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Report 2 – OFEV (Contract 19.0061.PJ / 3DA231CCE)

Compatibility between morel isolates of different origins

Final report

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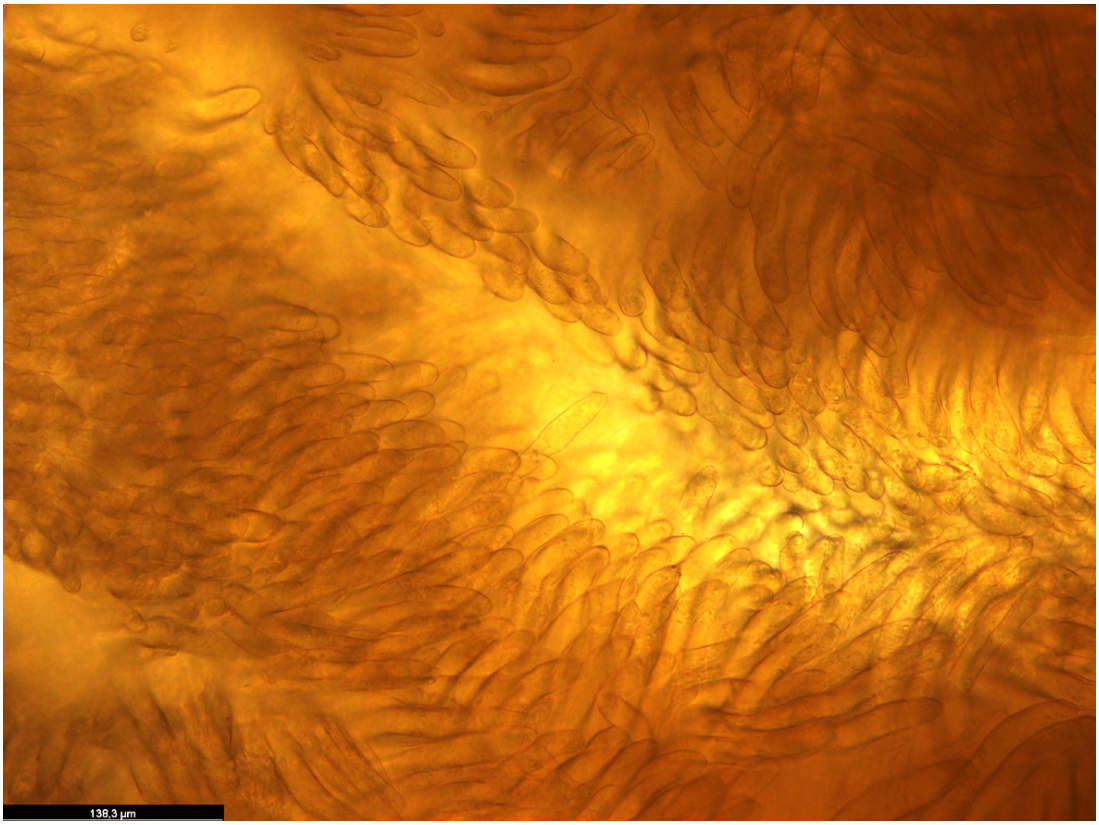

Light microscopic picture of black morel asci. © Melissa Cravero

Laboratory of Microbiology
Tél : +41 (0) 32 718 22 44
pilar.junier@unine.ch
saskia.bindschedler@unine.ch
www.unine.ch/lamun/home.html

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The FOEN is an agency of the Federal Department of the Environment, Transport, Energy and Communications (DETEC).
Contractor: Laboratory of Microbiology, University of Neuchatel
Author: Pilar Junier, Saskia Bindschedler, Melissa Cravero, Patrick Hilpisch
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Executive summary – English version

The new assessment of the diversity of morels in Switzerland yielded very interesting outcomes. The results highlighted that the majority of the strains obtained corresponded to the Esculenta clade (58.42% of the fruiting bodies identified as belonging to yellow morel species). Although genetic characterization at clade level could be reliably conducted with any method, species-level identification was more complex. Standard identification using only ITS sequencing with BLAST pairwise alignment with the GenBank database provided incorrect results (data not shown). This approach is starting to be questioned as at least two third of the morel sequences in the GenBank database are wrongly attributed at the species level (Du et al. 2012). In this report, we used instead a polyphasic (multi-locus) analysis consisting of consensus from pairwise alignments to a trustworthy database that was created especially for morel identification (https://morchella.mycobank.org/) (Du et al. 2015). A reliable database and the use of multiple genetic markers is crucial. In addition, for species identification, morphology and ecology must be considered (Loizides et al. 2022). Our multi-locus genetic analyses revealed that at least 17 of our morel specimens could belong to new or undescribed (*Morchella* sp. Mes-24 and Mel-13) species. This highlights the poor knowledge of European, and more specifically Swiss morel populations, as compared to North American and Chinese ones. In the light of species invasiveness and establishment of foreign cultivars in Switzerland, it is important to highlight that *M. sextelata* was already collected in Northern America, Mexico, and Asia, while it is grown commercially in China (Du et al. 2012). To our knowledge, we report for the first time the occurrence of this species in Europe (specimen M21-82). In the Canton Valais, at least one specimen (M20-3) corresponded to *M. importuna*, another black morel that is widely cultivated in China but native to the USA. In addition, our two-locus analysis indicated that seven specimens could belong to the North American *M. angusticeps*. But when investigating four genes in the specimens that were collected at the same area, morels were attributed to an unknown species. Knowing that specimens collected at the same area generally belong to the same species (Dalgleish and Jacobson 2005), we can hypothesize that the Swiss specimens that were initially identified as *M. angusticeps* actually belong to another closely related species native from Europe (or even Switzerland), which has not been described yet. The detection of these two (*M. importuna* and *M. sextelata*) or three (in addition to *M. angusticeps*) species could suggest not yet described biological invasion events that had already occurred within Swiss populations. However, regarding the risks of importing American or Asian morel species in Switzerland, our results indicate that a massive invasion and/or the colonization of ecological niches that would lead to a loss of Swiss morels biodiversity is not likely, or at least geographically limited.

Another important aspect required to understand the invasive potential of morels is their biotic interactions with other organisms in soils. Black morels are mostly saprophytic, while yellow morels often need interactions with plants for the fructification to appear (Du et al. 2015). Thus, black morels should be better adapted in terms of wide-range invasiveness. To better understand how yellow morels could interact with plants, we co-inoculated maize with two different strains from the Esculenta clade in a greenhouse. Although we observed mycelium in close proximity to plant roots, NGS revealed no *Morchella* in soil or root samples. We conducted this experiment in spring and reiterated it in winter with pine trees (data not shown). However, due to an invasive insect attack on the plants, pines had to be destroyed. When they were alive, no fruiting body appeared, and no visual difference was noticed between control pines and those co-inoculated with morels. Regarding interactions with microorganisms in soils, a very recent article has investigated the microbial soil communities associated with *M. sextelata* in cultivation in greenhouse systems (Benucci et al. 2019). Interestingly, these authors found that bacteria such as *Pedobacter, Pseudomonas, Stenotrophomonas*, and *Flavobacterium* were found to comprise the core bacteriome of *M. sextelata* ascocarps. Many of these groups were found in the ascocarps of the wild Swiss morels from 2019. We also demonstrated that the associated bacterial communities (bacteriomes) of mycelium and sclerotia differed strongly from those of ascocarps, in particular due to the high abundance of *Pseudomonas*. Bacteria are considered to be a factor promoting primordia differentiation and ascocarp growth, and may help suppress diseases that could affect crop yield in cultured systems (Q. Liu et al. 2018). Therefore, understanding the relationship of morels with bacteria is of high interest. Some of these *Pseudomonas* could be cultured as pure cultures and they

established different types of interactions (from beneficial to antagonist) with diverse morel hosts. These interactions were also tested with the Chinese strains that were co-inoculated with *Pseudomonas* spp. isolated from Swiss morel mycelia. The results indicated that one of the *Pseudomonas koreensis* strains inhibited mycelial growth in all the tested strains, while the other had antagonistic interactions (mycelial growth inhibition and triggering melanin and sclerotia production) with only one cultivar. The other bacterial strains had no antagonistic effect on any of the cultivars. These interactions are similar to those observed in the case of Swiss morels and therefore, it can be expected that bacterial-fungal interactions would not hinder the development of Chinese morels in Swiss soils. To further study the interactions between morel mycelium and bacterial strains isolated from Swiss specimens, two of them (*P. koreensis* B33.4 and VD-NE ext white) were tagged with GFP and co-inoculated with bacteria-free mycelia originating from the single-ascospore cultures. These experiments will be continued beyond the duration of this mandate.

Executive summary – French version

La nouvelle évaluation de la diversité des morilles en Suisse a donné des résultats très intéressants. Tout d'abord, elle a mis en évidence que la majorité des spécimens de morilles obtenus correspondaient au clade Esculenta (58,42% des fructifications identifiées comme appartenant à des espèces de morilles jaunes). Bien que la caractérisation génétique au niveau du clade ait pu être menée de manière fiable, l'identification précise au niveau de l'espèce était plus complexe. L'identification standard utilisant uniquement les séquences ITS, avec les alignements par BLAST dans la base de données GenBank, a fourni des résultats incorrects et/ou imprécis (données non présentées). Cette approche avait déjà été remise en question, car au moins deux tiers des séquences de morilles dans la base de données GenBank ne correspondent pas à la bonne espèce de morille (Du et al. 2012). Dans ce rapport, nous avons donc utilisé une analyse polyphasique (multi-locus) consistant en un consensus à partir d'alignements BLAST à une base de données fiable qui a été créée spécialement pour l'identification des morilles (https://morchella.mycobank.org/) (Du et al. 2015). Une base de données fiable et l'utilisation de marqueurs génétiques multiples sont cruciales. De plus, pour l'identification des espèces de champignons, la morphologie de la fructification et l'écologie doivent être prises en compte (Loizides et al. 2022). Nos analyses génétiques polyphasiques ont révélé qu'au moins 17 de nos spécimens de morilles pourraient appartenir à des espèces nouvelles ou encore non décrites (*Morchella* sp. Mes-24 et Mel-13). Ceci met en évidence la faible connaissance des populations de morilles européennes, et plus spécifiquement suisses, par rapport aux populations nord-américaines et chinoises. Concernant l'établissement de cultivars étrangers en Suisse, il est important de souligner que *M. sextelata* a déjà été collectée en Amérique du Nord, au Mexique et en Asie, alors qu'elle est cultivée commercialement en Chine (Du et al. 2012). A notre connaissance, nous rapportons pour la première fois la présence de cette espèce enEurope (spécimen M21-82). Dans le canton du Valais, au moins un spécimen (M20-3) correspondait à *M. importuna*, une autre morille noire largement cultivée en Chine mais en réalité originaire des Etats-Unis. En outre, notre analyse à deux locus a indiqué que sept spécimens pourraient appartenir à l'espèce nord-américaine *M. angusticeps*. Mais en examinant quatre gènes dans des spécimens collectés dans la même région, les morilles ont été attribuées à une espèce inconnue. Sachant que les spécimens collectés dans la même zone appartiennent généralement à la même espèce (Dalgleish et Jacobson 2005), nous pouvons émettre l'hypothèse que les spécimens suisses qui ont été initialement identifiés comme *M. angusticeps* peuvent appartenir à une autre espèce étroitement apparentée originaire d'Europe (ou même de Suisse), qui n'a pas encore été décrite. La détection de ces deux (*M. importuna* et *M. sextelata*) ou trois (en plus de *M. angusticeps*) espèces pourrait également suggérer des événements d'invasion biologique encore non décrits qui se seraient déjà produits au sein des populations suisses. Afin d'étudier ce point plus en détail, une approche utilisant des outils de génétique des populations tels que les microsatellites devrait être réalisée. Cependant, en ce qui concerne les risques d'importation d'espèces de morilles américaines ou asiatiques en Suisse, nos résultats indiquent qu'une invasion massive et/ou la colonisation de niches écologiques qui conduirait à une perte de la biodiversité des morilles suisses ne s'est pas encore produite ou est du moins limitée géographiquement.

Un autre aspect important nécessaire pour comprendre le potentiel invasif des morilles est leurs interactions biotiques avec d'autres organismes dans les sols. Les morilles noires sont principalement saprophytes, tandis que les morilles jaunes ont souvent besoin d'interactions avec les plantes pour que la fructification apparaisse (Du et al. 2015). Ainsi, les morilles noires devraient être mieux adaptées en termes d'invasivité à grande échelle. Pour mieux comprendre comment les morilles jaunes pourraient interagir avec les plantes, nous avons co-inoculé du maïs avec deux souches différentes du clade Esculenta en serre. Bien que nous ayons observé du mycélium à proximité des racines des plantes, le NGS (Next Generation Sequencing) n'a révélé aucune morille dans les échantillons de sol ou de racines. Nous avons mené cette expérience au printemps et l'avons réitérée en hiver mais avec des pins (données non présentées). Cependant, en raison d'une attaque d'insectes invasifs sur les plantes, les pins ont dû être détruits. Lorsqu'ils étaient vivants, aucun corps fructifiant n'est apparu, et aucune différence visuelle n'a été remarquée entre les pins contrôle et ceux co- inoculés avec des morilles. Concernant les interactions avec les microorganismes dans les sols, un article récent a étudié les communautés microbiennes du sol associées à *M. sextelata* en

culture dans des systèmes de serre (Benucci et al. 2019). Il est intéressant de noter que ces auteur·es ont constaté que des bactéries telles que *Pedobacter, Pseudomonas, Stenotrophomonas* et Flavobacterium constituaient le bactériome central des ascocarpes de *M. sextelata*. Plusieurs de ces groupes ont été trouvés dans les ascocarpes des morilles sauvages suisses de 2019. Nous avons également démontré que les communautés bactériennes associées au mycélium et aux sclérotes différaient fortement de celles des ascocarpes, notamment en raison de la forte abondance de *Pseudomonas*. Les bactéries sont considérées comme un facteur favorisant la différenciation des primordia et la croissance des ascocarpes, et peuvent aider à réduire les maladies qui pourraient affecter le rendement des cultures dans les systèmes cultivés (Q. Liu et al. 2018). Par conséquent, la compréhension de la relation des morilles avec les bactéries est d'un grand intérêt. Certains de ces *Pseudomonas* ont pu être cultivés en culture pure et ils ont établi différents types d'interactions (de bénéfiques à antagonistes) avec diverses morilles. Ces interactions ont également été testées avec les isolats de morilles chinoises qui ont été co-inoculés avec des *Pseudomonas* isolés de mycéliums de morilles suisses. Les résultats indiquent qu'une des souches de *Pseudomonas koreensis* a inhibé la croissance mycélienne de toutes les souches testées, tandis que l'autre a eu des interactions antagonistes (inhibition de la croissance mycélienne et déclenchement de la production de sclérotes et de mélanine) avec un seul cultivar. Les autres souches bactériennes n'ont eu aucun effet antagoniste sur aucun cultivar. Ces interactions sont similaires à celles observées dans le cas des morilles suisses et, par conséquent, on peut s'attendre à ce que les interactions bactérie-champignons n'entravent pas le développement des morilles chinoises dans les sols suisses. Afin d'étudier plus en détail les interactions entre le mycélium de morille et les souches bactériennes isolées de spécimens suisses, deux d'entre elles (*P. koreensis* B33.4 et VD-NE ext white) ont été transformées pour exprimer de la GFP (*Green Fluorescent Protein*) et co-inoculées avec des mycéliums exempts de bactéries provenant des cultures d'ascospores. Ces expériences seront poursuivies au-delà de la durée du présent mandat.

Executive summary – German version

Die neue Bewertung der Morchelvielfalt in der Schweiz hat sehr interessante Ergebnisse geliefert. Zunächst einmal zeigte sich, dass die Mehrheit der erhaltenen Morcheln der Esculenta-Klade angehörte (58,42 % der Fruchtkörper wurden als Gelbmorchelarten identifiziert). Obwohl die genetische Charakterisierung auf Kladenebene zuverlässig durchgeführt werden konnte, war die genaue Identifizierung auf Artniveau komplexer. Die Standardidentifizierung nur anhand von ITS-Sequenzen mit paarweisem BLAST-Abgleich in der GenBank-Datenbank lieferte falsche und/oder ungenaue Ergebnisse (Daten nicht gezeigt). Infolgedessen wird dieser Ansatz zunehmend in Frage gestellt, da mindestens zwei Drittel der Morchelsequenzen in der GenBank-Datenbank fälschlicherweise der Art zugeordnet werden (Du et al. 2012). In diesem Bericht haben wir stattdessen eine polyphasische (Multi-Locus) Analyse verwendet, die aus dem Abgleich der Sequenz zu einer vertrauenswürdigen Datenbank besteht, die speziell für die Identifizierung von Morcheln erstellt wurde (https://morchella.mycobank.org/) (Du et al. 2015). Eine zuverlässige Datenbank und die Verwendung mehrerer genetischer Marker sind entscheidend. Darüber hinaus müssen für die Artbestimmung bei Pilzen die Morphologie des Fruchtkörpers und die Ökologie berücksichtigt werden (Loizides et al. 2022). Unsere genetischen Multi-Locus Analysen ergaben, dass mindestens 17 unserer Morchelproben zu neuen oder noch unbeschriebenen Arten (Morchella sp. Mes-24 und Mel-13) gehören könnten. Dies unterstreicht die unzureichende Kenntnis der europäischen und insbesondere der schweizerischen Morchelpopulationen im Vergleich zu den nordamerikanischen und chinesischen. Im Hinblick auf die Invasivität der Art und die Etablierung ausländischer Kultursorten in der Schweiz ist es wichtig zu betonen, dass M. sextelata bereits in Nordamerika, Mexiko und Asien gesammelt wurde, während sie in China kommerziell angebaut wird (Du et al. 2012). Unseres Wissens berichten wir zum ersten Mal über das Vorkommen dieser Art in Europa (Exemplar M21-82). Im Kanton Wallis entsprach mindestens ein Exemplar (M20-3) der M. importuna, einer anderen Schwarzmorchel, die in China weit verbreitet ist, aber eigentlich in den USA heimisch ist. Darüber hinaus zeigte unsere Zwei-Lokus-Analyse, dass sieben Exemplare zur nordamerikanischen Art M. angusticeps gehören könnten. Bei der Untersuchung von vier Genen bei diesen Exemplaren, die im gleichen Gebiet gesammelt wurden, wurden die Morcheln jedoch einer unbekannten Art zugeordnet. Da wir wissen, dass Exemplare, die im selben Gebiet gesammelt wurden, im Allgemeinen zur selben Art gehören (Dalgleish und Jacobson 2005), können wir die Hypothese aufstellen, dass die Schweizer Exemplare, die zunächst als M. angusticeps identifiziert wurden, zu einer anderen, eng verwandten, in Europa (oder sogar in der Schweiz) heimischen Art gehören könnten, die noch nicht beschrieben wurde. Der Nachweis dieser zwei (M. importuna und M. sextelata) oder drei (zusätzlich zu M. angusticeps) Arten könnte auch auf noch unbeschriebene biologische Invasionsereignisse hindeuten, die bereits in Schweizer Populationen stattgefunden haben. Um dies genauer zu untersuchen, sollte ein Ansatz mit populationsgenetischen Methoden wie Mikrosatelliten verwendet werden. In Bezug auf die Risiken der Einfuhr amerikanischer oder asiatischer Morcheln in die Schweiz deuten unsere Ergebnisse jedoch darauf hin, dass eine massive Invasion und/oder die Besiedlung ökologischer Nischen, die zu einem Verlust der biologischen Vielfalt der Schweizer Morcheln führen würde, noch nicht stattgefunden hat oder zumindest geografisch begrenzt ist.

Ein weiterer wichtiger Aspekt, der zum Verständnis des invasiven Potenzials von Morcheln erforderlich ist, sind ihre biotischen Interaktionen mit anderen Organismen im Boden. Schwarze Morcheln sind meist saprophytisch, während gelbe Morcheln oft Interaktionen mit Pflanzen benötigen, damit die Fruchtbildung stattfinden kann (Du et al. 2015). Schwarze Morcheln sollten daher besser an eine weiträumige Invasion angepasst sein. Um besser zu verstehen, wie gelbe Morcheln mit Pflanzen interagieren könnten, haben wir Mais im Gewächshaus mit zwei verschiedenen Stämmen aus der Esculenta-Gruppe impfen lassen. Obwohl wir Myzelien in unmittelbarer Nähe von Pflanzenwurzeln beobachteten, wiesen NGS keine Morcheln in Boden- oder Wurzelproben nach. Wir führten diesen Versuch im Frühjahr durch und wiederholten ihn im Winter, allerdings mit Kiefern (Daten nicht gezeigt). Aufgrund eines invasiven Insektenbefalls der Pflanzen mussten die Kiefern jedoch vernichtet werden. Als sie noch lebten, erschienen keine Fruchtkörper, und es wurde kein visueller Unterschied zwischen den Kontrollkiefern und den mit Morcheln geimpften Kiefern festgestellt. Was die Interaktionen mit Mikroorganismen im Boden betrifft, so

wurden in einem kürzlich erschienenen Artikel die mikrobiellen Bodengemeinschaften untersucht, die mit M. sextelata beim Anbau in Gewächshäusern assoziiert sind (Benucci et al. 2019). Interessanterweise fanden diese Autoren heraus, dass Bakterien wie *Pedobacter, Pseudomonas, Stenotrophomonas* und *Flavobacterium* das Kernbakteriom von *M. sextelata* Ascocarps zu einem grossen Teil das Bakeriom ausmachen. Viele dieser Gruppen wurden auch in den Ascocarps der Schweizer Wildmorcheln von 2019 gefunden. Wir konnten auch zeigen, dass sich die assoziierten Bakteriengemeinschaften (Bakteriome) von Myzelien und Sklerotien stark von denen der Ascocarps unterscheiden, insbesondere aufgrund der hohen Abundanz von Pseudomonas. Bakterien gelten als ein Faktor, der die Differenzierung der Primordien und das Wachstum des Ascocarps fördert, und können dazu beitragen, Krankheiten zu unterdrücken, die den Ernteertrag in Kultursystemen beeinträchtigen könnten (Q. Liu et al. 2018). Daher ist das Verständnis der Beziehung zwischen Morcheln und Bakterien von großem Interesse. Einige dieser Pseudomonas Spezies konnten als Reinkulturen kultiviert werden und zeigten verschiedene Arten von Wechselwirkungen (von symbiontisch bis antagonistisch) mit verschiedenen Morchelwirten. Diese Wechselwirkungen wurden auch mit chinesischen Morchel-Isolaten getestet, die mit Pseudomonas spp. aus Schweizer Morchelmyzelien geimpft wurden. Die Ergebnisse zeigten, dass einer der *Pseudomonas koreensis* Stämme das Myzelienwachstum bei allen getesteten Stämmen hemmte, während die anderen antagonistischen Wechselwirkungen (Hemmung des Myzelienwachstums und Auslösung der Sklerotienproduktion und der Melaninproduktion) mit nur einer Sorte zeigte. Die anderen Bakterienstämme hatten keine antagonistische Wirkung auf eine Sorte. Diese Wechselwirkungen ähneln denen, die im Fall von Schweizer Morcheln beobachtet wurden, und daher ist zu erwarten, dass Bakterien-Pilz-Interaktionen die Entwicklung von Chinesischen Morcheln in Schweizer Böden nicht behindern werden. Um die Wechselwirkungen zwischen Morchelmyzelium und Bakterienstämmen, die aus Schweizer Exemplaren isoliert wurden, weiter zu untersuchen, wurden zwei von ihnen (*P. koreensis* B33.4 und VD-NE ext white) mit GFP markiert und mit bakterienfreien Myzelien aus den Einzelsporenkulturen co-inokuliert. Diese Versuche werden über die Dauer dieses Mandats hinaus fortgesetzt.

