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Final report: Microbial invasions, with focus on Morels (*Morchella* spp.)

Image of a native morel (*Morchella elata*) in a forest near Chaumont, Neuchâtel, Switzerland (A. Lohberger)

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SUMMARY

Current knowledge about the invasive potential of microorganisms, and in particular of non-pathogenic microbial species, is very limited. A French company (France Morilles) is currently offering in the market a colonized substrate that farmers can buy to produce morels. This substrate is based on developments made in China on the culturing of morels and presumably contains a Chinese fungus. This Chinese strain of the fungal genus *Morchella* is intended for the production of comestible morels (Elata morel). In this mandate, the implantation of this allochthonous species and its potential effect on native populations will be used as a case study to assess the invasive potential of microbes and the risk they pose to native species in Switzerland. The genetic identity of the strain (or strains) in the substrate, its relationship to native strains, and its invasive potential are unknown. In addition, bacteria or other pathogenic fungi associated to this allochthonous culture are also unknown. In this final report, we provide an extended review of the state-of-art in the literature concerning the invasive potential of microbial species. We then present a review of the biology of morels, with particular emphasis in their reproduction. Finally, we present the results of a pilot study in which 1) we assessed the genetic identity of two strains isolated by one farmer from a colonized substrate provided by France Morilles and 2) we evaluated the potential of these two strains for hybridization with native morel strains isolated from the Canton of Neuchâtel. Our investigations indicate that *in vitro*, the two putative Chinese strains are neither more competitive, nor more aggressive than the Swiss strains. However, co-cultures of a Chinese and a Swiss strain has shown that a potential of hybridization may exist. Therefore, at this stage, a risk of displacing native morel population by introducing Chinese morels cannot be excluded. Noteworthy, extrapolating *in vitro* results to an environment context may be taken with caution. Therefore, assessing the competitiveness and hybridization potentials of Chinese and Swiss strains in soil would be required to confirm these results. Finally, investigating the life-strategies of different morel strains into more detail would allow assessing more thoroughly the environmental risk posed by the introduction of non-native morel species.

MICROBIAL INVASIONS, WITH FOCUS ON FUNGI

Invasive alien species: general aspects

Invasive alien species are defined by the Convention on Biological Diversity as “species whose introduction and/or spread outside their natural past or present distribution threatens biological diversity”. Introduction of invasive alien species into a new environment is often accidental, but it can also be the result of human-related activities (for instance transport and trade). To date, most of the research in the field of invasive alien species has been focused in animal and plant invasive species, and only little attention has been given to the potential for invasion of microbes. In particular, non-pathogenic (free-living or symbiotic) microbial species have been largely neglected. One reason for this is that the impact of pathogens on macro-organisms is easier to detect. For instance, the bacterium *Erwinia amylovora*, is an example of an introduced plant pathogen introduced in Europe in the 20th century and that is still causing great damage to fruit tree orchards[1]. Nevertheless, invasive non-pathogenic microbes have also the potential to modify ecosystem function and threaten local biodiversity[2]. For instance, *Cylindrospermopsis raciborskii* is a freshwater cyanobacterium originating from the tropics that has spread to temperate zones over the last few decades because of global warming and which can displace local populations thanks to its ability to fix atmospheric nitrogen and to its efficient use of other limiting resources[3]. Likewise, Rodríguez-Echeverría[4] has reported the detection of exotic rhizobia that readily associated to native plants from Portugal, but which were possibly introduced along with an invasive Australian plant host (*Acacia longifolia*).

Investigating microbial invasion presents a major scientific challenge. A number of key questions remain unanswered and a common framework to investigate invasiveness of non-pathogenic microbial species is still lacking. The most significant question concerns the definition of a microbial invasive species. Litchman[2]

proposes to define invasive microbes as “microorganisms (viruses, archaea, bacteria, protist and fungi) that proliferate in a new geographical area and impact local communities and ecosystems”. However, this definition relates the notion of invasive microbial species with the autochthonous or allochthonous origin of the organism, something that is difficult to assess in the microbial realm. Because of their ability to disperse using various means (water, air, attached to a moving solid, etc.) and life-forms (active versus dormant cells), it has long been assumed that microbes present a global pattern of biogeographical distribution[5]. However, recent studies have demonstrated that some microbial species have restricted distribution patterns, which will suggest a potential for invasiveness within the microbial world[2]. A second major challenge consists in the notion of a microbial species, which is a highly controversial and divisive topic in microbiology[6-8]. Nevertheless, aside the lack of a clear definition of an invasive microbial species, other pressing questions in the field include aspects such as how to detect microbial invasions? What is the effect of invasive species on resident microbial communities, as well as on ecosystem functioning? How to prevent microbial invasion? Addressing all these questions is key in terms of establishing the risk that invasive microbial species could pose to native biodiversity and to the functioning of ecosystems.

It is proposed that invasion by a microbial species follows a typical route consisting of introduction, establishment, growth and spread, and impact[9]. Microbes can be typically transported into new areas by air and water currents. However, more recently, human activity is contributing at an unprecedented scale with the movement (intentional or not) of microbes across the globe via waste disposal, trade of foods and goods, and tourism[10,11]. Once introduced, many traits are thought to be associated to invasiveness. Litchman[2] mentions that traits such as high growth rate, efficiency in resource utilization, and competitive capabilities will favor the invasiveness of an introduced microbial species. In addition, the same author claims that, as in the case of invasive plant and animal species, non-pathogenic invading microorganisms are often similar to native species and will tend to spread in low diversity communities given their enhanced performance traits. Moreover, under the premises of global climatic change and its effect on the environmental parameters at a given ecosystem, it is likely that the process favoring microbial invasion under specific conditions (for instance, change in water temperature or soil moisture content) will increase. Finally, it is important to consider that the effect an invasive microbial species eventually has on ecosystem functioning is probably as important as its impact on biodiversity, as microbial taxonomic diversity does not necessarily mirror the functional diversity[12,13].

Invasive fungi

Fungal invasions are easily detectable when the fungus is pathogenic, because pathogenic fungi can have a large impact on plant or animal host populations. Moreover, in the case of pathogens, the negative impact on native biocoenosis (an essential component of the definition of an invasive species) is often beyond doubt. Although only 2% of the research in invasive species investigates fungi, there are remarkable examples that illustrate the invasive potential of pathogenic fungal (or fungi-like protist) species[14,15]. Those include *Cryphonectria parasitica* (Ascomycota) causing chestnut blight[16] or *Phytophthora infestans* (Oomycota) causing late blight in potatoes[17]. In some cases, the route of introduction is relatively well-accepted. For instance, *Ophiostoma ulmi* (Asctomycota), was accidentally introduced into America and Europe from Asia through wood infested with *Scolytus* spp., and its introduction has resulted in the devastation of the native elm populations[18]. Another dramatic example is *Batrachochytrium dendrobatidis* (Chytridiomycota), which most likely originates from East Asia and has spread throughout the world probably due to the trade of amphibians, devastating local populations in the Americas and Europe[19,20]. A final example of a well-documented case of invasion by a pathogenic fungus is the spread of a clonal population of *Pseudogymnoascus destructans* (formerly known as *Geomyces destructans*; Ascomycota), which is devastating bat populations in North America[21]. This psychrophilic fungus is broadly distributed in Europe, but its presence has never been associated with mass mortality among bat populations, suggesting it is native to this continent[22]. In all of the above-mentioned examples, the negative impact on local biological diversity is evident beyond doubt.

