FUNGAL BIOLOGY 120 (2016) 711–728

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Paxillus rubicundulus (*Boletales*, *Paxillaceae*) and two new alder-specific ectomycorrhizal species, *Paxillus olivellus* and *Paxillus adelphus*, from Europe and North Africa

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ARTICLE INFO

Article history:
Received 20 November 2015
Received in revised form
23 February 2016
Accepted 24 February 2016
Available online 2 March 2016
Corresponding Editor:
Martin I. Bidartondo

Keywords:
Alnus
Ectomycorrhizal fungi
Molecular phylogeny
Taxonomy

ABSTRACT

Paxillus rubicundulus P.D. Orton has been shown to be a complex of at least three ectomycorrhizal taxa strictly associated with alders (*Alnus*) in Europe, *P. rubicundulus s. str.*, and two undescribed clades. To assess the taxonomic status of these three clades and their phylogenetic relationships, phylogenetic analyses of two independent gene regions (ITS and *gpd*), combined with macro- and micromorphological comparisons of genetically identified specimens, were carried out. A total of 85 sequences were successfully obtained from basidiomata and alder mycorrhizae collected in France and Algeria and combined with GenBank and UNITE sequences. The phylogenetic results and estimates of genetic diversity confirmed that the three clades are distinct species, often found in sympatry. As a result, *P. rubicundulus s. str.* was redefined based on the revision of type material, and *Paxillus adelphus* and *Paxillus olivellus* are introduced as new Linnaean names. The often used name *Paxillus filamentosus* is rejected since it could not be applied to any of the new species. The three species are distinguished micromorphologically by spore size and shape. They are widely distributed in Europe, North Africa and western Asia; *P. rubicundulus* is rare, and all species have a limited host range.

Introduction

The genus *Paxillus* Fr.: Fr. was established by Fries (1821) for agaricoid fungi with decurrent lamellae and a soft hymenophore separable from the pileus, a unique character amongst brown-spored fungi. Because it is easy to grow and cultivate in the laboratory, the ectomycorrhizal species *Paxillus involutus* (Batsch: Fr.) Fr has been widely used for

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http://dx.doi.org/10.1016/j.funbio.2016.02.008
1878-6146/

physiological and metabolic studies of nutrient uptake and mobilization in investigations about plant-fungus mycorrhizae (Smith & Read 2008; Rineau *et al.* 2013). For several decades, *P. involutus* was the only ectomycorrhizal species recognized in the genus. However, several attempts were made over time to delimit another ectomycorrhizal species that was strictly associated to alders (the genus *Alnus* Mill.), and tentatively named *Paxillus leptopus* (Bresadola 1930: pl. 678), *Paxillus filamentosus* (Kotlaba & Pouzar 1960; Kühner 1962) or *Paxillus rubicundulus* (Orton 1969; Hahn 2000). As a first step towards the recognition of the real diversity within this genus, Fries (1985) recognized intersterility groups within *P. involutus* that formed the basis for the genetic species concepts of later authors. Then, both morphological (Hahn & Agerer 1999) and molecular studies (Hedh *et al.* 2008, Vellinga *et al.* 2012; Jargeat *et al.* 2014) identified four species in the *P. involutus* complex in Europe: *P. involutus*, *Paxillus ammoniavirescens*, *Paxillus obscurisporus*, and the more recently described *Paxillus cuprinus*. It also appeared that *P. rubicundulus* is a complex of several species (Gelardi *et al.* 2014) encompassing the European species *P. rubicundulus*, and at least two well-supported unnamed phylogenetic clades (A1.2 and A1.3). Gelardi *et al.* (2014) accommodated these European species and *Paxillus orientalis* from China in the subgenus *Alnopaxillus*.

In Gelardi *et al.*'s study (2014), *P. rubicundulus* is represented by the type collection, previously sequenced (Hedh *et al.* 2008), but the A1.2 and A1.3 clades are only represented by sequences from environmental DNA samples (ectomycorrhizal tips) or from basidiomata identified as '*P. rubicundulus*' or '*P. filamentosus*' by their collectors (Gelardi *et al.* 2014). Independently, seven *Paxillus* MOTUs (Molecular Operational Taxonomic Units) namely '*Paxillus #1*' to '*Paxillus #7*' were defined in a biogeographical study of the worldwide distribution of ectomycorrhizal taxa associated with alders, based on amplification and sequencing of the nuclear ribosomal Internal Transcribed Spacer (nrITS) of ectomycorrhizal fungal symbionts (Põlme *et al.* 2013). No attempt has been made so far to relate these *Paxillus* MOTUs to the phylogenetic species or clades delimited by Gelardi *et al.* (2014) and to morphological taxonomy. A similar lack of assignment at the species level exists for the '*P. rubicundulus*' isolate Ve08.2h10 whose genome was recently made public (http://genome.jgi-psf.org/Paxru2/Paxru2.home.html) and for a '*P. rubicundulus*' strain used to highlight differential responses to acidity and high nitrate concentrations in the environment of *Alnus*-associated and generalist *Paxillus* (Huggins *et al.* 2014).

The major aims of the present study are to: (i) delimit the phylogenetic species of the *P. rubicundulus* complex inferred from the analysis of two genes, and propose Linnaean names for each of the two unnamed clades, (ii) assess whether phylogenetically distinct species also have characteristic morphological features that can be used for taxonomic purposes, (iii) clarify relationships among European species of the subg. *Alnopaxillus*. Finally, we also mined metadata to clarify MOTU taxonomic issues and *Paxillus* isolate identification. The results provide a better understanding of species diversity in subg. *Alnopaxillus*, their geographical distribution, and host range.

Materials and methods

Isolates

From 2008 to 2014, we analysed 46 *Paxillus* collections that matched the macroscopic and ecological characteristics of the *Paxillus rubicundulus* complex, and four alder mycorrhizal tips (AK, Are, All, LBH). Overall, 32 locations were represented, 31 in France, and 1 in Algeria (Table 1). The '*P. rubicundulus*' strain used by Huggins *et al.* (2014), coded as ALB08.24, is included. In addition, one half of the *P. rubicundulus* holotype from the Kew herbarium (K(M) 190474) was also examined.

After morphological investigation, the specimens were dried, except for a small amount of fresh tissue from the basidiome cap that was frozen at −20 °C until DNA extraction.

Voucher specimens are available upon request from the Evolution et Diversité Biologique (EDB) laboratory, Université Paul Sabatier, Toulouse 3 (France), and the LIP herbarium, Faculté de Pharmacie, Université Lille 2 (France).

Morphological observations and statistical analyses

Freshly collected basidiomata were observed and described; colours were coded according to Cailleux & Taylor's 'Code Expolaire' (1963). Chemical tests were done on fresh basidiomata with 5 % KOH, TL4 (Henry's reagent), 50 % NH_4OH or NH_3, and PDAB (Paradimethylaminobenzene). Fresh spore print colours were coded according to Kornerup & Wanscher (1967). Microscopy observations were made under a Nachet Andromede 018 light microscope. Sections of hymenophore, pileipellis, and stipitipellis from exsiccata were revived and observed in 5 % KOH solution and Melzer's reagent. Pileipellis and hymenophores from fresh collections were mounted in water. In the descriptions, estimated spore dimensions are given as: (lower observed value) − 1^{st} decile − average value − 9^{th} decile − (higher observed value).

Quantitative spore characters, including spore length (apiculus excluded), spore width and the ratio between spore length and spore width (Q), were analysed to test whether they were statistically different among alnicolous *Paxillus* species, and to examine intraspecific variation. Spore sets of 412, 752, and 106 spores yielded by the spore prints of 7, 9, and 1 specimens of *Paxillus adelphus*, *Paxillus olivellus*, and *Paxillus rubicundulus* (type specimen), respectively, were analysed (Table 1). Univariate statistical analyses were performed to determine the mean, median, and variance values of the measurements. Mann−Whitney's nonparametric U-tests with Bonferroni correction were used to assess the statistical significance of interspecies differences for all the spore parameters. Statistical analyses were performed using PAST software program (Hammer *et al.* 2001).

Molecular methods and phylogenetic analysis

Fungal DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, Charbonnières-les-Bains, France). The final pellet was resuspended in 40 μl of sterile ultra-high quality (UHQ) water. ITS rDNA, including the 5′ end of the 28S gene, and regions of the glyceraldehyde-3-phosphate dehydrogenase

Table 1 – Collections and sequenced isolates included in the study. In bold, specimens used for the statistical analysis of spore parameters.