Abstract

The aim of this follow-up mandate performed by the laboratory of microbiology was to continue the research to assess the invasive potential of Chinese morel strains. In this mandate we started by extending the collection of Swiss morels by sampling different geographic regions in Switzerland. The goal was to evaluate a potential endemism as well as the distribution of specific clades among Swiss morel populations. While Chinese species used for outdoor cultivation belong to the black morel clade, we observed an over-representation of yellow morels in our initial sampling in Canton Neuchâtel. In addition, we followed with the study of the bacteria associated to Swiss morels in comparison to allochthonous strains. We also assessed the potential of the Chinese morel strains to interact with Swiss morel-associated soil bacteria. In this final report, we present the results of the four Modules included in this mandate, namely: Module 5 - Isolation of additional morel strains from population of different Swiss climatic regions; Module 6 - Compatibility test between Chinese and Swiss strains, as well as between Swiss strains of different geographic origins; Module 7 - Identification of bacteria naturally associated to Swiss morels and comparison with Chinese strains; and Module 8 - Testing the interaction of the Chinese morels with bacteria associated to Swiss morels. From 2019 to 2021, we were able to collect 143 black and yellow morel fruiting bodies from eight different cantons (NE, FR, VD, JU, BE, SO, LU, TI), in addition to four Chinese cultivars. This biological material was used both for experiments presented in "Report 1 - Analysis of the invasive potential of Morels, Modules 1-4" and in the present report (Modules 6-8). All the experiments we have carried out aimed at better defining the invasiveness and hybridization potential of non-native species, in particular focusing on Chinese cultivars that are already used by Swiss farmers. In addition to this aspect, bacterial communities associated with ascocarps, mycelia and sclerotia of Swiss morels were characterized. We were also able to isolate bacterial strains from mycelial isolates and we confronted them to both Swiss and Chinese morel isolates. This revealed the existence of beneficial and antagonist interactions. In addition to the objectives initially defined in this mandate, we investigated maize-morel interactions in a greenhouse, in order to highlight a potential mycorrhizal lifestyle in a selection of Swiss morel isolates. The experiments and knowledge acquired to fulfill this report led to the redaction of one scientific publication in which the contribution by the FOEN is acknowledged (Cailleau et al. 2022, *in preparation*).

1. Module 5: Isolation of additional morel strains from populations of different Swiss climatic regions

1.1 Rationale

As reviewed in the OFEV report "Microbial invasions, with focus on Morels (*Morchella* spp.); 2019", evaluating the risks and impacts of the introduction of alien species in a country is crucial. In the light of biodiversity conservation, this evaluation is important for pathogenic species that can cause significant damage to the environment, but also for non-pathogenic species, such as morels. Indeed, non-pathogenic fungi can cause changes in the environment by affecting ecological interactions and/or replacing native species via competition for the natural resources. In case of intentional introduction, as it is the case for Chinese morel cultivars, the monitoring of the cultivation sites is even more crucial, along with a global survey of morels biodiversity in Switzerland (Report OFEV-20191206). For this reason, we pursued morel sampling in 2020 and 2021.

Even though the sampling campaign planned for spring 2020 had to be largely canceled due to mobility restrictions in the first phase of the Covid-19 pandemic, we got in contact with morel hunters in the Cantons Valais, Vaud, and Jura. Through this initial collaboration we received nine fruiting bodies (six from Cantons Valais, two from Jura and one from Vaud). In spring 2021, the Swiss Mycologic Societies Union (https://www.vsvp.com) was contacted to call for morel fruiting bodies from all over Switzerland. Thanks to the collaboration of its members and other direct collaborations, we received 91 morel ascocarps from Cantons Neuchâtel (58 specimens), Fribourg (6), Vaud (6), Jura (1), Bern (6), Solothurn (1), Luzern (8) and Ticino (5). In total, for the morel hunting seasons 2019-2021, this resulted in 143 Swiss morel specimens (indicated as M19-#; M20-#; M21-#, with the # corresponding to the respective ascocarp specimen). In addition, we also obtained four Chinese

cultivars (PYL, PYT, NEU142, NEU143) directly from morel farmers. Finally, this resulted in a total of 147 biological specimens. Each morel specimen was genotyped for species determination. Our analysis revealed that yellow morels were predominant in Switzerland (80/143 Esculenta species). In addition, 38 specimens could not be determined to the species level based on the genetic markers used so far, indicating they could potentially belong to new species. Based on a state-of-the-art consensus on morel phylogeny (Loizides et al. 2022), further morphological (micro- and macroscopic) and genetical analyses will be needed to confirm this hypothesis.

1.2 Methods

Processing of the fruiting bodies

Ascocarps that were received in the lab were processed as follows: (1) imaging of the fruiting body, (2) description of the ascocarp, (3) sampling of two pieces of fungal tissue (one for direct DNA extraction and one for inoculation on culture media in order to isolate a mycelial phase) and (4) freezing of the rest of the ascocarp for further analyses.

ITS, *RPB1, RPB2* and *TEF-1α* amplification

Each fruiting body was identified to the species level initially based on sequence identity of the internal transcribed spacer (ITS) region including the 5.8S rDNA. For species that could not be identified with this locus only, the genes encoding the RNA polymerase II large subunits A (*RPB1*) and B (*RPB2*) and translation elongation factor 1-α (*TEF-1α*) were sequenced to be used in a four-locus phylogenetic analysis. DNA was directly extracted from about 10 mm^3 pieces of fresh or dry hymenia using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the protocol provided by the manufacturer. Eluted DNA was quantified with a Qubit kit (Invitrogen, USA) using the Broad Range buffer and reagent to determine the quantity of double-stranded DNA contained in each sample. DNA was then diluted with PCR-grade water to a concentration of 2 ng/µL to be used as a template in polymerase chain reactions (PCR). The primer sequences are available in Table 1. For each sample, the PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix (HighQu, Germany), 0.2 µM forward and 0.2 µM reverse primer and 1 µL of 2 ng/µL DNA. Amplifications were performed in a Thermo Scientific Arktik thermal cycler. The following parameters were used: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 62°C [ITS]; 55°C [*TEF1-α*]; 50°C [*RPB1* and *RPB2*] for 15 sec, 72°C for 15 sec), final elongation at 72°C for 2 min, end at 20°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100 mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator. PCR products were then purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified by Qubit. Final concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing.

Sequence analysis

The forward and reverse sequences obtained after Sanger sequencing were manually trimmed and assembled using BioEdit 7.2. The four gene sequences were analyzed with the polyphasic identification tool of the Westerdijk Fungal Biodiversity Institute (https://morchella.mycobank.org/page/Morchella_scenario), which consists in the pairwise alignment of the query sequences with a reliable *Morchella* database (Du et al. 2015). Queries of species indicating the higher similarity were used to identify the species of each morel specimen, using one to four genes.

Four Maximum Likelihood (ML) phylogenetic trees (black morels from 2019/2020; and 2021; yellow morels from 2019; and 2021) were computed following the General Time Reversible model (GTR) with Gamma distributed with Invariant sites (G+I) using 1000 bootstrap replicates. These trees were constructed using the ITS sequences generated, in addition to reference sequences (RS). Trees were rooted with a *Morchella rufobrunnea* as reference sequence. The most statistically supported trees

were kept. Specimens that were not segregated within any reference sequence were further analyzed; a four-locus phylogeny using the ITS-*RPB1-RPB2-TEF-1a* concatenated dataset was generated. The same parameters used for constructing the ITS phylogenies were used, but with only 100 bootstrap values due to CPU limitations. All phylogenies were generated with the software Mega version 11 (Kumar et al. 2018).

Table 1: *primer sequences used for the genetic analysis.*

Primer ID	Target region	Primer sequence 5'-3'	Reference
ITS1-forward	ITS 5.8S	CTTGGTCATTTAGAGGAAGTAA	(White et al. 1990)
ITS4-reverse	ITS 5.8S	TCCTCCGCTTATTGATATGC	(Gardes and Bruns 1993)
RPB1B-forward	*RPB1*	AACCGGTATATCACGTYGGTAT	(Du et al. 2012)
RPB1B-reverse	*RPB1*	GCCTCRAATTCGTTGACRACGT	(Du et al. 2012)
RPB2-7c-forward	*RPB2*	ATGGGYAARCAAGCYATGGG	(Liu et al. 1999)
RPB2-3053-reverse	*RPB2*	TGRATYTTRTCRTCSACCATRTG	(Reeb et al. 2004)
EF-2-forward	*TEF-1a*	AACATGATSACTGGTACYTCC	(Machuca et al. 2021)
EF-2218-reverse	*TEF-1a*	ATGACACCRACRGCRACRGTYTG	(Machuca et al. 2021)

1.3 Results and discussion

Species identification

Species identification within the genus *Morchella* is complex because it requires micro- and macromorphological characteristics, along with genetic analysis to distinguish cryptic species and genetically close species (Loizides et al. 2022). In addition to this, species definition in kingdom Fungi is still an open debate (Steenkamp et al. 2017). Morphology of the ascocarp varies greatly depending on environmental factors and developmental stages (Pilz et al. 2007). However, genetic analysis can also be biased depending on the dataset quality, the loci analyzed (Loizides et al. 2022), and the reference database (Du et al. 2012). For instance, it was established that the ITS is only able to identify 77.4% of the morel species globally, and that two thirds of the *Morchella* sequences uploaded in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) are misidentified (Du et al. 2012). To ameliorate genetically based identification, a polyphasic analysis tool specifically designed to identify *Morchella* species was generated by Du et al. (2015). In recent papers publishing new morel species, genes used for the genetic analysis include the ITS, *RPB1, RPB2* and the large subunit (LSU 25-28S) (Clowez et al. 2020) or the *TEF-1a* (Baroni et al. 2018). Generally, the ITS alone is enough to distinguish yellow morels (*Morchella* sp. sect. *Morchella*) (Du et al. 2012), however *M. fluvialis*, for instance, could only be distinguished from *M. esculenta* analyzing the *TEF-1a* (Clowez et al. 2014).

To identify our *Morchella* specimens collected in 2019, we re-analyzed the ITS sequences generated previously and started to generate RPB1, RPB2, *TEF-1a*, SSU (small subunit 18S) and LSU sequences for each of the 143 specimens. The specimens that were suspected to belong to a new or a non-native species will be precisely analyzed morphologically. To date, one (ITS) to four genes (ITS; *RPB1; RPB2; TEF-1a*) were used for species identification using the polyphasic identification tool (https://morchella.mycobank.org/page/Morchella_scenario). In addition, ITS-based phylogenies were generated.

Results varied depending on the genes that were considered for the analysis with the morel identification tool. For instance, specimen M19-41 was identified with 99.17% identity to the undescribed phylospecies Mel-13 using the four genes. However, based on the ITS and *RPB1* alone, M19-41 had a 100% identity to *M. deliciosa*. Analysis based on the *RPB2* sequence alone assigned the

same specimen to *M. pulchella, M. purpurascens, M. deliciosa*, *M. eximioides*, *Morchella* sp. Mel-13, Mel-14, Mel-21, Mel-34 (Elata clade) and even *Morchella* sp. Mes-18 (Esculenta clade), equally with 100% identity. Finally, based on the *TEF-1α* sequence, M19-41 had 100% identity with *M. pulchella, Morchella* sp. Mel-21 and Mel-34. Combining *RPB2* and *TEF-1α* only *Morchella* sp. Mel-21 and Mel-34 were retained. Including the sequence of *RPB1*, the identity to *Morchella* sp. Mel-34 was reduced by 0.13%. When including the ITS, the results changed and revealed the specimen belongs to *Morchella* sp. Mel-13 with 99.17% identity with two Chinese specimens (HKAS62891 and HKAS62892). This case-study was representative of the other morel specimens and revealed the importance of carrying multi-locus analyses for accurate species determination. In the future, the four genes (ITS; *RPB1; RPB2; TEF-1α*) in addition to the 18S and the 25-28S regions will be amplified to ensure correct species determination. Nonetheless, for clade (Black and Yellow morels) determination, the results of the ITS were considered accurate enough.

Over the 143 Swiss morel specimens (Figure 1 and Table 2), 63 belonged to the Elata (black morels) and 80 to the Esculenta (yellow morels) clades. So far, 57 specimens were determined with at least two genes. They belonged to the following species: *M. angusticeps* (7 specimens), *M. esculenta* (4), *M. eximioides* (3), *M. fluvialis* (1), *M. importuna* (1) and *M. pulchella* (1). In addition, the best match from five *Morchella* sp. specimens considering three to four genes was *M. hispaniolensis* (< 95%), which is a black morel species reported from the Dominican Republic (Baroni et al. 2018). However, as sequence identity was lower than 97%, these Swiss specimens could belong to a new species (Xu 2020). The same appears to be the case for seven specimens closely related to *Morchella* sp. M424-2, a morel species described from India for which only the ITS sequence is currently available. Results from the polyphasic determination also indicated 21 specimens (2 black and 19 yellow morels) had no species similarity with any of the specimens in the morel database. Thus, they could potentially correspond to new species, as well. Finally, five specimens were attributed to the undescribed species *Morchella* sp. Mel-13, the higher match being 99.51% similarity for the four genes. These morels were sampled in at least three populations from cantons Neuchâtel and Luzern, indicating we would have enough data to formally describe this as a new species (Loizides et al. 2022). In the 86 specimens that have been currently identified using only the ITS sequence, five *Morchella* sp. could not be attributed to any species and 11 had no similarity with any entry in the database. Two specimens were attributed to *M. tridentina*. As expected from Clowez et al. (2014), the ITS alone was able to distinguish two yellow morel species: *M. esculenta* and *M. fluvialis*. However, identity was the same for the two species with our queries. One black morel was determined as *M. sextelata* using the ITS region.

The ITS phylogenetic analyses (Figures 2-5) provided contrasted results compared to the polyphasic pairwise alignment. However, it allowed to precise the taxonomic affiliation of several specimens. M19-14, which had no similarity with any morel specimen in the Westerdijk database, was clustered with the undescribed *Morchella* sp. Mes-24. M19-15, which could also not be attributed to any species with the pairwise alignment, was defined as *M. palazonii* based on its position in the phylogenetic tree. M19-28 was clearly determined as *M. deliciosa*, although it was equally affiliated to *Morchella* sp. Mel-13 and *M. deliciosa* by the ITS-RPB2 alignments (100%). M20-7 segregated with *M. eohespera*. M21-7 matched with *M. fluvialis*, which could not be differentiated between *M. esculenta* and *M. fluvialis* in the pairwise alignment. As expected by the alignment, M21-20 segregated with *M. conifericola*, a European species that is still not completely resolved taxonomically (Loizides et al. 2022). M21-35, which was determined based on three genes as *Morchella* sp., could be attributed to *Morchella* sp. Mel-21 based on its position in the phylogenetic tree. Two specimens that were close to *M. hispaniolensis* (< 95% similarity) were close to *M. septentrionalis* in the phylogeny. The four-locus phylogeny (Figure 6) indicated M21-1 (*Morchella* sp. Mel-13) and M21-21 (*Morchella* sp.) were closely related to another group formed by M20-8 and M21-90 (*Morchella* spp.). M19-41 (Mel-13) was basal to these two subgroups, and closely related to *M. deliciosa* and *Morchella* sp. Mel-13, as expected. M19-2 and M19-11 (*Morchella* sp.) were segregated close to *M. eximioides*. Finally, M19-30 and M21-91 (*Morchella* sp.) were grouped near *M. brunnea*.

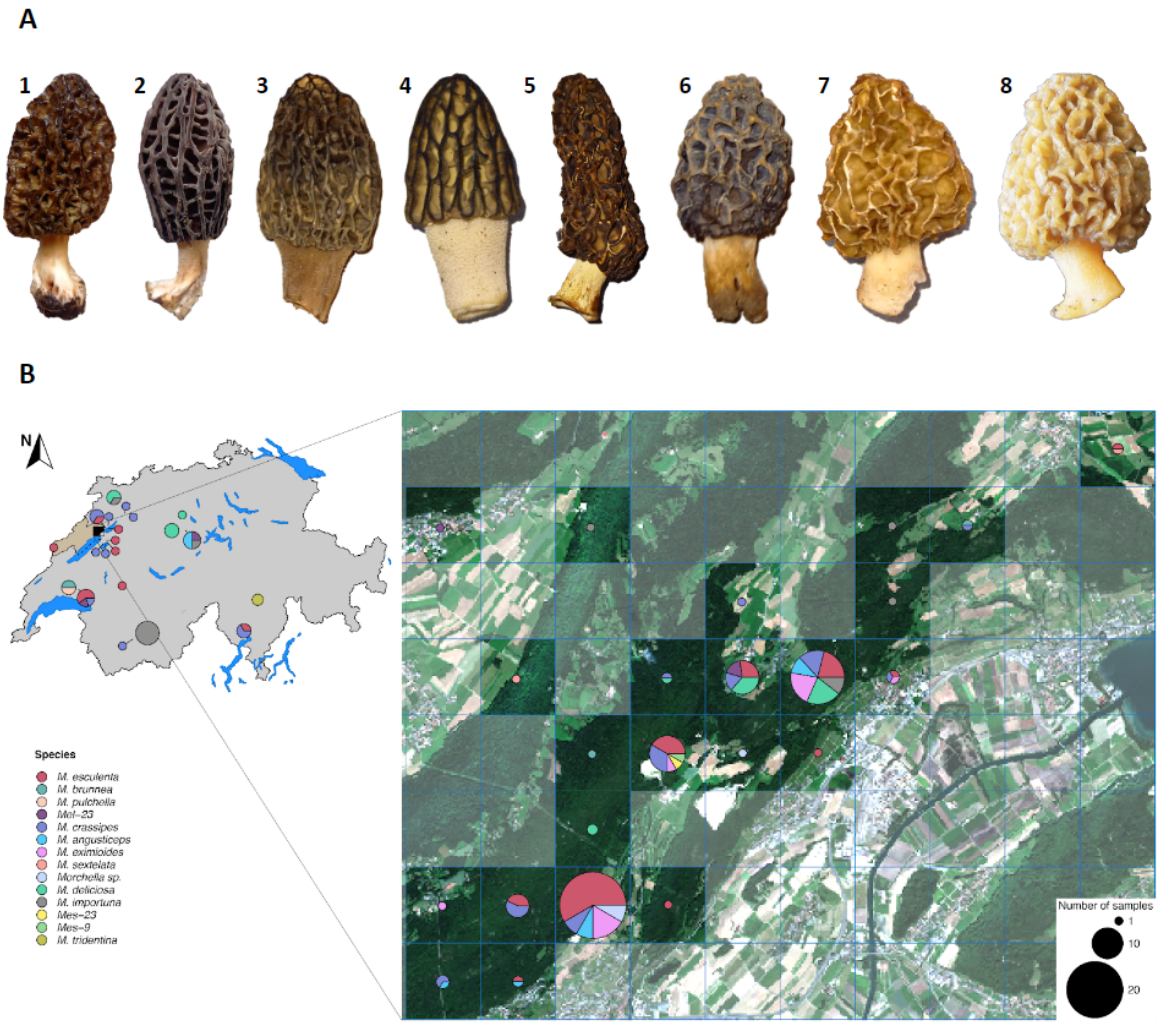

Figure 1: *A= Eight of the 143 ascocarps that were collected between 2019 and 2021 in Switzerland. Images are not to scale. 1=* Morchella *sp. M21-1; 2=* Morchella *sp. M21-2; 3=* Morchella *sp. M21-20; 4=* Morchella *sp. M21-48; 5=* M. esculenta *M21-89; 6=* M. fluvialis *M21-7; 7=*M. esculenta *M21-62; 8=* Morchella *sp. M19-23. B= Overview of morel sampling sites in Switzerland 2019-2021. Preliminary species identification obtained by ITS-based BLAST analysis is shown to indicate the distribution of the different species in the sampling locations. Canton Neuchâtel was magnified, since a majority of specimens originate from there.*

Table 2. *List of the morel specimens obtained between 2019 and 2021. The putative species of each biological specimen is indicated (PPIT= Polyphasic identification tool), in addition to the genes that were used for this identification. Clade, origin of the specimen and year of acquisition are given. For the Swiss strains (CH), the corresponding Canton is indicated.*

ID	PPIT-based species	ITS	RPB1	RPB2	TEFA	Clade	Origin	Year
PYL	*M. sextelata*	√	X	X	X	Elata	China	2020
PYT	*N.A.*	X	X	X	X	Elata	China	2020
NEU142	*M. sextelata*	√	X	X	X	Elata	China	2018
NEU143	*Morchella* sp. *Mel-8 < 88%*	√	X	X	X	Elata	China	2018
M19-1	*M. angusticeps 100%*	√	X	√	X	Elata	CH - NE	2019
M19-2	*M. hispaniolensis < 95%*	√	√	√	√	Elata	CH - NE	2019
M19-3	*M728 <95%*	√	X	√	X	Elata	CH - NE	2019
M19-4	*M. angusticeps*	√	X	√	X	Elata	CH - NE	2019
M19-5	*M. angusticeps*	√	X	√	X	Elata	CH - NE	2019
M19-6	*M. angusticeps*	√	X	√	X	Elata	CH - NE	2019
M19-7	*M. angusticeps*	√	X	√	X	Elata	CH - NE	2019

