MORCHELLA FLUVIALIS sp. nov. (*ASCOMYCOTA*, *PEZIZALES*): A NEW BUT WIDESPREAD MOREL IN SPAIN

P. CLOWEZ [1], P. ALVARADO [2], M. BECERRA [3], T. BILBAO [4], P.-A. MOREAU [5]

[1] 56 place des Tilleuls, F-60400 Pont-l'Evêque, France.
pharmacie.clowez@wanadoo.fr

[2.] ALVALAB, C/ La Rochela 47, E-39012 Santander, Cantabria, Spain
pablo.alvarado@gmail.com

[3.] Boa. San Miguel n° 1, E-29370 Benaoján, Málaga, Spain

[4.] C/ Mª Díaz de Haro, 20-6º Izq. E-48013 Bilbao, Bizkaia, Spain

[5.] Département des Sciences Végétales et Fongiques, Faculté des Sciences Pharmaceutiques et Biologiques, Univ. Lille 2, BP 83, F-59000 Lille, France
pierre-arthur.moreau@univ-lille2.fr

Summary. CLOWEZ P., P. ALVARADO, M. BECERRA PARRA, T. BILBAO VILLA, P.-A. MOREAU (2014). *Morchella fluvialis* sp. nov. (*Ascomycota*, *Pezizales*): A new but widespread morel in Spain. *Bol. Soc. Micol. Madrid* 38:253-00.
The authors propose to name a new morel species, sister to *M. esculenta* and already identified by previous phylogenetic studies as "*Mes*-18". The number of collections and geographical distribution of *Morchella fluvialis sp. nov.* suggest that this species is widespread along rivers of Spain, associated to riparian forests composed of *Fraxinus angustifolia* or *F. excelsior* and occasionally *Ulmus minor*. This habitat is shared by other morel species of wider European distribution, especially *M. vulgaris* and *M. esculenta* from which it differs by an assemblage of morphological features. Its apparent restriction to riparian habitats in Spain makes it a vulnerable species which requires monitoring.

Key words: riparian, Mediterranean, edible fungi, phylogeny

Resumen. CLOWEZ P., P. ALVARADO, M. BECERRA PARRA, T. BILBAO VILLA, P.-A. MOREAU. *Morchella fluvialis* sp. nov. (*Ascomycota*, *Pezizales*): Una nueva morilla extendida en España. *Bol. Soc. Micol. Madrid* 38: 253-00.
Los autores proponen una nueva especie de colmenilla para acomodar uno de los múltiples linajes de este género identificados por autores anteriores, previamente identificado como "*Mes*-18". El número de colecciones halladas y su distribución geográfica sugieren que esta especie es bastante común en los ríos de España. *Morchella fluvialis* parece asociada con los bosques riparios compuestos por *Fraxinus angustifolia* o *F. excelsior*, así como *Ulmus minor*. Este hábitat es compartido por otras especies como *M. esculenta*, que se asemeja mucho a *M. fluvialis*, pero tiene diferente distribución geográfica y una combinación de caracteres morfológicos distinta.

Palabras clave: ripario, Mediterráneo, hongos comestibles, filogenia

INTRODUCTION

The genus *Morchella* Dill. ex Pers.: Fr. has been extensively studied with molecular tools in the current decade (TAŞKIN & *al*., 2010; O'DONNELL & *al*., 2011; DU & *al*., 2012a, 2012b; TAŞKIN & *al*., 2012). In these works, a great number of lineages within the two major sections of this genus have been identified. Those related to *M. esculenta* (L.) Pers. (sect. *Morchella*, or "yellow morels") have been named Mes-1 to Mes-27, while those related to *M. elata* Fr.: Fr. (sect. *Distantes* Boud., or "black morels") where labeled Mel-1 to Mel-34. However, before Kuo & *al*. (2012) none of these phylogenetic species were linked with Linnean names, neither those created in the first 200 years of existence of this genus, nor the modern additions proposed by CLOWEZ (2010). KUO & *al*. (2012) proposed 11 new names for American lineages, for only 2 attributed to old-time published names (*M. angusticeps* Peck and *M. punctipes* Peck). Some of the present authors (RICHARD & *al*., unp.) recently contributed to a proposal for a unified taxonomy for European and North American morels, based on all available molecular data and a wide European sampling effort, including many Spanish collections. In this study, all but 3 phylogenetic species recognized from Europe in former works were claimed to be attributed valid Linnaean names. However, most others reported from Asia, including Turkey but not reported yet in Europe remain unnamed, pending to be ascribed to existing species or described as new taxa.