Phylogenetic species	Isolates	Sampling year	Location	Recorded trees	Tissue	Herbarium number	Accession numbers	
							ITS	*gpd*
P. olivellus	**AK11.01**	2011	Algeria, Ain Khiar	*Alnus glutinosa*	Basidiome	Alg11.02	KU163463	–
P. olivellus	Ala12.1	2012	France, Alata, Corse	*Alnus glutinosa*	Basidiome	Cor-010	KU163465	KU163513
P. olivellus	ALB08.11	2008	France, Amélie-les-Bains, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	ALB08.11	KU163467	KU163515
P. olivellus	AST08.4	2008	France, Arles-sur-Tech, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	Arl08.4	KU163476	KU163520
P. olivellus	**AST13.11**	2013	France, Arles-sur-Tech, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	Arl13.11	KU163477	–
P. olivellus	**AST13.5**	2013	France, Arles-sur-Tech, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	Arles13.5	KU163478	–
P. olivellus	**Auc10.1**	2010	France, Auch, Gers	*Alnus glutinosa*	Basidiome	TL127-0004	KU163479	KU163521
P. olivellus	Cer09.17	2008	France, Céret, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*	Basidiome	Cer09.17	KU163483	KU163525
P. olivellus	**Cer12.5**	2012	France, Céret, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*	Basidiome	Cer12.5	KU163485	–
P. olivellus	**Cond09.1**	2009	France, Condette, Pas-de-Calais	*Alnus glutinosa*	Basidiome	PAM09110704	KU163486	KU163527
P. olivellus	Des09.1	2009	France, Desvres, Pas-de-Calais	*Alnus glutinosa*	Basidiome	PAM09090701	KU163487	KU163528
P. olivellus	EM07.1	2007	France, Evin-Malmaison, Pas-de-Calais	*Alnus incana*	Basidiome	PAM07083001	KU163488	KU163529
P. olivellus	EM07.2	2007	France, Evin-Malmaison, Pas-de-Calais	*Alnus incana*	Basidiome	PAM07103001	KU163489	KU163530
P. olivellus	Har09.1	2009	France, Hardelot, Pas-de-Calais	*Alnus glutinosa*	Basidiome	PAM09090901	KU163491	KU163532
P. olivellus	**LBH06.1**	2006	France, Le-Bourget-en-Huile, Savoie	*Alnus glutinosa*	Basidiome	PAM06082623	KU163492	KU163533
P. olivellus	LBH10.1	2010	France, Le-Bourget-en-Huile, Savoie	*Alnus glutinosa*	Basidiome	PAM10082908	KU163494	KU163535
P. olivellus	Les99.1	1999	France, Lesponne, Hautes-Pyrénées	*Alnus glutinosa*	Basidiome	GC03091901	KU163495	KU163536
P. olivellus	LMS08.1	2008	France, La-Motte-Servolex, Savoie	*Alnus glutinosa*	Basidiome	PAM08082904	KU163496	KU163537
P. olivellus	**Mar06.1**	2006	France, Marchiennes, Nord	*Alnus glutinosa*	Basidiome	PAM06102606	KU163497	KU163538
P. olivellus	Mar14.1	2014	France, Marchiennes, Nord	*Alnus glutinosa*	Basidiome	PAM14090806	KU163498	KU163539
P. olivellus	Ore09.1	2009	France, Orezza, Corse	*Alnus glutinosa*	Basidiome	PAM09101304 - Co09	KU163501	KU163542
P. olivellus	PH09.1	2009	France, Paris-l'Hôpital, Saône-et-Loire	*Alnus glutinosa*	Basidiome	PH09.1	KU163503	KU163543
P. olivellus	Pha14.1	2014	France, Phalempin, Nord	*Alnus glutinosa*	Basidiome	PAM14011601	KU163504	KU163544
P. olivellus	PL08.1	2008	France, Ponte-Leccia, Corse	*Alnus glutinosa*	Basidiome	PAM08090502	KU163505	KU163545
P. olivellus	Seb12.7	2012	France, Sébrazac, Aveyron	*Alnus glutinosa*	Basidiome	Av12.7	KU163473	KU163517
P. olivellus	SLC11.5	2011	France, Saint-Laurent-de-Cerdans, Pyrénées-Orientales	*Alnus glutinosa*	Basidiome	SLC11.5	KU163508	KU163548
P. olivellus	**SQ06.1**	2006	France, Saint-Quentin, Aisne	*Alnus glutinosa*	Basidiome	PAM06102207	KU163509	KU163549
P. olivellus	STB09.1	2009	France, Saint-Trojan-les-Bains, Charente-Maritime	*Alnus gultinosa*	Basidiome	Ole09.1	KU163500	KU163541
P. olivellus	Tec08.5	2008	France, Le Tech, Pyrénées-Orientales	*Alnus glutinosa*	Basidiome	Tec08.5	KU163511	KU163551
P. rubicundulus	LBH08.m1	2008	France, Le-Bourget-en-Huile, Savoie	*Alnus glutinosa*	Mycorrhiza	BMI_1	KU163493	KU163534
P. rubicundulus	*Prub* Orton 2905	1969	United Kingdom	*Alnus glutinosa*	Basidiome	K(M)190474	KU163502	–

(continued on next page)

Table 1 – (continued)

Phylogenetic species	Isolates	Sampling year	Location	Recorded trees	Tissue	Herbarium number	Accession numbers	
							ITS	*gpd*
P. *adelphus*	AK11.m1	2011	Algeria, Ain Khiar	*Alnus glutinosa*	Mycorrhiza	II6_16	KU163464	–
P. *adelphus*	ALB08.24[a]	2008	France, Amélie-les-Bains, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Mycelium	ALB08.24	KU163466	KU163514
P. *adelphus*	**ALB09.10**	2009	France, Amélie-les-Bains, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	ALB09.10	KU163468	–
P. *adelphus*	**ALB13.13**	2013	France, Amélie-les-Bains, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	ALB13.13	KU163471	–
P. *adelphus*	**ALB13.3**	2013	France, Amélie-les-Bains, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	ALB13.3	KU163469	–
P. *adelphus*	**ALB13.9**	2013	France, Amélie-les-Bains, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*, *Salix*	Basidiome	ALB13.9	KU163470	–
P. *adelphus*	All09.m4	2009	France, Allemont, Isère	*Alnus incana*	Mycorrhiza	AL4	KU163472	KU163516
P. *adelphus*	Are09.m2	2009	France, Arêche, Savoie	*Alnus incana*	Mycorrhiza	Areche myc1a	KU163475	KU163519
P. *adelphus*	**Auc12.1**	2012	France, Auch, Gers	*Alnus glutinosa*	Basidiome	Auc12.1	KF261361	KF261434
P. *adelphus*	Beau10.1	2010	France, Beaufort, Savoie	*Alnus incana*	Basidiome	PAM10082803	KU163580	KU163522
P. *adelphus*	Beau14.1	2014	France, Beaufort, Savoie	*Alnus incana*	Basidiome	PAM14082602	KU163481	KU163523
P. *adelphus*	BV04.1	2004	France, Bessans-Villaron, Savoie	*Alnus incana*	Basidiome	MD08100124	KU163482	KU163524
P. *adelphus*	Cer09.4	2009	France, Céret, Pyrénées-Orientales	*Alnus glutinosa*, *Populus*	Basidiome	Cer09.4	KU163484	KU163526
P. *adelphus*	**Gue09.1**	2009	France, Guemps, Pas-de-Calais	*Alnus cordata*	Basidiome	PAM09111401	KU163490	KU163531
P. *adelphus*	**Mer10.1**	2010	France, Merlimont, Pas-de-Calais	*Alnus glutinosa*	Basidiome	PAM10102104	KU163499	KU163540
P. *adelphus*	PM12.1	2012	France, Poggio Marinaccio, Corse	*Alnus glutinosa*	Basidiome	Co12.08	KU163506	KU163546
P. *adelphus*	Seb12.58	2012	France, Sébrazac, Aveyron	*Alnus glutinosa*	Basidiome	Av12.58	KU163474	KU163518
P. *adelphus*	See09.1	2009	France, Séez, Savoie	*Alnus incana*	Basidiome	Alp09.1	KU163507	KU163547
P. *adelphus*	Tec08.1	2008	France, Le Tech, Pyrénées-Orientales	*Alnus glutinosa*	Basidiome	Tec08.1	KU163510	KU163550
P. *adelphus*	Ve08.2	2008	France, Le Vernet, Haute-Garonne	*Alnus glutinosa*	Basidiome	TL127-0003	KU163521	KU163552
P. *adelphus*	Ve08.2h10[b]	2008	France, Le Vernet, Haute-Garonne	*Alnus glutinosa*	Monocaryotic mycelium		KF261421	KF261491

a Strain studied by Huggins *et al.* (2014), provided by P. Jargeat.
b Genome-sequenced strain, obtained from spore germination.

(*gpd*) gene were PCR-amplified as in Jargeat *et al.* (2010). The PCR products were sequenced by GENOSCREEN (Lille, France), MilleGen (Labège, France) or Eurofins-MWG (Ebersberg, Germany), using amplification primers as sequencing primers. Sequence chromatograms were manually checked, and corrected using BioEdit software program (Hall 1999). The sequences are available from the GenBank database under accession numbers KU163463–KU163552.

To study the delimitation of the species, two dataset were created, one of ITS sequences and the other of *gpd* sequences. The *gpd* sequence dataset only included sequences from this study and the *gpd* sequence from the sequenced strain Ve08.2h10 (KF261491) from GenBank whereas the ITS dataset was complemented with the publicly available ITS sequences retrieved from GenBank and UNITE originating from basidiomata (Table 2) and from the sequenced strain Ve08.2h10 (KF261421). For both datasets, sequences from the four European species of the *Paxillus involutus* complex (Table 2) were used as out-group. For studying the identity of the ITS sequences amplified from *Alnus* ectomycorrhizae by Põlme *et al.* (2013, Table S1), Roy *et al.* (2013), and Tedersoo *et al.* (2009), a third dataset including these sequences as well as sequences from the type specimens was created.

Multiple sequence alignments were conducted in SeaView v4.0 (Galtier *et al.* 1996) using MUSCLE v3.7 (Edgar 2004). Alignments are available on treebase.org under the following link: http://purl.org/phylo/treebase/phylows/study/TB2:S18309.

The best evolutionary model of nucleotide substitution was selected by using jModelTest-2.1.4 (Darriba *et al.* 2012). Phylogenetic analyses were performed using Bayesian inference with MrBayes v3.2 (Ronquist *et al.* 2012) with six simultaneous Markov Chain Monte Carlo (MCMC) run over 2 000 000 generations, under GTR + gamma evolutionary model. Trees were saved every 500 generations, and the first 25 % were discarded as 'burn-in'. For the remaining trees, a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities. Phylogenetic trees were visualized and edited with FigTree v1.4.0, available at http://tree.bio.ed.ac.uk/software/figtree/.

Genetic distances were calculated with the two first sequence datasets. For both ITS (675 positions) and *gpd* (643 positions) datasets, genetic distances, including indels or not, between groups (number of base substitutions per site from estimated net average between groups of sequences) and within groups (number of base substitutions per site from averaging all sequence pairs within each group) were calculated using the Jukes–Cantor model (Jukes & Cantor 1969), with Treecon (Van de Peer & De Wachter 1994). In ITS sequences, partial 28S sequence (170 bp) was removed.

Results

Phylospecies delimitation and relationships within Alnus*-associated* Paxillus *species*

ITS sequences (823 bp) were obtained for 44 samples including the *Paxillus rubicundulus* holotype, whereas *gpd* sequences (643 bp) were generated from 41 samples. Bayesian analyses of the ITS dataset produced three well-supported clades (Fig 1A). A first clade included the ITS sequence from the *P. rubicundulus* holotype, and was therefore referred to as the *P. rubicundulus s. str.* clade. It also included one ectomycorrhizal tip sequence (LBH08.m1) yielded by this study, and one sequence from an Estonian specimen recorded as *Paxillus filamentosus* in the UNITE database. A second clade was a sister of the *P. rubicundulus* clade. This group includes four GenBank or UNITE sequences currently named *P. filamentosus* and previously assigned to the A1.2 clade, the sequence of the sequenced strain Ve08.2h10 and 16 sequences from our collections (14 from basidiomata, one from a mycorrhiza collected in a native *Alnus glutinosa* stand in Algeria, and the sequence from the strain ALB08.24, used by Huggins *et al.* (2014) as '*P. rubicundulus*'). The third clade was composed of eight sequences from GenBank or UNITE named *P. filamentosus* or *P. rubicundulus* and previously assigned to the A1.3 clade. These sequences were clustered with those of 26 of our samples, including an Algerian basidiome. The *gpd* tree (Fig 1B) also revealed three well supported clades, and clustered our samples similarly to the ITS tree.

