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*Original paper*

# Morels on the sand dunes of the Emilia-Romagna coast (Northwestern Adriatic Sea, Italy)

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**Abstract**
*Morchella* species are known as famous and prized edible fungi due to their culinary flavor and medicinal properties. The asomata are collected throughout the temperate regions of the northern hemisphere. *Morchella* spp. taxonomy has long been debated as a result of the high phenotypic plasticity charaterizing the genus. Most morels are considered saprobic but some species has been reported to interact with roots of many plant species forming different types of associations. In Emilia-Romagna (Italy), morels became a part of the culinary tradition, especially for the populations of the coastal areas. This work aimed to describe and identify the ascomata collected on the white dune habitat as well as to verify the extent of the interaction with plant species growing in the same area. All ascomata collected since 2001 shared a similar morphology with a range of variability mainly due to the harvesting period. Ascomata collected in 2017 were grouped within the Mes-17 clade, in the Esculenta group, based on their ITS rDNA sequences. Nine different plant species were found to be connected with morel ascomata through characteristic sand-mycelial structures never described before. The amount of the ascomata collected on the sand dunes demonstrates that the study area is particularly suitable for morel fruiting. Therefore, the protection of the dune system is imperative for preserving *Morchella* genetic diversity within the local population.

**Key words:** *Morchella vulgaris*; *Morchella esculenta*; *Morchella dunensis*; white dunes; host plant

## Introduction

True morels (*Morchella*, Ascomycetes) are among the most valuable edible mushrooms, which also have medicinal properties (Ajmal et al., 2015). Most of the morels in the market are harvested from the wild but recent attempts to cultivate these mushrooms were successful (Du et al., 2017). Before this success, other cultivation strategies were developed around the world but the low reproducibility and the high costs prevented large-scale cultivation (Peng et al., 2015). Species in the genus *Morchella* are collected throughout the temperate regions of the northern hemisphere where they produce ascomata during a few weeks' time period in the springtime (Du et al., 2015).

Taxonomy of morels has long been debated among mycologists due to the high phenotypic plasticity of their ascomata, which often do not reflect their phylogeny (Kellner et al., 2005). Moreover, these fungi exhibit a variety of trophic habits as well as a broad range of growth habitats. Most morels are considered saprobic because they can complete their life-cycle without host plants, but a number of species have been reported to interact with roots of many plant species forming different types of associations (Pilz et al., 2007). Morels can be found in many forest types, meadows, burned sites, coastal dunes and a broad range of disturbed soils and man-made environments (Pilz et al., 2007).

In Italy, morels are locally appreciated but have not yet gained a significant market share among edible mushrooms. Most of the morels commercialized in Italy come from Hungary, Turkey,

Romania and Bulgaria and their wholesale price is 20-28 € per kg whereas the retail price is 30-40 € per kg (Luigi Dattilo, Appennino Food Group SpA, personal communication). Unlike other high-priced edible mushrooms such as truffles (*Tuber* spp.) and porcini (*Boletus edulis* s.l.), the ecology and biology of morels in Italy have been poorly investigated. These mushrooms are particularly sought-after in the littoral area of the Emilia-Romagna region where their ascomata are collected either from the coast or from the inland pine and hardwood forests. In this work, we aimed i) to barcode the morels collected on the sand dunes of the Emilia-Romagna coast and ii) to investigate the type and extent of their interactions with the local plants.