M19-8	*M. eximioides 100%*	√	X	√	X	Elata	CH - NE	2019
M19-9	*M. angusticeps 100%*	√	X	√	X	Elata	CH - NE	2019
M19-10	*M. angusticeps 100%*	√	X	√	X	Elata	CH - NE	2019
M19-11	*M. hispaniolensis < 95%*	√	√	√	√	Elata	CH - NE	2019
M19-12	*M. eximioides*	√	X	√	X	Elata	CH - NE	2019
M19-13	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-14	*No similarity*	√	√	√	√	Esculenta	CH - NE	2019
M19-15	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-16	*No similarity*	√	X	√	√	Esculenta	CH - NE	2019
M19-17	*No similarity*	√	√	√	√	Esculenta	CH - NE	2019
M19-18	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-19	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-20	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-21	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-22	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-23	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-24	*M. esculenta*	√	X	√	X	Esculenta	CH - NE	2019
M19-25	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-26	*M. esculenta*	√	X	√	X	Esculenta	CH - NE	2019
M19-27	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-28	*Morchella* sp. *Mel-13 /deliciosa 100%*	√	X	√	X	Elata	CH - NE	2019
M19-29	*Mel-13*	√	X	√	X	Elata	CH - NE	2019
M19-30	*M. hispaniolensis < 95%*	√	√	√	√	Elata	CH - NE	2019
M19-31	*M424-2 Morchella* sp. *< 95%*	√	√	√	√	Elata	CH - NE	2019
M19-32	*No similarity*	√	X	√	X	Elata	CH - NE	2019
M19-33	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-34	*M. esculenta*	√	X	√	X	Esculenta	CH - NE	2019
M19-35	*M. esculenta 100%*	√	X	√	X	Esculenta	CH - NE	2019
M19-36	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-37	*Morchella* sp.	X	X	√	X	Esculenta	CH - NE	2019
M19-38	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-39	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-40	*No similarity*	√	X	√	X	Esculenta	CH - NE	2019
M19-41	*Mel-13 99.17%*	√	√	√	√	Elata	CH - NE	2019
M19-42	*M. fluvialis*	√	√	√	X	Esculenta	CH - NE	2019
M19-43	*M. eximioides*	√	X	√	X	Elata	CH - NE	2019
M20-1	*Morchella* sp.	√	X	X	X	Elata	CH - VS	2020
M20-2	*Morchella* sp.	√	X	X	X	Elata	CH - VS	2020
M20-3	*M. importuna China 100% / Germany 99.87%*	√	√	X	√	Elata	CH - VS	2020
M20-4	*Morchella* sp.	√	X	X	X	Elata	CH - VS	2020
M20-5	*Morchella* sp.	√	X	X	X	Elata	CH - VS	2020
M20-6	*Morchella* sp.	√	X	X	X	Elata	CH - VS	2020
M20-7	*Morchella purpurascens 100%*	√	X	X	X	Elata	CH - VD	2020
M20-8	*M424-2 Morchella* sp. *96.11%*	√	√	√	√	Elata	CH - JU	2020
M20-9	*M. deliciosa*	√	X	X	X	Elata	CH - JU	2020
M21-1	*Morchella* sp. *Mel-13 99.51%*	√	√	√	√	Elata	CH - LU	2021
M21-2	*Morchella purpurascens 100%*	√	X	X	X	Elata	CH - LU	2021

M21-3	*Morchella purpurascens 100%*	√	X	X	X	Elata	CH - LU	2021
M21-4	*No similarity*	√	X	X	X	Elata	CH - LU	2021
M21-5	*No similarity*	√	X	X	X	Esculenta	CH - TI	2021
M21-6	*No similarity*	√	X	X	X	Esculenta	CH - TI	2021
M21-7	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - TI	2021
M21-8	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-9	*No similarity*	√	X	X	X	Elata	CH - NE	2021
M21-10	*M. eximioides/angusticeps*	√	X	X	X	Elata	CH - NE	2021
M21-11	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-12	*M. eximioides/angusticeps*	√	X	X	X	Elata	CH - NE	2021
M21-13	*M. eximioides/angusticeps*	√	X	X	X	Elata	CH - NE	2021
M21-14	*M424.2 Morchella sp. 96.11%*	√	X	√	√	Elata	CH - NE	2021
M21-15	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-16	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-17	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-18	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-19	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-20	*M. pulchella/conifericola*	√	X	X	X	Elata	CH - NE	2021
M21-21	*M424-2 Morchella sp. 96.11%*	√	√	√	√	Elata	CH - LU	2021
M21-22	*M. deliciosa 100%*	√	X	X	X	Elata	CH - LU	2021
M21-23	*M. deliciosa 100%*	√	X	X	X	Elata	CH - LU	2021
M21-24	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-25	*M. fluvialis*	√	X	X	X	Esculenta	CH - NE	2021
M21-26	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-27	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-28	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-29	*M. eximioides/angusticeps*	√	X	X	X	Elata	CH - NE	2021
M21-30	*M. eximioides/angusticeps*	√	X	X	X	Elata	CH - NE	2021
M21-31	*M. fluvialis 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-32	*M. fluvialis 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-33	*M. tridentina*	√	X	X	X	Elata	CH - TI	2021
M21-34	*M. tridentina*	√	X	X	X	Elata	CH - TI	2021
M21-35	*M424-2 Morchella sp. 96.08%*	√	√	√	X	Elata	CH - NE	2021
M21-36	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-37	*Morchella sp. Mel-13/deliciosa 100%*	√	√	√	X	Elata	CH - NE	2021
M21-38	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-39	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-40	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-41	*M424-2 Morchella sp. 96.11%*	√	√	√	√	Elata	CH - NE	2021
M21-42	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-43	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - BE	2021
M21-44	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - BE	2021
M21-45	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - BE	2021
M21-46	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-47	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-48	*M. pulchella*	√	√	√	X	Elata	CH - NE	2021
M21-49	*No similarity*	√	X	X	√	Esculenta	CH - BE	2021

M21-50	*M424-2 Morchella* sp. *96.10%*	√	X	√	X	Elata	CH - NE	2021
M21-51	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-52	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-53	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-54	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-55	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-56	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-57	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-58	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-59	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-60	*M. deliciosa*	√	X	X	X	Elata	CH - NE	2021
M21-61	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-62	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-63	*M. deliciosa 100%*	√	X	X	X	Elata	CH - NE	2021
M21-64	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-65	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-66	*M. deliciosa*	√	X	X	X	Elata	CH - LU	2021
M21-67	*No similarity*	√	X	X	X	Esculenta	CH - VD	2021
M21-68	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - VD	2021
M21-69	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - VD	2021
M21-70	*No similarity*	√	X	X	X	Esculenta	CH - VD	2021
M21-71	*No similarity*	√	X	X	X	Esculenta	CH - FR	2021
M21-72	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - FR	2021
M21-73	*No similarity*	√	X	X	X	Esculenta	CH - FR	2021
M21-74	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - FR	2021
M21-75	*M. esculenta 100%*	√	X	X	X	Esculenta	CH - BE	2021
M21-76	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-77	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-78	*No similarity*	√	X	X	X	Esculenta	CH - NE	2021
M21-79	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - SO	2021
M21-80	*No similarity*	√	X	X	X	Esculenta	CH - BE	2021
M21-81	*M. fluvialis*	√	X	X	X	Esculenta	CH - NE	2021
M21-82	*M. sextelata*	√	X	X	X	Elata	CH - FR	2021
M21-83	*Morchella* sp.	√	X	X	X	Elata	CH - FR	2021
M21-84	*M. deliciosa 100%*	√	X	X	X	Elata	CH - JU	2021
M21-85	*M. fluvialis/esculenta 100%*	√	√	X	√	Esculenta	CH - NE	2021
M21-86	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-87	*No similarity*	√	X	X	X	Esculenta	CH - NE	2021
M21-88	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-89	*M. fluvialis/esculenta 100%*	√	X	X	X	Esculenta	CH - NE	2021
M21-90	*M. hispaniolensis < 95%*	√	√	√	√	Elata	CH - VD	2021
M21-91	*No similarity*	√	√	√	√	Elata	CH - VD	2021

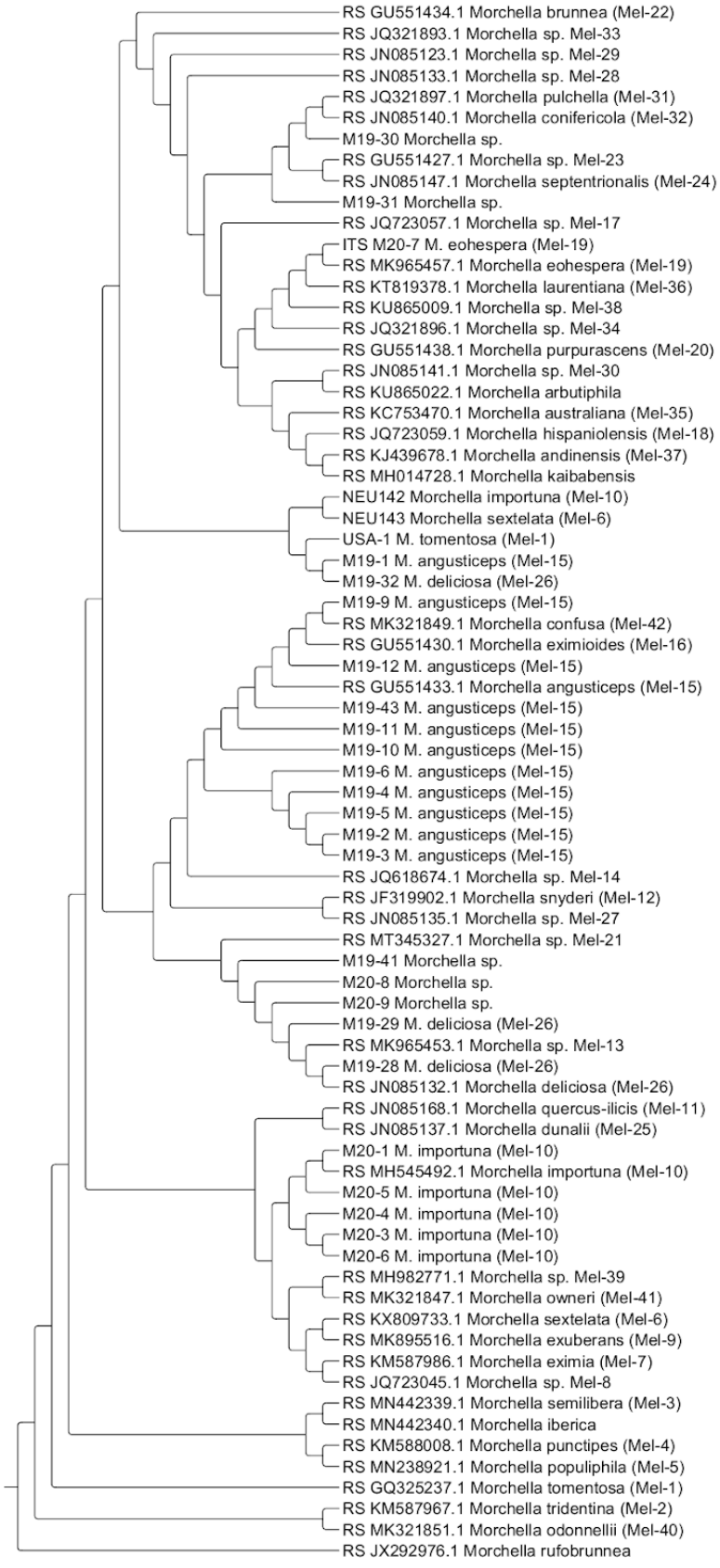

Figure 2: *ITS phylogeny of black morels sampled in 2019 (M19) and 2020 (M20) in Switzerland, in addition to reference sequences (RS). The putative species determined by the ITS alone is provided next to sample ID. This is a maximum Likelihood (ML) phylogenetic tree generated with the GTR + G + I model with 1000 bootstrap replicates. The tree was rooted with* M. rufobrunnea *RS.*

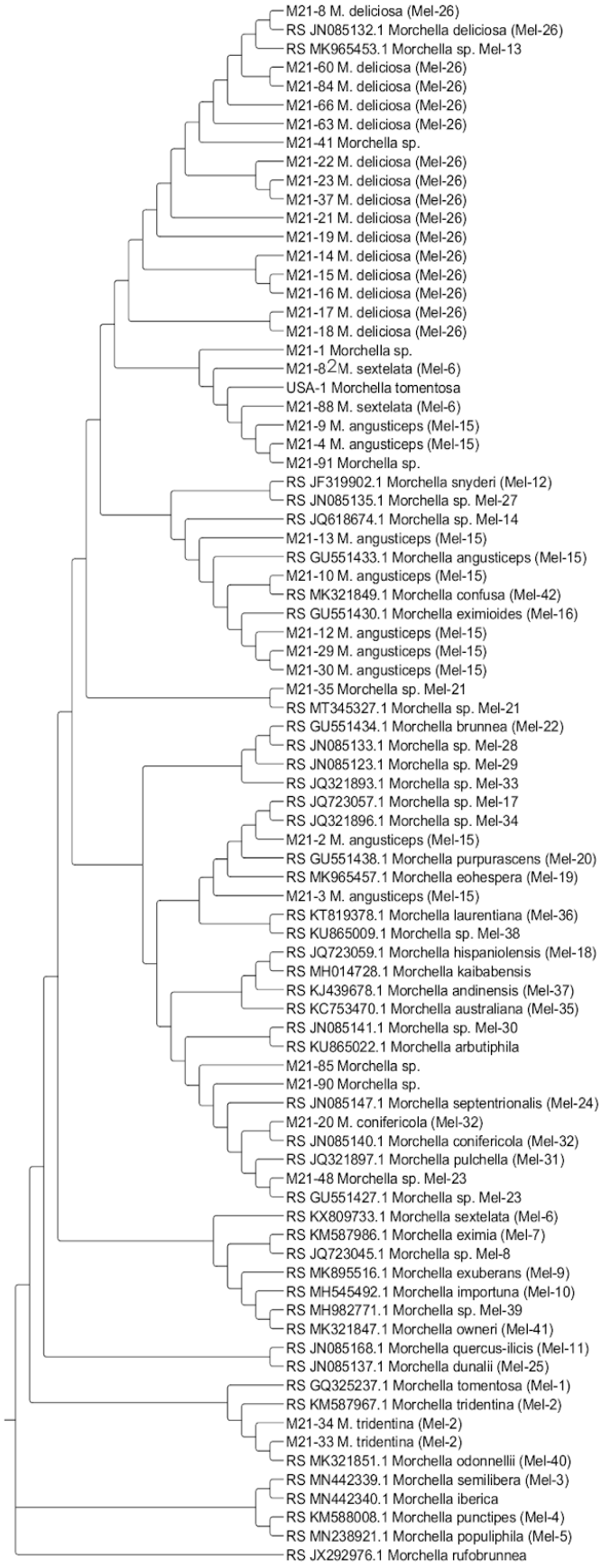

Figure 3: *ITS phylogeny of black morels sampled in 2021 (M21) in Switzerland, in addition to reference sequences (RS). The putative species determined by the ITS alone is provided next to sample ID. This is a maximum Likelihood (ML) phylogenetic tree generated with the GTR + G + I model with 1000 bootstrap replicates. The tree was rooted with* M. rufobrunnea *RS.*

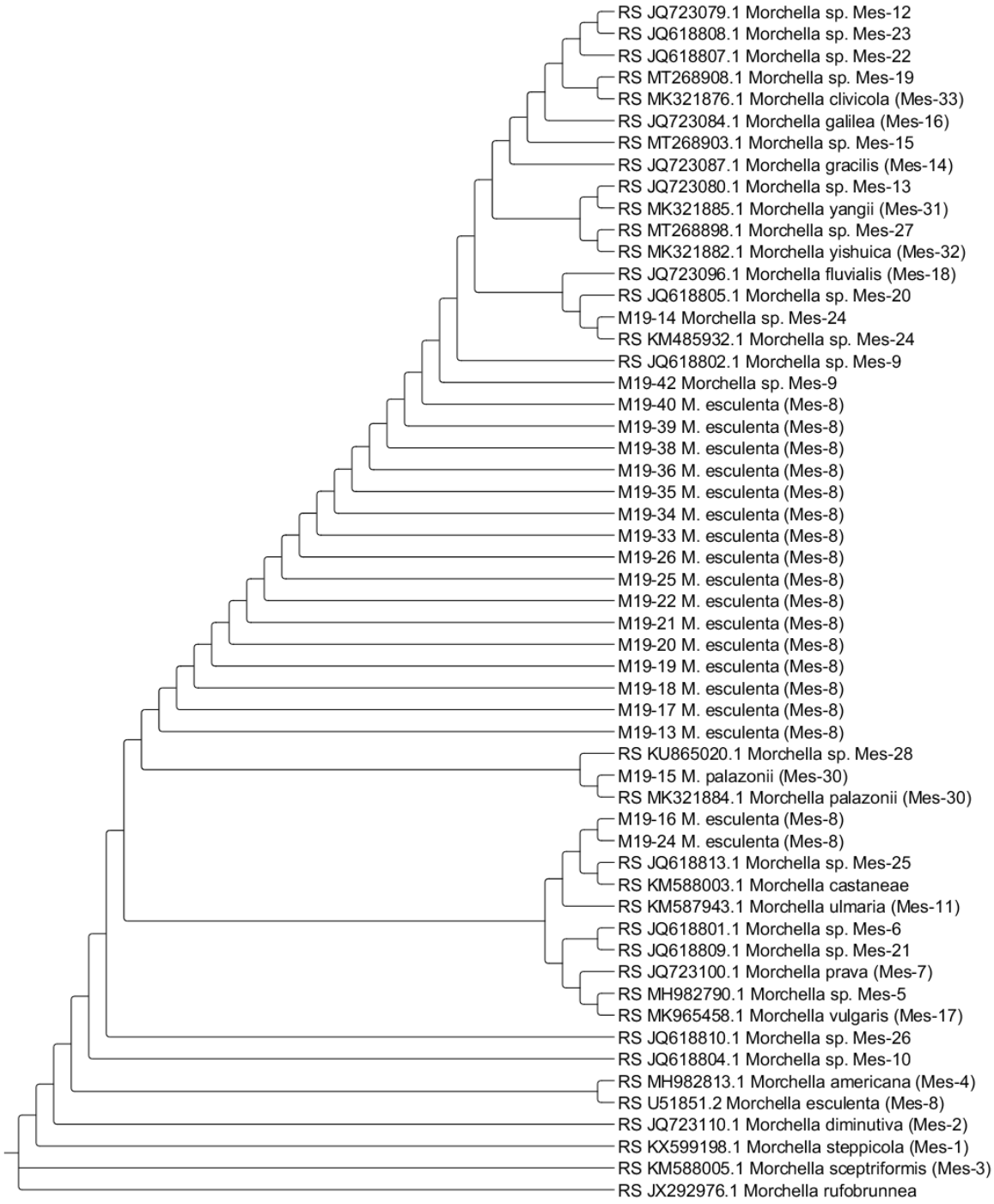

Figure 4: ITS phylogeny of yellow morels sampled in 2019 (M19) in Switzerland, in addition to reference sequences (RS). The putative species determined by the ITS alone is provided next to sample ID. This is a maximum Likelihood (ML) phylogenetic tree generated with the GTR + G + I model with 1000 bootstrap replicates. The tree was rooted with M. rufobrunnea *RS.*

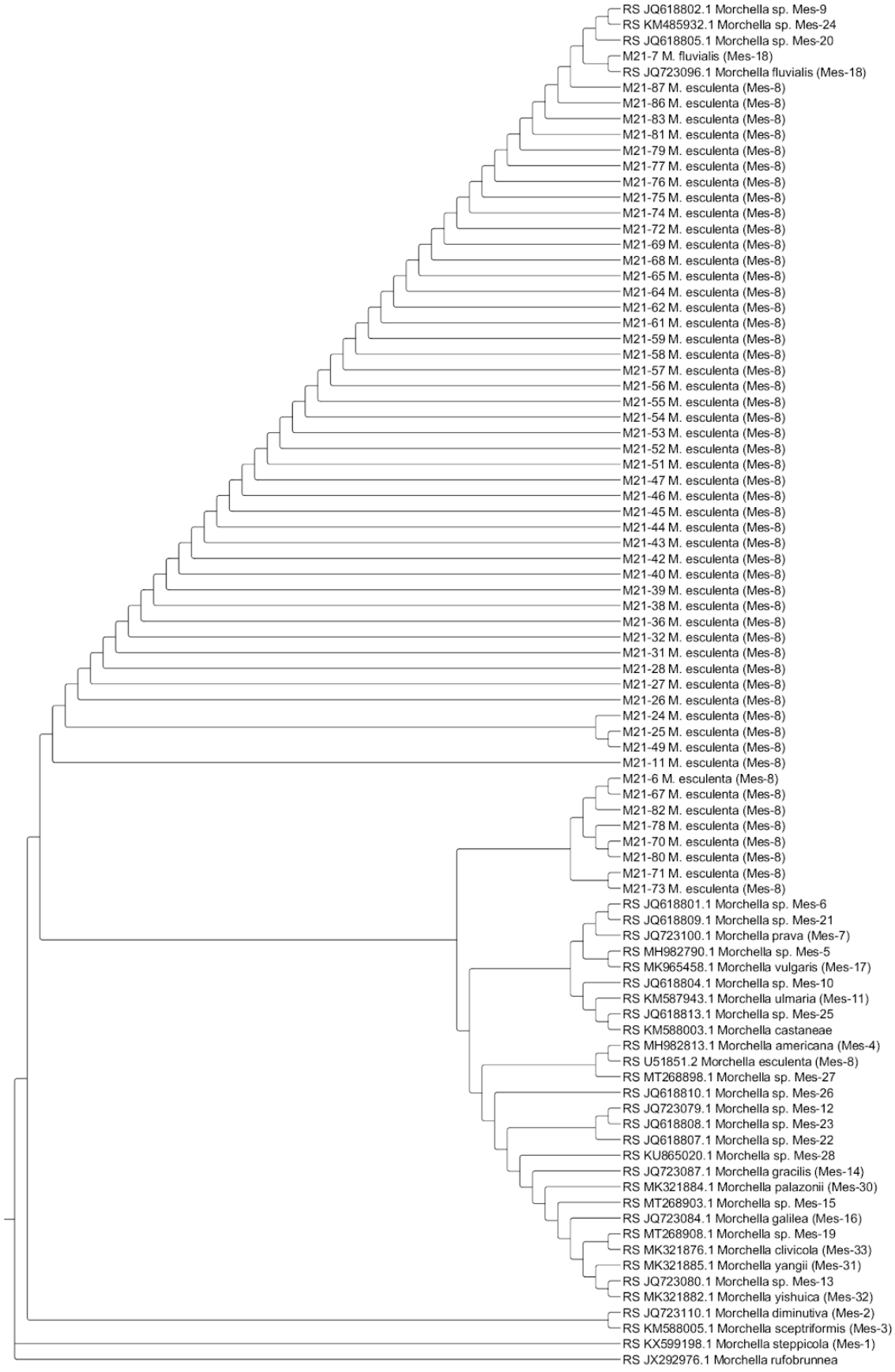

Figure 5: *ITS phylogeny of yellow morels sampled in 2021 (M21) in Switzerland, in addition to reference sequences (RS). The putative species determined by the ITS alone is provided next to sample ID. This is a maximum Likelihood (ML) phylogenetic tree generated with the GTR + G + I model with 1000 bootstrap replicates. The tree was rooted with* M. rufobrunnea *RS.*

