Morchella nipponensis sp. nov. (Ascomycota, Pezizales): a paleoendemic species of section Morchella discovered in Japan

Philippe Clowez\textsuperscript{a}, Takumi Izumi\textsuperscript{b}, Paul-Bill Lamiable\textsuperscript{c}, Koichi Shibakusa\textsuperscript{d}, Camelia Minculeasa\textsuperscript{e}, Pablo Alvarado\textsuperscript{f}

\textsuperscript{a} 56 place des Tilleuls, F-60400 Pont-l’Evêque, France
\textsuperscript{b} 321-0965, Miya Cycle Station, 632-17, Kawamuko-cho, Utsunomiya, Tochigi, Japan
\textsuperscript{c} 5-9-8-404, Minato-gawa-cho, Hyogo-ku, Kobe-city, 652-0041, Japan
\textsuperscript{d} 180-0011, 4-28-24, Yahata-cho, Muashino-City, Tokyo, Japan
\textsuperscript{e} ALVALAB, Dr. Fernando Bongera st., Severo Ochoa bldg. 51.04 33006 Oviedo, Spain

ABSTRACT
A previously unknown morel species apparently endemic to Japan is here described. \textit{Morchella nipponensis} is proposed for this species. This new taxon displays archaic features recalling section \textit{Rufobrunnea} (pileus lanceolate, few primary alveoli), and a yellow pileus similar to subsection \textit{Sceptriformis} of section \textit{Morchella}. The phylogenetic analysis of ITS rDNA, as well as \textit{RPB1}, \textit{RPB2} and \textit{TEF1} genes from up to four collections suggests that this morel species represents a basal branch of section \textit{Morchella} (yellow morels), for which the new subsection \textit{Japonicae} is proposed.

Keywords: Morchellaceae, morel, phylogeny

Article history: Received 6 June 2022, Revised 26 August 2022, Accepted 29 August 2022, Available online 21 October 2022.

1. Introduction
The systematics of genus \textit{Morchella} Dill. ex Pers. have been much clarified with the aid of molecular biology (Masaphy, Zabari, & Goldberg, 2009; O’Donnell et al., 2011; Du, Zhao, O’Donnell, Rooney, & Yang, 2012a; Kuo et al., 2012; Tağkin, Büyükalaca, Han- sen, & O’Donnell, 2012; Richard et al., 2015; Du, Wu, He, & Li, 2019; Clowez & Moreau, 2020). Before the DNA sequencing era, morphological and ecological features were the main basis to identify taxa, often leading to the introduction of many names for species of morels that are difficult to discriminate from each other (Boudier, 1897, 1907, 1909; Lagarde, 1923; Jacquetant, 1984; Clowez, 2010). This traditional approach did not provide satisfactory solutions to problems such as the great variability of colors and shapes of some morel species over time. Conversely, some authors considered that most of these names were actually synonyms, accepting only five or less independent species (Dennis, 1978; Breit enbach & Kränzlin, 1981). The incorrect visualization of spore ornamentations contributed to complicate the determination of the different species of morels until the development of new staining procedures and the widespread use of the scanning electron microscopy (Clowez & Moreau, 2018a, 2018b). Currently, the genus \textit{Morchella} has about 80 verified species, but the actual number could be closer to 100, considering the most recent adds (Machuta et al., 2021), as well as unpublished species in herbaria.

O’Donnell et al. (2011) proposed a major taxonomic revision supported by a multigene-based phylogeny consisting in three major clades: “archaic” morels (section \textit{Rufobrunnea}, also called ’Rufobrunnea clade’), “yellow” morels (section \textit{Morchella}, also called ’Esculenta clade’) and “black” morels (section \textit{Distantes}, also called ’Elata clade’). These authors suggested also that certain morels are only present at the sub-continental scale, a phenomenon referred by them as “continentalism” and “provincialism”. Tağkin et al. (2012) found also that some species of section \textit{Distantes} are confined to certain regions of Turkey, while Clowez, Alvarado, Becerra, Bilbao, & Moreau (2014) and Clowez, Bellanger, De la Osa, and Moreau (2015) found a similar pattern among species of section \textit{Morchella} in the Iberian Peninsula. Loizides et al. (2021) discovered also possibly endemic morels in Cyprus, a Mediterranean island where endemism could have been driven by geographic isolation (Kier et al., 2009).