The net value of between-group mean genetic distance was calculated using a total of 63 ITS sequences and 46 *gpd* sequences. For the ITS region, the genetic distance, including indels or not, between clades A1.2 and A1.3, and A1.3 and *P. rubicundulus* was above 3 % but between clade A1.2 and *P. rubicundulus* it was below 2 % (Table 3). For the *gpd* region, it was between 1.6 % and 2.8 %. By contrast, the mean genetic distance value within each clade and for both regions, was always below 0.7 %.

Overall, these results show that clades A1.2 and A1.3 of Gelardi *et al.* (2014) are two different phylospecies. Consequently, clades A1.2 and A1.3 are hereafter named *Paxillus adelphus* and *Paxillus olivellus*, respectively. Thus, the sequenced strain Ve08.2h10 and the ALB08.24 strain used by Huggins *et al.* (2014) are *P. adelphus* isolates.

Identification of Paxillus *species from alder ectomycorrhizal tips using GenBank and UNITE environmental ITS sequences*

Bayesian analysis carried out with ITS sequences of environmental ectomycorrhizae attributed to alder-associated *Paxillus* and with ITS sequence of type specimens allowed us to identify the fungal species. Thirty-six sequences (out of the 38 sequences we studied) were attributed to *Paxillus* species in subg. *Alnopaxillus*, and two to species in subg. *Paxillus* (Fig 2). The sequences identified as *Paxillus* #5 by Põlme *et al.* (2013) could be assigned to *Paxillus rubicundulus*. Furthermore, *Paxillus* #3 from Europe and *Paxillus* #4 from Turkey (Põlme *et al.* 2013) were clearly attributed to *Paxillus adelphus*. However, *P. rubicundulus* from Iran and *P. adelphus* from Turkey were genetically distinct from the European holotype specimens (Fig 2), suggesting either subspecies or distinct highly differentiated populations. *Paxillus* #2 (Põlme *et al.* 2013) is *Paxillus olivellus*. Ectomycorrhizae analysed by Roy *et al.* (2013) and Tedersoo *et al.* (2009), from France and Estonia, belong to *P. rubicundulus*, *P. adelphus* or *P. olivellus*. MOTU from China, named *Paxillus* #6 (Põlme *et al.* 2013) is most likely conspecific with *Paxillus orientalis* and MOTU *Paxillus* #7 (Põlme *et al.* 2013) from North Korea is closely related to *P. orientalis* but is likely to represent an undescribed species. Finally, sequences of

Table 2 – Voucher sequences, from GenBank and UNITE databases, included in this study.

	Phylogenetic species	Name in sequence databases	Country	Host species	Clade[a]	Accession numbers		Reference
						ITS	*gpd*	
Subclade *Alnopaxillus*	*P. olivellus*	*P. filamentosus* AT2000042	Sweden	nd	A1.3	UDB000667		
	P. olivellus	*P. filamentosus* 050518-09	Scotland	*Alnus glutinosa*	A1.3	UDB001612		
	P. olivellus	*P. filamentosus* IA06-3	Scotland	*Alnus glutinosa*	A1.3	UDB002374		
	P. olivellus	*P. filamentosus* TU110278	Estonia	nd	A1.3	UDB008237		
	P. olivellus	*P. filamentosus* TU110287	Estonia	nd	A1.3	UDB008238		
	P. olivellus	*P. rubicundulus* TU110297	Estonia	nd	A1.3	UDB008240		
	P. adelphus	*P. filamentosus* TU110298	Estonia	nd	A1.2	UDB008241		
	P. olivellus	*P. filamentosus* PAN 760	Poland	nd	nd	KM085406		
	P. olivellus	*P. filamentosus* TU118205	Estonia	nd	A1.3	UDB015382		http://www.europeana.eu/portal/record/11617/_MYCOLOGICALCOLLECTION_UTNHM_ESTONIA_TU118205.html
	P. olivellus	*P. filamentosus* TU118607	Estonia	nd	A1.3	UDB018013		http://www.europeana.eu/portal/record/11617/_MYCOLOGICALCOLLECTION_UTNHM_ESTONIA_TU118607.html
	P. rubicundulus	*P. rubicundulus* Orton_2905	Scotland	*Alnus* sp.	A1.1	EU084667		Hedh *et al.* 2008
	P. rubicundulus	*P. filamentosus* TU110325	Estonia	nd	A1.1	UDB008243		
	P. adelphus	*P. filamentosus* 304	Germany	*Alnus* sp.	A1.2	AF167687		Jarosch & Bresinsky 1999
	P. adelphus	*P. filamentosus* 133	Germany	*Alnus* sp.	A1.2	AF167688		Jarosch & Bresinsky 1999
	P. adelphus	*P. filamentosus* Pf01DE	Germany	*Alnus* sp.	A1.2	EU084664		Hedh *et al.* 2008
	P. orientalis	*P. orientalis* HKAS63615	China	Fagaceae, *Pinus*		KF372876		Gelardi *et al.* 2014
	P. orientalis	*P. orientalis* ZT13437	China	*Pinus yunnanensis*		KF372877		Gelardi *et al.* 2014
Subclade *Paxillus* Outgroup		*P. involutus* Can12.1 Type	France			KF261441	KF261368	Jargeat *et al.* 2014
		P. cuprinus Cas10.3 Type	France			KF261450	KF261379	Jargeat *et al.* 2014
		P. obscurisporus Bel12.1	France			KF261439	KF261367	Jargeat *et al.* 2014
		P. ammoniavirescens Tou12.1	France			KF261490	KF261420	Jargeat *et al.* 2014

nd: not determined.

a According to Gelardi *et al.* (2014).

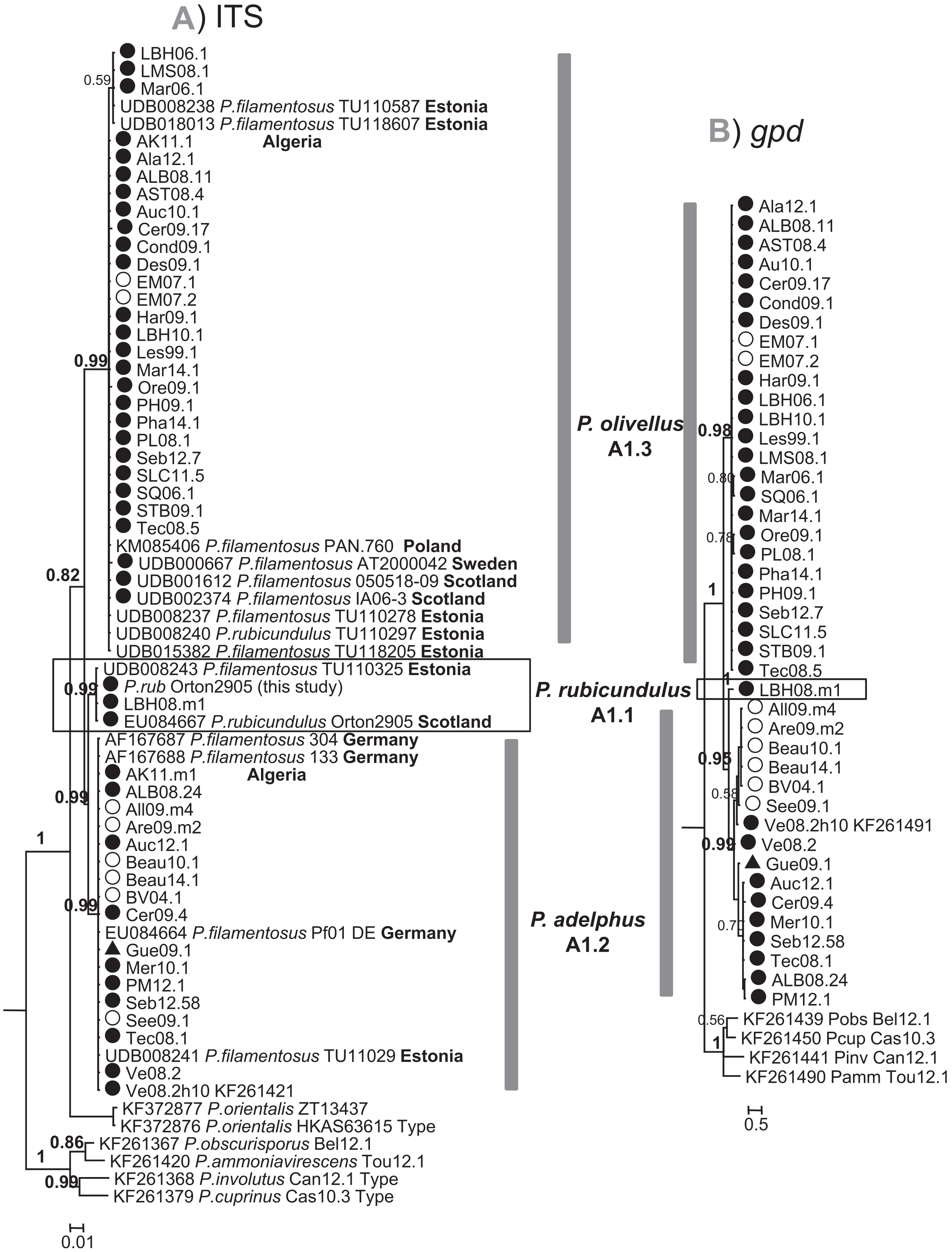

Fig 1 – **Phylogeny of *Paxillus rubicundulus* and allies derived from (A) ITS and (B) *gpd* sequences using Bayesian analyses. Bayesian PPs above 0.5 are shown above branches. The tree was rooted using sequences from the *P. involutus* group. The scale shows the expected number of changes *per* nucleotide. Three phylogenetically different species are identified. All strains and gene accession numbers are presented in Tables 1 and 2. A1.1, A1.2, and A1.3 are clades from Gelardi *et al.* (2014). Host plants are (●) *Alnus glutinosa*, (▲) *Alnus cordata*, and (○) *Alnus incana*.**

Table 3 – Genetic distances calculated at the intra- and interspecific levels (Juke–Cantor method), with indels not taken into account/taken into account.

