling and morphological methods

Seven species and 12 specimens were included in the molecular phylogenetic analyses. These specimens were from the Farlow Herbarium (FH), the Cornell University Plant Pathology Herbarium (CUP), the Herbarium Mycologium, Institute of Microbiology, Chinese Academy of Sciences (HMAS), and the Herbarium of Cryptogams of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS). Collections with voucher information and GenBank accession numbers are given in Table 1. In our discussion and figures we use the clade terminology applied by Hansen and Olariaga (2015). For morphological examination 14 dried specimens were used (Table 2). These were rehydrated in distilled water for two hours and sectioned using a freezing microtome. Measurements of structures and photographs were made with an Olympus BX40 light microscope with a digital camera (DP50, Olympus, Japan). All measurements were made in water. Melzer's reagent (MLZ) and 10% KOH were used to observe the reaction of resinous exudates and color changes as outlined by Olariaga et al. (2015). When present, basal mycelia were examined and the reaction to MLZ and 10% KOH was recorded.

Genomic DNA isolation

A small piece of apothecia was excised from the dried specimen and ground in an Eppendorf tube using a Fastprep FP120 Cell Disruptor (BIO 101, Carlsbad, CA, USA). Genomic DNA was isolated using the DNeasy Plant Mini kit (Qiagen, Germantown, MD, USA) according to the modified protocol of Costa and Roberts (2014). Optimal dilutions of the genomic DNA were used for PCR amplification of the nuclear ribosomal DNA internal transcribed spacers 1 and 2 plus 5.8S rRNA gene (ITS), the nuclear ribosomal large subunit rDNA gene (LSU), the translation elongation factor-1 alpha gene (*TEF1*-α) and the DNA-dependent RNA polymerase II gene (*RPB2*) regions.

PCR and sequencing

Amplification of the ITS region used the primers ITS1F (Gardes and Bruns, 1993), ITS2, ITS3 and ITS4 (White et al. 1990). The 5′ end of the LSU rDNA region was amplified using the primers LR0R, LR5 (http://www.biology.duke.edu/fungi/mycolab/primers.htm), LR3 and LR3R (Moncalvo et al. 2000). To address problematic samples, *Otidea*-specific primers (Table S1) were employed for amplification of *TEF1*-α, *RPB2*, ITS or LSU.

For PCR, 2 μL of diluted genomic DNA was used as template and 1.25 μL DMSO was added in 25 μL of the PCR master mix. For amplification of ITS and LSU gene sequences, a modified touch-down PCR (TD-PCR) program and Econo *Taq* DNA polymerase (Lucigen, Meddleton, WI, USA) were used. PCR cycling parameters were as follows: initial denaturation at 95 °C for 5 min, followed by 10 cycles including denaturation at 95 °C for 60 s, annealing at 62 °C (decreasing 1 °C each cycle or every three cycles) for 60 s and extension at 72 °C for 60 s, then 35 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 60 s, final extension at 72 °C for 7 min and hold at 12 °C. The PCR amplification parameters for *TEF1*-α and *RPB2* were as described by Hansen et al. (2005) and used Platinum *Taq* DNA polymerase High Fidelity (Thermo Fisher, Carlsbad, CA, USA). All PCR reactions were done in a Peltier Thermal cycler PTC-200 (MJ Research, Watertown, MA, USA).

PCR products were purified either directly or after band excisions using the QIAquick PCR purification kit (Qiagen, Germantown, MD, USA) or Gel Extraction kit (Qiagen, Germantown, MD, USA), and sequenced as described in Hansen et al. (2005). Sequencher 4.6 (GeneCodes, Ann Arbor, MI, USA) was used to edit and assemble the DNA sequences obtained.

Sequence analyses

Alignment of the sequences was done using MAFFT webserver (http://mafft.cbrc.jp/alignment/server, Katoh and Standley 2013) using default parameters and manually optimized in MEGA v6.0 (Tamura et al. 2013). The best-fit evolutionary model for each dataset was determined using jModelTest version 0.1 (Posada 2008) and then Bayesian inference (BI) was calculated with MrBayes3.1.2 (Ronquist and Huelsenbeck 2003). Four Markov chains were run for 2 runs from random starting trees for 1 million generations. Trees were sampled every 100 generations. The first one-fourth of the generations were discarded as burn-in. A majority rule consensus tree of all remaining trees was calculated. Maximum-Likelihood (ML) analysis was conducted with RAxML-HPC2 on Abe through the Cipres Science Gateway (www.phylo.org; Miller et al. 2010) using the default parameters. The GTRCAT model for bootstrapping phase was selected and a majority rule consensus tree of all remaining trees was calculated. Outgroup taxa were specified for each of the analyses and are listed in the respective figure legends. Branches that received bootstrap support for Bayesian posterior probabilities (BPP) and ML greater than or equal to 0.95 of BPP and 70% of ML-BP were considered as significantly supported.

Table 1 Collections used in the molecular phylogenetic analyses, with voucher information and GenBank accession numbers

Species	Collection or Herbarium	Geographic origin, year and collector	GenBank accession no.			
			ITS	LSU	*TEF1-α*	*RPB2*
Otidea alutacea	WZ 2123 (FH 00464722, HMAS 72058)	China, Sichuan province, 20-Aug-1997 D. S. Hibbett & Z. Wang	KU987013	KU987025	–	–
Otidea alutacea	WZ 2128 (FH 00464723, HMAS 72057)	China, Sichuan province, 20-Aug-1997 D. S. Hibbett & Z. Wang	KU987014	KU987026	–	–
Otidea hanseniae	WZ 2202 (FH 00464708); paratype	China, Sichuan province, 04-Sep-1997 D. S. Hibbett & Z. Wang	KU987012	KU987024	KU987033	–
Otidea hanseniae	XF 007 (FH); holotype	China, Gansu province, 07-Aug-2012 S. X. Wang	KU987016	KU987028	KU987035	KU987038
Otidea korfii	Z. W. Ge 1913 (FH, holotype; HKAS 53998, isotype)	China, Sichuan province, 19-Aug-2007 Z. W. Ge	KU987017	KU987029	KU987036	–
Otidea olivaceobrunnea	Q. L. Hu 232 (HMAS 23948)	China, Sichuan province, 24-Aug-1958 Q. L. Hu	KU987010	KU987022	–	–
Otidea propinquata	HMAS 83564	China, Xinjiang province, 01-Aug-2003 W. Y. Zhuang & Y. Nong	KU987009	KU987021	–	–
Otidea purpureogrisea	WZ 2157 (FH 00464724, holotype; HMAS 72805, isotype)	China, Sichuan province, 23-Aug-1997 D.S. Hibbett & Z. Wang	KU987015	KU987027	KU987034	–
Otidea purpureogrisea	Z. W. Ge 863 (FH 00286577, holotype; HKAS 49358, isotype)	China, Sichuan province, 11-Aug-2005 Z. W. Ge	KU987011	KU987023	KU987032	KU987037
Otidea subpurpurea = *Otidea bicolor*	Z. L. Yang 5156 (HKAS 54453, holotype; HMAS 188415, isotype)	China, Yunnan province, 16-Aug-2008 Z. L. Yang	KU987008	KU987020	–	–
Otidea subpurpurea	Z. L. Yang 4602 (HMAS 97530, holotype; HKAS 49443, isotype)	China, Yunnan province, 08-Oct-2005 Z. L. Yang	KU987019	KU987031	–	–
Otidea subpurpurea	Z. L. Yang 5152 (HKAS 54449)	China, Yunnan province, 16-Aug-2008 Z. L. Yang	KU987018	KU987030	–	–