## Materials and Methods

The area under investigation consists of a series of sand dunes extended along the coastline from Punta Marina and Marina di Ravenna (Ravenna, northeastern Italy). The collection sites are approximately 100 m from the sea at an elevation of 2 to 5 m asl, within the EU habitat 2120 [*Echinophoro spinosae-Ammophiletum australis* (Br.-Bl. 1933) Géhu, Rivas-Martinez & R. Tx. 1972 (Géhu et al., 1984)]. In this area, the dune system is extremely fragmented and threatened by touristic facilities (Sytnik and Stecchi, 2015). The dune vegetation of the study area is dominated by *Calamagrostis arenaria* (L.) Roth [=*Ammophila arenaria* (L.) Link subsp. *australis* (Mabille) Laínz], *Echinophora spinosa* L., *Eryngium maritimum* L., *Euphorbia paralias* L., *Medicago marina* L., *Cyperus capitatus* Vand., *Pancratium maritimum* L., *Stachys maritima* Gouan and *Spartina juncea* auct., non (Michx.) Willd., often associated to allochthonous species such as *Cenchrus incertus* M.A.Curtis (=*Cenchrus spinifex* Cav.), *Ambrosia psilostachya* DC (=*Ambrosia cronopifolia* Torr. & A. Gray), *Yucca gloriosa* L. and *Oenothera stucchii* Soldano (Merloni et al., 2015). A coastal *Pinus* spp. forest extends in the inland area behind the dune system.

Ascoma production was assessed every week during the fruiting season (from mid-March to mid-April) from 2001 to 2017. Voucher specimens were deposited in the private herbarium of the first to authors. The range of interactions between morels and host plant species was investigated by following the mycelial structures connecting the base of the stipe and the host roots. A trench was dug in proximity of each ascoma by using a trowel and the mycelial connecting structure was carefully unearthed with a paint brush. A total of 68 trenches were opened throughout the study period, most of them on the sand dunes but some also in the immediate surrounding anthropized areas (Table 1).

**Table 1 -** List of the surveys in which sand-mycelial structures were unearthed. Host plant and number of ascomata collected in the same day of excavation are reported

| Survey date | Locality | Number of trenches | Host plant |
|---|---|---|---|
| 8 April 2001 | Marina di Ravenna | 3 | *Cyperus capitatus* |
| | | 1 | *Hypochaeris radicata* |
| 14 April 2001 | Punta Marina | 2 | *Yucca gloriosa* |
| 8 April 2004 | Punta Marina | 2 | *Reseda alba* |
| 7 April 2006 | Punta Marina | 6 | *Yucca gloriosa* |
| | | 8 | *Tamarix gallica* |
| | | 4 | *Elaeagnus angustifolia* |
| 30 March 2008 | Punta Marina | 1 | *Elaeagnus angustifolia* |
| 3 April 2008 | Punta Marina | 2 | *Pinus pinaster* |
| 5 April 2008 | Punta Marina | 5 | *Yucca gloriosa* |
| 11 April 2008 | Punta Marina | 4 | *Yucca gloriosa* |
| | | 3 | *Hypochaeris radicata* |
| 19 April 2010 | Punta Marina | 3 | *Yucca gloriosa* |
| | | 2 | *Sedum* sp. |
| 10 April 2015 | Punta Marina | 7 | *Hypochaeris radicata* |
| | | 2 | *Cyperus capitatus* |
| 1 April 2016 | Punta Marina | 4 | *Ambrosia cronopifolia* |
| | | 6 | *Hypochaeris radicata* |
| 24 March 2017 | Marina di Ravenna | 3 | *Cyperus capitatus* |

Macro and micro-morphology of the specimens were assessed through the analysis of fresh ascomata. Micro-morphological analyses were carried out under a Laborlux K microscope (Leitz) on sections of the hymenia and the sterile ridges mounted either in water or Congo red. Measurements were made with an ocular micrometer at × 400 magnification for asci, paraphyses and elements of sterile ridges and × 1000 magnification for spores. One hundred measures were taken for each taxonomic character in two out of five ascomata collected in 2017.

