Otidea saliceticola (*Pezizales*) a new species from the Italian Alps

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Abstract: *Otidea saliceticola*, a species collected in arctic-alpine habitats of the Italian Alps, is proposed as new to science. The new species is supported by its morphology, genetic profile and ecology. A discussion on the affinities with members of the so-called "*bufonia* clade" is presented. Color pictures of both fresh and dried samples as well as microscopic features are provided.
Keywords: Ascomycota, ITS, 28S rDNA, *Otidea bufonia*, *O. mirabilis*, *O. smithii*, *O. brunneoparva*, phylogeny, taxonomy.

Riassunto: *Otidea saliceticola*, una specie rinvenuta tra la micro-selva delle Alpi italiane, viene descritta nuova per la scienza. La nuova specie è supportata per la sua morfologia, genetica ed ecologia. Vengono discusse le sue affinità con i membri del cosiddetto "clado *bufonia*". Il tutto viene corredato da fotocolor di esemplari freschi e secchi, nonchè di microscopia.
Parole chiave: Ascomycota, filogenia, ITS, 28S rDNA, *Otidea bufonia*, *O. mirabilis*, *O. smithii*, *O. brunneoparva*, tassonomia.

Introduction

As reported by Hansen & Olariaga (2015), species of *Otidea* (Pers.) Bonord. should be considered ectomycorrhizal, although still only few molecular ectomycorrhizal community studies have documented *Otidea* from root samples (Toljander *et al.*, 2006; Tedersoo & Smith, 2013) and direct evidence is lacking for most taxa. Most species of *Otidea* seem to be strictly associated with broadleaved or coniferous trees, and only few with both.

At best of our knowledge, no *Otidea* species have been recorded in arctic-alpine environments so far, with the exception of an *Otidea* sp. reported by Jamoni (2004), tentatively named "*fusconigra*" ad int. He did not state if it was new or maybe just an adaption to alpine dwarf *Salix* habitats of an already known species belonging to what he called "*bufonia-umbrina-grandis* group". Unfortunately, no color images were published although a good greyscale drawing and a detailed description were provided.

Olariaga *et al*. (2015) cited this collection in the comments of *Otidea brunneoparva* K. Hansen, M. Carbone, Olariaga & Van Vooren, and indicated it was nested in the so-called *bufonia*-clade as a sister species of *Otidea smithii* Kanouse.

During the summer of 2017 we had the chance to find many samples among the dwarf *Salix* and *Dryas* in high altitude of the Italian Alps in two occasions. It was first collected and photographed on 7th August by M. Cartabia and also on 27th August in the same place by M. Cartabia and M. Carbone. Since the morphological features of our samples matched perfectly Jamoni's collection, we decided to further investigate these samples and review their taxonomic status.

Material and methods

Morphological study

The microscopic studies were based on both fresh and dried specimens. Two optical microscopes were used: Paralux monocular and Olympus CX41 trinocular with plan-achromatic objectives 10×, 40×, 60×, 100× oil immersion. The following main reagents were used: Melzer's reagent, cotton blue, Congo red, 5% KOH. Water mounts were used for the observation of the pigmentation and measurements. At least 30 ascospores naturally discharged from the asci were measured from each apothecium.

Phylogenetic study

Total DNA was extracted from dry specimens employing a modified protocol based on Murray & Thompson (1980). PCR amplification was performed with the primers ITS1F and ITS4 (White *et al*., 1990, Gardes & Bruns, 1993) for ITS region, while LR0R and LR5 (Vilgalys & Hester, 1990; Cubeta *et al*., 1991) were used to amplify the 28S rDNA region, PCR reactions were performed under a program consisting of a hot start at 95 °C for 5 min, followed by 35 cycles at 94 °C, 54 °C and 72 °C (45, 30 and 45 s respectively) and a final 72 °C step 10 min. PCR products were checked in 1% agarose gels, and positive reactions were sequenced with one or both PCR primers. Chromatograms were checked searching for putative reading errors, and these were corrected. BLAST (Altschul *et al*., 1997) was used to select the most closely related sequences from INSD public databases (mainly from Hansen & Olariaga, 2015). Sequences first were aligned in MEGA 5.0 (Tamura *et al*., 2011) software with its Clustal W application and then corrected manually. Aligned loci were subjected to MrModeltest 2.3 (Nylander, 2004) in PAUP* 4.0b10 (Swofford, 2002), and the best models implemented in Mr.Bayes 3.1 (Ronquist & Huelsenbeck, 2003), where a Bayesian analysis was performed (ITS and 28S rDNA data partitioned, two simultaneous runs, six chains, temperature set to 0.2, sampling every 100th generation) until convergence parameters were met after about 0.35M generations, standard deviation having fell below 0.01. Finally, a full search for the best-scoring maximum likelihood tree was performed in RAxML (Stamatakis, 2006) using the standard search algorithm (data partitioned, 2000 bootstrap replications). Significance threshold was set above 0.95 for posterior probability (PP) and 70% bootstrap proportions (BP).