Table 2 Morphological comparison of *O. cantharella* clade and *O. bufonia-onotica* clade

		Ascospores (μm)	L_m (μm)	W_m (μm)	Q_m	Resinous exudates			Basal mycelium		
						In water	in MLZ	In KOH	In water	in MLZ	In KOH
O. cantharella clade	*O. hanseniae*	15–18.5 × 7.5–12	16.7–17.7	9.5–9.6	1.7–1.8	Brown to dark brown	Partially dissolving and becoming yellow	Unchanged	Pale brown with resinous exudates	Partially dissolv-ing	Becoming yellow
	O. propinquata	19–21 × 10–12.5	19.3–20	10.9–11.6	1.6–1.7	Yellow brown to brown	Dissolving	Becomes brownish red	Pale brown with resinous exudates	Dissolving	Partially dissolving
	O. cantharella	18–21 × 10–11.5	17.7–20	10.4–11.4	1.7–1.8	Yellow to yellow brown	Dissolving into amber drops	–	Very pale yellowish brown, smooth or with very small resinous exudates	Dissolving	Partially dissolving
	O. brunneoparva	11.5–14 × 6.5–8.5	11.7–13.8	7.1–8.3	1.6–1.7	Yellowish to reddish brown	Dissolving into amber drops	–	Very pale yellowish brown, smooth or with very small resinous exudates	Dissolving	Unchanged
O. bufonia-onotica clade	*O. korfii*	14.5–17 × 6.5–9	15.6	8.1	1.94	Brown to dark brown	Partially dissolving and becoming yellowish brown	Partially dissolving and becoming light brown	Pale brown and smooth	Becomes yellow	Unchanged
	O. bufonia	13–16.5 × 6–7.5	12.4–16.1	6.3–7.3	1.9–2.5	Dark brown	Partially dissolving and converting into small reddish particles	Partially dissolving	Hyaline to brown with oily, light brown drops on the surface	–	–
	O. bicolor	10–12 × 5–6.5	11.3	5.6	2.05	Brown to dark brown	Unchanged	Becomes reddish brown	Brown with resinous exudates	Unchanged	Dissolving and becoming yellowish brown
	O. subpurpurea	9–12 × 4.5–6	10–11.5	5.0–5.8	2.0–2.3	Dark brown	Unchanged	Becomes yellowish Brown	Brown with resinous exudates	Unchanged	Dissolving and becoming yellowish brown
	O. smithii	12–14 × 6–7.5	12.5–13.6	6.4–7.1	1.9–2.0	Dark brown	Partially dissolving and becoming reddish	–	Light to darker brown with small brown resinous exudates	–	–
	O. mirabilis	13.5–16 × 6–7	14.1–15.4	6.3–6.9	2.1–2.3	Dark brown	Partially dissolving and becoming reddish	–	Light yellow to light brown with small dark brown resinous exudates	–	–
	O. olivaceobrunnea	12.6–15.5 × 6–8	14.5	7.2	2.02	Brown to dark brown	Unchanged	Partially dissolving and becoming yellowish brown	Yellowish brow and smooth	Unchanged	becoming brown to dark brown
	O. purpureogrisea	11–16 × 6–7.5	12.8–15	6.4–6.6	2–2.3	Dark brown	Partially dissolving and becoming amber	Becomes brown	Pale brown and with resinous exudates	Unchanged	Dissolving and becoming yellowish brown
	O. onotica	12–13.5 × 6–7	12.1–13.3	6.2–6.8	1.8–2.0	Yellow		–		Dissolved	

Table 2 (continued)

	Ascospores (µm)	L_m (µm)	W_m (µm)	Q_m	Resinous exudates			Basal mycelium		
					In water	in MLZ	In KOH	In water	in MLZ	In KOH
						Dissolving into amber drops		Hyaline to very light yellow with resinous exudates		Partially dissolving and becoming yellow
O. purpurea	8–10 × 4.5–6	9.0	5.0	1.85	Yellowish brown	Becoming yellow	Unchanged	–	–	–

Results

Molecular recognition of three new species

The phylogenetic trees based on combined sequences of LSU, *TEF1-α*, and *RPB2* placed the three proposed new species, *O. hanseniae* Pfister, F. Xu, & S.X. Wang (represented by XF 007 and WZ 2202), *Otidea korfii* Pfister, F. Xu & Z. W. Ge (represented by a single collection, Z. W. Ge 1913) and *O. purpureogrisea* Pfister, F. Xu & Z. W. Ge (represented by Z.W. Ge 863 and WZ 2157) in separate lineages within previously recognized clades of *Otidea* (Fig. 1). Phylogenetic reconstruction based on ITS (Fig. 2), and single gene data (Fig. S1, S2, S3) placed the two specimens of *O. hanseniae* in the *O. cantharella* clade as a sister taxon to *O. propinquata* supported by Bayesian and ML analyses. The results are in agreement with Genealogical Concordance Phylogenetic Species Recognition (GCPSR, Taylor et al. 2000). *Otidea purpureogrisea* was highly supported as a sister taxon to *O. olivaceobrunnea* Harmaja (represented by HMAS 23948) in the *O. bufonia-onotica* clade based on the ITS rDNA phylogeny (Fig. 3). *Otidea korfii* resolved as a separate lineage in the *O. bufonia-onotica* clade in the ITS (Fig. 3) and LSU rDNA sequence phylogenies (Fig. S1). Phylogenetic reconstruction based on the *TEF1-α* gene sequence placed this species in the *O. bufonia-onotica* clade as a sister taxon to *O. smithii* Kanouse, but with low BP support (Fig. S2). We could not resolve the species delimitation through Genealogical Concordance Phylogenetic Species Recognition (GCPSR, Taylor et al. 2000) because there was only one collection of this species. It is considered to be a new species because it is genetically distinct from the other species in the *O. bufonia-onotica* clade (Fig. 3).