Non-pathogenic fungal invasions are much more difficult to evaluate, as no or only small effects are observed on the ecosystem. Nevertheless, there are examples in the literature showing that non-pathogenic fungal

species can also have an invasive behavior. For instance, a European cultivar of *Agaricus bisporus* (Basidiomycota) has become established in southern Canada and northwestern USA. All indigenous population have been invaded by this fungal cultivar, and hybridization occurred between indigenous population and the European cultivar, although outcrossing may not be frequent[23,24]. Another example of a saprophytic fungus invading an indigenous niche is *Favolaschia calocera*, a basidiomycete species that was first described from Madagascar, but is now spreading to many countries. The expansion of the distribution range of this species has been attributed to specific reproductive traits such as selfing (see below), polyphagy, and to the production of antimicrobial compounds[25]. The ecological effects of invasion by non-pathogenic fungi are often difficult to estimate. Issues regarding the conservation of the genetic diversity of indigenous populations seem obvious, but effects on ecosystem functioning are very difficult to assess. Some ectomyccorhizal fungal species are considered invasive species, as they were inadvertently introduced with their plant host. Their presence can in some cases facilitate plant invasion, thereby representing an example of an altered ecosystem functioning as a result of fungal invasion[26]. Other invasive fungi have deleterious effects on native fungi, which may in turn affect ecological interactions these species have within the ecosystem (e.g. mycorrhizae). For instance, it is thought that the native community of *Trichoderma* spp. (Ascomycota) in Tenerife island has been suppressed by strains coming from other regions. Invasive *Trichoderma* spp. were shown to be more competitive and display a larger antagonistic spectrum compared to native strains[27]. Similar observations were made in Sardinia, where invasive *Trichoderma* spp. may have been introduced by humans and/or aeolian transport[28]. However, invasiveness of fungal species such as Trichoderma that produce large amounts of asexual spores that can disperse over long distances[29] is difficult to assess. In addition to this, assessing how this may impact soil functioning is delicate.

Fungal reproduction, genetic diversity and invasiveness potential

Some pre-adaptations such as high phenotypic plasticity can favor the establishment of fungi in a novel environment[30]. However, specific adaptations after the phase of introduction might tilt the scale in favor of an invading fungal species and some of those traits can be acquired through sexual reproduction[31]. Reproduction modes and mating systems in fungi are extremely diverse and play a significant role in the invasiveness potential of a particular species[32,33]. Besides asexual (clonal) reproduction, fungi can reproduce sexually through different mating systems (reviewed by Billiard et al.[33]). A genomic region called the mating type locus (*MAT*) plays a major role in the sexual cycle of fungi[34]. This system can be bipolar (one locus with two opposite alleles), or tetrapolar (two unlinked loci, often multiallelic). To reproduce, mating partners must have different mating types. Fungi that possess both mating types in the same individual are called homothallic fungi and can reproduce sexually through selfing (i.e. fusion of gametes from a single individual). Fungi that possess only one mating type are called heterothallic fungi and reproduce by outcrossing (i.e. cytoplasmic and nuclear fusion between two individuals with opposite mating types). While asexual reproduction allows for a rapid increase in population size and transmission of adapted genotypes, sexual reproduction generates genetic diversity that is useful for adaptation to the environment. Many fungal species can reproduce both asexually and sexually[31]. Accordingly, simulations have shown that species with a mixed mating system, but predominantly reproducing by asexual reproduction, had the most potential to invade new niches[32].

Moreover, fungi can mate not only with conspecifics but sometimes also with interspecifics, giving rise to hybrids. Hybridization depends on the relatedness between two conspecifics, their physical proximity and the potential advantage conferred by genetic exchange[35]. Hybrids can display novel phenotypes such as increased virulence, switched lifestyle (i.e. pathogen to mutualist), and new host range[36]. Interestingly, it has been shown that hybridization was a common event in species that did not coexist and could give rise to new fungal pathogens[35,37]. Such interspecific hybridization has been observed for instance in *Ophiostoma* spp., the ascomycetes that cause Dutch elm disease[18]. When hybrids backcross with their parents, they can introduce new genetic material in the genetic pool of the parental line, a process called introgressive hybridization.

A last process that could help fungi to acquire new genes is the process of horizontal gene transfer (HGT), i.e. transfer of genetic material between species. There are hundreds of HGT events identified in fungi, with the

genes originally originating from prokaryotes and other fungi. These transfers can also favor the invasiveness of fungi[38].

Mycorrhizal fungi and biological invasion

Mycorrhizal fungi are a functional group of fungal species with a high potential of dissemination outside their local range of distribution. They are ubiquitous symbionts of roots of the vast majority of plant species and thousands of plants species have been, and continue to be, exported across countries. In the case of plants, an intact root system and its surrounding soil are required for viability. Therefore, plant-associated fungi are likely moved along with their plant host[39]. Examples illustrating the invasive potential of ectomycorrhizal fungi include *Amanita phalloides (*Basidiomycota*),* a fungus native to Europe but that has been introduced to many regions worldwide, and it appears to have become invasive in North America[40]. Likewise, *Aureoboletus projectellus* is a boletus species reported to originate in the Americas that has been recently spreading in Europe and forming ectomycorrhizal associations with native European pines[41].

As mycorrhizal fungi are known to provide plants with nutrients in exchange of photosynthetically derived carbon, this fungal group has generated great interest in agriculture[42]. For instance, arbuscular mycorrhizal fungi (AMF; Phylum Glomeromycota) are known to form symbiosis with most plants, and to provide them with nutrients, which can lead to an increase in plant productivity[43]. Whereas the efficacy of commercial AMF in the field is still debated[44-46], AMF have not been studied in the context of their potential impact as invasive species and their effect on local microbial communities. Nevertheless, Schwartz et al.[47] had warned that AMF have the potential to persist and invade new habitats, and may different effects on plant growth, local fungal and plant communities, as well as in ecosystem processes. In greenhouse experiments, researchers have found that commercial AMF of a foreign origin decreased diversity or abundance of local AMF communities[48]. A similar result has been shown in a field experiment, where a non-native AMF was dominant over native AMF in roots 3 months post-inoculation. Moreover, the non-native AMF survived as root symbiont 2 years post-inoculation, but declined after this time period[49]. Therefore, a commercial AMF inoculum with the potential to establish in soil, could also spread beyond the intended host plant, as AMF propagules are known to disperse through air, soil and water[44]. Moreover, it is possible that foreign strains have the potential to hybridize with native strains, as AMF from the same species but genetically distinct can exchange their genetic material through anastomosis[50]. Hence, there is a real need to assess the environmental risks of fungi used in agriculture and to establish regulations policies concerning the global distribution of AMF inocula and other microbial products used in agriculture[44].

