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ORIGINAL RESEARCH PAPER

Two species of true morels (the genus *Morchella*, *Ascomycota*) recorded in the Ojców National Park (south Poland)

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Krakow, Al. 29 Listopada 46, 31-425 Krakow, Poland* Corresponding author. Email: baran.jakub84@gmail.com**Abstract**

In this paper, we present results of survey on the occurrence of true morels, conducted in the Ojców National Park (ONP). The first data about true morels from the area of the ONP date back to the nineteenth century. However, despite multiple surveys in the next decades, their presence has not been confirmed. Therefore, true morels were thought to be extinct in the ONP. In 2014 and 2015, two morphotypes of true morel fruiting bodies were discovered in two sites, identified as black morels and yellow morels. In 2016, we collected three fruiting bodies for detailed morphological characterization and molecular identification. According to our results, these morels were identified as *Morchella deliciosa* and *Morchella esculenta*. Our finding is the first confirmation of the occurrence of both species (as they are presently recognized) in Poland.

Keywordsrare fungi; *Ascomycota*; fungi; nature conservation; edible fungi; mycobiota of Poland**Introduction**

The Ojców National Park (ONP) is the smallest national park in Poland, it has only 2146 ha, however it is characterized by unique biodiversity and the occurrence of almost 10.4 thousand species of fungi, plants, and animals that have been reported here [1]. Unfortunately, for many species of fungi these records are outdated and not confirmed recently. Therefore, these species have been deemed extinct in the ONP [2]. True morels *Morchella* spp. are among interesting and rare fungi, which occurrence have not been confirmed in the ONP, for more than 100 years.

True morels are sac fungi belonging to *Morchellaceae* family. Their fruiting bodies appear generally in spring, albeit sometimes even from early spring to mid-winter [3–5]. They are saprotrophs or form biotrophic relationships with trees [6]. True morels belong to the most valuable and widely recognized, edible fungi [7]. Five species of true morels have been reported from Poland: *Morchella esculenta*, *M. elata*, *M. conica*, *M. gigas*, and *M. crassipes*, all of them are under partial protection [8,9].

True morels are highly polymorphic, what impedes their taxonomic distinction [10–12]. They can differ in head shape and color, ridges and pits arrangement, and stalk to head ratio [11,13]. General agreement exists that two groups can be morphologically distinguished: the first comprises taxa with darken, grey or black regular ribs (“black morels”, *Elata* clade), while the second one encompasses taxa with yellow or cream to tan, irregular ribs (“yellow morels”, *Esculenta* clade) [14,15].

There are only two reports from the nineteenth century of true morels from the area of the ONP, i.e., *Morchella esculenta* and *M. deliciosa* [16]. Because there were no further reports of the occurrence of true morels from this area, they were considered extinct in the ONP [2,17,18]. On the list of protected and endangered fungi of Poland [19] there is

no information about true morels occurring in the area of ONP, although information exists about nearby sites, e.g., Krakow.

During the surveys of the area of the ONP in 2014 and 2015, two sites with occurring true morels, one site with black and one site with yellow morels, were discovered. In 2016, fruiting bodies in those sites have not been found, but three additional sites with occurring true morels were discovered: one with black and two with yellow morels. Fruiting bodies from these locations were sampled for detailed analyses and identification. In this paper, we present the results of morphological analyses and molecular identification of two species of true morels, recorded in the Ojców National Park.

Material and methods

The area of the ONP was surveyed for the occurrence of true morels in 2016. For each site where true morels occurred, detailed site conditions were recorded, including: slope inclination, exposition, and plant species composition. Additionally, information on the soil type was verified according to the map of soils of the ONP [20]. All occurring fruiting bodies were counted, measured, and photographed. A single fruiting body was collected from each site for further analyses conducted in the laboratories of the Department of Forest Pathology, Mycology and Tree Physiology, Faculty of Forestry, University of Agriculture in Krakow.