Phylogenetic results

Overall topology of the tree obtained after the analysis of the combined ITS–28S rDNA dataset agreed with that obtained by Hansen & Olariaga (2015) for the *O. bufonia-O. onotica*, *O. concinna* and *O. unicisa* clades. Five major lineages were significantly supported in the *O. bufonia-O. onotica* clade, matching the species *O. bufonia*, *O. mirabilis*, *O. onotica*, *O. smithii*, and an unnamed lineage where the arctic-alpine Otidea sp. samples collected in the present study where nested. Up to four distinct genetic lineages of *O. bufonia* where found to be significantly different from each other. Two of them were already found by Hansen & Olariaga (2015) in Europe, another one is composed of samples collected in East Asia, and a fourth one has been found in the present work. A significant relationship between *O. mirabilis* and *O. smithii* was also found, although not recovered by Hansen & Olariaga (2015), maybe due to greater number of DNA markers employed by these authors. *Otidea smithii* was significantly related to the unnamed *Otidea* sp. lineage composed of the Italian arctic-alpine samples. Therefore, we here propose a new species name to accommodate this lineage.

Table 1 – Samples used in the phylogenetic analysis, in bold the newly generated for the present study, GenBank codes.

Taxon	Voucher/Strain Number	ITS	28S rDNA
Otidea borealis	S-F242694	KM010023	KM823197
Otidea bufonia	**MCVE 29367**	**MG383799**	**MG383807**
Otidea bufonia	**MCVE 29368**	**MG383800**	**MG383808**
Otidea bufonia	**MCVE 29369**	**MG383801**	**MG383809**
Otidea bufonia	**MCVE 29370**	**MG383802**	**MG383810**
Otidea bufonia	**MCVE 29371**	--------	**MG383811**
Otidea bufonia	**MCVE 29372**	**MG383803**	**MG383812**
Otidea bufonia (as *Otidea* sp.)	MFLU 16-0611	KY498604	KY498609
Otidea bufonia (as *Otidea* sp.)	HKAS 87865	KY498603	KY498608
Otidea bufonia (as uncultured)	T-oo	AB506108	--------
Otidea bufonia (as uncultured)	P09151	AB587756	--------
Otidea bufonia	JS150904-08	KX963782	--------
Otidea bufonia	KH.09.249 (S)	KM010079	KM823201
Otidea bufonia	KH.09.248 (S)	JN942766	JN941084
Otidea bufonia	NV 2009.11.01 (S)	JN942765	JN941085
Otidea bufonia	K(M)81773	EU784384	--------
Otidea bufonia	K(M)91376	EU784387	--------
Otidea bufonia	K(M)135070	EU784386	--------
Otidea bufonia	K(M)57971	EU784383	--------
Otidea bufonia	KH.07.37 (S)	JN942767	JN941098
Otidea bufonia	C-F-94240	KP119674	--------
Otidea bufonia	JS.08.55 (S)	KM010078	KM823469
Otidea bufonia	KH.09.172 (S)	JN942764	JN941097
Otidea caeruleopruinosa	MT 10082601	KM010030	KM823203
Otidea caeruleopruinosa	H6010805	KF717575	KM823202
Otidea concinna	KH.09.250 (S)	JN942775	JN941095
Otidea concinna	KH.09.183	KM010032	JN941089
Otidea kaushalii	T. Læssøe 6236 (C)	KM010119	AF335111
Otidea minor	KH.98.84 (C)	KM010041	KM823217
Otidea minor	CL 950914-01	KM010044	KM823220
Otidea mirabilis	**MCVE 29373**	**MG383804**	-------
Otidea mirabilis	**MCVE 29374**	**MG383805**	**MG383813**
Otidea mirabilis	GMFN 1951 (S)	KF717580	KM823483
Otidea mirabilis	MC201008 (S)	KM010095	KM823482
Otidea mirabilis (as *O. umbrina*)	KH.01.09 (C)	JN942769	AY500540
Otidea mirabilis	NV 2008.09.14	JN942768	JN941094
Otidea mirabilis	KH.09.188 (S)	JN942770	JN941086
Otidea mirabilis	NV 108 (S)	JN942768	JN941094
Otidea mirabilis	KH.10.285 (S)	KM010094	KM823221
Otidea onotica	OSC 56759	--------	JN941088
Otidea onotica	KH.10.284 (S)	KP006505	KM823229
Otidea oregonensis	OSC 56745	AF072089	KM823232
Otidea oregonensis	Moorefun 58 (OSC, S)	KM010048	KM823231
Otidea papillata	H6003547	KF717582	KM823234
Otidea papillata	TUR 102134	KM010105	KM823233
Otidea phlebophora	JV06-385 (C)	KM010049	KM823236
Otidea rainierensis	A.H. Smith 30553 (MICH)	KF717583	KM823245
Otidea saliceticola	**MCVE 29365**	**MG383806**	**MG383814**
Otidea saliceticola (as *O. fusconigra*)	GMFN 2293 (S)	KM010037	KM823476
Otidea smithii	Ecv3345 (S)	JN942771	JN941093
Otidea smithii	OSC 56799	AF072063	JN941087
Otidea unicisa	KH.06.06 (FH)	--------	KC012693
Warcupia terrestris	CBS 891.69	--------	DQ220467