Another example of an invasive mycorrhizal fungus that has considerable economic implications is the Chinese truffle (*Tuber indicum*, Ascomycota). This species has been introduced to Italy via mycorrhized seedlings in truffle plantations. It is now invasive in Italy and threatens the indigenous populations of the Perigord black truffle (*Tuber melanosporum*), which has higher organoleptic properties and is therefore much more commercially valuable. *In vitro* experiments have shown that *T. indicum* is dominant, competitive and more aggressive than *T. melanosporum*, which is of great concern[51]. Aside the major consequences in terms of the economic value of this fungal crop, the ecological consequences of such an introduction in Europe are still unknown. The existence of *T. indicum* fruiting bodies is also reported in USA, with a similar introduction mode than in the case of Italy. The Chinese truffle is supposed to have been introduced via the import of mycorrhized seedlings thought to be inoculated with *T. melanosporum*[52]. Both *Tuber* species have been identified as heterothallic, meaning that two opposite mating types are required for reproduction[53,54]. Thus, sexual compatibility between *T. indicum* and *T. melanosporum* is not excluded. This last example shows that aside the ecological considerations, invasive species present a significant threat in key economic areas such as agriculture. Although, the absolute cost of this could be highest for the biggest agricultural global producers (China and the United States), other countries, and in particular developing countries appear to be the most vulnerable in relative terms. In addition, it was recently suggested that China and the United States represent the greatest sources of invasive species for the rest of the world[55], but global economy and transport of goods and products means that every country should be concerned.

Moreover, the soil biota is increasingly recognized as playing a crucial role in shaping the plant and animal communities above ground via a complex network of species interactions. Therefore, major modifications of the soil biota driven by microbial invasion, can have multiplying effects on the modification and invasiveness of other organisms[56,57]. Accidental transport of fungi, bacteria, viruses, and protists in terrestrial, freshwater, and marine systems can have a dramatic and often catastrophic effect on the populations of plants and animals, in particular if they lack prior evolutionary contact. Also, given the ability of microbial taxa to undergo swift genetic modification, either through natural selection or via horizontal gene transfer, can result in elevated virulence, the ability to infect new hosts, or the emergence of entirely new invasive species[56], and therefore, investigating the invasive potential of microorganisms should become an area of high priority for scientist and governmental institutions.

CASE STUDY: ADDRESSING THE INVASIVENESS POTENTIAL OF THE GENUS *MORCHELLA*

Morchella is a genus of fungi from the phylum Ascomycota, represented by a high diversity of species or species complexes. It is highly prized for its gustative qualities and of great economic importance. In the field of conservation biology, some authors had equal morels to emblematic macro fauna, as morels are easily recognized and appreciated by the general public[58]. They are collected and exported intensively in China, India, Turkey, Mexico, and the USA[59]. However, little is known about the population biology and life cycle of morels[60]. The systematics and taxonomy of the group is problematic, and there are numerous cryptic species. Moreover, morphology-based species identification of morels is limited and prone to confusion[61]. Hence, molecular studies appear to be the most appropriate approach for the identification of species assigned to the genus. However, an analysis of the ITS-rDNA locus, which is a genetic marker traditionally used to identify *Morchella* species, revealed that at least 66% of the sequences identified as *Morchella* in the GenBank database are misidentified[62]. More recently, the phylogeny of *Morchella* was determined using multilocus molecular analyses[58,63]. In contrast to morphologically based species concepts, multilocus studies suggest that *Morchella* spp. comprises around 50 phylogenetically distinct species[64]. According to phylogenetic analyses, species within the genus *Morchella* can be grouped in three clades: the Elata Clade (black morels, i.e. the one commercialized by France Morilles), the Esculenta Clade (yellow morels) and the rufobrunnea Clade (white morels)[65]. *Morchella* species are believed as being able to form ectomycorrhizal associations while others are saprophytic[66].

Morchella spp. exhibit a high level of continental endemism and provincialism[65], which is relevant in terms of the invasiveness potential of introduced cultivars. Richard et al.[61] found that only seven species were found in both Europe and north America, while high endemism for each continent was the most prevalent feature of the investigated species. A survey conducted in Turkey by Taşkın[58] also showed endemism, and a recent survey in Australia revealed a new endemic species[67]. This endemism could be explained by the reproduction cycle of this fungus, and the fact that it appears to support badly dispersal by natural means (water or air)[65]. Interestingly, one third of the strains identified as *M. elata* in a strain collection from Turkey, were endemic to North America, which suggest that many of these morels have been introduced into Turkey by human intervention[64]. In Europe and Switzerland, *M. esculenta, M. elata,* and *M. importuna* are reported as the most common species[68].

In the last few years, China started outdoor artificial cultivation of morels, and the annual export of dried morels increased five fold from 181 tons in 2010 to 900 tons in 2015, averaging $160 US dollars per kilogram[69]. As a comparison, India only exports 70 tons of dried wild morels per year[70]. 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, mainly *M. importuna*), with 80-90% of the production[71]. However, production is unstable, which is probably the result of the complex sexual reproduction of morels (see below)[72,73]. However, many other abiotic parameters can influence fruiting body formation, such as temperature, humidity and nutrients. The latter could be improved in natural habitats thanks to wild fires, which benefit post-fire fungi[73]. Regarding biotic factors, some soil microbes are thought to support morel growth. Liu et al[73] hypothesize that bacteria associated to the fungus, and in particular *Pseudomonas* spp., may have an effect on morel primordial differentiation, which is the key to the formation of the fruiting body throughout the morel life cycle. Similar benefits provided by bacteria have also been suggested for black truffles, where bacteria could play a role in the development, maturation and even final aroma of the black truffle[74]. In addition, *Morchella* are potentially reported to harbor endobacteria when establishing tripartite associations with Basidiomycete ectomycorrhizae[75]. Observations in our laboratory also suggest that some *Morchella* species directly harbor endobacteria in their hyphae (unpublished results), but the role of these endobacteria in morel physiology remains unknown.

Morchella reproduction

Throughout the life cycle, the mycelium of morels is mostly haploid. When primary mycelium germinates from an ascospore, it can form sclerotia to survive harsh environmental conditions. In spring, sclerotia can form a carpogenic mycelium and eventually fruiting bodies (haploid fruiting), although this is rare and no ascospores are produced. It is more likely that fruiting body formation is the result of the encounter of two compatible haploid mycelia, which will fuse to form a heterokaryotic mycelium and eventually the fruiting body[71]. Indeed, some studies indicate that the genus *Morchella* is a heterothallic filamentous ascomycete, as in the above example of truffles[60,76]. This means that morels have a single *MAT* locus (*MAT1)* with two idiomorphs (*MAT1-1* and *MAT1-2*)[77].

As haploid fruiting (without an opposite mating-type partner) is rare, O' Donnell et al.[65] claim that morels will be poorly adapted at invading novel niches and to sustain long-distance dispersal. Once a spore germinates, the chances of finding a colony with the opposing mating-type and finishing the sexual reproduction cycle are limited, thus the dispersal is restricted to asexual ascospores. This might partly explain the problem of reliable cultivation of morels for commercial purposes. Du et al.[76] speculate that the predominance of a mating type and spatial segregation of mating types are factors that reduce the chances of compatible organisms to mate and hence form fruiting bodies. Apart from intraspecific mating, it has been shown that hybridization or horizontal gene transfer occurred between two sympatric species[72]. These researchers have found that sympatric distribution was common in *Morchella* spp. hence allowing possible hybridization if a non-native species is introduced (for instance through cultivation as in the case of France Morilles) to a new environment.

