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The Amidella clade in Europe (Basidiomycota: Amanitaceae): clarification of the contentious *Amanita valens* (E.-J.Gilbert) Bertault and the importance of taxon-specific PCR primers for identification

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The Amidella clade in Europe (Basidiomycota: Amanitaceae): clarification of the contentious *Amanita valens* (E.-J.Gilbert) Bertault and the importance of taxon-specific PCR primers for identification

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ABSTRACT
The species in *Amanita* ser. *Amidella* (E.-J.Gilbert) Neville & Poumarat form a well-defined clade, but some taxa remain difficult to discriminate. In particular, the concept of *Amanita valens* (E.-J.Gilbert) Bertault remains controversial. To understand the phylogenetic placement of a set of collections from southern Portugal with a novel nrDNA barcode, we have obtained nrDNA sequences for previously unassessed type collections. The taxon formerly described as *A. curtipes* f. *pseudovalens* Neville & Poumarat is interpreted as a separate species, *A. pseudovalens* comb. nov., stat. nov., and is genetically indistinct from those Portuguese collections, thus clarifying their taxonomic context. However, the morphology and ecology of our collections are distinct (respectively, ellipsoid to oblong basidiospores and occurrence on schist soils), and are proposed as a new variety, *A. pseudovalens* var. *tartessiana* var. nov. These developments also enable a better diagnosis of the taxa of series *Amidella* in Europe, a progress that is most decisive for the late winter to spring collections, and identification keys are proposed. Still, the co-occurrence and morphological similarity of the new variety with the prized edible *A. ponderosa* Malençon & R.Heim could leave some collections of either taxon unresolved. Thus, a molecular marker approach was developed, to provide a clear and cost-effective identification aid to complement the keys. The proposed diagnostic tools can be applied toward a review of European *Amidella* taxa chorology from existing records, conserved materials, and future collections.

KEY WORDS
Portugal,
phylogeny,
taxonomic probes,
evolutionary convergence,
new combination,
new status,
new variety.

RÉSUMÉ
Le clade Amidella en Europe (Basidiomycota: Amanitaceae) : clarification du controversé Amanita valens *(E.-J.Gilbert) Bertault et importance de l'utilisation de primers PCR spécifiques aux taxons pour l'identification.*
Les espèces de *Amanita* ser. *Amidella* (E.-J.Gilbert) Neville & Poumarat forment un clade bien défini, mais certains taxons restent difficiles à discriminer. En particulier, le concept d'*Amanita valens* (E.-J. Gilbert) Bertault reste controversé. Pour comprendre le placement phylogénétique d'un ensemble de collections du sud du Portugal avec un nouveau barcode nrDNA, nous avons obtenu des séquences nrDNA pour des collections type non évaluées auparavant. Le taxon précédemment décrit comme *A. curtipes* f. *pseudovalens* Neville & Poumarat est interprété comme une espèce distincte, *A. pseudovalens* comb. nov., stat. nov., et est génétiquement indistinct de ces collections portugaises, clarifiant ainsi leur contexte taxonomique. Cependant, la morphologie et l'écologie de nos collections sont distinctes (respectivement, basidiospores ellipsoïdes à oblongues et occurrence sur des sols schisteux), et sont proposées comme une nouvelle variété, *Amanita pseudovalens* var. *tartessiana* var. nov. Ces développements permettent également de mieux diagnostiquer les taxons d'*Amidella* en Europe, progrès décisif pour les récoltes de la fin de l'hiver au printemps, et des clés d'identification sont proposées. Malgré tout, la cooccurrence et la similitude morphologique de la nouvelle variété avec la très appréciée et comestible *A. ponderosa* Malençon & R.Heim pourrait laisser certaines collections non résolues. Ainsi, une approche de marqueurs moléculaires a été développée, afin de fournir une aide claire et économique à l'identification pour complémenter les clés. Les outils de diagnostic proposés peuvent être appliqués à une révision de la chorologie des taxons d'*Amidella* européens à partir des enregistrements existants, des matériaux conservés et des collections futures.

MOTS CLÉS
Portugal,
phylogénie,
sondes taxonomiques,
convergence évolutive,
combinaison nouvelle,
statut nouveau,
variété nouvelle.

INTRODUCTION

Amidella (E.-J.Gilbert) Neville & Poumarat was originally proposed as a genus in the family Amanitaceae E.-J.Gilbert, to encompass a group of species with basidiomes initially white, some of them unchanging and others transitioning to pinkish, ochre or brown tones with maturation, or where rubbed, presenting a non-striated pileus, very friable partial veil leaving a fugacious annulus on the stipe and appendiculate remains in the pileus margin, a thick membranous saccate volva with a friable inner layer, and ellipsoid to subcylindrical amyloid spores (Neville & Poumarat 2004). Gilbert soon made it a subgenus of *Amanita* Pers., and currently the name applies, as a series within *Amanita* subgenus *Lepidella* (E.J.Gilbert) Beauseign. emend. Corner & Bas, only to the species that change colour, while the unchanging ones (*A. ovoidea* (Bull.) Link and allies) belong now to *Amanita* section *Roanokenses* Singer (Cui *et al.* 2018; Riccioni *et al.* 2019). Worldwide there are nearly 30 *Amidella* species present on several continents. The type species is the North American *A. volvata* (Peck) Lloyd.

In Neville & Poumarat's (2004) review of European *Amidella*, three species are recognised: *Amanita curtipes* E.-J.Gilbert, *A. lepiotoides* Barla, and *A. ponderosa* Malençon & R.Heim. For each of these species, they created divergent forms: on one hand, *A. lepiotoides* f. *subcylindrospora* Neville & Poumarat, for the high variability of spore morphology in this species; on the other hand, *A. ponderosa* f. *valens* (E.-J.Gilbert) Neville & Poumarat and *A. curtipes* f. *pseudovalens* Neville & Poumarat, because of the forms of intermediate size between the small *A. curtipes* and the typically robust *A. ponderosa*.

The epithet *valens* was originally created by E. J. Gilbert in 1941 as a variety of *Amanita lepiotoides*, before *A. curtipes* and *A. ponderosa* were known, and later raised to the species rank by Bertault, to fill the size gap between these two (Neville & Poumarat 2004). However, the spore morphology of *A. valens* (E.-J.Gilbert) Bertault differs markedly from the original form described by Gilbert, so that Neville & Poumarat (2004) proposed that the epithet *valens* was being applied to two taxa, the original as the form of *A. ponderosa* mentioned above, in line with the opinions of Malençon (date unknown; Neville & Poumarat 2009) and Pinho-Almeida (1994), while Bertault's *A. valens* was reclassified as *A. curtipes* f. *pseudovalens*. A consensus on this matter ought to be met with genetic information on the type specimens, thus the present study investigates the nrDNA from the types for the three divergent forms created by Neville & Poumarat. The results presented here support the concepts of *A. ponderosa* f. *valens* and (provisionally) of *A. lepiotoides* f. *subcylindrospora*, but *A. curtipes* f. *pseudovalens* is shown to be a well separated species, thus, a new combination is proposed, *A. pseudovalens* comb. nov., stat. nov.

Furthermore, we describe the Portuguese collections belonging to *A. pseudovalens* comb. nov., stat. nov. as a new variety, *A. pseudovalens* var. *tartessiana* var. nov., and due to its very difficult separation from smaller specimens of the prized edible *A. ponderosa*, a molecular marker approach was developed, to provide robust determinations.

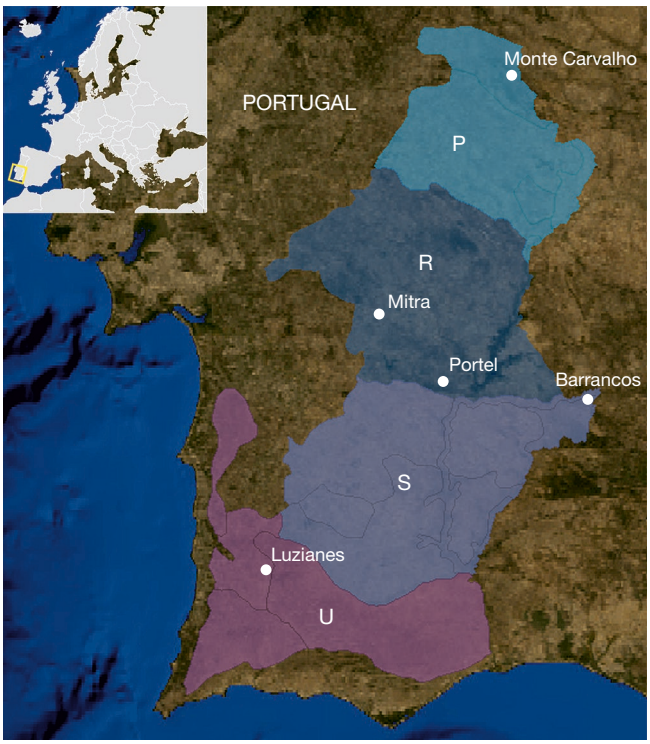

Fig. 1. — Locations of the collections used in this study (see Table 1 for details). The letters designate Landscape Units: **P**, Alto Alentejo; **R**, Alentejo Central; **S**, Baixo Alentejo; **U**, Serras do Algarve e do Litoral Alentejano. The area is outlined on the inset with a **yellow rectangle**. Source: DGT, Carta de Unidades de Paisagem (CUP), https://www.dgterritorio.gov.pt/dados-abertos

MATERIAL AND METHODS

Collections

Field sampling was conducted in southern Portugal during two spring seasons (2010 and 2015) and complemented with herbarium collections, including type specimens from taxa that remained unsequenced (Fig. 1; Table 1). The spring 2010 collections are from the Barrancos (38°10'11"N, 6°58'58"W; ten samples) and Portel (38°19'47"N, 7°41'46"W; ten samples) counties. The 2015 collections comprise 12 samples from Luzianes (Odemira county) and one from Monte Carvalho (Portalegre county). The Luzianes sites were designated as A (37°36'26"N, 8°26'59"W), B (37°36'12"N, 8°26'49"W), and C for a neighbouring site (precise location unknown). The single Monte Carvalho collection was made on a path, with a sandy texture on the surface, at the edge of a *Quercus suber* L. grove located in Alto da Quinta Nova (39°20'10"N, 7°24'57"W). Additional material was studied: three *Amanita ponderosa* specimens offered by traditional collectors in spring 2015, from uncharacterised locations (*Ode13-15*); and three *A. curtipes* collections made during spring 2010 and Autumn 2016 in the Mitra university campus, Évora (38°31'39"N, 8°01'21"W), in association with evergreen oaks on sandy soil. The 16 specimens from the 2015 field campaign were deposited in the PO Herbarium (Natural History and Science Museum of the University of Porto, MHNC-UP), with consecutive codes between PO-F2132 and PO-F2147.

The herbaria material comprised type specimens cited by Neville & Poumarat (2004) ceded by the Conservatoire & Jardin botaniques de la Ville de Genève (Geneva, Switzerland, http://www.ville-ge.ch/musinfo/bd/cjb/chg): the *Amanita lepiotoides* f. *subcylindrospora* holotype (G[G00126408], SIB: 270556/1), the *A. ponderosa* f. *valens* neotype (collection number *SGP 94.03.206*, G[G00561773], SIB: 383077/1) and the *A. curtipes* f. *pseudovalens* holotype (*SGP 93.05.15.203*, G[G00561774], SIB: 382650/1). Materials from two further collections were ceded by the University of Montpellier – Institute of Botany (MPU) herbarium (https://science.mnhn.fr/institution/mnhn/search), one labelled as *Amanita valens* from the Landes department, France (MPU[MPUC00085]) and the other labelled *Amanita eliae* Quél. from Larache, Morocco (G. Malençon collection number 5225, MPU[MPUC00038]). Finally, one *A. lepiotoides* from the Aranzadi Science Society herbarium (ARAN[ARAN-Fungi 5040268]), collected in Navarra and reported by Arrillaga & Mayoz (2005), Spain, was provided by Pedro Arrillaga.