Taxonomy

Otidea hanseniae **Pfister, F. Xu & S.X. Wang, sp. nov. (**Fig. 4**).**

MycoBank 816082.

Holotype: CHINA, Gansu province, Zhangye. 7 Aug 2012. Shouxian Wang, XF 007 (FH).

Apothecia gregarious, 10–18 mm high, 5–12 mm wide, initially ear-shaped, margin subacute, split, broadly ear-shaped at maturity, seldom almost cup-shaped, entire, stipitate. *Hymenium* yellowish brown to olivaceous brown, when dried pale brown to dark brown. *Receptacle surface* concolorous, pale brown to olivaceous brown when dried, then pale brown to dark brown, furfuraceous, sometimes pustulate, finely warted at the base. Pustules hemispherical, gregarious, brown. *Stipe* 5–10 × 2–4 mm. Basal tomentum and mycelium white to pale brown, when dried pale brown to ochre brown. *Spores* ellipsoid to broadly ellipsoid, sometimes very slightly inequilateral, with one to two large

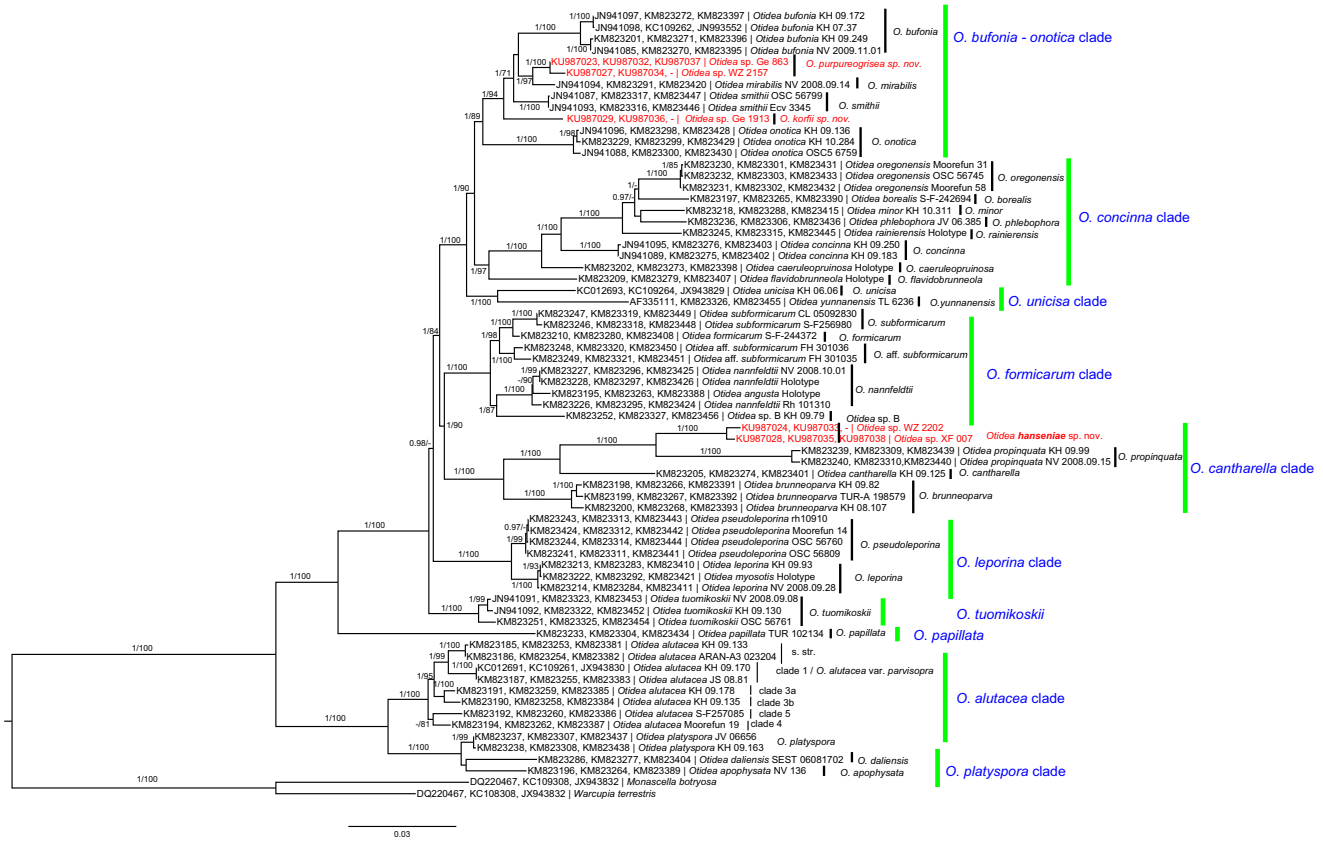

Fig. 1 Phylogeny of *Otidea* produced from Bayesian analysis based on combined LSU, *TEF1-α* and *RPB2*, indicating the placement of the new species: *O. hanseniae, O. korfii and O. purpureogrisea*. Sequences of *Monascella botryosa* Guarro & Arx and *Warcupia terrestris* Paden & Cameron were included as outgroups. Only values above 0.95 of Bayesian posterior probabilities (BPP) and 70% of Maximum Likelihood bootstrap (ML-BP) are shown

guttules, seldom with several additional smaller granules, smooth, hyaline, 15–18.5 (19.5) × 7.5–12 µm (L_m = 16.7–17.7 µm, W_m = 9.5–9.6 µm, Q_m = 1.7–1.8, n = 30). *Paraphyses* curved to hooked of uniform width or slightly enlarged at the apices to 2.7–3.3 µm wide. *Asci* cylindrical, 8-spored, 150–230 × 11–15 µm. Apothecial flesh 700–