Five ascomata collected on March 2017 were barcoded by sequencing the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA). Few sterile hyphae were used directly as target material for DNA amplification through polymerase chain reaction (PCR), bypassing the need for DNA isolation (Iotti and Zambonelli, 2006). ITS region was amplified in a 50-µl volume reaction using the primer pair ITS1F-ITS4 (White et al., 1990; Gardes and Bruns, 1993) and a SimpliAmp thermal cycler (ThermoFisher). PCR reactions contained BioMix mastermix (Bioline), 400 nM for each primer and 30 µg of bovine serum albumin. Thermal cycler conditions were the following: 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with an initial denaturation at 94 °C for 8 min and a final extension at 72 °C for 10 min. Amplicons were purified with the NucleoSpin Extract II kit (Macherey-Nagel) and sequencing was performed with ITS5 and ITS4 as forward and reverse primers at GATC Biotech AG (Constance, Germany). ITS sequences were aligned and edited manually with BioEdit 7.0.5.3 (Hall, 1999) and consensus sequences were compared to those deposited in GenBank (www.ncbi.nlm.nih.gov) and Morchella MLST (http://www.cbs.knaw.nl/morchella/) databases. Genotyped specimens were deposited in Herbarium Mycologicum Aquilanum (AQUI9839 to AQUI9841) and their ITS sequences submitted to GenBank (accession numbers MK388505 to MK388509).

The phylogenetic analysis of the genotyped ascomata was inferred by using the maximum likelihood (ML) estimation implemented in Raxml (version 8.2; Stamatakis, 2014) and MrBayes. Sequences generated in this study were analyzed together with other ITS sequences from GenBank. Alignment was performed by CLUSTAL W (Thompson et al., 1994) and gaps and ambiguous sites were excluded prior to phylogenetic analyses. Hasegawa-Kishino-Yano (HKY) model was identified as the best fit-model of nucleotide substitution by using JModelTest v2.1.4 (Darriba et al., 2012). Branch support of ML tree was assessed by bootstrap analysis of 1,000 replicates. Bayesian analyses were conducted using the software package MrBayes v3.2.2 (Ronquist et al., 2012). To ensure convergence, two independent runs were conducted, each with four chains and for 5,000,000 generations, sampling every 100 generations. A 50% majority-rule consensus tree was generated after the exclusion of the first 250 trees and the posterior probabilities of clades were computed.

## Results

During the period of survey, about 200 ascomata were collected on sand dunes and their immediate surroundings. All the ascomata collected during the period of investigation shared the same micro and macro-morphology. The main source of morphological diversity was due to the collection period (Fig. 1). In general, the earlier fruiting strains (first half of March) generated little and sterile ascomata with darker pits while the ascomata collected in April were yellowish with a larger size and mature ascospores.

### *Macro-morphological description*

Ascomata 50-130 mm high. Hymenophore 30-80 mm high and 20-60 mm wide at the widest point; irregularly spherical up to conical with a rounded apex; pitted and ridged; adnate at the point of attachment. Ridges glabrous; light gray when young, becoming ochre-yellowish, ochre-grayish or light brown with maturity; rusty-brown or orange brown when exposed to the sun or sea air; widely rounded to nearly flat when young, becoming thinned in age. Pits glabrous; usually darker than ridges. Stipe 20-50 mm high, almost entirely buried in the soil; 15-30 mm wide; more or less equal, occasionally lacunose and basally subclavate; glabrous or slightly pruinose; whitish to beige,

sometimes with brown spots; 1.5-2 mm thick, becoming thickened near the base. Context whitish; sterile inner surface whitish and finely warty. Flesh firm, elastic, with slight spermatic typical odour.

