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Analysis of the invasive potential of non-native Morels

Final report

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Picture of a black morel collected in Switzerland in 2019. © Blaise Hofer

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Executive summary

Morchella spp. are highly prized fungi whose edible fruiting body can be harvested in the wild ,mostly between March and June in the northern hemisphere (Pilz et al. 2007). Morels show high levels of endemism and provincialism (O'Donnell et al. 2011), however at least 33% of the species are actually found in more than one continent. Such as transcontinentalism could be explained by different hypotheses such as anthropogenic introduction, long distance spore dispersal, and a common Pleistocene origin (Loizides 2017). The first hypothesis was confirmed for some species (Richard et al. 2015; Baroni et al. 2018), but further studies are necessary to evaluate the two other hypotheses. In the light of species invasiveness, anthropogenic introductions are the central preoccupation. In China, the increased demand for morels motivated the cultivation of these mushrooms as outdoor crops, thus leading to the selection of efficient cultivars (Liu et al. 2018). These latter have also been exported to France and Switzerland since a few years for morel cropping, mostly through France Morilles SAS. To obtain the edible ascocarp, morels must undergo sexual reproduction, which involves, in heterothallic species, two partners with opposite mating types. However, some morel species from both Elata and Esculenta clades are also able to reproduce by secondary homothallism, in which an individual is self-fertile (Du and Yang 2021). Hence, this would indicate that some species are more prone to reproduce sexually and produced ascocarps, as they do not need to encounter a complementary sexual partner to mate. For this reason, *M. importuna* can be seen as the most problematic species because it has been described as being able to reproduce by heterothallism and secondary homothallism; in addition to the fact that it is extensively cultivated in China. Two of the cultivars that we obtained from morel farmers corresponded to this species. However, it is possible that this species is already present in Switzerland. Of the 143 fruiting bodies that were collected between 2019 and 2021, at least one of those (originating from the canton of Valais; Report 2, Module 5), appears to belong to the species *M. importuna*. Interestingly, the first occurrence of this species in Switzerland was already reported from canton Valais in 1986 (Richard et al. 2015). As a result, we can hypothesize that if the invasive potential of *M. importuna* was important, other individuals of this species should have been found in other locations in Switzerland. Nevertheless, since we did not perform a systematic sampling over several years at several locations across Switzerland, this cannot be entirely affirmed. Therefore, a continued collection of fruiting bodies in Valais, and elsewhere around this canton, could be important to investigate the extent of the colonization by *M. importuna*. In addition, our confrontation experiments *in vitro* and soil-like substrates indicated that *M. sextelata* could be more concerning in term of invasiveness than *M. importuna*. The Chinese cultivars from *M. sextelata* (PYL and NEU143) performed better than any other morel in terms of medium colonization in both soil-like conditions and chemically defined media. However, only one fruiting body belonging to *M. sextelata* was collected in Switzerland within the three years of monitoring performed during this mandate (Report 2, Module 5). This could indicate that the species could need particular environmental or ecological factors to establish in the wild in the long-term, and that those features may be limited in Switzerland. This could be evaluated by the continuous and systematic monitoring of selected plots in which the two Chinese species have been detected. This is relevant as the cultivation of *M. sextelata* was already possible in Switzerland the last two years, and likely the species will still be cultivated in the future until a Swiss cultivar is available and performs as well as the Chinese ones. For this reason, in order to evaluate this possibility, based on mycelial growth and sclerotia production, we selected three Swiss morel specimens that were provided to a morel farmer in order to conduct the first outdoor cultivation of Swiss-native *Morchella* spp. in Cantons Bern, Fribourg and Vaud. The inoculations were made in autumn 2021 but did not lead to the emergence of fruiting bodies as expected in May 2022. The presence of a potential fungal pathogen (or competitor) was observed and was also deleterious to fruiting body formation in the Chinese cultivars. Therefore, in addition to hybridization, dispersal, and germination potential, susceptibility to disease is another aspect that needs to be considered in the evaluation of the invasive potential of a non-pathogenic fungal species such as morels.

In the future, it will be important to monitor the surroundings of the current areas in which morels are cultivated in Switzerland, in order to verify whether cultivated morels are able to colonize soils with their mycelia. It will be particularly important to conduct these experiments around non-native morel crops, to track in addition a potential emergence of hybrids. In China, where different black morel

species are cultivated (*M. importuna*, *M. sextelata, Morchella eximia, Morchella exuberans*, Mel-13 and Mel-21), genetic flows were commonly observed between wild and cultivated populations. Interestingly, the genetic variation was not correlated with the geography, probably due to human interventions such as the exchange of cultivars between different regions. This would result in a decrease in the genetic variability among the different morel populations. In addition, hybridization events were noticed between at least seven different black morel species (*M. exuberans, M. importuna*, Mel-13, *M. eohespera, M. eximioides*, Mel-21 and Mel-34) (Du et al. 2019). The hybridization events reported by Du et al. (2019) occurred not only between closely related species, such as *M. eximia* with M. *exuberans*, but also phylogenetically distant ones (*M. sextelata* with Mel-21) (O'Donnell et al. 2011). Until now, no hybrid of *M. importuna/M. sextelata* were discovered in crops or in the wild (Du et al. 2019). However, such hybridization event was achieved experimentally by PEG-induced double inactivated protoplast fusion. This would have resulted in individuals containing heterokaryons that underwent subsequent meiosis. The hybrid lines were grown *in vitro* and as crops. The fruiting bodies of the hybrids were of three types: (I) similar to parent 1, (II) similar to parent 2 and (III) mix of both parental lines (cap from parent 1 and stipe from parent 2). The phylogenetic analyses showed that the hybrids were clustered between the parental taxa. Interestingly, the *in vitro* confrontations between the hybrids and the parental lines resulted in somatic incompatibility demonstrated by the formation of a barrier line (He et al. 2020), which was also characterized in our experiments from Module 1. The confrontation line was also observed between both parental specimens (*M. importuna* and *M. sextelata*). However, it is unclear if these confrontation lines are at all related to plasmogamy and exchange of nuclei between different individuals.

To further evaluate hybridization potential, indoor morel cultivation is required. The discussions and collaborations with a morel farmer provided us the knowledge necessary to succeed in cultivating morels in a climatic chamber, which will be tested during 2022. So far, we used wild morel fruiting bodies to collect the sexual spores (ascospores) and investigate those. We studied their germination and nuclear content and compared it with asexual spores (conidia). Along with this, we developed a method to trigger conidial production *in vitro*. This method was, however, only successful for some isolates. Ascospores were used to produce single-ascospore derived mycelial cultures which supposedly contained a single haploid genetic material. The mating type(s) and then the reproductive mode of these isolates were determined genetically to be used in multiple experiments such as confrontation and adaptability assays. These experiments revealed that mating types were generally not associated with a visible phenotype, in both mono and co-cultures. However, MAT1-2 was overrepresented in single ascospore isolates obtained from the black morel specimen M21-48 and these isolates seemed better adapted at early mycelial stage as compared with MAT1-1 isolates. Chinese studies revealed that maternal tissues (fungal hyphae of the stipe and cap) was prominently of MAT1-1 rather than MAT1-2 (Du and Yang 2021). The opposite was observed in Swiss morel specimens (Cravero et al. 2022*, submitted*). In truffles it has been shown that both types can provide the maternal tissue and this could also be the case in morels. As a result, we hypothesized that mating types could have different ecological roles in a natural environment, which should also be considered while investigating the invasive potential of non-native fungal species.

Executive summary – French version

Les morilles (*Morchella* spp.) sont des champignons très prisés, dont la fructification comestible peut être récoltée à l'état sauvage, principalement entre mars et juin dans l'hémisphère nord (Pilz et al. 2007). Les morilles présentent des niveaux élevés d'endémisme et de provincialisme (O'Donnell et al. 2011), bien qu'au moins 33% des espèces peuvent être trouvées dans plusieurs continents. Un tel transcontinentalisme pourrait être expliqué par différentes hypothèses, telles que l'introduction anthropogénique, la dispersion des spores sur de longues distances et une origine commune au Pléistocène (Loizides 2017). La première hypothèse a été confirmée pour certaines espèces (Richard et al. 2015 ; Baroni et al. 2018), mais des études supplémentaires sont nécessaires pour évaluer les deux autres théories. Concernant les espèces invasives, les introductions anthropiques sont au centre des préoccupations. En Chine, la demande accrue de morilles a motivé la culture de ces champignons en extérieur, conduisant ainsi à la sélection de cultivars (Liu et al. 2018). Ces derniers sont également exportés en France et en Suisse depuis quelques années pour la culture de morilles, principalement via France Morilles SAS. Pour obtenir l'ascocarpe comestible, les morilles doivent entreprendre une reproduction sexuée, qui implique, chez les espèces hétérothalliques, deux partenaires aux types d'accouplement opposés (mating types ; MAT). Cependant, certaines espèces de morilles appartenant aux clades Elata et Esculenta sont également capables de se reproduire par homothallisme secondaire (individu auto-fertile) (Du et Yang 2021). Cela indiquerait donc que certaines espèces sont plus enclines à se reproduire sexuellement et à produire des ascocarpes, car elles n'ont pas besoin de rencontrer un partenaire sexuel complémentaire pour s'accoupler. Pour cette raison, *M. importuna* pourrait être considérée comme l'espèce la plus problématique, car elle a été décrite comme étant capable de se reproduire par hétérothallisme et homothallisme secondaire ; en plus du fait qu'elle est largement cultivée en Chine. Deux des cultivars que nous avons obtenus auprès de cultivateurs de morilles correspondaient à cette espèce. Il est également possible que cette espèce soit déjà présente en Suisse dans la nature. Sur les 143 fructifications qui ont été collectées entre 2019 et 2021, au moins une d'entre elles (provenant du canton du Valais ; rapport 2, module 5), semble appartenir à l'espèce *M. importuna*. Il est intéressant de noter que la première occurrence de cette espèce en Suisse avait déjà été signalée dans le canton du Valais en 1986 (Richard et al. 2015). Par conséquent, nous pouvons émettre l'hypothèse que si le potentiel invasif de *M. importuna* était important, d'autres individus de cette espèce auraient dû être trouvés dans d'autres endroits en Suisse. Néanmoins, comme nous n'avons pas effectué un échantillonnage systématique sur plusieurs années et à plusieurs endroits en Suisse, ceci ne peut être entièrement affirmé. Par conséquent, une collecte continue de fructifications en Valais pourrait être utile pour étudier l'étendue de la colonisation par *M. importuna*. De plus, nos expériences de confrontation *in vitro* et sur des substrats de type sol ont indiqué que *M. sextelata* pourrait être plus préoccupante, en termes d'envahissement, que *M. importuna*. Les cultivars chinois de *M. sextelata* (PYL et NEU143) ont été plus efficaces que toutes les autres morilles, en termes de colonisation du milieu, aussi bien dans du substrat contenant du sol que dans des milieux définis chimiquement. Cependant, une seule ascocarpe appartenant à *M. sextelata* a été collecté en Suisse au cours des trois années d'échantillonnage effectuées dans le cadre de ce mandat (rapport 2, module 5). Cela pourrait indiquer que l'espèce pourrait avoir besoin de facteurs environnementaux ou écologiques particuliers pour s'établir à long terme dans la nature, et que ces caractéristiques sont peut-être limitées en Suisse. Ceci pourrait être évalué par la surveillance continue et systématique de parcelles sélectionnées dans lesquelles les deux espèces chinoises ont été détectées. Ceci serait pertinent, car la culture de *M. sextelata* est déjà possible en Suisse, et il est probable que l'espèce sera encore cultivée dans le futur jusqu'à ce qu'un cultivar suisse performant soit disponible. Afin d'évaluer cette possibilité, sur la base de la croissance mycélienne et de la production de sclérotes, nous avons sélectionné trois spécimens de morilles suisses qui ont été fournis à un cultivateur de morilles afin de mener la première culture en plein air de *Morchella* spp. d'origine suisse. Cela a été fait dans les cantons de Berne, Fribourg et Vaud. Les inoculations ont été faites en automne 2021 mais n'ont pas conduit à l'émergence de fructifications. La présence d'un champignon pathogène potentiel (ou concurrent) a été observée et s'est avérée également délétère pour la formation des fructifications dans les cultivars chinois. Par conséquent, en plus de l'hybridation, de la dispersion et du potentiel de germination, la sensibilité aux maladies est un autre

aspect qui doit être pris en compte dans l'évaluation du potentiel invasif d'une espèce fongique non pathogène telle que la morille.

Dans le futur, il sera important de surveiller les environs des zones actuelles de culture de morilles en Suisse, afin de vérifier si les morilles cultivées sont capables de coloniser les sols avec leurs mycéliums. Il sera particulièrement utile de mener ces expériences autour de cultures de morilles non indigènes, pour suivre une éventuelle émergence d'hybrides. En Chine, où différentes espèces de morilles noires sont cultivées (*M. importuna, M. sextelata, M. eximia, M. exuberans*, Mel-13 et Mel-21), des flux génétiques ont été couramment observés entre les populations sauvages et cultivées. Il est intéressant de noter que la variation génétique n'était pas corrélée à la géographie, probablement en raison d'interventions humaines telles que l'échange de cultivars entre différentes régions. Cela a entrainé une diminution de la variabilité génétique entre les différentes populations de morilles. En outre, des événements d'hybridation ont été remarqués entre au moins sept espèces différentes de morilles noires (*M. exuberans, M. importuna,* Mel-13*, M. eohespera, M. eximioides,* Mel-21 et Mel- 34) (Du et al. 2019). Les événements d'hybridation rapportés par Du et al. (2019) se sont produits non seulement entre des espèces étroitement liées, comme *M. eximia* avec *M. exuberans*, mais aussi des espèces phylogénétiquement éloignées (*M. sextelata* avec Mel-21) (O'Donnell et al. 2011). Jusqu'à présent, aucun hybride de *M. importuna/M. sextelata* n'a été découvert dans les cultures ou dans la nature (Du et al. 2019). Cependant, un tel événement d'hybridation a été réalisé expérimentalement par une double fusion de protoplastes inactivés induite par du PEG (polyéthylène glycol). Cela aurait donné lieu à des individus contenant des hétérocaryons ayant subi une méiose ultérieure. Les lignées hybrides ont été cultivées *in vitro* puis utilisées pour produire des ascocarpes. Les fructifications hybrides étaient de trois types : (I) similaires au parent 1, (II) similaires au parent 2 et (III) mélange des deux lignées parentales (chapeau du parent 1 et pied du parent 2). Les analyses phylogénétiques ont montré que les hybrides étaient regroupés entre les taxons parentaux. Il est intéressant de noter que les confrontations *in vitro* entre les hybrides et les lignées parentales ont entraîné une incompatibilité somatique, démontrée par la formation d'une ligne de confrontation (He et al. 2020), ce qui a également été caractérisée dans nos expériences du module 1. La ligne de confrontation a également été observée entre les deux spécimens parentaux (*M. importuna* et *M. sextelata*). Cependant, ces lignes de confrontation pourraient soit être liées à la plasmogamie, soit à l'échange de noyaux entre différents individus.

Pour évaluer plus précisément le potentiel d'hybridation, la culture de morilles en intérieur est nécessaire. Les discussions et collaborations avec un cultivateur de morilles nous ont fourni les connaissances essentielles pour réussir à cultiver des morilles dans une chambre climatique, ce qui sera testé au cours de l'année 2022. Jusqu'à présent, nous avons utilisé des fructifications de morilles sauvages pour recueillir les spores sexuelles (ascospores) et les étudier. Nous avons étudié leur germination et leur contenu nucléaire et les avons comparés aux spores asexuées (conidies). En parallèle, nous avons développé une méthode pour déclencher la production de conidies *in vitro*. Cette méthode n'a toutefois été efficace que pour certains isolats. Les ascospores ont été utilisées pour produire des cultures mycéliennes dérivées d'ascospores uniques, censées contenir un seul matériel génétique haploïde. Le(s) type(s) d'accouplement (MAT) et le mode de reproduction de ces isolats ont été déterminés génétiquement pour être ensuite utilisés dans de multiples expériences, telles que des tests de confrontation et d'adaptabilité. Ces expériences ont révélé que les types d'accouplement n'étaient généralement pas associés à un phénotype visible, tant dans les monocultures que dans les cocultures. Cependant, le MAT1-2 était surreprésenté dans les isolats d'ascospores uniques obtenus à partir du spécimen de morille noire M21-48, et ces isolats semblaient mieux adaptés au stade mycélien précoce par rapport aux isolats MAT1-1. Des études chinoises ont révélé que les tissus maternels (hyphes fongiques du pied et du chapeau) étaient principalement de MAT1-1 plutôt que de MAT1-2 (Du et Yang 2021). Le contraire a été observé dans des spécimens de morilles suisses (Cravero et al. 2022, *soumis pour publication*). Chez les truffes, il a été démontré que les deux types peuvent être présents dans le tissu maternel, et cela pourrait également être le cas chez les morilles. Par conséquent, nous avons émis l'hypothèse que les types d'accouplement pourraient avoir des rôles écologiques différents dans un environnement naturel, ce qui devrait également être pris en compte lors de l'étude du potentiel invasif des espèces fongiques non-natives.