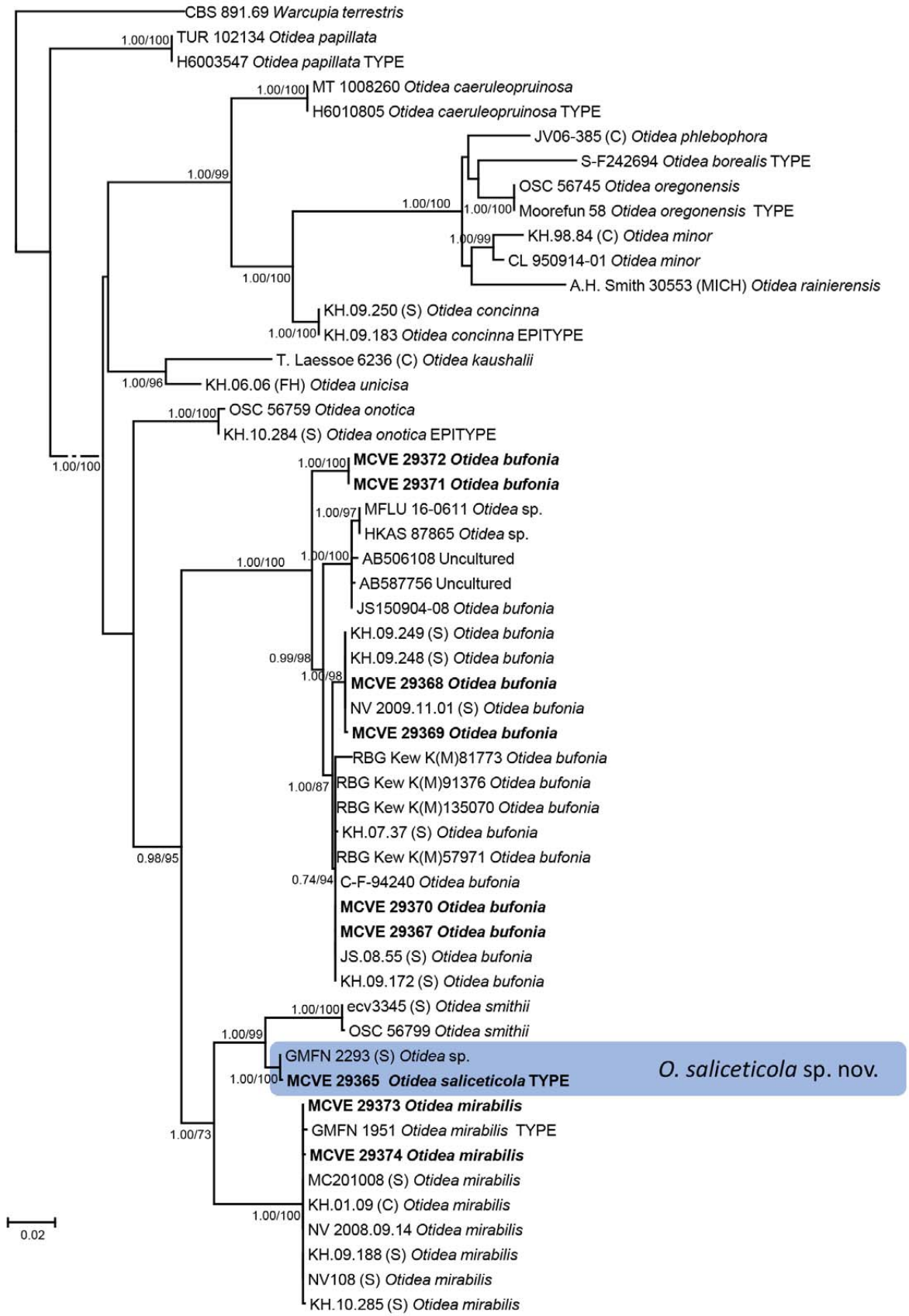

Fig. 1 – Best-scoring maximum-likelihood (ML) combined ITS-28S rDNA phylogram of obtained in RAxML from 2000 bootstrapped trees. Nodes were annotated if supported by >0.95 Bayesian PP (left) or >70% ML BP (right).

Taxonomy

Otidea saliceticola Cartabia, M. Carbone & P. Alvarado, *sp. nov.* MB 823262 – Plate 1-5

Diagnosis: Besides its unique genetic profile, it differs from its sister species *Otidea bufonia*, *O. smithii* and *O. mirabilis* mainly in the much smaller size, lower spore length/width ratio, and arctic-alpine habitat.

Holotype (selected): Italy, Baceno (VB), Alpe Devero, 2290 m a.s.l. (GPS coord. 46.294955° N, 8.228085° E), in the shadow part of a very moist slope, among *Salix reticulata* L., *Salix herbacea* L. and *Dryas octopetala* L., 27.VIII.2017, *legit* M. Cartabia & M. Carbone, MCVE 29365. Paratype: *ibidem*, 07.VIII.2017, *legit* M. Cartabia, MCVE 29366.

Etymology: Inhabitant of *Salix*, due to its growth among dwarf *Salix*.

Synonym: *Otidea fusconigra* Jamoni *ad int.*, *Funghi e Ambiente*, 94-95: 10 (2004), *nom. inval*.

Macroscopical features

Apothecia gregarious, up to 20 mm high and 10 mm wide, heterogeneous in shape, some ear-shaped with rounded or truncated apical part, some others wider, more deeply cup-shaped, split, most lacking a true stipe but some distinctly substipitate. **Hymenium** mainly pale alutaceous-greyish (almost pure whitish in one sample) but becoming slightly darker with age, finally almost blackish in some sun-exposed samples; orangish when dried in the palest samples. **Receptacle surface** finely furfuraceous-warty, slightly hygrophanous, not perfectly uniform in color, mainly dark brown, sometimes rusty brown, with lighter orangish-brown spots; brown when dried. **Basal tomentum** and mycelium white and abundant in some samples whilst almost absent in others, light yellowish only in old samples.