Characterization of the sites

The Barrancos and Portel sites belong to a geomorphological unit dominated by Paleozoic siliceous materials, mainly schists or granites. The territory has a dry to sub-humid ombrotype, and a thermotype spanning the thermomediterranean to mesomediterranean bioclimatic belts (Costa *et al.* 1998; Monteiro-Henriques *et al.* 2016; Rivas-Martínez *et al.*

TABLE 1. — Collections examined in this study. Abbreviations: ***curtipes***, *A. curtipes* E.-J.Gilbert; ***lepiotoides***, *A. lepiotoides* Barla; ***ponderosa***, *A. ponderosa* Malençon & R.Heim; ***pseudovalens***, *A. pseudovalens* comb. nov., stat. nov.; ***subcylindrospora***, *A. lepiotoides* f. *subcylindrospora* Neville & Poumarat; ***tartessiana***, *Amanita pseudovalens* var. *tartessiana* var. nov.; ***valens***, *A. ponderosa* f. *valens* (E.-J.Gilbert) Neville & Poumarat. *, the MPU2 collection, labelled *A. eliae* Quél., was interpreted by G. Malençon as *A. curtipes* (Neville & Poumarat 2004: 654).

Set	Site	Date	Code	Collector	Epithet	ITS	LSU
Spring 2010	Barrancos	2010.04.07	*B03*	L. N. M.	*tartessiana*	KJ950803	KJ950782
Spring 2010	Barrancos	2010.04.07	*B04*	L. N. M.	*ponderosa*	KJ950787	KJ950779
Spring 2010	Barrancos	2010.04.07	*B05*	L. N. M.	*ponderosa*	KJ950788	KJ950780
Spring 2010	Portel	2010.04.07	*P01*	R. A.-C.	*tartessiana*	KJ950804	KJ950783
Spring 2010	Portel	2010.04.22	*P09*	R. A.-C.	*ponderosa*	KJ950801	KJ950781
Spring 2010	Mitra	2010.03.24	*M02*	R. A.-C.	*curtipes*	KJ950805	KJ950784
Spring 2010	Mitra	2016.12.14	*M04*	P. O.	*curtipes*	MW538529	MW494958
Spring 2010	Mitra	2016.12.16	*M05*	P. O.	*curtipes*	MW494955	MW494959
Spring 2015	Luzianes A	2015.03.21	*Ode01*	A. C. S.	*tartessiana*	–	–
Spring 2015	Luzianes A	2015.03.21	*Ode02*	A. C. S.	*tartessiana* isotype	–	–
Spring 2015	Luzianes A	2015.03.21	*Ode03*	A. C. S.	*tartessiana*	–	–
Spring 2015	Luzianes A	2015.03.28	*Ode04*	A. C. S.	*tartessiana*	–	–
Spring 2015	Luzianes B	2015.04.04	*Ode05*	A. C. S.	*tartessiana*	MW431349	MW431339
Spring 2015	Luzianes B	2015.04.04	*Ode06*	A. C. S.	*tartessiana*	–	–
Spring 2015	Luzianes B	2015.04.04	*Ode07*	A. C. S.	*tartessiana*	MW431348	MW431338
Spring 2015	Luzianes A	2015.04.04	*Ode08*	A. C. S.	*tartessiana*	–	–
Spring 2015	Luzianes C	2015.04.10	*Ode09*	Manuel Fontes	*ponderosa*	–	–
Spring 2015	Luzianes B	2015.04.12	*Ode10*	A. C. S.	*tartessiana*	–	–
Spring 2015	Luzianes B	2015.04.12	*Ode11*	A. C. S.	*tartessiana*	MW431350	MW431340
Spring 2015	Luzianes A	2015.04.12	*Ode12*	A. C. S.	*tartessiana* holotype	MW431351	MW431341
Spring 2015	Portel	2015.04.22	*Ode13*	R. A.-C.	*ponderosa*	–	–
Spring 2015	Arraiolos	2015.04.22	*Ode14*	Unknown	*ponderosa*	–	–
Spring 2015	Arraiolos	2015.05.03	*Ode15*	Unknown	*ponderosa*	–	–
Spring 2015	Portalegre	2015.04.27	*Ode16*	Nuno Alegria	*tartessiana*	–	–
Herbaria	Alpes-Maritimes	1998.07.10	*G08*	D. Auzias	*subcylindrospora* holotype	MW431346	MW431336
Herbaria	Hérault	1994.03.27	*G73*	Serge Poumarat	*valens* neotype	MW431345	MW431335
Herbaria	Landes	1993.05.15	*G74*	Mesplède	*pseudovalens* holotype	MW431354	MW431344
Herbaria	Landes	1965.05.19	*MPU1*	J. Beller	*pseudovalens*	MW431352	MW431342
Herbaria	Larache	1964.05.04	*MPU2*	G. Malençon	*pseudovalens**	MW431353	MW431343
Herbaria	Navarra	2006.08.03	*Eusk*	I. Mayoz	*lepiotoides*	MW431347	MW431337
GenBank	Lugo	2018.07.18	*ECC 18071801*	J. Alonso	*lepiotoides*	MN497357	MN497358
GenBank	Segovia	2004.10.24	*AH31924*	J. M. Barrasa	*curtipes*	EF653963	EF653960
GenBank	Madrid	2002.04.20	*AH19699*	G. Moreno	*ponderosa*	EF653962	EF653959
GenBank	Cáceres	2000.05.06	*AH19752*	G. Moreno & E. Arrojo	*ponderosa*	AY486234	EF653958

2017; Capelo & Vila-Viçosa 2021). The potential vegetation is a oak woodland co-dominated by cork oak (*Quercus suber*) and round-leaf oak (*Q. rotundifolia* Lam.) that, by anthropic action, result in an open landscape and patch-like mosaic known as Montado/Dehesa (Blanco-Castro *et al.* 2005). All collections were performed in skeletical soils that are disturbed by agricultural and silvicultural activities. The prevailing understory is composed of grasslands and shrubland associations dominated by *Cistaceae*, *Lamiaceae* and *Fabaceae* species (e.g. *Cistus ladanifer* L., *C. salviifolius* L., *C. crispus* L., *Lavandula* sect. *Stoechas* Ging., *Genista* L. and *Ulex* L.). These shrub associations belong to *Ulici eriocladi-Cistetum ladaniferi* and *Genisto hirsutae-Cistetum ladaniferi* (Costa *et al.* 2012) and they represent the late regressive and pioneer stages resulting from erosion of the soil upper layer, intensive grazing, or fires (Castro & Freitas 2009; Mendes *et al.* 2015).

The collections from Luzianes sites A and B were on compact, eroded and acidic clay soils derived from schistose bedrock, at the edge of *Eucalyptus globulus* Labill. plantations. The vegetation type is a shrubland dominated by *Cistus ladanifer* (*Cisto ladaniferi-Ulicetum argentei*) with an estimated vegetation cover of 70-80%, representing an early regressive stage of cork oak forests (*Lavandulo viridis-Quercetum suberis*, Quinto-Canas *et al.* 2010).

MYCOLOGICAL DESCRIPTIONS

Descriptions of the basidiomes collected during spring 2015 were made on site and completed in the laboratory by following the guidelines of a specifically designed observation form. Basidiome photographs were taken on site and on arrival to the laboratory. Reagents used for microscopy were prepared as described in Clémençon (2009). Basidiospore measurements were made from spore print samples preferably, or from lamellae preparations. Basidiospores were measured with a 100× objective, and basidia with a 40× objective. To study the structure of the universal veil in dried unfixed specimens, hand sections of the volva were made and observed either unstained (GSM mounting medium, Clémençon 2009) or stained with Congo Red SDS. All measurements were made using calibrated microscope eyepieces, for the most part an

AM-423X Dino-Eye USB digital camera coupled with image processing DinoCapture software (AnMo Electronics Corp., Taiwan). Statistical analyses and testing were made using JASP (JASP Team 2020, https://jasp-stats.org).

Reference sporographs were drawn based on the guidelines of Tulloss (1984), plotting basidiospore lengths and widths, and using the values in Neville & Poumarat (2004). The colour reactions to 10% $FeSO_4$ were observed under the dissecting microscope, on rehydrated samples of stipe context.

DNA extraction, PCR sequencing and phylogenetics

DNA was extracted from basidiome samples using a modification of the method for filamentous fungi described by Stirling (2003). Amplification of the nuclear ribosomal DNA internal transcribed spacers (ITS) was made using different combinations of primers, primarily the NSA3-NLC2 (Martin & Rygiewicz 2005) or the V9D-LS266 (Gerrits van den Ende & de Hoog 1999) primer pairs. For the initial 1 Kb of nuclear large subunit (28S) ribosomal DNA (LSU), the LR0R-LR5 primer pair (Vilgalys & Hester 1990; Rehner & Samuels 1994; Hopple & Vilgalys 1999) was used. All amplifications yield a single product with at least 1 Kb, using a protocol of 30 cycles with annealing temperatures of 61°C, 57°C and 54°C, respectively, in the presence of 0.8 mM dNTPs and 1.5 mM $MgCl_2$. The herbaria material produced very fragmented (and possibly nicked) DNA. Thus, successful amplifications were obtained mostly with shorter segments or, to avoid the amplification of fungal contaminants in some extracts, with taxon-specific primers (Osmundson *et al.* 2013; Appendix 3).

The PCR solutions were extracted with chloroform and ethanol-precipitated in the presence of ammonium acetate, using linear polyacrylamide as carrier (Fregel *et al.* 2010) and sent to a sequencing service (STAB VIDA, Portugal). The sequencing chromatograms were inspected to trim the sequence ends and analyse ambiguous positions, using Applied Biosystems Sequence Scanner (Thermo Fisher Scientific, United States) and Ugene version 42.0 (Okonechnikov *et al.* 2012). Contigs were assembled using the CAP3 (Huang & Madan 1999) module in UGene. The sequences are deposited in GenBank (Table 1).

Sequence alignments were made using the MUSCLE (Edgar 2004) module in UGene, and phylogenetic analyses using MEGA-X (Kumar *et al.* 2018), with tree rendering from the Newick export using the iTOL web tool (Letunic & Bork 2019). In the concatenated ITS-LSU regions, the partial deletion option was selected at minimum 70% site coverage (i.e., allowing the aligned sites with fewer than 30% alignment gaps, missing data, and ambiguous bases, 1121 sites), while for the ITS alone 65% was selected (563 sites). A search for the Species Hypothesis codes at the UNITE database version 08FU (Kõljalg *et al.* 2013) was made based on the ITS sequences obtained. Alignments are available at Figshare (https://doi.org/10.6084/m9.figshare.19727074.v2).

Molecular markers

Taxon-specific PCR primers targeting the ITS and LSU regions were developed for the quick detection of *Amanita pseudovalens* comb. nov., stat. nov. among *A. ponderosa* collections (Table 2; Appendix 1). The strategy assumes the following: 1) DNA extracted from fresh basidiomes; 2) PCR primers at concentration 0.25 µM each; 3) one of the primers is general for fungi, and the other is taxon-specific; 4) the taxon-specific primer has the lower melting temperature (IDT OligoAnalyzer tool, https://www.idtdna.com/calc/analyzer/); and 5) the PCR annealing temperature is equal to the taxon-specific melting temperature. Negative controls use water, and positive controls include one *A. pseudovalens* comb. nov., stat. nov. reference DNA, and/or a parallel reaction to detect false negatives, either by using primers specific for *A. ponderosa* or matching two general fungal primers.