	No of sequences		*P. adelphus* A1.2		*P. olivellus* A1.3		*P. rubicundulus*		*P. involutus* group	
	ITS	*gpd*	ITS	*gpd*	ITS	*gpd*	ITS	*gpd*	ITS	*gpd*
P. adelphus A1.2	21	16	0/0.1	0.6/0.7						
P. olivellus A1.3	32	25	3.3/4.4	2.7/2.8	0.5/0.6	0/0				
P. rubicundulus	4	1	1.2/1.8	1.6/1.8	3.1/4.1	2.1/2.2	0/0.1	n.r.		
P. involutus group	4	4	12.5/14.8	7.7/7.9	11.9/14.7	7.6/7.8	11.7/14.1	7.5/7.8	4.3/5.2	3.8/3.9

n.r. = Not relevant.

MOTU *Paxillus* #1 (Põlme *et al.* 2013) cluster with the sequence from the holotype of *Paxillus cuprinus*, the only species in subg. *Paxillus* known to occasionally associate with alder roots.

Geographical distribution, host, and habitat

Paxillus adelphus was found in 14 different locations in France (including the island of Corsica – PM collection), and elsewhere in Europe (Estonia, Germany, Austria), in North Africa (Algeria), and in Western Asia (Turkey) (Figs 1A and 2). Similarly to *P. adelphus*, *Paxillus olivellus* appeared to be widespread in France (22 sites, including the islands of Corsica –Ala collection and Oléron – STB collection). It is also present throughout Europe: Estonia, Sweden, Scotland, Poland, Romania, and in North Africa (Algeria) (Figs 1A and 2). In our study, *P. adelphus* and *P. olivellus* coexisted in six sites out of 32. Basidiomata of the two species were collected side-by-side at the same time at five sites (AK, ALB, Cer, Seb, Tec) (Table 1) and at the same site but not the same year in one site (Auc). *Paxillus adelphus* and *P. olivellus* often fruited together with *Gyrodon lividus*, another strictly alnicolous ectomycorrhizal species, in the *Paxillaceae*. Their fruiting habitats were mainly river banks or edges of forest streams, usually near *Alnus glutinosa* trees (Fig 1). On the other hand, *P. adelphus* was found fruiting near *Alnus incana* trees in the Alps (All, Are, Beau, BV, See sites), associated to *Alnus orientalis* in Turkey and to ornamental *Alnus cordata* in North of France (Gue collection) (Figs 1 and 2). *Paxillus olivellus* was also collected from *A. incana* plantation at EM site (North of France) (Fig 1).

Once redefined, *Paxillus rubicundulus* was found to be less abundant than the two other clades, at least in France. Despite an extensive sampling effort of basidiomata and analysis of ectomycorrhiza ITS sequences, we only detected it in the French Alps (LBH site), associated with *A. glutinosa* (Fig 1). However, sequence analysis based on GenBank and UNITE data also revealed its presence in Estonia, Scotland, and Finland (Figs 1A and 2) under *A. glutinosa* but also in Western Asia (Iran), associated with *Alnus subcordata* and *A. glutinosa* (Fig 2).

In Eastern Asia, *Paxillus orientalis* and the undescribed *Paxillus* #7 (Põlme *et al.* 2013) were found associated with *Alnus cremastogyne*, *Alnus nepalensis* and *Alnus hirsuta* (Fig 2).

Comparison of spore parameters

Spore length, width and ratio of length and width (Q) were statistically analysed (Fig 3). These characters were chosen because they are good representatives of spore shape. The longest spores were observed in *Paxillus rubicundulus* (6.6–8.5 μm), while *Paxillus adelphus* had the shortest (6.1–7.3 μm). No significant difference in spore width was found between *P. rubicundulus* and *Paxillus olivellus*, but their spores were significantly wider than those of *P. adelphus*. Spore measurements also showed that *P. adelphus* had the smallest spores among the three species, and *P. rubicundulus* the largest. However, there was considerable variability in spore size within species (particularly significant for *P. olivellus*), and a large amount of size overlaps between species. As indicated by Q values (Fig 3, Table 4), *P. olivellus* spores are mostly ovoid (lowest Q values). By contrast, *P. adelphus* spores and most of *P. rubicundulus* spores are cylindrical, with the highest Q values for *P. rubicundulus* spores.

Taxonomy

Paxillus adelphus J.-P. Chaumeton, H. Gryta, P. Jargeat & P.-A. Moreau, **sp. nov.**
MycoBank No.: MB815076
Figs 4 and 5A

Etymology: from the Greek αδελφός (*adelphós*): brother, double; in reference to its high similarity with its sister species *Paxillus rubicundulus*.

Diagnosis: basidiomata solitary to gregarious, medium- to large-sized, with usually robust stipe; pileus viscidulous to sticky, at first bronze-grey to putty-brown with olivaceous shade soon turning wine-red to rusty-brown, early dissociated in large adpressed fibrillose scales on an ochre-yellow background. Lamellae cream to pale lemon yellow when young, more brown and olivaceous with age. Context bright yellow, quickly reddening then turning brown. Spores 6.1–7.3 × 4.0–4.7 μm, subcylindrical to slightly allantoid. Pleurocystidia rather abundant, thick-walled with brown content. Cheilocystidia scattered, slightly thick- or thin-walled, sometimes with colourless content. Under *A. glutinosa*, *A. incana*, and *A. cordata* in riparian forests and plantations. Eurasia, North Africa.

Holotype: **France**, Haute-Garonne 31, Le Vernet, elev. 167 m, under *A. glutinosa* on river bank, leg P. Jargeat and H. Gryta, 19 Oct., 2008, Ve08.2. Deposited at the Université Paul Sabatier Toulouse 3 herbarium, TL127-0003. Isotype: LIP.

Macroscopic description

Pileus 6–15 cm diam., at first subhemispherical to convex, soon flattened with a depressed centre, later somewhat

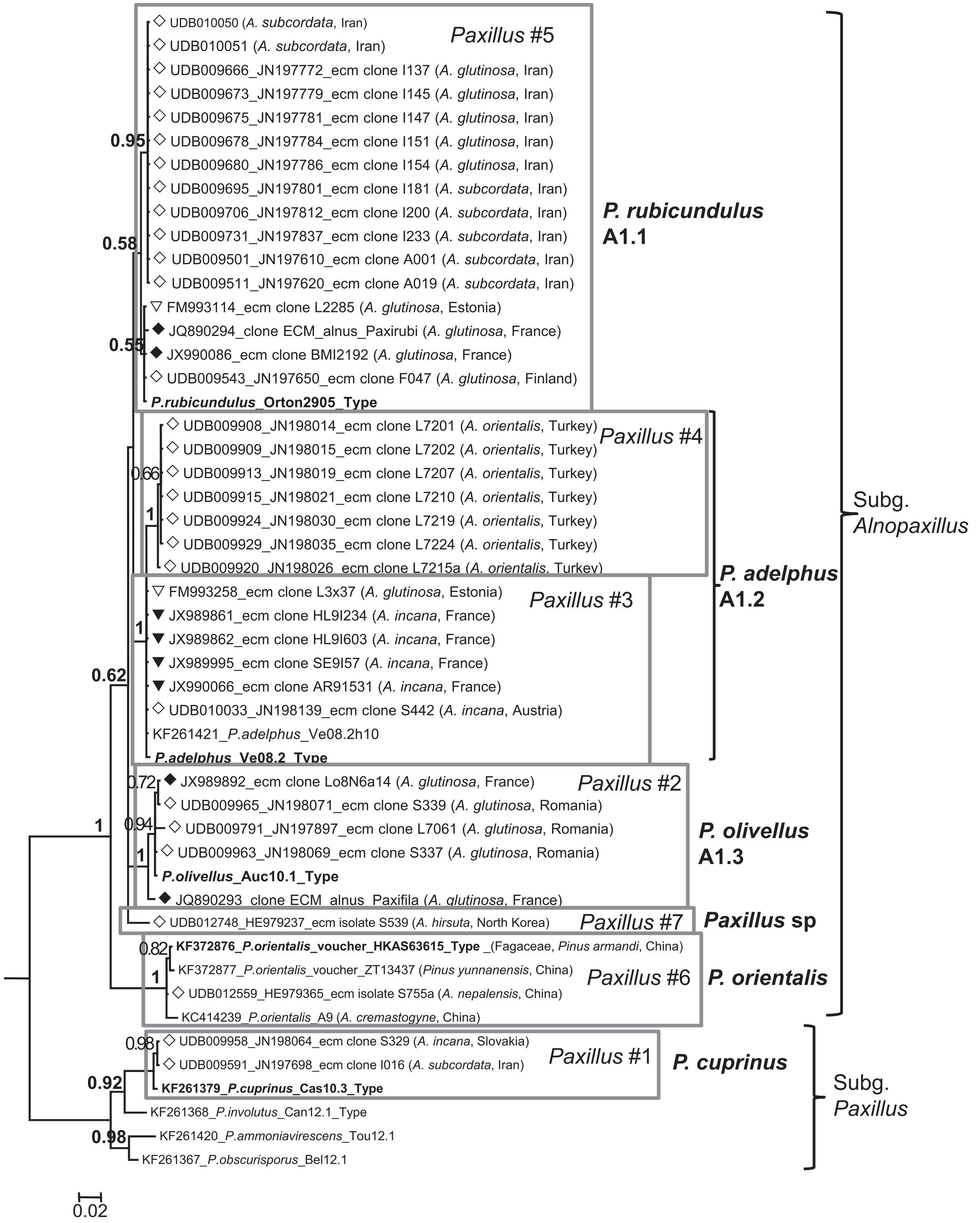

Fig 2 – Bayesian phylogenetic analysis of environmental ectomycorrhizal tip ITS sequences from GenBank and UNITE, previously attributed to alnicolous *Paxillus* species. Sequences were generated by (◇) Põlme *et al.* (2013), (◆) Roy *et al.* (2013), and (▽) Tedersoo *et al.* (2009). A1.1, A1.2, and A1.3 are clades from Gelardi *et al.* (2014). *Paxillus* #1–#7 names are from Table S1 from Põlme *et al.* (2013). Bayesian PPs above 0.5 are shown above branches. The tree was rooted using sequences from the *P. involutus* group. The scale shows the expected number of changes *per* nucleotide.

funnel-shaped, rarely with a persistent low umbo, not or only weakly costate at margin; margin slightly decurved, often lobed-undulating, later expanding and reflexed in some places, surface not sulcate, viscidulous to slimy or sticky when moist, scurfy, early dissociated in broad radial fibrillose tatters at mid-radius, more rarely cracked in adpressed scales towards margin, surface colour at first bronze-grey, putty brown to olivaceous buff, underlying context ochre-yellow where excoriated, turning wine-red where touched or with age; margin pale yellow to pinkish grey, minutely pruinose at first, soon unrolled and obtuse, undulate and lobate when old.