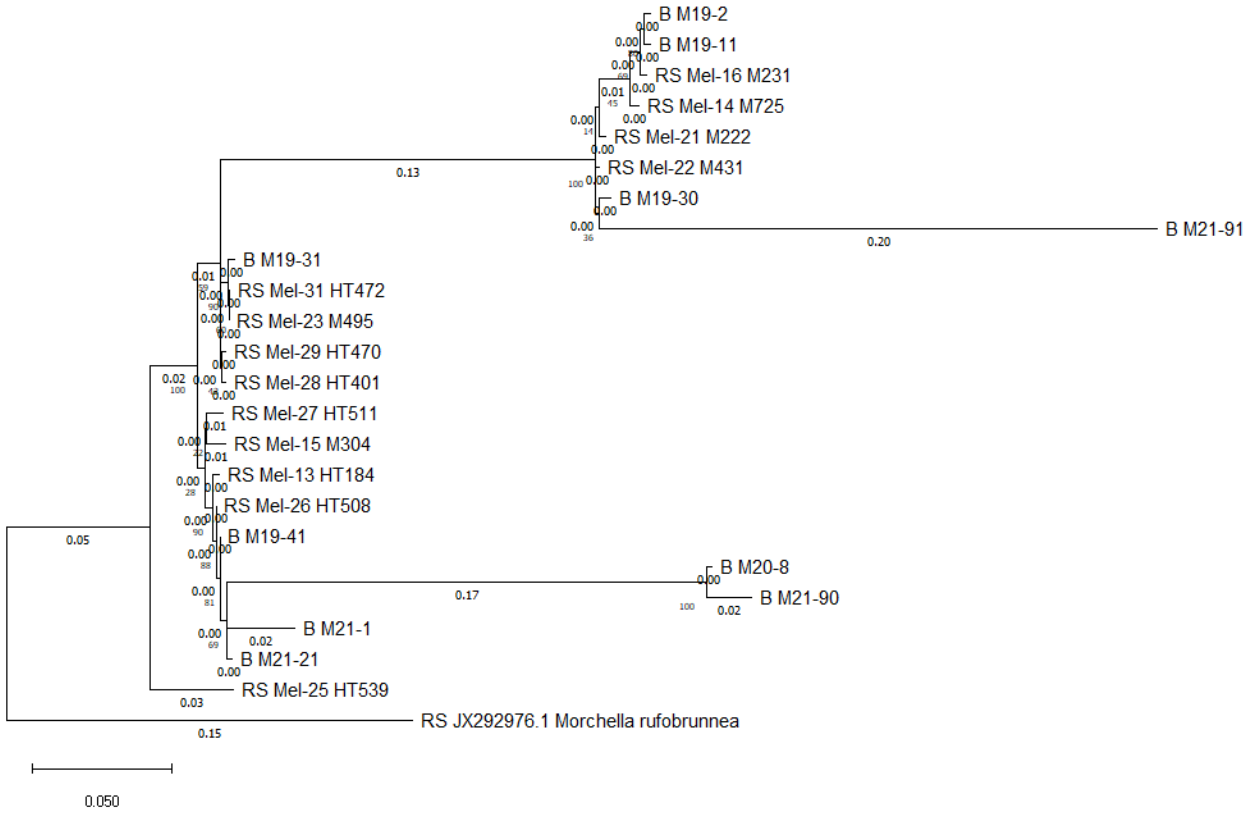

Figure 6: *Multi-locus phylogeny (ITS-RPB1-RPB2-TEF-1α) of suspected new species (indicate by the prefix B), in addition to reference sequences (RS). This is a maximum Likelihood (ML) phylogenetic tree generated with the GTR + G + I model with 100 bootstrap replicates. The tree was rooted with* M. rufobrunnea *RS.*

1.4 Conclusion

The sampling and genotyping of fruiting bodies from various Swiss regions highlights the importance of expanding the geographical range of the study (**Milestone M2-1**). In Cantons Bern and Fribourg, only yellow morels were obtained, while in Jura, Luzern and Valais only black morels were collected. The largest fraction of our collection (2019-2021) comes from Canton Neuchâtel, where most of the strains collected corresponded to the yellow (Esculenta) clade (59/101). Species determination varied significantly depending on the method used (**Milestone M2-2**). Our analyses coupled to the literature available to-date indicate that combined genetic and morphological methods are the most relevant in the case of morels. In addition, using multiple genes instead of a single marker for the genetic analysis appears required (Loizides et al. 2022). To determine morel species with precision, six genetic markers (ITS; *RPB1; RPB2; TEF-1α*; SSU rRNA; LSU rRNA) will be sequenced for each species. The species that were suspected to be new to science (M19-30, M19-31, M20-8, M20-9, M21-14, M21-21, M21-35, M21-41, M21-48, M21-50, M21-85, M21-90) or undescribed (M19-14, *Morchella* sp. Mes-24; M19-28, M19-29, M19-41, M21-1 *Morchella* sp. Mel-13) will be analyzed into more details concerning their morphology (cap and stipe, ascospores and hyphae), in order to prepare specific scientific publications associated with their description and detection in Switzerland. Finally, these biological specimens also led to the successful isolation of morel strains from different geographical regions in Switzerland (Milestone M2-1). This material is used in further experiments in this mandate and in mandate 1.

2. Module 6: Compatibility test between Chinese and Swiss isolates

2.1 Rationale

In order to establish sexual compatibility between two morel isolates three elements must be considered: (1) the reproductive strategy (heterothallism or secondary homothallism) of the species, (2) the mating types of the individual strains, and (3) the phylogenetic distance between them. The process of sexual reproduction in *Morchella* spp. is precisely described in "Report 1 - Analysis of the invasive potential of Morels", Module 2. Briefly, both black and yellow morels appear to be able to reproduce by three reproductive strategies: heterothallism (requiring the encounter of two distinct sexually compatible individuals), secondary homothallism (self-fertility), and unisexual reproduction (haploid meiosis; production of sterile ascocarps). To be compatible, two heterothallic individuals need to have opposite mating types (MAT; MAT1-1 or MAT1-2), to produce a fruiting body resulting from sexual reproduction (Du and Yang 2021).

To determine the two first compatibility parameters, mycelial cultures derived from a single ascospore must be generated. In those, which must correspond to a haploid genome, the characterisation of the MAT locus can be precisely done. Mating type detection can be done by MAT-PCR followed by gel electrophoresis. The generation of single-ascospore cultures and MAT determination are described in Report 1, Module 3 (**Milestone M2-2**). However, we were able to generate ascospore-derived cultures from the Swiss morels only, as the Chinese specimens (PYL, PYT, NEU142, NEU143) were provided directly as mycelial inocula and not as fruiting-bodies. The MAT analysis of the single-ascospore cultures revealed that the black morel *M. angusticeps* M21-2 was heterothallic with a 1:2 (MAT1-1:MAT1-2) ratio among the ascospores of a single fruiting-body. In contrast, another black morel, *Morchella* sp. M21-48, was mainly heterothallic but contained ascospores carrying both MAT types (3/20 spores analyzed), putatively indicating secondary homothallism. The mating type analysis in the inocula-derived mycelial isolates was also conducted in Report 1 (Module 2). Briefly, among the Chinese inocula, *M. sextelata* NEU143 and PYL contained both MAT, while *M. importuna* NEU142 and PYT contained MAT1-2 only. These results indicate that the *M. sextelata* inoculum is potentially a secondary homothallic cultivar, hence a self-fertile mycelium. On the other hand, *M. importuna* would need to encounter individuals carrying the opposite mating-type, i.e., a MAT1-1 individual. However, since *M. sextelata* possesses both MAT, it may theoretically be compatible with closely-related native black morels carrying either of the opposite mating type.

The third point that is important to consider about sexual compatibility is the phylogenetic relationship between the confronted individuals. Two specimens belonging to the same species will indeed be compatible. However, interspecific reproduction exists, and gives rise to hybrid lines. So far, hybridization events were observed between seven black morel species (*M. exuberans, M. importuna*, Mel-13, *M. eohespera, M. eximioides*, Mel-21 and Mel-34) (Du et al. 2019) but have never been reported within the Esculenta clade, or between black and yellow morels. Thus, we considered as potentially sexually compatible only the species within the same clade. To test this, Chinese isolates were confronted to Swiss isolates in three to four different chemically defined media (Angle pH6, Angle pH7, Angle and milk, Malt agar) and in a soil-like substrate. The details of the experimental procedure and the results are presented in Report 1, Module 2 (**Milestone M2-3 and M2-4**). Therefore, in this module, in order to go further into compatibility testing, we evaluated the ability of sexually compatible morel isolates to form a symbiotic ectomycorrhizal relationship with plants, in order to assess whether this stage may be required for fruiting-body formation in yellow morels. Ectomycorrhizal symbiosis, or at least an interaction with plant roots, has been reported as an important feature in the life cycle of yellow morels (Du et al. 2015). Mycorrhiza-like interactions with pines (Dahlstrom et al. 2000) and herbaceous plants were observed and may promote fruiting body formation (Baynes et al. 2012; Yu et al. 2016). To do this, we first placed our effort in developing a method to produce a spawn that could be used for further tests in soil and plant colonization, in order to approach natural conditions. This spawn was then used to inoculate maize plants in a greenhouse to study morel-maize interactions.

2.2 Methods

Selection of the morel specimens

For these experiments, two isolates from the Esculenta clade (M19-16 and M19-34) were used. In the genotyping, isolate M19-34 appeared to preferentially carry the MAT-1-1 idiomorph, while strain M19-16 carried the complementary idiomorph (MAT-1-2; Report 1).

Spawn production

The substrate to prepare a spawn was prepared by mixing cooked wheat with soil that was collected in proximity to morels ascocarps in 2019. Filter-bearing autoclavable plastic bags especially designed for fungal growth were used. Each bag was filled with 90 g of cooked wheat, 40 g of soil, and 25 mL of distilled water. This mixture was then sterilized by autoclaving. For the inoculation, pieces of mycelium previously grown on potato dextrose agar medium (PDA; 39 g/L) were cut and transferred into the bags. Three bags were inoculated with strain M19-34, three with strain M19-16 and three with both strains. After 12 days of growth at 23°C in the dark, spawns were ready to be used as inoculants. Samples were kept for microscopic observations.

Sweet corn cultivation and spawn inoculation

For the mycorrhization assays, sweet corn was selected as the host plant. This choice was made since effective mycorrhization between this plant species and *Morchella* has been reported in the literature (Yu et al. 2016). In addition to this, the initial testing started in late spring, which was not an ideal timing to work with tree seedlings. The substrate for sweet corn growth was composed of 50% compost, 30% wood fiber (mainly conifers), and 20% sand and was autoclaved twice before sowing. Sweet corn seeds (strain Sansonnet d'Or) were germinated in 1 L pots in a greenhouse at the Botanical Garden of Neuchâtel in early June 2020. After 14 days of plant growth, the pots were inoculated with the 12-day-old spawns by placing 2 teaspoons of spawn at 1 cm depth at the two sides of the plants (Figure 7) and covered with the displaced substrate. Twenty pots were inoculated with the spawn from each individual strain and the co-culture. The remaining pots were not inoculated and served as controls.

Figure 7: *Inoculation of morel spawn next to a sweet corn plant.*

Investigation of plant-fungal interactions

Plant roots were sampled at 7, 15, 36, and 58-days post inoculation (dpi). At the first three sampling time points, four plants per treatment were randomly chosen on site to be analyzed: their roots were separated from the aerial parts, washed under running water to remove soil particles and longitudinal and cross sections were used for the preparation of microscope slides in order to detect (ecto)mycorrhizal structures. For 7 and 15 dpi root samples, a direct staining with cotton blue lactophenol was used. For 36 and 58 dpi samples a technique that was initially developed for arbuscular mycorrhizae was used (Vierheilig et al. 1998) : roots were boiled in KOH 10% for 5 min, rinsed under running water, boiled in a solution of 5% ink-vinegar (5 mL blue ink in 100 mL vinegar)

for 3 min, and then immersed in acidified water (water with a few drops of acetic acid) for 20 min. Root hairs were then placed on microscope slides and fixed with glycerol. All slides were observed with a light microscope to detect mycorrhizal structures. At 58 dpi, aerial parts of healthy plants were cut and weighted on site. Flowering individuals were counted. Three pots per treatment were randomly chosen to collect substrate and roots. The latter were washed and used both to prepare microscope slides (as described above) and for DNA extractions.

Genetic analysis

The frozen roots obtained in the fourth sampling (58 dpi) were thawed. 250-500 mg of wet biomass were randomly sampled for molecular analyses. The same mass of soil was also collected, as well as mycelia from the two *Morchella* isolates that were grown in potato dextrose agar (PDA). DNA extractions were performed using the Fast DNA Extraction Spin Kit for Soil (MP Biomedicals, USA). The extracted genetic material was then amplified with PCR using the fungal ITS1-F and ITS4 primers (Module 5, Methods) and the amplification was verified by gel electrophoresis (1.2% agarose). 25µL of each PCR product concentrated at 4ng/µL was sent to Fasteris (Geneva, Switzerland) for Next Generation Sequencing (NGS).

2.3 Results and discussion

Spawn production

The two *Morchella* isolates used for this experiment formed visually different spawns. Isolate M19-34 produced a dense white mycelium that covered all the substrate, while the mycelium of isolate M19-16 was less dense and brownish. Both formed mycelial-soil aggregates of about 0.5 to 2 cm in diameter. In the spawn inoculated with the two strains, the white mycelium of isolate M19-34 was widespread, while the darker mycelium of isolate M19-16 formed only few aggregates (Figure 8). In addition, isolate M19-34 formed some soft brownish tissues and isolate M19-16 formed several orange/brown sclerotia. Stereoscope and light microscope observations showed that these structures consisted of dense aggregates of mycelium (Figure 9).

Figure 8: *Pictures of the three spawns that were produced. A= M19-34; B= M19-16; C= M19-34 + M19-16.*

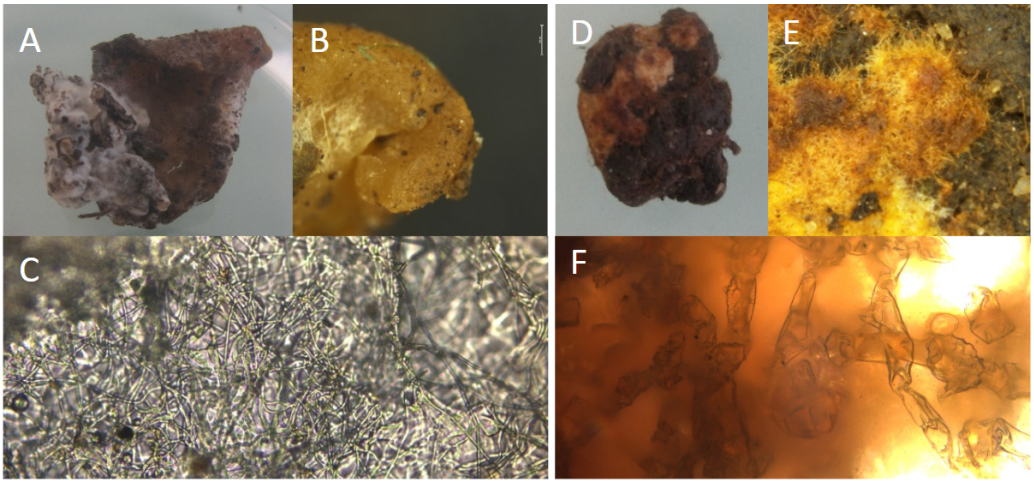

Figure 9: *Pictures of the three spawns that were produced. A= M19-34, naked eye; B= M19-34, stereomicroscope 0.75x; C= M19-34, light microscope 10x, DI water; D= M19-16, naked eye; E= M19-16, stereomicroscope 0.75x; E= M19-16, light microscope 20x, DI water.*

Effect of morel spawn on maize growth

No significant difference in the wet aerial biomass of the plants was observed between the treatments, but controls (i.e., non-inoculated plants) showed a larger variability (Figure 10). The number of flowering plants per treatment out of the seven individuals varied from one (control) to four (inoculation with individual strains). For the co-culture, three plants flowered.

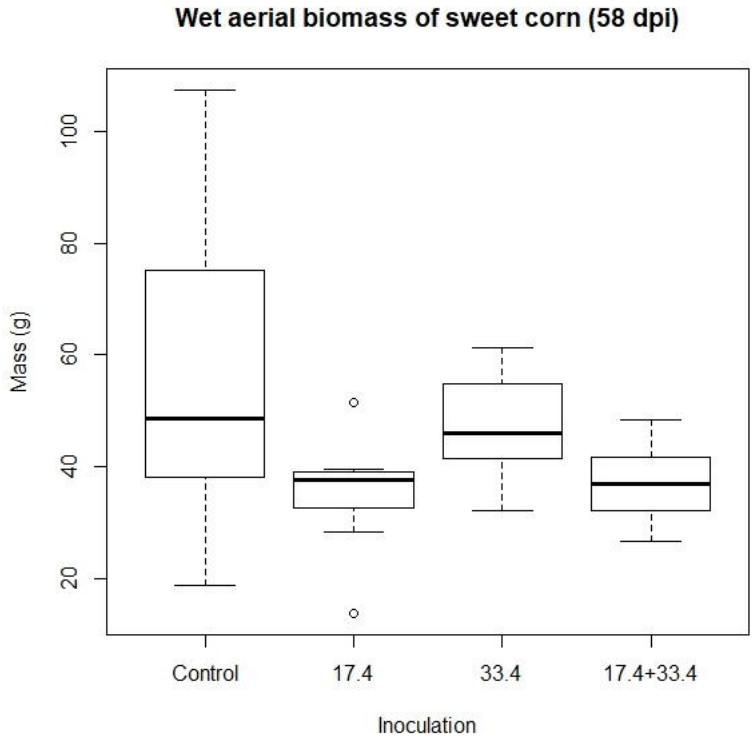

Figure 10: *Wet weight aerial biomass of sweet corn at 58 days post-inoculation (dpi). The sweet corn plants were inoculated with spawns of Morchella esculenta strains M19-16 (17.4) and M19-34 (33.4), as well as co-cultures of the two strains (M19-16 + M19-34). A control without inoculation was also prepared. The values correspond to means and standard deviations of 7 individual measurements.*

Plant-fungal interactions

No fungal colonization of roots was detected by microscopic observations of the root sections of the first two samplings (7 and 15 dpi), indicating the growth period tested might be too short for the establishment of a mycorrhiza-like interaction. In the third sampling (36 dpi), fungal hyphae were detected in close contact with the roots in all treatments, including controls (Figure 11). This indicated that the mycelium observed probably did not belong to the inoculated *Morchella*. No clear structures suggesting either a parasitic or a mutualistic interaction were highlighted.

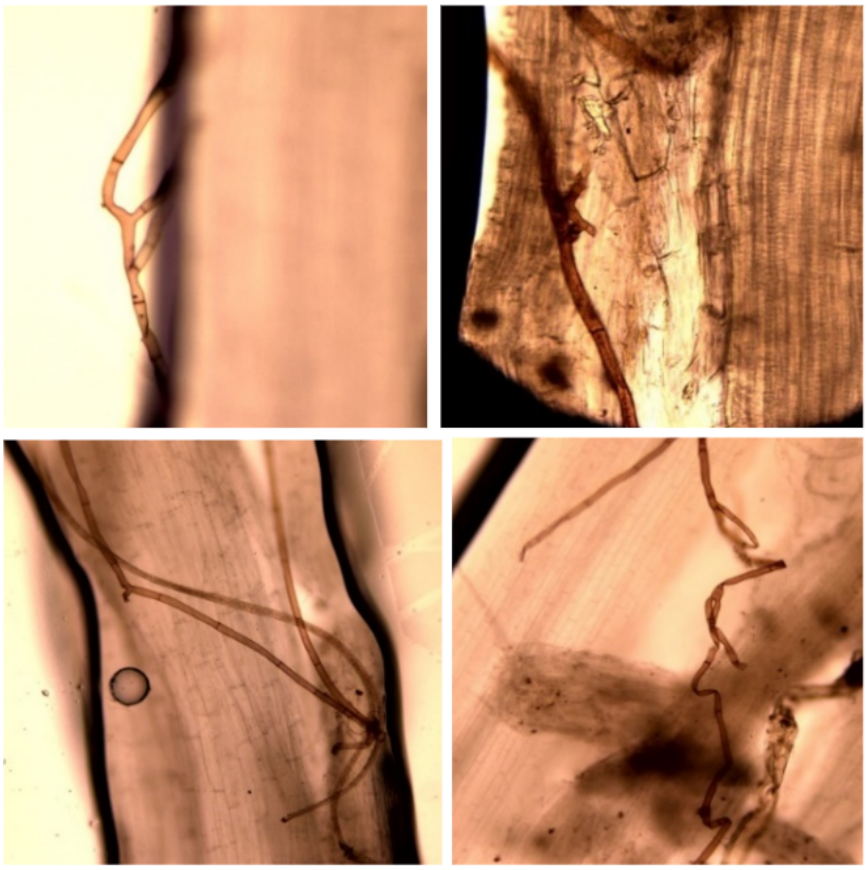

Figure 11: *Microscopic observations of fungal hyphae in close contact with roots at 36 dpi. From top left, clockwise, examples of root sections from treatments M19-16, M19-34 , M19-16 + M19-34 , and control.*

Genetic analysis

The profiles corresponding to the pure cultures of both strains (positive controls; bottom right in the Figure 12) showed a bright band corresponding to an amplified product of a length between 500-750 base pairs (bp). Almost all root samples had bands of equal length in their profiles, except for one sample from the co-culture. Concerning soil samples, bands of equal length were present in treatments M19-34 (2/3 replicates), M19-16 + M19-34 (2/3 replicates) and control (1/3 replicates), while the three profiles of treatment M19-16 were empty. In order to obtain further insights on the taxonomic identity of the fungi corresponding to these positive signals, samples were sequenced using next-generation sequencing (NGS). This allowed identifying the fungal taxa that were associated with the roots and in the soil. Unexpectedly, NGS revealed no *Morchella* in any of the soil or root samples (Figure 13). Instead, *Cephalotrichum* and *Mortierella* were the most represented genera in soil while Fusarium and *Cephalotrichum* were the most represented in roots. *Cephalotrichum* spp. are Sordariomycetes (Ascomycetes) mainly found in soil and rhizosphere soils from forests, crops or grassland*; Mortierella* spp. are Mortierellomycetes (Mortierellomycota) mainly found in soil and roots in forests and grasslands; *Fusarium* spp. also are Sordariomycetes, mostly found in soil, roots and shoots in forests, grasslands and croplands (https://globalfungi.com/). No significant difference was noticed between the three different treatments, in both sample types. The *Morchella* inocula probably died early in the culture, because of unsuitable environmental conditions or were over-competed by the fungal genera cited above.