Since Turkey is included in the biogeographical area of the Mediterranean basin (BLONDEL & *al*., 2010), as well as North Africa and Southern Europe, it was predictable that some of the phylogenetic species identified by TAŞKIN & *al*. (2012) from Southern Turkey had a pan-Mediterranean distribution. In the present work we deal with one such species, first revealed from a single collection (HT-519) found in Turkey by TAŞKIN & *al*. (2012) labeled as "Mes-18". It was reported to have been found under *Pinus nigra* in a Mediterranean habitat near Feke, at Adana province. Its phylogenetic position was found to be basal in a monophyletic clade together with Mes-8 and Mes-9. Later, DU & *al*. (2012a, 2012b) found also the by now exclusively Chinese lineage Mes-20 is related to this group. DNA analyses revealed seven new Spanish morel collections from different origins could be considered conspecific to Mes-18, and are here accommodated in the new species *Morchella fluvialis*, since all of them were collected in riparian forests. Descriptions, illustrations, phylogenetic position and comparisons with *M. esculenta* and other similar species are provided.

MATERIALS & METHODS

Description

The collections studied here were collected in Spain, with macroscopic images taken in the field, and appropriately dried afterwards. Some collections were sent to P. Clowez and are deposed partly in the herbarium LIP (Faculté de Pharmacie, Lille, France), and partly in the fungal collections of the CEFE (Centre d'Ecologie Fonctionnelle et Evolutive, 1919 Route de Mende, 34293 Montpellier, France) from which DNA extractions have been processed. The other collections were sent to P. Alvarado or collected by himself and are deposited at AH (Universidad de Alcalá, Alcalá de Henares, Madrid, Spain). The macromorphological description proposed below is a compilation of observations from all available pictures of molecularly confirmed collections. Microscopical observations were conducted by P. Clowez on exsiccata, on hand-made sections revived and observed in congo red + 10 % ammonium aqueous solution. Description and measurements of spores were made only on the most mature specimens, on free spores before crushing.

DNA extraction, amplification and sequencing

Total DNA was extracted from dry specimens blending a portion of them with the aid of a micropestle in 600 µL CTAB buffer (CTAB 2%, NaCl 1.4 M, EDTA pH 8.0 20 mM, Tris-

HCl pH 8.0 100 mM). The resulting mixture was incubated for 15 min. at 65ºC. A similar volume of chloroform: isoamilalcohol (24:1) was added and carefully mixed with the samples until their emulsion. It was then centrifugated for 10 min at 13.000 g, and the DNA in the supernatant was precipitated with a volume of isopropanol. After a new centrifugation of 15 min at the same speed, the pellet was washed in cold ethanol 70%, centrifugated again for 2 min and dried. It was finally resuspended in 200 µL de ddH_2O. PCR amplification was performed with the primers ITS1F (5'– CTTGGTCATTTAGA GGAAGTAA– 3') and ITS4 (5'– TCCTCCGCTTATTGATATGC– 3') for ITS (WHITE & *al*., 1990; GARDES & BRUNS, 1993), while EF1-983 F (5' –GCYCCYGGHCAYCGTGAY TTYAT– 3') and EF1-1567R (5' – ACHGTRCCRATACCACCRATCTT– 3') were used to amplify the translation elongation factor 1-α (*tef1*) gene (REHNER & BUCKLEY, 2005). PCR reactions were performed under a program consisting of a hot start at 95 ºC for 5 min, followed by 35 cycles at 94 ºC, 54 ºC and 72 ºC (45, 30 and 45 s respectively) and a final 72 ºC step 10 min. PCR products were checked in 1% agarose gels, and positive reactions were sequenced with primer ITS4. Chromatograms were checked searching for putative reading errors, and these were corrected.

Phylogenetic analyses

Sequences were aligned with the closest matches obtained with BLAST queries through the INSD public databases. Sequences came mainly from DU & *al*. (2012a, 2012b). Sequences first were aligned in MEGA 5.0 (TAMURA & *al*., 2011) software with its Clustal W application and then corrected manually. The final alignment included 459/1139 variable sites. The aligned loci were subjected to MrModeltest 2.3 (NYLANDER, 2004) in PAUP* 4.0b10 (SWOFFORD 2001). Model GTR+Γ+I was selected and implemented in MrBayes 3.1 (RONQUIST & HUELSENBECK 2003), where a Bayesian analysis was performed (two simultaneous runs, six chains, temperature set to 0.2, sampling every 100th generation) until average standard deviation of split frequencies fell below 0.01 after about 450 000 generations. Finally a full search for the best-scoring maximum likelihood tree was performed in RAxML (STAMATAKIS. 2006) using the standard search algorithm (2000 bootstrap replications). Significance threshold was set above 0.95 for posterior probability (PP).