Lamellae slightly arcuate to moderately decurrent, fairly crowded (9–15 per cm at margin) with few lamellulae (up to 4 per lamella), at first thick, mostly forked, intervenose, often crispate-anastomosed at insertion; at first cream to pale lemon yellow, then yellow-brown, finally olivaceous brown, immediately purplish brown when bruised; edge obtuse, smooth to serrulate, pale yellow remaining paler than faces.

Stipe rather short, 1.5–4.5 (6) × (0.6) 0.8–1.8 (2.2) cm, central to eccentric or almost lateral, cylindrical to obconical, usually short rooting down to 1.5 cm deep; surface minutely wrinkled and fibrillose, lemon yellow, marbled by glaucous fibrils on lower part, soon dark purplish from the base. Mycelium whitish to pinkish, more or less abundant, forming pale brown rhizomorphs.

Context uniformly bright yellow, quickly red-marbled, then turning brown after a few minutes, purplish black in stipe base; hypophyllum thin, glaucous hyaline.

Smell faintly fruity, taste fungoid.

Chemical reactions: 5 % KOH: purplish black on pileus, pinkish on context; TL4 (Henry's reagent): none on pileus, slate grey in 10 s on context; 50 % NH_4OH solution: greyish-brown to blackish-grey or brownish on pileus surface, distinctly (although fleeting) greyish-green on the slimy part in one collection (Auc12); NH_3: reddish brown, slightly purplish.

Spore print ochraceous-reddish or chocolate (6E7) in fresh deposit, without greenish shade, duller, and more ochraceous after storage (5D7–5E7).

Microscopic description

Spores (n = 412, 7 coll.): (5.5) 6.1–6.6–7.3 (8.3) × (3.7) 4.0–4.3–4.7–(5.2) μm, Q: (1.28) 1.42–1.54–1.64 (1.85), short cylindrical to slightly allantoid, light yellowish in KOH, mostly uniguttulate, with large droplet; wall 0.2–0.3 μm thick.

Basidia 25–37 × 6.5–8 μm, mostly 4-spored, cylindrical to clavate when nearly mature, clavate, guttulate when mature, pale to colourless.

Subhymenium 10–15 μm thick, pseudoparenchymatous, including reddish-yellow vesicular 10–15 μm wide hyphae.

Hymenial cystidia: Pleurocystidia rather abundant, 2000–9000 per mm^2, 35–60 (90) × 8–12 μm, cylindrical to fusiform or lageni-fusiform, often with tapering apex, wall usually 0.3–0.5 μm thick, not collapsing; smooth, all with yellow-brown to amber-brown content. Edge fertile; cheilocystidia scattered (5–20 per mm), 35–85 × 9–15 μm, thin to slightly thick-walled, cylindrical to fusiform, sometimes branched towards apex, with colourless to pale yellow content.

Hymenophoral trama (mediostratum) parallel, narrow, 30–45 μm wide, yellow-brown, made of sarcoid 8–15 μm diam. hyphae; hymenopodium thick, gelatinized, colourless, 12–20 μm thick, parallel to bilateral towards edges, made of cylindrical 5–12 μm wide hyphae, wall up to 1.5 (–2) μm thick; thromboplerous hyphae with yellowish content locally frequent towards subhymenium.

Pileipellis a weakly gelatinized ixocutis, when young made of cylindrical 3.5–7 μm wide hyphae with abundant obtuse terminal articles 80–250 μm long, wall thickened up to 0.5 μm,

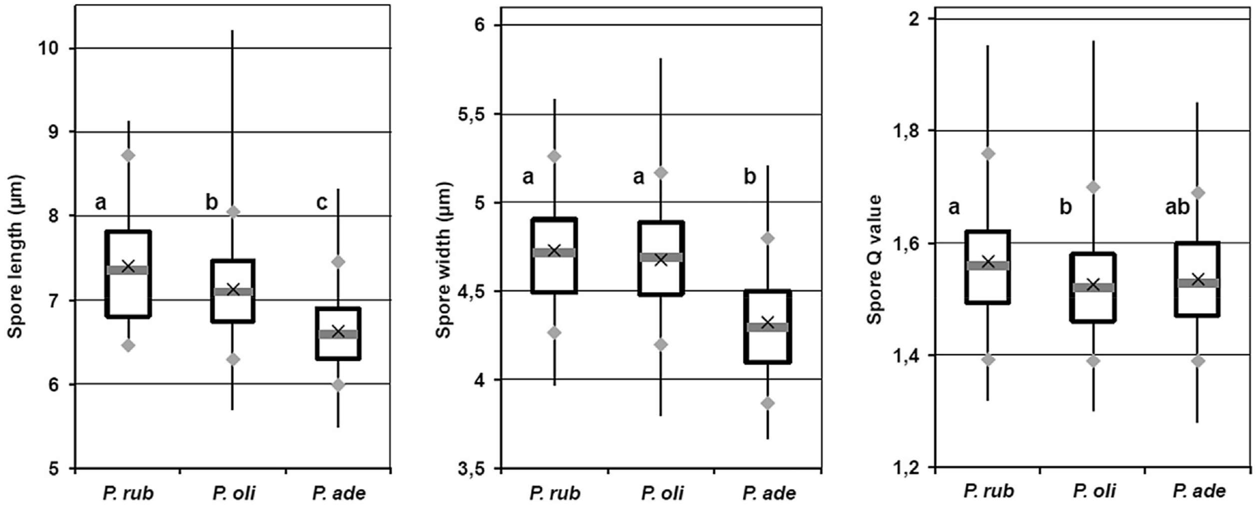

Fig 3 – Statistics of spore parameters (length, width, and Q = length-over-width ratio) measured from 412, 752, and 106 spores of *P. adelphus* (Pade), *P. olivellus* (Poli), and *P. rubicundulus* (Prub), respectively. Each white box shows the interquartile range (50 % of the data) and grey diamonds indicate the 5th and 95th percentiles. Median and mean values are shown with a horizontal line inside the box and an × symbol, respectively. Vertical lines are drawn between the minimum and maximum values. Different letters indicate significant differences ($P < 0.01$) between species for each parameter (Mann–Whitney U-tests with Bonferroni correction).

Table 4 – Comparison of microscopic elements.

	P. adelphus	*P. olivellus*	*P. rubicundulus*
Spore size (μm)	(5.5) 6.1–7.3 (8.3) × (3.7) 4.0–4.7 (5.2)	(5.7) 6.4–7.9 (10.2) × (3.8) 4.3–5.1 (5.8)	(6.4) 6.6–8.5 (9.1) × (4.0) 4.4–5.1 (5.6)
Spore length/ width ratio (Q)	(1.28) 1.42–**1.54**–1.64 (1.85)	(1.30) 1.42–**1.53**–1.64 (1.96)	(1.32) 1.42–**1.57**–1.73 (1.95)
Spore shape	Short cylindrical to slightly allantoid	Ovoid to ellipsoid	Cylindrical without allantoid tendency
Basidia (μm)	25–37 × 6.5–8	(15) 22–32 × 5.5–8	18–38 × 5.5–8
Pleurocystidia (μm)	35–60 (90) × 8–12	62–68 × 11–16	70 × 7 (base) × 4 (apex)
Cheilocystidia (μm)	35–85 × 9–15	40–55 × 5.5–11	35 × 7 (base) × 3.5 (apex)

The mean Q values are represented in bold.

with yellowish granular content, later showing subjacent orange to deep yellow-brown, mostly slender 2.0–4.5 μm wide hyphae, more or less arranged in prostrate fascicles, thin-walled, smooth.

Stipitipellis towards apex covered with small and soon collapsed caulocystidia, 15–40 × 8–12 μm, clavate, embedded in an orange-yellow mucus. Superficial hyphae 3–6 μm wide, smooth.