Microscopic morphological analysis was performed using a Zeiss Axiophot light microscope with differential interference contrast (DIC) illumination and an AxioCam MRc5 camera (Carl Zeiss, Germany). For each fruiting body, at least six slides containing preparations of the stalk and apothecium were analyzed. H₂O was used for microscopic investigation. Dimensions of microscopic structures were based on 30 measurements. Ascospores were measured after release from asci, in brackets there are occasional extreme values. Herbarium specimens were deposited in the mycotheca of the Department of Forest Pathology, Mycology and Tree Physiology, Faculty of Forestry, University of Agriculture in Krakow.

Pure culture isolations were performed using malt extract agar medium supplemented with tetracycline [MEA+T; 20 g L⁻¹ malt extract (Difco, USA), 15 g L⁻¹ agar Difco supplemented with 200 mg L⁻¹ tetracycline (Tetracyclinum TZF Polfa, Poland)]. For each fruiting body, 24 explants, ca. 2 × 2 × 2 mm, excised from the stalk and apothecium after removal of the superficial tissue, were cultured. Cultures were incubated at 20°C in the dark for 2 weeks, after that time, regular observations of the culture morphology began. Obtained *Morchella* pure mycelia were subcultured using fresh MEA+T plates.

Next, the molecular identification was performed for each studied fruiting body. Ca. 2 cm³ of the upper part of the apothecium was used for DNA extraction. Mycelium was pulverized in liquid nitrogen in a mortar and total genomic DNA was extracted using the modified CTAB protocol of Gawel and Jarret [21]. One-hundred-fold diluted (20–100 ng µL⁻¹) DNA extracts were used as templates in the amplification of three target sequences – ITS region (primers ITS1 and ITS4 [22]), a fragment of *LSU* gene (primers LR7 and LR0R [23]), and a fragment of *rpb2* gene (primers RPB2-9f and RPB2-3r [24]). The PCRs, clean-up of amplification products, and bidirectional sequencing were performed as described in other survey [25]. Sequences were processed with Chromas Pro 1.6 software (Technelysium, Australia) and queried against the NCBI GenBank database with the BLAST search tool to retrieve the most similar sequences. The best representatives of each unique ITS, *LSU*, and *rpb2* sequences were deposited in NCBI GenBank with the accession numbers KY792586–KY792591.

Results

Molecular identification

Based on sequence analysis of ITS, *LSU*, and *rpb2*, we identified two different species of true morels: a black morel *Morchella deliciosa* Fr.: Fr. in Fries occurring at one site,

and a yellow morel *Morchella esculenta* (L.: Fr) Pers. occurring at two sites. All three target fragments for both species were identical with their respective GenBank accessions (Tab. 1).

Collected specimens

Morchella deliciosa

DESCRIPTION OF ASCOCARP. Ascocarp 120 mm high, hymenophore 90 mm high, 35 mm wide at widest point, dark brown conical, pitted and ridged with vertical ridges (Fig. 1a). Stipe 30 mm high; 29 mm wide, whitish. The cap-adnate section was the widest part of the stipe. Asci cylindrical, 280–320 × 17–20 µm, eight-spored. Ascospores mostly uniseriate, sporadically irregularly biseriate, single-celled, hyaline, broadly ellipsoid 21–23(26) × 12–15 µm (Fig. 1b). Paraphyses ramified at the base, septate, sometimes constricted near septa.

Tab. 1 Results of molecular identification of specimens with ITS, *LSU*, and *rpb2* sequences.