Executive summary – German version

Morcheln *Morchella* spp. sind sehr geschätzte Pilze, deren essbare Fruchtkörper in der freien Natur, meist zwischen März und Juni auf der Nordhalbkugel geerntet werden können (Pilz et al. 2007). Morcheln weisen ein hohes Maß an Endemismus und Provinzialität auf (O'Donnell et al. 2011), aber mindestens 33 % der Arten kommen tatsächlich auf mehr als einem Kontinent vor. Dieser Transkontinentalismus könnte durch verschiedene Hypothesen erklärt werden, wie z. B. anthropogene Einführung, Sporenverbreitung über große Entfernungen und einen gemeinsamen pleistozänen Ursprung (Loizides 2017). Die erste Hypothese wurde durch einige Arten bestätigt (Richard et al. 2015; Baroni et al. 2018), doch sind weitere Studien erforderlich, um die beiden anderen Hypothesen zu bewerten. Im Hinblick auf die Invasivität von Arten stehen anthropogene Einschleppungen im Mittelpunkt des Interesses. In China motivierte die gestiegene Nachfrage nach Morcheln den Anbau dieser Pilze als Freilandkulturen, was zur Selektion effizienter Sorten führte (Liu et al. 2018). Diese werden seit einigen Jahren auch für den Morchelanbau nach Frankreich und in die Schweiz exportiert, meist über France Morilles SAS. Um das essbare Ascokarp zu erhalten, müssen sich Morcheln sexuell fortpflanzen, was bei heterothallischen Arten zwei Partner mit entgegengesetzten Paarungstypen erfordert. Einige Morchelarten aus den Kladen Elata und Esculenta sind jedoch auch in der Lage, sich durch sekundären Homothallismus fortzupflanzen, bei dem ein Individuum selbstbefruchdend ist (Du und Yang 2021). Dies würde darauf hindeuten, dass einige Arten eher dazu neigen, sich sexuell fortzupflanzen und Ascocarps zu produzieren, da sie zur Paarung nicht auf einen komplementären Sexualpartner angewiesen sind. Aus diesem Grund kann *M. importuna* als die problematischste Art angesehen werden, da sie in der Lage ist, sich durch Heterothallismus und sekundären Homothallismus fortzupflanzen. Zusätzlich wird sie in China in großem Umfang angebaut. Zwei der Sorten, die wir von Morchelzüchtern erhalten haben, gehörten zu dieser Art. Es ist jedoch möglich, dass diese Art bereits in der Schweiz vorkommt. Von den 143 Fruchtkörpern, die zwischen 2019 und 2021 gesammelt wurden, scheint mindestens einer (aus dem Kanton Wallis; Bericht 2, Modul 5) zur Art *M. importuna* zu gehören. Interessanterweise wurde das erste Vorkommen dieser Art in der Schweiz bereits 1986 aus dem Kanton Wallis gemeldet (Richard et al. 2015). Daher können wir die Hypothese aufstellen, dass, wenn das invasive Potenzial von *M. importuna* gross wäre, weitere Individuen dieser Art an anderen Orten in der Schweiz hätten gefunden werden müssen. Da wir jedoch keine systematischen Probenahmen über mehrere Jahre an verschiedenen Standorten in der Schweiz durchgeführt haben, kann dies nicht vollständig bestätigt werden. Daher könnte eine fortgesetzte Sammlung von Fruchtkörpern im Wallis und anderswo in diesem Kanton wichtig sein, um das Ausmaß der Besiedlung durch *M. importuna* zu untersuchen. Unsere Konfrontationsversuche in vitro und mit bodenähnlichen Substraten deuteten zudem darauf hin, dass *M. sextelata* in Bezug auf die Invasivität bedenklicher sein könnte als *M. importuna*. Die chinesischen Züchter von *M. sextelata* (PYL und NEU143) schnitten in Bezug auf die Besiedlung des Mediums sowohl unter bodenähnlichen Bedingungen als auch in chemisch definierten Medien besser ab als alle anderen Morcheln. Allerdings wurde in den drei Jahren der Überwachung im Rahmen dieses Mandats nur ein Fruchtkörper von *M. sextelata* in der Schweiz gefunden (Bericht 2, Modul 5). Dies könnte darauf hindeuten, dass die Art bestimmte Umwelt- oder ökologische Faktoren benötigt, um sich langfristig in der freien Natur zu etablieren, und dass diese Merkmale in der Schweiz möglicherweise nur begrenzt vorhanden sind. Dies könnte durch die kontinuierliche und systematische Überwachung ausgewählter Parzellen, auf denen die beiden chinesischen Arten nachgewiesen wurden, beurteilt werden. Dies ist insofern von Bedeutung, als der Anbau von *M. sextelata* in der Schweiz bereits in den letzten beiden Jahren möglich war und die Art wahrscheinlich auch in Zukunft angebaut werden wird, bis eine schweizerische Sorte verfügbar ist, die ebenso gut wie die chinesische Sorte funktioniert. Aus diesem Grund haben wir, um diese Möglichkeit anhand des Myzelwachstums und der Sklerotienproduktion zu bewerten, drei Schweizer Morcheln ausgewählt, die einem Morchelbauern zur Verfügung gestellt wurden, um den ersten Freilandanbau von in der Schweiz heimischen *Morchella* spp. in den Kantonen Bern, Freiburg und Waadt durchzuführen. Die Inokulationen wurden im Herbst 2021 durchgeführt, führten aber nicht zum Auftreten von Fruchtkörpern, welche im Mai 2022 erwartet wurde. Die Anwesenheit eines potenziellen Pilzerregers (oder Konkurrenten) wurde festgestellt und wirkte sich auch negativ auf die Bildung von Fruchtkörpern bei den chinesischen Sorten aus. Durch diese Erkenntnisse ist neben der Hybridisierung, der Ausbreitung und dem Keimungspotenzial die Krankheitsanfälligkeit ein weiterer

Aspekt, der bei der Auswahl der Sorten berücksichtigt werden muss. Um deren invasiven Potenzials einer nicht-pathogenen Pilzart wie der Morchel abzuschätzen.

In Zukunft wird es wichtig sein, die Umgebung der aktuellen Morchelanbaugebiete in der Schweiz zu beobachten, um zu überprüfen, ob die Kulturmorcheln in der Lage sind, die Böden mit ihren Myzelien zu besiedeln. Es wird besonders wichtig sein, diese Experimente in der Nähe von nicht einheimischen Morchelkulturen durchzuführen, um auch ein mögliches Auftreten von Hybriden nachzuverfolgen. In China, wo verschiedene schwarze Morchelarten angebaut werden (*M. importuna, M. sextelata, M. eximia, M. exuberans*, Mel-13 und Mel-21), wurden häufig genetische Veränderungen zwischen wilden und angebauten Populationen beobachtet. Interessanterweise korrelierte die genetische Variation nicht mit der geografischen Lage, was wahrscheinlich auf menschliche Eingriffe wie den Austausch von Kultursorten zwischen verschiedenen Regionen zurückzuführen ist. Dies würde zu einer Abnahme der genetischen Variabilität zwischen den verschiedenen Morchelpopulationen führen. Darüber hinaus wurden Hybridisierungsereignisse zwischen mindestens sieben verschiedenen Schwarzmorchelarten (*M. exuberans, M. importuna*, Mel-13, *M. eohespera, M. eximioides*, Mel-21 und Mel-34) festgestellt (Du et al. 2019). Die von Du et al. (2019) berichteten Hybridisierungsereignisse traten nicht nur zwischen eng verwandten Arten auf, wie *M. eximia* mit *M. exuberans*, sondern auch zwischen phylogenetisch weit entfernten Arten (*M. sextelata* mit Mel-21) (O'Donnell et al. 2011). Bisher wurden keine Hybriden von *M. importuna/M. sextelata* in Kulturpflanzen oder in der freien Natur entdeckt (Du et al. 2019). Ein solches Hybridisierungsereignis wurde jedoch experimentell durch PEG-induzierte doppelte inaktivierte Protoplastenfusion erreicht. Dies hätte zu Individuen mit Heterokaryonen geführt, die eine anschließende Meiose durchliefen. Die Hybridlinien wurden in vitro und als Kulturpflanzen angebaut. Die Fruchtkörper der Hybriden waren von dreierlei Art: (I) ähnlich wie bei Elternteil 1, (II) ähnlich wie bei Elternteil 2 und (III) eine Mischung aus beiden Elternlinien (Hut von Elternteil 1 und Stiel von Elternteil 2). Die phylogenetischen Analysen zeigten, dass die Hybriden zwischen den elterlichen Taxa angesiedelt waren. Interessanterweise führten die In-vitro-Konfrontationen zwischen den Hybriden und den Elternlinien zu somatischer Inkompatibilität (He et al. 2020), die sich auch in unseren Experimenten (Modul 1) zeigten. Die Konfrontationslinie wurde auch zwischen den beiden Elternexemplaren (*M. importuna* und *M. sextelata*) beobachtet. Es ist jedoch unklar, ob diese Konfrontationslinien in irgendeiner Weise mit Plasmogamie und dem Austausch von Kernen zwischen verschiedenen Individuen zusammenhängen.

Zur weiteren Bewertung des Hybridisierungspotenzials ist der Anbau von Morcheln in Innenräumen erforderlich. Die Gespräche und die Zusammenarbeit mit einem Morchelzüchter haben uns das nötige Wissen vermittelt, um Morcheln unter Laborbedingungen zu kultivieren, die im Jahr 2022 getestet werden sollen. Bisher haben wir Fruchtkörper von Wildmorcheln verwendet, um die sexuellen Sporen (Ascosporen) zu sammeln und zu untersuchen. Wir haben ihre Keimung und ihren Kerngehalt untersucht und sie mit ungeschlechtlichen Sporen (Konidien) verglichen. Parallel dazu entwickelten wir eine Methode, um die Konidienproduktion in vitro auszulösen. Diese Methode war jedoch nur bei einigen Isolaten erfolgreich. Mit Hilfe von Ascosporen wurden Myzelkulturen hergestellt, die vermutlich ein einziges haploides genetisches Material enthielten. Der/die Paarungstyp(en) und dann die Fortpflanzungsweise dieser Isolate wurden genetisch bestimmt, um sie in verschiedenen Experimenten wie Konfrontations- und Anpassungsversuchen zu verwenden. Diese Experimente zeigten, dass die Paarungstypen im Allgemeinen nicht mit einem sichtbaren Phänotyp verbunden waren, sowohl in Mono- als auch in Co-Kulturen. Allerdings war MAT1-2 in einzelnen Ascosporenisolaten aus der Schwarzmorchelprobe M21-48 überrepräsentiert, und diese Isolate schienen im frühen Myzelstadium besser angepasst zu sein als MAT1-1 Isolate. Chinesische Studien ergaben, dass das mütterliche Gewebe (Pilzhyphen des Stiels und der Kappe) eher von MAT1-1 als von MAT1-2 gebildet wurde (Du und Yang 2021). Das Gegenteil wurde bei Schweizer Morchelproben beobachtet (Cravero et al. 2022, eingereicht). Bei Trüffeln hat sich gezeigt, dass beide Typen das mütterliche Gewebe liefern können, und dies könnte auch bei Morcheln der Fall sein. Daher stellten wir die Hypothese auf, dass die Paarungstypen in einer natürlichen Umgebung unterschiedliche ökologische Funktionen haben könnten, was auch bei der Untersuchung des invasiven Potenzials nicht einheimischer Pilzarten berücksichtigt werden sollte.

Abstract

The goal of this mandate was to assess the invasive potential of Chinese morel species that are used for commercial farming. For this, we established their ecological features in comparison to native Swiss morels and we attempted to complete a full life cycle of potential hybrids between the Chinese and Swiss species, as a way to establish reproductive success of a putative invasive species. This final report presents the results of the first four modules of the mandate, namely, Module 1: Characterization of the *in vitro* competitive abilities of the Chinese morel specimens; Module 2: Morphological and genetic characterization of the hybridization areas between sexually compatible specimens; Module 3: Stimulation of the formation of asexual spores, sclerotia, and fruiting bodies by simulation of stress conditions; genetic analysis of the resulting structures; and, Module 4: Evaluation of the germination and dispersal abilities of any reproductive structures (asexual spores and fruiting bodies) obtained in module 3. The experiments performed in Module 1 demonstrated that morel specimens from a Chinese origin display a faster colonization of the substrates (both media and soil-like substrates) and therefore, could potentially overtake native Swiss morels while growing as saprotrophs in soils. Concerning their reproductive strategies (Module 2 and 3), the morel species amenable to cultivation in China are described as heterothallic, secondary homothallic, and unisexual (haploid meiosis). The genetic characterization of four Chinese cultivars revealed the simultaneous detection of two mating type idiomorphs in *Morchella sextelata* and one (MAT1-2) in *Morchella importuna*. Thus, in order to produce fruiting bodies from these, the Chinese cultivars could represent cultivars with pseudo homothallic (*M. sextelata*) and unisexual (*M. importuna*) reproductive strategies, but the absence of fruiting body formation does not allow to verify this. Even though mycelial cultures obtained from both Chinese cultivars and Swiss specimens were potentially sexually compatible or self-fertile, the stimulation of sexual structures *in vitro* was not possible, even in controlled essays in a growth chamber (Chinese cultivars) or in greenhouse and the field (Swiss strains). Nonetheless, a suite of methods to characterize resulting sexual structures and mycelium to identify the existence of hybrids that might arise was established and could be used in the future when indoor/outdoor production of fruiting bodies is successful. In module 3, a method was developed to stimulate the production of conidia. They were compared (germination, morphology) with the ascospores that were collected on wild morel fructifications. Moreover, we provided three Swiss morel cultivars (mycelial isolates with both MAT idiomorphs) to a morel grower for fruiting body production to be harvested in May 2022. Unfortunately, none of the cultivars (including the traditional Chinese cultivar planted alongside by the grower) produced fruiting-bodies in Spring 2022. This highlights once more the complexity of establishing morels as a crop. At the end of the report, we provide our recommendations about imported cultivated morels management and follow-up in Switzerland. The experiments and knowledge acquired to fulfill this report led to the production of one scientific publication in which the financial support by the FOEN is acknowledged (Cravero et al. 2022, *submitted*).

1. Module 1: Characterization of the *in vitro* competitive abilities of Chinese morels

1.1 Rationale

The indoor cultivation of morels was developed in the USA in the eighties, with the successful growth of a fruiting body of *Morchella rufobrunnea* (referred to as *Morchella esculenta* in Ower 1982). Ten years ago, China followed up with the large-scale outdoor cultivation of black morels (Elata clade). The Chinese area cultivated with morels increased from 200 ha in 2011 to more than 1200 ha in 2015. The most cultivated species are the saprotrophic black morels (of which *Morchella importuna* is largely dominant), with approximately 90% of the production. Yellow morels can be cultivated as well, but the technique is complex and unstable as these species often need to be associated with trees. In China, dry morels exports annually make an equivalent of 144 million CHF (Liu et al. 2018). The indoors production of morels is thus clearly a highly lucrative business. This has stimulated developments in other countries. For instance, in Europe the Danish Morel project (https://thedanishmorelproject.com/the-morel-project/) is a 40-year project aiming at the full development of an all-year-round method for the controlled indoor cultivation of black morels. Slowly, these cultivars are making their way to other countries by the interest of farmers and

amateurs for the production of this crop. Therefore, it is crucial to assess whether foreign cultivars can be invasive in the light of biodiversity conservation, specifically because *Morchella* spp. exhibit a high level of continental endemism. Morel's endemism could be explained by their ecology (see below), their complex reproduction cycle, and the fact that morels appear not to be dispersed easily supporting long-range water-borne or air-borne dispersal (O'Donnell et al. 2011).

The consequences of morel's introduction in non-native countries have already been reported. For instance, *M. importuna* has shown a great potential to adapt to new environments as suggested by its inadvertent introduction by humans from North America to Europe and Asia (Richard et al. 2015), where it is now extensively cultivated (Liu et al. 2018). Other black morels such as *Morchella tridentina* (Baroni et al. 2018) and *Morchella populiphila* (Richard et al. 2015) were introduced from Europe to South America and from North America to Europe, respectively, along with trees. These introduced species all belong to the Elata clade (black morels). Interestingly, black morels are considered as pioneer saprotrophs (e.g., fire morels) that colonize highly specific ecological niches, while yellow morels are generalists found in ecologically stable environments (Loizides 2017). In addition, yellow morels are more likely to establish interactions with trees (ectomycorrhiza/root endophytes or parasites). In contrast, black morels are mostly saprophytic, even though mycorrhizae and parasitic interactions have also been reported (Liu et al. 2018). Their habitat preference also varies. For instance, yellow morels prefer temperate deciduous forests at low altitudes (<1200m), while black morels often live in coniferous forests at high altitudes (>2000m), at least in Asia (Du et al. 2015).

Black morels being likely to be introduced in new environments, it is important to investigate whether the Chinese cultivars that are being imported and grown in Europe, including Switzerland, can constitute a major threat to native morel species by over competing them. To test this, we were able to obtain four cultivars from China to perform tests in our laboratory. The genetic analyses revealed that they belong to two black morel species: *M. importuna* (NEU142 and PYT) and *Morchella sextelata* (NEU143 and PYL). They were used in this module to assess the competitive abilities of the commercial cultivars compared to different mycelial isolates obtained from wild morel specimens collected in Switzerland. All isolates were identified by sequencing of three genetic markers (ITS, 18S and 28S rRNA genes).

1.2 Methods

Adaptability of the specimens on different media

To test and compare the adaptability of Chinese and Swiss mycelial cultures to various nutritive conditions (Chinese: *M. importuna* PYT, *M. sextelata* PYL; Swiss: *M. esculenta* M19-42, *Morchella* sp. M19-41, *M. angusticeps/eximioides* M19-43), mycelial growth rate was recorded on chemically defined media that contained four different carbon sources (glucose, sucrose, maltose, and starch) and three different nitrogen sources (glycine, urea, and ammonium hydrogen carbonate). For this, Brock's medium (Brock 1951) was adapted as indicated in Table 1. After inoculation under sterile conditions, plates were cultivated at room temperature (21-23°C) in darkness. The radial growth of the mycelium was measured after 3, 6, and 9-day post-inoculation by calculating the mean between the vertical and the horizontal diameter of mycelium from the inoculum.

Table 1: *Composition of the modified Brock's medium supplemented with different carbon and nitrogen sources.*

Medium ID	Basal medium	Carbon source	Nitrogen source
Glucose	0.5g [MgSO4·7H2O] 1g [K2HPO4] 0.01g [FeSO4] 1L [H2O] 15g [Technical agar]	12 g [$C_6H_{12}O_6$]	-
Glucose + glycine		12 g [$C_6H_{12}O_6$]	0.25 g [NH_2CH_2COOH]
Glucose + urea		12 g [$C_6H_{12}O_6$]	0.25 g [$CO(NH_2)_2$]
Glucose + NH4		12 g [$C_6H_{12}O_6$]	0.25 g [NH4HCO3]
Starch + glycine		12 g [(C6H10O5)n]	0.25 g [NH_2CH_2COOH]
Maltose + glycine		12 g [C12H22O11]	0.25 g [NH_2CH_2COOH]
Sucrose + glycine		12 g [C12H22O11]	0.25 g [NH_2CH_2COOH]

Confrontation in culture media and in soil-like substrate

In order to assess the competitive advantage of Chinese morels over Swiss morels, confrontation assays were conducted. Chinese and Swiss mycelial cultures (Table 2) were confronted *in vitro* in: (A) Petri dishes containing chemically defined agar-based media and (B) glass tubes containing an organic substrate. Experiments A were performed using four media: (1) malt agar medium (MA; 12 g/L); Angle medium, mimicking the low nutrient conditions in soils (Angle and Chaney 1991) at pH 6; (3) Angle medium at pH 7; and, (4) Angle medium supplemented with skimmed milk (SM; 10 g/L). For each pair of mycelial cultures to be confronted, the two strains were inoculated each on one side of the Petri dish to allow mycelium to grow and to encounter somewhere at the middle of the dish. Experiments B were conducted in an organic substrate mimicking soil (autoclaved soil supplemented with cooked wheat grains) used for spawn production in the process of morel cultivation. The substrate was kindly provided by a Swiss morel farmer. In these experiments, one mycelial isolate was inoculated at the bottom of the tube, and the other one at the top. The tubes were then incubated horizontally to allow the mycelium to grow evenly. All the cultures were maintained at room temperature (21-23°C) in darkness for two months. Macroscopic (naked eye) and microscopic (stereo and light microscopic) observations were regularly conducted to evaluate the advance and the phenotypes of the confronted mycelial isolates.

Table 2: *List of the species used in the confrontation assays. All strains consist of mycelial isolates obtained as the commercial cultivar from morel farmers (China) or from fruiting-bodies collected in the wild (Switzerland). Their origin and clade are indicated.*

Species	Isolate ID	Origin	Clade
Morchella sextelata	NEU143	China	Elata
Morchella importuna	NEU142	China	Elata
Morchella sextelata	PYL	China	Elata
Morchella importuna	PYT	China	Elata
Morchella deliciosa	M19-29	Switzerland	Elata
Morchella sp.	M19-41	Switzerland	Elata
Morchella angusticeps/eximioides	M19-43	Switzerland	Elata
Morchella esculenta	M19-14	Switzerland	Esculenta
Morchella esculenta	M19-16	Switzerland	Esculenta
Morchella esculenta	M19-20	Switzerland	Esculenta
Morchella esculenta	M19-23	Switzerland	Esculenta
Morchella esculenta	M19-34	Switzerland	Esculenta
Morchella esculenta	M19-39	Switzerland	Esculenta
Morchella esculenta	M21-38	Switzerland	Esculenta
Morchella deliciosa	M21-16	Switzerland	Elata
Morchella sp.	M21-41	Switzerland	Elata
Morchella sextelata	M21-83	Switzerland	Elata

1.3 Results and discussion

Adaptability of the Chinese and Swiss morels to different carbon and nitrogen sources

The comparison of mycelial growth in media containing diverse sources of carbon and nitrogen showed that *M. sextelata* PYL performed better than all the other isolates in glucose, glucose-urea, and glucose-NH_4 media. Conversely, in glucose-glycine and starch-glycine, the Swiss *M. esculenta* had a better growth. Finally, both isolates had a similar growth in maltose-glycine and sucrose-glycine (Figure 1A). Limited growth was observed for 4 out of the 5 isolates in presence of glucose and glycine, so that the addition of this simple amino acid gave an advantage to the Swiss *M. esculenta* isolate. This effect was however compensated with maltose and sucrose as C sources. Except for the Swiss *M. esculenta*, both Chinese isolates outcompeted the other two Swiss isolates on the different media. Interestingly, the addition of a nitrogen source was not necessary for mycelial growth, since all isolates were able to grow in a medium containing glucose only. However, it is noteworthy that the Chinese *M. sextelata* PYL isolate clearly outgrew all other isolates in this condition.