Microscopic features

Ascospores smooth, hyaline, with walls up to 0.8 µm thick, very heterogeneous in shape and in the number/size of guttules, the most regular spores are slightly subfusoid with rounded poles and with two guttules of equal size, but most frequently ellipsoid and slitghly inequilateral with two guttules of equal or different size or only one big guttule (present in living spores); a few ovoid spores and some with a single oil droplet were also observed; spore size seems to be very variable: (11–)12.5–15(–17) × (6.5–)7–8(–11) µm, most of them measuring between 14–15 × 7.5–8 µm, but abnormal ovoid spores up to 17 × 11 µm have been found, Q=(1.7–)1.75–1.87(–2). **Paraphyses** cylindrical, septate, branched at the base, sometimes anastomosing, 2 µm wide, tips slightly bent to broadly curved or hooked, equal or slightly enlarged up to 3.5 µm wide, some with a low notch on the underside. **Asci** 150–180 × 11–12 µm, 8-spored, operculate, pleurorhynchous. **Subhymenium** visible as a small darker zone, composed of cylindrical to swollen cells, densely arranged, with scattered brownish resinous exudates at septa. **Medullary excipulum** arranged as a *textura intricata*, hyphae thick-walled, 5–8(–13) µm wide, constricted at septa, hyaline to very light brown, often with resinous brown exudates at the hyphal septa. **Ectal excipulum** of *textura globuloso-angularis*, cells slightly thick-walled, light brown, up to 25 µm in diameter. Most of the external part (i.e. the warts) is composed by chains of 4-6 subglobose to elongated cells, constricted at the septa, the basal ones the largest with a size up to 30 × 20 µm. Resinous exudates abundant, brown, partly dissolving and converting into small reddish particles in Melzer's. **Basal mycelium** of 3–4 µm wide, composed by hyaline to light brown hyphae, walls up to 0.8 µm thick, with an encrusted extracellular pigment.

Plate 1 – *Otidea saliceticola*. Holotype. Photo: M. Carbone.

Plate 2 – *Otidea saliceticola*. Holotype. Photo: M. Carbone.