Species	Target fragment	Accession number	100% match with / reference / country of origin
<i>M. deliciosa</i>	ITS	KY792586	GU551429 / [14] / NS DQ257334 / DS / China KM588006 / [12] / France JQ321873 / [33] / China JQ618584 / [34] / NS JQ618624 / [34] / NS KR073753 / [35] / China KR073758 / [35] / China KR073764 / [35] / China KR073766 / [35] / China KR073767 / [35] / China KR073770 / [35] / China KR073771 / [35] / China KR073772 / [35] / China KR073777 / [35] / China JN085110 / [36] / Turkey JN085131 / [36] / Turkey
	<i>LSU</i>	KY792587	KM587891 / [12] / France
	<i>rpb2</i>	KY792588	KM588029 / [12] / France JN085248 / [36] / Turkey JN085247 / [36] / Turkey JN085226 / [36] / Turkey HM056473 / [24] / Turkey
<i>M. esculenta</i> (two specimens)	ITS	KY792589	AJ623265 / [10] / Montenegro JQ691475 / [38] / France JQ691496 / [38] / Ukraine JQ618814 / [33] / NS KM588016 / [12] / France KM588023 / [12] / France KM587925 / [12] / France KM587951 / [12] / Spain
	<i>LSU</i>	KY792590	KM587902 / [12] / France
	<i>rpb2</i>	KY792591	HM056508 / [24] / Turkey GU551313 / [14] / Czech Republic GU551684 / [14] / Czech Republic

DS – no reference / direct submission; NS – not specified in GenBank submission.

DESCRIPTION OF CULTURES (NO. 21106). Colony fast growing, after 5 days at 20°C, reached 90 mm in diameter, with radial arranged hyphae. Initially medium adhered, hyaline to light beige, dark brown with time, with puffy aerial mycelia (Fig. 1c). On the surface of some colonies, reddish-brown stromatic structures, composed of large (33–39 × 23–30 μm) pseudoparenchymatic cells were formed (Fig. 1d). Hyphae variable in color and dimensions: (i) hyaline, up to 6 μm in diameter, regular in shape (Fig. 1e), (ii) brown, 12 to 20 μm in diameter, smooth or rough (Fig. 1f), and (iii) monilioid hyaline and brown, oval cells 32–50 × 20–32 μm (Fig. 1g). After about 6 weeks, a few branched conidiophores were observed for some colonies, producing circular or oval, hyaline conidia 6–11 × 6–10 μm in size (Fig. 1h).