The risk of importing morels on the biodiversity of native species from Switzerland

Despite the apparent absence of competitiveness of *Morchella* spp. to invade new niches suggested by some authors, imported morel cultivars could possibly hybridize with native species, as in the previous reports of hybridization or horizontal gene transfer between two sympatric species[72]. If so, introducing a Chinese cultivar into a new territory for its commercial exploitation would be a major issue as it could interbreed (and potentially displace) indigenous species. As the proposed Chinese inoculum is reported to correspond to the Elata clade morels, which is one of the clades of species commonly found as indigenous in Europe and Switzerland, they could represent possible invaders that could replace native genetic species. Ecological and economic consequences of such an introduction and invasion are unknown. Increasing our knowledge of the biology of cultivated morels is essential to investigate its invasive potential. This include not only its competitive abilities, survival and establishment in soil, but also their reproductive capabilities (i.e. mating type). Apart from morel invasion, there could also be a risk of morel-associated-microbes invasion, as well. Examples of those could be the endobacteria potentially associated to morels hyphae (unpublished results), as well as the pathogenic fungi currently affecting production in China. In China, *Morchella* spp. has been subjected to different fungal diseases affecting yield. Those include stipe rot disease[78], white mold[79], pileus rot disease[80]. Several of these diseases could be the result of an endophytic/mycoparasitic switch, in which, depending on conditions, a saprophytic fungus living in close association with *Morchella* could become pathogenic. Currently, the effect of those pathogens on native populations is unknown and represents another potential risk of the unwanted introduction of non-native morels.

Key questions that need to be addressed concerning the introduction of a Chinese cultivar by producers such as France Morilles are: can we expect a similar situation with morels as the current situation reported for truffles in Italy and the USA (displacement of native populations by less commercially valuable species)? Is the Chinese *Morchella* strain more competitive and aggressive of indigenous strains? Could import of Chinese morel strains pose a risk in terms of importing pathogens as well? Would the cultivated Chinese strain hybridize with indigenous species? In order to start addressing all these questions, the laboratory of Microbiology performed a pilot study in which the aims were: i) to identify two strains reported by a farmer as isolated from the inoculum provided by France Morilles in order to determine their phylogenetic affiliation; ii) to establish a collection of native morels from the Canton of Neuchâtel; iii) to determine the mating type of the different strains

in the newly created collection, as well as the putative strains from France Morilles and strains from referenced culture collections; iv) to perform preliminary trials of interbreeding under culture conditions between strains of complementary mating types.

Genetic identification of the strains from France Morilles

Two strains were collected from the farmer “Morilles du Lac” in the Savoie region (France). The strains were isolated by the farmer from soil in which the substrate provided by France Morilles was inoculated. The strains were maintained on Petri dishes with malt agar (MA) or potato dextrose agar (PDA) medium. Only two to three cycles of re-culturing were made before the strains were obtained. A genetic analysis based on ITS1-4 regions of the ribosomal gene cluster suggested that the two strains are related to *M. sextelata* (100% identity with *M. sextelata* strain HL-1), while the second strain was closely related to *M. importuna* (100% identity with *M. importuna* strain YAAS1392). Therefore, this analysis confirms that both of the putative strains from France Morilles correspond to species within the Elata clade that are known to be “domesticated” in China and to be amenable for cultivation under controlled conditions (*M. sextelata* and *M. importuna* strain SCYDJ1-A1[71,81]). Nevertheless, based on this genetic analysis only, it is difficult to conclude unambiguously if the strains are indeed from a Chinese origin. This brings an important subsidiary question concerning the culture of morels and it is the compliance with the Nagoya protocol. The Nagoya protocol is an international agreement that aims at sharing the benefits arising from the utilization of genetic resources in a fair and equitable way. While France and Switzerland had ratified the protocol (in 2016 and 2014, respectively), and are dealing with all the associated regulations that are required to fulfill its commitments, China is currently still in the phase of accession to the protocol (as for to 2016). The acquisition and commercial use of strains originally generated from China, therefore might infringe the protocol, if the required measures were not taken by the providing party.

As the initial provider, France Morilles should provide information relevant to the Nagoya treaty (the current distribution license is protected by a Chinese patent, and public information is highly limited). From the best of our knowledge, the farmer that provided the strains was entirely unaware of any potential legal issue concerning the access to genetic resources from China. After the discussion with the Federal Office for the Environment (FOEN) in Switzerland, it was concluded that the French legislation applies in the case of the strains used for the genetic identification originate from France, given the fact that the strains were isolated in France. Accordingly, to the French legislation, the genetic resources originated from domesticated or cultivated species are excluded from the measures to apply the Nagoya treaty, and therefore, there was a potential by which, if considered a domesticated mushroom, morels could be excluded. Therefore, we transferred our request to the contact point in France and got in contact with the people involved in implementing the national ABS legislation. This legislation regulates the access to French genetic resources for their utilization. The "utilization" is defined, under article L. 412-4 of the French environmental code, as "the research and development activities regarding the genetic or biochemical composition[...]". There is a general scheme and 5 specific ones, including one on genetic resources from domesticated or cultivated species, which are defined as "any species in which the evolutionary process has been influenced by humans to meet their needs" (article L. 412-5 of the French environment code). Indeed, no measures are needed regarding that specific scheme. However, there is no list of such cultivated species, so it is a case by case study. Furthermore, the fact that a population of mushrooms is cultivated by a farmer does not make the species to which they belong cultivated or domesticated *per se*: it has to fit the definition of article L. 412-5. However, in order to clearly determine if the morels proposed by France Morilles fulfill the definition, information by the former is required, but could not be obtained. In our case, we could declare the strains as part of a R&D project without any direct commercial purpose, but in the case of any farmer in direct contact with the company, it is unclear what the precise procedure should be as in this case the use of the strains has a commercial purpose. Overall, this issue highlights the importance, in the case of a wish to develop a commercial endeavor in Switzerland, of focusing on developing a cultivation system based on native strains and the Swiss genetic resource.

Establishing a collection of Swiss morels from the Canton of Neuchâtel

During the spring season (March to April 2019) a collaboration was established with a morel hunter to get access to morels collected in the canton of Neuchâtel. Investigating native morels is not necessarily something easily achievable, as morel hunters usually prefer to keep the localization of their hunting areas secret. Nonetheless, thanks to this collaboration we were able to obtain 34 populations of Morels identified by the hunter as *M. conica* (20 sets of samples; 61 individuals), *M. elata* (3 sets of samples; 15 individuals), *M. esculenta* (8 sets of samples; 26 individuals), and *M. vulgaris* (3 sets of samples; 12 individuals). In addition, 2 individuals of *M. semilibera* (the half-free morel) and 5 populations with 15 individuals 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 (*V. bohemica*) also belongs to the Morchellaceae family and is used as an outgroup to reconstruct *Morchella* phylogenies[65].

Upon collection, several analyses were performed on the individuals. First, we collected material for genetic identification from the fruiting bodies directly. Second, in those individuals with an intact fruiting body, isolation of mycelia colonies either from spores (Figure 1) or directly from the fruiting body was tested. Third, we investigated the mating type of both, fruiting bodies and the mycelia isolated, using the primers defined by Du et al[76]. Finally, we performed competition experiments between strains from the field, and also between the putative strains from France Morilles and those collected from the field possessing a complementary mating type (see below).