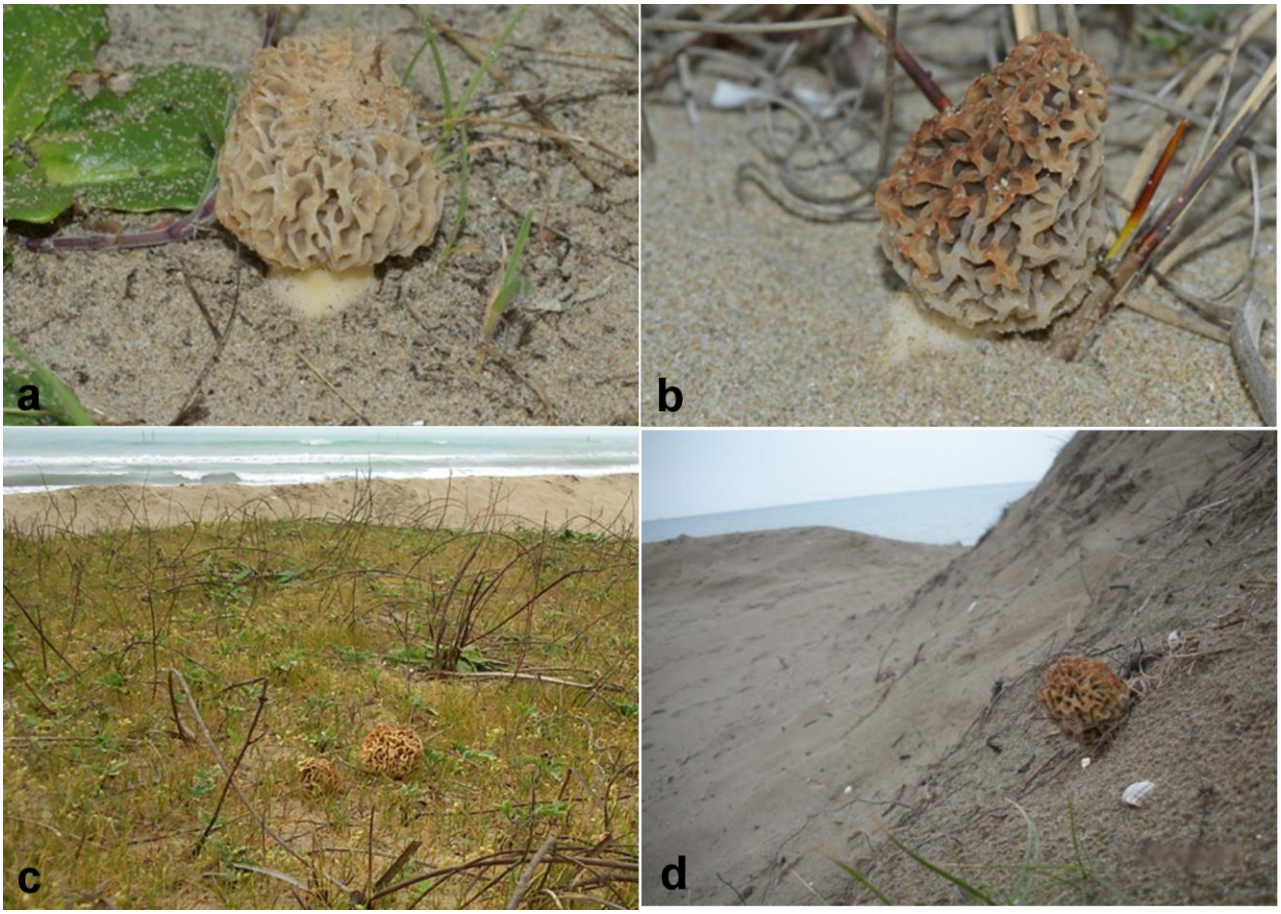


**Fig. 1 -** Ascomata collected in the study area during the period of investigation. a) immature specimen (Mvu10) hosted by *Hypochaeris radicata*, collected on March 24, 2017; b) specimen (Mvu37) hosted by *Cyperus capitatus*, collected on March 24, 2017: the darker side of the hymenophore faced the sea with < 10% of spore-containing asci; c) mature specimen collected on April first, 2016; d) mature specimen collected on April 17, 2010

*Micromorphological description*

Ascospores pale yellowish in deposit, (13.2) 16.7-20.4 (23.2) µm × (8.2) 10.2-12.3 (13.9) µm; Q (1.1) 1.5-1.8 (2.3); elliptical; smooth; thin-walled; hyaline; contents homogeneous, sometime microguttulate at poles. Asci 275-325 x 15-20 µm; eight-spored; cylindrical to subclavate, sinuose at the base; thin-walled; operculate; hyaline; uniseriate; inamyloid. Paraphyses 200-250 x 5-9 µm; cylindrical; hyaline; apices rounded to subcapitate; thick-walled; 2-3-septate; sometimes branched in the lower half. Elements on sterile ridges 140-150 x 12-25 µm; septate; terminal cell clavate to capitate; thick-walled; hyaline or with granular content.