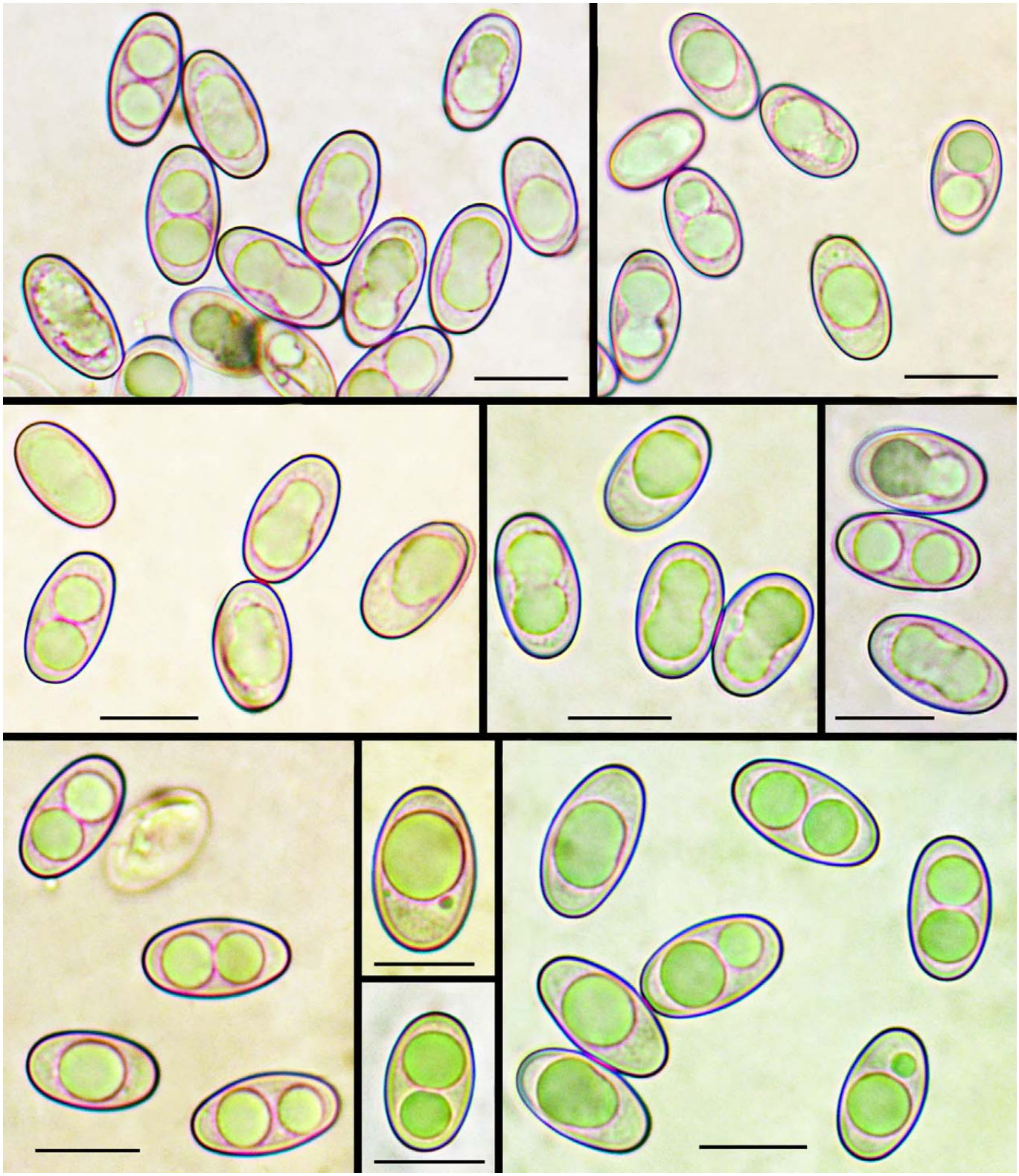

Plate 3 – *Otidea saliceticola*. Holotype. Photo: M. Carbone.
All spores in water mounts. Bars = 10 µm.