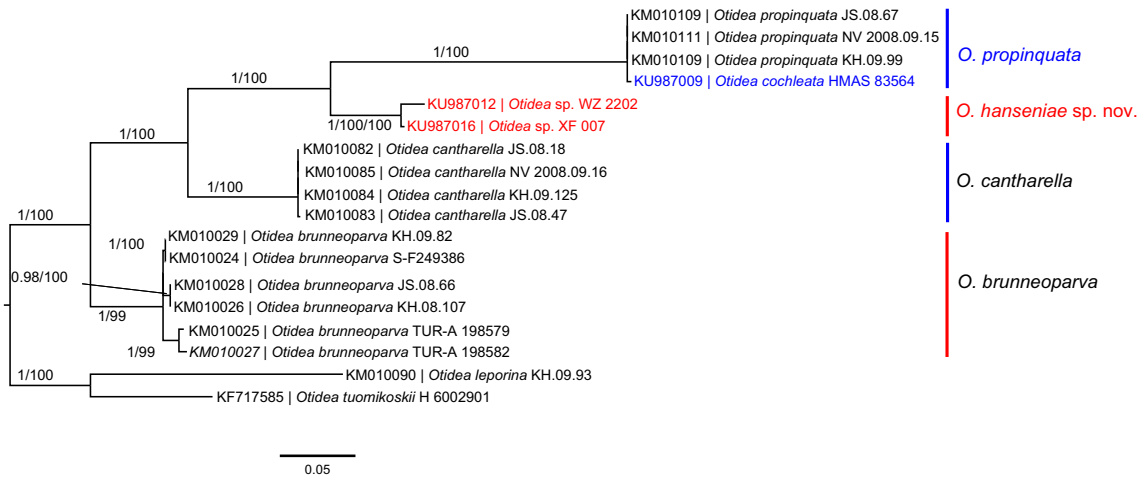

Fig. 2 Phylogeny of *Otidea cantharella* clade produced from Maximum-Likelihood (ML) analysis based on ITS sequence data, indicating the placement of *Otidea hanseniae*. Sequences of *Otidea leporina* (Batsch) Fuckel and *Otidea tuomikoskii* Harmaja were included as outgroups. Only values above 0.95 of Bayesian posterior probabilities (BPP) and 70% of ML-BP are shown

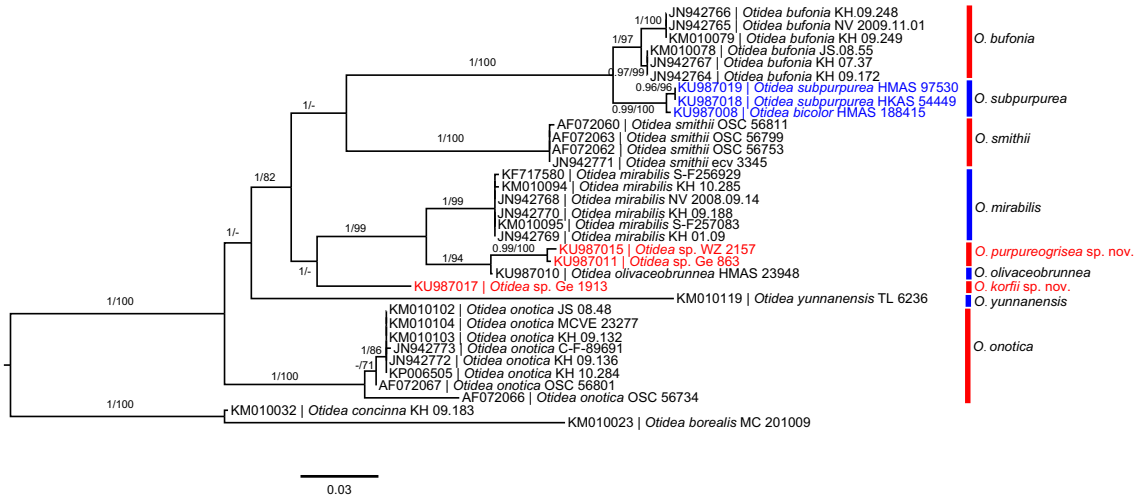

Fig. 3 Phylogeny of *Otidea bufonia-onotica* clade produced from Maximum-Likelihood (ML) analysis based on ITS sequence data, indicating the placement of *Otidea korfii* sp. nov. and *Otidea purpureogrisea* sp. nov. Sequences of *Otidea borealis* W.Y.Zhuang & Zhu L. Yang and *Otidea concinna* (Pers.) Sacc. were included as outgroups. Only values above 0.95 of Bayesian posterior probabilities (BPP) and 70% of Maximum-Likelihood bootstrap (ML-BP) are shown here

1100 μm thick. *Subhymenium* ca. 60–160 μm thick, visible as a yellow brown zone of densely arranged cylindrical to swollen cells, with scattered brown resinous exudate at septa. *Medullary excipulum* of *textura intricata*, 400–650 μm thick, formed by loosely woven cylindrical to slightly swollen thick-walled hyphae, 3–7.5 μm wide, pale brown. *Ectal excipulum* of *textura angularis*, 40–50 μm (excluding pustules), cells thick-walled, brown to dark brown, 17–30 × 14–18 μm, pustules 30–50 μm high. *Resinous exudates* abundant on the outer surface, brown to dark brown, partially dissolving in MLZ and turning yellow, unchanged in KOH. *Basal mycelium* of interwoven hyphae, 2.8–3.8 μm wide, septate, pale brown, turning yellow in KOH, smooth or with very small granules of resinous exudates on the hyphal surface, partially dissolving in MLZ.

Etymology: This species is named in honor of Karen Hansen in recognition of her work on this genus and for her helpful suggestions in the preparation of this paper.

Additional specimen: CHINA, Sichuan province, Luhuo. 4 Sep 1997. David S. Hibbett & Zheng Wang, WZ 2202 (FH 00464708).

Notes: Morphologically this species shares characters with the *O. cantharella* clade but differs in the size of ascospores. Among the species in this clade, ascospores of *O. hanseniae* are bigger than those of *O. brunneoparva* Harmaja but smaller than those of *O. propinquata* and *O. cantharella* (Fr.) Quél. (Table 2). *Otidea hanseniae* is also characterized by smaller apothecia and ascospores with few or no small guttules. The molecular phylogenetic reconstruction (Fig. 1) strongly supports it as a separate lineage in this clade. This species is closely related to *O. propinquata*. The smaller ascospores, color of the resinous exudates, reaction of the resinous exudates to KOH and reaction of basal mycelium to KOH clearly differentiates it from *O. propinquata* (Table 2).

***Otidea korfii* Pfister, F. Xu & Z.W. Ge, sp. nov. (**Fig. 5**).** Mycobank 816085.

Holotype: CHINA, Sichuan Province, Hongyuan. 19 Aug 2007. Z. W. Ge 1913 (FH).