Herbarium code	Personal code	Origin	GenBank ITS	GenBank *tef1*
LIP 0900141	PhC112	Spain, Málaga	KM252952	
LIP 0900155	PhC127	Spain, Sevilla	KM252949	
LIP 0900033	PhC165	Spain, Bizkaia	KM252950	
LIP 0900032	PhC167	Spain, Bizkaia	KM252951	
LIP 0900034	PhC168	Spain, Burgos	KM252948	
LIP 0000234	ALV3114	Spain, Asturias	KM252945	KM252954
LIP 0000232	ALV3172	Spain, Cantabria	KM252946	KM252953
LIP 0000233	ALV3286	Spain, Huesca	KM252947	KM252955

TAXONOMY

Morchella fluvialis Clowez, P. Alvarado, M. Becerra, Bilbao & P.-A. Moreau, *sp. nov*.
MycoBank MB 809796, Figs. 1-2
Etymology: from latin *fluvialis*, pertaining to rivers (root: *fluvius*, stream, river), because of the habitat preference of this species, appearing at river margins.

Diagnosis: A yellow morel close to *Morchella esculenta*, differing from it by its more slender habit with a stipe longer than the pileus (sometimes twice as long as it), and pileus typically conical, often tilted to one side at the top. Sinus absent at the base of the pileus. Primary pits of the pileus rounded or elongated, crests pale staining orange when damaged, slightly longitudinally oriented. Secondary crests absent. Under *Fraxinus excelsior*, *F. angustifolia*. and *Ulmus minor* sometimes with *Alnus glutinosa*, and rarely associated with *Populus nigra* or *Eucalyptus camaldulensis*, in riparian forests of Mediterranean basin countries.
Holotype: SPAIN: Euskadi, Bizkaia, Villaro, river side under *F. angustifolia* with *Alnus glutinosa*, leg. T. Bilbao Villa, IV-2012, PhC165 (LIP 0900033).

Description: Pileus at first conical, generally with acute apex, elongated with age, 5-7 cm high when mature, 3-5 cm wide at the base; only primary crests, mainly longitudinally oriented, some short transversal ones forming polygonal, irregularly shaped pits, isodiametrical or longitudinally elongated; pits pale when young evolving to dark umber-grey, early fading to light ochraceous-brown to pale yellow-orange with age; crests blunt, thick and white when fresh, turning ochraceous to reddish-orange when damaged or drying. Sinus absent. Stipe slender, 5-12 × 1-4 cm when mature, 1-2 times longer than pileus height, and 1-3 times longer than wide, when young regularly enlarged from apex to base, occasionally equal to almost tapering, wrinkled or grooved at base; surface at first pure white, quickly staining ochraceous to yellow-orange, uniformly dirty ochraceous on dried specimens. Spores elliptical, hyaline, measuring (20-)23-24 × (12-)13-14 µm. Asci up to 300-360 × 15-20 µm (at the apex) or × 10-15 µm at the base, base tapering and sinuous, with a small crozier. Paraphyses abundant, cylindrical, branched at base, septate with 1-4 items per branch, filled with refractive oil droplets; terminal element longer than the others, 100-120 × 13-15 µm, irregularly verrucose, moniliform, measuring 100(120) × 13-15 µm, apex rounded, obtuse to somewhat clavate, wall thickened at apex; lower elements regularly cylindrical, 25-50 × 7-10 µm. Crests sterile, with hairs 30-150 µm long forming an unequal palisade, 1-2(-3)-septate, with elements measuring 60-110 × 13-18 µm (1-septate hairs) to 30-80 × 7-15 µm (2-septate hairs), some filled with oily droplets, rarely embedded in an encrusting pigment; terminal element cylindrical to somewhat irregularly inflate to moniliform apex rounded, obtuse to slightly inflated; lower elements shorter, usually cylindrical; basal elements shorter and cylindrical, rarely dimorphic. Stipe cortex structured as an epithelium of sphaerocysts measuring 30-35 × 35-45 µm. From this cortex arises an irregular palisade of hairs, measuring about 40 × 12 µm, some of them non-septate, but most 1-septate with elements 30-75 × 15 µm, or 2-septate (30-65 × 10-15 µm), the terminal element the largest, basal elements always with bulbous to vesicular base.