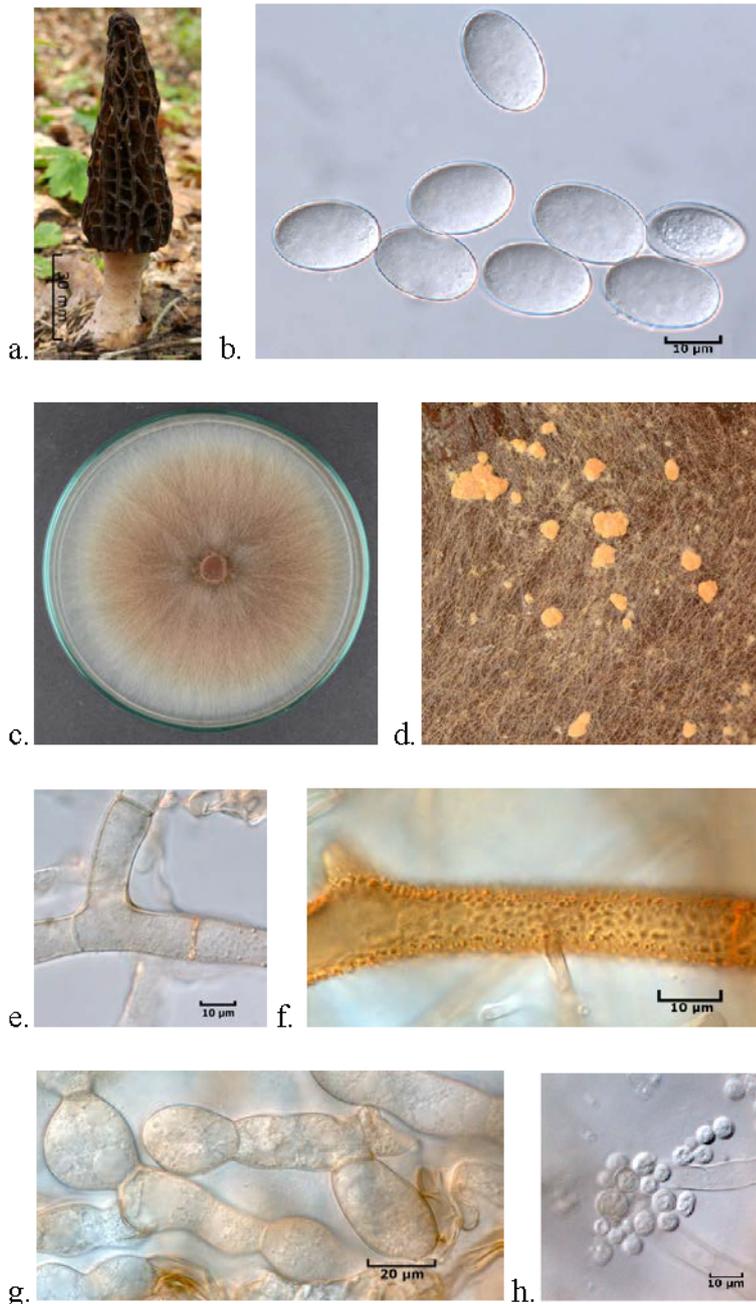


Fig. 1 *Morchella deliciosa*. **a** Fruiting body (photo by J. B.). **b** Released ascospores in H₂O. **c** Five-day-old colony on MEA+T. **d** Stromatic structures on the colony surface. **e** Hyaline hyphae from the mycelial culture. **f** Brown hyphae with rough surface from the mycelial culture. **g** Monilioid hyphae from the mycelial culture. **h** Conidia of anamorphic stadium on the colony (**b–h** photos by Tadeusz Kowalski).

ANALYZED MATERIAL AND DESCRIPTION OF LOCALITY. Three specimens, one mature, two decayed, were found on May 2, 2016 (herbal specimen No. 46.16). The site was located in the central part of “Skalbania” ravine, on the ravine slope, on the old forest track (SE exposure, inclination 25°, track inclination 4°). Soil brown rendzina. The overstory, 50% cover, included mostly *Abies alba* and *Picea abies*, with small share of *Fagus sylvatica* and *Tilia platyphyllos*. The shrub layer, 10% cover, included *Tilia platyphyllos*, *Acer pseudo-platanus*, *Picea abies*, *Euonymus verrucosa*, and *Fraxinus excelsior*. The herb layer, 60% ground cover, included *Galeobdolon luteum*, *Ranunculus lanuginosus*, *Impatiens noli-tangere*, *Impatiens parviflora*, *Viola reichenbachiana*, *Brachypodium sylvaticum*, *Oxalis acetosella*, *Stachys sylvatica*, *Geum urbanum*, *Urtica dioica*, *Mercurialis perennis*, *Veronica montana*, *Chaerophyllum temulum*, *Cerasus avium*, *Carex silvatica*, *Asarum europaeum*, *Galium odoratum*, *Athyrium filix-femina*, *Stelaria nemorosa*.

Morchella esculenta

DESCRIPTION OF ASCOCARPS. Ascocarps 50 to 110 mm high. Hymenophore 25–60 mm high, 23–60 mm wide at the widest point, subglobose to ovoid (some slightly conical); light beige or yellowish scattered pits and ridges. Stipe 25–50 mm high, 15–28 mm wide, light beige or whitish. Ascocarps did not darken with age (Fig. 2a,b). Micromorphological analysis based on the specimen from the Site 1: asci cylindrical, 260–340 × 16–25 μm, eight-spored (Fig. 2c). Ascospores mostly uniseriate, sporadically irregularly biseriate, single-celled, hyaline, broadly elliptical, smooth, 16–22 × 10–12 μm (Fig. 2d). Paraphyses ramified at the base, septate, slightly broadened at the top. Despite slight morphological difference between fruiting bodies on Site 1 and 2, they proven to be genetically uniform for all three target sequences, ITS, LSU, and *rpb2*.