In terms of the macroscopic appearance of the mycelia, no notable differences between the different isolates was highlighted, except in glucose-urea cultures where the mycelium was particularly dense (Figure 1B). Sclerotia only appeared in *M. importuna* in glucose-NH_4 medium, at the periphery of the dish at nine days post-inoculation. Sclerotia was initially white (Figure 1C) and turned brown later on.

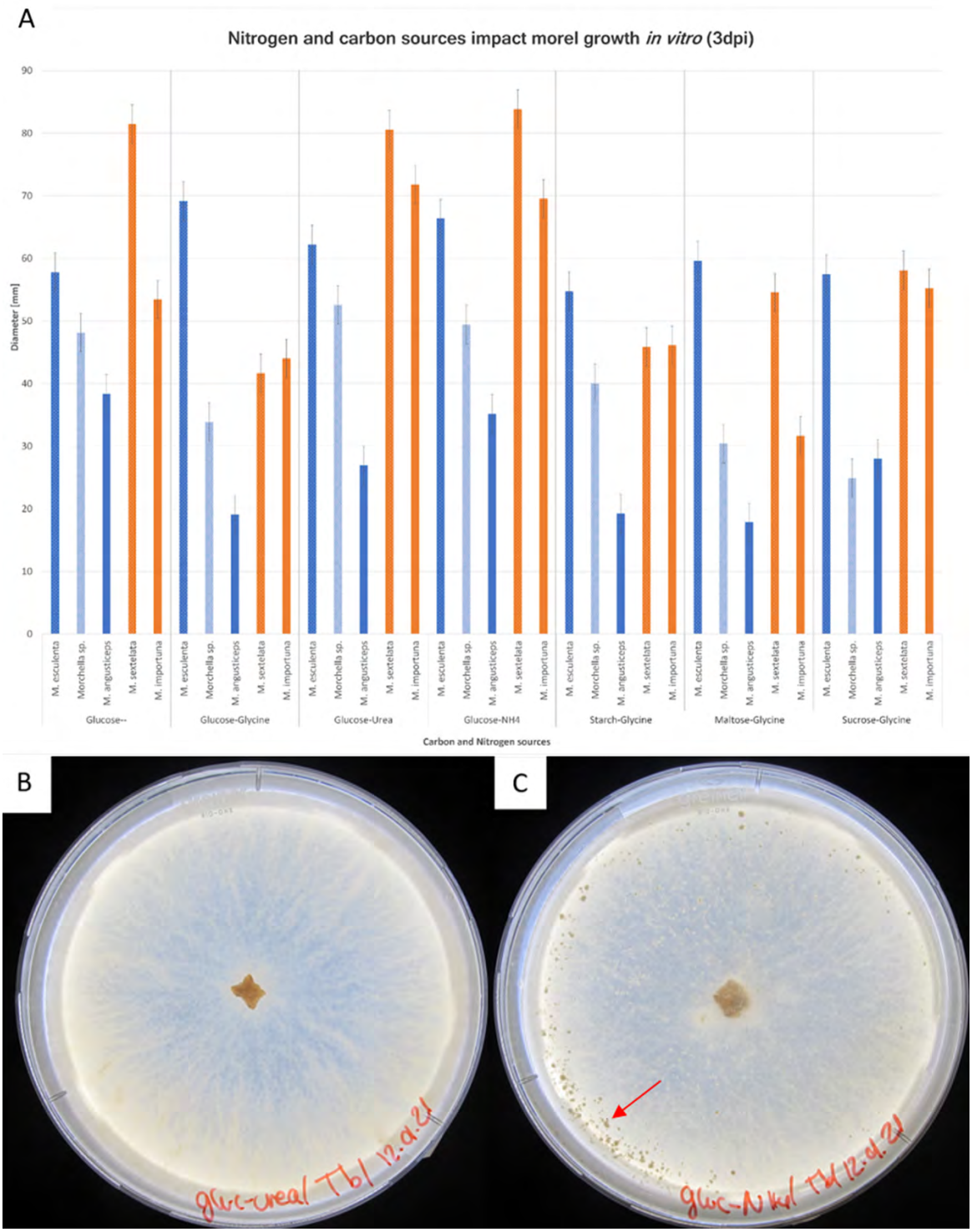

Figure 1: *(A) Mean distances colonized by mycelia 3 days post inoculation (Y axis) for isolates of* Morchella spp. *Swiss isolates (blue):* M. esculenta *M19-42,* Morchella *sp. M19-41, and* M. angusticeps/eximioides *M19-43. Chinese isolates (orange):* M. sextelata *PYL and* M. importuna *PYT. Specimens were inoculated in vitro in seven culture media (X axis) containing four different carbon sources (glucose, starch, maltose and sucrose) and three different nitrogen sources (glycine, urea and ammonium hydrogen carbonate). Standard deviations are provided. (B)* M. importuna *PYT in glucose-urea, (C)* M. importuna *PYT in glucose-NH4. Sclerotia are shown by the red arrow.*

Confrontation assays

Different types of interactions (Figures 2-4) were observed in the confrontation experiments. Those interactions ranged from the formation of a very clear confrontation line to an exclusion zone (summary in Annex 1). Overall, the results showed that the Swiss isolates are poor competitors to the Chinese isolates, particularly in confrontation with *M. sextelata* NEU143 (Figure 3). The only condition in which *M. sextelata* NEU143 did not overgrow the isolate to which it was confronted was in the confrontation with the Chinese *M. importuna* NEU142 isolate, in Angle medium (Figure 3; top panels). In malt agar medium (MA), a tissue-like structure formed at the confrontation lines. This struture was transplanted to a fresh MA medium in order to attempt to induce the formation of additional structures suggesting hybridization between the isolates. After the transplantation to fresh medium, the pairs *M. sextelata* NEU143 vs *M. esculenta* M19-23 and *M. sextelata* NEU143 vs *M. esculenta* M19-20 produced an unidentified tissue (Figure 5). This tissue had a macroscopic appearance compatible with the one of a fruiting-body primordium, thus potentially constituting an evidence of hybridization. However, these structures did not progress into forming an ascocarp. Hybridization and the formation of these structures was not expected since the two isolates in confrontation belonged to different morel clades (i.e., Elata and Esculenta). Microscopy observations showed rounded cells resembling those inside sclerotia indicating they could also be a resistance structure of undetermined nature (Figure 5E). The formation of sclerotia was mainly observed in the medium supplemented with organic nitrogen (skimmed milk) and in MA. Interestingly, the putative sclerotia produced in the confrontations (Figures 5 and 6) were different (smoother surface, round and regular shape) from the ones observed in single cultures (exemplified in Figure 7). However, once more, this did not progress to the formation of primordia. At the end, we were not able to stimulate the formation of any sexual structures as a result of a hybridization event *in vitro* (Milestone M1-7).

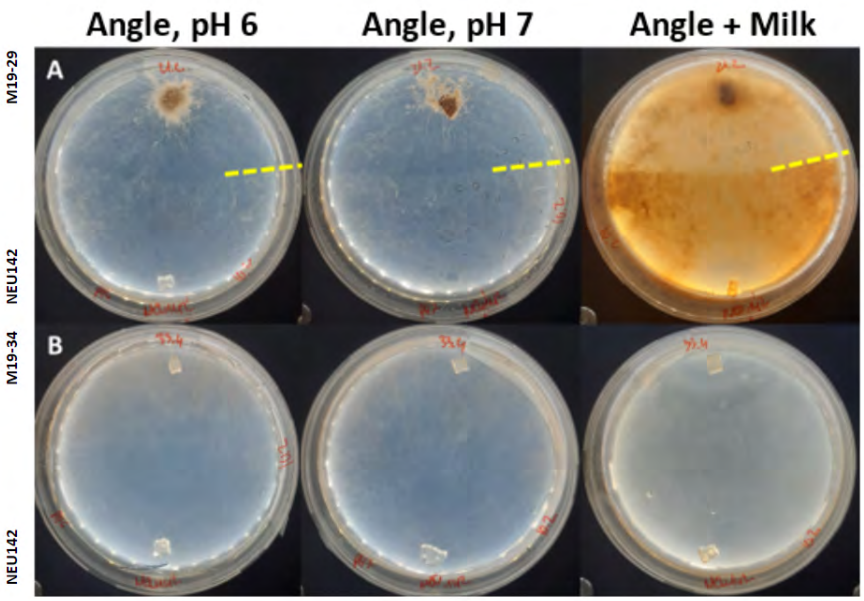

Figure 2: Confrontation experiments between the Chinese isolate M. importuna *NEU142 (inoculated at the bottom) and Swiss isolates (inoculated at the top)* M. importuna *M19-29 (A) and M. esculenta M19-34 (B). The experiments were performed in Angle medium at pH 6, pH 7 and in Angle medium supplemented with skimmed milk. Pictures were taken five months after inoculation. The dashed yellow lines show the position of a confrontation line.*

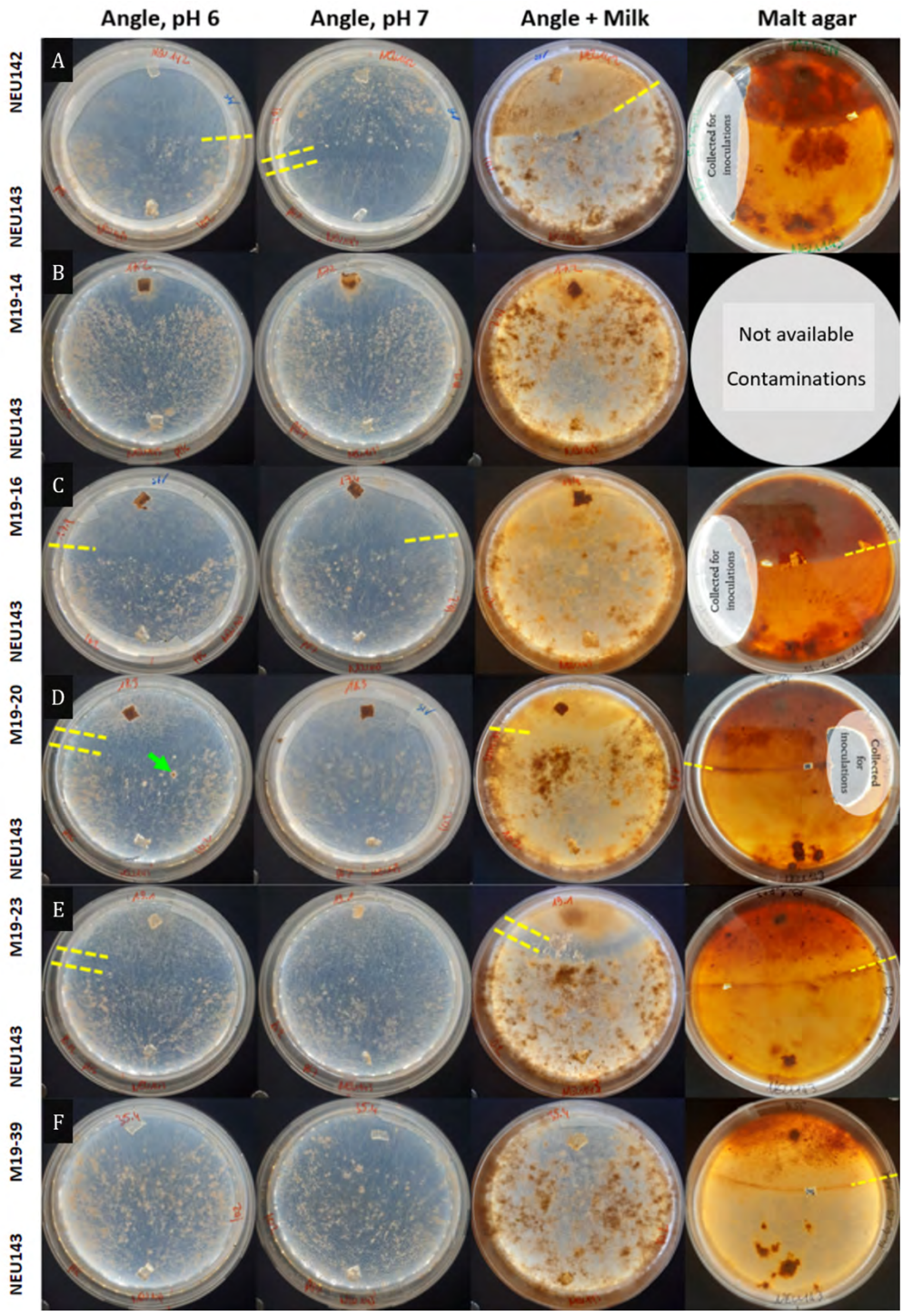

Figure 3: *(A) Confrontations between* M. sextelata *NEU143 (inoculated at the bottom) and* M. importuna *NEU142 (inoculated at the top) and (B to F) between* M. sextelata *NEU143 (inoculated at the bottom) and Swiss isolates (inoculated at the top)* M. esculenta *M19-14 (B),* M. esculenta *M19-16 (C),* M. esculenta *M19-20 (D),* M. esculenta *M19-23 (E), and* M. esculenta *M19-39 (F). The experiments were performed in Angle medium at pH 6, pH 7 and in Angle medium supplemented with skimmed milk and in malt agar. Pictures were taken five months after inoculation. The dashed yellow lines show the position of a confrontation line.*

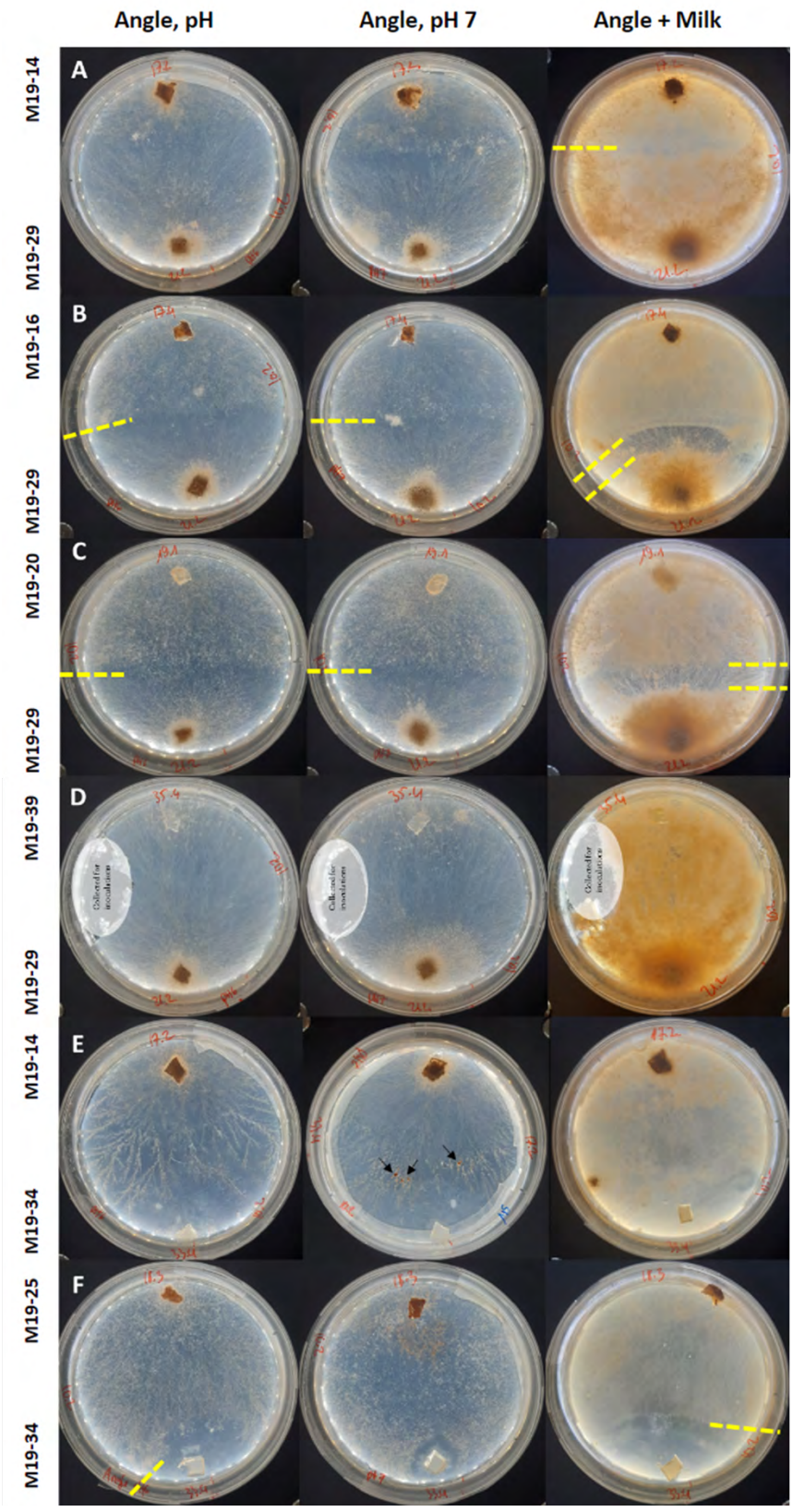

Figure 4: *(A to D) Confrontations between the Swiss isolate* M. importuna *(M19-29) (inoculated at the bottom) and other Swiss isolates (inoculated at the top)* M. esculenta *M19-14 (A)* M. esculenta *M19-16 (B),* M. esculenta *M19-20 (C) and* M. esculenta *M19-39 (D). (E-F) Confrontations between the Swiss specimen* M. esculenta *M19-34 (inoculated at the bottom) and other Swiss specimens (inoculated at the top)* M. esculenta *M19-14 (E) and* M. esculenta *M19-20 (F). The experiments were performed in Angle medium at pH 6, pH 7 and in Angle medium supplemented with skimmed milk. Pictures were taken five months after inoculation. The dashed yellow lines show the position of a confrontation line.*