Abbreviations

nrDNA	nuclear ribosomal DNA region;
ITS	nrDNA internal transcribed spacer;
LSU	nuclear ribosomal large subunit DNA region;
NUTS	nomenclature of territorial units for statistics.

RESULTS

Phylogenetic analyses

The phylogenetic analysis of the concatenated ITS-LSU regions revealed four distinct clades corresponding to *Amanita ponderosa*, *A. lepiotoides*, *A. curtipes,* and a sister clade of *A. curtipes* containing the type specimen of *A. curtipes* f. *pseudovalens* (Fig. 2). The latter clade also includes the provisionally named *A.* aff. *curtipes* that had been collected in 2010 among other collections of *A. ponderosa* (specimens *B03* and *P01*), along with the MPU and Ode sequences. The same result was obtained separately for the ITS and the LSU regions (not shown). This clade is thus suggested to represent a separate taxon at species level that can be collected both in southern France and in Portugal, henceforth designated *A. pseudovalens* comb. nov., stat. nov.

To provide further support to the proposal of a separate taxon at species level, the ITS region was studied for genetic divergence estimates. Using the Net Between Group Distances estimator (Tamura & Nei 1993) in MEGA, the between-node distances for each clade pair were calculated. The net evolutionary divergence (i.e., the number of substitutions per site) between the *curtipes* and *pseudovalens* nodes is positive, albeit lower than other comparisons as expected (Table 3); even without corrections, the divergence estimate (p) for the ITS, between the *curtipes* and the *pseudovalens* nodes, was estimated at 6.2%. The *curtipes* and *pseudovalens* ITS sequences corresponded unambiguously to different Species Hypothesis clusters (Kõljalg *et al.* 2013) even at the 3% dissimilarity threshold (SH1184304.08FU and SH1184305.08FU, respectively, Table 4).

The average Within-Group distance (Tamura-Nei substitution model, Table 4) for the *pseudovalens* clade (containing the

TABLE 2. — List of selected taxon-specific primers, showing the sequences, annealing temperatures (T_a), matching and positive control universal primers. *Apolf2* is for *Amanita ponderosa* Maleçon & R.Heim, the remainder are for *A. pseudovalens* comb. nov., stat. nov. [a], White *et al*. 1990; [b], Gardes & Bruns 1993; [c], NLC2R is the reverse complement of NLC2 (Martin & Rygiewicz 2005); [d], Hopple & Vilgalys 1999.

ID	Sequence	T_a (°C)	Matching	Control
Apolf2	5' GAGTGTCATTCATATTCTC 3'	50.3	ITS4[a]	ITS3[a]
Apslr1	5' TTGTTCATTAACAATTGTCTTTC 3'	54.7	ITS1F[b]	ITS4
Apslr3	5' GACACAAATTCATTTAGAAAG 3'	51.5	ITS3	ITS4
ApsLr2	5' AATGAATGGCCTGGCGA 3'	59.1	NLC2R[c]	LR5[d]

TABLE 3. — Net evolutionary divergence (base substitutions per site, standard errors in brackets) between groups of sequences (Tamura 1992).

Cluster pair	ITS (TN93 model)	ITS (uncorrected p)
curtipes-pseudovalens	0.0695 (0.0144)	0.0622 (0.0117)
curtipes-lepiotoides	0.1631 (0.0216)	0.1348 (0.0148)
curtipes-ponderosa	0.2211 (0.0283)	0.1698 (0.0159)
pseudovalens-lepiotoides	0.1799 (0.0281)	0.1436 (0.0176)
pseudovalens-ponderosa	0.2342 (0.0341)	0.1764 (0.0183)
lepiotoides-ponderosa	0.1597 (0.0223)	0.1304 (0.0143)

TABLE 4. — Estimates of average Within-Group distances (nucleotide substitutions per site, standard errors in brackets, Tamura-Nei model) and the Species Hypotheses (**SH**) for the *curtipes* and the *pseudovalens* clusters defined in this study.

Cluster	Within-Group	1.5% SH	3% SH
curtipes	0.0019 (0.0012)	SH1562916.08FU	SH1184304.08FU
pseudovalens	0.0109 (0.0031)	SH1562918.08FU	SH1184305.08FU

Portuguese and French collections) was estimated at 0.0109 nucleotide substitutions per site (± 0.0031 standard error).

Phylogenetic analyses of worldwide collections belonging to series *Amidella* indicate that the European species, apart from the close relationship between *Amanita curtipes* and *A. pseudovalens* comb. nov., stat. nov., have separate origins (data not shown, but see Moreno *et al.* 2008), with *A. ponderosa* close to the American *A. whetstoneae* Tulloss, Goldman & Kudzma nom. prov., *A. lepiotoides* f. *subcylindrospora* close to the Asiatic *A. rufobrunnescens* W.Q.Deng & T.H.Li, and *A. curtipes* with *A. pseudovalens* comb. nov., stat. nov. sharing a branch with the Asiatic *A. brunneomaculata* Zhu L. Yang, Y.Y.Cui & Q.Cai.

Family AMANITACEAE E.-J.Gilbert
Genus *Amanita* Pers.

Amanita pseudovalens
(Neville & Poumarat) R.Arraiano-Castilho, A.C.Silva, C.Vila-Viçosa, M.R.Castro, L.Morgado & P.Oliveira
var. ***pseudovalens*** comb. nov., stat. nov.

Basionym: *Amanita curtipes* f. *pseudovalens* Neville & Poumarat, *Fungi Europaei* 9: 656 (Neville & Poumarat 2004).

SPECIMENS EXAMINED. — **France**. Landes, 15.V.1993, Mesplède, *G74*; 19.V.1965, J. Beller échantillon 2937, *MPU1*.
Morocco. Larache, 04.V.1964, G. Malençon échantillon 5225, *MPU2* (DNA only).

MYCOBANK. — MB 845581.

INDEX FUNGORUM. — IF 559870.

NOTES
This taxon corresponds to the French specimens (see Discussion for other possible locations). The basionym diagnosis (Neville & Poumarat 2004) remains unaltered for this variety (Table 6).

Amanita pseudovalens var. ***tartessiana***
R.Arraiano-Castilho, A.C.Silva, C.Vila-Viçosa, M.R.Castro, L.Morgado & P.Oliveira var. nov.

HOLOTYPE. — **Portugal**. Alentejo, Beja district, Odemira, Luzianes A, 12.IV.2015, A. C. Silva, *Ode12* (holo-, PO[PO-F2143]).

ISOTYPE. — **Portugal**. Alentejo, Beja district, Odemira, Luzianes A, 21.III.2015, A. C. Silva, *Ode02* (iso-, PO[PO-F2133]).

ADDITIONAL SPECIMENS EXAMINED. — **Portugal**. Alentejo, Beja district, Odemira, Luzianes A, 21.III.2015, A. C. Silva, *Ode01*; *Ode03*; 28.III.2015, A. C. Silva, *Ode04*; 04.IV.2015, A. C. Silva, *Ode08*; Luzianes B, 04.IV.2015, A. C. Silva, *Ode05*; *Ode06*; *Ode07*; 12.IV.2015, A. C. Silva, *Ode10*; *Ode11*; Portalegre district, Portalegre, 27.IV.2015, N. Alegria, *Ode16*; Évora district, Portel, 07.IV.2010, R. Arraiano-Castilho, *P01* (for basidiospores only).

ETYMOLOGY. — The epithet refers to the ancient Tartessian civilization (Ταρτησσός) located in the south-west of the Iberian Peninsula (Celestino Pérez & López-Ruiz 2016).

PHENOLOGY. — Late winter and spring.

HABITAT. — Mediterranean, in association with *Cistus* spp., typically on compact, acidic and eroded soils, corresponding to regressive shrubland stages of evergreen oak forests (Fig. 5).

DISTRIBUTION. — **Portugal**. Reported from the NUTS III regions of Alentejo Litoral, Baixo Alentejo, Alentejo Central and Alto Alentejo.

MYCOBANK. — MB 845582.

INDEX FUNGORUM. — IF 559871.

NOTES
This taxon corresponds to the Portuguese specimens described in this study. It differs from the autonym by its habitat (on acidic schist soils, with *Cistus* spp.), the ellipsoid to oblong, infrequently subcylindric basidiospores, and longer basidia (Table 6). Such differences are not considered to be at the rank of form. A species rank is currently not supported, due to the lack of genetic resolution of the nrDNA sequences.

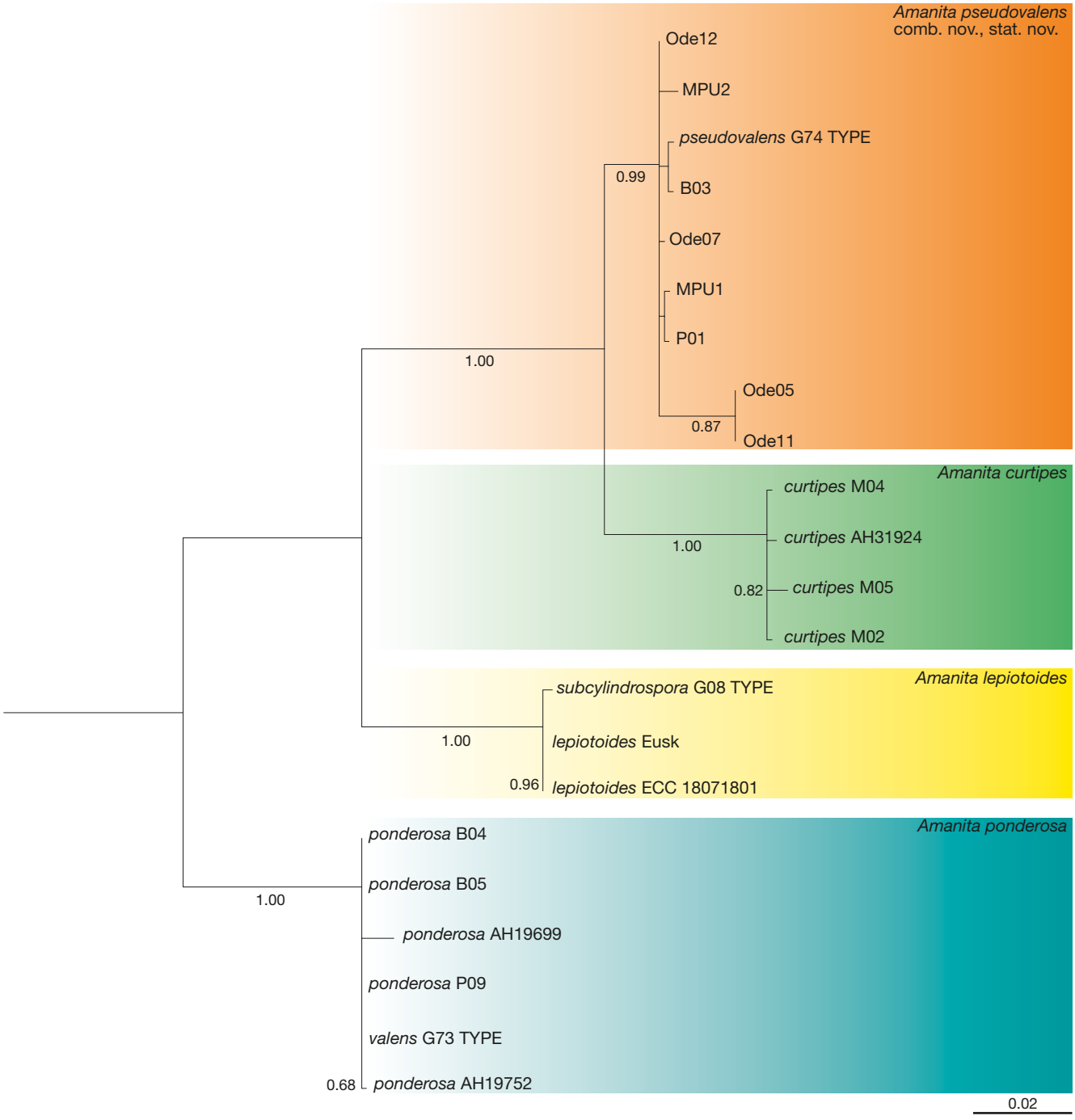

Fig. 2. — Phylogenetic placement of the type nrDNA sequences, in relation to ITS + LSU sequences from european *Amanita* Pers. taxa belonging to the Amidella clade (only the terminal epithets are shown). Using a 70% coverage cutoff, a total of 1121 aligned positions were analysed with the Maximum Likelihood method and the Tamura-Nei (Tamura & Nei 1993) substitution model, with a discrete Gamma distribution to model evolutionary rate differences among sites (five categories [gamma parameter 0.7526]), allowing for some sites to be evolutionarily invariable (40.90% sites). The percentage bootstrap support for each node (1000 replicates) is shown next to the branches. The support for each species clade is 99% (*Amanita pseudovalens* comb. nov., stat. nov.), 100% (*Amanita curtipes* E.-J.Gilbert), 100% (*Amanita lepiotoides* Barla) and 100% (*Amanita ponderosa* Maleçon & R.Heim). The scale indicates 0.02 substitutions per site.