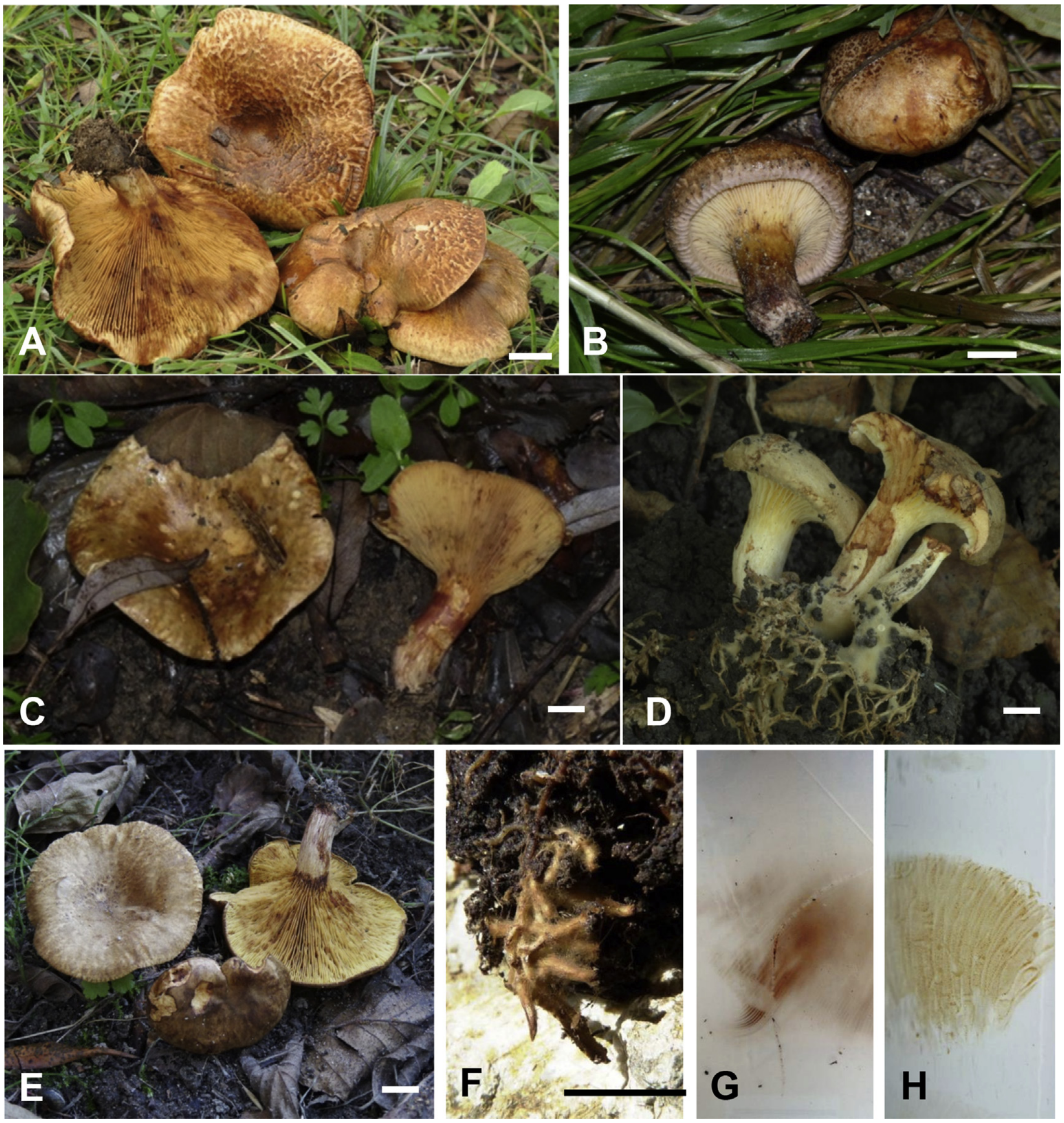

Fig 4 – Various aspects of *Paxillus adelphus* sp. nov. (A–C), *P. olivellus* sp. nov. (D and E) basidiomata, aspect of *P. olivellus* mycorrhizae on *Alnus glutinosa* root (F) and fresh spore print of *Paxillus adelphus* sp. nov. (G), and *Paxillus olivellus* sp. nov. (H). Scale bar = 1 cm.

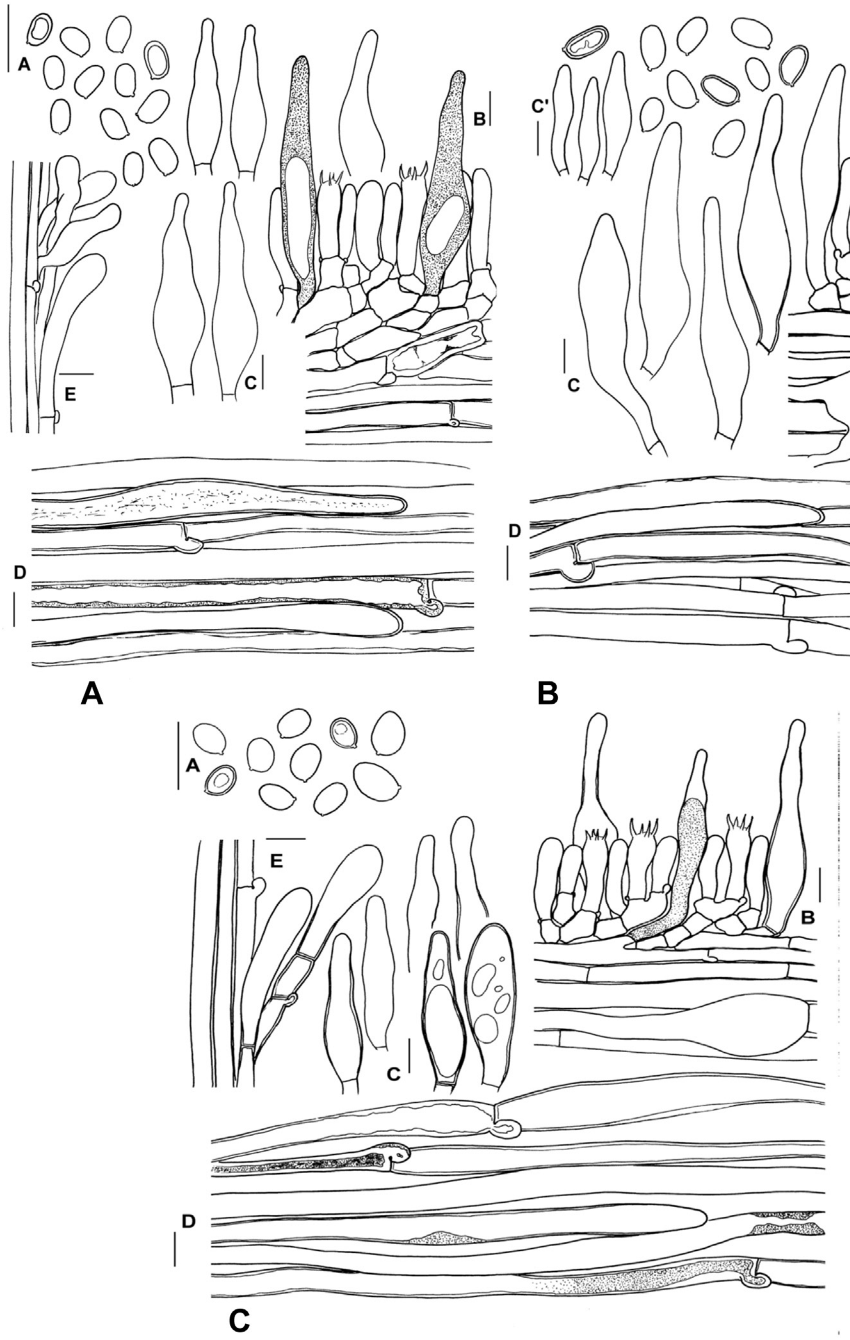

Fig 5 – Microscopic features of (A) *Paxillus adelphus* sp. nov., (B) *P. rubicundulus*, and (C) *P. olivellus* sp. nov. A. Basidiospores; B. Hymenium with basidia and cystidia; C. Pleurocystidia; D. Pileipellis (cutis). Scale bars = 10 μm.

Clamps present at all septa.

Ecology and distribution

In groups and sometimes in dense clusters, sometimes by two or three connate basidiomata, in bright places on river banks and along streams. Growing in association with *Alnus glutinosa*, *A. incana*, *A. cordata*, and *A. orientalis*. Present in Europe, Western Asia, and North Africa. Late summer and autumn.

Paxillus olivellus P.-A. Moreau, J.-P. Chaumeton, H. Gryta & P. Jargeat **sp. nov.**

MycoBank No.: MB815077

Misappl. names: *Paxillus filamentosus* (Scop.) Fr. *sensu* Kotlaba & Pouzar (1960), Kühner (1962), Bon (1977), *etc.*; *Paxillus leptopus* Fr. sensu Bresadola (1930: pl. 678).

Figs 4 and 5C

Etymology: from Latin *oliveus*, olive-coloured, after the greenish colour of the cap in fresh specimens; and the diminutive – *ellus* for the small basidiome (the smallest European *Paxillus* on average).

Diagnosis: basidiomata solitary to gregarious, small- to medium-sized, usually with slender, brittle stipe; pileus covered with a thin gelatinized pileipellis, light ash-grey with olivaceous shade, reddening when bruised, forming reddish adpressed scales at the centre with age; lamellae whitish to light butter yellow when young, with pale lemon yellow edge; context pale yellow, greyish in lower stipe, quickly staining rusty. Spores 6.4–7.9 × 4.3–5.1 μm, mostly ovoid to ellipsoidal. Cheilocystidia frequent in mature specimens, slightly thick-walled at apex; pleurocystidia slightly thick-walled, with brownish content with age and in interlamellar hymenium. Under *A. glutinosa* and *A. incana* on wet soils. Europe, Asia, North Africa.

Holotype: **France**, Gers 32, Auch, elev. 133 m, under *A. glutinosa* on river bank, leg. J- P Chaumeton, 15 Oct., 2010, Auc10.1. Deposited at the Université Paul Sabatier Toulouse three herbarium, TL127-0004.

Macroscopical description

Pileus 2–12 cm diam., at first flattened then depressed, sometimes with low and obtuse umbo when young; pileipellis a thin gelatinized pellicle removable when wet, sticky, tomentose-hairy by fascicles or short brownish hairs at first, later confined to margin and arranged in radial crests, ash grey with slight greenish tone (C52, E63), when drying or ageing usually dissociated in adpressed scales from centre, turning reddish brown (H44) when touched or damaged; margin thin, shortly enrolled until full maturity, minutely spiny-tuberculate at first then glabrous, pale yellow then concolourous, later grooved or sulcate on 0.5–1 cm to centre.

Lamellae 40–50 reaching the stipe, 4–5 series of lamellulae, usually thin and crowded, deeply decurrent, occasionally forked towards mid-radius; rarely or not distinctly anastomosing at insertion, on some fleshy specimens thicker and intervenose; at very first cream white to pale yellow, quickly butter yellow (A78–C76), often lighter towards margin, finally ferruginous brown (towards D56); edge thin, smooth, pale lemon yellow.

Stipe 2.5–5 × 0.6–1.5 (2) cm, equal to slightly attenuate at base, faintly pruinose-punctuate then glabrous or slightly fibrillose, rarely scaly when old, originally pale yellow, turning reddish brown to dark purplish red from base when touched or with age and drying. Mycelium usually abundant, pale yellow and profuse like a byssus, turning pinkish when drying, with thin rhizomorphs in the substrate, staining dark brown and collapsing quickly.

Context at very first whitish, then pale lemon yellow (A78–A78) in pileus and upper side of stipe, towards base pale greyish, turning rusty 2 min after section, dirty red-brown in lower half stipe.

Smell faintly fruity, taste fungoid.

Chemical reactions: KOH on pileipellis: red brown; PDAB: immediately salmon-pink on pileipellis, pale lemon yellow elsewhere; guaiac: positive on context after 30 s; 50 % NH_4OH solution: leaden grey to blackish-grey or grey-brownish on the pileus surface; NH_3: immediately and persistently pink-brown (D34).

Spore print: fresh deposit ochraceous-brown to rusty-brown with a greenish hue (5D7–5D8–5E8), duller, and more ochraceous after storage (5D7–5E7).

Microscopical description

Spores (n = 752, 9 coll.): (5.7) 6.4–7.1–7.9 (10.2) × (3.8) 4.3–4.7–5.1 (5.8) μm, Q: (1.30) 1.42–1.53–1.64 (1.96), ovoid to ellipsoid, light yellow in KOH, usually uniguttulate, wall 0.3–0.5 μm thick.