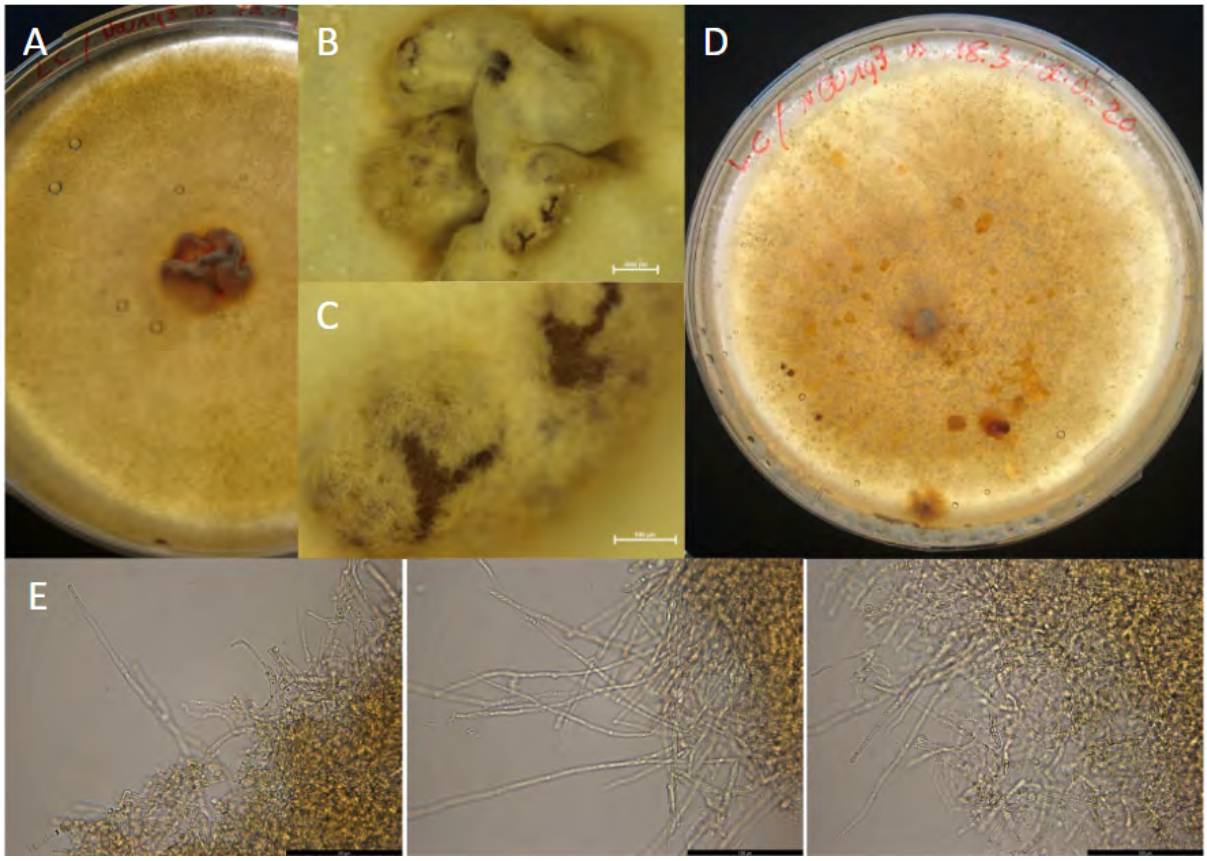

Figure 5: *Tissue-like structures produced after transplanting the confrontation lines of co-cultures of the Chinese* M. sextelata *NEU143 vs (A) the Swiss isolates* M. esculenta *M19-2 and (D)* M. esculenta *M19-20 (confrontation in Figure 3D; Malt agar). (B-C) Close-up images and (E) microscopic images at 35 days post inoculation of the structures observed in A. E images show thick and rounded shapes of the hyphae cells, similar to the ones observed in sclerotia.*

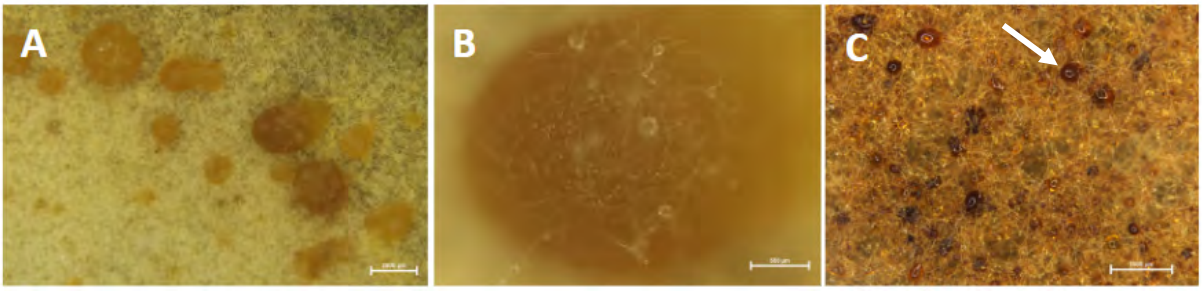

Figure 6: *(A) Tissue-like structures produced after transplanting the confrontation lines of co-cultures of the two Chinese isolates:* M. importuna *NEU142 and* M. sextelata *NEU143, which could be potentially compatible (compatible mating types and belonging to the same clade). (B) close-up image of the structures shown in A. (C) The accumulation of brown-coloured exudates, potentially containing melanin, may be seen (white arrow).*

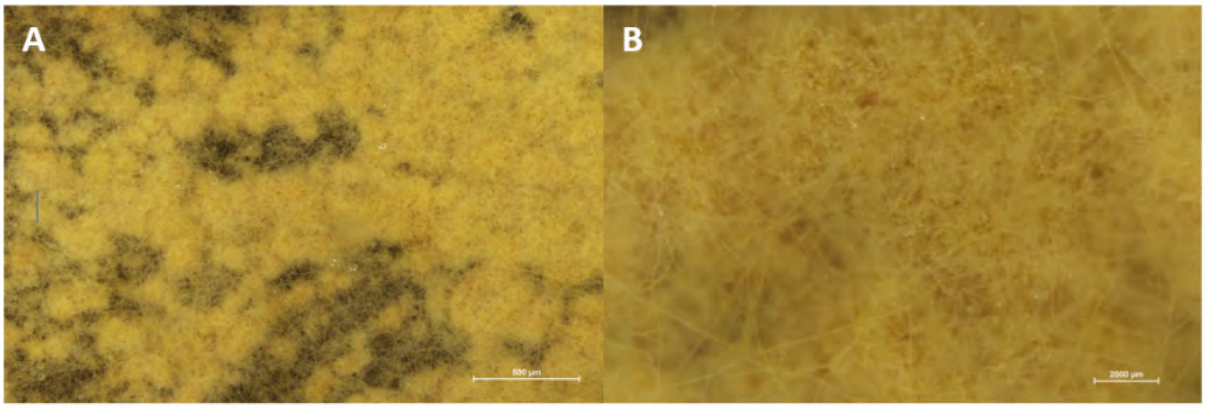

Figure 7: *Sclerotia (darker yellow masses) in a mycelial culture from cultivation by germination of individual ascospores (isolate 18) obtained from the Swiss specimen Morchella* sp. *M21-48 on PDA. (A) sclerotia magnified with a stereomicroscope (10 x); (B) sclerotia magnified with a stereomicroscope (50 x). Pictures were taken 15 days post inoculation.*

Confrontation in soil-like substrate

In a soil-like substrate, all the isolates produced visible mycelial fronts that progressed from the inoculation zones (each extremity of the tube) towards the center (Figure 8A-B). As soon as the two mycelial fronts encountered (between 9- and 14-days post inoculation, depending on the isolates paired; Figure 8C-D), it became difficult to ascribe the hyphae to one or the other isolate in competition. Most of the isolates had similar growth rates (*Morchella* sp. M19-41, *M. angusticeps/eximioides* M19-43, *M. importuna* PYT, *M. sextelata* NEU143), with the exceptions of *M. importuna* NEU142 and *Morchella sextelata* PYL, which were the slowest and the fastest one, respectively (Figure 8). The mycelia were initially white, then turned to brown-reddish (melanized phase; for instance, 21- and 52 dpi; Figure 8D and 8E). The time-point of the color shift varied depending on the isolates, but it generally happened between the second and fourth week after inoculation and after the mycelial fronts had already encountered. The position of the mycelial fronts over time is shown in Table 3. The encounter zones were described according to three categories, which were defined based on the comparison of mycelial density between this zone and the rest of the tube: denser mycelium (+), less dense mycelium (-) or no clear difference (=) ("Encounter zone"; Table 3). Sclerotia formation was observed in two isolates (*M. angusticeps/eximioides* M19-43 and *M. importuna* PYT) but this was not systematic ("Sclerotia"; Table 3). The sclerotia were first white and then turned brown reddish simultaneously to the color shift of the mycelium. As an example, one tube presented all the features mentioned above: melanization of the mycelium, formation of sclerotia by M19-43 (bottom), and denser mycelium at the encounter zone (M19-43 vs NEU142; Figure 9A). When comparing the direct competition between the Chinese isolates (*M. sextelata* NEU142 and PYL, *M. importuna* NEU143 and PYT) and the two Swiss isolates (*M. angusticeps* M19-43 and *M. deliciosa* M19-41) (Figure 10), colonization of the substrate by *M. sextelata* (both isolates) and *M. importuna* PYT were faster than for the Swiss isolates. In soils, this might result in the exclusion of mycelia from native species by the mycelium of a Chinese cultivar. Concerning *M. importuna* (NEU143 isolate), the colonization rate was slower than the three other Chinese isolates and the Swiss *M. deliciosa* isolate was able to overgrow this particular Chinese isolate. An analogous experiment was performed using Swiss isolates obtained after the 2021 sampling season and Chinese PYL and PYT isolates. Briefly, the mycelial growth of the Swiss *M. sextelata* M21-83 was slower than the one of the same species from China (isolate PYL). Furthermore, intra-specific differences were observed in sclerotia formation: the Chinese *M. sextelata* PYL produced more sclerotia than the Swiss one (M21-83).

Table 3: *Summary of quantitative and qualitative observations of the confrontation assays performed in soil-like substrate. Swiss isolates are highlighted in light grey and Chinese isolates in dark gray. Position of the mycelial front of the isolate inoculated at the bottom of the tube is indicated at 7, 9, 14, 21, and 52-day post-inoculation (dpi). The characteristics of the encounter zone, compared to the rest of the tube, is indicated as: "+" when the mycelium appears denser, "-" when the mycelium appears less dense, and "=" when the mycelium does not appear as clearly different in the encounter zone.*

Bottom isolate	Top isolate	Position of the mycelial front of the bottom specimen [cm]					Encounter zone	Sclerotia
		7 dpi	9 dpi	14 dpi	21 dpi	52 dpi		
M19-41	M19-41	3.5	6	Encounter	Encounter	Encounter	=	
M19-41	M19-43	6	8	Encounter	Encounter	Encounter	-	M19-43
M19-41	PYL	5	Encounter	Encounter	Encounter	Encounter	-	
M19-41	PYT	5	8	Encounter	Encounter	Encounter	+	
M19-41	NEU 142	5	Encounter	Encounter	Encounter	Encounter	+	
M19-41	NEU 143	6.5	Encounter	Encounter	Encounter	Encounter	+	
M19-43	M19-41	5	Encounter	Encounter	Encounter	Encounter	=	M19-43
M19-43	M19-43	3.5	4.5	Encounter	Encounter	Encounter	=	
M19-43	PYL	5	Encounter	Encounter	Encounter	Encounter	=	
M19-43	PYT	5	7	Encounter	Encounter	Encounter	=	M19-43
M19-43	NEU 142	5	7	Encounter	Encounter	Encounter	+	M19-43
M19-43	NEU 143	5	Encounter	Encounter	Encounter	Encounter	-	M19-43
PYL	M19-41	7.5	Encounter	Encounter	Encounter	Encounter	+	
PYL	M19-43	7.5	Encounter	Encounter	Encounter	Encounter	+	
PYL	PYL	7	Encounter	Encounter	Encounter	Encounter	=	
PYL	PYT	7.5	Encounter	Encounter	Encounter	Encounter	-	
PYL	NEU 142	9	Encounter	Encounter	Encounter	Encounter	-	
PYL	NEU 143	8	Encounter	Encounter	Encounter	Encounter	=	
PYT	M19-41	5.5	8	Encounter	Encounter	Encounter	=	
PYT	M19-43	5	7	Encounter	Encounter	Encounter	=	PYT, M19-43
PYT	PYL	6	7.5	Encounter	Encounter	Encounter	-	
PYT	PYT	4	6	Encounter	Encounter	Encounter	=	
PYT	NEU 142	5	7.5	Encounter	Encounter	Encounter	-	PYT
PYT	NEU 143	5.5	Encounter	Encounter	Encounter	Encounter	=	PYT
NEU142	M19-41	3.5	5	Encounter	Encounter	Encounter	+	
NEU142	M19-43	3.5	5	7	Encounter	Encounter	-	
NEU142	PYL	4	5	Encounter	Encounter	Encounter	-	
NEU142	PYT	4	5.5	Encounter	Encounter	Encounter	-	PYT
NEU142	NEU 142	2.5	4.5	7	Encounter	Encounter	-	
NEU142	NEU 143	3.5	5	Encounter	Encounter	Encounter	-	
NEU143	M19-41	6	8.5	Encounter	Encounter	Encounter	-	
NEU143	M19-43	5.5	8.5	Encounter	Encounter	Encounter	-	
NEU143	PYL	6	Encounter	Encounter	Encounter	Encounter	-	
NEU143	PYT	5.5	Encounter	Encounter	Encounter	Encounter	=	PYT
NEU143	NEU 142	6.5	9	Encounter	Encounter	Encounter	-	
NEU143	NEU 143	4.5	Encounter	Encounter	Encounter	Encounter	-	

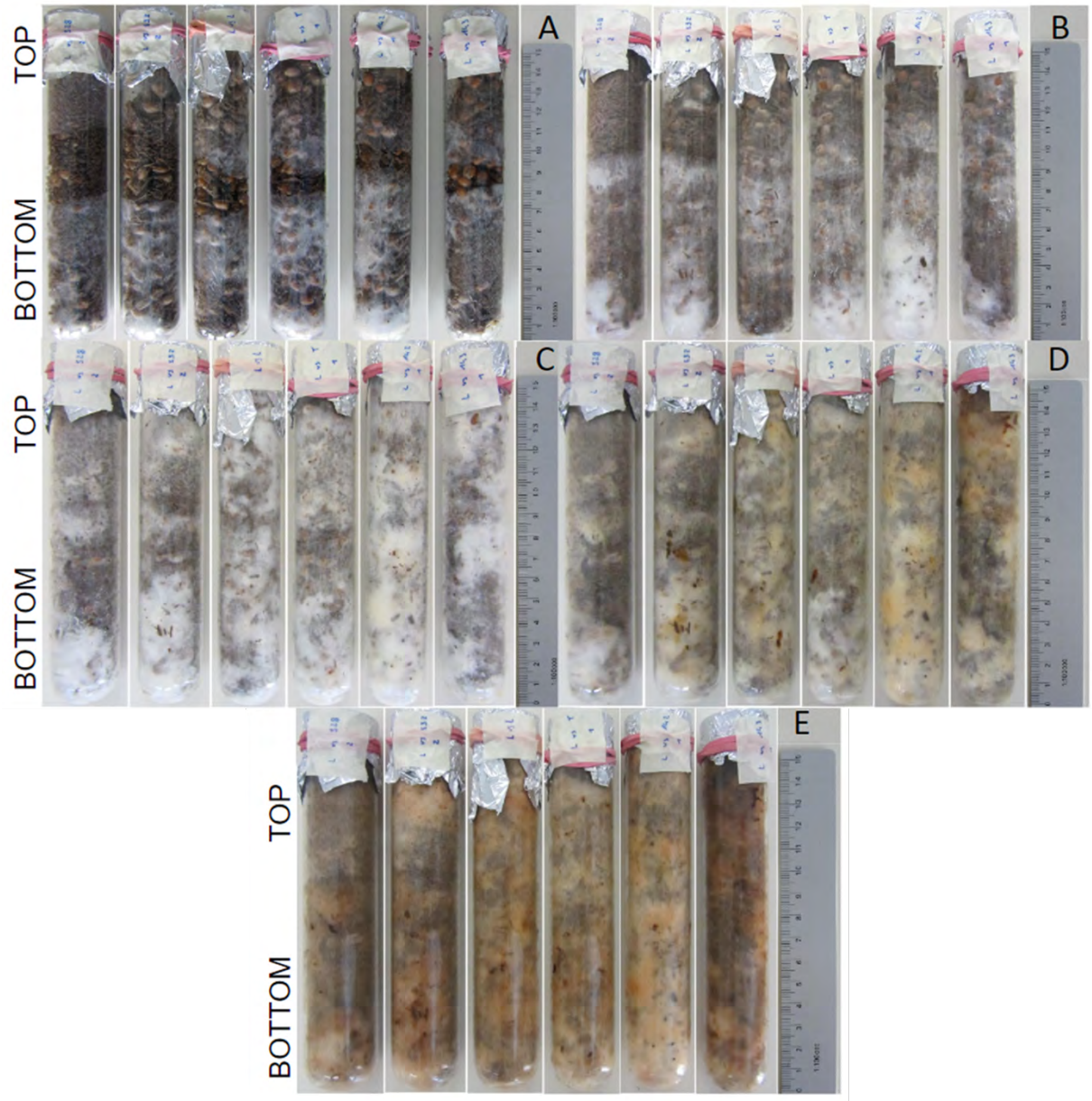

Figure 8: Confrontation experiments between M. sextelata *PYL and all the other specimens in the following order (left-to-right):* M. deliciosa *M19-41,* M. angusticeps/eximioides *M19-43*, M. sextelata *PYL*, M. importuna *PYT,* M. importuna *NEU142, and* M. sextelata *NEU143. The images were taken at 7 (A), 9 (B), 14 (C), 21 (D) and 52 (E) days post inoculation.*

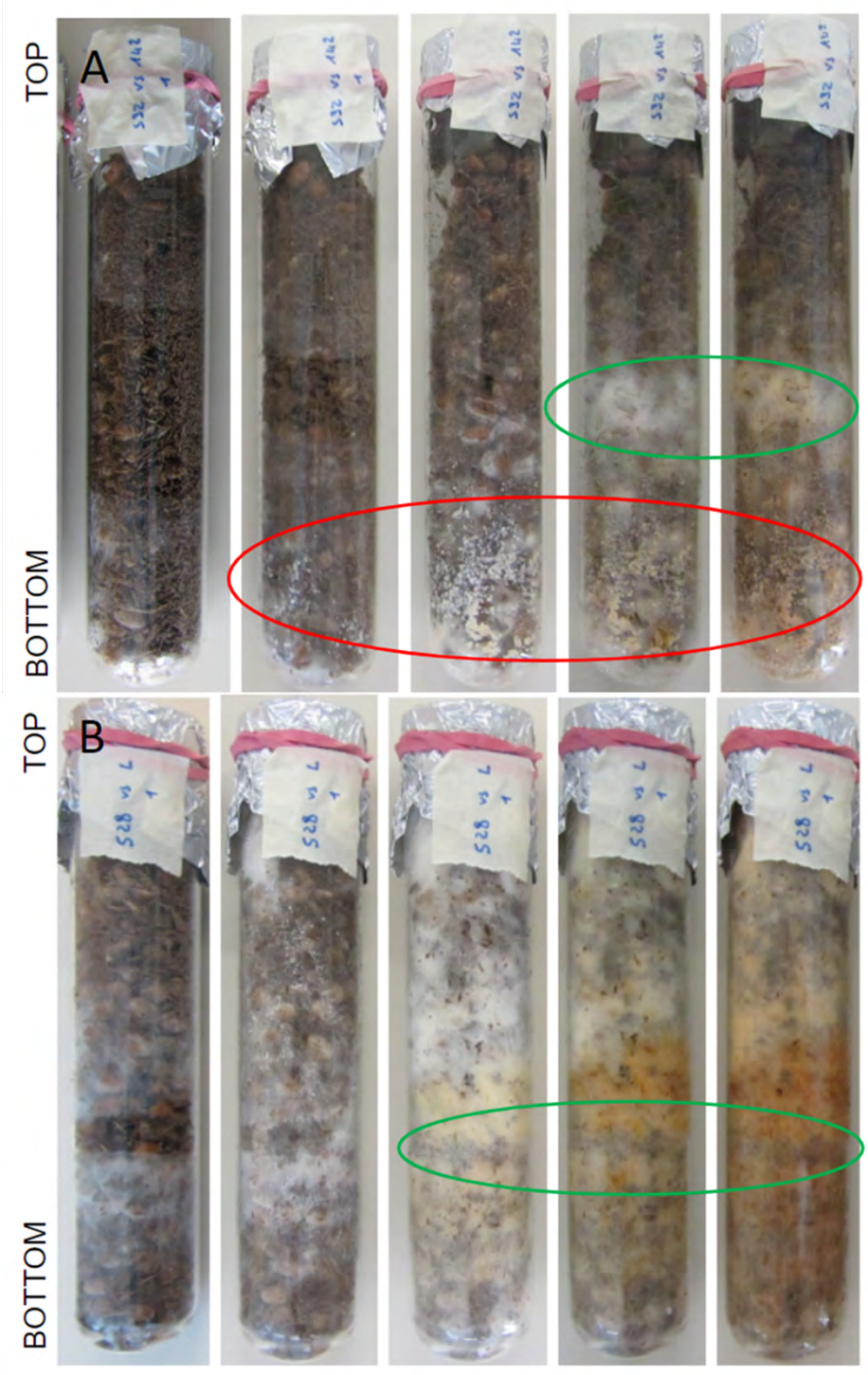

Figure 9: *Swiss vs Chinese isolate confrontation; pictures taken, from left to right, at 7, 9, 14, 21, and 52 dpi. (A) Tube with* M. angusticeps/eximioides *M19-43 inoculated at the bottom and* M. importuna *NEU 142 at the top. The green circle highlights the encountering zone, in which a strip of denser mycelium formed. The red circle indicates the zone where M19-43 formed numerous sclerotia. (B) tube with* M. deliciosa *M19-41 inoculated at the bottom and* M. importuna *PYL at the top. The green circle highlights the encountering zone, in which a strip of less dense mycelium formed.*