In the present work, another apparently endemic species of \textit{Morchella} is found in Japan. The new taxon is described, providing morphological and molecular data, and accommodated in a new subsection on the basis of phylogenetic results.
2. Materials and methods

2.1. Morpho-anatomical studies

The macroscopic description of *M. nipponensis* was based on specimens and photographs provided by T. Izumi. Microscopic characters were studied in dried specimens. The naturally expelled spores were measured in an aqueous solution of chlorazol black aqueous SDS, and the visualization of the spore ornamentations was done with the help of PhC2 staining. The asci, paraphyses, acroparaphyses and pyramids (clusters of spherocysts in the stipe, defined in Clowez, 2016) were preferably re-inflated in water or in a 5% KOH solution (the latter re-inflates and sometimes deforms the paraphyses too quickly), and later stained in chlorazol black. Measurements are reported in the following format: (lowest value–)average minus standard deviation–(highest value).

2.2. Phylogenetic analysis

2.2.1. DNA extraction, amplification and sequencing

Total DNA was extracted from dry specimens employing a modified protocol based on Murray and Thompson (1980). PCR reactions (Mullis & Faloona, 1987) included 35 cycles with an annealing temperature of 54 °C. The primers ITS1F and ITS4 (White, Bruns, Lee, & Taylor, 1990; Gardes & Bruns, 1993) were employed to amplify the ITS rDNA region, LR0R and LR5 (Vilgalys & Hester, 1990; Cubeta, Echandi, Abernethy, & Vilgalys, 1991) were used for the 28S rDNA region, EFI-728F, EFI-1983F, EFI-1567R and EFI-2218R (Carbone & Kohn, 1999; Rehner & Buckley, 2005) for the translation elongation factor 1a (*TEF1*) gene, RPB1-9F and RPB2-3AR for the RNA polymerase II second largest subunit (*RPB2*) gene (Takan, Büyükalaca, Doğan, Rehner, & O’Donnell, 2010), as well as RPB1-Af (Stillier & Hall, 1997) and RPB1-Cr (Matheny, Liu, Ammirati, & Hall, 2000) for RNA polymerase II largest subunit (*RPB2*) gene. PCR products were checked in 1% agarose gels, and ampliﬁcons were sequenced with one or both PCR primers at Stab Vida (Portugal). Sequences were corrected to remove reading errors in chromatograms.

2.2.2. Phylogenetic analyses

BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) was used to select the most closely related sequences from the International Nucleotide Sequence Database Collaboration public database (INSDC; Cochrane, Kamits-Mizrahi, & Nakamura, 2011). The sequences retrieved (Supplementary Table S1) were mainly from studies conducted by O’Donnell et al. (2011), Du et al. (2012a, 2012b, 2019), Takan et al. (2010, 2012, 2016), Clowez et al. (2014); Loizides et al. (2015), Richard et al. (2015), and Loizides, Bellanger, Clowez, Richard, and Moreau (2016), among others. Sequences first were aligned in MEGA 5.0 (Tamura et al., 2011) with its Clustal W application and then realigned manually as needed to establish positional homology. The final alignment included 258/586 variable/totals sites in ITS (199/452 after GBlocks; Castresana, 2000), 84/584 in LSU, 400/1416 in *TEF1*, 234/890 in *RPB2*, and 259/799 in *RPB1*. The aligned loci were loaded in MrBayes 3.2.6 (Ronquist et al., 2012), where a Bayesian analysis was performed (five partitions: ITS, LSU, *TEF1*, *RPB1*, *RPB2*; two simultaneous runs, four chains, temperature set to 0.2, sampling every 100th generation) until the average split frequencies between the simultaneous runs fell below 0.01 after 2.3 M generations. Finally, a full search for the best-scoring maximum likelihood tree was performed in RAxML 8.2.12 (Stamatakis, 2014) using the standard search algorithm (same partitions, GTRGAMMAI model, 2000 bootstrap replications). The significance threshold was set above 0.95 for posterior probability (PP) and 70% bootstrap proportions (BP).