Apothecia gregarious, 10–45 mm high, 5–15 mm wide when dried, ear-shaped, split, seldom almost cup-shaped, entire, stipitate. *Hymenium* ochre to yellowish brown, when dried yellowish brown. *Receptacle surface* olivaceous brown, furfuraceous, finely pustulate at the base. Pustules hemispherical, gregarious, dark brown. *Stipe* 6–20 × 2–8 mm. Basal tomentum and mycelium pale brown. *Spores* ellipsoid to broadly ellipsoid, sometimes very slightly inequilateral, with one to two large guttules, seldom with several additional smaller granules, smooth, hyaline, 14.5–17 × 6.5–9 μm (L_m = 15.6 μm, W_m = 8.1 μm, Q_m = 1.94, n = 15). *Paraphyses* curved to hooked, of uniform width or slightly enlarged at the apices to 2–2.5 μm. *Asci* cylindrical, 8-spored, 180–220 × 9–12 μm. Apothecial flesh 650–900 μm thick. *Subhymenium* ca. 80–135 μm thick, visible as a brown zone, of densely arranged cylindrical to swollen cells, with scattered brown resinous exudate at septa. *Medullary excipulum* of *textura intricata*, 300–500 μm thick, formed of loosely woven cylindrical to slightly swollen thick-walled hyphae, 4.5–9 μm wide, pale brown. *Ectal excipulum* of *textura angularis*, 40–80 μm (excluding pustules), cells thick-walled, brown, 15–30 × 12–20 μm, pustules 35–60 μm high. *Resinous exudate* abundant on the outer surface, brown to dark brown, partial

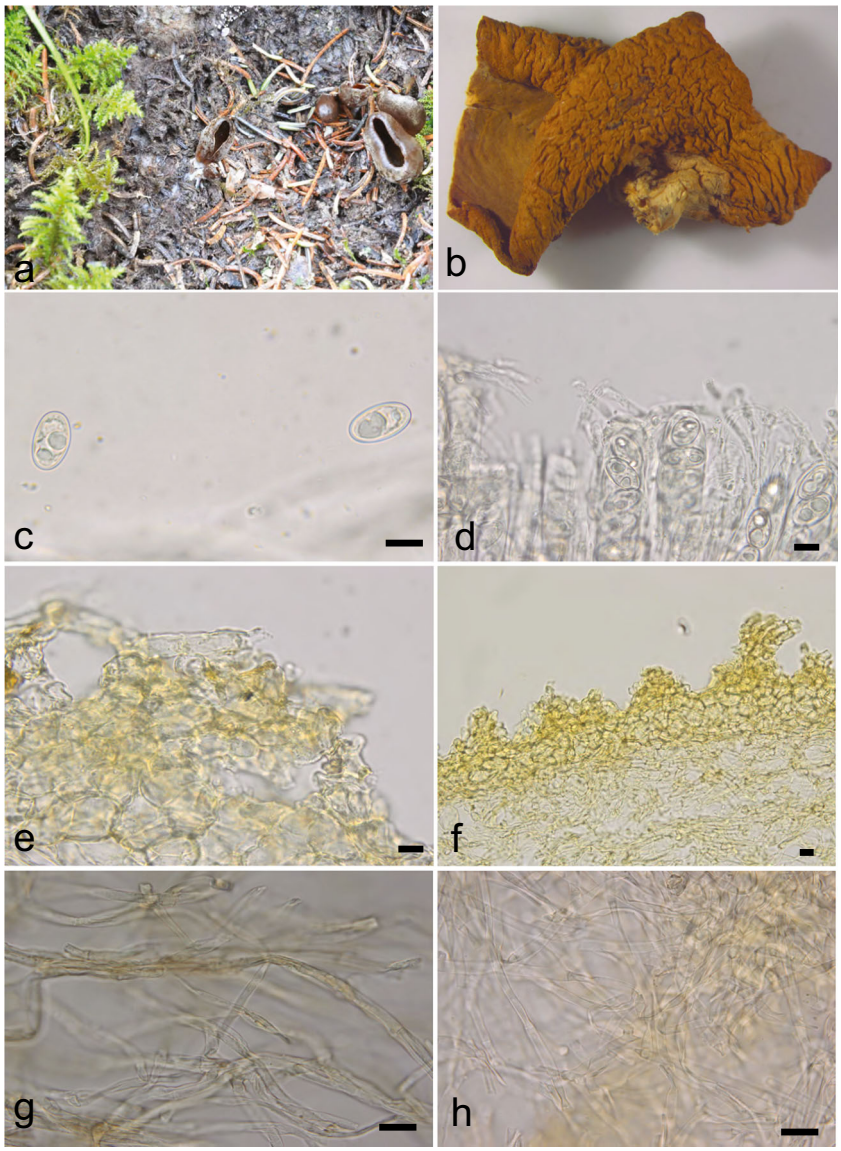

Fig. 4 *Otidea hanseniae*. a, Apothecia of XF 007; b, dried apothecium of WZ 2202; c, Ascospores in water; d, Paraphyses and asci in water; e, Pustule on the outer surface of receptacle in water; f, Pustule in Melzer's reagent; g, Basal mycelium in KOH; h, Basal mycelium in Melzer's reagent. Scale bars, 10 μm

dissolving in MLZ and turn yellowish brown, partially dissolving in KOH, becoming light brown. *Basal mycelium* of interwoven, 3.5–5 μm wide, septate, pale brown hyphae, unchanged in KOH, smooth, turning yellow in MLZ.

Etymology: This species is dedicated to Richard P. Korf, student of discomycetes and mentor to many.

Notes: This species shares the morphological characters of those in the *O. bufonia-onotica* clade. Ascospore size distinguishes it from *O. brevispora (W.Y.Zhuang) Olariaga & K. Hansen*, *O. bicolor*, *O. subpurpurea W.Y. Zhuang*, *O. smithii*, *O. onotica (Pers.) Fuckel*, *O. purpurea* (M. Zang) Korf & W.Y. Zhuang and *O. purpureogrisea*. Reaction of the resinous exudate in the ectal excipulum to MLZ and KOH in *O. bufonia (Pers.) Boud.*, *O. mirabilis* Bolognini & Jamoni and *O. olivaceobrunnea,* all members of this clade (Table 2), distinguishes them from *O. korfii*. The unique spore size distinguishes *O. korfii* from the other species described from China (Zhuang 2014). Molecular phylogeny supports *O. korfii* as a separate lineage in this clade, which is closely related to *O. smithii, O. mirabilis* and *O. olivaceobrunnea*. It differs from them in microscopic characters, reaction of resinous exudates to MLZ, and in having smooth basal mycelium.

***Otidea purpureogrisea* Pfister, F. Xu & Z.W. Ge, sp. nov. (**Fig. 6**).**

Mycobank 816084.

Holotype: CHINA, Sichuan Province, Sêrtar. 11 Aug 2005. Z.W. Ge 863 (FH 00286577), isotype: HKAS 49358.