Both taxa share, aside from the characters in common with other species of series *Amidella*, the vernal fruiting season, the medium to small size, and the indistinct odour (Table 6), and can be confirmed with the *Aps* diagnostic PCR primers described in Table 2.

Description

To describe the new species, we used fresh materials obtained from sites A and B near Luzianes in spring 2015 (*Ode01-12* except *Ode09*) and the basidiospores from a collection in the Portel county (*P01*, near Monte Novo, spring 2010) and another from São Mamede Park near Monte Carvalho,

Portalegre county (*Ode16*, spring 2015). All specimens used in this description were deposited in the PO herbarium (see Material and Methods).

Pileus
Flat to slightly depressed, convex at the margin, expanding to a diameter of 7.5 cm. Most collections whitish *in situ*, turning rose/ochre with either aging, handling, or scratching. A pale grey plaque from the universal veil is frequently present. Some collections present brownish scales close to the margin (Fig. 3B, C). Margin thinly appendiculate.

Hymenophore
Adnexed ascending, white, turning rose/ochre with either aging, bruising or scratching, with lamellulae.

Stipe
Almost cylindrical, slightly tapering toward the apex, non-bulbous, base obconical. Concolorous with the pileus, with a very fugacious annulus (Fig. 3H). A scale covering can be seen below the annulus region (Fig. 3A; C; E; F). Height not longer than the diameter of the expanded pileus, thickness 2.2 cm at the most.

Veil
Universal veil leaving a sac-like thick volva with a lobed margin, pale grey, with an internal ridge raised in contact with the stipe (Fig. 3H); often it remains also as a single pale grey plaque on the pileus.

Partial veil leaving a fugacious non-membranous annulus at roughly two-thirds of the stipe height, and narrow remnants on the pileus margin.

Context
Concolorous with the surface, homogeneous, relatively compact, non-putrescent. Odour indistinct. Reaction with 10% $FeSO_4$ on rehydrated samples from the stipe develops an immediate change to greenish grey that lasts a few minutes.

Basidiospores
White, amyloid, ellipsoid to oblong, average length 11.78 µm, average width 6.97 µm, average length/width ratio (Q) 1.696 (Table 5), overlapping the *A. ponderosa* sporograph but not the one for *A. curtipes* f. *pseudovalens* (Fig. 4). Due to the lack of spore print, collections *Ode01* and *Ode16* were not included in the summary calculations. Statistical testing rejected the hypothesis of homogeneity among the collections included in the summary statistics, for all three variables (Appendices 4; 5). Indeed, the heterogeneity among collections was the rule (Table 5; Appendix 8): *Ode10* had longer spores and higher Q, bordering on standard *A. pseudovalens* comb. nov., stat. nov. limits; *Ode08* had wider spores and lower Q, even more than *A. ponderosa*, while *Ode11* had spores of average Q values but small size. Nested ANOVA (within and between sites) also suggested heterogeneity within sites for the three variables, but only for length and width between sites. Site A collections have on average significantly higher values (Appendix 6).

Basidia
Clavate, with 4 sterigmata, base unclamped, average length 56.9 µm (equal to the median), range 41.0-73.4 µm, n = 123. The measurements were obtained from collections *Ode02b*, *Ode05*, *Ode06a*, *Ode07*, *Ode08*, *Ode10*, *Ode11* and *Ode12*, revealing a normal distribution of the global data (Shapiro-Wilk's W = 0.990, *P* = 0.555). On average, similar basidia sizes were observed across all collections, although *Ode11* had a higher average length of 63.9 ± 5.0 µm (Appendix 7).

Universal veil
Sagital sections (*Ode02*, *Ode07*, *Ode11*, *Ode12*) revealed a thin outer layer, 80-100 µm deep, composed of slightly interwoven, longitudinally oriented hyphae, very compact and 10 µm wide (as measured in transversal sections), from which thinner hyphae, very loose, projected outwards (not found in *Ode11*). Many of the latter hyphae had a slightly widened, bulbous termination. No clamp connections were observed. The remainder of the structure was composed of more loosely packed longitudinal, slightly wavy hyphae, with very conspicuous lacunae interpreted as remnants of larger inflated, ellipsoid to oblong, hyphal elements. Measurement of these lacunae in Congo Red SDS stained sections (*Ode02*, *Ode07*, n = 41), under low magnification, gave an estimate of 40-71 µm length (average 54 µm, C. V. 17%) by 20-46 µm width (average 33 µm, C. V. 16%).

Comparison with *Amanita curtipes* f. *pseudovalens* and *Amanita ponderosa*

When comparing the descriptions of all European taxa of series *Amidella*, based on Neville & Poumarat (2004), as shown in Table 6, the Portuguese specimens of *Amanita pseudovalens* comb. nov., stat. nov. did not conform with the description for the genetically indistinct *A. curtipes* f. *pseudovalens* (Neville & Poumarat 2004), diverging in their soil and vegetation affinities and in the microscopy. Therefore, we understand them as representing a separate taxon, however at infraspecific level.

Moreover, Table 6 shows that the *tartessiana* variety resembles *A. ponderosa* in almost every aspect – the obvious difference being the so far unknown occurrence of large and heavy specimens in the former – being, by comparison, much less similar to the conspecific *A. pseudovalens* comb. nov., stat. nov. collected in France.

Molecular probes for discrimination from *Amanita ponderosa*

The use of ITS and LSU primers specific for *Amanita pseudovalens* comb. nov., stat. nov. on the *Ode01-Ode16* samples indicated that all DNA extracts from the Luzianes A and B collections (*Ode1-8* and *Ode10-12*) and from the Portalegre collection (*Ode16*) were *A. pseudovalens* comb. nov., stat. nov. (Fig. 6). All extracts that did not amplify with these primers amplified with the positive control primer sets (not shown). Similar results were obtained with some of the herbarium material (Appendix 2).

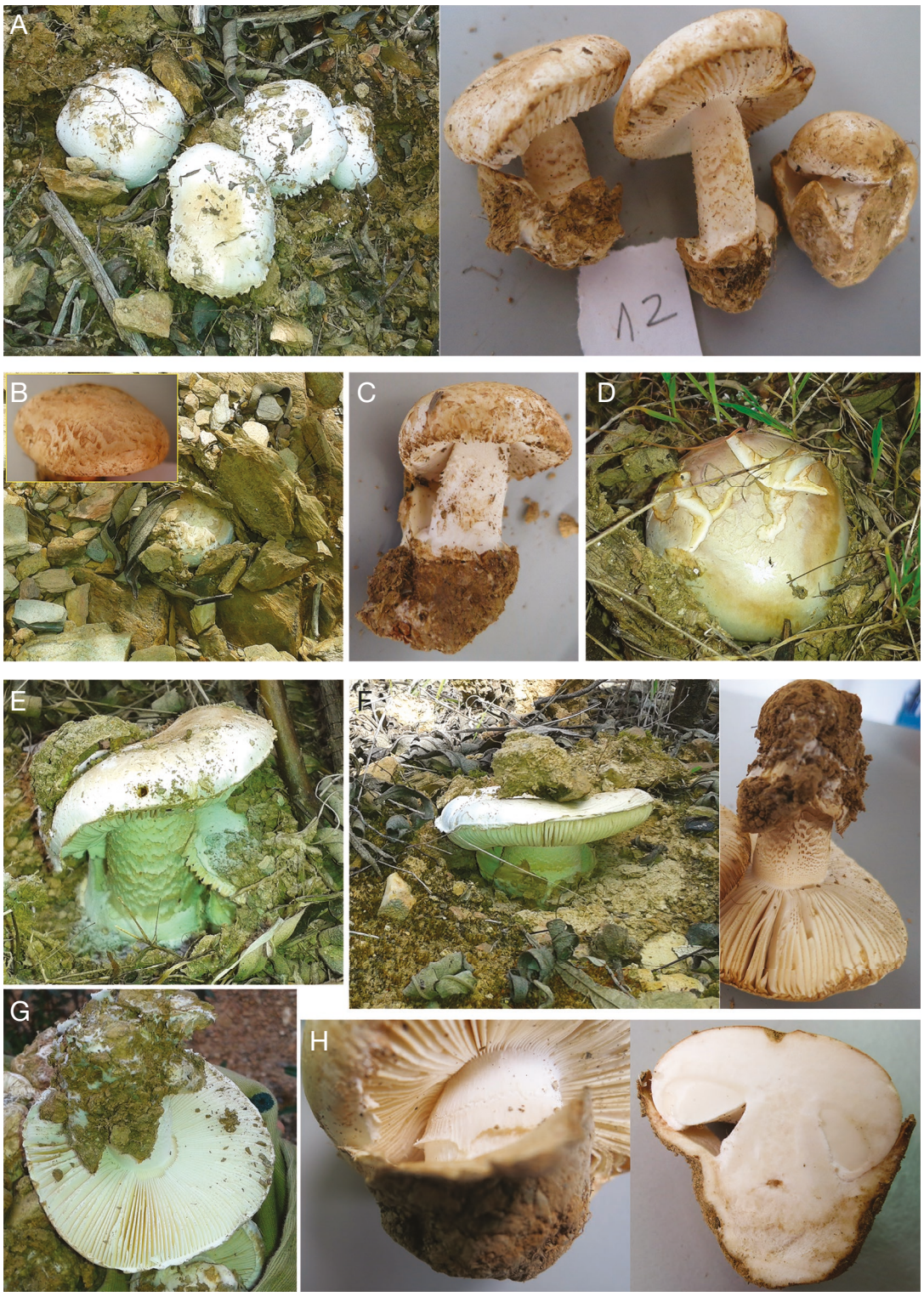

FIG. 3. — Photographs of *Amanita pseudovalens* var. *tartessiana* var. nov. specimens from Odemira. Some images taken in the field are matched with corresponding ones taken in the laboratory: **A**, grouped basidiomes (*Ode12*) and their appearance upon arrival at the laboratory; **B**, outcropping away from the vegetation, showing the coarse soil texture in this case (*Ode02*); inset shows squamulose inner cuticle remains; **C**, another specimen with cuticle squamules (*Ode11*); **D**, specimen with cracked cuticle (*Ode05*); **E**, example with stipe squamules (*Ode06*); **F**, side view of an emerging basidiome (*Ode03*) and the same specimen showing the darkened squamules on the stipe; **G**, view of the hymenophore and the appendiculate pileus margin (*Ode02*, specimen different from **B**); **H**, details of an immature basidiome (*Ode06*, specimen different from **E**) showing the annulus and insertion of the lamellae (**left**), and longitudinal section (**right**).