3. Results

3.1. Phylogenetic results

The present phylogeny based on ITS, 28S rDNA, *RPB1*, *RPB2* and *TEF1* suggests that the samples analyzed represent a basal branch of section *Morchella* that diverged before all others (excepting *M. steppicola*) with a significant statistical support (Fig. 1). It is here hypothesized that this clade is formed by samples reproducibly isolated from those of other clades, therefore deserving to be considered a distinct species. A new name, *Morchella nipponensis*, is proposed below for this species, which is accommodated in a new subsection due to the basal position of this clade within section *Morchella*, and the distinct morphology of the new species.

3.2. Taxonomy

*Morchella subsection Japonicae* Clowez & P. Alvarado, subsect. nov.


Diagnosis: section *Morchella*, medium to large ascomata. Pileus ochraceous yellow to orange brown, lancelolate or rarely ovoid, few flat-bottomed pits, fine ridges. Stipe cylindrical and punctuate, turning brown with age. Associated with different trees species in Japan.

Type: *Morchella nipponensis*, Mycobank: MB 842570

Etymology: *Japonica*, from Japan, the country where the type species, *M. nipponensis*, was found.

Notes: The acharic features of *M. nipponensis* (large size, few cells) resemble those of species in section *Rafobrunnea* (*M. anatolica* and *M. rafobrunnea*), while the yellow color of its pileus recall subsection *Sectiformis* of section *Morchella* (*M. sectiformis, M. diminutiva*). These mixed features are here considered enough to propose a new subsection for this lineage.


Diagnosis: Morel with medium-sized to large yellow ascomata, morphologically close to the smaller *M. sectiformis* and *M. diminutiva*. The pileus is lancelolate, rarely ovoid, dark yellow-brown at first but quickly turning yellow, yellow-ochraceous or yellow-orange, sometimes even orange-brown when very mature. Few large primary pits, 9 to 12 (15) by side, triangular or sometimes trapezoidal, shallow and flat. Ridges thin, concolorous, locally orange-brown when mature. Pileus annexed to the stipe or presenting a shallow sinus. Stipe cylindrical, turning orange-red at the base if bruised or with age, presenting in this case many thin small brown granules. Found under *T. standishii* and also sometimes under bamboos or broadleaved trees in Japan.

Type: JAPAN, Tochigi Prefecture, near Oshiraji Falls, 1025 m a.s.l., under *Thuja standishii*, 26 May 2021, leg. T. Izumi, Phc 353 (holotype, LIP 0902163).

Gene sequences ex-holotype: OM230111 (ITS rDNA), OM230104 (28S rDNA), OM179895 (*TEF1*), OM179887 (*RPB1*), OM179891 (*RPB2*).
Fig. 1 – A 50% majority rule ITS (DNA-28S) DNA-TEF1-RPB2-RPB1 consensus phylogram of Morchella sections Morchella and Distantes (with section Rufobrunnea as outgroup) obtained using MrBayes from 17250 sampled trees. Nodes were annotated if they were supported by $\geq 0.95$ Bayesian posterior probability (left) or $\geq 70\%$ maximum likelihood bootstrap proportions (right). Sequences newly generated in this study are in bold.

doi: 10.47371/mycosci.2022.08.005
Fig. 2 – A. Ascomata of *M. nipponensis* (drawn by Xavier Carteret).
Fig. 3 – *Morchella nipponensis* (A, B: PhC 363; C, E, F: holotype PhC 353). A–C. Ascomata. D. Habitat of *M. nipponensis* under *T. standishii* (by Takumi Izumi). E. Stipe with brownish granules (by Philippe Clowez). F. Thin pit ridge. G. Primary pit with almost flat bottom and diffuse brownish grooves. Bars: A 5 cm; C 5 cm; E–G 1 cm.

doi: 10.47371/mycoscl.2022.08.005
Etymology: *nippoinensis*, from Nippon (Japan), the country where this species was first found.