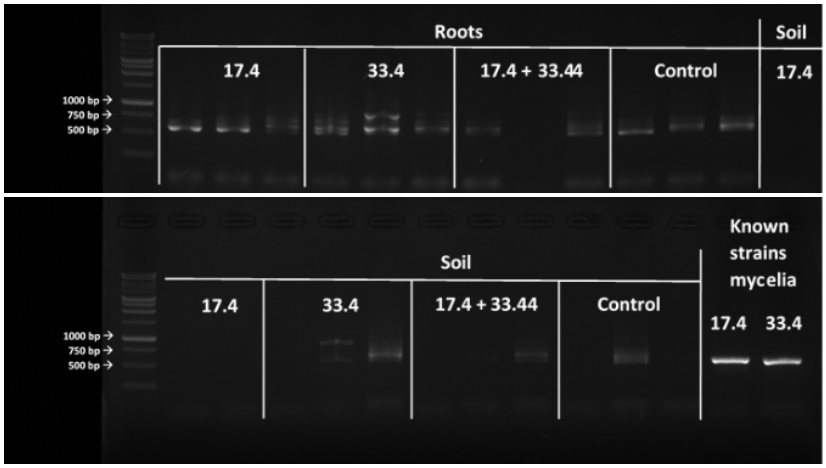

Figure 12: *Gel electrophoresis profiles of roots and soil samples after ITS-PCR. The scale references at top and bottom left were obtained with BenchTop 1kb DNA ladder. In the figure, strain M19-16 corresponds to 17.4 and strain M19-34 corresponds to 33.4.*

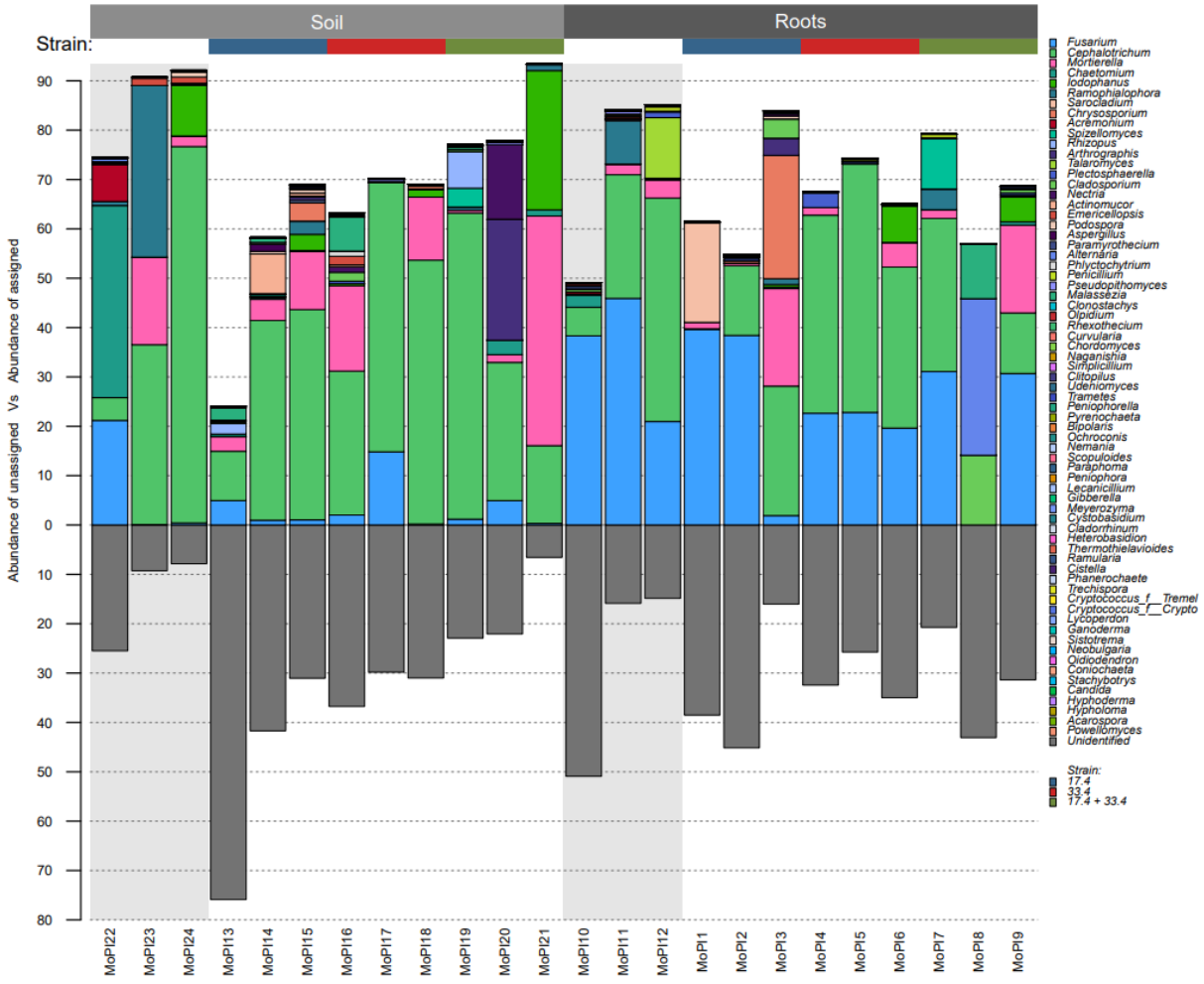

Figure 13: *Next Generation ITS Sequencing of soil and root samples from* Morchella*-maize co-cultures.*

2.4 Conclusion

The initial objectives from Module 6 were already fulfilled by the experiments conducted in Report 1, Module 2. For this reason, we investigated an important aspect of the yellow morel life cycle: plant-

fungal interactions. Mycorrhizal-like interactions and mutualism were reported between *Morchella* and plants (Dahlstrom et al. 2000; Bayes et al. 2011, Yu et al. 2016). We produced morel spawn in a soil-like substrate and used it as inoculum in maize plant cultures. Although we observed hyphae in close contact with the roots, these interactions were found in the control cultures where no morels were inoculated. To verify the species to which the mycelia belonged to, we used next generation sequencing on both root and soil samples from the inoculated cultures and the controls. The results were coherent with the microscopic observations as those did not indicate the presence of ectomycorrhizal structures potentially belonging to *Morchella,* but likely of other fungi common between the inoculated and non-inoculated soils. Therefore, this experiment did not allow to conclude on a potential mycorrhizal lifestyle in yellow morels. To do so, root colonization experiments in simpler systems could be a solution.

3. Module 7: Identification of bacteria naturally associated to Swiss morels and comparison with Chinese strains

3.1 Rationale

The experiments that were initially conducted to fulfill the aims of this module led to numerous remarkable results and further analyses, leading to their compilation in a scientific article that will be submitted to a peer-reviewed journal within the next month. The abstract is copied below and for more details, please refer to the paper in Annex 1 (Cailleau et al. 2022, *in preparation*).

The fungal genus *Morchella* is an iconic fungal group with great ecological and economic relevance. In this study, the bacterial communities associated with different types of fungal tissues (fruiting bodies, mycelium, and sclerotia) was analyzed in a field collection of morels that included yellow and black specimens, as well as in strains from a culture collection. *Pseudomonas* spp. were the most prevalent associate bacterial group in mycelium and sclerotia regardless of the clade investigated and origin of the material (field specimen or culture strain). Together with *Ralstonia* spp. (mycelium and sclerotia) and *Methylobacterium* spp. (sclerotia), they constituted the core associated bacterial community. In contrast, a highly diverse bacterial community was found to be associated with fruiting bodies. Representatives of *Pedobacter* spp., *Deviosa* spp. and *Bradyrhizobium* spp. constituted the core bacterial community in fruiting bodies (**Milestone M2-5**). Furthermore, multiple *Pseudomonas* spp. strains were isolated from mycelia during fungal mycelial culturing (**Milestone M2-6**). These culturable *Pseudomonas* spp. corresponded to amplicon sequence variants (ASVs) representing minor members of the fungal bacteriome, while the highly abundant and prevalent *Pseudomonas* ASVs belonging to the core bacteriome could not be isolated from the fungus. Confrontation assays of these bacterial isolates with different *Morchella* spp. resulted in either beneficial or antagonistic interactions. A comparative genomic analysis of these fungi-associated *Pseudomonas* spp. allowed the identification of putative gene clusters relevant to those interactions, including clusters involved in the metabolism of ectoin, or in the production of insecticidal toxins, toxin-antitoxin systems involved in stress response, chitinases, and adhesion and secretion systems.

4. Module 8: Testing the interaction of the Chinese morels with bacteria from Swiss soils associated to morels

4.1 Rationale

Bacteria can both have antagonistic and beneficial associations with morels (Cailleau et al. 2022, *in preparation*). For instance, associate bacteria are necessary to trigger the formation of fruiting bodies (Q. Liu et al. 2018) while antagonists impair fungal growth (Cailleau et al. 2022, *in preparation*). Testing the interactions between bacteria isolated from Swiss morels and Chinese morels was then necessary to verify whether the non-native cultivars could benefit or be inhibited by native bacteria associated to Swiss morels.

4.2 Methods

Confrontation assays

The interactions between Chinese *Morchella* cultivars and bacteria isolated from Swiss morel isolates were tested. The bacterial strains that were used corresponded to three *Pseudomonas* spp. isolated from Swiss morel isolates (M19-29, M19-34 and M20-7), a strain of *Pseudomonas baltica* previously isolated from *M. crassipes* M84 (Pion et al. 2013) and a reference strain of *Pseudomonas putida* tagged with the green-fluorescent protein (GFP; strain KT2440) (Table 3).

Table 3: *Specimens used in the confrontation assays between Swiss bacteria and Chinese morels.*

Group	Strain name/ID	Origin
Fungi	*Morchella sextelata* NEU143	China
	Morchella importuna NEU142	China
	Morchella sextelata PYL	China
	Morchella importuna PYT	China
Bacteria	*Pseudomonas putida* B188	*Morchella esculenta* M19-34 (Esculenta)
	Pseudomonas koreensis B33.4	*Morchella esculenta* M19-34 (Esculenta)
	Pseudomonas koreensis VD-NE White	*Morchella* sp. M20-7 (Elata)
	Pseudomonas baltica B84	*Morchella crassipes* M84 (Esculenta)
	Pseudomonas putida KT2440	Reference strain

Bacteria and morels were co-inoculated in PDA (39 g/L) plates and each combination was replicated twice. The cultures were incubated in the dark at 22°C. Pictures of the petri dishes were taken at three, seven, and 15-days post inoculation (dpi).

4.3 Results and discussion

Confrontation assays

P. koreensis VD-NE White suppressed the growth of all the *Morchella* isolates around the zone of bacterial inoculation (Figure 14), suggesting an inhibitory (antagonist) interaction. *P. koreensis* B33.4 influenced the growth of *M. importuna* NEU142 in various ways: initially, mycelial growth was inhibited around the zone of bacterial inoculation; then, the mycelium melanized in this particular area (Figure 15), suggesting a stress-response (Cordero and Casadevall 2017). Interestingly, the other morel strains were not affected by *P. koreensis* B33.4. No significant interaction was observed between *Pseudomonas baltica* B84 and *P. putida* KT2440 with any of the morel isolates, because the mycelia covered the whole surface of the dishes without being inhibited or melanized (Figure 16).

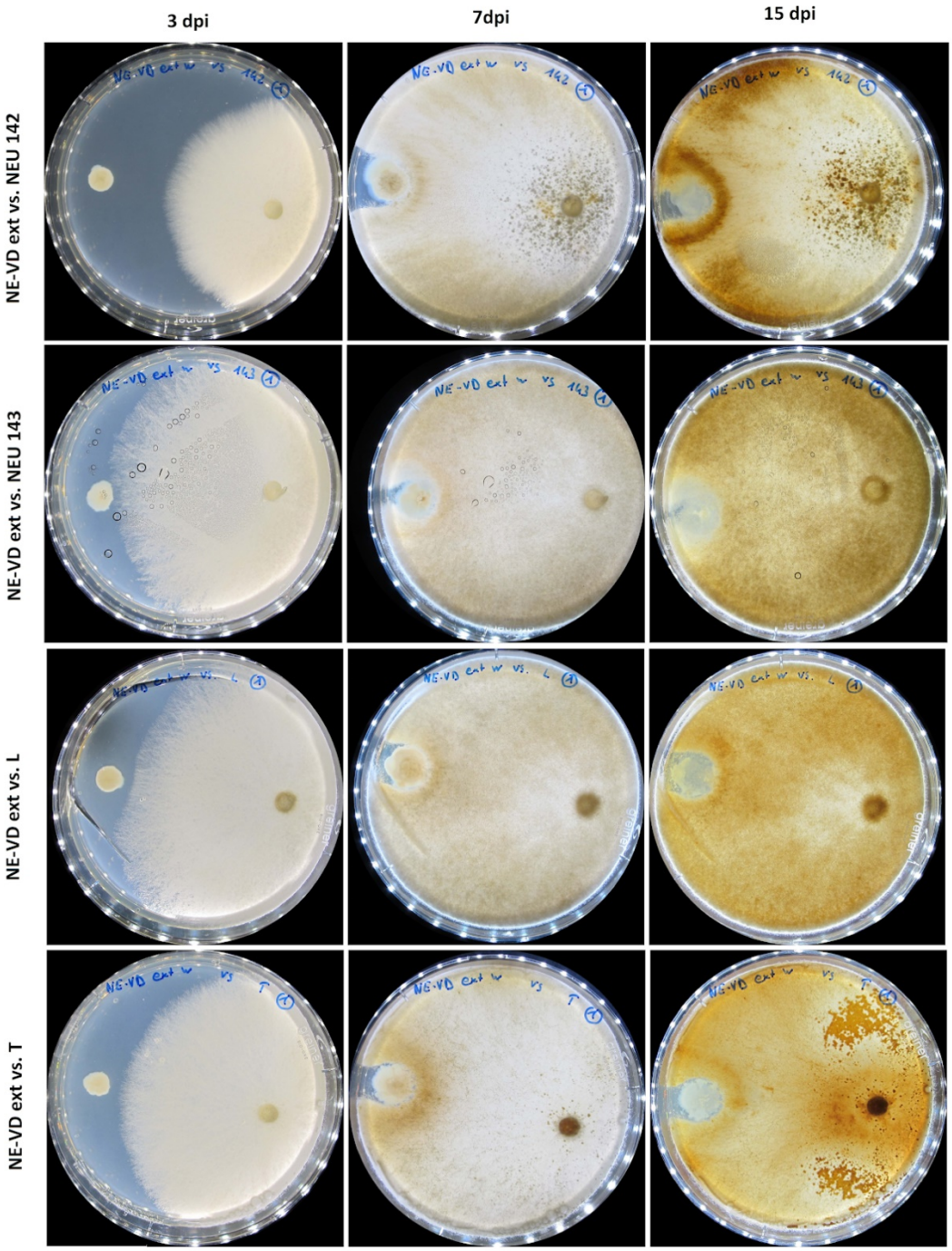

Figure 14 : Confrontation assays between Pseudomonas koreensis *NE-VD ext (inoculated on the left side of the Petri dishes) and Chinese morel cultivars NEU142 and PYT (*M. importuna*); NEU143 and PYL (*M. sextelata*) (inoculated on the right side of the Petri dishes). Pictures were taken at three, seven and 15 dpi, from left to right.*

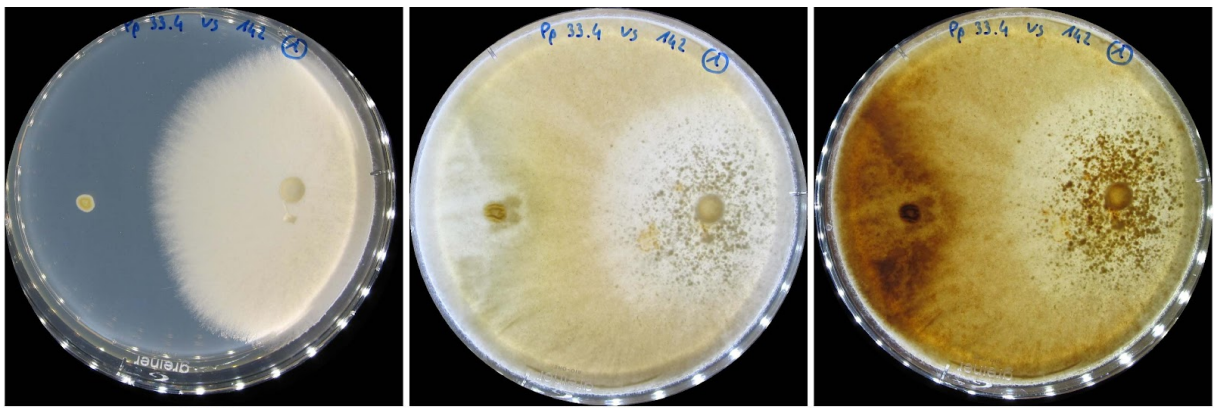

Figure 15: *Confrontation assays between* Pseudomonas koreensis *B33.4 (inoculated on the left side of the Petri dishes) and the Chinese cultivar* M. importuna *NEU142 (inoculated on the right side of the Petri dishes). Pictures were taken at three, seven and 15 dpi, from left to right.*

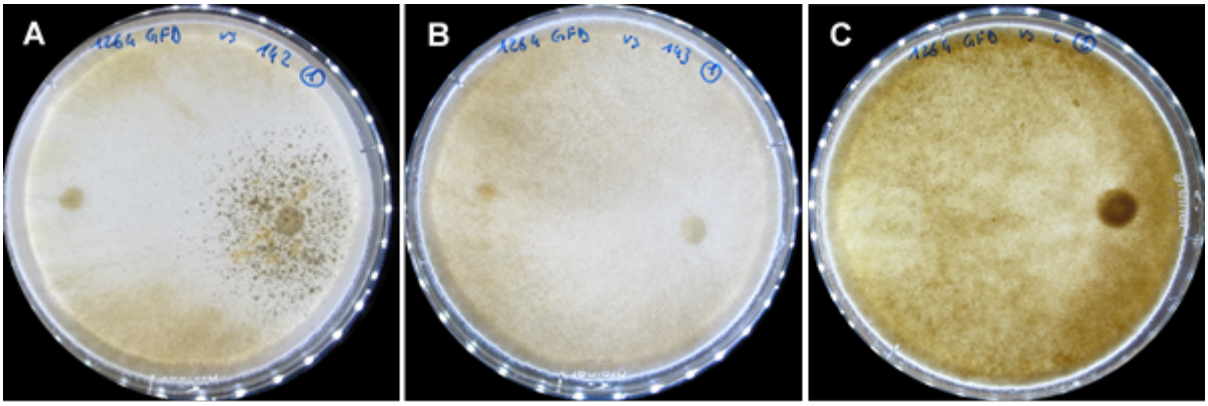

Figure 16: *Confrontation assays between* Pseudomonas putida *KT2440 (inoculated on the left side of the Petri dishes) and the Chinese cultivar* M. importuna *NEU142 (A);* M. sextelata *NEU143 (B) and* M. sextelata *PYL (C). Pictures were taken at 15 dpi.*

4.4 Conclusion for Modules 7 and 8

The characterization of the bacteria associated with Swiss morels allowed the identification of bacteria from the genus *Pseudomonas* as key members of the natural bacteriome in mycelia and sclerotia in morels (**Milestone M2-5**). Some members of this bacteriome can be isolated and cultured without the fungal host (**Milestone M2-6**). Interactions of the Chinese isolates, originating from cultivars used by morel farmers, with the isolated bacteria were always antagonistic against *M. importuna* (NEU142 and PYT) but beneficial with *M. sextelata* (NEU143 and PYL) (**Milestone M2-7**), which would indicate that *M. sextelata* would be better adapted than *M. importuna* in Swiss soils.

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Manuscript in preparation

Tissue-specific bacteriome analysis reveals *Pseudomonas* spp. as a natural fungal-associated bacterium of *Morchella* spp.

Authors:
Guillaume Cailleau[1], Melissa Cravero[1], Sami Zhioua[1], Patrick Hilpish[1], Celia Ruiz[1], Aaron Robinson[2], Julia M. Kelliher[2], Buck H. Hanson[2], Demosthenes Morales[2], La Verne Gallegos-Graves[2], Karen Davenport[2], Gregory Bonito[3], Patrick S. Chain[2], Saskia Bindschedler[1], Pilar Junier[1]

[1]Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, CH-2000, Switzerland.
[2]Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA
[3]Department of Plant, Soil and Microbial Sciences, Michigan State University, USA

Highlights:

- Comparison of the bacteriome in diverse tissues (fruiting bodies, mycelium, sclerotia) obtained from black and yellow morels from Switzerland and China.
- Detection of *Pseudomonas* as the most abundant associated bacterium in mycelium and sclerotia (core bacteriome).
- Detection of a diverse bacterial community associated with fruiting bodies.
- Isolation and characterization (genomics and interactions with Morchella spp) of multiple *Pseudomonas* strains obtained from mycelia.

Abstract
The fungal genus *Morchella* is an iconic fungal group with great ecological and economic relevance. In this study, the bacterial communities associated with different types of fungal tissues (fruiting bodies, mycelium, and sclerotia) was analyzed in a field collection of morels that included yellow and black specimens, as well as in strains from a culture collection. *Pseudomonas* spp. were the most prevalent associate bacterial group in mycelium and sclerotia regardless of the clade investigated and origin of the material (field specimen or culture strain). Together with *Ralstonia* spp. (mycelium and sclerotia) and *Methylobacterium* spp. (sclerotia), they constituted the core associated bacterial community. In contrast, a highly diverse bacterial community was found to be associated with fruiting bodies. Representatives of *Pedobacter* spp., *Deviosa* spp. and *Bradyrhizobium* spp. constituted the core bacterial community in fruiting bodies. Furthermore, multiple *Pseudomonas* spp. strains were isolated from mycelia during fungal mycelial culturing. These culturable *Pseudomonas* spp. corresponded to amplicon sequence variants (ASVs) representing minor members of the fungal bacteriome, while the highly abundant and prevalent *Pseudomonas* ASVs belonging to the core bacteriome could not be isolated from the fungus. Confrontation assays of these bacterial isolates with different *Morchella* spp. resulted in either beneficial or antagonistic interactions. A comparative genomic analysis of these fungi-associated *Pseudomonas* spp. allowed the identification of putative gene clusters relevant to those interactions, including clusters involved in the metabolism of ectoin, or in the production of insecticidal toxins, toxin-antitoxin systems involved in stress response, chitinases, and adhesion and secretion systems.