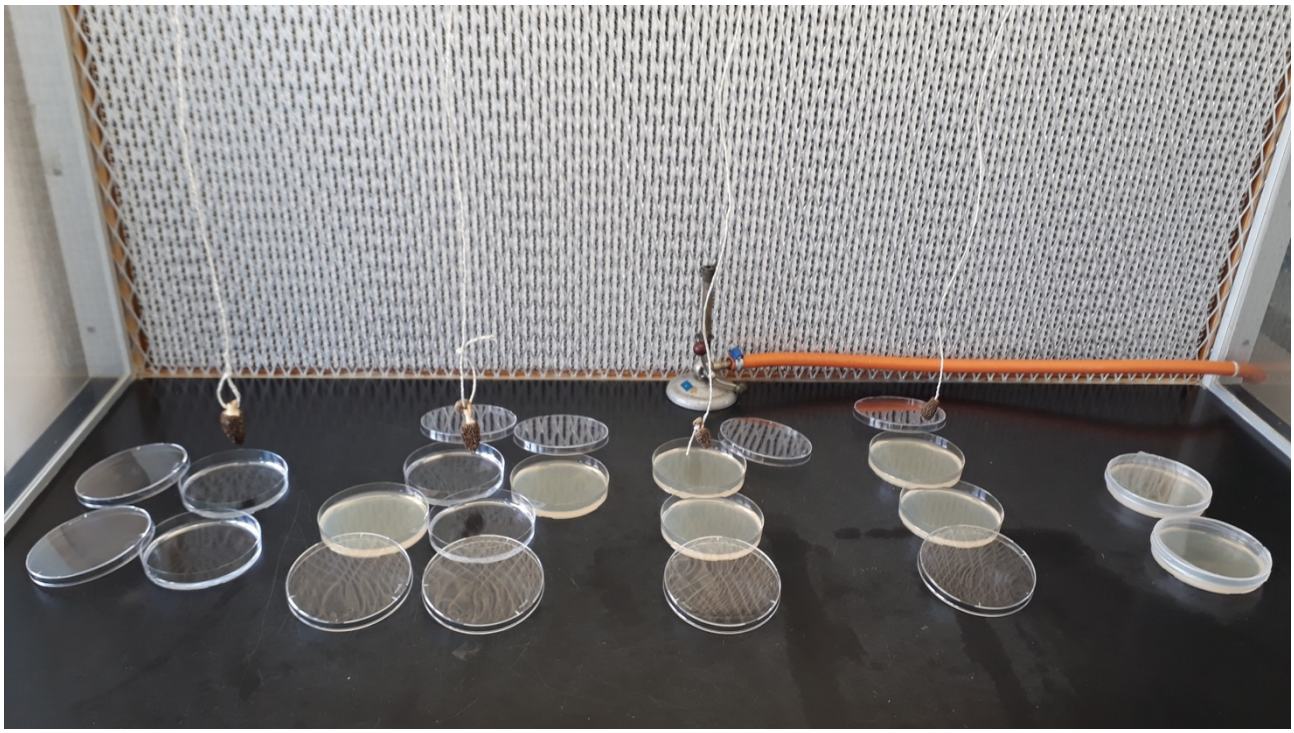

Figure 1. Collection of spores from mature *Morchella* ascocarps to establish mycelial cultures from spores. Ascocarps were hanged in a horizontal laminar flow hood and spores retrieved in empty Petri dishes (left side of the image) or directly on a culture medium (Malt- or potato dextrose-agar).

In terms of the identification based on direct sequencing from the fruiting bodies, data only for 28 individuals was deemed as acceptable, and from those, 4 identifications had poor confidence values (Table 1). Four species were identified from the fruiting bodies (but with a clear dominance of *M. esculenta/M. crassipes*), and from those we finally could isolate 10 new cultures (7 strains identified as *Morchella angusticeps* -formerly

known as *M. elata*[82]-; 2 strains of *M. esculenta*; and 1 *Morchella deliciosa*). Examples of the macroscopic mycelial morphology of the strains in the collection are shown in Figure 2. These results highlight clearly that morphology-based species identification of morels is limited and prone to confusion[61]. However, our strains were identified based on the ITS region (ITS 1 and 2, including the 5.8S rRNA gene), which may lead to misinterpretations as suggested by Du et al[62]. Therefore, refining ITS database entries with a multilocus approach should be performed in order to investigate properly the diversity and phylogeny of *Morchella* species.

Table 1. Summary of the identification of the collected morels.

Sample	Alignment scores	Species according to NCBI	Species according to CBS	Corresponding mycelium	Visual identification
6.1	> 200	*Morchella angusticeps*	*Morchella angusticeps*	*No mycelium*	*M. conica*
7.2	> 200	*Morchella* sp.	*Morchella* sp.	*No mycelium*	*M. elata*
7.3	80 - 200	*M. elata*	*No data (too short sequence)*	*No mycelium*	*M. elata*
7.5	> 200	*M. eximioides*	*M. angusticeps*	*No mycelium*	*M. elata*
8	> 200	*M. angusticeps*	*M. angusticeps*	*No mycelium*	*M. conica*
17.1	> 200	*M.esculenta OR M.crassipes*	*M.esculenta OR M.crassipes*	*Trichoderma sp.**	*M. esculenta*
17.2	> 200	*M.esculenta OR M.crassipes*	*M.esculenta OR M.crassipes*	*M. crassipes*	*M. esculenta*
17.3	> 200	*M. crassipes OR esculenta*	*M. crassipes OR esculenta*	*No mycelium*	*M. esculenta*
17.4	> 200	*M. crassipes OR esculenta*	*M. crassipes*	*M. crassipes*	*M. esculenta*
18.1	50 - 80	*M. esculenta*	*No data (too short sequence)*	*M. angusticeps*	*M. vulgaris*
18.2	> 200	*M. crassipes*	*M. crassipes OR esculenta*	*M. angusticeps*	*M. vulgaris*
18.3	> 200	*M. crassipes*	*M. crassipes*	*M. angusticeps*	*M. vulgaris*
18.4	> 200	*M. esculenta*	*M. esculenta*	*M. angusticeps*	*M. vulgaris*
18.5	> 200	*M. esculenta*	*M. esculenta*	*No mycelium*	*M. vulgaris*
19.1	50 - 80	*M. esculenta*	*No data (too short sequence)*	*M. angusticeps*	*M. esculenta*
19.2	> 200	*M.esculenta OR M.crassipes*	*M.esculenta OR M.crassipes*	*No data*	*M. esculenta*
19.3	> 200	*M. crassipes*	*M. crassipes OR esculenta*	*No mycelium*	*M. esculenta*
19.5	80 - 200	*M. esculenta*	*Morchella* sp.	*M. deliciosa*	*M. esculenta*
21.1	> 200	*M. costata*	*Morchella* sp.	*No mycelium*	*M. conica*
21.2	> 200	*Morchella deliciosa OR M.costata*	*Morchella deliciosa*	*No data*	*M. conica*
23.1	> 200	*Morchella* sp.	*M. deliciosa*	*No data*	*M. elata*
25.1	> 200	*Morchella* sp.	*Morchella* sp.	*No mycelium*	*M. conica*
33.4	> 200	*M. crassipes OR esculenta*	*M. crassipes*	*Nectria sp. but short sequence*	*M. esculenta*
35.1	> 200	*M. crassipes*	*M. esculenta*	*M. angusticeps*	*M. vulgaris*
35.2	> 200	*Morchella* sp.	*M. esculenta*	*No data*	*M. vulgaris*
35.3	> 200	*M. crassipes*	*M. crassipes OR esculenta*	*No mycelium*	*M. vulgaris*
35.4	> 200	*M. crassipes*	*M. crassipes OR esculenta*	*M. angusticeps*	*M. vulgaris*
36	> 200	*M. crassipes OR esculenta*	*M. crassipes OR esculenta*	*No mycelium*	*M. esculenta*