Figure 10: Confrontation experiments between the Chinese M. sextelata *isolates (NEU142 and PYL) and* M. importuna *isolates (NEU143 and PYT) against two Swiss isolates (*M. angusticeps *M19-43 and* M. deliciosa *M19-41). Growth direction for the respective Chinese (CN) and Swiss (CH) isolates is indicated on the right. Also, the position of the area of interaction is shown by a yellow line.*

1.4 Conclusion

The experiments of this module demonstrated that *M. sextelata* PYL, and *M. importuna* PYT to a lesser extent, colonized most of the chemically defined media more rapidly than the Swiss isolates (**Milestone M1-1**). Mycelial isolates of both Chinese species (*M. sextelata* and *M. importuna*) outcompeted Swiss isolates of black morels in terms of distance colonized. However, in media containing glycine, the yellow morel isolate of *M. esculenta* was better adapted than, or equivalent to, the Chinese isolates. The ability of *M. sextelata* PYL to outcompete all the other isolates was also demonstrated in confrontation assays, where it was always colonizing the substrate (both in chemically defined media and in a soil-like substrate) faster than the other isolates (**Milestones M1-2 and 1-3**). This high adaptability was also demonstrated by the absence of sclerotia in *M. sextelata* PYL in the soil-like substrate. Indeed, sclerotia are highly resistant resting structures that can be considered as indirect evidence of stress (Liu et al. 2018). Due to these features, this species/strain could potentially be problematic in the wild as it could outcompete Swiss native species (especially Black morels) in various growth conditions. In contrast to *M. importuna*, which has already shown to have a great potential to adapt to new environments (Taskin et al. 2010; Richard et al. 2015), there are no literature reports yet about the range expansion and adaptability of *M. sextelata*.

2. Module 2: Morphological and genetic characterization of the hybridization areas between sexually compatible isolates

2.1 Rationale

In Ascomycetes, sexual reproduction is genetically controlled by the so-called mating-type locus (MAT locus). At this location, two variants exist: MAT1-1 or MAT1-2, which can be found either in a single individual (homothallic species) or in different individuals (heterothallic species). Each MAT variant consists in different genetic structure and therefore the different versions are not considered alleles but rather idiomorphs. Morel species are usually reported to be heterothallic. Hence, to accomplish sexual reproduction, individuals with opposite mating types have to encounter and fuse (plasmogamy, followed by karyogamy) (Du and Yang 2021). Therefore, in theory, after meiosis, an ascospore gives rise to a new haploid individual and must contain only one mating type. However, the sexual reproduction in morels appears as more complex than previously thought and some species, such as *M. importuna*, can use three different mating strategies (i.e., heterothallism, primary and secondary homothallism) to reproduce sexually and then produce the structure (ascocarp) that releases the ascospores (Du and Yang 2021). Primary and secondary homothallic morels are self-fertile as both MAT loci are present in a single individual, either in a single genome/nucleus or in two distinct nuclei in a single cell-compartment (heterokaryosis). Heterokaryotic ascospores have been detected in wild specimens, with a ratio of 12.1% (four heterokaryotic ascospores detected from 33 investigated ascospores). The ratios of heterokaryotic:homokaryotic ascospores were approximately 10% and 1.2% in *M. sextelata* and *Morchella* sp. Mes-15, respectively, when single wild ascocarps were analyzed (Du and Yang 2021). A different strategy consists in the formation of a sterile ascocarp from a haploid mycelium (unisexual reproduction). Unisexual reproduction is a rare and poorly described phenomenon and the resulting ascocarp contains empty asci (i.e., no ascospores) (Du and Yang 2021).
In Ascomycetes, the creation of a functional dikaryon between sexually compatible individuals cannot be visualized as in the Basidiomycetes by detecting clamp connections, because such structures do not exist for Ascomycetes (Krings et al. 2011). However, microscopically, it is possible to identify mycelial cells containing pairs of nuclei that could be the result of a sexual anastomosis event between two haploid mycelia (creation of the dikaryon). This technique was demonstrated in morels by Volk and Leonard (1989) a few decades ago. They generated confrontation lines between non-sister morel isolates from which they sampled and stained the mycelium. Compared to the vegetative hyphae that contained randomly distributed nuclei, the aerial hyphae coming from the confrontation line comported paired nuclei (3-5 pairs per cell). This was the evidence that morels were able to form heterokaryons *in vitro*. Another technique that can be used to detect hybridization events is the analysis of simple sequence repeats (SSR) microsatellites. This technique was tested on samples from wild and cultivated black Chinese morels by Du et al. (2019). Hybridization events

occurred between wild and cultivated samples of multiple species from the Elata clade: *M. exuberans, M. importuna*, Mel-13, *M. eohespera, M. eximioides*, Mel-21 and Mel-34 (Du et al. 2019). Finally, *in situ* fluorescence hybridization (FISH) can be also used to identify the co-occurrence of genetically distinct nuclei within single cells (Kuhn et al. 2001). FISH is a molecular method often used in bacteria but that can be applied to filamentous fungi, although its use is more limited. The technique consists in using fluorescent probes that can complement and hybridize with a target RNA or DNA sequence contained in a cytological preparation (Tsuchiya and Taga 2010). The co-occurrence of genetically different nuclei in a single individual was evidenced by DNA-DNA FISH in arbuscular mycorrhizal fungi (AMF) targeting variable areas of the internal transcribed spacer (ITS) region (Kuhn, Hijri, and Sanders 2001). We wanted to test whether it was possible to track morel mating types at nuclear level within the mycelium (precisely in the potential hybridization zones), to be able to determine genetically the presence of heterokaryons. Standard FISH applications target RNA or DNA sequences present in multiple copies in a genome (Tsuchiya and Taga 2010). As the mating type region does not exist in multiple copies in fungal genomes (Zou et al. 2019), standard FISH could not be applied here. Instead, single molecule FISH (smFISH) should be used. This technique combines multiple probes that hybridize to a single RNA molecule to amplify the fluorescent signal (Chen et al. 2018). Using two different probes, smFISH could help to distinguish nuclei containing the MAT1-1, MAT1-2 idiomorphs, or both. Thus, we investigated whether it was possible to apply this technique to track morel mating types in potential hybridization areas.

In this module, we first attempted the identification of heterokarotic mycelia by fluorescence microscopy. We also determined the mating types of the mycelial isolates in our collection, both for the Chinese and Swiss samples. This allowed inferring the sexual compatibility of the various isolates and interpreting the results obtained from the hybridization assays. If applicable, we also assessed the mating types present in the potential hybridization areas. Identifying morel mating types must be done genetically, as no visible difference can be observed between MAT1-1 and MAT1-2 isolates (Du et al. 2017; 2020). For this, two master regulator genes (*MAT1-1-1* and *MAT1-2-1,* diagnostic of the MAT1-1 and MAT1-2 variants, respectively) can be amplified by polymerase chain reaction (PCR) using appropriate primers. Amplicons can then be visualized after gel electrophoresis. Although, we were able to amplify morel mating types in wild Swiss morels using *Morchella* spp. MAT-specific primers published by a Chinese research group (Du et al. 2017), our results have exposed clear issues regarding the primer design and performance of previously published primers to study mating in morels (Cravero et al. 2022, *submitted*). Therefore, the lack of a reliable genomic template for the design of smFISH probes for the MAT1-1 idiomorph limited our ability to develop this method further within the timeframe of this mandate. Nonetheless, a proof-of-concept of fluorescence microscopy is presented here.

2.2 Methods

Mycelial compartment staining

To test whether heterokaryons could form between two different morel isolates/species, and then demonstrate hybridization can occur, we sampled and stained mycelial portions coming from confrontation lines obtained in Module 1 (Figure 3C), as in Volk and Leonard (1989), and optimized the method further. Mycelium was first picked with a sterile scalpel and deposited on a clean microscopy glass slide. In order to highlight the fungal cell wall, the mycelium was then rinsed with phosphate-buffered saline (PBS) and one drop of Calcofluor White Stain and one drop of 10% Potassium Hydroxide was added to the preparation. In addition, 2 µL of 10% SYBR Green ($C_{32}H_{37}N_4S$) in a deionized water droplet was added to highlight the DNA contained in nuclei. The sample was incubated 10 min in darkness and then washed three times with PBS before microscopy observations under UV light.

Determination of the mating types

To determine the mating types of various fungal tissues, DNA extractions were performed with the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the protocol provided by

the manufacturer. For mycelial cultures of the initial Chinese cultivars provided by the morel farmers, biomass for DNA extraction was obtained by scrapping the surface of a fully colonized medium. DNA was also obtained from about 25 mm^3 agar plugs sampled in the confrontation lines between *M. sextelata* NEU143 and Swiss *M. esculenta* isolates M19-16; M19-20; M19-23; M19-39 and between the Chinese isolates *M. sextelata* NEU143 and *M. importuna* NEU142. In addition, DNA was also extracted from about 25 mm^3 of the tissues-like structures that appeared after the transplantation of the confrontation lines between *M. sextelata* NEU143 vs *M. esculenta* M19-23 and *M. sextelata* NEU143 vs *M. importuna* NEU142. Eluted DNA was then measured by Qubit quantification (Invitrogen, USA) using the Broad Range kit to obtain the quantity of double-stranded DNA contained in each sample. DNA was then diluted with PCR-grade water at 2 ng/µL and stored at 4°C until further use. Partial PCR amplifications of the genes *MAT1-1-1* and *MAT1-2-1* were performed in to identify the mating types MAT1-1 and MAT1-2, respectively. The two following primer pairs were used: MAT11L/R (MAT11L: CCACCTTCTGAGTCCATTAT; MAT11R: GTTATTCTCGACAAGGTGTG) and MAT22L/R (MAT22L: TTATTAGACCATGTTCCTCG; MAT22R: CAGTATTATCACCAACCGTA) (Du et al. 2017). The PCR mix was adapted from Du et al. 2017 and contained: PCR-grade water, 1.2 mg/mL bovine serum albumin (BSA), 1X polymerase buffer (1.5 mM MgCl2), 0.4 µM forward (MAT11L or MAT22L) and 0.4 µM reverse (MAT11R or MAT22R) primer, 0.2 mM TaKaRa dNTPs and 1.5 U of TaKaRa TaqTM DNA Polymerase (Takara Bio Inc., Japan) and 1µL of DNA at 2 ng/µL. A gradient PCR ranging from 50°C to 60°C was tested to optimize annealing temperatures for both primer pairs. The thermocycling parameters were then adapted from Du et al. (2017) as follows: initial denaturation at 94°C for 3min, 35 cycles of denaturation, annealing and elongation (94°C for 1 min, 54°C (MAT11) or 51.5°C (MAT22) for 30sec, 72°C for 1 min), final elongation at 72°C for 10 min, end at 20°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100mV, 30 min). Amplicons were then visualized under UVs in a Genoplex VWR transilluminator to detect the mating types.

2.3 Results and discussion

Microscopic observations of the potential hybridization areas

Nuclei pairs have been proposed as structures resulting from the sexual fusion of compatible mycelia in *M. esculenta* (Volk and Leonard 1990). To track such phenomena as a potential hybridization event between different morel isolates, we optimized a staining protocol allowing us to investigate the nuclear content of hyphal compartments. This methodology for microscopic observations consists in a dual staining using calcofluor white for the fungal cell wall and SYBR Green for the nuclei (Milestone M1-4). The first tests performed with mycelia sampled from the confrontation experiments between Chinese and Swiss isolates (Module 1) suggested the potential existence of nuclei pairs (Figure 11). However, as hybridization events are not expected to occur between morels of the Elata and Esculenta clades, the pairs of nuclei might represent another phenomenon, such as the existence of heterokaryons already from the germination of ascospores containing multiple genetically distinct haploid nuclei. In addition, histological sections of the confrontation lines result in dense mycelial bundles in which it was difficult to distinguish individual hyphae with a regular microscope (non-confocal). In order to overcome this limitation, we used a new experimental procedure that was developed in our laboratory (Buffi et al., *in preparation*) to better track individual hyphae. This method dubbed “drop-system” combines the use of Petri dishes specially designed for cell culturing with the growth of filamentous fungi in multiple discrete drops of liquid medium. Different mycelia are placed in drops at a fixed distance and their behavior can be monitored live using an inverted light microscope. This allows the direct observation of hyphae growing on the Petri dish surface in between the drops without the need to sample them (Figure 12). This method can be combined with staining procedures to monitor processes occurring at the hyphal scale after the encounter of sexually compatible mycelia, including the fusion of hyphae (plasmogamy) and the fate of nuclei during and after this process (preliminary work in Milestone M1-5). However, in our observations of morels, hyphal encounter or plasmogamy were never observed.

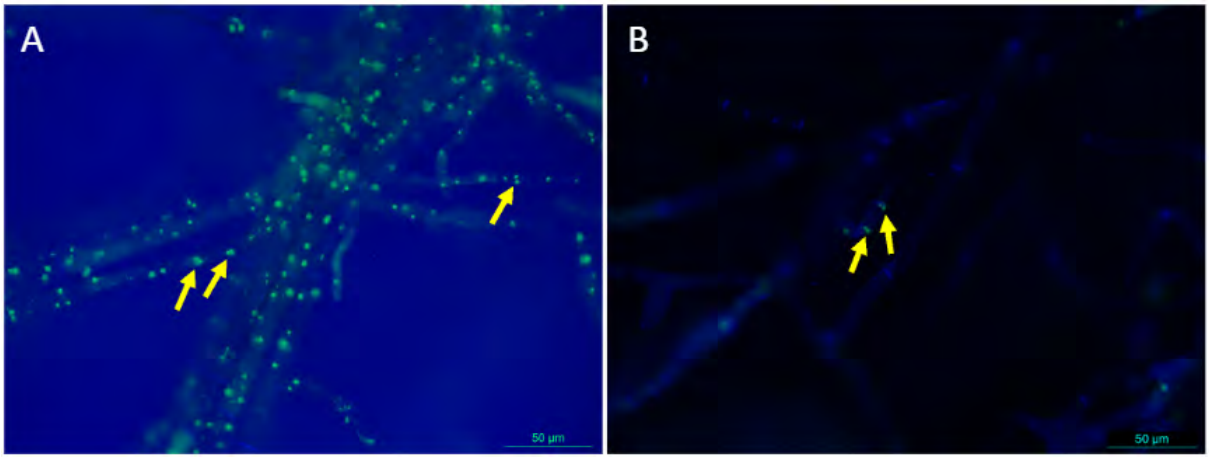

Figure 11: *Microscopic images showing the potential detection of pairs of nuclei after the confrontation of mating type compatible specimens. A= Mycelium of* Morchella *sp. M19-29 in confrontation with* M. importuna *NEU142 (confrontation not shown); B= Mycelium of* M. esculenta *M19-16 in confrontation with* M. sextelata *NEU143 (confrontation in Figure 3C; Malt agar). The cell wall was stained with calcofluor white and the nuclei were stained with SYBR Green. Yellow arrows indicate the pairs of nuclei in the images.*

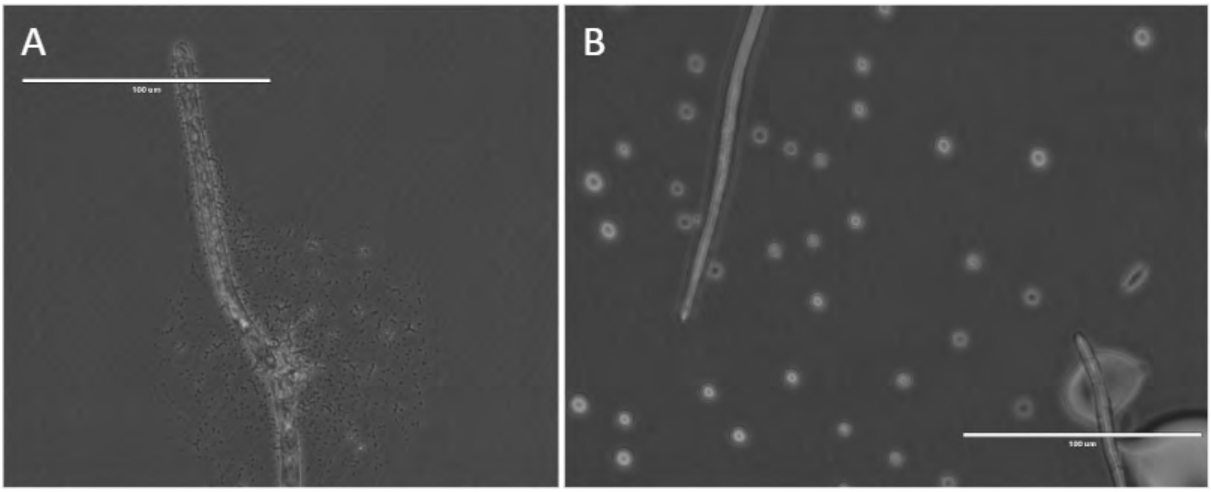

Figure 12: *Microscopy images of hyphae using the "drop-system" culture method developed in our laboratory (Buffi* et al*, in preparation). (A)* M. importuna *NEU142 at 11 dpi;(B)* M. sextelata *NEU143 (right) vs* M. esculenta *M19-39 (left) at 3 dpi.*

Genetic characterization of the confrontation lines

In Module 1, the *in vitro* confrontations between different morel isolates in malt agar medium did result in confrontation lines and tissue-like structures. The genetic characterization of these potential hybridization regions was done, in order to determine the mating type(s) present at this location. The aim was to determine whether the structures formed contained only one mating type, as in sclerotia (Du et al. 2017), or whether their genetic makeup was different. To compare, mating types were also analyzed in sclerotia that grew randomly in malt agar cultures. The mating types initially present in the mycelial isolates used to generate the confrontation lines were also determined. For the results concerning the mycelial isolates and the sclerotia, please refer to the paper appended as Annex 2 (Cravero et al. 2022, *submitted*). Briefly, sclerotia contained one mating type, except for one sample where both MAT were amplified. In the mycelia, both MAT were generally amplified. In the Chinese inocula, *M. sextelata* NEU143 and PYL contained both MAT, while *M. importuna* NEU142 and PYT only contained MAT1-2. Concerning the potential hybridization areas, both MAT1-1 and MAT1-2 were present in each confrontation line. For the pair *M. sextelata* NEU143 vs *M. esculenta* M19-23, no mating type could be amplified in any of the tissue-like structures (Figure 13). The mating types'

determination on the confrontation lines, the transplanted tissue-like structures, and the monocultured sclerotia clearly demonstrated that these three structures were genetically distinct.
Although we were able to amplify and sequence mating types in both black and yellow morels, the methodology is not completely reliable as no MAT could be amplified in 17.37% of the 167 samples that were investigated. In addition, one of the primer pairs to detect MAT1-1 (MAT11L/R) can potentially amplify off-target genetic regions. This was indeed observed after gel electrophoresis, where non-specific signals were present in the MAT11L/R amplicons (see Figure 13A, lane 2). For these reasons, we concluded that it is not possible to develop a smFISH method targeting mating types, until trustworthy probes for both MAT idiomorphs are developed. To do so, complete and annotated morel genomes must be openly available to investigate the entire mating type region. Currently, only seven genomes are available in the GenBank database (https://www.ncbi.nlm.nih.gov/genome/?term=morchella) and none of those contains a trustworthy MAT1-1-1 region (Cravero et al. 2022, *submitted*). We are working on the generation and annotation of full genomes from two single ascospore cultures in which putatively we have obtained both idiomorphs. However, this work cannot be completed within the framework of this mandate.