*Molecular analyses*

PCR amplification of the five specimens collected in 2017 generated ITS1f/ITS4 amplicons of 1225 bp. Two ITS1-5.8S-ITS4 types with 99.64% similarity (4 variable positions out of 1099 bp) were obtained after sequencing. Blastn search against Morchella MLST database yielded the best hit to a *M. esculenta* sequence (JQ723102), having > 99.5% similarity with both ITS types. According to the clustering criteria formulated by Kõljalg et al. (2013), ITS sequences generated in this study fallen into the *M. spongiola* species hypothesis (SH181478.07FU) within the UNITE database. Phylogenetic analyses placed all the sequences generated in this work within the clade Mes-17 identified by Du et al. (2012a). Phylogenetic tree from this work (Fig. 2) was generated by using the

full-length ITS1-5.8S-ITS2 sequences of Mes-17 clade deposited in Genbank and a sequence of the closely related clade Mes-6 (JQ322060) as outgroup.

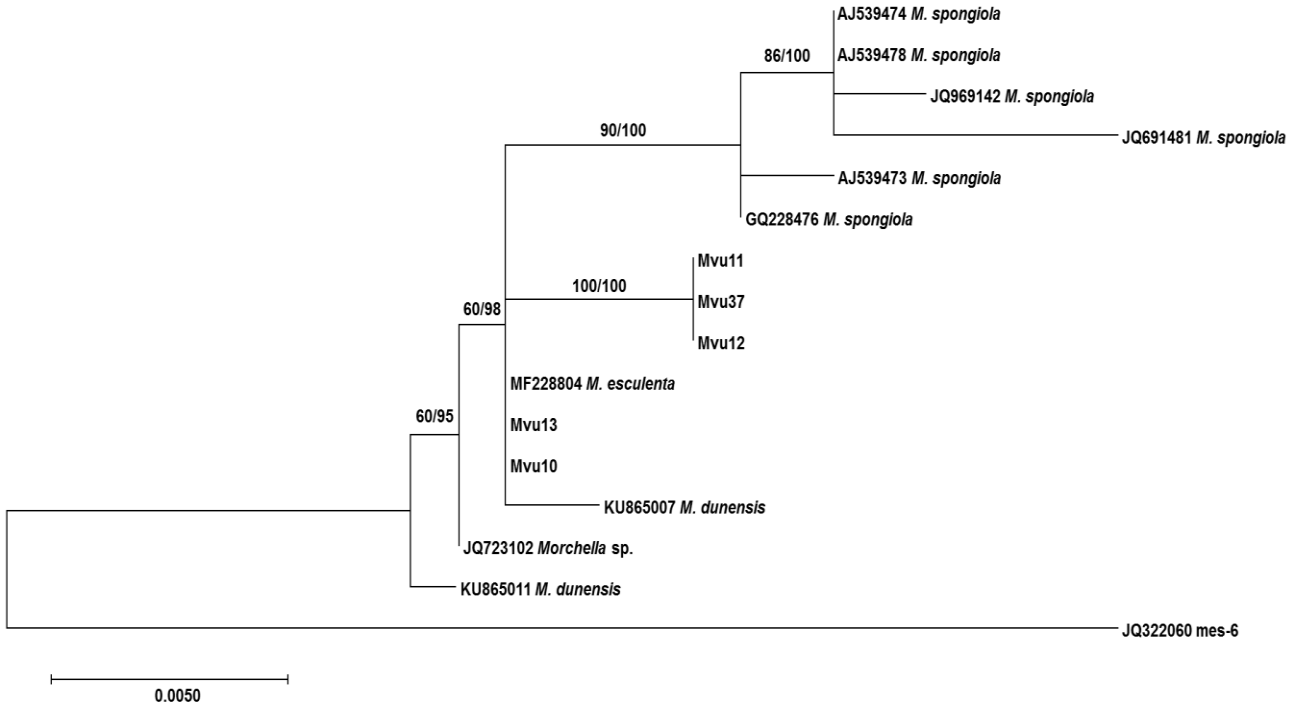


**Fig. 2 -** Maximum Likelihood (ML) tree generated from full ITS1-5.8S-ITS2 sequences (1015 bp) of Mes-17 clade after Du et al. (2012a). Tree topologies from ML and Bayesian analyses were congruent at all supported nodes. Bootstrap values (1000 replications) and Bayesian posterior probabilities are reported on each branch. Ascomata genotyped in this work are labelled by their isolation code while the specimens from GenBank are identified by their accession number and the associated species epithet. The tree was edited using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/)

*Host plant connections*

Characteristic mycelial structures (Fig. 3) were found to connect the base of the morel stalks to the roots of nine different plant species (Table 1). They appeared as a sand-mycelium cord, simple or ramified, brittle to the touch, 5 to 30 mm in diameter and 20 to 100 cm long, composed of entangled hyphae encrusted by sand grains. Fine roots were completely embedded within these mycelial structures whereas the surface of the rhizomes and conductive roots (> 0.5 cm in diameter) was only partially colonized. During the field surveys, any other type of fungal structure (e.g. sclerotia) was found on the roots of host plants or in the soil.