1. Introduction

Morchella is an iconic fungal genus in Ascomycota, represented by a high diversity of species or species complexes. The fruiting bodies (ascocarps or fructifications) of morels are highly prized for their gustative qualities and are of great economic and cultural importance. The systematics and taxonomy of the group are problematic, and there are numerous cryptic species. Moreover, morphology-based species identification of morels is

limited and prone to confusion (Richard, Bellanger et al. 2015). Hence, molecular studies appear to be the most appropriate approach for the identification of species assigned to the genus. Phylogenies based on multilocus molecular analyses (Taskin, Buyukalaca et al. 2012, Baroni, Beug et al. 2018) suggest that the genus *Morchella* comprises at least 65 phylogenetically distinct species (Richard et al. 2014), that can be grouped in three clades: Elata (black morels), Esculenta (yellow morels) and Rufobrunnea (grey morels) (O'Donnell, Rooney et al. 2011). Wild morels are collected and exported intensively in China, India, Turkey, Mexico, and the USA (Pilz 2007). In the last few years, China started outdoor artificial cultivation of morels. The Chinese area cultivated with morels increased from 200 ha in 2011 to more than 1200 ha in 2015. The most commonly cultivated species are black morels (of which, *Morchella importuna* is largely dominant), with about 90% of the production (Liu, Ma et al. 2018). However, ascocarp production is unstable, which is probably the result of the highly complex life cycle and ecology of morels (Du, Zhao et al. 2016, Li, Xiong et al. 2017).

Many abiotic parameters, such as temperature, humidity and nutrient availability, are known to influence fruiting body formation. Nutrient availability improves in natural habitats after wildfires, which are known to benefit post-fire fungi (Li, Xiong et al. 2017). Regarding the biotic factors affecting morels in the wild, the interaction with other soil microorganisms is thought to influence growth and the development of mature ascocarps. Li et al (Li, Xiong et al. 2017) hypothesized that bacteria associated to the fungus, and in particular *Pseudomonas* spp., may have an effect on morel primordial differentiation, which is a key stage in the formation of fruiting bodies. Similar benefits provided by bacteria have been suggested for black truffles, where bacteria could play a role in the development, maturation and even the final aroma of the black truffle (Antony-Babu, Deveau et al. 2013; https://pubmed.ncbi.nlm.nih.gov/24903279/). A recent study investigating the microbiota in soils beneath ascospores of black morels cultivated in greenhouses (*Morchella sextelata*), suggested that the bacterial communities in morel ascocarp tissues differ significantly from those of the surrounding soils (Benucci GMN, Longley R, Zhang P, Zhao Q, Bonito G, Yu Fuqiang. 2019. Microbial communities associated with the cultivated black morel Morchella sextelata in greenhouses. PeerJ. doi: 10.7717/peerj.7744).

Although there has been growing interest in understanding the associations between morel fungi and bacteria, a systematic study analyzing bacterial communities in association with different structures produced during the life cycle of morels is not yet available. In this study, we investigated the composition of bacterial communities present in different tissues (fruiting bodies, mycelium, and sclerotia) derived from a diverse collection of morel specimens collected in the wild, as well as derived mycelial cultures and mycelium from reference strains from culture collections. In addition, whenever possible sclerotia, which are survival structures involved in primordia formation (Ref), were also included. Considering the differential chemistry and function of these tissues, we hypothesized that the associated bacterial communities will differ between those tissues. We also hypothesized that differences in the core bacteriome will be equally significant between compared tissues and between distinct phylogenetic morel clades. Our results indicate that *Pseudomonas* spp. are a major component of the morel bacteriome, particularly in vegetative tissues (mycelium and sclerotia), and less so in fruiting bodies. This core bacteriome was common to different morel clades. Moreover, we isolated and identified several *Pseudomonas* spp. during morel cultivation and tested the type of interaction established with the fungal host. These bacteria were either beneficial or antagonistic, but the degree of interaction varied across multiple hosts. Genome sequencing of the associated bacteria revealed the existence of unique sets of genes potentially involved in different types of interactions. This study offers the first evidence connecting observations

from soils and confrontation studies indicating the relevance of *Pseudomonas* spp. on the physiology and development of morels.

2. Material and methods

Collection and processing of the fruiting bodies
During the spring season (March to June, 2019) morel ascocarps were obtained from 34 locations in the canton of Neuchâtel, Switzerland. The fruiting bodies were initially identified based on their morphology as *Morchella conica* (20 sets of samples; 61 specimens), *Morchella elata* (3 sets of samples; 15 specimens), *Morchella esculenta* (8 sets of samples; 26 specimens), and *Morchella vulgaris* (3 sets of samples; 12 specimens). In addition, 2 specimens of *Morchella semilibera* (the half-free morel) and 5 populations with 15 specimens identified as *Verpa bohemica* (early false morel) were also collected. The first species (*M. semilibera*) is closely related to black morels from the Elata clade. The second species (*Verpa bohemica*) belongs to the Morchellaceae family and is often used as an outgroup to reconstruct *Morchella* phylogenies (O'Donnell, Rooney et al. 2011). This initial morphological identification was confirmed through sequence analysis of the ITS region (see below).

Culturing and isolation of mycelia
Upon collection of the ascocarps, several analyses were performed. First, material for genetic identification from the ascocarps was collected directly. Second, in those individuals with an intact fruiting body, fungal isolation was attempted either by germinating spores or by direct culturing pieces of hymenia. Isolation was performed on potato dextrose agar (PDA, Potato infusion powder, Sigma-Aldrich, 4 g/L + D(+) - Glucose Monohydrat, Roth, 20 g/L + Agar-Agar, Merck, 15 g/L; which was autoclaved at 121°C before preparation) and pure strains were obtained by successive plating in the same medium. In addition, two Chinese cultivars (NEU142 and NEU143), one yellow morel specimen from our collection (M84), and one black morel collected in spring 2020 in the canton of Vaud (M20-7) were cultivated *in vitro* in PDA. A total of 51 biological specimens were used for genetic analysis and generation of mycelial cultures. From those, 43 wild morels were identified by the prefix “M19” followed by an Arabic number and used to construct phylogenetic trees along with M20-7, NEU142 and NEU143. Four fruiting body specimens (MC36, MC41, MC42, MC43) were only used for species identification.

DNA extraction and fungal identification
DNA was extracted either directly from fruiting bodies by cutting a piece of the hymenia, from mycelia by scraping hyphae from the surface of seven-day cultures on PDA, or from sclerotia sampled on mycelial cultures grown on malt agar (Malt extract, Fluka, 12 g/L + Agar-Agar, Merck, 15 g/L; MA). All DNA extractions were made with Quick-DNA Fungal/Bacterial Miniprep Kits (ZymoResearch®) following the providers’ protocol. After extraction, eluted DNA was quantified with a Qubit kit (Invitrogen, USA) using the Broad Range buffer and reagents. DNA was then diluted with PCR-grade water to a concentration of 2 ng/µL. Clades and potential species were assigned by sequencing comparison of the internal transcribed spacer (ITS) amplified using the ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (White et al. 1990) and ITS4 (5'-TCC TCC GCT TAT TGA TA TGC-3') (Gardes and Bruns 1993) primers. The PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix and 0.2 µM forward and reverse primers, adding finally 1 µL of the DNA template concentrated at 2 ng/µL. The PCRs were performed in a Thermo Scientific Arktik thermal cycler with the following parameters: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 62°C for 15 sec, 72°C for 15 sec), final elongation at 72°C for 2 min, end at 20°C. Amplicons were purified in a MultiScreen® filter PCR µ96 (Millipore Corporation, USA) plate as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells

until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified by Qubit. Final concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing. After sequencing, the 51 biological specimens of *Morchella* we used in this study were attributed to a species using the ITS sequences and a pairwise alignment tool from the Westerdijk Institute (https://wi.knaw.nl/page/Pairwise_alignment) that aligns query sequences to a curated reference set. The reference species with the higher query cover and similarity were used to assign species to our isolates.

Identification of Morchella spp. phylogenetic analysis
Initially, we used 46 ITS sequences from *Morchella* specimens from our collection (M19-1 to M19-43) and eight reference sequences (RS) (*M. rufobrunnea* JX292976.1; *Morchella* sp. Mes-8 U51851.2; *Morchella* sp. Mes-17 MK965458.1; *Morchella* sp. Mes-19 MT268908.1; *Morchella* sp. Mel-12 JF319902.1; *Morchella* sp. Mel-15 GU551433.1; *Morchella* sp. Mel-16 GU551430.1; and *Morchella* sp. Mel-20 GU551438.1) retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank). ITS from specimens M19-23 and M19-37 were removed from the dataset because they were shorter and decreased the tree's reliability. The remaining 52 sequences were separated into two groups (Elata/Esculenta) and aligned with MUSCLE (Neighbor Joining). The alignment was used to generate Neighbor Joining trees (NJ) with statistical support of 1000 bootstrap values. The phylogenetic analysis was performed in the software Mega version 11 (Kumar et al. 2018).

Analysis of the associated bacterial community
The analysis of associated bacteria was based on the same fungal DNA extracts prepared as indicated above. To test for surface contamination, one mycelium (*M. esculenta* M19-23) was grown on a medium containing 100 µg/ml streptomycin. The composition of associated bacteria was examined via amplicon sequencing of the V3-V4 region of the 16S rDNA. PCR amplification from the fungal DNA extracts followed methods described previously (Robinson et al., 2021). Briefly, a nested PCR approach was used to increase the amplification efficiency for bacteria with the relative exclusion of fungal mitochondria. The primers EUB9-27 5'-GAG TTT GAT CCT GGC TCA G-3' (Ref) and 907R 5'-CCG TCA ATT CCT TTG AGT TT-3' (Lane, 1991) were used to amplify the V1-V5 region of the 16S rDNA. Purified PCR products from this initial amplification were sent to Fasteris (Geneva, Switzerland) for a nested amplification with primers to amplify the V3–V4 region (universal primers Bakt_341F 5'-CCT ACG GGN GGC WGC AG-3' and Bakt_805R 5'-GAC TAC HVG GGT ATC TAA TCC-3') (Herlemann, Labrenz et al. 2011). PCR amplification was performed along with sample barcoding to allow multiplexing and adapter ligation for sequencing on an Illumina MiSeq platform (2 x 300bp paired end reads). No-template controls (starting with the DNA extraction) were also included in the sequencing run.

Sequence analysis
Demultiplexed and trimmed sequences provided by Fasteris were analyzed using the QIIME2 pipeline (Bolyen, et al., 2019). Read lengths were truncated to 488 bp to optimize total nucleotide lengths (based on quality scores). This allowed the joining of denoised paired-end reads by at least 12 identical bp. In order to obtain these sequences, the truncated, unmerged, paired-end reads were denoised using the DADA2 plugin (Callahan et al. 2016) which denoises paired-end sequences, dereplicates them, and filters chimeras. This step yields Amplicon Sequence Variants (ASVs), which are expected to better represent the biological diversity present in the samples. As the DADA2 filtering step is based on quality score, there is no need for a quality filtering step prior to this. The ASVs were then taxonomically classified using QIIME2's VSEARCH-based consensus taxonomy classifier (Rognes et al., 2016) with the SILVA database release 132 (Quast et al., 2013), that was previously trimmed to the same V3-V4 region produced by the sequencing primers

used. Further data analysis was performed using the phyloseq package (version 1.30.0) and R (version 3.6.2). First, any ASV represented in the no-template control samples were excluded. Any ASVs not assigned to the Bacteria Kingdom were discarded. The resulting dataset was normalized using a Total-sum scaling (TSS) implemented by the metagMisc package (version 0.0.4). At this step, non-relevant genus rank taxonomies were merged with unassigned ones as "unassigned" ASV to be plotted against the "assigned" ASVs. Finally, samples were grouped by species and ordered by sample types to be plotted as cumulative barplots.

Core community analysis

Data of relative abundance of the bacteriome for the three sample types (mycelium, sclerotia, and fructification) were used to define the core bacteriome. Given that the core community can be defined based on multiple parameters, two parameters were considered here: i) prevalence or occupancy (percentage of samples in which a given ASV is present), and ii) relative abundance threshold above which an ASV is considered as present in a given sample. The core microbiome was computed using the microbiome package (version 1.8.0) and R (version 3.6.2). As there is no consensus on the prevalence and relative abundance thresholds that define the core community, we used an approach that defines consecutive values of interest for both parameters. Consequently, prevalence values were set from 30 to 100 with steps of 5%. Detection thresholds for the relative abundance were set to 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.75, 1.00, 2.00, 5.00, and 10.00%. For each combination of prevalence and relative abundance threshold, a core community was calculated and the associated members were recorded. Thereafter, based on the increasing number of ASVs included as core members, a Venn diagram was produced using R package nVennR (version 0.2.3) for each sample type.

Opportunistic bacterial isolation and characterization

Six bacterial strains were obtained during cultivation of the fungal mycelia (Supplementary Table S1). Two of the strains were isolated during re-plating of two yellow morel specimens (*M. esculenta* M84 and *M. esculenta* M19-34). In one case, bacteria were detected following confrontation with a mycelium from another fungal specimen (strain B33.4; Figure S1). A second bacterial strain (strain B188) was detected from the same fungus, *M. esculenta* M19-34, but it was considered as a potential contaminant. Three strains (strains VD-NEext, VD-NEins, VD-NEwhite) were all obtained from mycelium of *Morchella* sp. M20-7. Among those, the bacterial strain VD-NEext was associated with the fungus after cultivation on streptomycin. All the strains were initially identified by partial sequencing of the 16S rDNA amplified using the GMF3 (5'-AGA GTT TGA TC(AC) TGG C-3') and GM4R (5'-TAC CTT GTT ACG ACT T-3') primers (Muyzer et al. (1995), Arch. Microbiologie 164: 165-172).

Table S1. Origin of the bacteria isolated and the *Morchella* spp. host

Bacteria	Fungus	Origin of the fungus	Comments
Pseudomonas sp. B84	*M. crassipes* M84	Switzerland (unknown region)	Isolated during re-platting
Pseudomonas sp. B188	*M. esculenta* M19-34	Switzerland (Neuchâtel)	Potential contaminant
Pseudomonas sp. B33.4	*M. esculenta* M19-34	Switzerland (Neuchâtel)	Present from the first purification of the fungal specimen. Always associated with the mycelium, even on streptomycin medium

Pseudomonas sp. VD-NE Ext	*Morchella* sp. M20-7	Switzerland (Vaud)	Associated with the mycelium from the first inoculation of the fungus.
Pseudomonas sp. VD-NE Ins	*Morchella* sp. M20-7	Switzerland (Vaud)	Associated with the mycelium from the first inoculation of the fungus.
Pseudomonas sp. VD-NE White	*Morchella* sp. M20-7	Switzerland (Vaud)	Associated with the mycelium from the first inoculation of the fungus.

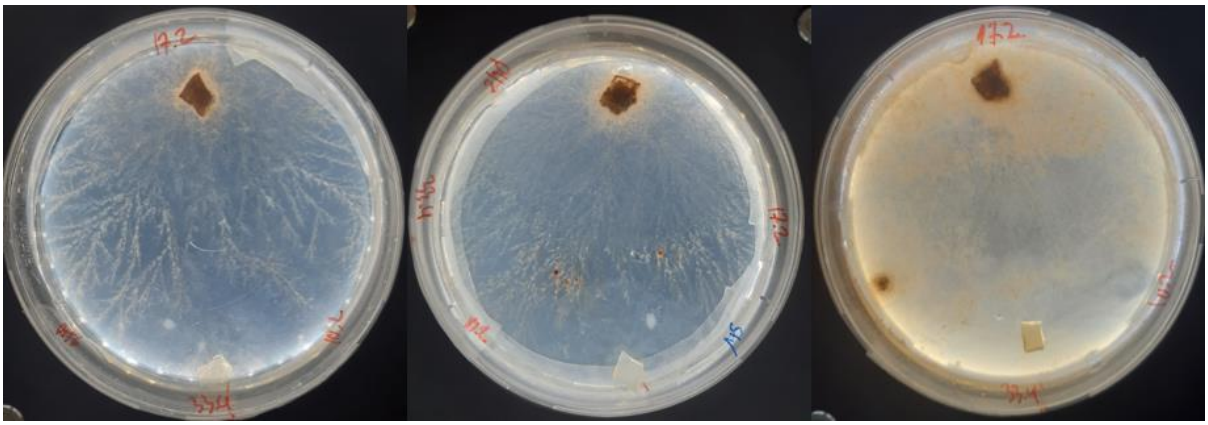


Figure S1. Confrontation experiment between *M. esculenta* strains M19-14 (top) and M19-34 (bottom) leading to the isolation of the bacterium 33.4. The bacterium was observed emerging from the mycelium of strain M19-34 when confronted with the other strain in different media.

Genomic DNA extraction, sequencing, and genome assembly

Bacterial genomic DNA was extracted using either QIAGEN® Genomic-tip 20/G (#10223 QIAGEN®) or the Wizard® HMW DNA Extraction Kit (#A2920 Promega Switzerland). Protocols from QIAGEN® or Promega® were used and adapted to obtain sufficient quantities of high molecular weight genomic DNA. All bacteria were grown overnight in nutrient broth for genomic DNA extraction. The following modifications from the Promega kit were applied: two additional steps of cleanup with 1 ml PBS was done to remove any EPS in the pellet; 20 µl of RNAse A (10 mg/ml) was added to each sample and incubated at 37°C for 30 min; Proteinase K was incubated for 30 min; after the protein precipitation, the sample was centrifuged at 16'000g for 20 min at 4°C; 0.7 volume of isopropanol was added to the volume extracted after the protein precipitation step; after that the pellet was washed with ethanol, centrifugation was done at 4°C for 5 min at 16'000g; Finally, 50 µl of rehydration solution was added. DNA quality tests were done by casting a 0.7% agarose gel (TAE running buffer) containing 3 µl StainIN ™ GREEN Nucleic acid stain. The gels were run 1h40 min at 70V. If DNA was present in the gel, quality measurements were performed using a fluorospectrometer (#ND-3300 Thermo Scientific ™). DNA quantification was measured using a fluorometer (Qubit 2.0 Thermo Scientific ™). Pure genomic DNA samples with sufficient concentrations were sequenced with PacBio® at the sequencing facility of the University of Lausanne, Switzerland. High molecular weight DNA was sheared with Megaruptor (Diagenode, Denville, NJ, USA) to obtain 10-15 kb fragments. After shearing the DNA size distribution was checked on a Fragment Analyzer (Advanced Analytical Technologies, Ames, IA, USA). Then, 500 ng of the DNA was used to prepare a SMRTbell library with the PacBio SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA) according to the manufacturer's recommendations. The resulting library was pooled with other libraries processed the same. The pool was size-selected with

Ampure PacBio beads to eliminate fragments <3kb. Libraries were sequenced with v2.0/v2.0 chemistry and diffusion loading on a PacBio Sequel II instrument (Pacific Biosciences, Menlo Park, CA, USA) at 900 min movie length, pre-extension time of 120 min using one SMRT cell 8M. Genome assembly was performed using the protocol Microbial Assembly in SMRT Link Version 10.1

Comparative genomic analysis
In order to perform genome comparisons, the six assembled genomes from *Pseudomonas* were imported into anvi'o (version 7) environment (Eren et al., 2021). Following the outlined pangenomic workflow described by the authors, a contig database that was populated using DIAMOND (Buchnfink et al., 2015) was created in 'fast' mode to calculate the similarity of each amino acid sequence in every genome and across all our genomes. This step allowed us to define gene clusters based on similarities. At this step weak similarities were filtered out using minbit scores (default to 0.5) REF. Then, cluster granularity in amino acid sequence similarity was determined using MCL algorithm (van Dongen and Abreu-Goodger, 2012) with default parameters. At this step, the anvi-run-kegg-kofams program and then anvi-estimate-metabolism program were run to investigate the predicted metabolic capabilities. Briefly, the anvi-run-kegg-kofams program uses the KEGG database to annotate functions and metabolic pathways with HMM hits from KOfam, a database of KEGG Orthologs (KOs). Then anvi-estimate-metabolism program assessed metabolic KEGG modules completeness with a default threshold of 0.75 (i.e., 75% of the steps required in the KEGG module are present in a genome). The level of completeness for a given KEGG module (Kanehisa et al. 2014; Kanehisa et al. 2017) in our genomes (based on the previous KEGG annotation) was then assessed. Several modifications were done to organize gene clusters and genomes in the anvi'o interactive interface. At this point manual bining selections were performed in order to highlight meaningful gene cluster groups. These bins were then exported to assess if metabolic capabilities were exclusive to a single or a group of genomes. This assessment was performed as follows: for each genome, for all KEGG modules estimated as complete in a given genome, each group of gene callers belonging to a complete KEGG module were compared to gene callers isolated in a bin. This allowed us to define if all the gene callers necessary to satisfy a module completeness (as computed with anvi-estimate-metabolism) are present in the specific bin of interest and thus if a potential metabolic pathway is unique to a bin.

Bacterial-fungal confrontations
Confrontations between *Pseudomonas* spp. and *Morchella* spp. were performed in 60 mm Petri dishes in PDA. Inocula for the fungal specimens were prepared on MA. Inocula for the bacterial strains were prepared from overnight liquid cultures grown in nutrient broth (NB) on a rotary shaker (120 rpm at room temperature). For the confrontation, a disc of agar from the edge of the fungal colony was cut and placed in the center of the Petri dish. To inoculate similar concentrations of bacteria, cells were initially counted using a Neubauer chamber. For this, after centrifugation of the overnight culture, the pellet was mixed in physiological water until the solution was homogenous. An aliquot of the solution was diluted 100x and 5 µl of the solution was transferred to the Neubauer chamber. Bacterial cells were counted at 400 x magnification under the microscope. All strains were diluted to obtain an equal number of bacteria cells (77'000 cells/ul) to be inoculated in the confrontations. Inoculation was performed using a glass 10 µl pipette. A 5 µl drop of bacterial culture was inoculated at the far periphery of the Petri dish. Pictures of the confrontations were taken at different days post inoculation. After approximately 22 days, confrontations were stored at 4°C until further use. Different variables were observed in the confrontations. Scores were given to each variable (0 = negative interaction; 1/2/3 = increasing interactions). All confrontations were performed in triplicates. If a confrontation was contaminated, *NA* (not applicable) was recorded. Sclerotia formed during

confrontation assays were transferred onto fresh MA plates to observe whether bacteria were associated with them. If no sclerotia were observed, *NA* was listed as the observation. If bacteria grew, they were isolated in NA with the fungicide cycloheximide (#C7698-5G from Sigma-Aldrich) to avoid fungal growth. If bacteria did not grow after isolation, *NA* was listed as the observation. Furthermore, samples from the initial bacteria inoculation sites were taken and replated on NA with cycloheximide to observe if bacteria survived the interaction. The same was done by inoculating a sample from the opposite site of the initial bacterial inoculation site. Further inoculations were made in NA to isolate bacterial colonies. If a bacterial isolate did not grow, *NA* was listed as the observation. All isolations were scored on a binary scale of 0 (no growth) to 1 (successful growth).