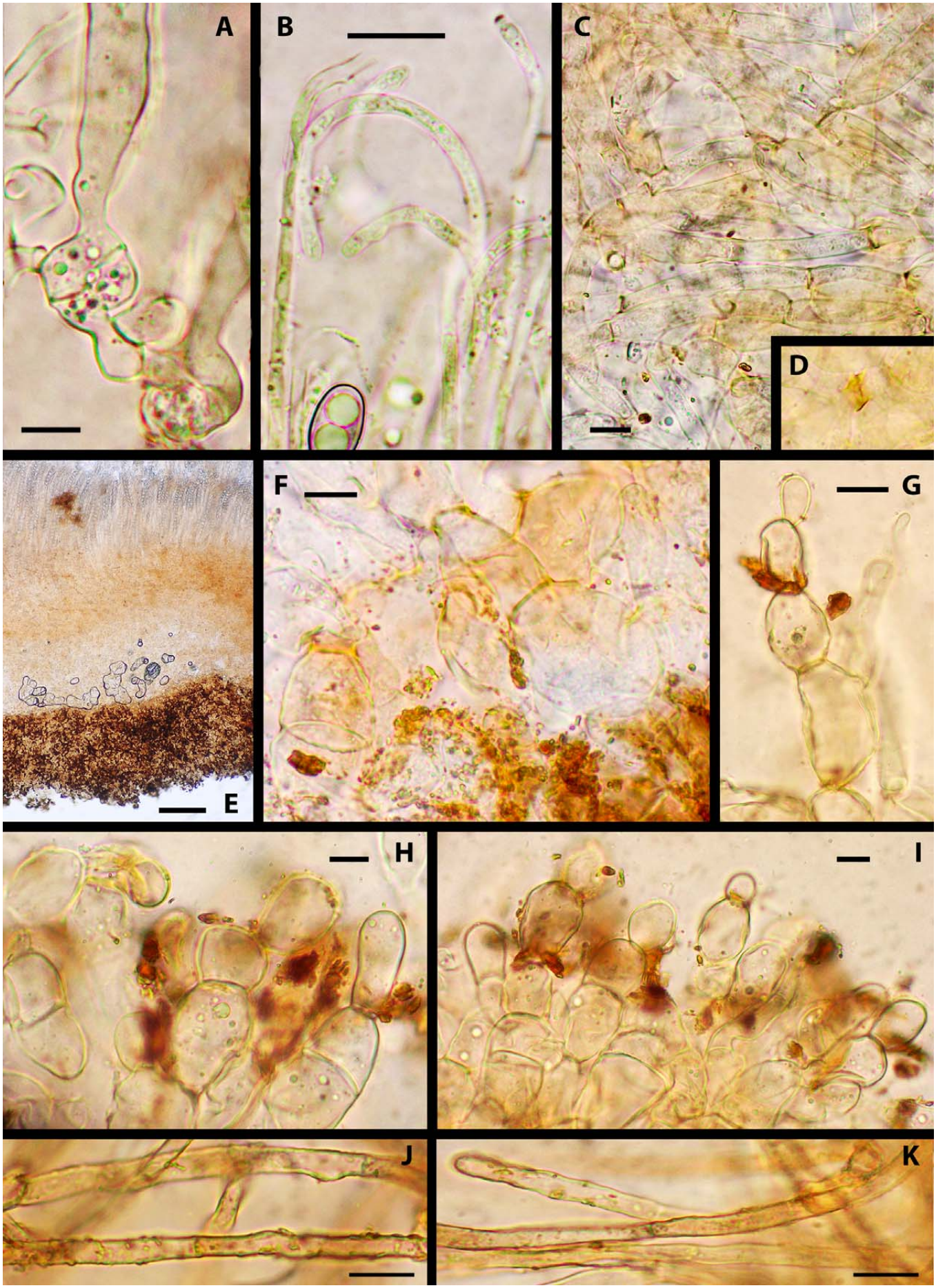

Plate 4 – *Otidea saliceticola*. Holotype. Photos: M. Carbone.
A: pleurorhynchous ascus base; B: paraphyses tips; C: medullary excipulum hyphae with some thickening septa with brown exudates deposit; D: brown exudates deposit at septa in the medullary excipulum hyphae; E: apothecium section; F: elements of the ectal excipulum; G-I: chained element of the external surface warts: J-K: basal mycelium. Bars = 10 µm, except E = 100 µm. All pictures in water mounts.

Plate 5 – *Otidea saliceticola*. Holotype. Photo: M. Carbone.
Dried apothecia. Bar = 1 cm

Ecology

All the samples were located in the shaded area of a small, very steep and moist (trickeling water) area. They were found among moss, *Salix* ssp. (*S. herbacea* L. and *S. reticulata* L.) at the lower edge of a *Dryas octopetala* L. belt growing in the drier and most exposed part of the slope above. The environment was strictly arctic-alpine with no other trees nor plants. The rocky substrate is predominantly formed by calcschists with some quartzite inclusions (Albertini, 1991).