Regarding the mating type distribution, we used two primers pairs specific for the two *MAT* loci (*MAT1-1-1* and *MAT1-2-1*) of black morel species[76]. Despite the fact that optimal conditions could be easily establishes to test for both loci, the primers should be improved, as we could not eliminate non-specific amplification for the negative controls (Example in Figure 3). Nevertheless, our results show that most fruiting bodies harbored both *MAT* loci, as this may be expected for a heterothallic fungus (Table 2). However, two samples amplified only for *MAT1-2-1*. On the other hand, the vast majority of mycelial colonies isolated in culture harbored only one *MAT* locus, as expected for a monokaryotic vegetative state. Interestingly, out of the 26 isolates analyzed, 17 had *MAT1-2-1*, only 3 had *MAT1-1-1* and 5 had both *MAT* loci. The fact that one MAT locus dominates the monokaryotic vegetative state of Chinese wild morels was already highlighted by Du et al[76]. However, in their study *MAT1-1-1* dominated over *MAT1-2-1* in wild morels, while the ratio was more balanced in cultivated morels. For this reason, they speculated that the predominance of a mating type and spatial segregation of mating types are factors that reduce the chances of compatible organisms to mate and hence form fruiting bodies. However, this raises several questions, such as how do wild morels achieve sexual reproduction if one

mating type is less common than the other one? Alternatively, does one of the mating types has a vegetative growth form that is different than a mycelial colony (sexual dimorphism)? This may be supported by the observation of apparently pure cultures that actually harbored both MAT loci, where one MAT locus is present in a mycelial growth form and the other one as a physical reduced state.

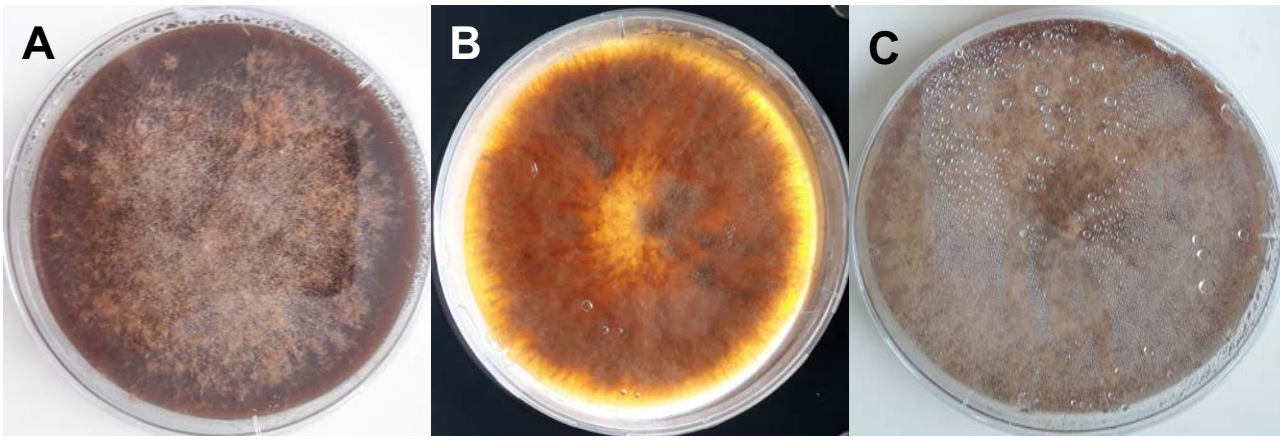

Figure 2. Macroscopic images illustrating the aspect of *M. esculenta* (A; strain 17.4), *M. angusticeps* (B; strain 18.3) and *M. deliciosa* (C; strain 19.5).

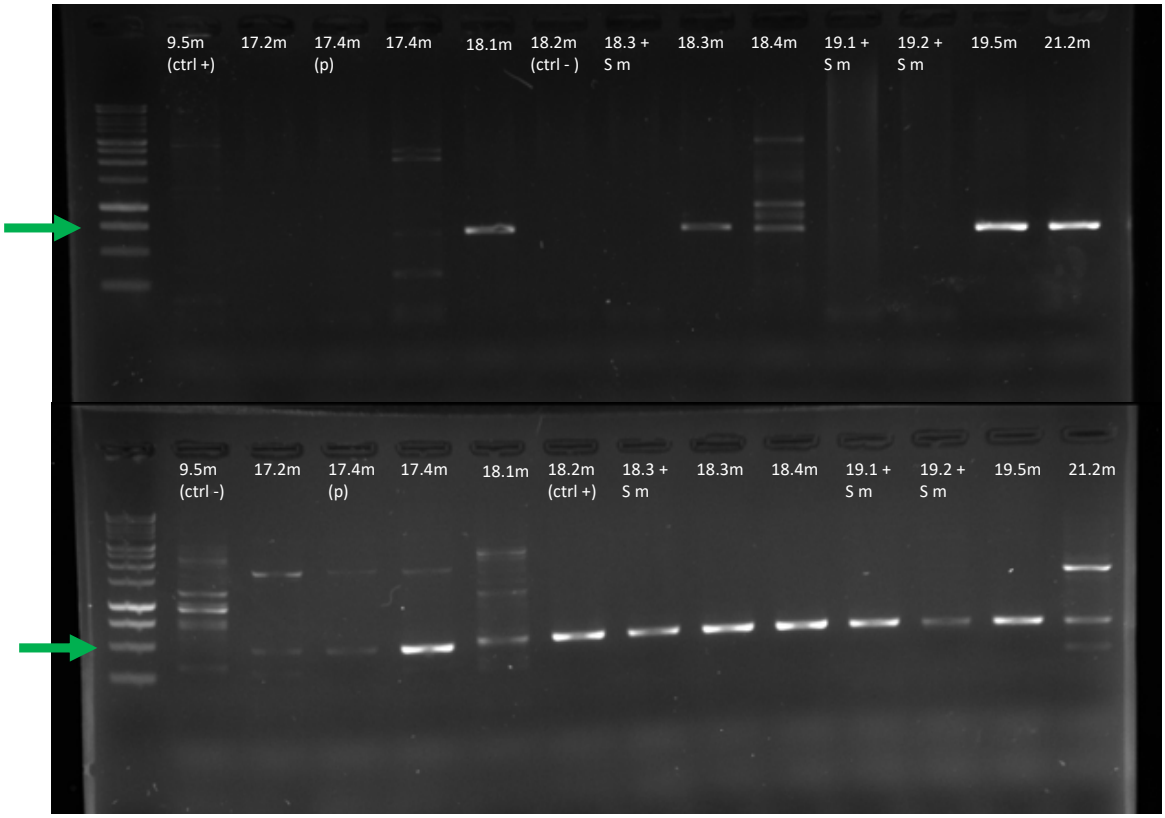

Figure 3. Example showing the comparison of the PCR results for the determination of the mating type using *MAT* loci specific primers[76] (*MAT1-1-1* -top image- and *MAT1-2-1* -bottom image-).