## Discussion

In this work, morels from white dunes of the Italian coast were genotyped for the first time. A number of *Morchella* species have been reported to grow on sand dunes throughout Atlantic (Rotheroe, 1993; Castañera and Moreno, 1996; Guinberteau, 2012) and Mediterranean coasts (Loizides, 2016; Melis et al., 2017). However, the phylogeny and ecology of morels growing in this habitat have been poorly investigated. The morels of the coastline from Punta Marina and Marina di Ravenna (Ravenna, northeastern Italy) were previously described by Snabl and Guidori (2015) and identified as *M. esculenta* based on morphological traits of their ascomata. Morphology of these morels is shared by different species in the *Morchella* genus which can be found on coastal sand dunes [*M. dunensis* (Castañera, J.L. Alonso & G. Moreno) Clowez] but also in other habitats [*M. vulgaris* (Pers.) Gray and *M. esculenta* (L.) Pers.]. The growth habitat has often been considered as a key character in morel taxonomy.

**Fig. 3 -** Sand-mycelium cord of an ascoma associated to *Cyperus capitatus*

In a recent work, *M. dunensis* was described as a xerophilous and sand-inhabiting species, with rufescent ascomata, ochraceous or ochraceous-brown in color, misshapen stipe and coarse ridges (Loizides et al., 2016). As main differences with *M. dunensis*, *M. vulgaris* shows gray tinges and contrasting dark pits whereas *M. esculenta* displays a stipe rather smooth, cylindrical and weakly inflated at the base, regularly polygonal pits with more or less smooth ridges. Long-term monitoring of ascomata collected in the study site highlighted a substantial range of morphological variation attributable to seasonal climate dynamics and maturity stage. In this context, intraspecific variability of ascoma morphology masked the differences among closely related morel species.