The year 2017 was characterized by a limited amount of snow in winter and scarce rains in spring. Interestingly, alpine fungi in the Alpe Devero area fruited a couple of weeks before the average fruiting dates of previous years.

Other studied and sequenced collections in the present study

Otidea bufonia. Italy. Falcade (BL), Fraz. Caviola, in a mixed forest, 06.IX.2008, *leg*. E. Bizio (MCVE 29367). Cesarò (ME), Contrada Bufali, under *Quercus*, 26.X.2013, *leg*. M. Carbone (MCVE 29368). Caorle (VE), Brussa, Vallevecchia, under *Pinus pinea* close to the sea, 04.XI.2001, *leg*. E. Campo (S-F257089 and MCVE 29369). Vinadio (CN), San Bernolfo, mixed forest, 29.IX.2008, *leg*. M. Carbone (MCVE 29370). Finland. Kuusamo, Oulanka National Park, Ampumavaara, under *Picea abies*, *Pinus sylvestris*, *Alnus incana* and *Betula*, 14.VIII.2010, *leg*. M. Carbone (MCVE 29371). *Ibidem*, 20.VIII.2010, *leg*. M. Carbone (MCVE 29372). *Otidea mirabilis*. Italy. Falcade (BL), Fraz. Caviola, under conifers, 25.VIII.1998, *leg*. E. Bizio (MCVE 29373). Falcade (BL), Colmean, under conifer, 04.IX.2008, *leg*. E. Bizio (MCVE 29374).