Table 2. Summary of the mating types obtained for the samples collected. N.D.= not determined.

Sample	Strain	Species	Mating type
Mycelium	9.5	*M. angusticeps*	MAT1-1-1
	17.2	*M. crassipes or esculenta*	MAT1-2-1
	17.4p	*M. esculenta*	MAT1-2-1
	17.4	*M. esculenta*	MAT1-2-1
	18.1	*Morchella* sp.	MAT1-1-1, -MAT1-2-1
	18.2	*M. crassipes or esculenta*	MAT1-2-1
	18.3 + S	*M. crassipes*	MAT1-2-1
	18.3	*M. crassipes*	MAT1-2-1
	18.4	*M. esculenta*	MAT1-2-1, -MAT1-1-1
	19.1 + S	*M. esculenta*	MAT1-2-1
	19.2 + S	*M. crassipes or esculenta*	MAT1-2-1
	19.5	*Morchella* sp.	MAT1-1-1, -MAT1-2-1
	21.2	*M. deliciosa*	MAT1-1-1
	23.1	*Morchella* sp.	N.D.
	35.1	*M. crassipes or esculenta*	MAT1-2-1, -MAT1-1-1
	35.2	*Morchella sp.*	MAT1-2-1
	33.4	*M. esculenta*	MAT1-1-1
	35.4	*M. crassipes or esculenta*	MAT1-2-1
	20A	*M. crassipes* (Netherlands)	MAT1-2-1
	17A	*M. crassipes*	MAT1-2-1
	29A	*M. crassipes*	MAT1-2-1
	Me4	*M. rufobrunnea*	MAT1-2-1
	84A	*M. crassipes*	MAT1-2-1
	NEU142	*M. importuna*	MAT1-2-1
	NEU143	*M. sextelata*	MAT1-1-1
	90A	*M. rufobrunnea*	MAT1-2-1
Fructiications	19.5	*Morchella sp.*	MAT1-2-1, -MAT1-1-1
	21.2	*M. deliciosa*	MAT1-2-1, MAT1-1-1
	23.1	*Morchella sp.*	MAT1-2-1, MAT1-1-1
	33.4	*M. esculenta*	MAT1-2-1, -MAT1-1-1
	35.1	*M. crassipes or esculenta*	MAT1-2-1, -MAT1-1-1
	35.2	*Morchella sp.*	MAT1-2-1, -MAT1-1-1
	17.2	*M. crassipes or esculenta*	MAT1-2-1, -MAT1-1-1
	17.4	*M. crassipes or esculenta*	MAT1-2-1
	18.1	*Morchella sp.*	MAT1-2-1
	18.2	*M. crassipes*	MAT1-2-1, -MAT1-1-1
	18.3	*M. crassipes*	MAT1-2-1, -MAT1-1-1
	18.4	*M. esculenta*	MAT1-2-1, -MAT1-1-1
	35.4	*M. crassipes or esculenta*	MAT1-2-1, -MAT1-1-1
	19.1	*M. esculenta*	MAT1-1-1, MAT1-2-1
	19.2	*M. crassipes or esculenta*	MAT1-2-1, -MAT1-1-1

Finally, competition experiments were conducted and showed that strains of similar mating types are not compatible, as exemplified by an un-colonized zone at their encounter (Figure 4). On the contrary, strains of opposite mating types (including the two strains obtained from the farmer, which are putatively of Chinese origin) led to the creation of highly melanized lines (Figure 5). This can be interpreted as the manifestation of the formation of a heterokaryon[83]. These experiments are currently ongoing and further analyses are required to confirm this.

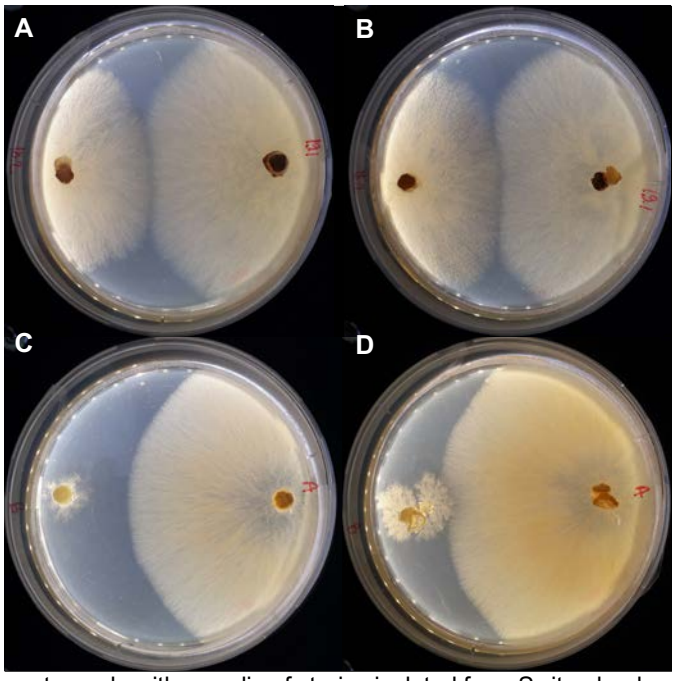

Figure 4. Competition experiments made with mycelia of strains isolated from Switzerland and with the same mating type. On the top panel images corresponding to the competition between two strains of *M. angusticeps* (strains 18.2, on the left, and 19.1, on the right) at 2 (A) and 5 (B) days post-inoculation are shown. Both of the strains possess the *MAT1-2-1* locus and are clearly incompatible. On the bottom panel two non-identified strains were put in competition.