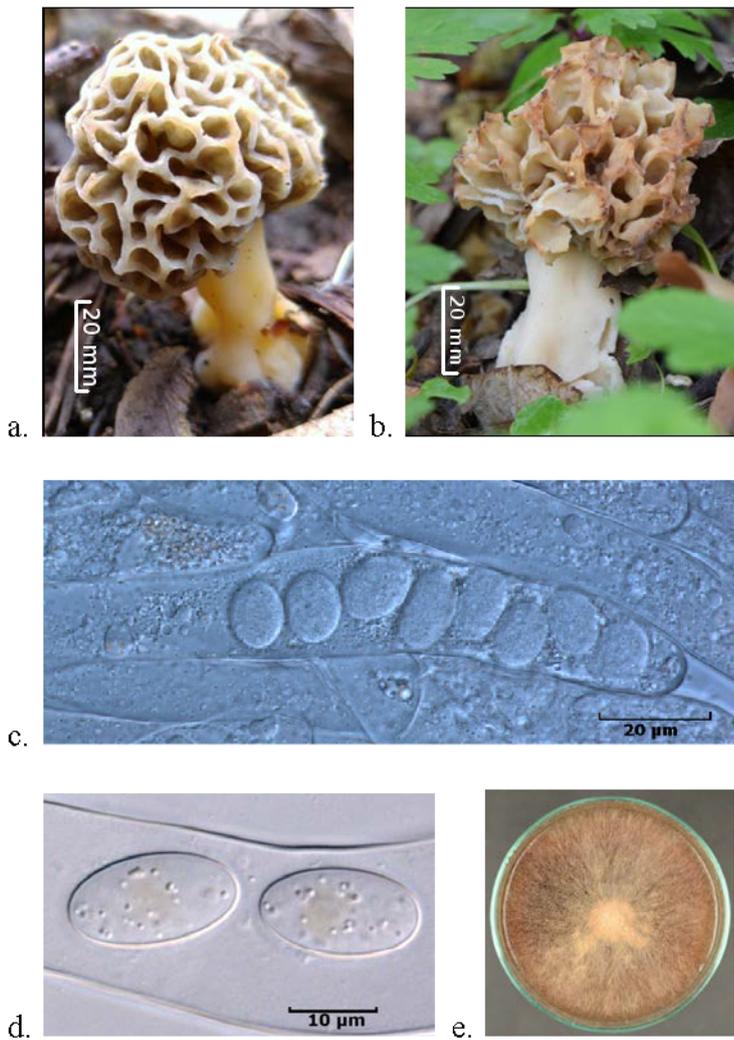


Fig. 2 *Morchella esculenta*. **a** Fruiting body collected in the Site 1 (photo by J. B.). **b** Fruiting body collected in the Site 2 (photo by Dariusz Wawro). **c** Asci containing ascospores in H₂O. **d** Ascospores inside asci in H₂O. **e** Seven-day-old colony on MEA+T (c–e photos by Tadeusz Kowalski).

DESCRIPTION OF CULTURES (NO. 21105) (obtained from the fruiting body collected on the Site 1). Colony generally similar to *M. deliciosa*, fast growing, after 5 days at 20°C reached 90 mm in diameter. Initially light beige and medium adhered, dark brown with time (Fig. 2e). On the superficies of some colonies stromatic structures were formed (as shown in Fig. 1d for *M. deliciosa*). Variable hyphae in the aerial mycelium: (i) hyaline up to 6 µm in diameter, (ii) brown 12 to 23 µm in diameter, smooth or rough, hyphae with unicellular, oval lateral ramifications up to 30 × 15 µm or with seta-like ramification up to 175 × 10 µm, and (iii) monilioid hyphae with oval cells, mostly 42–75 × 27–32 µm. No conidia production was observed.