Apothecia gregarious, 20–40 mm high, 10–22 mm wide when dried, ear-shaped, entire, stipitate. *Hymenium* purple-brown, when dried ochraceous brown to dark brown. *Receptacle surface* dark purple-brown, near base purple-gray, when dried ochraceous brown to dark brown, furfuraceous, sometimes finely pustulate at the base. Pustules

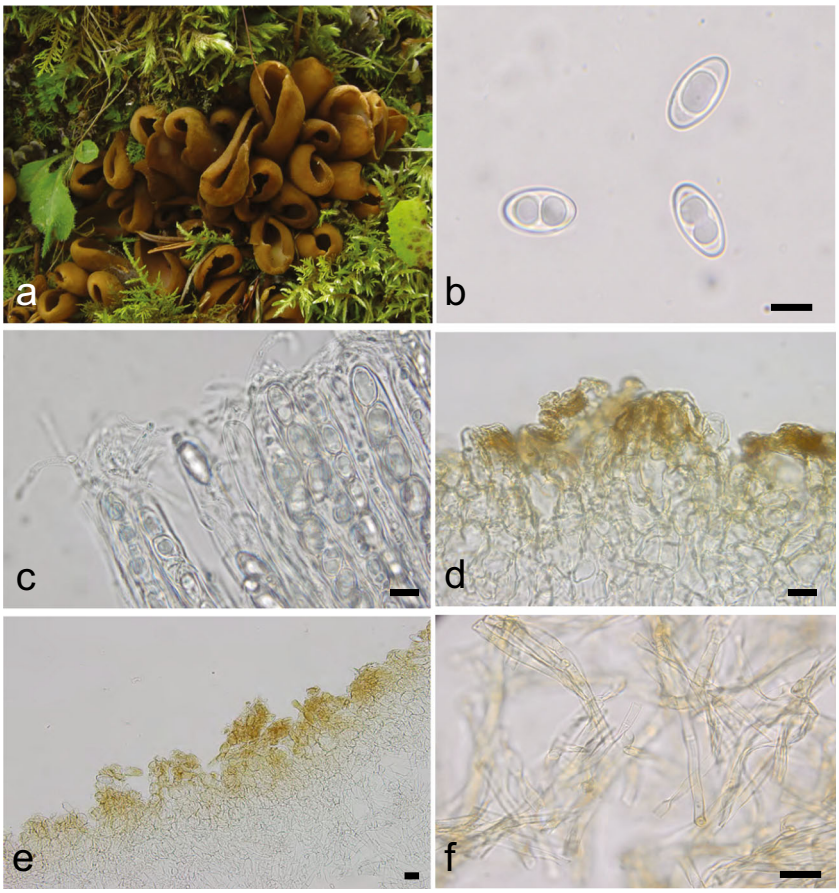

Fig. 5 *Otidea korfii*. a, Apothecia of Z.W. Ge 1913; b, Ascospores in water; c, Paraphyses and asci in water; d, Pustules on the outer surface in water; e, Pustules in KOH; f, Basal mycelium in KOH. Scale bars, 10 μm

hemispherical, gregarious, dark brown. *Stipe* 10–15 × 2–8 mm when dried. Basal tomentum and mycelium pale brown when dried. *Spores* ellipsoid, sometimes very slightly inequilateral, with one to two large guttules, sometimes with several additional smaller guttules, smooth, hyaline, 11–16 × 6–7.5 μm (L_m = 12.8–15 μm, W_m = 6.4–6.6 μm, Q_m = 2–2.3, n = 31). *Paraphyses* curved to hooked of uniform width or slightly enlarged at the apices, 3.3–4.7 μm wide at apex, 2–3 μm below. *Asci* cylindrical, 8-spored, 115–190 × 9–13 μm. Apothecial flesh 600–950 μm thick. *Subhymenium* ca. 50–80 μm thick, visible as a brown zone of densely arranged cylindrical to swollen cells with scattered brown resinous exudate at septa. *Medullary excipulum* of *textura intricata*, 250–500 μm thick, formed of loosely woven cylindrical to slightly swollen thick-walled hyphae, 3–6.5 μm wide, pale brown. *Ectal excipulum* of *textura angularis*, 50–60 μm thick, cells thick-walled, dark brown, 10–20 × 8–17 μm, pustules 30–90 μm high. *Resinous exudate* abundant on the outer surface, dark brown, partial dissolving in MLZ and turning amber and brown in KOH. *Basal mycelium* of interwoven, 2.3–6 μm wide, septate, pale brown hyphae, dissolving and turn yellowish brown in KOH, with very little resinous exudates on the surface, unchanged in MLZ.

Etymology: The name refers to the purple-gray tone of receptacle surface near base.

Additional specimen: CHINA. Sichuan Province, Luding. 23 Aug 1997. David S. Hibbett & Zheng Wang. WZ 2157 (FH 00464724 = HMAS 72805).

Notes: This species is characterized by its purple brown hymenium, brown receptacle surface, purple-gray tone of the receptacle surface near the base and the color reaction of resinous exudates to MLZ and KOH. It shares morphological characters with species in the *O. bufonia-onotica* clade, but the reaction of the resinous exudate in the ectal excipulum in MLZ and KOH differentiate it from the other members of the *O. bufonia-onotica* clade (Table 2). The molecular phylogenetic reconstruction strongly supports the placement of *O. purpureogrisea* in this clade, which is close to *O. olivaceobrunnea* based on ITS analysis (Fig. 3). It differs from *O. olivaceobrunnea* in color of the hymenium and receptacle, thicker medullary excipulum and its obvious stipitate form.

The examination of the holotype of *O. purpurea* (HKAS 5670 = CUP-CH 2520) shows spores 8–10 × 4.5–6 μm (L_m = 8.98 μm, W_m = 5.01 μm, Q_m = 1.85, n = 11), a yellow MLZ reaction and resinous exudates unchanged in KOH. This distinguishes *O. purpurea* from *O. purpureogrisea*. In spore size