TABLE 5. — Basidiospore measurements (µm) and Q values, represented as the central 90% distribution intervals (or minimum-maximum ranges (**min-max**) where indicated), with averages in **italic**. Summary distributions do not include *Ode01* and *Ode16* (no spore print available).

Collection	Length	Width	Q	Comment
P01	10.2-*11.80*-13.4	6.3-*7.35*-8.4	1.38-*1.61*-1.84	n = 30
Ode01	9.5-*12.41*-15.3	5.7-*6.58*-7.5	1.61-*1.88*-2.07	n = 15 (min-max)
Ode02b	10.7-*12.02*-13.4	6.2-*7.03*-7.9	1.45-*1.71*-1.98	n = 30
Ode03	10.7-*12.95*-15.2	6.4-*7.29*-8.2	1.56-*1.78*-2.00	n = 36
Ode04	10.7-*12.43*-14.2	5.6-*7.03*-8.5	1.42-*1.78*-2.14	n = 34
Ode05	9.6-*11.07*-12.6	5.9-*6.69*-7.5	1.43-*1.66*-1.88	n = 41
Ode06a	10.0-*11.53*-13.1	6.4-*7.27*-8.1	1.39-*1.59*-1.79	n = 44
Ode07	10.9-*12.32*-13.7	6.1-*6.88*-7.7	1.51-*1.80*-2.08	n = 32
Ode08	8.7-*11.27*-13.9	6.3-*7.35*-8.4	1.24-*1.54*-1.83	n = 31
Ode10	9.1-*11.74*-14.4	5.5-*6.56*-7.6	1.40-*1.79*-2.19	n = 31
Ode11	7.7-*10.33*-13.0	5.1-*6.15*-7.2	1.26-*1.68*-2.11	n = 31
Ode12	10.8-*12.11*-13.5	6.5-*7.06*-7.7	1.52-*1.72*-1.92	n = 32
Ode16	11.8-*12.62*-13.5	5.2-*6.17*-6.6	1.81-*2.05*-2.46	n = 15 (min-max)
Summary	9.50-*11.78*-14.06	5.83-*6.97*-8.12	1.37-*1.70*-2.01	N = 372

TABLE 6. — Comparisons of the described Odemira collections (*Amanita pseudovalens* var. *tartessiana* var. nov.) with the descriptions made by Neville & Poumarat (2004) of other taxa of series *Amidella* (E.-J.Gilbert) Neville & Poumarat.

	A. curtipes* f. *curtipes	***A. curtipes* f. *pseudovalens***	***A. pseudovalens* var. *tartessiana* var. nov.**	***A. ponderosa* f. *ponderosa***	***A. ponderosa* f. *valens***
Phenology	Autumn (mostly)	Spring	Winter-Spring	Winter-Spring	Winter-Spring
Vegetation	*Pinus*, *Quercus* & others	*Pinus*, *Calluna*	*Cistus*	*Cistus*, *Quercus*	*Cistus*
Soil	Sandy	Sandy humic	Schists	Compact	Schists
Pileus	3-7.5 cm	7-10 cm	3.5-7.5 cm	5-15 cm	4.5-7 cm
Stipe	2-6.7 × 0.5-1.6 cm	6-11 × 1-4 cm	2-6 × 1-2.2 cm	5-13 × 2-4 cm	8 × 2.5 cm
Odour	Indistinct	Indistinct	Indistinct	Earthy	Recalls young *A. ovoidea* ("sea", seafood) but weaker
Taste	Indistinct	Initially insignificant, then of hazelnut, slightly bitter	Aftertaste slightly bitter	Soft	Soft
Basidiospores	12.05 × 6.31 µm, Qm = 1.91	13.26 × 6.03 µm Qm = 2.20	11.78 × 6.97 µm Qm = 1.696	11.39 × 6.75 µm Qm = 1.69	10.86 × 7.20 µm Qm = 1.51
Basidia	52.1 µm	52.6 µm	56.9 µm	57.6 µm	59.28 µm

DISCUSSION

The present study introduces evidence supporting the reclassification of *Amanita curtipes* f. *pseudovalens* to *A. pseudovalens* comb. nov., stat. nov., as well as defining a new variety for the Portuguese collections of the species, *A. pseudovalens* var. *tartessiana* var. nov. The data provide a clarification of the diagnostic criteria among the European species of *Amanita* series *Amidella*, supplemented by the design of a straightforward molecular approach to discriminate the new variety from smaller specimens of *A. ponderosa*. The need for this approach is underlined by what appears to be an evolutionary convergence process.

TAXONOMIC RESOLUTION OF THE EUROPEAN *AMIDELLA* SPECIES

The proposed reclassification to *Amanita pseudovalens* comb. nov., stat. nov. is well supported by the nrDNA sequences, with a net distance of at least 6.2% to the *A. curtipes* clade (Table 3), ruling against the previous notion that it is merely a robust form of *A. curtipes* (Neville & Poumarat 2009: 68). This branch within *Amanita* series *Amidella* contains the *A. pseudovalens* comb. nov., stat. nov. holotype and other herbarium specimens collected in France that conform to its description, as well as an *A. ponderosa* look-alike collected in various locations of southern Portugal, here proposed to belong to *A. pseudovalens* comb. nov., stat. nov. The morphology and ecology of these Portuguese collections is so distinct from the original concept of *A. pseudovalens* comb. nov., stat. nov. (Table 6), that the conspecificity of these two taxa would not be thinkable without the genetic data. Yet, the phylogenetic resemblance suggested by the nrDNA data does not preclude that the new taxon, here ranked as variety, should be considered a new species (Badotti *et al.* 2017; Vu *et al.* 2019), but the latter hypothesis will require a definite genetic support.

The literature of *Amanita* series *Amidella* in Europe is relatively scarce and hampered by diverging interpretations that remain unresolved to this day, notwithstanding the decisive advances made by Neville & Poumarat (2004). It is clear to us that basidiome size (aside perhaps from the extreme rep-

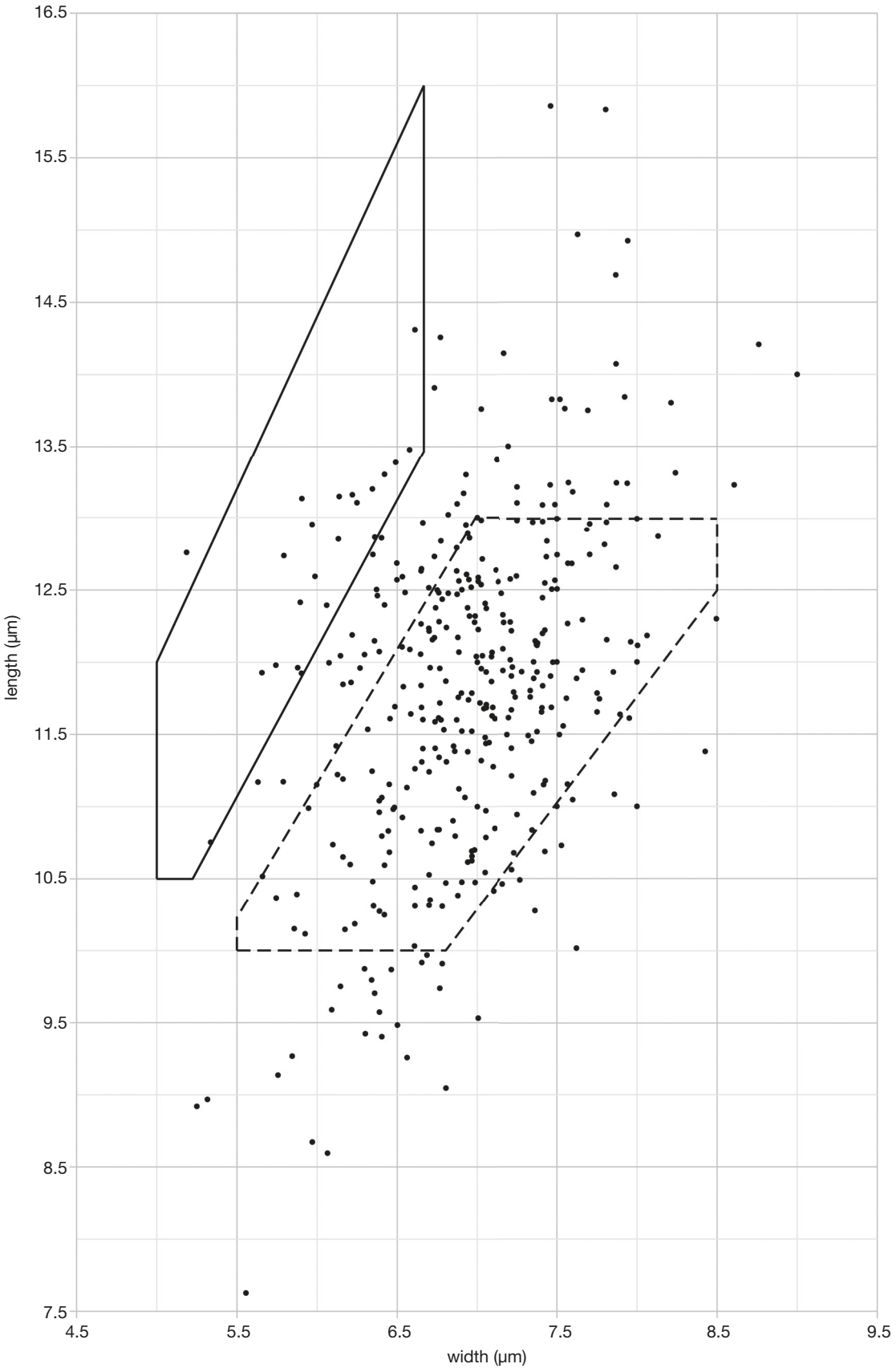

Fig. 4. — Plotting of the measurements summarised in Table 5 (**dots**), comparing with the limits of the sporographs, based on the descriptions in Neville & Poumarat (2004) for *Amanita curtipes* f. *pseudovalens* Neville & Poumarat (**continuous line**) and *A. ponderosa* f. *ponderosa* Malençon & R.Heim (**dashed line**).