Basidia (15) 22–32 × 5.5–8 μm, 4-spored, cylindrical to slightly club-shaped when mature, scarcely guttulate, thin-walled, not especially collapsing after spore discharge.

Subhymenium 10–15 μm thick, pseudoparenchymatous.

Hymenial cystidia: Pleurocystidia abundant in mature specimens (4000–7000 per mm^2), 62–68 × 11–16 μm, 4–4.5 μm wide at apex, lageni-fusiform, slightly thick-walled in lower part, colourless towards margin or in young specimens, partly filled with yellow-brown content in older specimens or far from margin and in sinuses, especially those with tapering base arising from hymenopodium. Edge substerile, cheilocystidia 40–55 × 5.5–11 μm, narrowly ellipsoidal to slightly fusiform, with yellowish, slightly thickened wall usually well-revived on exsiccata, in fresh specimens partly covered with punctuate hyaline exudate; content occasionally filled with pale yellow droplets.

Hymenophoral trama narrow, attenuated, and almost missing at mid-length towards edges, parallel, pale when young or towards margin, then brownish, without thromboplerous hyphae; hymenopodium gelatinized and collapsing with age, parallel to distinctly bilateral towards edges, colourless.

Pileipellis an ixocutis made of cylindrical slender 4.0–9.5 μm wide hyphae, smooth, some short elements with a thickened, waved inner wall up to 3 μm thick, with amber-yellow content and yellow-brown wall up to 1.2 μm thick, and other elements with a distinctly waved inner wall; when older, dominated by slender 2.5–4 μm wide hyphae, broader towards subpellis, more distinctly incrusted.

Stipitipellis on 1/3 upper part covered with caulohymenium when young, then with sparse clusters of cystidia with thin to irregularly thickened walls, 17–25 × 4.5–8 μm, cylindrical, thin-walled, and soon collapsed, with pale yellowish content.

Stipititrama sarcodimitic with rather slender physalohyphae up to 8 μm wide, mixed with abundant thromboplerous 4–11 μm wide hyphae with pale yellow content.

Clamps present at all septa.

Ecology and distribution

In groups or sometimes by two or three connate basidiomata, in marshlands, on river banks, along streams, and in peat bogs. Growing mainly in association with *A. glutinosa*, occasionally with *A. incana*. Widespread and probably fairly common across Europe; present in North Africa. Late summer to early winter.

Observations

Paxillus olivellus basidiomata display a high phenetic variability, overlapping that of the apparently more stable *P. adelphus*, and thus preventing field identification. From our experience, *P. olivellus* occurs under two rather distinct phenotypes, which are not genetically distinct: 1) a slender habitus with pale lamellae not distinctly anastomosing, and with persistent olivaceous shades before the pileus becomes scaly at centre; 2) a stout and short-stiped habitus, with basidiomata often densely clustered, with deep yellow and distinctly anastomosing lamellae at stipe apex and in sinuses, with scales developing rather at mid-radius. All our collections matching phenotype 1 were correctly attributed to *P. olivellus* in the field, but before spore measurements and molecular analysis, the phenotype 2 was each time suspected as being distinct from the former and attributed to *P. adelphus*.

Paxillus rubicundulus P.D. Orton, Notes R. bot. Gdn Edinb. 29: 110 (1969)
Fig 5B

Material studied: UK: Scotland, Mid Perthshire, Rannoch, 22nd Oct., 1966, P.D. Orton 2905, K(M) 190474 (*P. rubicundulus* holotype, labelled as 'part of type').

Macroscopical description (holotype, from a half-exsiccatum, young mature basidiome)
Pileus 4 cm diam., broadly depressed; margin smooth, shortly enrolled; surface uniformly copper-brown, slightly more reddish at the very centre, minutely fibrillose sub-lens, smooth without any trace of scales.
Lamellae deeply decurrent, abundantly forked along the stipe, ochre brown with olivaceous shade.
Stipe 3 × 0.5 cm, strongly collapsed, light yellowish, reddish in places; base covered with yellow-ochre mycelium embedding some litter debris.

Microscopical description
Spores (n = 106, 1 coll.): (6.4) 6.6–7.4–8.5 (9.1) × (4.0) 4.4–4.7–5.2 (5.6) μm, Q: (1.32) 1.42–1.57–1.73 (1.95), cylindrical, no allantoid tendency, light yellow in KOH, wall 0.2–0.3 μm thick.
Basidia 18–38 × 5.5–8 μm, 4-spored, cylindrical, slender with straightened base, guttulate before maturity, wall thin, mostly collapsed after spore discharge.
Subhymenium 20–25 μm thick, pseudoparenchymatous.
Hymenial cystidia: Pleurocystidia 70 × 7 μm (base) × 4 μm (apex), rooting deeply, arising from hymenium up to 40–50 μm; irregularly frequent, more abundant towards the edge, approx. 200–600 per mm^2, partly thickened at base, with dark red-orange content in KOH, otherwise pale yellow to bright orange. Edge fertile cheilocystidia sparse (less than 20 per mm), 35 × 7 μm (base) × 3.5 μm (apex), colourless or with light yellowish content. Trama regular, 70–100 μm wide, made of long 3–16 μm wide hyphae, pale yellow, with thin smooth wall, mixed with sparse oleiferous 4–8 μm wide hyphae with yellow-orange content, and scattered 10–15 μm wide vesicles with pale yellow wall up to 0.8 μm thick, covered with amorphous deposits; hymenopodium not distinct.
Pileipellis an ixocutis made of slender 4–11 μm wide hyphae, with smooth slightly thickened wall up to 0.3 μm thick, often more at apex; terminal hyphae frequent, 80–150 × 6.8 μm, with a blunt apex, usually thickened. Subpellis not differentiated.
Stipitipellis a cutis of smooth-walled hyphae, caulocystidia not observed.
Clamps present at all septa.

Observations
It cannot be assessed whether Orton's (1969) original description is only based on the holotype or on several original collections, maybe including the much more common species *P. olivellus* and/or *P. adelphus*. Because no other annotated collection of *P. rubicundulus* is known to us, we cannot provide a full macroscopical description of this species, and cautiously refer to Orton's (1969) original description. However, the protologue fully matches our observations regarding microscopy. Morphologically, Orton's description depicts a fungus extremely similar to *P. adelphus*, except the stipe 'whitish or pale cream, soon pale sulphur or olive yellowish, often deeper yellow at apex'. This character went unobserved in all the other species, which displayed a uniformly yellowish stipe that turned reddish with age. We also observed that the hymenophore on the holotype is strongly decurrent, with abundant anastomoses all along the stipe, which might help as a field character for its recognition.

Discussion

Species delimitation, host range, and geographical distribution

In the present study, phylogenetic analyses and estimations of genetic diversity within and between lineages, combined with morphological studies, confirmed the three lineages of Gelardi *et al.* (2014) belonging to the *Paxillus rubicundulus* complex as distinct phylogenetic species. The *P. rubicundulus s. str.* clade is represented by the Orton's holotype collection (Orton 1969) and, in the absence of material linked to any type or formerly described species, no formal name was assigned by Gelardi *et al.* (2014) to clades A1.2 and A1.3, here respectively named *Paxillus adelphus* sp. nov. and *Paxillus olivellus* sp. nov. ITS sequences generated from our type specimens should contribute to enrich databases such as RefSeq (Schoch *et al.* 2014) and UNITE species hypothesis (Kõljalg *et al.* 2013) to a better identification of environmental samples. It is very important to have well identified references, especially when genetic distances are below 3 %, such as for *P. adelphus* and *P. rubicundulus*. The high level of genetic similarity between these two species (mean genetic distance around 1.5 %) suggests a recent divergence.

Our analyses illustrate the strengths and limitations of the use of morphological characters to identify *Paxillus* species. The three alnicolous species described here match the definition of the subg. *Alnopaxillus* proposed by Gelardi *et al.* (2014), *i.e.* basidiomata 'with a (sub)squamulose pileus surface, yellowish to deep yellow context and spores on average shorter than 8 μm'. As already shown by Hahn & Agerer (1999) and Hahn (2000), small spores are sufficient to characterize *P. rubicundulus s. lat.*, which encompasses indifferently the three species, although *Paxillus obscurisporus* (subg. *Paxillus*) and *P. rubicundulus* spore ranges may overlap. Two relevant features to define subg. *Alnopaxillus* could be added: 1) a thin mediostratum in the hymenophoral trama that fades out far from the gill edge, while the four European species in subg. *Paxillus* have a much thicker mediostratum with a sarcodimitic structure that almost reaches the edge; 2) the active reddish to purplish staining of the hymenophore when bruised. However, it is very difficult to tell apart the three species of the *P. rubicundulus* complex morphologically because most of the macroscopic and microscopic features tested as morphological divergences were found to overlap. It was only possible to identify young, fresh specimens macroscopically, but handled, mature, and older specimens were quite indistinguishable. The difficulty is enhanced by the fact that *P. adelphus* and *P. olivellus* often grow in sympatry, sometimes only a few centimetres away (authors' personal observations). We retained spore size and shape as the most reliable morphological markers for the three species (Table 4). To distinguish *P. olivellus* from *P. adelphus*, spore print colour could be an indicator since the fresh spore print of *P. olivellus* is usually olivaceous, while the spore print of *P. adelphus* is more chocolate-coloured. Thus, although these lineages have diverged, their morphological characters have not diverged as perceptible for human eyes. A comparable example in the same family *Paxillaceae* is provided by the gastroid genus *Alpova* (Moreau *et al.* 2013; Hayward *et al.* 2014), whereas most agaricoid alder-associated lineages (e.g. *Alnicola* or *Lactarius*) show morphological characteristics to indicate even faint genetic divergences (Rochet *et al.* 2011). A different evolutionary reaction to environmental pressure and host-dependant speciation between *Paxillaceae* (*Boletales*) and *Agaricales* lineages is a possibility to explore further.