Comments: The above description takes account of the high chromatic variability of ascomata with age, which is probably affected by growth conditions. When growing in moist situations such as river margins (e.g. ALV3114 or PhC165) ascomata are ochre-yellow even when young, while specimens collected in drier weather (such as PhC112) may remain dark grey-brown for some time. Color in old specimens seems to fade away naturally, although the oxidative yellow-orange staining observed in most collections may turn this originally dark morel with pale crests (resembling *M. americana*, *M. esculenta* or *M. vulgaris*) into a pale morel with yellow-brown crests. Local conditions and associated trees might also influence such variations. An outstanding collection under *Eucalyptus* (PhC127, Fig. 1g) molecularly matching the present *M. fluvialis* concept, exhibited a globose pileus, probably misshaped due to unusual habitat or climatic conditions.

Material studied: SPAIN: Asturias, Coalla, river side under *Fraxinus excelsior*, *leg*. V. López, L. López, P. Alvarado, 05-IV-2014, ALV3114 (LIP 0000234). Burgos, Villasuso, river side under *Fraxinus angustifolia* with *Alnus glutinosa*, *leg*. T. Bilbao Villa, IV-2012, PhC168 (LIP 0900034). Cantabria, San Roque de Riomiera, river side, *leg*. V. Castañera, 19-IV-2013, ALV3172 (LIP 0000232). Euskadi, Bizkaia, Villaro, river side under *F. angustifolia* with *Alnus glutinosa*, *leg*. T. Bilbao Villa, IV-2012, PhC165 (LIP 0900033, HOLOTYPE); *ibídem*, V-2012, PhC167 (LIP 0900032). Huesca, Puente la Reina, 690 m asl, margin of river Aragón-Subordán, under *Populus nigra*, *Fraxinus angustifolia* and *F. excelsior*, *leg*. A. Palazón, A. Hereza, L. Rubio, C. Monedero, J. Pérez, L. Sánchez, J. M. Galardi, 5-IV-2013, ALV3286 (LIP 0000233). Málaga, Arriate, Arroyo de La Ventilla, river side under *Ulmus minor*, *leg*. M. Becerra Parra, PhC 112 (LIP 0900141). Sevilla, La Puebla de los Infantes, Arroyo del Toril, 159 m asl, river side under *Eucalyptus camaldulensis* and *Oenanthe crocata*, *leg*. T.