Medium to large or very large ascomata (Figs. 2, 3A–C), 15–23–30 cm high × 7–10 cm diam. Pileus measuring 5–15 × 3–7 cm (height × width), mostly elongate, lanceolate or sometimes ovoid, tapering at the top; ocher-brown to ocher-beige in its youth, later ocher-yellow to orange-ocher or even turning brown when mature; made up of 9–12 (sometimes up to 14–15) primary alveoli per side, mostly longitudinal, trapezoidal or triangular, angled upwards, not closed at the base, practically without anastomoses, shallow with an almost flat bottom (Fig. 3G), slightly bumpy (resembling pleated paper), marbled with diffuse brownish grooves, cracked in the excisata; alveolar margins are thin, straight but sometimes slightly wavy, colorless, turning slightly russet with age or when injured; secondary alveoli absent or rarely aborted. Small sinuses, which helps in delimiting the pileus and the stipe. Stipe measuring 7–10(–15) × 1–3(–4) cm (height × diameter), regular and sometimes slightly thickened in the lower half, although the base can be sharply narrowed, sometimes slightly wrinkled, pruinose-white to uniformly dirty-white, rusting with age (starting from the base) or due to injuries, then pruinose-red); uniformly covered by brownish granules with age, often very evident on the whole stem (Fig. 3E). In some older specimens the brown stem greatly contrasts with the orange-yellow pileus. Odor not remarkable, taste not known.

Spores light golden-yellow in color; elongate to ellipsoid (type C (B) a2,4, (6) in Clowez & Moreau, 2018a), with negative ornamen-
tation often presenting thick bulges and moderately to fairly shallow grooves with a barbed wire-like appearance, rarely thinner and more or less regular with anastomoses, or coarser and irregular with numerous anastomoses (not furrowed and looking embossed, resembling cerebral convolutions) (Fig. 4E–I), or even more rarely smooth; measuring (18–)20–23.5(–24) × (10.5–)11.5–12.5(–13) μm, Q = 1.75, frequently 21–22 ×11.5–12 μm; with refractive granula-
tions at the poles of spores (spumositides, Fig. 4A, C). Asci cylindrical and regular at the tip, then thinned, often with a slight swelling at the base, medium-sized, 250–280 μm × 15/ 5–10/ 12–15 μm (tip/ middle/base) (Fig. 4A, C). Paraphyses fasciculate, numerous, sometimes also a single long section, 170–300 × 5–15 μm, with an elevation caused by a base of partitioned hypae with complex entanglements, difficult to measure, and with a size often equal to that of the asci; cylindrical in shape, slightly widened at the top, bifurcated and sometimes bident or trident, partitioned with a common base (20–60 × 6–10 μm), 1 partition (example of a bifurcated paraphyses: two articles from top to bottom 135 × 10 μm and 35 × 7 μm; 3-septate: top element 60–70/18–22/11–12 μm, second element 35–50/16–18/14–16 μm, third element 30–35/15–17/13–17 μm, and basal element 35–37/14–18/12–14 μm (Fig. 4B, K).

Habitat and distribution: *Morchella nipponensis* is until now only known in Japan. Mr. Izumi Takumi has been harvesting it for several decades in montane areas under *T. standishii* (Gordon) Carr. (ネズコ in Japanese, Fig. 3D), a species 20–35 m high, native to the mountains in southern Japan, whose age can exceed a thousand years. Further information about the ecological associations of this morel species occurring in the montane range (around 500 to 1000 m a.s.l.) are necessary, but it seems that *M. nipponensis* can be associated also with other trees (i.e. Alnus firma and several spe-
cies of bamboos).

4.1. Macroscopic comparisons with other known morels

*Morchella nipponensis* seems morphologically different from all other known morels. The large size of some ascomata (up to 30 cm) is notable, its often conical pileus resembles that of morel species in section *Distantes* (“black” morels). The small sinuses is also similar to that of certain “black” morels, such as *M. tridentina*, and the stipe is uniformly covered by brownish granules with age, much like those observed on *M. punctipes* (section *Distantes*). On the other hand, its ocher-yellow color recalls section *Morchella* (*“yellow”* morels). Two other smaller morel species in section *Morchella* subsection *Sceptriformis* have also a lancelolate pileus and few primary alveoli: *M. sceptriformis*, known from southeastern North America, and *M. diminutiva*, found in the northwest and west of the same continent. On the contrary, spore Q ratio of *M. nipponensis* is quite high for a yellow morel, the closest being *M. galilaea* with 1.72. Finally, the few primary alveoli of *M. nipponensis* point towards the archaic morel species in section *Ruf brunnea*, and the reddening stipe recalls also that of *M. ruf brunnea* (Guzmán, Torres, & Logemann, 1985; Guzmán & Tapia, 1998). To a lesser extent, *M. castanea* also has large alveoli with a more or less flat base, but it has an ovoid pileus, and it is endemic from Spain. *Morchella anatolica*, which is another small archaic morel species from the Mediterranean basin, shows a buff or pale grey pileus with faint lilac reflections when mature, resembling that of *M. nipponensis*. It would be interesting to compare it with morel species from tropical forests, such as *M. anteridiformis* R. Heim, found in New Caledonia, or also *M. rigidoides* R. Heim from New Guinea, both described by Heim (1966a, 1966b). Although having a somewhat similar general habit, a mid-mountain ecology and a punctuated stipe, these morel spe-