Colony PCR of Bacteria

For samples in which colony isolation was possible, bacteria grew sufficiently to perform colony PCR. However, in cases in which colonies were not obtained, samples from the opposite/inoculation bacterial-site were used to perform colony PCR. For the colony PCR, a sterile pipette-tip was used to place a bacterial colony in a PCR tube filled with 20 µl of Y-PER buffer (#78990 Thermo Scientific ™). Tubes were vortexed until homogenous then heated at 99°C for 10 min. Samples were centrifuged briefly to separate cell debris from genetic material. 4 µl of the supernatant was diluted in 60 µl PCR-grade water. Amplification of the 16S rDNA was done using GMF3 (5'-AGA GTT TGA TC(AC) TGG C-3') and GM4R (5'-TAC CTT GTT ACG ACT T-3') primers (Muyzer et al. (1995), Arch. Microbiologie 164: 165-172). The PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix and 0.2 µM forward and reverse primers, adding finally 1 µL of the DNA template (1:15 dilution). The PCRs were performed in a Thermo Scientific Arktik thermal cycler with the following parameters: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 56°C for 15 sec, 72°C for 30 sec), final elongation at 72°C for 2 min, end at 20°C. Amplicons were purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as described before. Once purified, the PCR products were quantified by Qubit. Final concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing. Sequences were compared to sequences in the NCBI database using the nucleotide BLAST® set to search for highly similar sequences. Queries of species indicating the higher percentages of query cover and similarity were used to identify the species of the morel specimen.

3. Results

Diversity of wild morel populations

In the spring of 2019, 47 morel fruiting bodies were collected in Switzerland (Figure 1A). They included 20 black and 27 yellow morels representing five different species based on the ITS genetic region. The species corresponded to *M. angusticeps* (13 specimens), *M. deliciosa* (3), *M. esculenta* (28) and three black morel specimens that could not be determined after sequencing of the ITS sequence and were then assigned to *Morchella* sp. until further investigation. In addition, two Chinese strains from our culture collection were attributed to *M. importuna* (NEU142) and *M. sextelata* (NEU143). The black morel specimen collected in 2020 (M20-7) could not be attributed to a species with the ITS sequence only. To visualize the genetic relationships between our specimens, 44 ITS sequences generated in this study and eight reference sequences were used to construct a Neighbor Joining phylogenetic tree (Figure 1B).

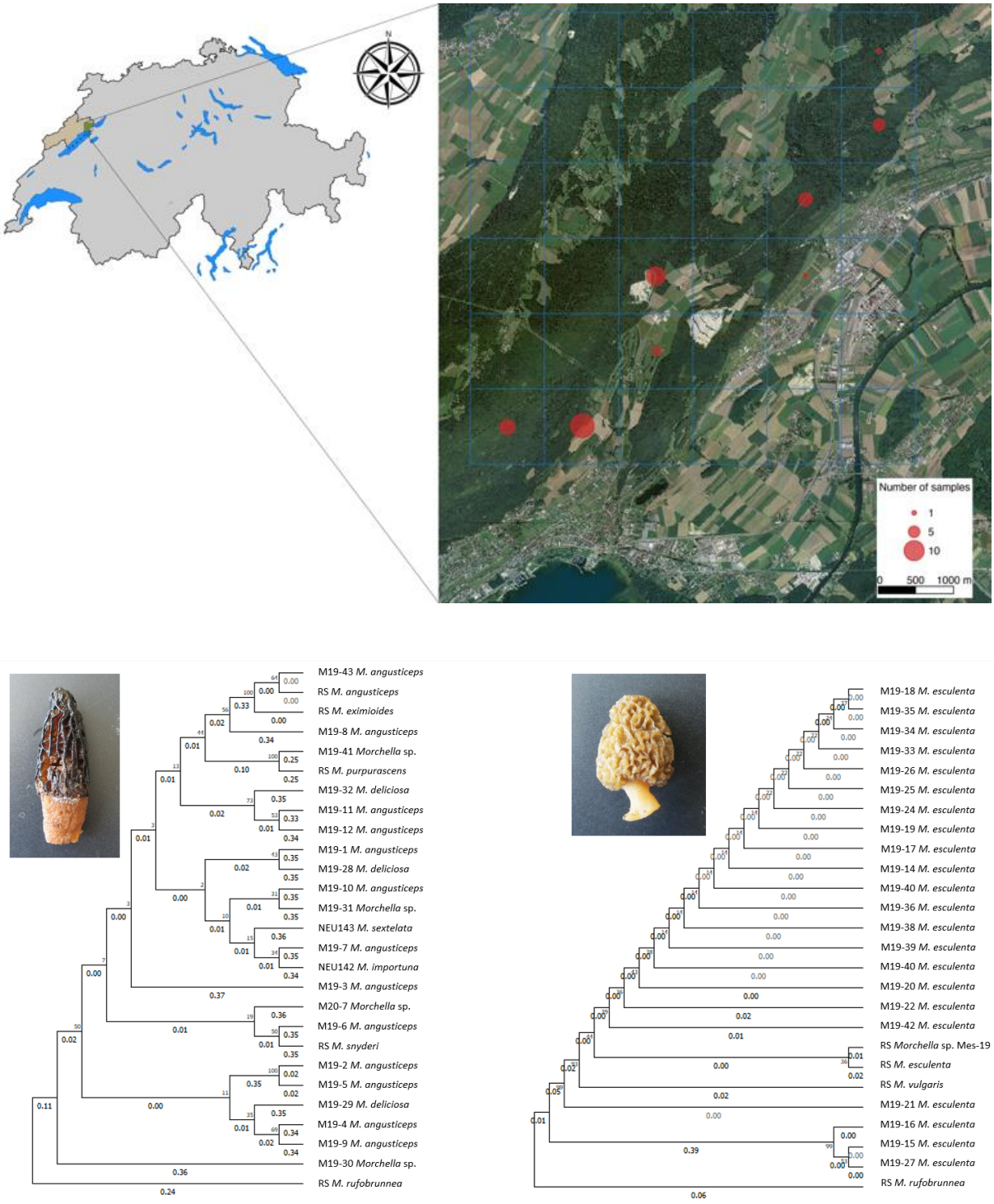


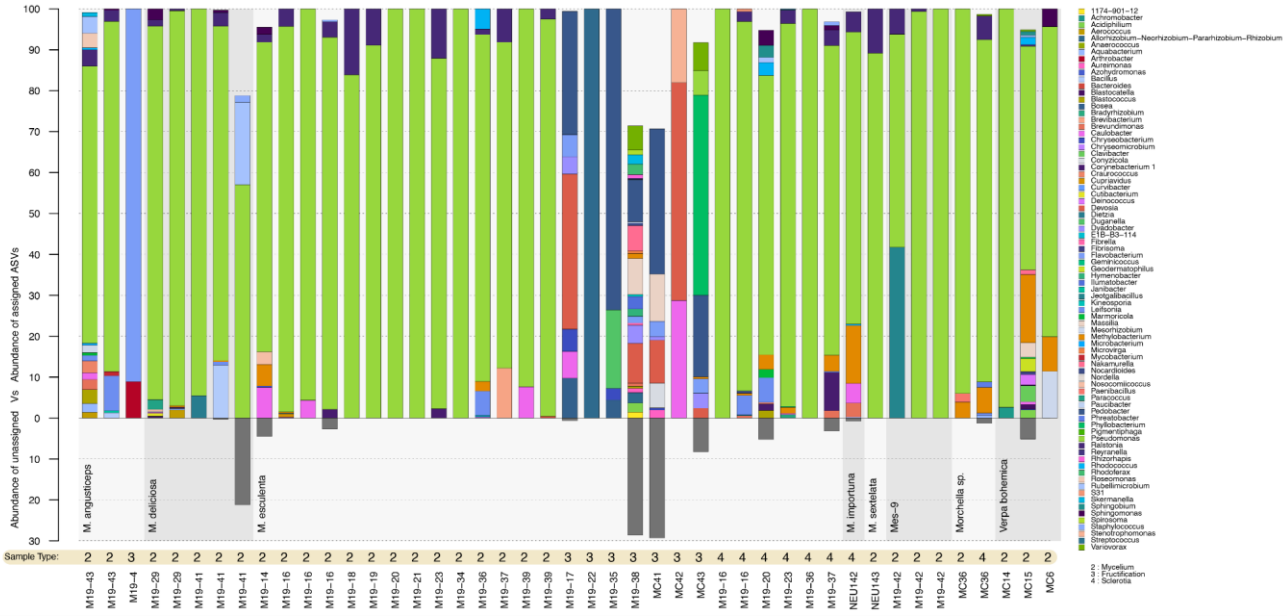
Figure 1. Morel populations under study. A. Sampling areas for collection of wild morels in Neuchâtel, Switzerland. The number of fruiting bodies obtained per sampling site is indicated by the size of the circle. B. Neighbor Joining (NJ) phylogenetic trees constructed with 27 (Elata, left) and 26 (Esculenta, right) ITS rDNA sequences (RS=Reference sequences). On the left of the phylogenetic trees, images of representative ascocarps are shown (Elata: M19-30; Esculenta: M19-23). The species determined by the pairwise alignment tool from the Westerdijk Institute is indicated next to each collected biological specimen.

Associated bacterial communities

Pseudomonas was the most abundant bacterial genus in the communities associated with mycelium and sclerotia regardless of the species investigated (Figure 2A). In contrast, the bacterial communities associated with fruiting bodies were highly diverse (Figure 2A), even within the fruiting bodies of *M. esculenta*, which were over-represented in the collection.

Another way to highlight the dominant members of the associated bacterial communities is through the analysis of the core community in each tissue type. This core bacterial community was defined based on the combination of prevalence and the threshold of detection (relative abundance) of a given ASV. As different arbitrary thresholds can be selected for each of these parameters, the core community was represented by integrating multiple prevalence and detection thresholds in the form of an ever more inclusive Venn diagram. This representation allowed representing both an inner core (highest prevalence and detection thresholds) and an outer core (lowest prevalence and detection thresholds). This analysis showed that the inner and outer core bacterial community associated with mycelia is composed of five ASV, three of which are affiliated to *Pseudomonas* (Figure 2B). The core bacterial community associated with sclerotia also contains three of *Pseudomonas* ASVs as part of the innermost core, but ASVs related to *Ralstonia* and *Methylobacterium* composed the outer core. As predicted, the core community from the fruiting bodies is more diverse and the prevalence of the ASVs in the innermost core (75%) is much lower the prevalence of the innermost core in both mycelia and sclerotia (95%). In the fruiting bodies ASVs related to *Pedobacter*, *Deviosa*, and *Bradyrhizobium* composed the innermost core community.

A



B

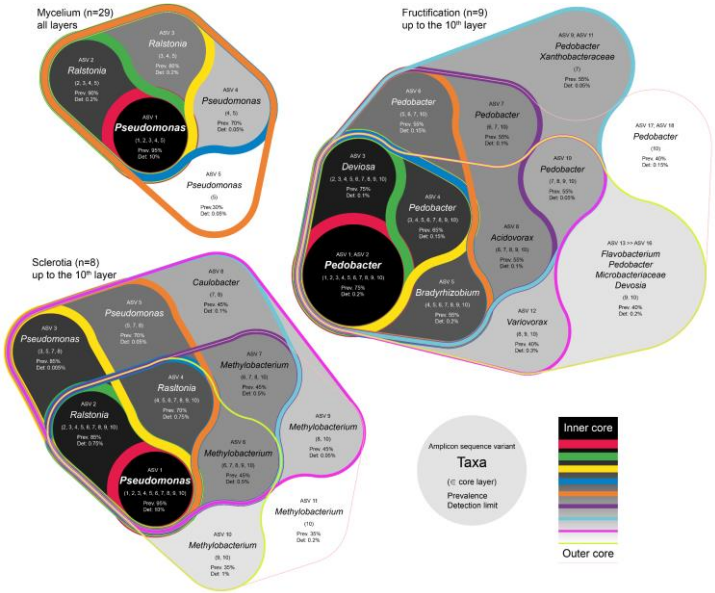


Figure 2. Bacterial communities associated with different types of tissues of wild morels and reference morel species. A. Bar charts displaying the bacterial composition (relative abundance) at the genus level. The sample types are indicated as follows: mycelium -2-, fructification -3-; sclerotia -4-. The transition between different species are highlighted by a change in the grey background. The proportion of unassigned ASVs is shown at the bottom of the graph by the dark grey bars. B. Representation of the core bacterial communities. The core community was determined by combining two parameters: prevalence and detection threshold (based on relative abundance). Different thresholds were applied to these parameters and the resulting core community was represented as a Venn diagram that encompasses the different thresholds of prevalence and detection of each ASV. The graphical representation highlights the concept of core layer, with the inner layer corresponding to the most ubiquitous taxa, while the outer layer refers to increasingly marginal taxa. For each core layer the taxa id, as an ASV number(s), its corresponding identification(s) and its subsequent presence in the lower core layers is provided in parenthesis. In addition, prevalence and detection parameters defining the core layer are provided.

Isolation and identification of associated Pseudomonas *spp.*

The detection of Pseudomonas as the most common bacterial genus associated with mycelium and sclerotia in morels was noteworthy as, in parallel to the molecular analysis, we were able to isolate six bacterial strains classified as *Pseudomonas* spp. based on their partial 16S rDNA sequences. The bacteria were isolated during the passaging of fungal specimens or were shed from apparently axenic mycelium in confrontation experiments between various fungal strains (Figure S1 and Table S1). Based on full genome sequencing, these isolated bacteria were classified as belonging to the *Pseudomonas koreensis* (strains B33.4, VD-NE ext, VD-NE ins, VD-NE white), *Pseudomonas baltica* (strain B84), and *Pseudomonas putida* (B188) subgroups (https://doi.org/10.3390/microorganisms9081766). A phylogenetic comparison of the bacterial isolates, reference bacterial strains, and the ASVs assigned to *Pseudomonas* within the associated bacterial communities, clearly shows all but strains B188 and B84 were closely related to ASVs (Figure 3). However, none of the strains corresponded to ASVs that were part of the innermost core bacterial community found within the fungal mycelium, but rather to ASVs that were less prevalent or abundant (Figure 3).

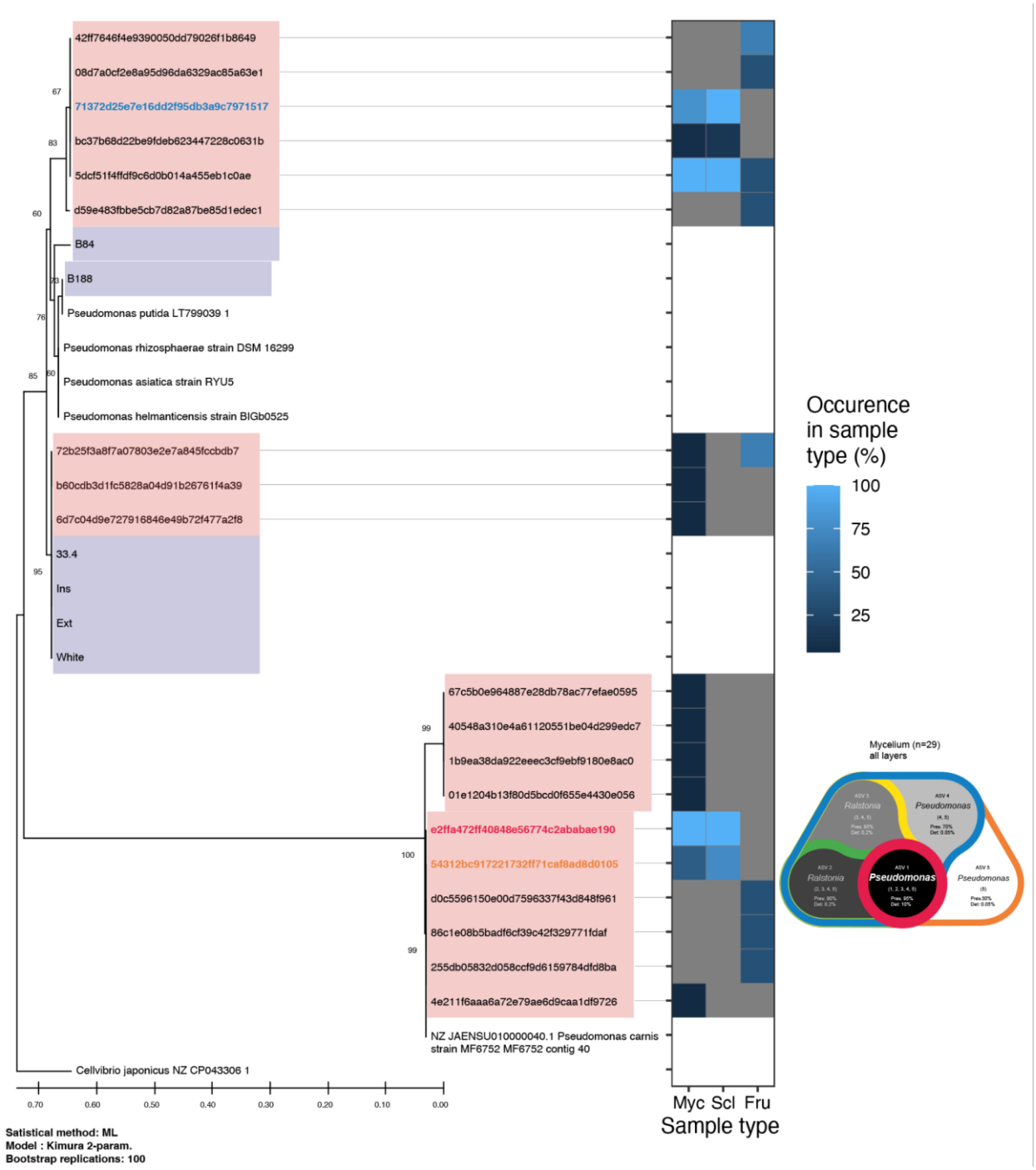


Figure 3. Phylogenetic classification of the bacterial strains isolated from morels. A. Maximum likelihood tree built from 16S rDNA extracted from the reference genomes (white), whole genomes sequencing from the isolates (violet) and V3-V4 16S rDNA regions obtained from *Pseudomonas*' ASVs (pink) from the associated bacterial communities. In front of the phylogeny, the occurrence of ASVs in different sample types is represented as a heatmap. The IDs of ASVs belonging to the core community in mycelium are highlighted in the same colors shown in the insert to the left. For the sample types Myc= mycelium, 29 samples; Scl= sclerotia, 8 samples; Fru= fruiting bodies, 9 samples.

Confrontation assays

We tested the interaction of these six bacterial strains with five morel strains, including representatives of the Elata and Esculenta clades. Both the bacteria and fungi developed in the medium, but the interactions established between different species varied and resulted in the modification of the way the fungus or the bacterium developed (Figure S2). Different interaction outcomes were observed and to better represent the effect of the confrontation, those were grouped in three categories related to the interaction phase (growth patterns of each partner, overlapping, melanization after contact), fungal survival (formation and positioning of sclerotia, association of bacteria to sclerotia), and bacterial survival (survival of the bacterium upon contact with the fungus established by reculturing). A total of 13 categories were established and for each category a score was given to the different combinations. With those data, the resulting pattern of interaction was represented as a heatmap including all the bacterial and fungal species tested (Figure 4A).

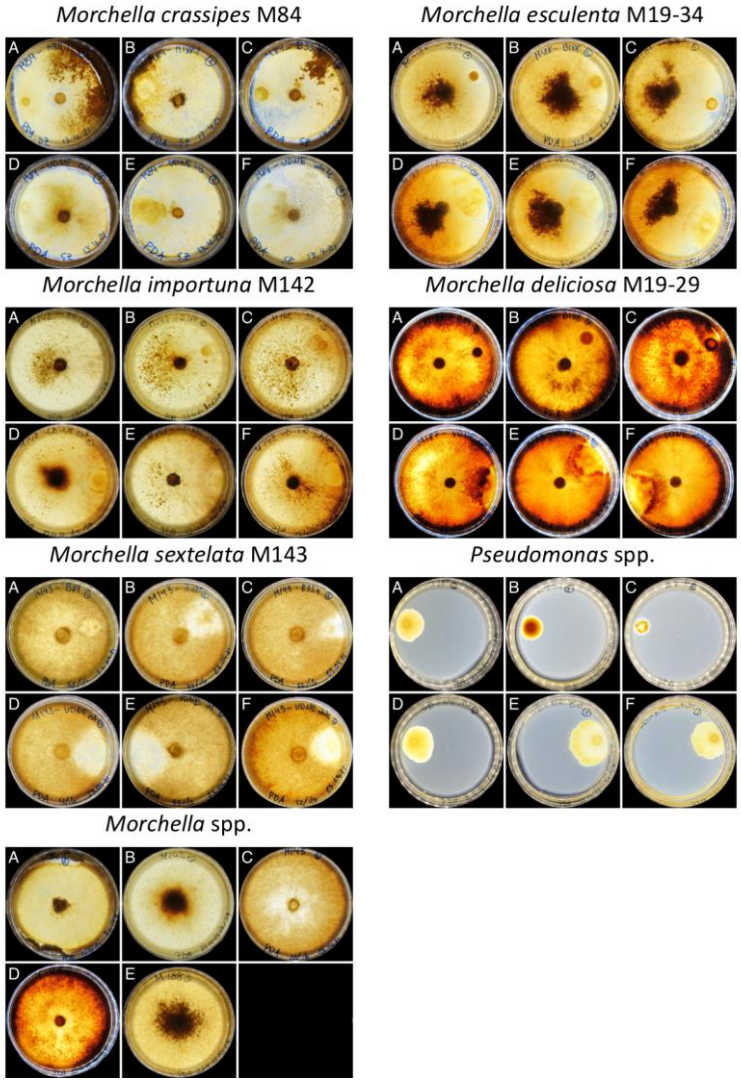


Figure S2. Interactions between different *Morchella* spp. and bacteria from the genus *Pseudomonas* isolated from mycelia. In each case, the bacterium was inoculated on one side of the plate and the fungus was inoculated as a plug in the center. Images of the interactions taken at 21 or 22-day post inoculation. For the bacterial strains: A= B84; B=B188; C=B33.4; D=VD-NE Ext; E=VD-NE Ins; F= VD-NE White. For the *Morchella* specimens control plates (bottom): A= M84, B= M142, C= M143, D= M19-29, E= M19-34.