ANALYZED MATERIAL AND DESCRIPTION OF LOCALITIES. Site 1: five specimens found on April 19, 2016 (herbal specimen No. 45.16). The site was located next to the “Smardzowicki” ravine, on a tourist trail and directly next to it (SWW slope exposure, inclination 20°). Three specimens grew on a wooden step on the trail, while the other two, 2 m next to the trail. Soil brown rendzina. The overstory, 90% cover, comprised *Carpinus betulus* as a dominant species. The shrub layer, 30% cover, included: *Corylus avellana*, *Lonicera xylosteum*, *Ribes uva-crispa*, *Fagus sylvatica*, and *Acer platanoides*. The herb layer, 50% ground cover, included: *Acer platanoides*, *Abies alba*, *Fraxinus excelsior*, *Brachypodium sylvaticum*, *Hepatica nobilis*, *Oxalis acetosella*, *Asarum europaeum*, *Primula veris*, *Aegopodium podagraria*, *Galeobdolon luteum*, *Viola reichenbachiana*, *Anemone nemorosa*, *Pulmonaria obscura*, *Neottia nidus-avis*. Site 2: there was one specimen, the site was located in the lower part of the Sąspowska

valley, near to a tourist trail. The site was flat, located a few meters from the southern slope. Soil leached brown. The overstory, 80% cover, included: *Carpinus betulus*, *Fraxinus excelsior*, and *Acer pseudoplatanus*. The shrub layer, 20% cover, included: *Padus avium*, *Ribes uva-crispa*, *Abies alba*, *Acer platanoides*, and *Sambucus nigra*. The herb layer, 60% ground cover, included: *Galeobdolon luteum*, *Ranunculus lanuginosus*, *Poa nemoralis*, *Viola reichenbachiana*, *Aegopodium podagraria*, *Geranium pheum*, *Pulmonaria obscura*, *Agrimonia eupatoria*, *Oxalis acetosella*, *Mercurialis perennis*, *Carex silvatica*, *Asarum europaeum*, *Galium odoratum*.

Discussion

The exact number of *Morchella* species is not clear. Index Fungorum lists 332 records within *Morchella* genus, and the MycoBank 296 records [26,27]. In the morphology-based classifications of the genus, the number of species varies from 3 to 52 (discussed in [10] and [12]). Some doubts concern two taxons: *Morchella conica* and *M. elata*, both of them regarded in Polish literature and legal regulations as separate species [8,9]. This status is, however, disputed as some authors regard *Morchella conica* as a distinct species [28–31], while the other as a synonym of *Morchella elata* (e.g., [32]). The uncertainty

described above is an example of problems arising from attempts to accommodate morphologically defined species names to molecularly defined monophyletic groups. The inaccuracy in using of morphologically defined binomial species names to describe genetic diversity detected with molecular methods resulted in adoption of provisional system of species designations (see [7,24,33–36]), where *M. deliciosa* is referred to as Mel-26 and *M. esculenta* as Mes-8. According to the newest data provided by Richard et al. [12], the name *Morchella conica* is illegitimate at the species rank, while the *Morchella elata* status needs further study to be definitively resolved. *Morchella deliciosa* is regarded a valid species, formerly sometimes under different names: *M. conica* var. *deliciosa*, *M. conica* var. *flexuosa*, *M. conica* var. *nigra*, *M. conica* var. *violeipes*, *M. conica*, or *M. intermedia* [12].