Paxillus adelphus and *P. olivellus* mainly fruit on river banks and flooded riparian forests colonized by *Alnus* trees, but *Salix* sp. and *Populus* sp. are often present in the same habitats, and confusions may arise from the concurrent presence of non alder-associated *Paxillus* species. For instance, *Paxillus ammoniavirescens*, a species known to be associated with *Populus* and *Salix*, was sometimes found with *P. adelphus* and *P. olivellus* at the same sites (e.g. ALB and Cer sites) (Jargeat *et al.* 2014). All three have a bright-coloured cap with age and yellow flesh. However, *P. ammoniavirescens* has a distinctive non-squamose pileus, a green reaction with ammonia on young areas of the pileus, and larger spores than *P. adelphus* and *P. olivellus* (Jargeat *et al.* 2014). Similarly, *Paxillus cuprinus* is associated to *Betulaceae* and was occasionally found under alders (Jargeat *et al.* 2014; Põlme *et al.* 2013 *Paxillus* #1). It resembles *P. olivellus* because of its brownish olivaceous cap on young specimens but it differs by a less squamose cap, the presence of pink tones at the stipe apex in young specimens, the absence of profuse bright yellowish mycelium, an ochraceous-reddish to chocolate-brown spore print, and finally distinctly larger spores.

Species from subg. *Alnopaxillus*, including the undescribed species from North Korea (MOTU *Paxillus* #7 in Põlme *et al.* 2013) and *Paxillus orientalis* that is not a strictly alnicolous species, appear to be associated to alders that belong to subg. *Alnus*, but not to alders belonging to subg. *Alnobetula* (green alders). Thus, they have never been detected on roots of *Alnus alnobetula* subsp. *alnobetula* or of subsp. *suaveolens* (Roy *et al.* 2013), and have never been found fruiting in pure stands of green alders (authors' personal observations). Similarly, in Asia (China, Taiwan, Japan), they were not found on alders from either subg. *Alnobetula* or subg. *Clethropsis* (Põlme *et al.* 2013). Possible explanations for niche differentiation include physiological adaptation to the host, weak competitive ability, dispersal limitations, and genetic barriers to host-fungus compatibility.

Subg. *Alnopaxillus* has a wide intercontinental geographical distribution throughout the Northern hemisphere. Thus, the three strictly alnicolous species described here are present in many places in Europe, but also in North Africa (Algeria) and Western Asia (Turkey, Iran). However, they have never been found in America so far, whether from mycorrhiza sampling (Kennedy & Hill 2010; Kennedy *et al.* 2011), or in surveys of basidiomata (E.C. Vellinga, pers. comm.). *Paxillus olivellus* appears to be the most common in Europe. At the local scale, it also fruits abundantly and regularly (authors' personal observations). By contrast, the lack of *P. rubicundulus* specimens in our collections suggests that this species is rare, at least at a regional scale: it has been found so far only in the north of Europe and in mountains in the south of Europe and the west of Asia. To fully investigate the morphological and genetic diversity of this species and its geographical boundaries, a thorough sampling in Scandinavia and Western and Central Asia would be required. Additionally, only one species, *P. orientalis*, has been described from China, but Asia (particularly Central Asia and Eastern Asia) has been poorly sampled as compared to Europe or North America. Our study also reveals that taxonomic diversity in Asia is underestimated (for instance *Paxillus* #7 (Põlme *et al.* 2013) from North Korea – Changbai Mountains Range is undescribed). Moreover, as Asia is the supposed centre of origin for *Alnus* (Rochet *et al.* 2011), adding new information from this region will significantly contribute to our understanding of the coevolutionary patterns of *Paxillus* and their hosts, and potential migration routes and barriers for the fungi.

Revision of Paxillus filamentosus *and* Paxillus leptopus

Before Orton (1969) published the new name *Paxillus rubicundulus*, *Alnus*-associated *Paxillus* collections were already documented under the names *Paxillus filamentosus* or, more occasionally, *Paxillus leptopus* Fr. (Bresadola 1930). The question whether *P. filamentosus* or *P. leptopus* could be applied to any *Alnus*-associated *Paxillus* species was treated by Kotlaba & Pouzar (1960), Kühner (1962), Orton (1969), Bon (1977), and by Hahn & Agerer (1999), who recommended to neglect these two names but did not discuss the pre-Friesian origins of the question. Such investigations are performed here, and they reach the same conclusion. Fries (1836–1838) described *P. filamentosus* referring to two elements: 1) Scopoli's *Agaricus filamentosus* (Scopoli 1771–1772) from which the epithet is taken, and 2) personal collections from Sweden, '*ad terram et inter ramenta lignes; circa Upsaliam raro*'. He also cited as comments an unpublished collection from Schumacher (as '*Agaricus entoxanthus*') and two illustrated references: Bolton (1788, pl. 55, '*Agaricus adscendibus*', curved Agaric) and Buxbaum (1733, pl. VIII Fig 1, '*Fungus spadiceus, arvensis*', collected in fields around Constantinople, now Istanbul, Turkey). Scopoli (1771–1772) did not illustrate his '*Agaricus *filamentosus*', but validated a Linnean name for a taxon briefly described by Micheli (1729): '*Fungus ex luteo rufescens, cute superne pileoli partis lacera, veluti squamosa*'. Based on these original elements, Fries described *P. filamentosus* as a wood-inhabiting fungus with a short, lateral stipe, bright yellow decurrent lamellae, not bruising when touched, and with a '*totus hirto-tomentosus*, *fusco-lutescens*' pileus (Fries 1836–1838), merging Micheli's and Scopoli's elements. Whether it could be referred to any well-defined species of *Gymnopilus* or *Tricholomopsis* is debatable, but it should be admitted that no objective element in Fries' protologue indicates that *P. filamentosus* can be included in the current definition of the genus *Paxillus* (Bresinsky *et al.* 1999). Later, as detailed by Hahn & Agerer (1999), Fries (1857) introduced *P. leptopus* Fr. as a name change because of a homonymy between the former one (for which he provided some additional observations in comparison with *Paxillus involutus*) and *Ag. filamentosus* Schaeff. (Schaeffer 1774). Fries later (1874) explained: '*vix est A. filamentosus* Scop. *nec* Schaeff. *et nomen ineptum*, *quare novum receptum*'. Nevertheless, according to the rules of the current Code of nomenclature (McNeill *et al.* 2011), *Ag. filamentosus* Scop. (1771) is legitimate, published two years earlier than Schaeffer's (1774) homonym, and therefore *P. leptopus* Fr. is a superfluous new name for *P. filamentosus* (Scop.) Fr. The interpretations of *P. filamentosus* and *P. leptopus* gradually drifted after Fries towards a small-sized, terrestrial species, becoming squarrose only late with age. In 1878, Cooke & Quélet wrote a booklet based on Fries' rather meddled descriptions where *P. leptopus* is cited. Then both authors followed their own way: Quélet (1886) first explicitly applied the name *P. leptopus*, relegated as a variety of *P. involutus*, to a small-sized *Paxillus*, without any ecological details, whereas Cooke (1888, pl. 929) illustrated it as a typical *Paxillus* species, but lignicolous, possibly plagiarized from Bolton's plate of *Ag. adscendibus*, cited above. *Paxillus leptopus* was figured later by Bresadola (1930, pl. 678) who undoubtedly illustrated one of the alder-associated species studied here, likely *P. olivellus* according to the squamulose cap and the elliptical spore shape. However, Bresadola's description is not consistent with the plate but directly inspired from Fries' protologue, including the lignicolous ecology. The name *P. leptopus* was used by Marchand (1974, pl. 237), amongst others, but most authors such as Moser (1967) had noticed Fries' mistake and mechanistically adopted the name *P. filamentosus* instead of *P. leptopus* for the same fungus. Bon (1977) seems to have been the first (and only) author who recognized more than one *Alnus*-associated *Paxillus* species (namely *P. filamentosus* and *P. rubicundulus*), with a comparative table showing typical cylindrical spores for the latter, and elliptical ones for the former, thus interpretable as *P. olivellus*. Unfortunately, ITS and *gpd* amplification of six collections labelled '*P. filamentosus*', '*P. leptopus*' or '*P. rubicundulus*' in Bon's herbarium (LIP) failed. As stated by Hahn & Agerer (1999), there is no nomenclatural or taxonomic justification for applying the name *P. filamentosus* to any *Paxillus* species in the current concept of this genus. Because no other name than *P. rubicundulus* in European literature was found to match any of the *Alnus*-associated species described above, we introduced new names, respectively *Paxillus adelphus* and *P. olivellus*, for clades A1.2 and A1.3 of Gelardi *et al.* (2014). Finally, recent bibliographical reports of '*P. rubicundulus*' or '*P. filamentosus*' are hardly interpretable, especially when no microscopic details are provided. Thus, it is currently very difficult to provide a historical background of the descriptions and illustrations for *P. adelphus* due to the confusion with the closely related *P. rubicundulus*, for which good colour illustrations of molecularly identified material are not available. Most authors merged *P. olivellus* with *P. adelphus* and/or *P. rubicundulus* under the names *P. filamentosus* or *P. rubicundulus* (e.g. Szczepka 1987; Hahn & Agerer 1999; Knudsen & Taylor 2012), and descriptions are likely a mixture of these different taxa. However, Kühner (1962) likely described *P. olivellus* (*sub nomine P. filamentosus*) under *Alnus incana* from Samoëns in the French Alps, and provided the best description for this species. Marchand's (1974) photograph and text of *P. filamentosus* also appear to refer to *P. olivellus*. Ludwig's (2001) illustration 61.3. A perfectly depicts the scaly form of this species in mature specimens. All these authors describe ovoid to ellipsoid spores less than 8.5 μm long, which are characteristic of *P. olivellus*, in contrast with the cylindrical-suballantoid spores of *P. adelphus*.

Acknowledgements

We thank Elisé Pitois, Gilles Corriol, Maurice Durand, and Jacques Guinberteau, who provided specimens. We are also grateful to Bryn Dentinger and Begoña Aguirre-Hudson (Kew Herbarium) for the loan of the holotype of *P. rubicundulus* kept at K(M). We thank Sophie Manzi for help with labwork. Algerian data were collected in the National Park of El Kala on the invitation of Dr Radia Djelloul (Centre universitaire El Tarf, Algeria) and with the authorization of the National Park director. We also thank all the L2 and L3 BOPE students who participated to the *Paxillus* story (Romuald Rouger, Pierre-Olivier Belon, Chloé Bourden, Raphaëlle Gareil, Bruno Abad, Emeline Bru, and Elena Schuster). Thanks are due to Annie Buchwalter for correcting the English. The authors are very grateful to the two reviewers for their contribution to the improvement of the manuscript through their attentive reading, numerous corrections, and helpful suggestions. The Université Paul Sabatier Toulouse 3, the Centre National de la Recherche Scientifique (CNRS), and the Labex TULIP provided financial support for this study.

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