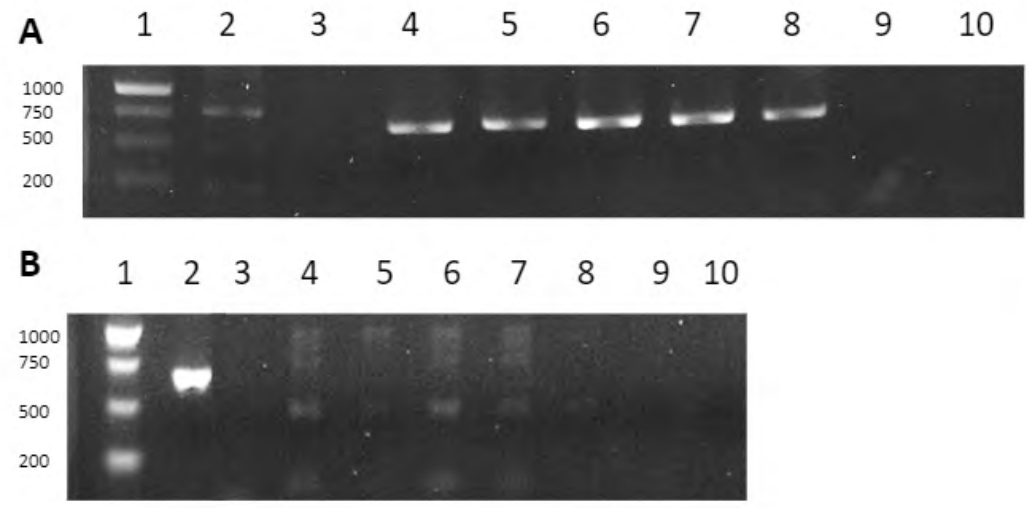

Figure 13: *Agarose gel electrophoresis showing examples of putative MAT1-1-1 (A; 600 bp) and MAT1-2-1 (B; 480 bp) PCR products amplified with the Elata MAT-specific primers (MAT11L/R (A) and MAT22L/R (B)). Lane 1= 1 Kb BrightMAX™ DNA ladder (1'000, 750, 500 and 250 bp); Lane 1=* Morchella *sp. mycelium isolate; Lane 2= white control; Lanes 3-8= confrontation lines; Lanes 9-10= tissue-like structures. These pictures demonstrate that both mating types were amplified in the confrontation lines but not in the tissue-like structures.*

2.4 Conclusion

We were able to produce a confrontation line that could potentially contain heterokaryons coming from the fusion of the two individuals (Volk and Leonard 1989; He et al. 2020), following the confrontation of two mycelial cultures of different morel specimens *in vitro*. We optimized a staining technique to be able to distinguish both cell wall and nuclei, in order to identify paired nuclei (**Milestones M1-4 and M1-5**). We found paired nuclei but could not confirm they came from the fusion of the two morel specimens that were confronted. To do so, it would be necessary to use an advanced technology (smFISH) that is for the moment unfeasible due to the lack of reliable probes targeting morel mating types. However, we were able to confirm that both mating types were present in the confrontation areas. On the contrary, the tissue-like structures that were produced when replating the confrontation lines produced in Module 1 did not contain any mating type. The mycelia generally contained both mating types, while the sclerotia produced in the cultures only contained one MAT in most cases. This showed that the confrontation lines, the sclerotia, and the tissue-like structures differ genetically (**Milestone 1-6**).

3. Module 3: Stimulation of sexual and asexual structures

3.1 Rationale

The results of the competition experiments performed so far highlighted the potential risk of introducing Chinese Morels into Swiss soils, where they could have a more competitive saprophytic growth than the natural populations, something that appears to be particularly significant in the case of *M. sextelata* NEU143 and PYL. However, one additional factor that can drive the displacement or replacement of the natural populations is both the hybridization between specimens from different origins and a better reproductive success of the invasive species. As indicated above, the experiments performed in soil-like substrates never resulted in the formation of a primordium or other structure involved in sexual reproduction (Module 1). However, areas with potential heterokaryons were formed when confronting some specimens *in vitro* (Module 2). This could be the result of hybridization between different morel species, this event being common in black morels (Du et al. 2019). To prove hybrids can be viable, the complete sexual life cycle must be accomplished. Unfortunately, despite multiple tests in a greenhouse, the Botanical Garden of Neuchâtel, and in controlled conditions in the laboratory, no fruiting bodies could be obtained either from the Chinese cultivars (only controlled *in vitro* experiments) or from our Swiss specimens (including the greenhouse experiments). Alternative methods were thus developed in order to advance in the potential production of ascocarps in the future. Those included: (a) the development of mycelial cultures originating from a single ascospore (haploid genetic material) and the detection of the mating type(s) in these cultures to identify sexually compatible mycelia; (b) the production of asexual spores (conidia and chlamydospores); and (c) testing the specimens' ability to produce sclerotia, a crucial component preceding fruiting body formation. The use of single-ascospore derived mycelia decreases variability factors linked to genetics, because they contain a haploid genome. Important phenotypic differences were noticed between single-ascospores cultures originating from the same ascocarp.

Conidia (asexual spores) are important structures for dispersal in filamentous fungi, because they produce clones of the original individual and their formation does not involve mating. Conidia are produced by specific aerial structures, the conidiophores. They are produced after vegetative growth and germinate when the environmental conditions are appropriate (Yuan et al. 2020). Conidia of *M. sextelata* appeared to contain only one mating type (Liu et al. 2022). Chlamydospores have the same purpose as conidia but are even more resistant and can undergo long-term stress survival (Yuan et al. 2020). Both conidia and chlamydospores were recently successfully induced in laboratory conditions on *M. sextelata* (Yuan et al. 2020). We tested the method proposed and developed a new one applicable to the material collected in Switzerland. Finally, we monitored the ability of some Swiss specimens to produce sclerotia in chemically defined media and in a soil-like environment. This feature, in addition to mycelial growth rate, was used to select the specimens that were most likely to produce fruiting bodies in culture. They were provided to a morel cultivator from canton Fribourg, who inoculated them in fields in Autumn 2021, to test if the overwinter could result in the production of sexual structures.

3.2 Methods

Single-ascospore cultures

To obtain ascospores, fresh morel fruiting bodies collected in the wild in Switzerland were placed in plastic jars in the dark and at 22°C, for one to three days. Sporulation occurred naturally in mature fructifications. With this method, ascospores were obtained for the following specimens: *Morchella* sp. M19-41, *M. esculenta* M19-42, *M. angusticeps/eximioides* M19-43, *Morchella* sp. M21-1, *Morchella* sp. M21-2, *M. esculenta* M21-43 and *Morchella* sp. M21-48. Some of these ascospores were immersed in DI water and photographed under a light microscope (Figure 14). The obtained ascospores were placed in hermetic empty Petri dishes and kept at room temperature in the dark until further use. Dried ascospores of *Morchella* sp. M21-48 were spread on PDA for one day to germinate (darkness, 23°C). Under a stereomicroscope, germinated single ascospores were picked with a Fine-ject©

needle (26Gx1, 0.45x25mm) and transferred on new PDA media to grow at room temperature with a natural light-darkness cycle. For each isolate obtained from a single ascospore, mycelium from a whole petri dish was scraped and DNA was extracted. The DNA extract was then used to determine which mating type was present in each single-ascospore isolate, using MAT-PCRs as indicated in Module 2. These cultures were then used as original material in order to compare the adaptive and sclerotia-producing abilities of MAT1-1 and MAT1-2 isolates using different media (glucose-NH_4, glucose-urea, glucose-glycine; Table 3) and potato dextrose agar (PDA; 39 g/L).

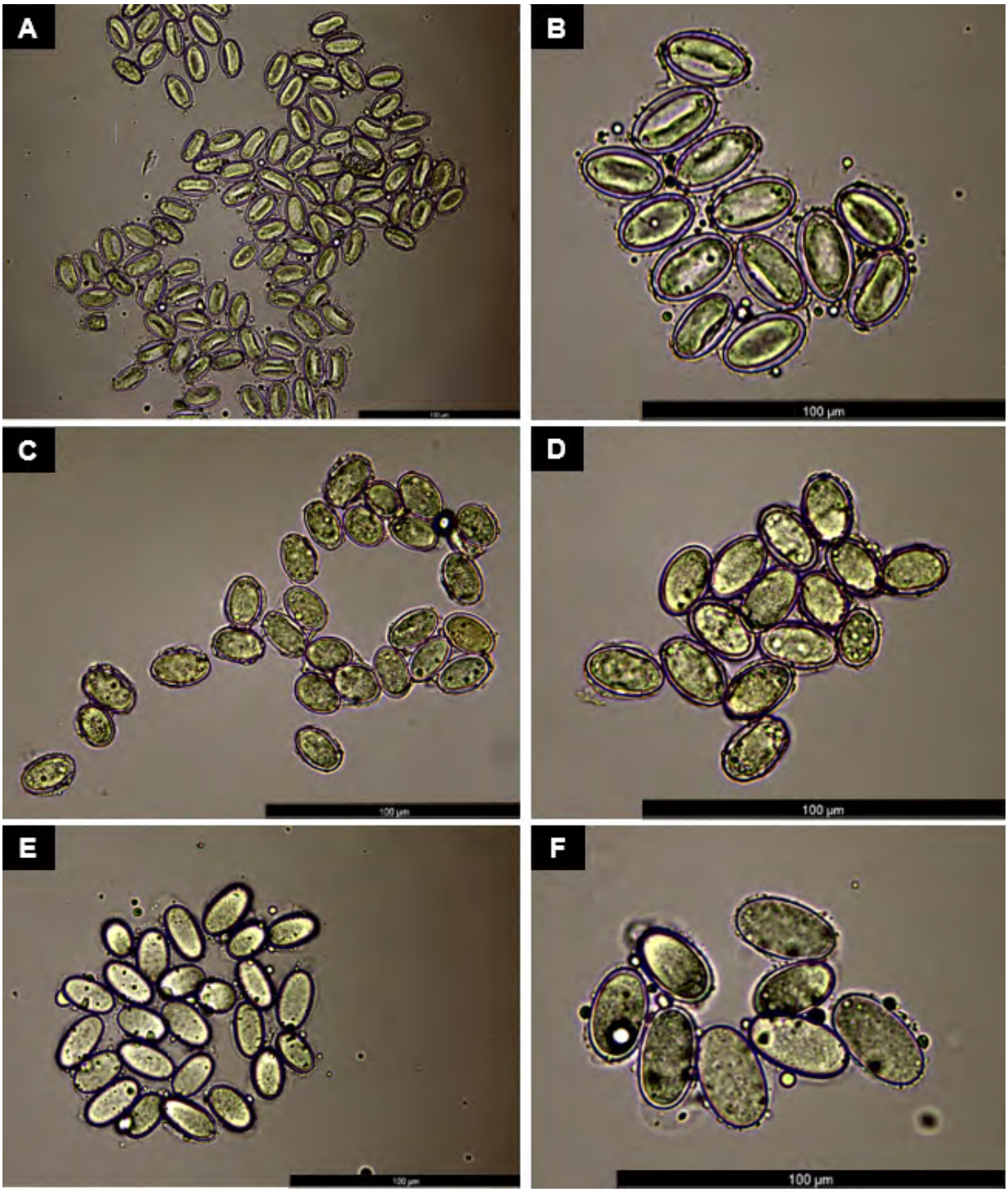

***Figure 14**: Ascospores of three* Morchella *species under light microscopy in DI water. A-B=* Morchella *sp. M19-41; C-D=* Morchella esculenta *M19-42; E-F=* Morchella angusticeps/eximioides *M19-43.*

Conidia production

Macroscopically, conidia have the aspect of a white or light brown powder on mycelium (Yuan et al. 2020). When such structures were observed on a culture, the latter was examined under a stereomicroscope and/or with a light microscope to verify the presence of conidia. To force the production of conidia, PDA plates containing the single-ascospore isolates 1 to 8 of specimen M21-48 were placed for one month in a climatic chamber. Microscopy glass coverslips were inserted vertically in the media to ease the microscopic observation of conidiophores growing on the slide as suggested previously (Yuan et al. 2020). The temperature was initially set at 19°C, was then decreased by 1°C every week and eventually maintained at 16°C for one month. Humidity was fixed at 65% with alternating cycles of 12 hours light and darkness. The same conditions were applied to the following isolates (non-single ascopores): *M. sextelata* PYL, *M. importuna* PYT, and *Morchella* sp. M19-41. Instead of PDA, isolates were inoculated on an organic substrate containing natural soil (autoclaved) and a morel substrate provided by a morel farmer. Fragment of mycelium growing on this organic substrate were regularly collected and checked under a stereomicroscope and/or a light microscope for the presence of conidia. In parallel, confrontations of single-ascospore isolates were conducted on a malt agar medium (incubation at 22°C, in darkness). The isolates used for co-cultures and the resulting phenotypes are described in Annex 3. The conidia that were produced were inoculated on PDA and incubated at room temperature in darkness. Mycelial phenotype was monitored for each culture.

Selection and cultivation of a Swiss morel cultivar

To select optimal isolates for cropping, ten Swiss isolates (M19-13, M19-23, M19-28, M19-39, M19-40, M21-20, M21-24, M21-38, M21-43, M21-82) were initially selected because they grew well in PDA. First, they were inoculated from PDA plates to glass tubes containing a soil-like substrate and incubated in the darkness at room temperature. Growth rate was monitored every day by measuring the distance traveled by the mycelium in the tube. The color of the mycelium and the formation of sclerotia were also tracked. Then, these ten specimens were provided to a swiss morel farmer, to be inoculated in glass bottles containing the soil-like substrate used to produce the first morel spawn utilized for morel cultivation.

3.3 Results and discussion

Assays with single-ascospore cultures

Single-ascospore cultures were generated in order to obtain mycelial isolates originating from haploid genetic material, thereby avoiding possible heterokaryons and reducing variability factors that may occur between individuals coming from the same biological specimen (i.e., one fruiting body). Six and seven isolates were obtained from specimens *Morchella* sp. M21-2 (Figure 15) and *M. angusticeps/eximioides* M19-43 (Figure 16), respectively. They were compared phenotypically and screened for their mating types. M21-2-1 isolate (isolate 1 of specimen M21-2; Figure 16A) differed from the others because it resulted in a mycelium with no sclerotia and no pigmentation. For this reason, we hypothesize it could be a melanin mutant (Rehnstrom and Free 1996). Isolates M21-2-2 and M21-2-3 only contained MAT1-1 (Figure 16B and C) while the others contained MAT1-2 only (Figure 16A, D, E, and F). No phenotypic differences were noticed between MAT1-1 and MAT1-2 isolates. In M19-43 isolates, three of them (M19-43-1, -5 and -7) showed a rapid and dense mycelial growth already at 6 dpi. At 14 dpi, all the cultures were fully grown and each of them showed diverse phenotypes: mycelia from isolates 1, 2, 3 and 5 were brown and dense in the center and white at the periphery (Figure 17A, B, C and E), while isolates 4 and 7 were homogeneously golden brown (Figure 17D and G). Mycelium from isolate M19-43-6 was completely white (Figure 17F). From those isolates, only M19-43-7 showed a positive result for the mating type which corresponded to MAT1-1 only. In the other isolates, none of the MAT-PCRs led to an amplification product.
Twenty isolates from specimen *Morchella* sp. M21-48 (black morel; Figure 17) were extensively investigated and used in different assays: (1) mating type analysis, (2) mycelial growth ability in different chemically defined media, (3) confrontations, and (4) ability to produce conidia.