According to our results, two species of true morels, *M. deliciosa*, and *M. esculenta*, were identified. This finding is the only report of the occurrence of true morels in the area of the Prądnik Valley, including ONP, for more than 100 years, as they were recorded previously only by Berdau [16]. For studied specimens macroscopic and microscopic features: sizes of fruiting bodies, asci, and ascospores, does not deviate from descriptions reported in literature for *M. conica* var. *deliciosa* and *M. esculenta* [28,33,37]. There are also no differences between these two species in microscopic features. Similarly, all sequences for all three target fragments for the studied specimens do not deviate from the previously reported. Additionally, the sequences detected in our specimens occurred also (100% match) in specimens collected in: Czech Republic, France, Montenegro, Turkey, Spain, and Ukraine – *M. esculenta* [7,10,12,24,38] and in: China, France, and Turkey – *M. deliciosa* [7,12,24,33–36]. This fact indicates that both morels species found in the ONP belong to widely distributed genotypes.

As for habitat requirements, the ONP morels generally follow the usual habitat pattern: yellow morels occur predominantly in deciduous forest, while black morels in conifer forest, however, both can also grow in different habitats [13,35,39,40]. Our observation supports this pattern, as two sites of *M. esculenta* were located in *C. betulus* dominated or *C. betulus*, *F. excelsor*, *A. pseudoplatanus* mixed stands, and *M. deliciosa* site was located in *A. alba* / *P. abies* dominated stand. It is known that some morels are considered saprotrophs, while other can form biotrophic relations with plants, some researchers even believe that morels can form mycorrhiza-like interactions with trees [6,41,42]. While we did not consider that aspect in our study, it could be, however, interesting subject for future studies.

Güeler and Arkan [43] pointed out that *Morchella mycelium* is the fastest growing of all mushrooms and has the same nutritive content and aroma as its ascocarps. The fast growth of the cultures was confirmed in our experiments for cultures isolated from the ONP specimens. Some researchers informed also about the formation of sclerotia in pure cultures of *Morchella* spp. [43,44]. Buscot [44] observed two types of sclerotia: the first type involves numerous sclerotia formed along periphery of the colony, 2 days after the colony reached the edge of a Petri dish, while the second type comprises the sparse sclerotia formed after 12 days and randomly distributed over the medium. However, the colony structure and development of *Morchella* spp., including sclerotia formation, depends strongly on the incubation temperature and medium composition [43,44]. Güeler and Arkan [43] used three medium types: potato dextrose agar, malt extract agar, and complete yeast medium prepared with casein, casamino acids, peptone, and sodium nitrate, while Buscot [44] used malt extract agar and yeast extract agar. We used only malt extract agar to culture our isolates. Perhaps for this reason, in colonies of both analyzed *Morchella* species sclerotia, as described above, were not observed. On the surface of some colonies, however, stromatic structures were formed, but dissimilar to described by Buscot [44].

The interesting observation is that in some *M. deliciosa* cultures, conidia were formed. The first author who observed the formation of anamorphic state was Molliard [45,46], who classified it in *Costantinella* genus. Later, Paden [47] obtained the *Costantinella* state for *M. elata* cultures. Another observation of conidial state found in *Morchella* cultures was reported by Buscot [44], while Barron [48] indicated the close relationship of *Costantinella* and *Nodulosporium* asexual states. The next step was the observation of *Costantinella* colonies in nature: on soil, moss, and woody debris in fall in the inland Pacific Northwest USA; from these colonies, pure cultures of *Costantinella* were isolated [49]. According to these authors, the widespread occurrence of asexual states

of morels, false morels, and allied taxa suggests that their life cycles are more complex than previously thought. Conidiophores and conidia observed on *M. deliciosa* cultures from the ONP corresponded to features of *Costantinella* [50].

The survival of true morels in the ONP seems to be unthreatened. Some of the sites are located next to tourist trails, however, mushroom picking in the ONP is forbidden [51]. Despite small area of the ONP and more than 200 years research history, it is still not totally explored place, especially concerning fungi. There is no doubt that the community of fungi in the ONP requires further studies.

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