IDENTIFICATION KEY TO *AMANITA* PERS. SERIES *AMIDELLA* (E.-J.GILBERT) NEVILLE & POUMARAT*

1. Pileus remaining convex for most of the development of the basidiome, sometimes with an umbo, diameter notably smaller than the length of the developed stipe, covered at least on the margin with heterogeneous scales from the inner layer of the universal veil, which become brown like the exposed context, stipe base bulbous, margin of the volva leaning towards the stipe, context discoloration moderate to intense. Rare occurrences, widely spaced in time (*A. lepiotoides*) 2
— Pileus becoming flat then depressed at the centre, diameter about the same as the stipe length, generally without scales, stipe slightly or not bulbous, margin of the volva free, pale context discoloration, rose then ochre. Occurrence generally annual 3

2. Context discoloration intense, reddish then dark brown, stipe base distinctly bulbous, basidiospores mostly ellipsoid (Qm < 1.65)..... *A. lepiotoides* f. *lepiotoides* Barla
— Context discoloration moderate, rose then ochre, stipe base slightly bulbous, basidiospores mostly oblong and cylindrical (Qm > 1.65) ..... *A. lepiotoides* f. *subcylindrospora* Neville & Poumarat

3. Occurrence in Autumn, on siliceous sandy soil ..... *A. curtipes* E.-J.Gilbert
— Occurrence from late winter (rarely in January) to spring, also early summer 4

4. Heavy habit, semi-hypogeous, typically with an earthy odour, associated with *Cistus*, basidiospores mostly ellipsoid to oblong (1.60 < Qm < 1.80) ..... *A. ponderosa* f. *ponderosa* Malençon & R.Heim
— Medium-sized (pileus diameter generally less than 10 cm)..... 5

5. On siliceous sandy soil, associated or not with *Cistus*, pines, etc., basidia with average length < 55 µm 6
— On heavy, often naked soil generally of schists, mostly associated with *Cistus*, basidia with average length > 55 µm 7

6. Relatively small (pileus diameter usually around 5 to 6 cm and below 8 cm), taste of the context indistinct, basidiospores mostly oblong (1.8 < Qm < 2.05) ..... *A. curtipes* E.-J.Gilbert
— Medium-sized (pileus diameter usually between 7 and 10 cm), taste can be slightly of hazelnut, then bitter, basidiospores mostly cylindrical (2.0 < Qm < 2.4)..... *A. pseudovalens* var. *pseudovalens* comb. nov., stat. nov.

7. Odour and/or taste typically distinct, semi-hypogeous habit (*A. ponderosa*) 8
— Odour indistinct, aftertaste slightly bitter, epigeous habit, pileus margin occasionally with brown scales .....
..... *A. pseudovalens* var. *tartessiana* var. nov.

8. Basidiospores broadly oblong (Qm = 1.60 – 1.80), strictly thermophile.....
..... *A. ponderosa* f. *ponderosa* Malençon & R.Heim
— Basidiospores long-ellipsoid (Qm = 1.45 – 1.55), also in cooler areas.....
..... *A. ponderosa* f. *valens* (Gilbert) Neville & Poumarat

*, only the European taxa within *Amanita* series *Amidella* are considered. Parts of the keys are from the work of Neville & Poumarat (2004).

resented by large specimens of *A. ponderosa*) is not a pivotal diagnostic character for the group. In this work we propose a set of identification keys for the European taxa in this series, where fruiting season, soil type and associated vegetation are critical for determination. Thus, it becomes possible to review previous collections, most notable being those in the extensive study by Castro (1997), compiling collections in Spain and Portugal identified as *A. curtipes* (including *A. ponderosa* as a variety of the former, a concept that subsequently has been rejected by Neville & Poumarat (2004) and rebutted by Moreno *et al.* (2008)). Of the 59 *A. curtipes* collections listed by Castro (1997), at least 24 (in nine Spanish provinces) were collected between the end of February and mid-July. Although *A. curtipes* is not exclusively autumnal (as exemplified by our specimen *M02*, see also the proposed key 6), a verification of the *A. pseudovalens* comb. nov., stat. nov. identity of some of Castro's collections, reckoned by Neville & Poumarat (2004: 660), would now have, given its species status, a high chorological interest. Probing those collections with our diagnostic primers (Table 6; Appendix 1) should provide a prompt answer and, together with the field annotations on the soil and vegetation that may exist for them, along with the individual statistics on basidiospore dimensions, could elucidate those collections and, in the ones confirmed to be *A. pseudovalens* comb. nov., stat. nov., enrich the knowledge on this species. Of note, the "Q = (1.5)-2-2.2" reported by Castro (1997) is suggestive of the Q values described for *A. pseudovalens* var. *pseudovalens* comb. nov., stat. nov. (Table 6, key 6), with the lower range (down to 1.5) possibly indicating the presence of other taxa, including the new variety *A. pseudovalens* var. *tartessiana* var. nov., especially if the soil and vegetation records are concordant. Moreover, the latter is so similar to comparatively small specimens of *A. ponderosa* (Table 6) that only a few nonmolecular characters are available to set them apart (identification key 7), and those characters might be difficult to ascertain in some collections. Thus, molecular probing of the smaller *A. ponderosa* collections in that study

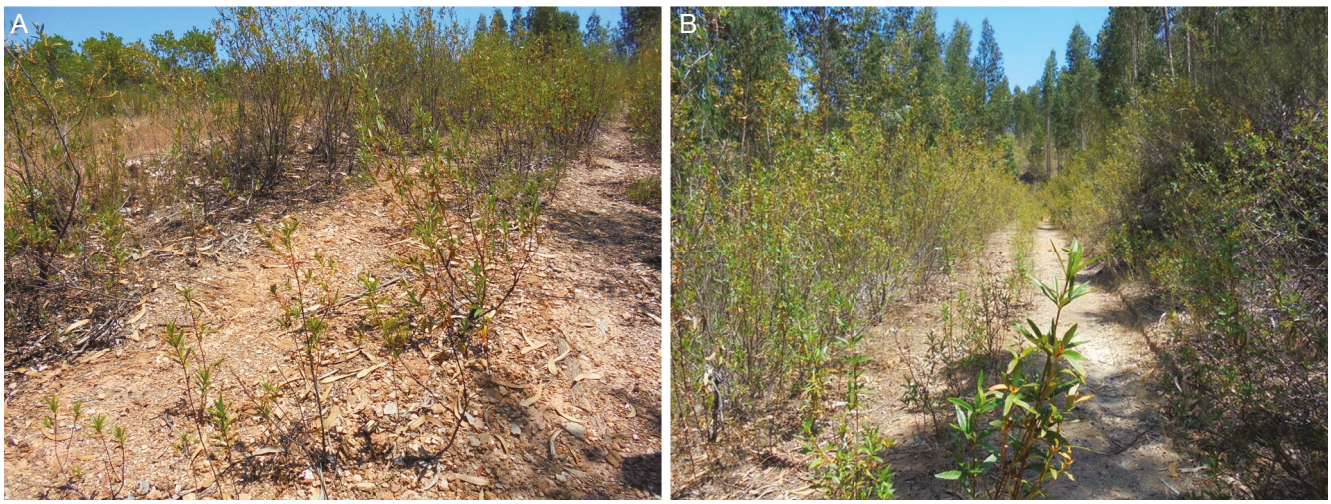

Fig. 5. — Ground view of the Luzianes locations, showing the *Cistus ladanifer* L. dominance: **A**, Luzianes A; **B**, Luzianes B. Both photos were taken in spring 2015 by Ana C. Silva.

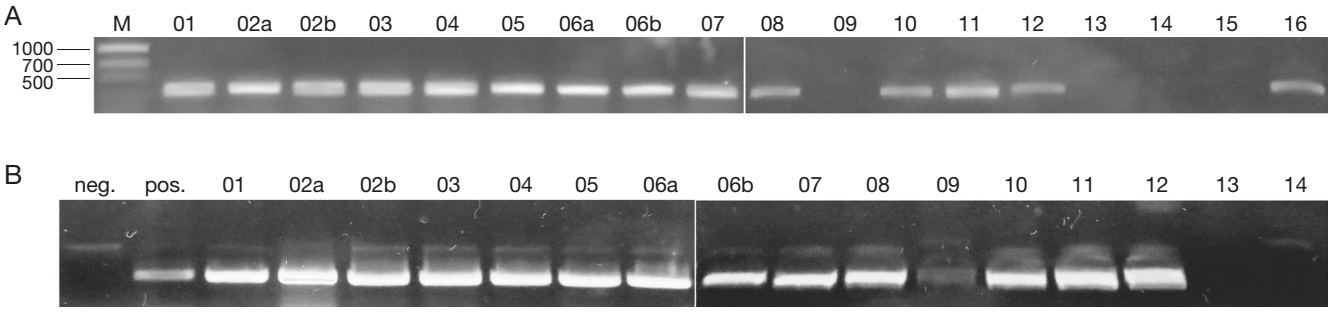

Fig. 6. — Results of PCR amplifications using discriminant probes for *Amanita pseudovalens* comb. nov., stat. nov.: **A**, ITS region: ITS3-*Aps*Ir3 (all samples positive with concurring control with primers ITS3-ITS4, not shown); **B**, LSU region: NLC2R-*Aps*Lr2 (all positive with control NLC2R-LR5); *Ode15* and *Ode16* were negative and positive, respectively, in another amplification (not shown); **M**, molecular marker; **neg.** designates the negative control (water); **pos.** designates the positive control (the *P01* extract).

might even reveal further *A. pseudovalens* var. *tartessiana* var. nov. collections.

Given its spore morphology and the sandy texture of the soil where it was collected, the *Ode16* specimen is not unlike the *Amanita pseudovalens* comb. nov., stat. nov. autonym. It is quite possible that, at least in Spain and Portugal, collections intermediate between the two varieties are found.

Concerning other parts of the *Amidella* phylogeny, a few remarks can be made. First, more collections of *Amanita ponderosa* f. *valens* are needed, and their DNAs studied – for one, *Ode14* may already belong here, given our measurements of the basidiospores: length = 11.8 µm, width = 7.8 µm, Q = 1.53 (averages of 14 spores). Second, *A. lepiotoides* f. *lepiotoides* is not yet represented in the databases, since all *A. lepiotoides* collections represented in Figure 2 are identifiable as the f. *subcylindrospora* (Neville & Poumarat 2004; Arrillaga & Mayoz 2005; Alonso-Díaz & Rigueiro-Rodríguez 2019); in this regard, two immature collections from Varese, Italy, dated 1999 (Neville & Poumarat 2004), may be good candidates for sequencing. Finally, several ITS sequences retrieved from Genbank were found to suggest a few novel clades between *A. curtipes* and *A. lepiotoides* f. *subcylindrospora* (Appendix 9).

Relevance of the molecular markers

In comparison with other European species of series *Amidella*, it is generally considered that marketed *Amanita ponderosa* specimens are large and sturdy enough to leave no doubts on their identification (see the first entry in the proposed key 4). Even so, our specimens from Portel, picked by traditionally trained mushroom collectors, included one *A. pseudovalens* var. *tartessiana* var. nov. (*P01*), and later collections have shown that this co-occurrence with *A. ponderosa* is far from negligible (Arraiano-Castilho 2013; Fátima Pinho-Almeida pers. comm.). In fact, the few characters available so far for discriminating them (key 7) might not be useful to ascertain some collections, either for being difficult to interpret or for being inconstant.

Thus, assessing the ecological, gastronomical, and economic impacts of such mix-ups is a significant question to be tackled. The genetic markers developed in this study seem to be quite stable across the varied materials examined (Appendix 2) and, combined with a simple DNA extraction method such as the one used in the present work (Stirling 2003), they provide a simple, scalable, and effective means toward monitoring the

presence of *Amanita pseudovalens* var. *tartessiana* var. nov. among the *A. ponderosa* collected for human consumption.

Evolution and plasticity of the new variety

Table 6 shows how much the new variety, *Amanita pseudovalens* var. *tartessiana* var. nov., resembles *A. ponderosa* much more than the species autonym, and this intraspecific divergence is found both in the microscopy and in the ecology. A likely evolutionary scenario is that the common ancestor of the *curtipes*/*pseudovalens* clade (Fig. 2) was adapted to sandy soil and producing (sub)cylindrical spores, a combination of characters retained by *A. pseudovalens* var. *pseudovalens* comb. nov., stat. nov. while *A. pseudovalens* var. *tartessiana* var. nov. adapted to schists, with the production of ellipsoid-oblong spores. Other from its lack of large specimens, the *tartessiana* variety has been converging with *A. ponderosa*, and the plasticity of spore morphology observed in this variety (Fig. 3; Table 5 and statistical analyses) may suggest that such process is still underway. More data on the *curtipes*/*pseudovalens* clade, including the Korean occurrence mentioned in Appendix 10, might shed some light on this process.