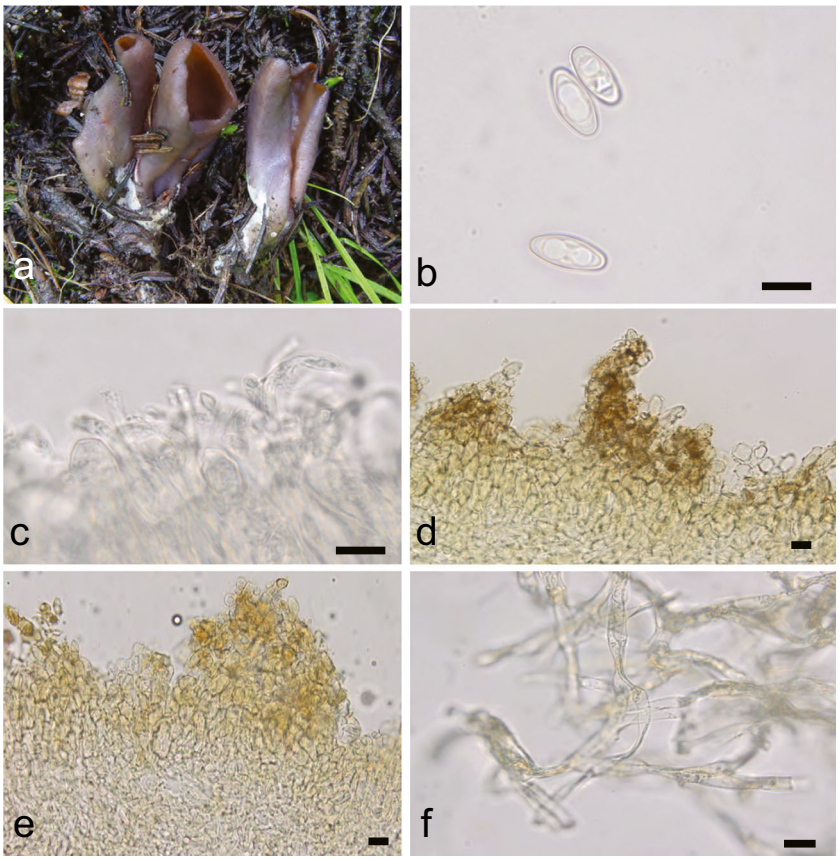

Fig. 6 *Otidea purpureogrisea*. a, Apothecia of Z.W. Ge 863; b, Ascospores in water; c, Paraphyses in water; d, Pustules on the outer surface in water; e, Pustules in KOH; f, Basal mycelium in KOH. Scale bars, 10 μm

this species differs from *O. brevispora*, *O. subpurpurea* and *O. bicolor*, the other Chinese species in this clade.

Olariaga et al. (2015) mentioned that two Chinese collections assigned to *O. leporina* (Batsch) Fuckel by Zhuang (2007) were resolved in the *O. mirabilis* clade. We included LSU sequences from these two specimens in the phylogenetic tree and confirm that they are in the *O. mirabilis* clade. Apothecial color differences and the larger ascospores further confirm that this species differs from Chinese material of *O. leporina*.

Discussions

Phylogenetic placement of O. bicolor *and* O. subpurpurea

Two specimens of *O. subpurpurea* (HMAS 97530; HKAS 54449) and one specimen of *O. bicolor* (HMAS 188415) were included in this study (Table 1). Phylogenetic reconstructions based on the ITS and LSU gene sequences (Fig. 3 and S1) indicated that *O. bufonia*, which is sister to *O. subpurpurea*, may represent two species. *Otidea subpurpurea* consistently is distinguished as a species in the LSU phylogeny (Fig. S1) and Bayesian and ML analysis using the full length of ITS sequences (Fig. 3) also supported *O. subpurpurea* as a separate lineage with 0.96 of BPP and 96% ML-BP. These two species differ in the reaction of the resinous exudate in KOH (Table 2), the color of the hymenium and the receptacle surface (Zhuang and Yang 2007; Zhuang 2010). We prefer to treat *O. bicolor* and *O. subpurpurea* as synonyms. *Otidea bicolor* is incompletely known and the most recently proposed name. We could locate only one specimen of *O. bicolor*; a deeper study with more collections and more loci will help to fully understand *O. bicolor* and its relationships.

Phylogenetic placement of O. olivaceobrunnea

Otidea olivacea J.Z. Cao & L. Fan was described by Cao et al. (1990) with one collection (HMAS 36970). It was renamed as *O. olivaceobrunnea* by Harmaja (2009a) because the epithet *olivacea* had already been used in *Otidea*. Zhuang (2014) redescribed this species and noted that the ascospores (13–15.5 × 6.5–7.5 μm) were smaller than those given in the original description (14–17 × 8–8.5 μm), but out of 5 specimens examined, two specimens (WZ 2128 = HMAS 72057 = FH 00464722 and WZ 2123 = HMAS 72058 = FH 00464723) proved to be in the *O. alutacea* (Pers.) Massee complex based on LSU and ITS analyses (Fig. S1 and S4). Re-examination and comparisons, particularly using the morphological features proposed by Hansen and Olariaga (2015), revealed that

the holotype (HMAS 36970) and HMAS 23948, should be considered to be the same species. Thus, specimen HMAS 23948 is treated as *O. olivaceobrunnea*. The phylogenetic placement of *O. olivaceobrunnea* was demonstrated to be in *O. bufonia-onotica* clade and is closely related to *O. purpureogrisea* (Fig. 3).

Phylogenetic placement of O. purpurea

There is only one collection of *O. purpurea* known (HKAS 5670, holotype; HMAS 58212, isotype; CUP-CH-002520; Zhuang and Korf 1987; Zhuang 2014). Unfortunately, only a partial ITS sequence was obtained from the holotype in this study. BLAST results indicate that the ITS sequence of *O. purpurea* has high similarity to *O. subpurpurea* in the *O. bufonia-onotica* clade (data not shown). Olariaga et al. (2015) treated *O. purpurea* in this clade based on its dark brown apothecia as well as its brown basal tomentum. They mentioned that *O. purpurea* should be compared with *O. subpurpurea*. Zhuang and Yang (2007) noted in the original description of *O. subpurpurea* that the two were similar but differed in hymenial color, receptacle surface, size of hyphae in medullary excipulum and ascus length. Re-examination of specimens of these two species regarding the reaction of the ectal excipulum to MLZ and KOH confirms that these are two distinct species (Table 2). In our analysis based on LSU *O. purpurea* falls in clade 6 of the *O. alutacea* complex (Fig. S1).

Occurrence of O. propinquata *in China*

Chinese collections of *O. cochleata* (L.) Fuckel were thought to be confused with *O. propinquata* because of their habitat (Zhuang 2014). The concepts of *O. cochleata* have varied over time and have led to confusion. One of the specimens of *O. hanseniae* was originally identified as *O. cochleata,* a name that was considered to be a *nomen ambiguum* by Parslow and Spooner (2013). Olariaga et al. (2015) treated *O. cochleata* as a synonym of *O. alutacea* based on both morphological and molecular evidence. Cao et al. (1990) listed *O. propinquata* from China citing two specimens, but Zhuang (2014) revised one of these specimens (MHSU 1804 = HMAS 61358) to *O. cochleata* because of its smaller ascospores compared with the original description. Unfortunately, no Chinese collections identified as *O. cochleata* were included in the phylogenetic study by Liu and Zhuang (2006). As stated above *O. cochleata* has been variously interpreted and now it has been referred to *O. alutacea*. Although ascospore size of *O. hanseniae* matches the taxon some have called *O. cochleata,* the concolorous receptacle and hymenium and different shape of the cells of the outer layer of ectal excipulum separate *O. hanseniae.* Further molecular examination of a specimen identified as *O. cochleata* (HMAS 83564 from Xinjiang province, China) showed that it should be referred to *O. propinquata* (Fig. 2, S1). *Otidea proqinquata* was thought to be found only in Europe but, through this study, is now confirmed to exist in China.

Another Chinese collection (HMAS 30799) was originally identified as *Peziza abietina* Pers. (a synonym of *O. propinquata*). It was collected from the same location as the holotype of *O. hanseniae*. Molecular analysis of this specimen indicated it was a member of the Helvellaceae (unpublished data).