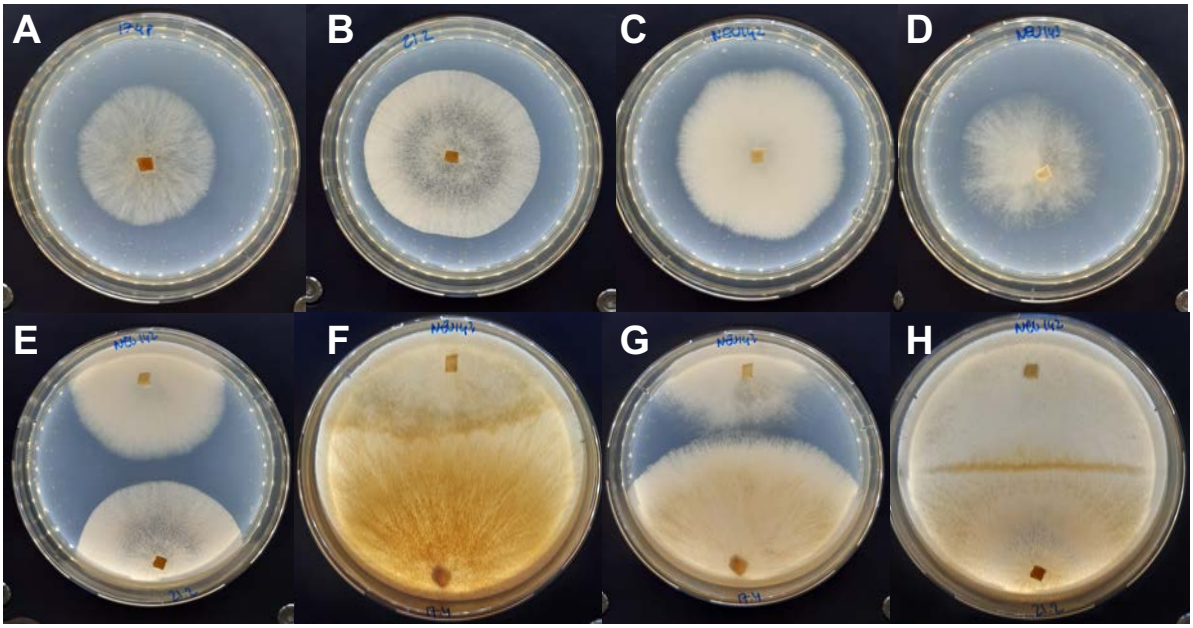

Figure 5. Competition experiments made with mycelia of *M. crassipes* strain 17.4 (A; *MAT1-2-1*) and *M. deliciosa* strain 21.2 (B; *MAT1-1-1*) isolated from Switzerland versus *M. importuna* NEU142 (C; *MAT1-2-1*) and *M. sextelata* NEU143 (D; *MAT1-1-1*). The competition experiments were made for complementing mating types as following: *M. deliciosa* strain 21.2 (*MAT1-1-1*) versus *M. importuna* NEU142 (*MAT1-2-1*) at 2 (E) and 5 (F) days post inoculation; and *M. crassipes* strain 17.4 (*MAT1-2-1*) versus *M. sextelata* NEU143 (*MAT1-1-1*) at 2 (G) and 5 (H) days post inoculation.

OUTLOOK AND FUTURE DIRECTIONS

The lack of detailed knowledge on the diversity of morels in Switzerland is one the limitations for the research, not only in the invasiveness potential of morels, but also is crucial for developing informed management practices that ensure this unique genetic resource is conserved[59]. Also, given the agronomic potential of this fungus, a better understanding of the biology of morels is crucial for formulating informed conservation policies directed at preventing species loss and ensuring a sustainable and ecologically sound exploitation of morels[58]. In order to achieve this, several new directions of research can be highlighted after this pilot study. First, a sound understanding of the sexuality of morels is acutely required. This can only be achieved by refining and ameliorating the current set of genetic markers used to determine mating types. As shown by our study, while the mating type *MAT1-1-1* could be unambiguously assigned to most of the strains in the collection obtained, determining *MAT1-2-1* was harder and this could not be simply solved by changing PCR conditions. Therefore, refining primer design is key, but for this, the generation of new genomes would be ideal. At the moment, there are three *Morchella* genomes released as part of the global initiative of the 1000 fungal genome sequencing project (http://1000.fungalgenomes.org/home/): two strains of *Morchella importuna* (strains SCYDJ1-A1 and CCBAS932), and one strain of *M. snyderi* (a post-fire morel species). *M. importuna* strain SCYDJ1-A1 was originally derived from an ancestor wild-collected mushroom in eastern Tibetan Plateau and was domesticated in the Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences, China. It is the most commonly farmed strain under controlled conditions. It is reported to form fruiting bodies in farm plots with steady yield, and is being promoted as a commercial mushroom strain[81]. The strain is maintained in a saprophytic life style, which contrasts to the ectomycorrhizal life style of many other species of *Morchella* with a perennial life-style, for which associations and interactions with plants are often essential at certain stages (potentially even during their sexual reproduction cycle). Along with this, the population genetics of wild morels is still poorly understood. Recent studies regarding genetic diversity of natural morel populations showed that both clonal and more diversified populations exist in nature[84,85]. This suggests that morels adopt different reproductive strategies, whether the population is present as a pioneer species (high inbreeding observed, secondary homothallism) or as a perennial species in a stable ecosystem (more genetic variation observed). Since our confrontation experiments indicated that, *in vitro,* the putative Chinese strains may have formed a heterokaryotic structure with Swiss strains (formation of melanized barriers), pointing to a plausible risk of hydridization, assessing whether Chinese morels are able to establish as pioneer species in the nature in Switzerland would be required. For this, investigating both pioneering and perennial morel sites with molecular markers specific to Chinese strains should be performed. This would allow evaluating properly the risk of hybridization between Chinese and Swiss strains, as well as the risk of displacement of native populations by less commercially valuable species. Therefore, generating additional genomes from native strains would allow us to compare not only their mating systems, but also other key ecological features (for example genetic complements for an ectomycorrhizal versus a pioneering lifestyle) of this remarkable fungal genus.
Another relevant life stage of Morels is sclerotia, which are resistant multi-cellular structures that are formed to resist harsh environmental conditions. It is believed that the sclerotia stage is required for fruiting[71], which may for instance explain why some *Morchella* species are observed after fire events[86]. This brings also an interesting explanation to the recurrent observation of morels in urban planted areas. While morels are considered as forest mushrooms, they may actually be collected in rather large amounts in urban areas that are freshly covered with fir and spruce wood chunks. For this reason, we contacted the “service des parcs et promenades de la ville de Neuchâtel” to get information on how these areas were maintained and eventually understand how morels may end up in cities. From this discussion, it appeared that morels are found only in areas covered with wood chunks from trunks that have over-wintered in the forest. This points to the implication of a sclerotial life-stage, as morel mycelium may have been present in the wood before cold conditions prevail and formed sclerotia as a cold-resistant stage during winter. This again raises questions as to the ecology of morels, as this points to an ability to degrade wood. Then, an additional important aspect, is that fruiting bodies appear only once the wood chunks are in contact with soil. This points to the importance of either, nutrients present in the soil, to the interaction of morel fungi with soil bacteria, or both. We have demonstrated that a common soil bacterium, *Pseudomonas putida*, is farmed by *Morchella crassipes* as an additional carbon source[87], but that this behavior is tightly linked to the nitrogen source[88]. Therefore, investigating further the

nutritional strategies and ecology of *Morchella* species and their interaction with the soil microbiota represent a fruitful field to develop the cultivation of morels.
Finally, in a long-term vision, if the culture of mushrooms continues to expand in Switzerland, the question of invasiveness should be opened to other cultivated fungal species such as *Pleurotus ostreatus*, *Lentinula edodes* (Shiitake), *Auricularia auricula-judae,* or *Flammulina velutipes*, for instance. Indeed, it has been demonstrated that cultivar of the commonly cultivated *Agaricus bisporus* has invaded natural populations in North America. However, to the best of our knowledge, the effect that this has on ecosystem functioning remains un-evaluated. Therefore, in order to prevent an unwanted effect on ecosystem functioning, estimating the potential of invasion of common and/or potentially cultivated mushroom species is of interest, before actually starting to disseminate these species in the environment.

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