Discussion

Otidea saliceticola is mainly characterized by small brown apothecia, white (fresh) to brownish (dry) basal mycelium, ellipsoid inequilateral (sub)fusoid or ovoid spores measuring on average 14–15 × 7.5–8 µm with Q=1.75–1.87, often with resinous brown exudates at the hyphal septa of the medullary excipulum, and growth in arctic-alpine habitat in connection with dwarf *Salix* and (possibly also) *Dryas*. It is not surprising that it belongs to the *bufonia*-clade because just from a first sight, and for a trained eye, it immediately recalls a dwarf form of *Otidea bufonia* (Pers.) Boud. [= *O. umbrina* (Pers.) Bres. = *O. grandis* (Pers.) Rehm *sensu* Boudier (1905) *et auct. plur.*] or a smaller and brown (not-purple) *Otidea mirabilis* Bolognini & Jamoni.

Otidea bufonia is a widespread species growing under both broadleaved trees and conifers from sea level to the alpine range. It is morphologically characterized by medium to big apothecia, mainly cup-shaped, split on a side, dark brown in color with possible purplish, greenish or lilac tinges; (sub)fusoid spores and elements of the hyphae in medullary excipulum partly with encrusting brown exudates (Olariaga *et al*., 2015).

Otidea mirabilis typically grows in coniferous forests and is mainly characterized by medium or small ear-shaped apothecia, with a blue-violet external surface and light ochraceous-yellow hymenium (at least in young and fresh apothecia), subfusoid spores, and absence of incrusted pigment in the hyphae of the medullary excipulum but, when present, biflabellate crystal-like exudates (Jamoni, 2001a, 2001b; Carbone *et al*., 2010; Olariaga *et al*., 2015).

Otidea smithii is a species so far known from Western North America only (Kanouse, 1949; Peterson, 1998), typically characterized by narrow ear-shaped, dark purple-brown apothecia, ellipsoid-subfu-

soid spores measuring 12–14(–14.5) × 6–7.5 µm (Qm=1.9–2.0), and rarely showing pigmented resinous exudates at the hyphal septa of the medullary excipulum (Olariaga *et al*., 2015). Besides the genetic relationship between *Otidea saliceticola* and *O. smithii*, it seems they also share some morphological features such as a lower spore Q (if compared with *O. bufonia* and *O. mirabilis*), and the pigmented resinous exudates at the hyphal septa of the medullary excipulum.

Regarding the variability in spore shape, *Otidea saliceticola* seems to show the greatest diversity in the whole clade. However, ovoid spores were also observed in one genetically confirmed collection of *O. bufonia* (Olariaga *et al*., 2015).

Finally, as already cited in the introduction, another similar small brown species is *Otidea brunneoparva*. Its macro- and micromorphology, as well as its genetic profile, are all well characterized and clearly differ from those of the *bufonia*-clade and the new species proposed in the present work [see for details Olariaga *et al*. (2015)].

Known distribution and period of growth

O. saliceticola is so far known only from the Italian Alps of the Piedmont region. In fact the only two other collections known come from Monte Rosa (Vercelli province) and Colle dell'Agnello (Cuneo province). The former is the well documented (and preserved) collection of Jamoni (2004), whilst the latter is just a personal communication by our friends Mario Filippa and Giorgio Baiano. According to their description and due to their excellent knowledge of Ascomycetes, and especially the arctic-alpine species, we have no doubts that they found *Otidea saliceticola*. Unfortunately, it is an old record and, at the time of the writing of this paper, both the slide and the dried samples could not be located. The first author knows the Cuneo place quite well and we can confirm that the habitat fits perfectly.

Due to the particular habitat and the different climate conditions of each year, it is not so easy to circumscribe the period of growth of *O. saliceticola*. Jamoni (2004) samples were collected at the beginning of August, and ours have been first detected by the second author on 7th August and keep on fruiting until the end of the month. However all the species growing in this habitat usually start fruiting in mid July until mid September with the first snow and the drastic decline in temperature.

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Plate 6 – *Otidea saliceticola*. Paratype. Photo: M. Cartabia.

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