A Chinese clade in O. alutacea *complex*

In our study, phylogenetic reconstruction based on LSU placed two specimens (WZ 2128 and WZ 2123), originally named *O. olivaceobrunnea,* in the *O. alutacea* complex with high BP support (Fig. S1). Parslow and Spooner (2015) found that two Chinese collections of *O. alutacea* (HMAS 52742 and HMAS 57844) formed a distinct clade with high BP (clade 6) in an ITS rDNA phylogeny. This clade was closely related to clade 3a and clade 3b (Fig. S4) as noted by Olariaga et al. (2015). In our study a dataset of LSU gene sequences of the *O. alutacea* clade and *O. platyspora* clade provided information on the phylogenetic relationship within the *O. alutacea* complex (Fig. S1). The two Chinese specimens (WZ 2123and WZ 2128) fell into the same clade as the previously examined Chinese *O. alutacea* specimens (HMAS 52742 and HMAS 57844) examined by Parslow and Spooner (2015) (clade 6 in Fig. S1). Thus, a new group in the *O. alutacea* clade, clade 6, includes four Chinese collections (WZ 2123, WZ 2128, HMAS 52742 and HMAS 57844). The group is characterized by spores in the range of 14–17.5 × 6–8 μm (L_m = 14–16 μm, W_m = 6.8–7.6 μm, Q_m = 2, n = 15). The spore sizes in these collections overlap with those of *O. alutacea* s. str. but is distinctly non-overlapping with *O. kunmingensis* W.Y. Zhuang described from China (Table S2). Although *O. kunmingensis* was considered to be in the *O. alutacea* complex by Olariaga et al. (2015) and Parslow and Spooner (2015), the small ascospore size, even smaller than *O. alutacea* var. *parvispora*Parslow & Spooner, as well as the geographic location, indicates that it may constitute a distinct taxon in the *O. alutacea* complex. It should be noted that one European collection (voucher C-F-48045) represents a separate lineage in both LSU and ITS phylogenetic tree (Fig. S1 and S4). We suggest that this be treated as an additional clade in the *O. alutacea* complex (clade 7 Fig. S1 and S4). The Chinese collections of the *O. alutacea* complex involve at least three taxa including *O. kunmingensis,* with the smallest ascospores in the complex (Table S2). To fully clarify species boundaries within this complex, wide sampling of additional collections both for molecular and morphological study is needed.

Conclusions

Three new species are recognized and their phylogenetic placements are confirmed by ITS primary sequence analysis. *Otidea hanseniae*, *O. purpureogrisea* and *O. korfii* are proposed. Phylogenetic placement of *O. subpurpurea*, *O. olivaceobrunnea* and *O. purpurea* are discussed and clarified. The occurrence of *O. propinquata* in China and a Chinese clade in the *O. alutacea* complex is confirmed. With the discovery of these three new species, and the expanded distribution of *O. subpurpurea*, *O. olivaceobrunnea*, *O. propinquata* and *O. purpurea* as occurring in China, the genus *Otidea* proves to be more diverse both genetically and geographically than previously recognized.

Acknowledgments We thank the curators Scott La Greca (CUP), Ende Liu (HKAS), Yijian Yao and Liu Yang (HMAS) for loan of specimens used in this study and Genevieve E. Tocci at FH for dealing with loans. We thank Shouxian Wang at Beijing Academy of Agriculture & Forestry Sciences for sharing his collection. We are especially grateful to Karen Hansen at Swedish Museum of Natural History for helpful suggestions. Partial funding of this research was supported by a grant from National Natural Science Foundation of China (NSFC 31401929) and China Scholarship Council (CSC) to Feng Xu.

References

Cao JZ, Fan L, Liu B (1990) Some species of *Otidea* from China. Mycologia 82:734–741

Costa CM, Roberts RP (2014) Techniques for improving the quality and quatity of DNA extracted from herbarium specimens. Phytoeuron 48:1–8

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Hansen K, Olariaga I (2015) Species limits and relationships within *Otidea* inferred from multiple gene phylogenies. Persoonia 35: 148–165

Hansen K, LoBuglio KF, Pfister DH (2005) Evolutionary relationships of the cup-fungus genus *Peziza* and *Pezizaceae* inferred from multiple nuclear genes, RPB2, β-tubulin, and LSU rDNA. Mol Phylogenet Evol 36:1–23

Hansen K, Perry BA, Dranginis AW, Pfister DH (2013) A phylogeny of the highly diverse cup-fungus family *Pyronemataceae* (*Pezizomycetes, Ascomycota*) clarifies relationships and evolution of selected life history traits. Mol Phylogenet Evol 67:311–335

Harmaja H (2009b) A note on *Otidea* (*Pezizales*, Fungi). Phytotaxa 2:49–50

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7, improvements in performance and usability. Mol Biol Evol 30:772–780

Liu CY, Zhuang WY (2006) Relationships among some members of the genus *Otidea* (*Pezizales, Pyronemataceae*). Fungal Diver 23:181–192

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. In, Proceedings of the gateway computing environments workshop (GCE), 14 Nov., 1–8. New Orleans, LA, USA

Moncalvo JM, Lutzoni FM, Rehner SA, Johnson J, Vilgalys R (2000) Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. Syst Biol 49:278–305

Olariaga I, Van Vooren N, Carbone M, Hansen K (2015) A monograph of *Otidea* (Pyronemataceae, Pezizomycetes). Persoonia 35:166–229

Parslow M, Spooner BM (2013) The British species of *Otidea*, overview and the large-spored species. Mycosystema 32:347–365

Parslow M, Spooner BM (2015) The British species of *Otidea* (2), *O. alutacea* and related taxa. Ascomycete.org 7: 295–302

Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25:1253–1256

Ronquist F, Huelsenbeck JP (2003) MrBayes 3, Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Smith ME, Healy RA (2009) *Otidea subterranea* sp. nov., *Otidea* goes below ground. Mycol Res 113:858–866

Smith ME, Healy RA (2016) Index Fungorum:297

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6, molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21–32

White TJ, Bruns TD, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols, a guide to methods and applications. Academic Press, San Diego, pp 315–322

Zhuang WY (2010) Taxonomic assessment of some pyronemataceous fungi from China. Mycotaxon 112:31–46

Zhuang WY (2014) Flora fungorum sinicorum vol. 48 - *Pyronemataceae*. 92–118. Science press, Beijing, China

Zhuang WY, Korf RP (1987) Some new species and new records of discomycetes in China. II. Mycotaxon 29:309–314

Zhuang WY, Yang ZL (2007) Some pezizalean fungi from alpine areas of southwestern China. Mycologia Montenegrina 10:235–249