A genome-wide exploration of the divergence between the two varieties of *Amanita pseudovalens* comb. nov., stat. nov. may be a productive model for identifying genotypic markers of the evolutionary divergence process and understanding functions associated with it.

CONCLUSION

The genetic comparison between the *Amanita* collections from southern Portugal described in this study and the type specimens of three forms established by Neville & Poumarat (2004) firmly suggests their conspecificity with *A. pseudovalens* comb. nov., stat. nov., although as a separate variety of a diverging ecology, here described as *A. pseudovalens* var. *tartessiana* var. nov. These taxonomic novelties helped clarify the diagnosis of European species within series *Amidella*, paving the way for a re-evaluation of vernal collections, henceforth supported by diagnostic molecular probes proposed in this study.

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Mr Manuel Nobre Francisco de Vale Fontes guided the authors to the Luzianes locations A and B. The helpful comments by two anonymous reviewers, as well as those kindly provided by P.-A. Moreau (Université de Lille, France) on a preprint version, are deeply appreciated. The authors dedicate this work to the Iberian mycologists Fátima Pinho-Almeida and Marisa Castro Cerceda.

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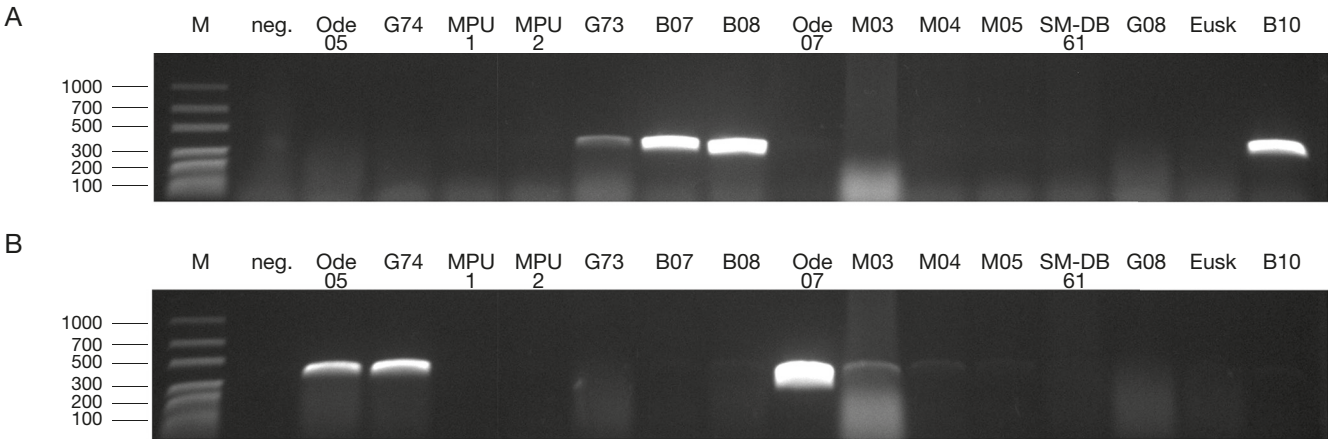
APPENDICES

Appendix 1. — List of taxon-specific primers, their matching and positive control universal primers and annealing temperatures (T_a), used either for sequencing or as molecular markers. * refers to the site number of the 5' terminal on the alignment used for the phylogenetic reconstruction in Figure 2.

Target	Region	ID	Strand	Position*	Sequence	T_a(°C)	Matching Control		Comment
Amanita curtipes	ITS	*Acu*If1	plus	97	5' AGCGGGCATGTGCACG	63.5	ITS2	ITS1	–
		*Acu*If2	plus	533	5' TGCATGAAGAGGAGTTTTTGGAC	54.3	ITS4	ITS3	–
	LSU	*Acu*Lr1	minus	1410	5' GCCAGCCCATTGAATGATCTG	55.0	NLC2R	LR3	–
Amanita lepiotoides	ITS	*Ale*If1	plus	276	5' GTTTTATATGAATGGCTATTG	51.0	ITS4	ITS3	–
		*Ale*Ir1	minus	572	5' GTCCAACAAACATCTCCAAC	56.9	ITS1F	ITS2	–
	LSU	*Ale*Lf1	plus	1195	5' GAAGTCAGTAGAGTTGGCTG	57.5	LR3	NLC2R	–
		*Ale*Lr1	minus	1301	5' CCTTAGCTTTTCTCTAGTG	57.5	NLC2R	LR3	–
Amanita ponderosa	ITS	*Apo*If1	plus	58	5' TTAAAACTCTGGCAAAG	49.4	ITS2	ITS1	–
		*Apo*If2	plus	499	5' GAGTGTCATTCATATTCTC	50.3	ITS4	ITS3	–
	LSU	*Apo*Lr1	minus	1399	5' CAAAGCCCAGCCTAGTAG	56.8	NLC2R	LR3	–
Amanita pseudovalens comb. nov.	ITS	*Aps*Ir1	minus	339	5' TTGTTCATTAACAATTGTCTTTC	54.7	ITS1F	ITS4	*A. curtipes* produces very faint bands; Ode11 is divergent
		*Aps*If1	plus	522	5' AGTCTTCTTCGTGACAAG	53.9	ITS4	ITS3	Variety *pseudovalens* has mismatches
		*Aps*Ir2	minus	691	5' CTTATTTCATGGTGTAAAACTTTATC	55.9	ITS3	ITS4	–
		*Aps*Ir3	minus	731	5' GACACAAATTCATTTAGAAAG	51.5	ITS3	ITS4	*A. curtipes* produces very faint bands
	LSU	*Aps*Lf1	plus	887	5' GCAGCGTCTGGTTGTCC	60.3	NLC2	LR0R	*A. curtipes* also positive (only one mismatch)
		*Aps*Lr1	minus	1301	5' CCTTAGCTTTTTTCTACCGGTCAGAA	62.9	NLC2R	LR3	–
		*Aps*Lr2	minus	1401	5' AATGAATGGCCTGGCGA	59.1	NLC2R	LR3	Some *A.curtipes* specimens display a distinctive set of slower-migrating bands

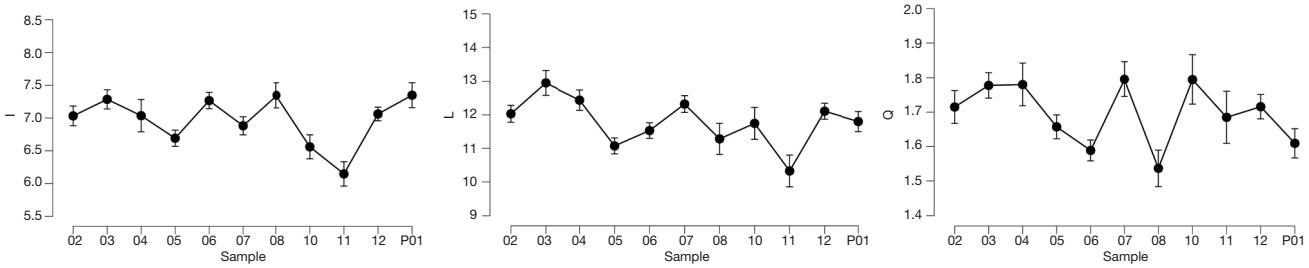
Appendix 2. — Example of a matched comparison of *Amanita ponderosa* Malençon & R.Heim (*Apo*If2-ITS4) and *A. pseudovalens* comb. nov., stat. nov. (ITS3-*Aps*Ir3) for the same set of samples representing these two species, along with others of *A. curtipes* E.-J.Gilbert and *A. lepiotoides* Barla, and an unknown sample (*SM-DB 61*). The whole contents of each 20 µL PCR reactions were loaded, to help detect faint signals. Negative controls (**neg.**) with water instead of DNA. *Amanita ponderosa* is represented by the valens type (*G73*) and three samples from Spring 2010 (*B07*, *P08*, *B10*). *Amanita pseudovalens* is represented by the *pseudovalens* type (*G74*), the two MPU samples, *Ode05* and *Ode07*. The *Amanita curtipes* samples (*M03*, *M04* and *M05*) may produce a weak co-migrating band with the ITS3-*Aps*IR3 primer pair, and the *A. lepiotoides* samples (*G08* and *Eusk*) were negative in both cases. Positive control reactions were not attempted because herbaria materials may be contaminated with other fungi.



Appendix 3. — Usefulness of the taxon-specific primers for the generation of PCR amplicons from herbarium specimens' DNA. **ITS-I** and **ITS-II** represent the first and second nrDNA intron, respectively, and **LSU** represents the proximal region of the 26S nrDNA. [a], https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi (accessed on May 15th 2021).

Taxon	Specimen	ITS-I	ITS-II	LSU	Comment
Amanita lepiotoides f. *subcylindrospora*	*G08*	ITS1F-*Ale*Ir1	*Ale*If1-ITS4B[a]	LR0R-*Ale*Lr1 *Ale*Lf1-LR3[a] *Ale*Lf1-LR5	–
Amanita ponderosa f. *valens*	*G73*	*Apo*If1-ITS2_KYO2	*Apo*If2-ITS4	NLC2R-*Apo*Lr1	–
Amanita pseudovalens comb. nov.	*G74*	SR6R[a]-*Aps*Ir1	*Aps*If1-NLC2	*Aps*Lf1-*Aps*Lr2	–
	MPU1	ITS1F-*Aps*Ir1	*Aps*If1-ITS4	LR0R-*Aps*LR1 NLC2R-*Aps*Lr2	All regions after reamplification
	MPU2	SR6R-*Aps*Ir1	*Aps*If1-ITS4	*Aps*Lf1-*Aps*Lr2	ITS-II region after reamplification

Appendix 4. — Descriptive (mean ± SE) plots for basidiospore length (**L**), width (**I**) and L/I ratio (**Q**). Sample identifiers refer to the *Ode* samples (Spring 2015) and *P01* (Spring 2010).



Appendix 5. — Results of the ANOVA tests for basidiospore length (**L**), width (**I**) and L/I ratio (**Q**). ***, P < 0.001.