There were two general patterns of growth of the bacterial colonies. Strains B188, B84 and B33.4 developed as a round colony that rarely exceeded 1 cm of diameter. In contrast, all the strain VD-NE grew as spreading colonies, although their growth was restricted in the interaction with the fungal strains M142 and M143 (both black morels from China). Fungal growth was less affected by the interaction, with the exception of *M. deliciosa* M19-29, which grew less in confrontation with the bacterial strains VD-NE ins and white, and the yellow morels M19-34 and M84, which grew less in confrontation with the bacterial strains B33.4 (isolated from the M19-34) and all the VD-NE strains, respectively. The area of contact with the bacterial inoculum was usually not melanized, but hyphae of two of the black morels (M142 and M19-29) were more melanized upon contact with bacteria. A conflict line was present in the interactions with the VD-NE strains for all the fungi but M84. In contrast, this was variable for the other three bacterial strains. Also, the fungus *M. esculenta* M19-34 always produced a conflict line in the interaction with the different bacterial strains. The interaction with bacteria inhibited the formation of sclerotia in *M. sextelata* M143 (all bacterial strains), *M. deliciosa* M19-29 (B188, B84, and B33.4), and *M. crassipes* M84 (VD-NEext and VD-NEwhite). In the other fungi, sclerotia were formed mostly opposite to the bacterial inoculum (always in the case of *M. esculenta* M19-34). When formed, those sclerotia were colonized by bacteria, although bacteria could not always be recovered after transfer to a medium with antibiotics (strains B188, B84 and B33.4; bacteria scletoria) and further subculturing (strains B188, B84 and B33.4; bacteria colony). The different bacteria always survived in the area of inoculation, and appeared in the majority of cases, to disperse in the Petri dish using the fungal mycelium (detection on the opposite site). However, the recovery of bacterial colonies in either area after culturing on medium with antifungals was never possible in the case of those bacteria in interaction with *M. crassipes* M84 and in some cases with *M. sextelata* M143 and *M. esculenta* M19-34 (Figure 4).

As the testing of the effect of the confrontation on bacterial viability was done by re-culturing, 153 bacterial subcultures (bacterial inoculum: 83 and opposite inoculum: 70) were obtained. The bacteria present in these subcultures were re-identified using colony PCR. Many of the re-cultured strains appear to be more closely related to other *Pseudomonas* ASVs of the associated bacterial communities, rather than to the original strains. However, it is important to indicate that in many cases, multiple strains were suspected to re-grow and co-exist after reculturing, but those could not be isolated individually afterwards (data not shown).

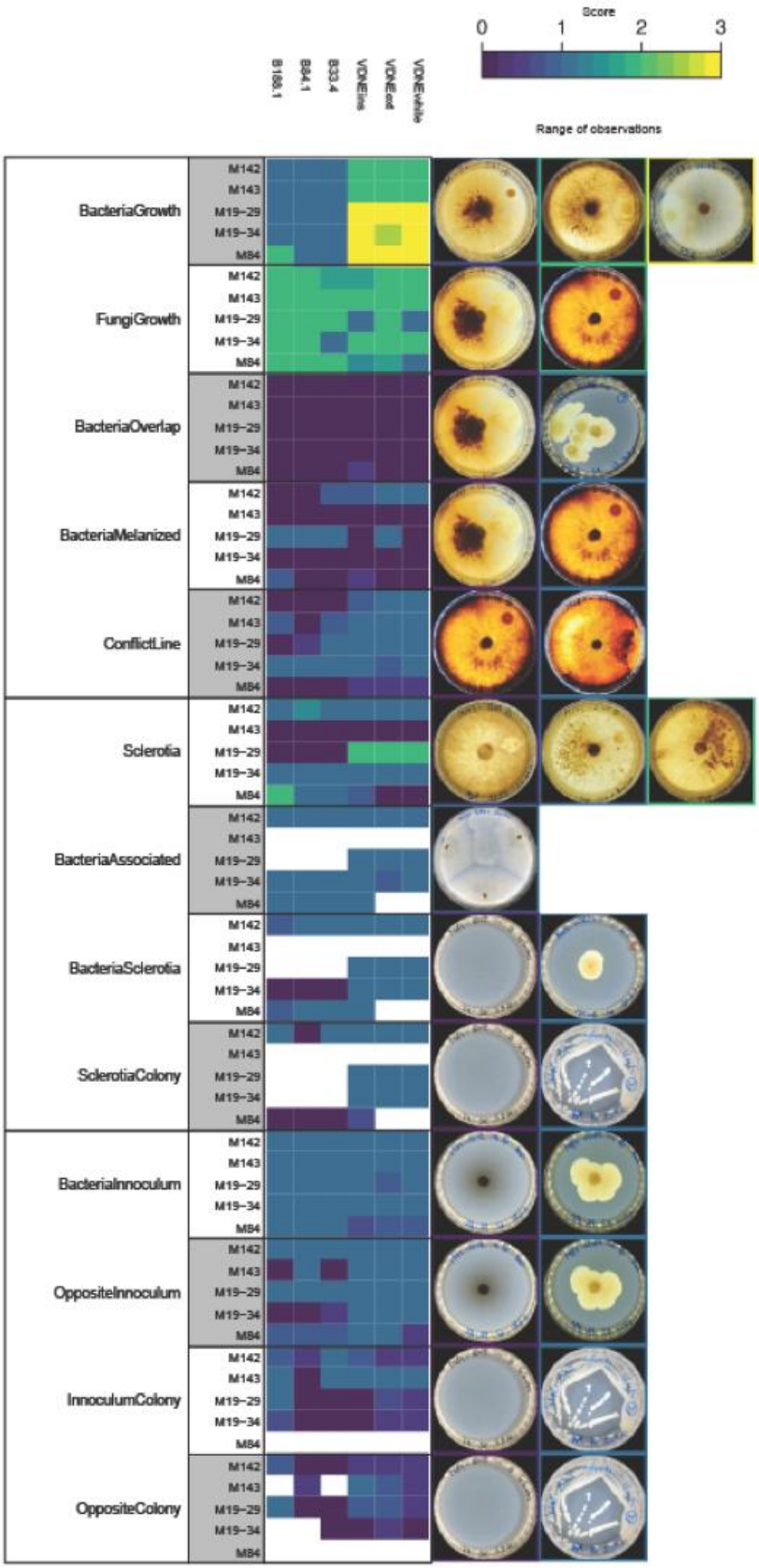


Figure 4. Heatmap illustrating 13 interactions between *Morchella* spp. and *Pseudomonas* spp. First 5 interactions illustrate the BFI, fungal properties and finally bacterial survival. Levels are represented with petri dishes and associated with color levels. BacterialGrowth has 4 levels, FungalGrowth and Sclerotia have 3 levels, all other interactions have two levels

Genomics of Morchella-associated Pseudomonas

The assembled genomes of the six Pseudomonas strains were analyzed. The size of the genome for all but strain B188 was in the range of 6.6 Mbp and coded for approximately 6000 CDS. The genome of strain B188 was smaller, and accordingly, encoded only 5700 CDS. The genome of B188 contained the largest number of tRNAs, while strain B84 contained the lowest number. The number of hypothetical proteins and those with a functional assignment was very similar between the strains (Table 1).

Table 1: Genome statistics

Genome	B188.1	B84.1	33.4	Ins	Ext1	White
Contigs	1	1	1	1	1	1
GC Content	61.51	61.41	59.15	59.22	59.22	59.22
Genome Length (bp)	6'191'592	6'630'422	6'604'192	6'696'054	6'696'047	6'695'895
CDS	5701	6029	5917	6080	6081	6084
tRNA	114	66	73	89	89	91
Repeat Regions	76	57	44	73	73	73
rRNA	22	19	19	19	19	19
Hypothetical proteins	1'102	1'411	1'323	1'407	1'412	1'414
Proteins with functional assignments	4'599	4'618	4'594	4'673	4'669	4'670
Proteins with EC number assignments	1'240	1'281	1'262	1'259	1'258	1'258
Proteins with GO assignments	1'060	1'097	1'088	1'086	1'085	1'085
Proteins with Pathway assignments	926	969	956	949	948	948
Proteins with PATRIC cross-genus family (PGfam) assignments	5'654	4'933	5'669	5'904	5'903	5'907

The core genome of the six strains corresponded to 2642 Gene Clusters (GCs), which is approximately half of the genome with a functional assignment. Accordingly, a large fraction of the carbohydrate metabolism was shared between the strains. In contrast, the completeness of other metabolic modules was more variable, including those involved in the metabolism of cofactors and vitamins (Figure 5A). The relatedness of the three VD-NE strains, which could be suspected from their morphology and effect on the fungal host, became very evident based on the comparative genomic analysis for the six genomes. Not only did they share all of the annotated metabolic modules, but also had a common core of 632 GCs. In contrast, for the other three strains, a unique set of specific genes was detectable. This set was largest for strain B84 (2169 GCs), and smallest for strain 33.4 (558 GCs), which was closely related to the VD-NE strains (Supplementary Figure S3). The differences and similarities of the strains can be also identified by comparing their gene content with the essential gene content of a model organism such as P. putida KT2440 (Figure 5B). While the essential gene repertoire of P. putida is not only fully present but also displays an extremely high sequence identity in strain B188, two, three and five essential genes are missing in strains 33.4, all the VD-NE strains and strain B84, respectively. Moreover, protein sequence identity was much lower than for strain B188.

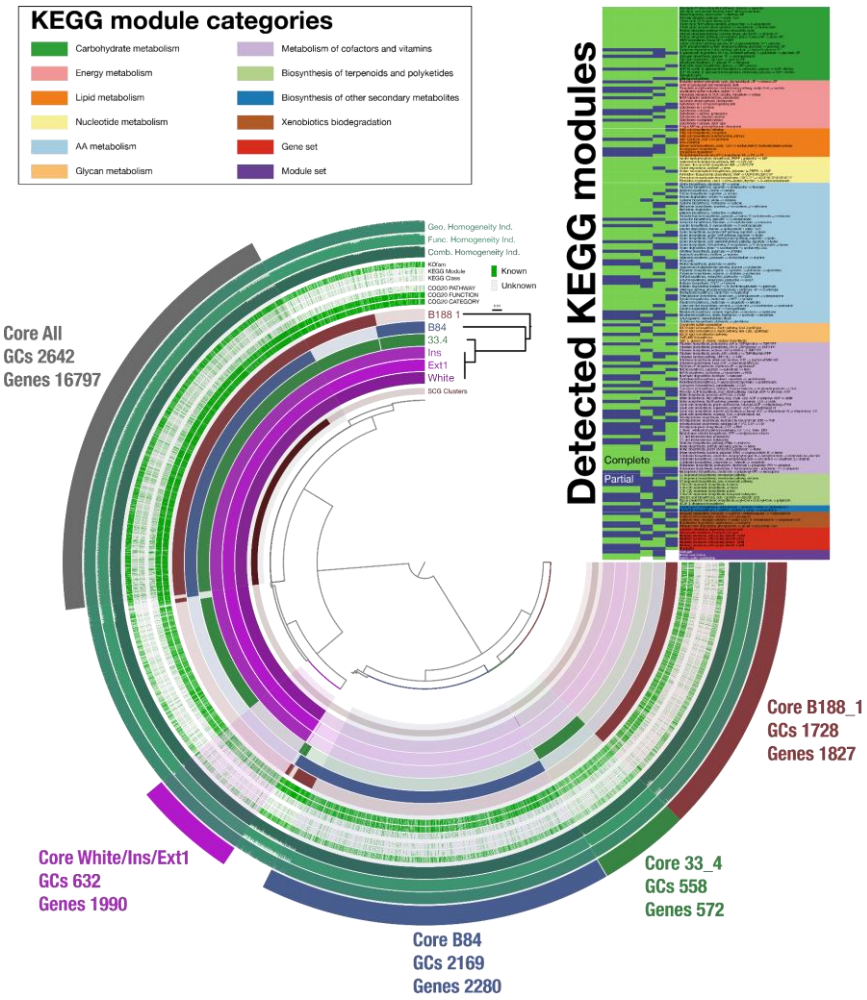


Figure 5A.

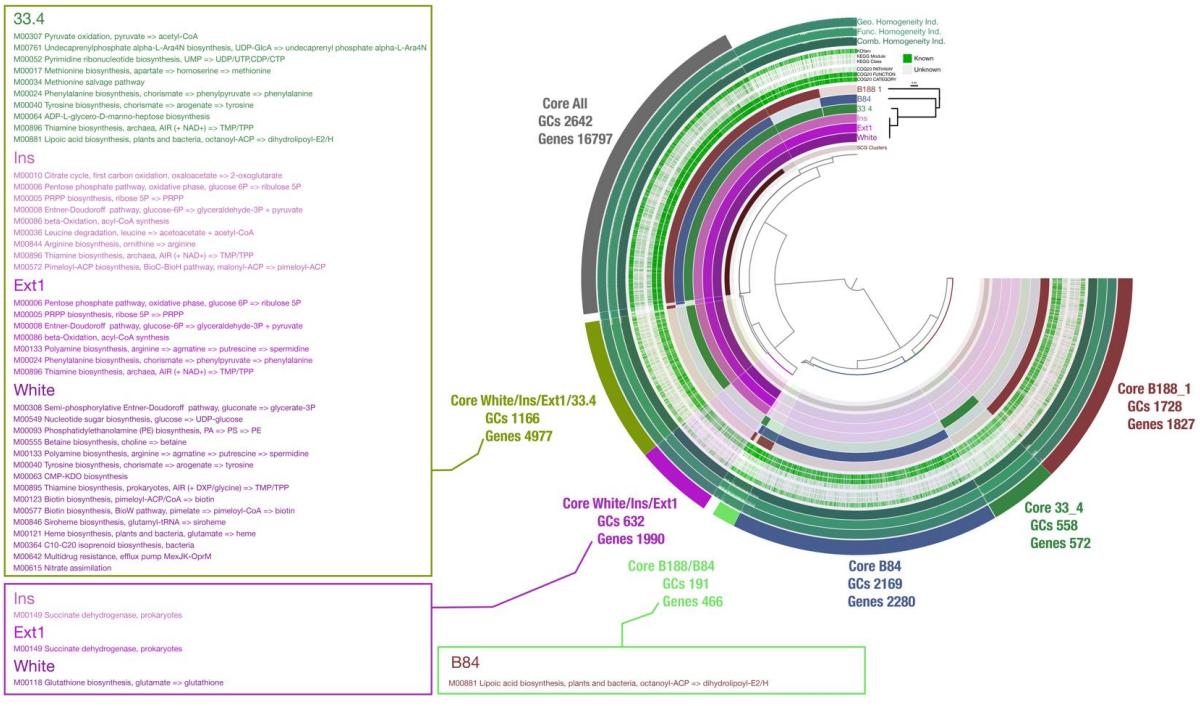


Figure S3.

An analysis of complete KEGG modules contained within the core genome was also performed (Figure 5B). The core genome of all the strains contained the complete KEGG modules corresponding to the biosynthesis pathway for the amino acid tyrosine, the degradation of pyrimidine, a component of the respiratory chain (cytochrome c oxidase).

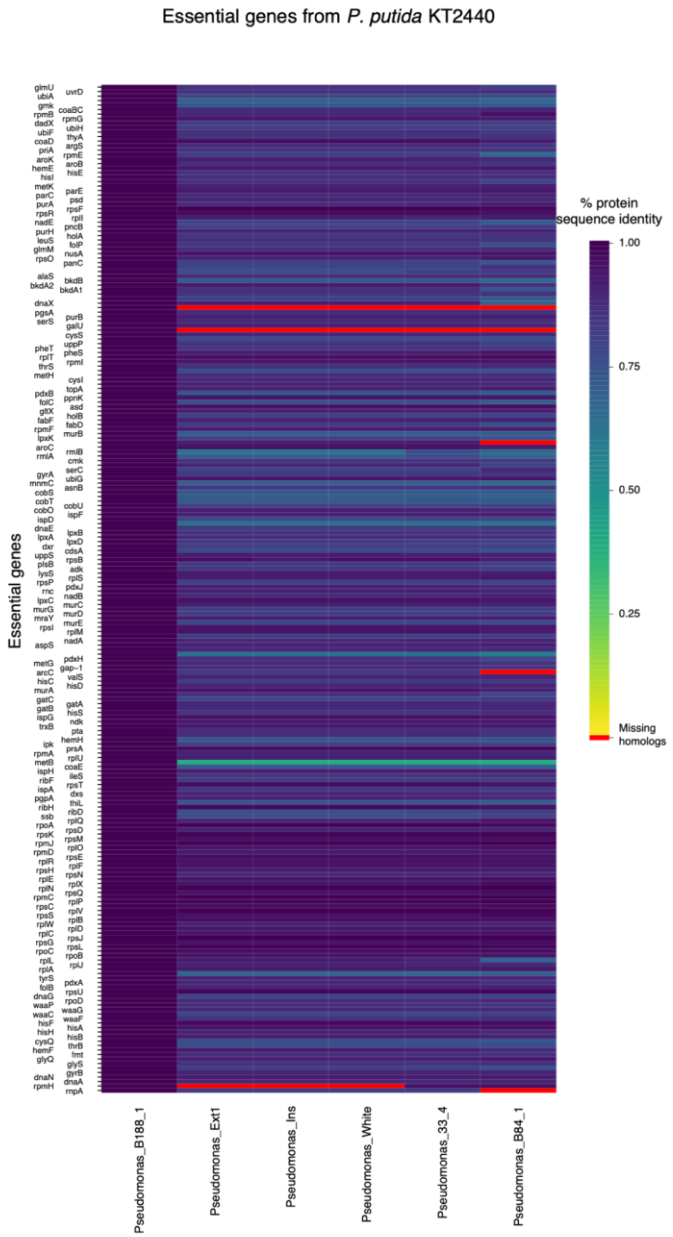
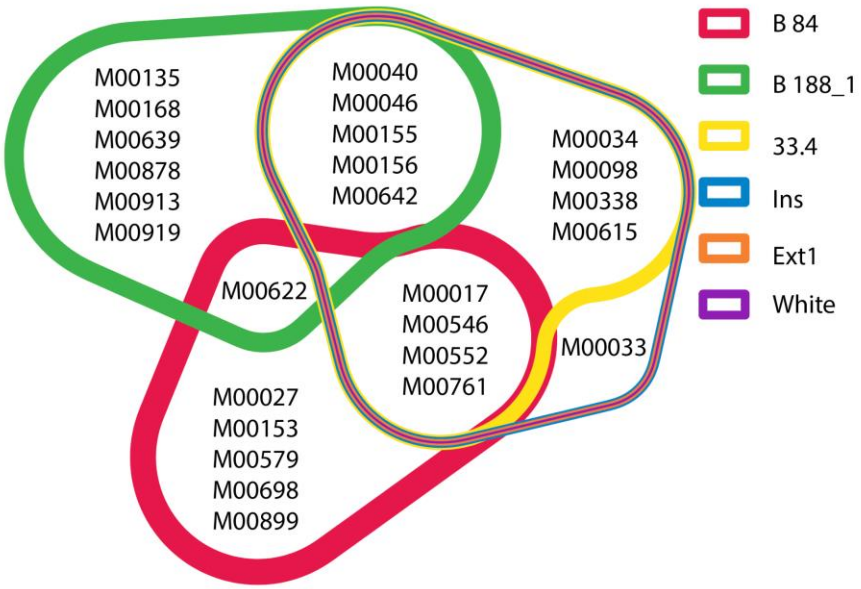


Figure 5B.

The strains B188 and B84 contained, as expected, the most unique complete KEGG modules (6 and 5, respectively). In the case of B188, two of these modules correspond to metabolic components of plant metabolism, including the GABA biosynthesis pathway (M00135) and the Crassulacean acid metabolism (M00168). Accumulation of GABA has been shown to be involved in the regulation of growth mode (planktonic versus biofilm) in Pseudomonas protegens (https://apsjournals.apsnet.org/doi/10.1094/MPMI-05-17-0120-R). In contrast, strain B84 contained a GABA shunt module (M00027). While all but strain B84 possessed the multidrug efflux pump MexJK-OprM (module M00642), which is typical of Pseudomonas (https://academic.oup.com/femsre/article/33/2/430/590421), strain B188 and B84 possessed in addition the efflux pumps MexCD-OprJ (M00639) and BpeEF-OprC (M00698), respectively.



4. Discussion

In this study, the bacteriome of morels was investigated in detail for the first time. In addition to the factors that can affect fruiting body formation (mating types, ecology, season), another important aspect required to understand the biology and life cycle of morels is their biotic interactions with other soil microorganisms. Bacteria are considered to be a factor promoting primordia differentiation and ascocarp growth, and may help to suppress diseases that could affect crop yield in cultured systems (Liu, Liu et al. 2017). Therefore, understanding the relationship of morels with their associated bacteria is of high interest. A previous article investigating the microbial soil communities associated with M. sextelata in cultivation in greenhouse systems found that bacteria such as Pedobacter, Pseudomonas, Stenotrophomonas, and Flavobacterium comprise the core microbiome in soils underneath M. sextelata ascocarps (Benucci, Longley et al. 2019). Many of these groups were also found in close association with the ascocarps of the wild Swiss morels investigated here. Moreover, here we demonstrate that the microbiome of mycelium and sclerotia differs from the microbiome of ascocarps. The main difference corresponded to the high abundance of Pseudomonas in both mycelial and sclerotia samples. *Pseudomonas* are ubiquitous bacteria that are often identified as fundamental components of bacterial communities and thus play essential ecological functions in the environment [24–26]. Furthermore, *Pseudomonas* are remarkable producers of secondary metabolites that are often directly involved in the support of a very diversified lifestyle in different species (e.g., iron scavenging, swarming motility, biofilm formation, pathogenicity, cooperation, or antagonism) [27,28]. In the past, Pseudomonas have been shown to associate with morels acting as a potential nutritional resource (Ref to Farming) or promoting better access to nutrients such as nitrogen (Ref Andrea). This has prompted authors to hypothesize that Pseudomonas spp. may have an effect on morel primordial differentiation, which is a key stage in the formation of fruiting bodies (Li, Xiong et al. 2017). Our results provide a clear indication that indeed, Pseudomonas are not only natural biological partners to morels, but that their distribution changes according to the tissue under consideration.

We also show that some of these Pseudomonas can be isolated from the fungal host and cultured as pure cultures. Those strains might represent more facultative associates, while the most intimately associated Pseudomonas (those constituting the core microbiota),

were not recovered in cultivation. The strains that could be cultivated were clearly distinct from those that were initially considered as potential contaminants (strain B188) and established different types of interactions with diverse morel hosts. The strains associated with *Morchella* were also divergent in terms of their genomes. The most divergent strain was B84.
In arbuscular endomycorrhizae, endosymbiotic bacteria have been reported within vegetative spores, germinating hyphae and mycelia, showing a symbiotic interaction at every fungal life stage (Kobayashi & Crouch, 2009).

5. Literature

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