In recent years, significant efforts have been made to improve the molecular-based taxonomy of true morels by genotyping specimens from North America and Asia (O'Donnell et al., 2011; Du et al., 2012b) and later from Europe (Taşkin et al., 2012; Richard et al., 2015; Loizides et al., 2016). Despite the increasing interest in phylogeny of the *Morchella* genus, the genetic diversity of the Italian specimens remains almost unexplored. To our knowledge, only three ascomata from alpine and sub-alpine habitats were barcoded before December 2017: *M. conica* Pers., *M. elata* Fr. (Osmundson et al., 2013) and *M. semilibera* DC. (Richard et al., 2015). No genetic data are available on morels growing in the rest of the peninsula and, in particular, in the littoral areas where our investigation was conducted.

All the ascomata genotyped in this work are nested within the clade Mes-17 identified by Du et al. (2012a). Phylogeny of the specimens within this clade is controversial and the high number of misidentified GenBank records within the Esculenta group (Du et al., 2012a) does not facilitate the

identification to species. Buscot et al. (1996) molecularly identified this species as *Morchella spongiola* Boud. and, later, a number of authors accepted this epithet (Kellner et al., 2005; Kanwal et al., 2011; Barseghyan et al., 2012). Based on priority criteria, Richard et al. (2015) applied the name *Morchella vulgaris* (Pers.) Gray to the collections nested within Mes-17 clade and indicated a number of synonyms: *M. dunensis* (Castañera & G. Moreno) Clowez, *M. acerina* Clowez & C. Boulanger, *M. andalusiae* Clowez & L. Romero, *M. anthracina* Clowez & Vanhille, *M. lepida* Clowez & Franç. Petit, *M. robiniae* Clowez and *M. spongiola*. In the same work, two members belonging to this clade were described from coastal areas of northern France, including the withe dune habitat with *A. arenaria*. Later, *M. dunensis* was elevated to species rank by Loizides et al. (2016) because most of the Mediterranean and coastal collections nested in a subclade within *M. vulgaris*.

Morels in the Esculenta group seem to feed on the roots of a broad range of plant species forming different types of structures such as mycorrhiza-like structure (Pilz et al., 2007) or mycelial muffs (Buscot and Roux, 1987). In the study site, plant/ascoma association through sand-mycelium cords seem to be the main nutritional strategy to support the fruiting phase. These structures were found to finish their growth on a variety of underground plant organs differing in size, type (roots or rhizomes) and species. Similar structures, called radisciscleroti, were found by Stefani et al. (2010) under ascomata of *Morchella tomentosa* M. Kuo a year after a forest fire in a black spruce forest in Alaska. Radisciscleroti were described as whitish, ramified and compact hypogeous structures, 5-15 mm in diameter, which represent time-resistant nutrient reservoir that could last a few years and support ascoma production. On the contrary, the fungus-host connective structures described in this work were fragile and ephemeral sand aggregates, with a likely seasonal lifetime. However, all the assumptions made on nutritional strategies of this morel group need to be confirmed and explored more in depth by molecular methods such as stable isotopes (Hobbie et al., 2016).

On white dunes, the ascomata were mostly associated with the rhizomatous plants *C. capitatus, A. cronopifolia* and *H. radicata* while a heterogeneous range of hosts, both herbaceous and woody plants, were found in the surroundings anthropized areas. Sand dunes represent an optimal condition to study the morel-plant interaction in the wild because the sandy soil allows to follow the growth of the sand-mycelium cords in the soil without damage these mycelial structures.

Considering the limited extension of the habitat under investigation and the amount of ascoma production, the study area seems to be particularly suitable for morel production. Coastal dune systems in Emilia-Romagna have an important role as ecosystem service to the coast. They provide a number of services such as coastal defense, groundwater and carbon storage, water purification and preserving biodiversity (Drius et al., 2016). Unfortunately, these systems are highly threatened by a number of factors such as urbanization and tourism (Sytnik and Stecchi, 2015). In Spain, Moreno et al. (2013) listed *M. dunensis* among the fungal species threatened by the loss of coastal ecosystems due to climate changes. Although these fungi are also able to colonize heavily anthropized environments, the protection of the dune system is imperative for preserving genetic diversity within the local population of wild morels.

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