Measurement	L	I	Q
ANOVA (uncorrected)	$F_{10361} = 19.583^{***}$	$F_{10361} = 21.031^{***}$	$F_{10361} = 13.684^{***}$
Levene's test (variance homogeneity)	$F_{10361} = 3.846^{***}$	$F_{10361} = 3.786^{***}$	$F_{10361} = 4.314^{***}$
ANOVA (Welch correction)	$F_{10141.108} = 16.760^{***}$	$F_{10141.158} = 19.401^{***}$	$F_{10140.459} = 15.368^{***}$
Maximum Effect size (Cohen's d)	2.207*** (*Ode03-Ode11*)	2.463*** (*Ode06-Ode11*)	1.907*** (*Ode03-Ode08*)

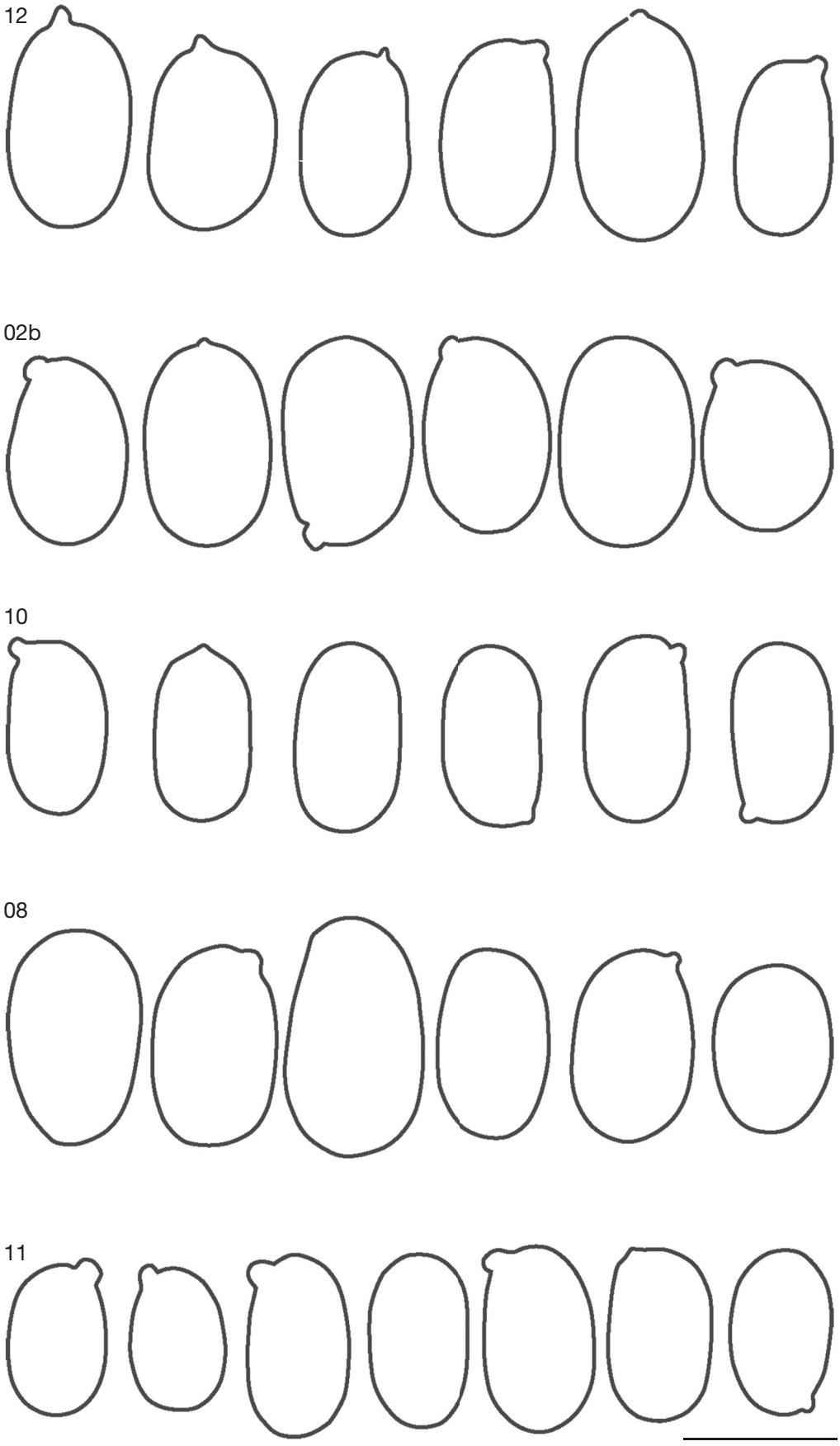
Appendix 6. — Differences between sites for basidiospore length (**L**), width (**I**) and L/I ratio (**Q**). ***, P < 0.001; *, P < 0.05; ns, P ≥ 0.05.

Measurement	L	I	Q
Averages ± SE, µm	12.192 ± 0.085 (A) 11.396 ± 0.086 (B)	7.156 ± 0.040 (A) 6.750 ± 0.043 (B)	1.709 ± 0.012 (A) 1.694 ± 0.013 (B)
Mean difference (A minus B) ± SE	0.796 ± 0.121, $t_{340} = 6.573^{***}$	0.406 ± 0.059, $t_{340} = 6.904^{***}$	0.015 ± 0.018, $t_{340} = 0.839^{ns}$
Effect size (Cohen's d)	0.712	0.747	0.091
Shapiro-Wilk normality test	W = 0.978* (A) W = 0.994ns (B)	W = 0.986ns (A) W = 0.995ns (B)	W = 0.987ns (A) W = 0.971*** (B)
Levene's test (variance homogeneity)	$F_1 = 2.313^{ns}$	$F_1 = 2.894^{ns}$	$F_1 = 1.029^{ns}$
Nested ANOVA, between sites	$F_{1132} = 58.985^{***}$	$F_{1132} = 64.524^{***}$	$F_{1132} = 0.937^{ns}$
Nested ANOVA, within sites	$F_{8332} = 16.527^{***}$	$F_{8332} = 16.028^{***}$	$F_{8332} = 15.068^{***}$

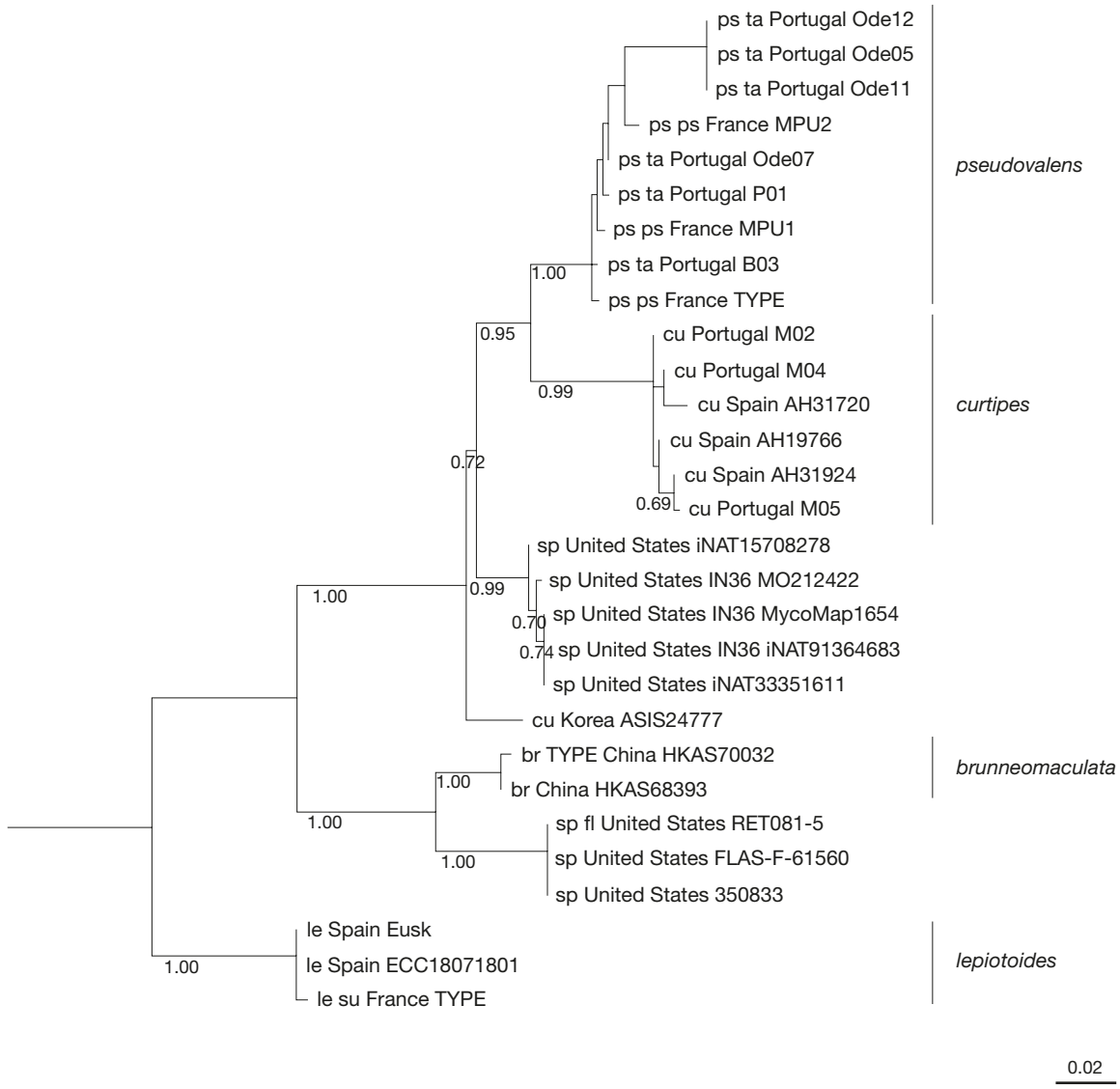
Appendix 7. — Analyses for basidia length.

Test	Result
ANOVA	$F_{7116} = 2.399$, P = 0.025 effect size $\omega^2 = 0.073$
Levene's test (variance homogeneity)	$F_{7116} = 1.267$, P = 0.273
Maximum Effect size (Cohen's d)	1.746, P = 0.009 (Ode11-Ode02b)
ANOVA without *Ode11*	$F_{6107} = 1.327$, P = 0.251 effect size $\omega^2 = 0.017$
Levene's test (variance homogeneity)	$F_{6107} = 1.256$, P = 0.284

Appendix 8. — Examples of basidiospore outlines from the Spring 2015 Luzianes collections. Scale bar: 10 µm.



Appendix 9. — Phylogenetic reconstruction of ITS sequences from *Amanita curtipes* related specimens collected in worldwide locations (Appendix 10), using *A. lepiotoides* Barla as outgroup. The known epithets are next to the brackets, sp designating unknown taxa. Using a 75% coverage cutoff, a total of 584 aligned positions were analysed with the Maximum Likelihood method and the General Time Reversible substitution model (Nei & Kumar 2000), with a discrete Gamma distribution to model evolutionary rate differences among sites (5 categories [gamma parameter 0.4648]). Bootstrap support values above 0.66 (200 replicates) are shown next to the relevant nodes.



Appendix 10. — Additional ITS sequences used for Appendix 9, approximately in the same order. Abbreviations: **MO**, Mushroom Observer (https://mushroomobserver.org); **iNAT**, iNaturalist (https://www.inaturalist.org/observations).

Taxon	Location	Country	Date	Site	Collection/strain	GenBank record	Contributors
A. curtipes	–	Spain	–	–	*AH19766*	AY486235	J. M. Bernedo *et al.*
A. curtipes	–	Spain	–	–	*AH31720*	AY486236	J. M. Bernedo *et al.*
Unknown	Morgan County, IN	United States	Aug. 2015	–	*MO212422*	MZ667952	S. D. Russell
Unknown	Marion County, IN	United States	Sep. 2016	–	*MycoMap1654*	MZ668151	S. D. Russell
Unknown	Porter County, IN	United States	Aug. 2021	–	*iNAT 91364683*	OM809189	S. D. Russell
Unknown	Brooklyn, NY	United States	Aug. 2018	Oaks	*iNAT 15708278*	MT241886	Sigrid Jakob
Unknown	Brooklyn, NY	United States	Sep. 2019	Oaks	*iNAT 33351611*	MN722424	Sigrid Jakob
A. curtipes?	–	South Korea	–	–	*ASIS24777*	KM052544	S. J. Seok *et al.*
A. brunneomaculata	Lijiang, Yunnan	China	Aug. 2010	Pinus (2600 m)	*HKAS68393*	MH508278	Y. Y. Cui *et al.*
A. brunneomaculata	Lijiang, Yunnan	China	Jul. 2011	Pinus (2283 m)	*HKAS70032*	MH508279	Y. Y. Cui *et al.*
Unknown	Leon County, FL	United States	Aug. 1985	–	*RET081-5 'floridella'*	OK316971	R.E. Tulloss *et al.*
Unknown	Putnam County, FL	United States	Sep. 2017	Pines and oaks	*FLAS-F-61560*	MH281877	Kaminsky *et al.*
Unknown	Brooks County, GA	United States	Sep. 2018	–	*MO350833*	ON080993	VSUFungi (Cantonwine *et al.*)