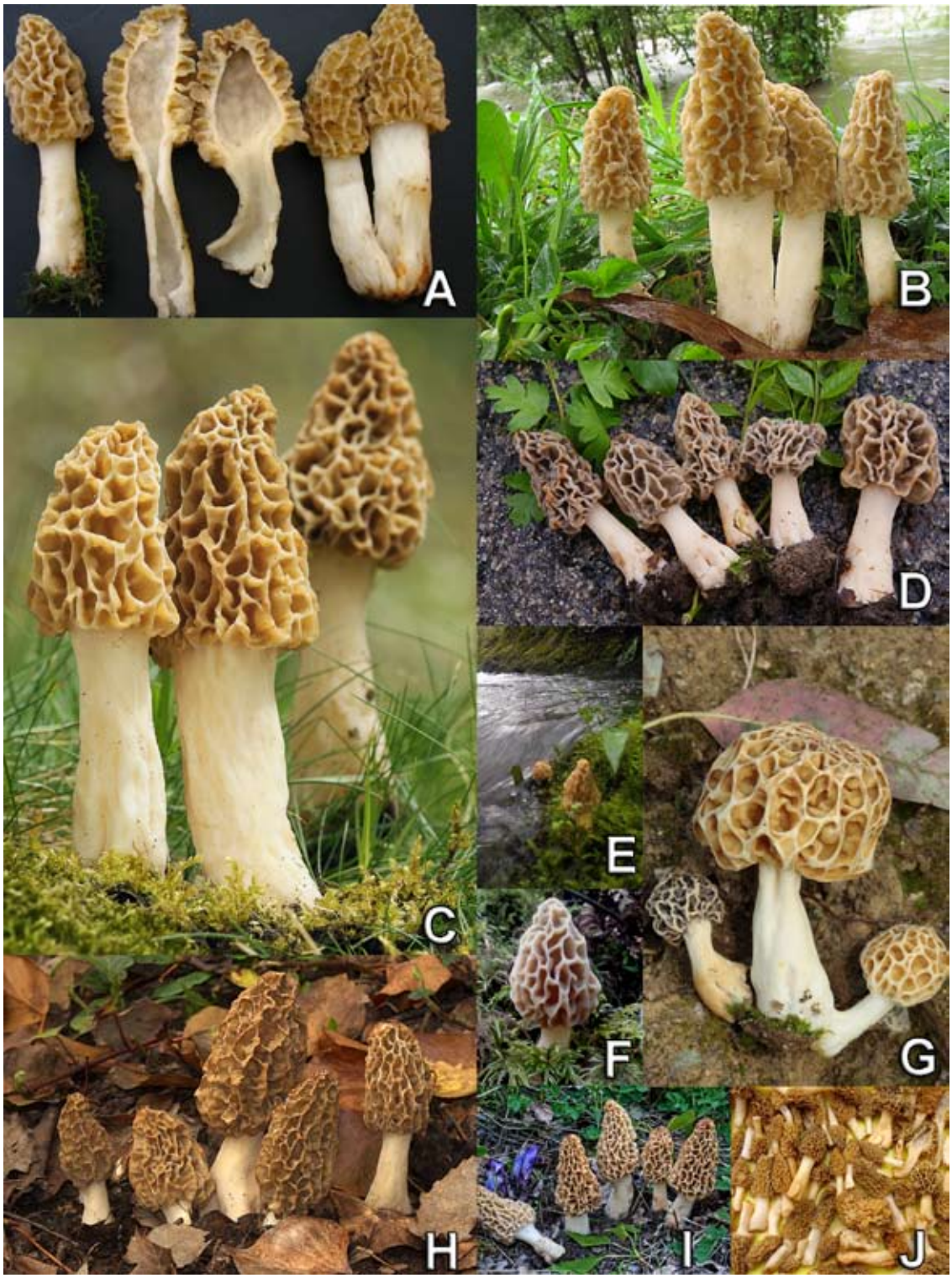

Fig. 1 – *Morchella fluvialis* at different developmental stages. A-C. PhC165 holotype; D. PhC168; E-F. ALV3114; G. PhC127 found under *Eucalyptus camaldulensis*; H. PhC112; I. ALV3286; J. PhC165 holotype.

Illescas, 16-IV-2011, PhC127 (LIP 0900155).

Ecology and distribution: Marked ecological preference for riparian forests with *Fraxinus* spp., *Alnus glutinosa* and *Ulmus minor*, in sandy river sides. The original report of "Mes-18" from Turkey by TAŞKIN & *al*. (2012) under *Pinus nigra*, if conspecific, seems to support the thermophilic distribution of this species, and suggests a wider host range, in the same way as other common Mediterranean species such as *M. tridentina* or *M. rufobrunnea* (LOIZIDES & *al*., in prep.). This is also confirmed by the collection of *M. fluvialis* under *Eucalyptus camaldulensis*, a likely substitutive host, in southern Spain.

RESULTS and DISCUSSION

The ITS phylogeny of *Morchella* sect *Morchella* (Fig. 3) agrees with the topologies already proposed by TAŞKIN & *al*. (2012) and RICHARD & *al*. (unp.). A monophyletic lineage encompassing Mes-8 (*M. esculenta*), Mes-9, Mes-18 and Mes-20 (the last three up to now known only from Asia) can be identified. The nine collections studied here can be considered as indistinct from the only known ITS sequence of lineage Mes-18 (JQ723096), with insignificant differences between all sequences. These differences arise from ambiguous base readings (maybe heteromorphic sites), and a few insertions in a poly-A region at the end of ITS1. It is worth mentioning that this poly-A region caused a persistent polymerase slippage problem when sequenced with ITS4 primer, this being a probable explanation why only 5.8S-ITS2 regions where obtained from the Turkish Mes-18 sample. ITS molecular differences between *M. fluvialis* and Mes-8, Mes-9 and Mes-20 are very subtle in ITS2 region (only 2 bp), but remarkable in ITS1 region (about 20 bp). The identity of the new collections was further confirmed by sequencing their *tef1* gene. BLAST results evidenced a 100% match (560/560) with Mes-18, and only 99% (555/560) with Mes-8.