doi: 10.47731/mycosci.2022.08.005
cies are small in size. In addition, *M. rigidoides* has a more rounded pileus, and *M. anteridiformis* has grayish to pinkish alveolar margins, and very few primary alveoli (less than 10) with a densely wrinkled bottom.

4.2. Illustrations of *M. nipponensis* in Japanese literature

*Morchella nipponensis* does not seem rare in Japan, as it appears to be represented in several Japanese books, especially from the middle of the 20th century, but it was often misidentified and given names of European morels. From this assessment, we will try to present some Japanese illustrations which could be morphologically close to *M. nipponensis*. The first Japanese mycologist who described this morel is Imai (p. 12; 1954), who attributed to it, without presenting a drawing or photograph, the name of *M. costata* with a Japanese name widely taken up later: “Hirome-no-togari-amigasa-take” (large conical morel). *Phallus costatus* Ventenat (= *M. costata*; Ventenat, 1797) on the basis of plate 85 fig. 3 of Michelí (1729), is a non-priority nomenclatural synonym of *M. elata* Fr. Several elements in the description of Imai are contradictory, indeed, *M. nipponensis* is a morcell of the section *Morchella* (“yellow” morels), whereas *M. elata* belongs to section *Distantes* (“black” morels). If the greenish shades of the pileus may correspond to the description of *M. elata*, it is not the case for the few alveoli. Following this first description, some illustrated Japanese mycological works could have represented *M. nipponensis* by attributing to it the names of *M. elata* or *M. costata*. We assume that this could be the case of Imazeki & Hongō (fig. 341, plate 58, *M. elata*, p. 174; 1965), Imazeki, Otani, and Hongō (photo p. 567, *M. costata*, p. 566; 1988), Hongō, Ueda, and Izawa (*M. costata*, p. 312; 1994).

4.3. Phylogeny of *Morchella*

It is probable that the lineage of *M. nipponensis* (subsection *Japonicae*) diverged early, like those of *M. steppicola*, *M. diminutiva* or *M. sceptriformis*, from the ancestor of *Morchella* section *Morchella*. The latest hypotheses about the geographical origin of the different sections of *Morchella* locate the apparition of sect. *Rufobrunnea* in the Mediterranean basin, section *Morchella* in Asia, and section *Distantes* in western North America (Loizides et al., 2021). However, the reconstruction of ancestral states is very sensitive to the information available of the oldest lineages, so these hypotheses could change dramatically after the discovery of early-diverging species in poorly sampled geographical areas. In this context, the search for such ancient lineages is critical to test or modify current hypotheses. The discovery of *M. nipponensis* in Japan is therefore of paramount importance, as it raises the possibility that there could be other related lineages too in Japan (paleoendemic morels) that could refine the current concepts of the evolutionary history of the genus *Morchella*.