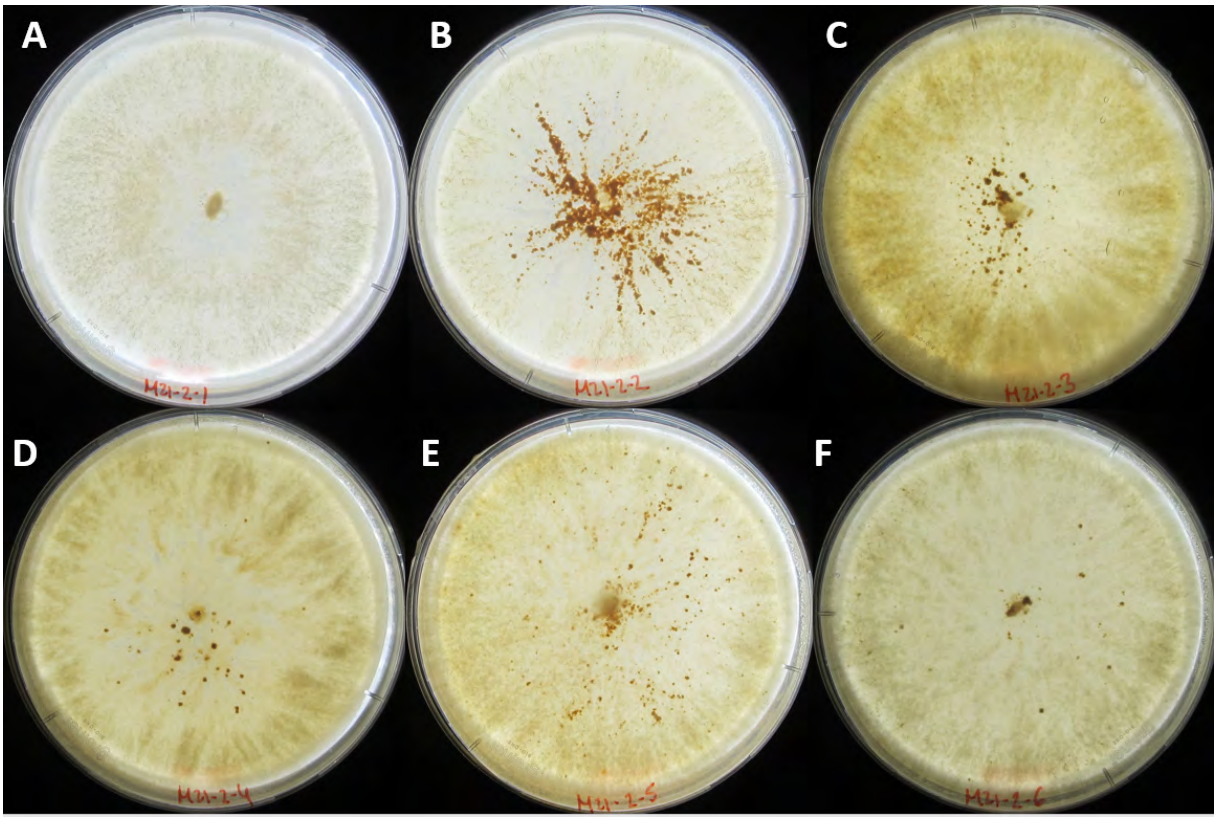

Figure 15: *Mycelial cultures of single-ascospore isolates 1 to 6 (A-F) of* Morchella *sp. M21-2 on potato dextrose agar. Pictures were taken 14 days post-inoculation.*

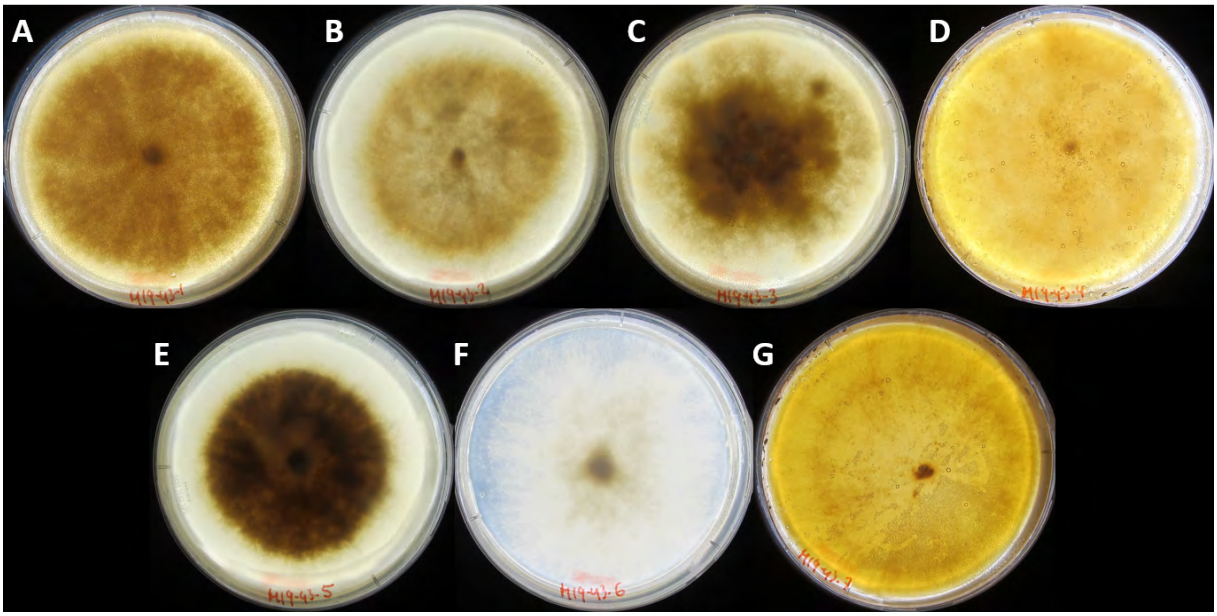

Figure 16: *Mycelial cultures of single-ascospore isolates 1 to 7 (A-G) of* M. angusticeps/eximioides *M19-43 on potato dextrose agar. Pictures were taken 14 days post-inoculation.*

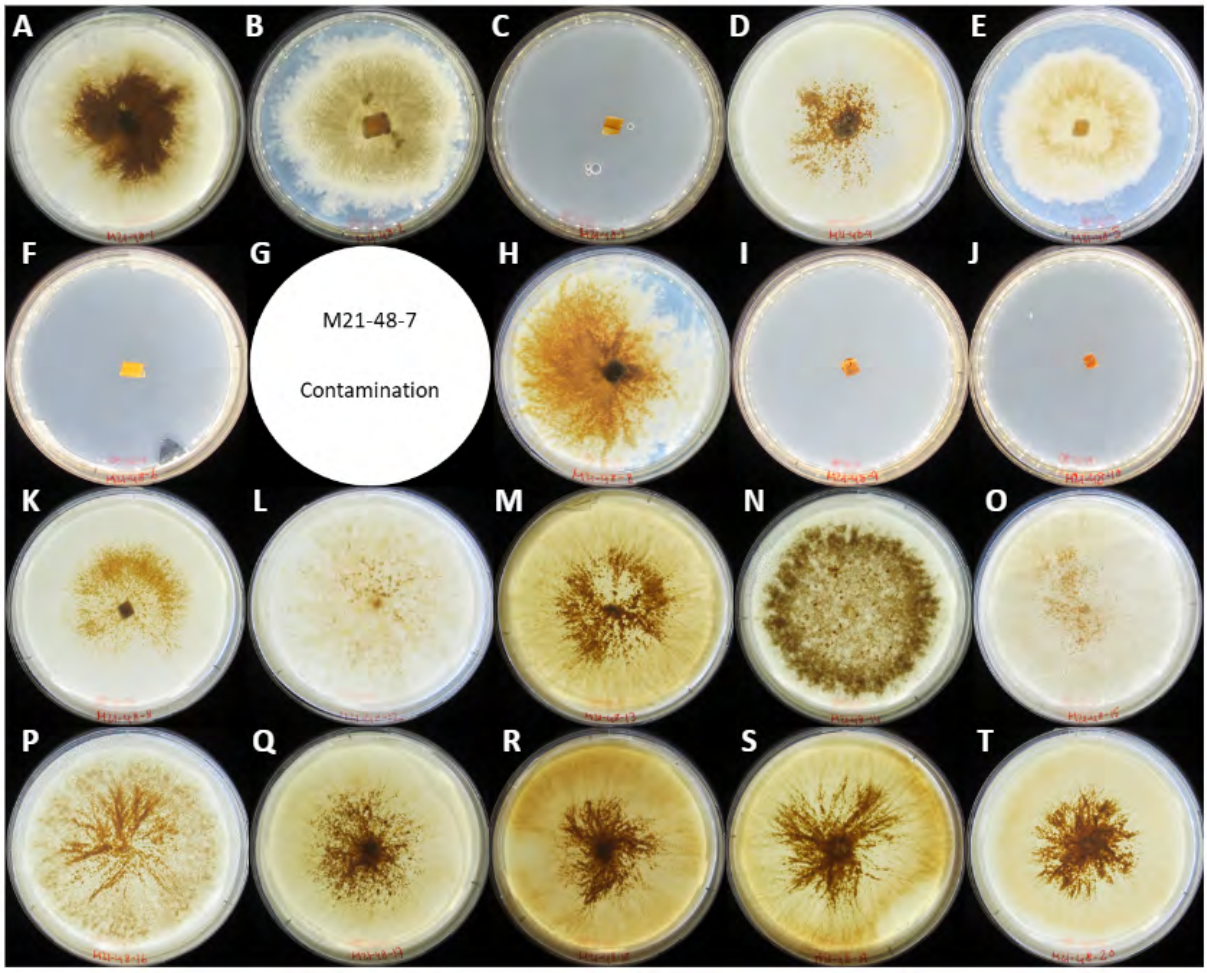

Figure 17: *Mycelial cultures of single-ascospore isolates of* Morchella *sp. M21-48-1 to M21-48-20 (A-T) on PDA. Pictures were taken 14 days post-inoculation.*

Mating type analysis

A clear unbalance on the mating type was observed among the 20 isolates, with 13/20 isolates carrying MAT1-2 only. Then, only two isolates (M21-48-1 and -14) carried MAT1-1 only and three isolates (M21-48-17, -18, -20) had both MAT. This was in strong contrast to the results published in Chinese Black and Yellow morels, in which a 1:1 ratio for MAT1-1:MAT1-2 between single ascospore cultures was used to validate a heterothallic reproduction mode (Du et al., 2017, Du et al., 2020). However, this was before the most recent detection of ascospores containing both MAT in morels (Du and Yang 2021), which is compatible with some of our findings. Mycelia from isolates containing MAT1-1 only (M21-48-1, Figure 17A and -14, Figure 17N) differed from the others phenotypically; melanin and sclerotia were spread more homogeneously and in a larger area. Two MAT1-2 isolates contained no sclerotia and grew denser than the other isolates, potentially indicating a lower stress response (M21-48-2, Figure 17B and -5, Figure 17E). Isolates M21-48-1 (MAT1-1) and -2 (MAT1-2) were replated in fresh PDA and mating types were determined again on the freshly obtained mycelia. Interestingly, M21-48-2 still harboured MAT1-2 only, but MAT1-2 could also be detected in fresh mycelium of M21-48-1, which originally contained only MAT1-1. We hypothesized that mating types could be cryptically present in low frequency nuclei containing the complementary mating type. Those nuclei could potentially expand when needed to generate a self-fertile secondary homothallic individual. As a result, the results obtained here do not confirm the system used by morels for sexual reproduction and open several questions regarding the mechanisms that lead to a successful sexual reproduction. Note that in the following experiments, only the mother culture containing MAT1-1 was used.

Mycelial growth

To compare the phenotypes and the adaptive abilities of MAT1-1 and MAT1-2 isolates of M21-48, fresh mycelia from eight isolates (M21-48-1 to -8) were transplanted into four different media containing different carbon and nitrogen sources: glucose-NH4, glucose-urea, glucose-glycine and potato dextrose agar (Module 1; Table 1). After three days, MAT1-2 isolates seem to grow denser as compared to MAT1-1 isolates, and this was valid for each media tested (Figure 18). After seven days, the mycelia reached the edges of the petri dish in all media, except for PDA. In the latter, the mycelium appeared denser than in the other media, where mycelia grew rapidly with few ramifications and density. The ramification degree is a measure of trophic stress (Agbagwa et al. 2020), the denser the mycelium is, the richer the medium. As a result, this indicated that PDA was the medium with the best nutritional conditions for the growth of both isolates. On the contrary, poor mycelial density was observed in sucrose-glycine media. Melanization is also an indicator of stress in filamentous fungi, mostly responding to abiotic and biotic stresses (Cordero and Casadevall 2017). In our experiments, no disparities were visually noticed between MAT1-1 and MAT1-2 isolates in the different media. Sclerotia were observed in each culture containing glucose-NH4 and glucose-urea (Figure 19). Overall, MAT1-2 isolates seemed better adapted than MAT1-1 isolates after three days of growth, because their growth was higher in all media. However, after seven days, the appearance of both isolates was similar on each media. The melanization and ramification of the mycelia was similar for the different cultures, but this could be verified with more replicates. After 16 days of growth, notable differences existed between the mycelia depending on the media but not on the isolate. In glucose-NH4 and glucose-glycine (Figure 18A, C, E and G), the culture media was not colored uniformly by brown pigments, contrarily to glucose-urea and PDA (Figure 18B, D, F and H). Sclerotia were present in each media, except in PDA (Figure 18D and H). Interestingly, one replicate of the sucrose-glycine medium of isolate MAT1-1 turned dark brown (Figure 19C) after 21 days. The reasons remained unknown, as the culture conditions were identical for each plate, but we hypothesized that this was probably a sign of stress.

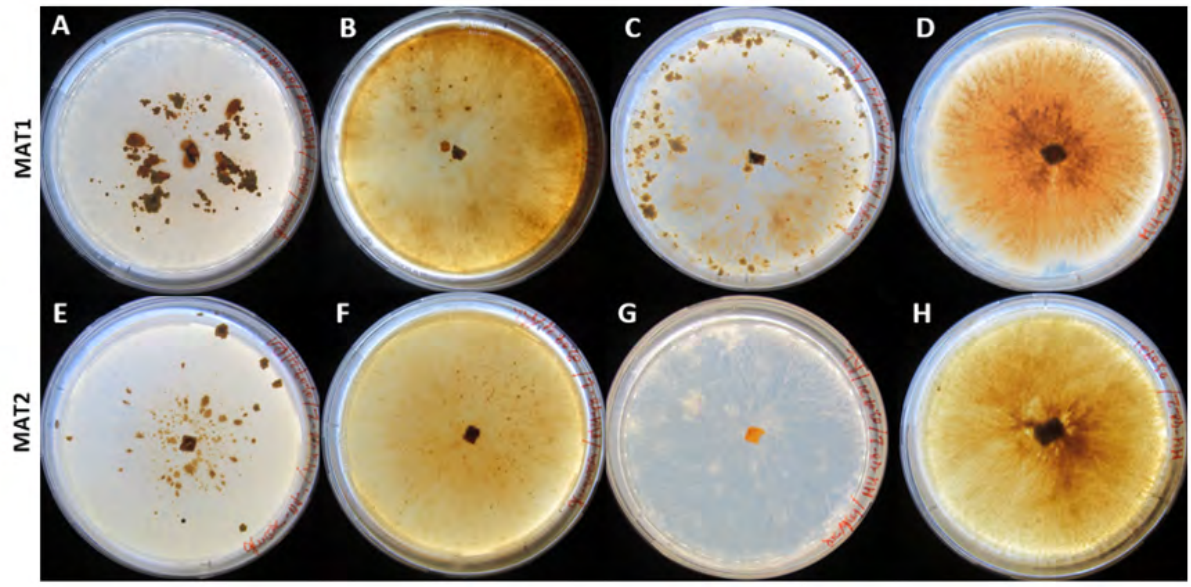

Figure 18: *Mycelial cultures of single-ascospore isolates 1 (MAT1-1, A-D) and 2 (MAT1-2, E-H) of* Morchella *sp. M21-48 on different media: A and E= glucose-NH4; B and F= glucose-urea; C and G= glucose-glycine; D and H= potato dextrose agar. Pictures were taken 16 days post-inoculation.*

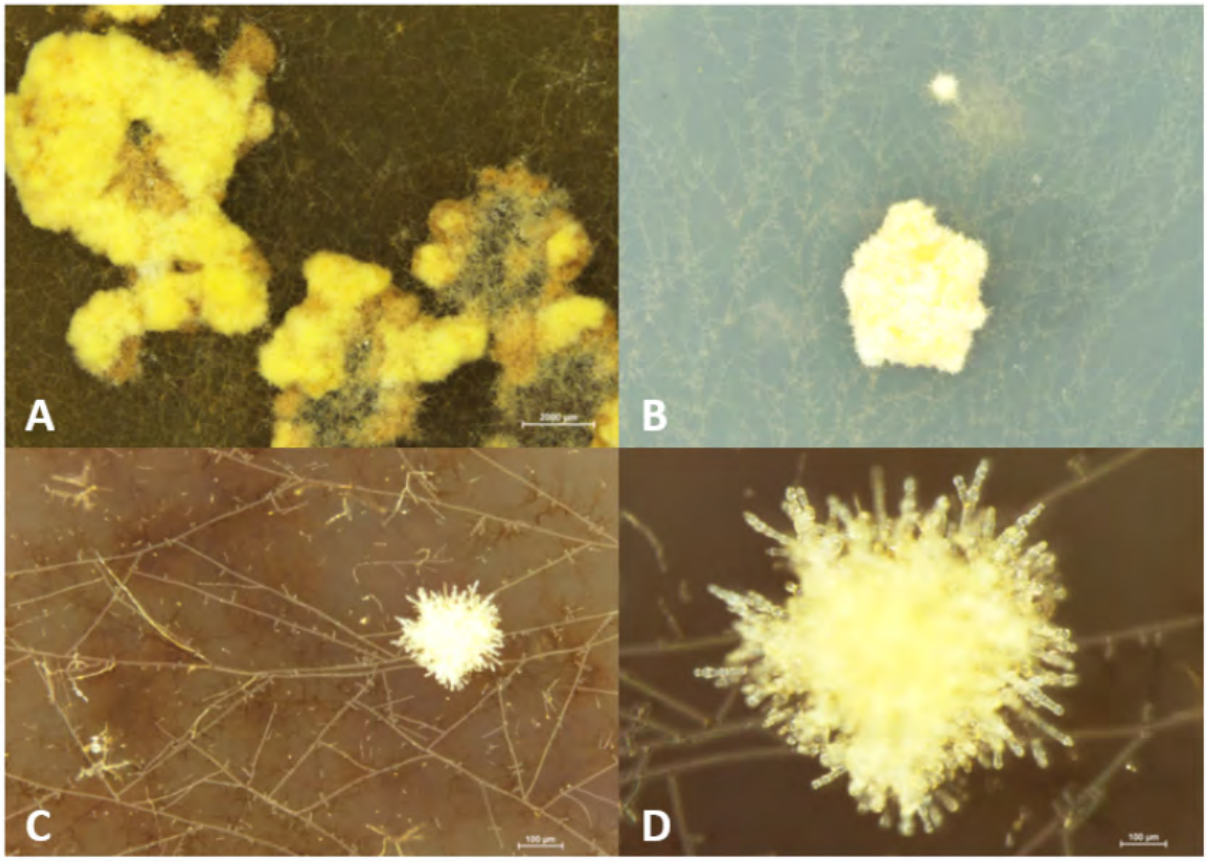

Figure 19: *Sclerotia in a mycelial culture of the single-ascospore isolate 1 of* Morchella *sp. M21-48 (M21-48-1) under a stereomicroscope. A= sclerotia on glucose-NH_4; B= sclerotia on sucrose-glycine medium; C= sclerotia on sucrose-glycine medium; D= magnification of sclerotia C. Pictures were taken 21 days post-inoculation.*

Confrontations

For the confrontation assays, the twenty M21-48 isolates were confronted to single-ascospore isolates from other morel specimens (M19-43, M21-2, M21-43) and between them. First, isolates M21-48-1 (MAT1-1) and M21-48-2 (MAT1-2) were confronted (Figure 20): the mycelia of M21-48-1 (MAT1-1) and M21-48-2 physically encountered five days post inoculation. However, no confrontation line nor tissue-like structures did form. On the contrary, a neat exclusion zone appeared between each isolate, even in the controls where both isolates were competing against themselves. MAT1-1 isolate was more melanized in each culture and produced crusts of sclerotia. MAT1-2 isolate produced small sclerotia and had a greater growth rate than MAT1-1. Then, the confrontations were reiterated with different isolates. The following features were assessed for each co-culture, after 35 days: the mating type present in each isolate, the production of sclerotia, the presence of melanin and the type of interaction between both isolates (exclusion area, confrontation line, tissue-like structure). All these results are presented in Annex 3. A few examples are shown in Figure 21. Overall, isolates M21-48-1 and -2, when confronted to other single-ascospore isolates, grew slowly. The only difference with these M21-48 isolates and the others is the fact they have already been manipulated (and then exposed to light) while the others remained in the culturing chamber in darkness. Four types of interactions were observed in the confrontation area: exclusion (mycelia are less dense or did not grow in the confrontation area), merged (the two mycelia merge and do not form a visible line), delimitation (a neat delimitation was observed but it did not form any particular structure) and tissue-like structure (a neat delimitation with a particular type of dense mycelium formed in the confrontation area). The type of interaction in the confrontation area was neither influenced by the mating types of the isolates in co-culture, nor the biological specimen from which the isolates originated. Most of the isolates showed an exclusion line in the confrontation area.