Since most of our findings were collected beside small streams, sometimes even at water level (Figs. 1b, 1e), we propose the name *Morchella fluvialis* for this apparently new species. Nevertheless it is not excluded that this lineage could be found in other habitats, as is suggested by the original finding by TAŞKIN & *al*. (2012) under *Pinus nigra* near Feke (Adana, Turkey) where no river is present (TAŞKIN pers. comm.). Another possibility is that the Turkish Mes-18 is specifically distinct from the Spanish *M. fluvialis*, in spite of the high similarity of their ITS and *tef1* markers. This possibility could be further explored by comparing new collections from Turkey and other Mediterranean regions, and conducting multigenic analyses on Spanish material, including additional gene markers such as *rpb1* and *rpb2* (O'DONNELL & *al*., 2011). However, even if multilocus analyses are necessary to solve species delimitations in sect. *Distantes*, ITS alone is usually enough for the delimitation of phylogenetic species in sect. *Morchella* (DU & *al*., 2012a; RICHARD & *al*., unp.). For this reason and in expectation of more precisions from Eastern collections, we are inclined to consider *M. fluvialis* as conspecific to *Mes*-18.

The most distinctive features of *Morchella fluvialis* in sect. *Esculenta* are undoubtedly the elongate-conical shape and elongate pits, with the generally longitudinal orientation of the crests. These features are sufficient for discriminating *M. fluvialis* from *M. esculenta* (as redefined by RICHARD & *al*., unp.), which forms isodiametrical pits and non-oriented crests, and usually a globose or ovoid pileus. Another common species in riparian forests, *Morchella vulgaris*, develops a conical pileus but pits are irregular, often tuberculate and with frequent secondary crests, and the stipe is usually thick with a fleshy, bulbous base. Ancient European literature mentions some other taxa with features comparable to those of *M. fluvialis*. In FRIES' (1822: 7) systematics, color and shape of the pileus are ignored in favor of the shape of pits, which can be either isodiametrical (*M. esculenta*), elongate (*M. deliciosa*) or vertical with transversal septa (*M. elata*). Fries introduced some transition forms between the first two as varieties of *M. esculenta*, such as his *M. esculenta* var. *fulva* Fr.: Fr., which is reported to be frequent in Southern and Eastern