4.4. Biogeography of *Morchella nipponensis*

While morel species associated to disturbed grounds often have an almost cosmopolite distribution (i.e. *M. importuna*, *M. elata*, *M. rufobrunnea*), those associated to plants (Clowez, 2016) are often restricted to the distribution areas of these species they associate to (i.e. *M. castanea*, *M. palazonii*, *M. sceptriformis*, *M. vulgaris*), generally occurring in a single continent unless they are artificially introduced by human activity (i.e. *M. americana*, which occurs in scattered populations of hybrid American poplars in Europe). Interestingly, some morels associated with multiple plant species are present also in several continents, further suggesting a relationship between geographical distribution and host range. For example, *M. vulgaris*, a species occurring from Europe to India and China, associates mainly with elm and ash, but also sometimes with Pinaceae and exceptionally with other plant species such as maples, *Paulownia* or grows saprotrophic on fruits such as apples or pears (Clowez, 1997; Du, Zhao, & Yang, 2015; Clowez & Moreau, 2020; Weholt, Alvarado, Kristiansen, & Gulden, 2021). In contrast, other species seem to be restricted to narrower geographical distributions, being considered endemics if they are absent from other regions with suitable habitats, i.e. those occurring only at specific areas of the Mediterranean basin (Taşkın et al., 2012; Clowez et al., 2014, 2015; Loizides et al., 2021).

All known collections of *M. nipponensis* have been located in montane areas of Japan, growing principally under *T. standishii*, a tree native to this country. Other Japanese morels being studied by the authors also have peculiar ecologies, like those harvested under *Ginkgo biloba* (*Ginkgoales* are mainly present in Laurasia and probably originated there; Zhou, 2009). Interestingly, most fungal species associated to another endemic tree in Japan, *Fagus crenata*, are not present in *F. sylvatica*, which occupies a similar ecological niche in Europe (Hosoya, Hosaka, & Nam, 2018), suggesting that these fungal species either associated with *F. crenata* after its establishment in Japan or followed a similar evolutionary history to this tree. Ectomycorrhizal fungi associated with *Castanopsis sieboldii*, another endemic tree of Japan, were shown to be partly correlated with the phylogeography of their host, indicating a putative co-migration (Matsukata et al., 2019). If morels followed a similar pattern, the presence of endemic species in Japan and other areas of the Pacific (i.e. *M. diminutiva* in eastern North America) could be linked to the evolution of relict flora from the Tertiary (Milne & Abbott, 2002). Breaks between the Tertiary flora of East Asia and North America depend on periods of cooling, the ice level, and land bridges over the Bering Strait (*O’Donnell et al.*, 2011). During the ice ages, the continent of East Asia was generally not covered by ice-sheets to the same degree as northern Europe and North America. Some plant taxa having originated at the Paleogene or even earlier during the Pleistocene survived and found themselves located in a large number of refuge areas in Japan during the Neogene/Quaternary, where the irregular orography contributes also to create diverse habitats favoring the survival of endemic flora (Tsukada, 1985; Ooi, 2016). This could be the case of relict lineages such as those of *Ginkgoales* and genus *Thuja*, which have been reduced to a few species or even to a single species in East Asia (*Manchester, Chen, Lu, & Uemura, 2009; Tang et al., 2018*). Finally, the volcanic character of the Japanese archipelago could have influenced also speciation processes there by maintaining lower levels of competition, a particularly ideal scenario for speciation of ascomycetes (Clowez, 1995).

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the countries where they were performed.

Acknowledgments

We would particularly like to thank Paul-Bill Lamiable, who allowed us to join “team Japanese morels” and thus created privileged contacts with Japan, for his contribution to the access, for reading and translation of Japanese mycological works; the mycologist Takumi Izumi, who shared with us his long experience of harvesting *M. nipponensis*; and Koichi Shibakusa, without whom...
we would not have been able to receive several samples in the form of high quality exsiccata. We would also like to thank Jean-Michel Bellanger (CNRS Montpellier, France) for his advice, Arash Jamali of University of Picardie Jules Verne, Amiens, France (scanning electron microscopy), Alain Petit (photographic montage) and Danielle Esposito (proofreading of English version).

References


Bellanger (CNRS Montpellier, France) for his advice, Arash Jamali of University of Picardie Jules Verne, Amiens, France (scanning electron microscopy), Alain Petit (photographic montage) and Danielle Esposito (proofreading of English version).

References


Bellanger (CNRS Montpellier, France) for his advice, Arash Jamali of University of Picardie Jules Verne, Amiens, France (scanning electron microscopy), Alain Petit (photographic montage) and Danielle Esposito (proofreading of English version).


doi: 10.4737/mycosci.2022.08.005

P. Clowez et al. / Mycoscience VOL.63 (2022) MYC590

—— 10 ——