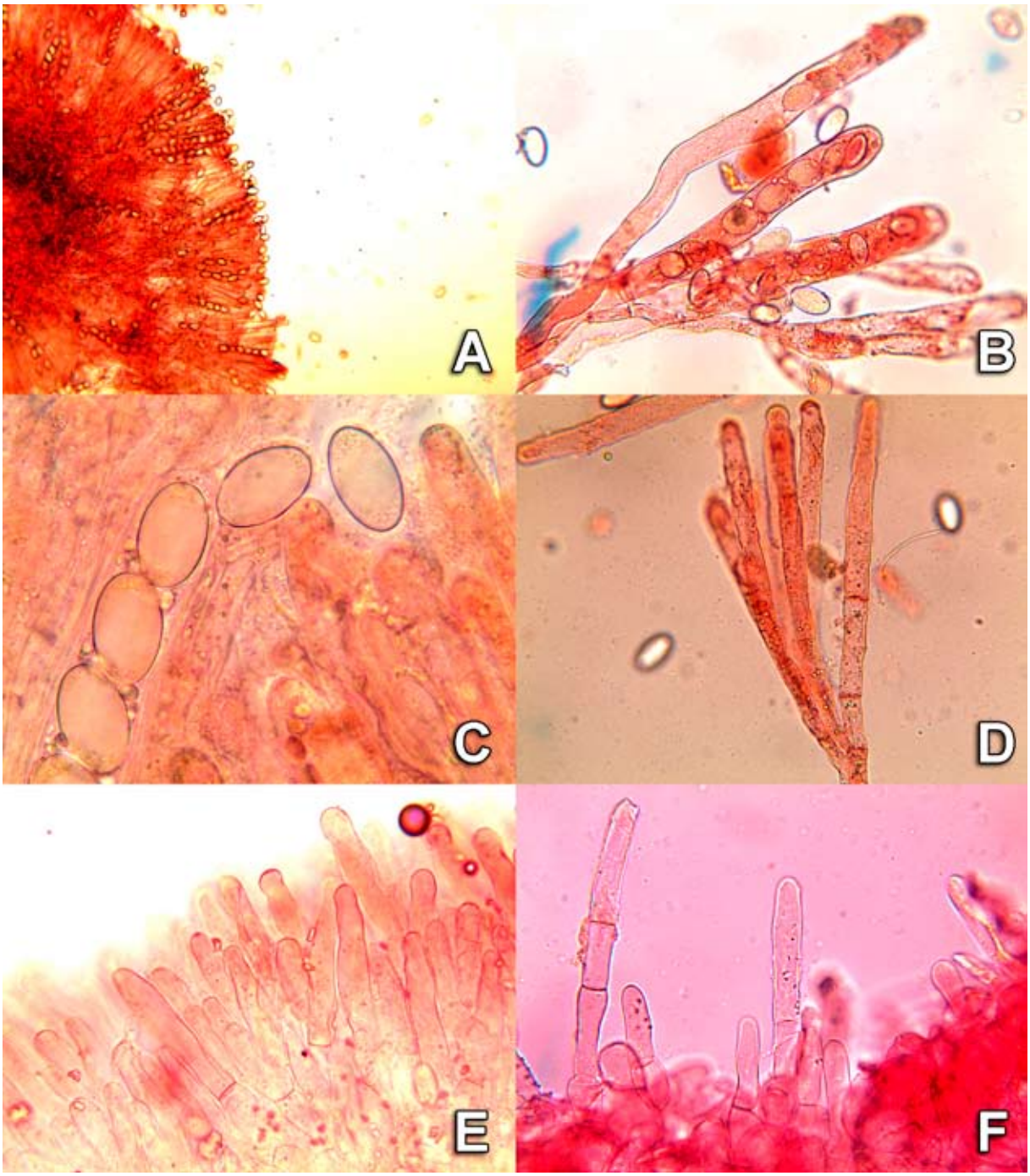

Fig. 2 – Microscopic studies of *Morchella fluvialis* in Congo red- ammonium. A. Hymenium 100x. B. Asci and paraphyses 400x. C. Spores 1000x. D. Paraphyses 400x. E. Hairs of the crests of primary pits 400x. F. Hairs of stipe 400x. (P. Clowez).

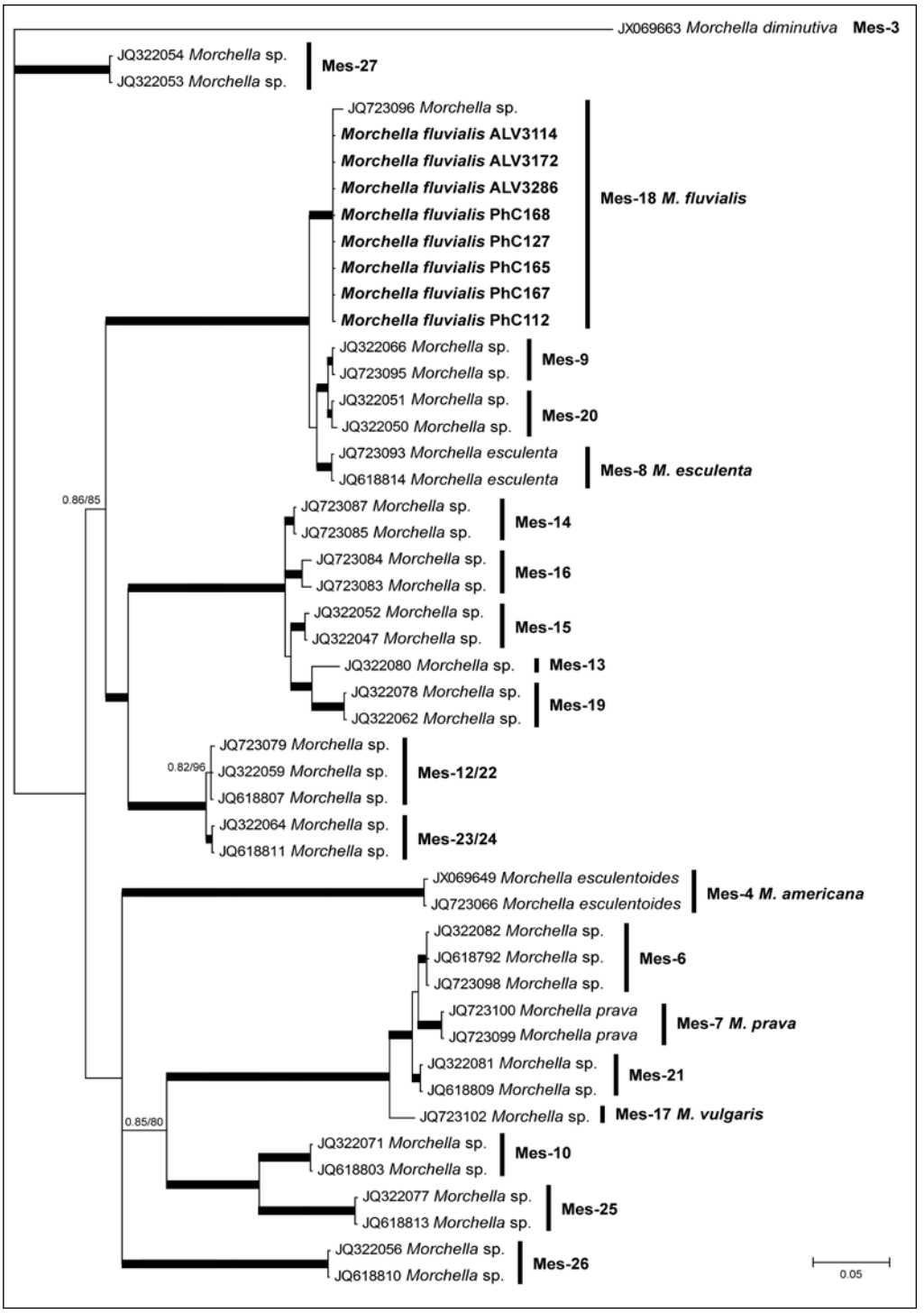

Fig. 3 – Consensus phylogram obtained in MrBayes 3.1 after the alignment and analysis of regions ITS1, 5.8S and ITS2. Bold bars represent nodes significantly supported by both maximum likelihood and Bayesian inference. Values next to nodes represent nearly significant posterior probabilities (PP) from Bayesian inference and bootstrap proportions (BP) from maximum likelihood. 'Mes' codes are indicated for each clade. Agreed species names are provided.

Europe, and presents a "*fulvus, fulvo-fuscus*" pileus with rhomboid pits. The iconographic references cited by Fries are enigmatic: the first (CLUSIUS, 1601: cclxiii, from Pannonia) is a rough illustration of a roundish and short-stiped morel (likely the first morel illustration published in literature), the second (MICHELI, 1729: pl. 85 fig. 3) is also cited by Fries (op. cit.) as the only iconographic reference for his *Morchella elata* Fr.: Fr., and the third (BATTARRA, 1759: pl. II fig. F) has a subglobose pileus with ellipsoidal pits. Unfortunately, these references do not give a clear idea of Fries' taxon. BOUDIER's (1909: 100, pl. 196) plate under "*M. rotunda* var. *fulva* Krombh." is much more informative and reminds *M. fluvialis*; moreover the specimens illustrated originated from J.-B. Barla from the Nice (France) area, a Mediterranean region with many riparian forests. Since no combination at the species rank has been proposed for *M. esculenta* var. *fulva* and because of the inconsistency of the protologue, we do not apply Fries' name for our species; moreover the epithet "*fulva*" does not depict accurately the color range of *M. fluvialis*.

KROMBHOLZ's (1834, pl.17 fig. 3-4) illustration of *M. esculenta* var. *fulva* is less convincing than Boudier's, as it depicts a fungus with globose pileus and rather regular pits. A more similar fungus to *M. fluvialis* is illustrated under the name *M. conica* var. *rigida* Krombh. (pl. 17 fig. 1-2), raised to species rank by Boudier. *Morchella rigida* (Krombh.) Boud. is a critical species: all specimens named as such by CLOWEZ (2012) revealed to be conspecific with *Morchella americana* Clowez & C. Matherly (RICHARD & *al*., unp.) on the basis of molecular analyses. *Morchella americana* (including *M. esculenta* ss. KELLNER & *al*., 2005) is a widespread species throughout the world, apparently common in Central Europe (RICHARD & *al*., unp.) but not yet documented from Mediterranean areas, and at present it is unlikely that *M. fluvialis* is present in Central Europe and could be observed by Krombholz. However, macroscopically *M. americana* is the most similar European morel to *M. fluvialis*, although they belong to distinct clades. A thorough comparative study between both species is required; *M. americana* differs at least by the absence or reddish staining when bruised (KUO & *al*., 2012).

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