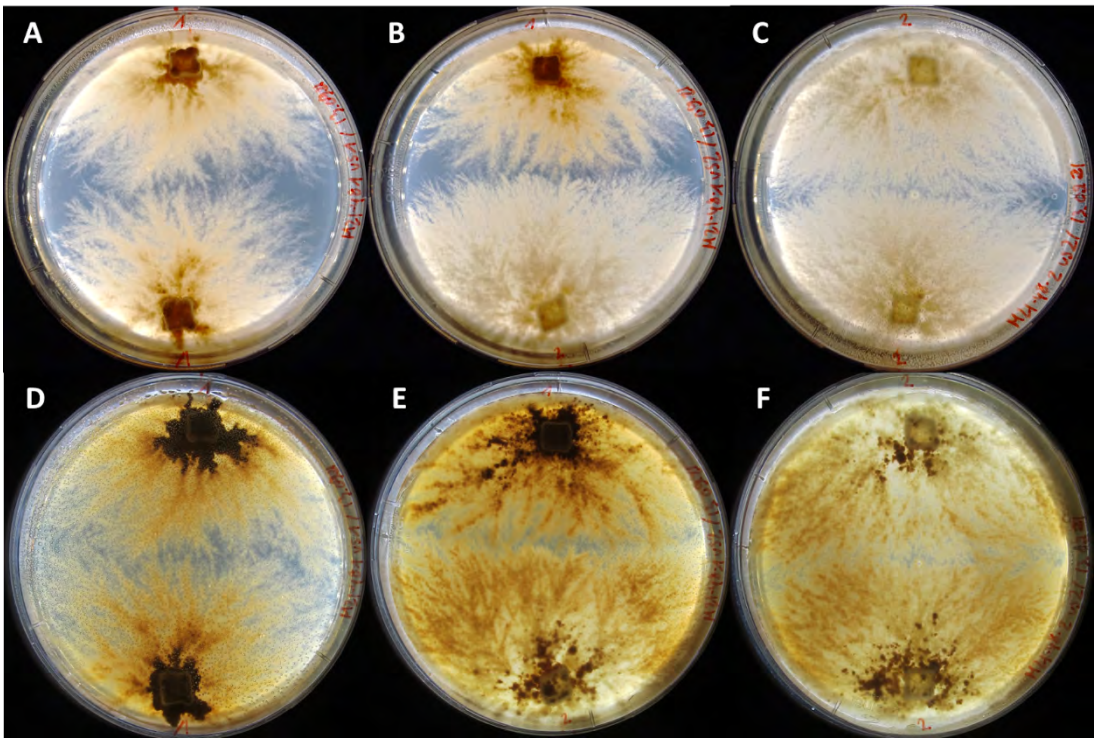

Figure 20: *Confrontation between single-ascospore isolates of* Morchella *sp. M21-48 on PDA media (M21-48-1 versus M21-48-2). A= control; isolate 1 (MAT1-1) vs isolate 1; B= isolate 1 (MAT1-1) vs isolate 2 (MAT1-2); C= control; isolate 2 (MAT1-2) vs isolate 2. Pictures A, B and C correspond to 8 days post inoculation; while D, E and F represent 27 dpi.*

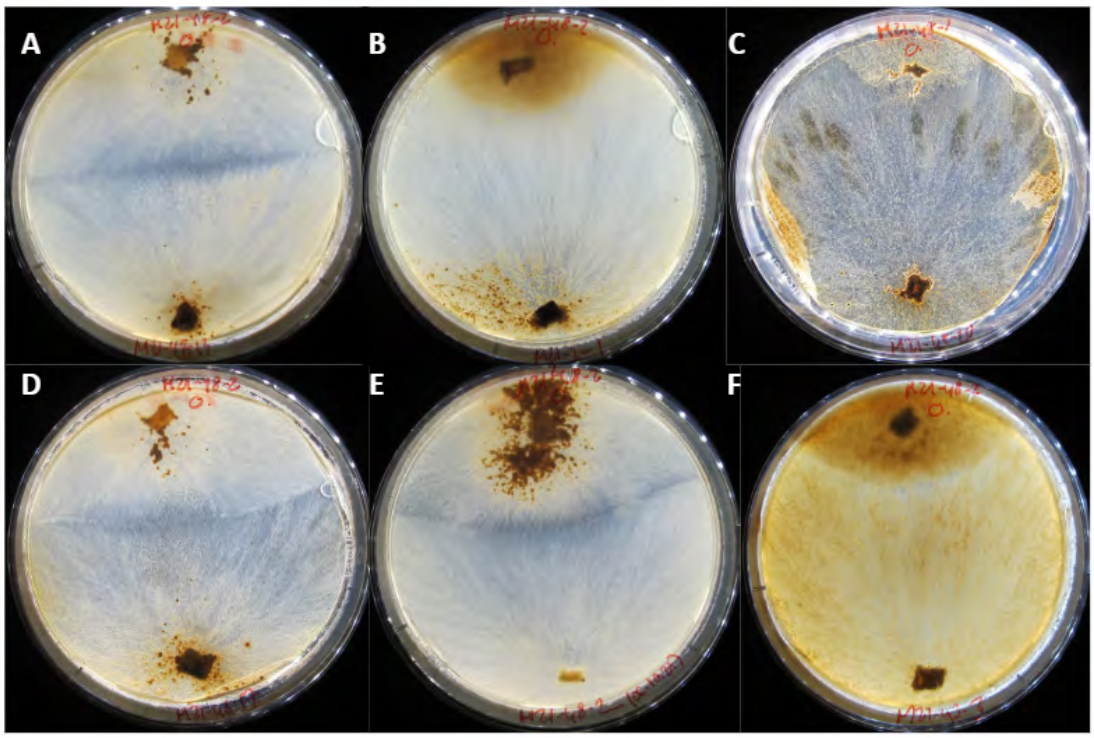

Figure 21: *Confrontation between single-ascospore isolates. Different types of interaction are shown: A= M21-48-2 (top) versus M21-48-13; Exclusion. B= M21-48-2 (top) versus M21-2-1; Merged. C= M21-48-1 (top) versus M21-48-20; Tissue-like structure. D= M21-48-2 (top) versus M21-48-17; Delimitation. E= M21-48-2 mother culture (top) versus M21-48-2 replated; Sclerotia. F= M21-48-2 (top) versus M21-43-3; Differential melanization. Pictures were taken 35 days post-inoculation.*

Conidia production

Microscopic observations of the confrontations unexpectedly revealed conidia were produced in isolate M21-48-2 (MAT1-2) when co-cultured with M21-48-4 (MAT1-2), -16 (MAT1-2), -12 (MAT1-2) and -18 (MAT1-1+MAT1-2; Figure 22A-C). Conidia were only produced in confrontations where the interaction between both isolates was an exclusion (Annex 3). Conidia were white and bear by conidiophores present at the tip or aerial hyphae, mostly near the confrontation area, near the inoculum and at the periphery of the Petri dish. The conidia that were collected and cultured generated mycelia that produced sclerotia more rapidly than in any other culture, after three days of growth only (Figure 22D). Co-cultures of isolates that produced conidia were replicated and always produced conidia when the Petri dish was not closed with a parafilm and incubated in the dark at 22°C. Hence, we hypothesized that decreasing water availability (i.e. drying medium) would trigger conidia production. In the other confrontation assays, Petri dishes were closed hermetically with parafilm that retained humidity, and conidia were not produced. In contrast, the coverslips method (Yuan et al. 2020) combined with growth in a climatic chamber with regular temperature decrease (19-16°C) applied to isolates M21-48-1 to M21-48-8 did not result in the formation of conidia but allowed instead a very clear observation of the mycelial architecture at the cell compartment level (Figure 23). Different shapes, sizes and color of cells were observed. The mycelial cells varied between 50-330 µm long and 5-20 µm large. Melanized, strongly melanized and not melanized hyphae were observed. As expected, cell walls were thicker in melanized cells. Some of the melanized cell compartments appeared to have a granular structure. Interestingly, completely transparent cells were adjoined highly melanized cells on the same hyphal branch. Growing mycelium was also observed. It was characterized by absence of septa, thin cell wall and no coloration (transparent). Cellular compartments of growing mycelium were regular and seemed soft, contrarily to the melanized cells that were straight and rigid. Some hyphae seemed old, as the cells were irregular, dark and looked dry and dead. Fusions between different hyphal branches were also observed.

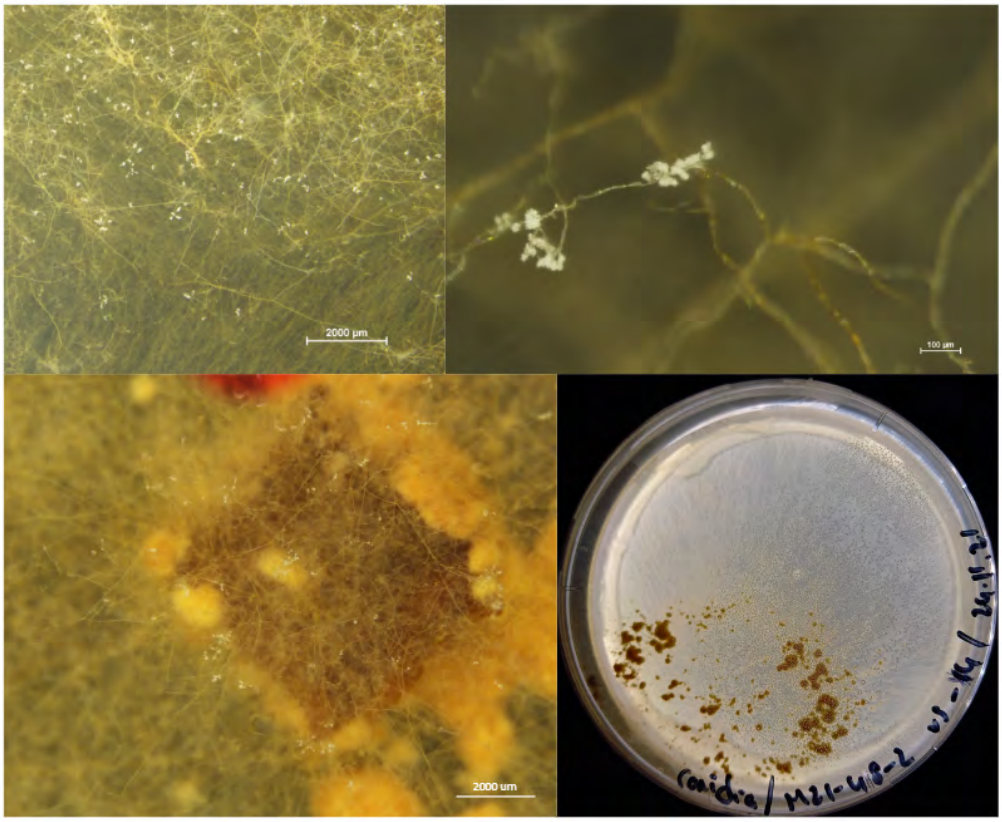

Figure 22: *A= Conidia (white aggregates) in M21-48-2 (confronted with M21-48-18) in malt agar; Stereo microscope 0.75x, 27 dpi. B= Magnification (13.5x) of conidia in picture A. C= Conidia (white aggregates) in M21-48-2 (confronted with M21-48-16); Stereo microscope 0.75x. D= Culture of the conidia produced in M21-48-2 (confronted with M21-48-4) in malt agar; 3dpi.*

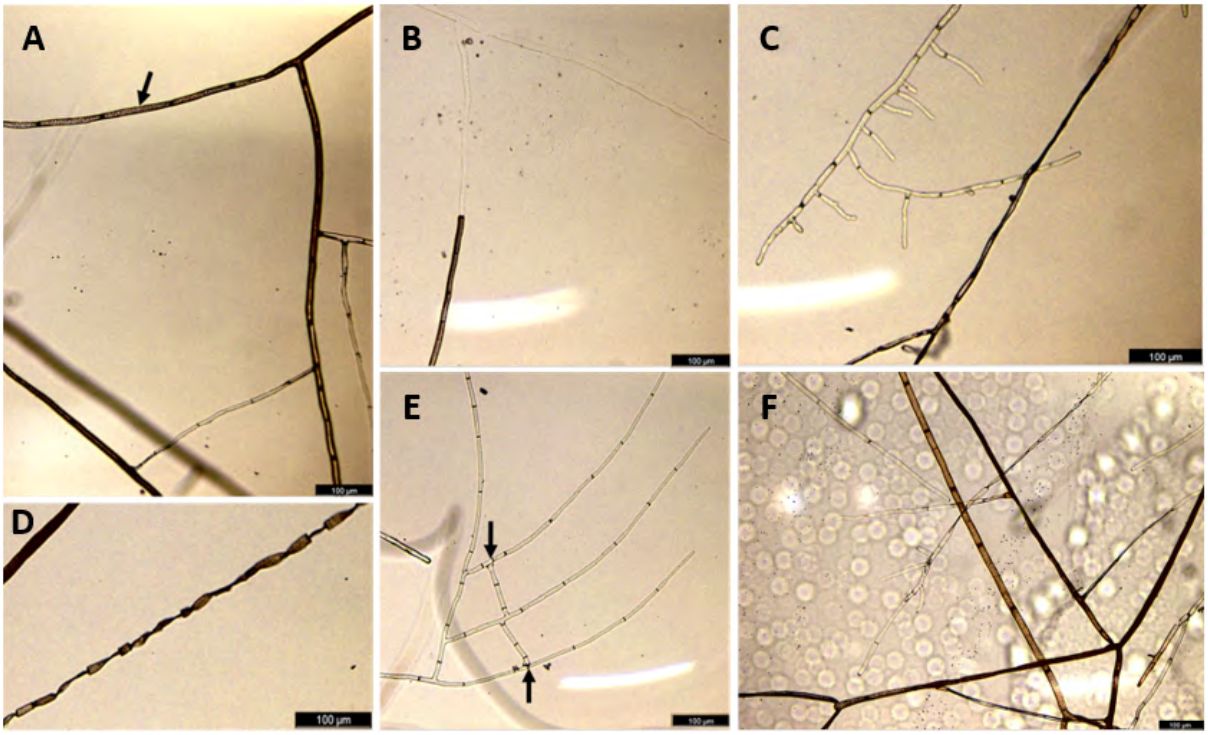

Figure 23: *Single-ascospore isolates of M21-48 growing on a microscope coverslip inserted vertically into PDA media. A= granular appearance in isolate 3; B= melanized and transparent cell compartments adjoined in isolate 4; C= growing mycelium (left) and older mycelium (right) in isolate 7; D= irregular, dry, and melanized hypha in isolate 1; E= hyphal anastomoses in isolate 8; F= non-melanized (transparent, left), melanized (brown, middle), and highly melanized (dark brown, right) hyphae in isolate 2. Mycelial samples were immersed in DI water and pictures were taken with an inverted light microscope.*

Production of conidia in a soil-like substrate

Conidia were obtained in a soil-like substrate incubated in a climatic chamber at 19°C and 65% RH, after 20 days. Macroscopic structures with an aspect corresponding to a white powder were visible with the naked eye. Observations with a stereomicroscope and a light microscope confirmed the presence of conidiophores and conidia (Figure 24). The conidia measured about 7 µm in diameter. As a result, conidition appear as smaller as compared to the sexual ascospores that have an average dimension of 25 x 15 µm (Figure 14). In addition, conidia were produced in glass tubes with a soil-like substrate where *M. esculenta* M21-38 was co-cultured with another isolate (*M. deliciosa* M21-16 and *M. sextelata* M21-83) or with itself (control M21-38 *vs* M21-38 ; Figure 25). These conidia were noticed 35 days post-inoculation (for the detailed methods, please refer to Module 1 “Confrontation in soil-like substrate”).

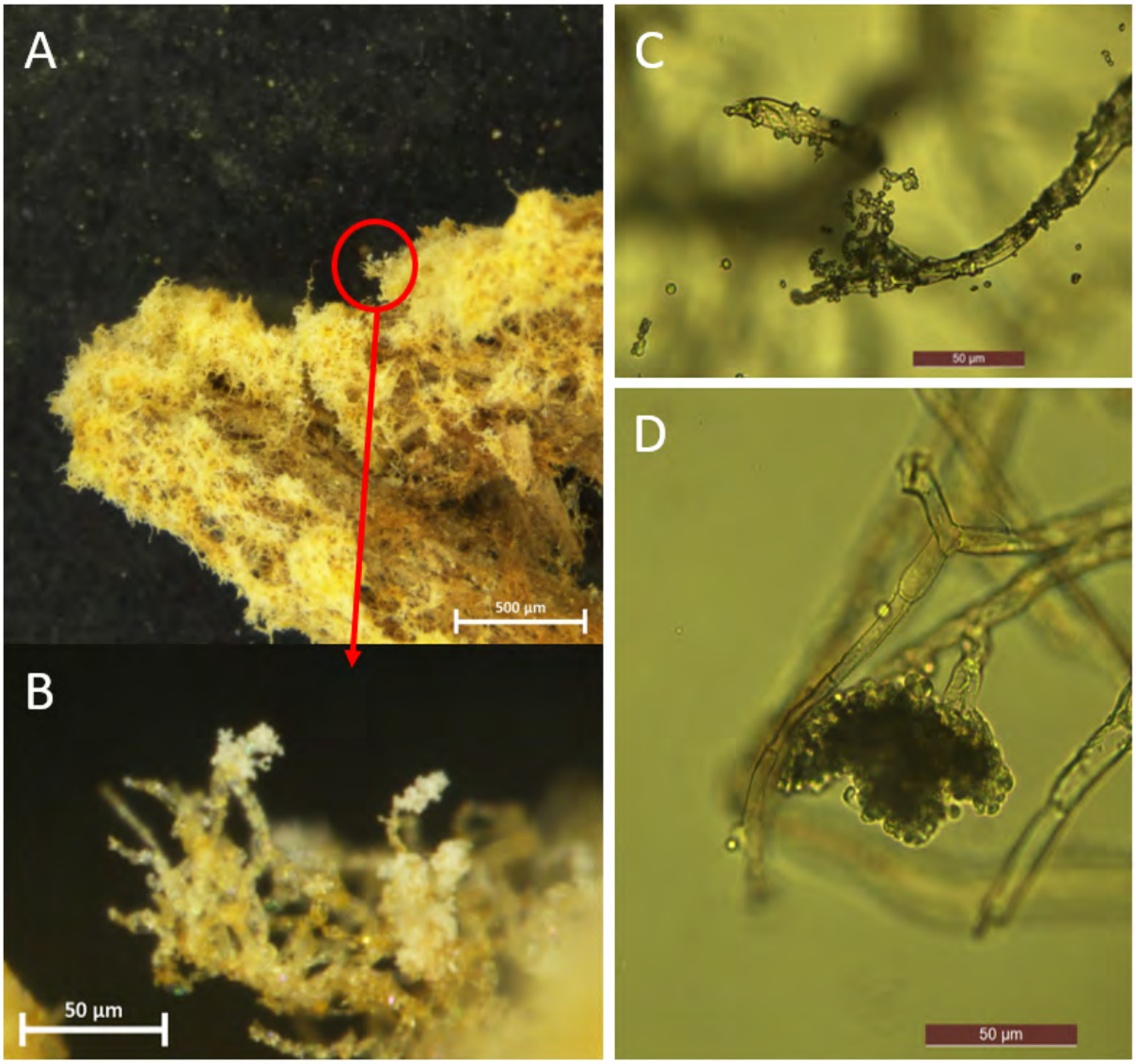

Figure 24: *Observations of conidiophores and conidia formed at the surface of the soil-like substrate in the climatic chamber. Pictures were taken 20 days post-inoculation with a stereomicroscope (A and B) and under a light microscope at (C and D).*

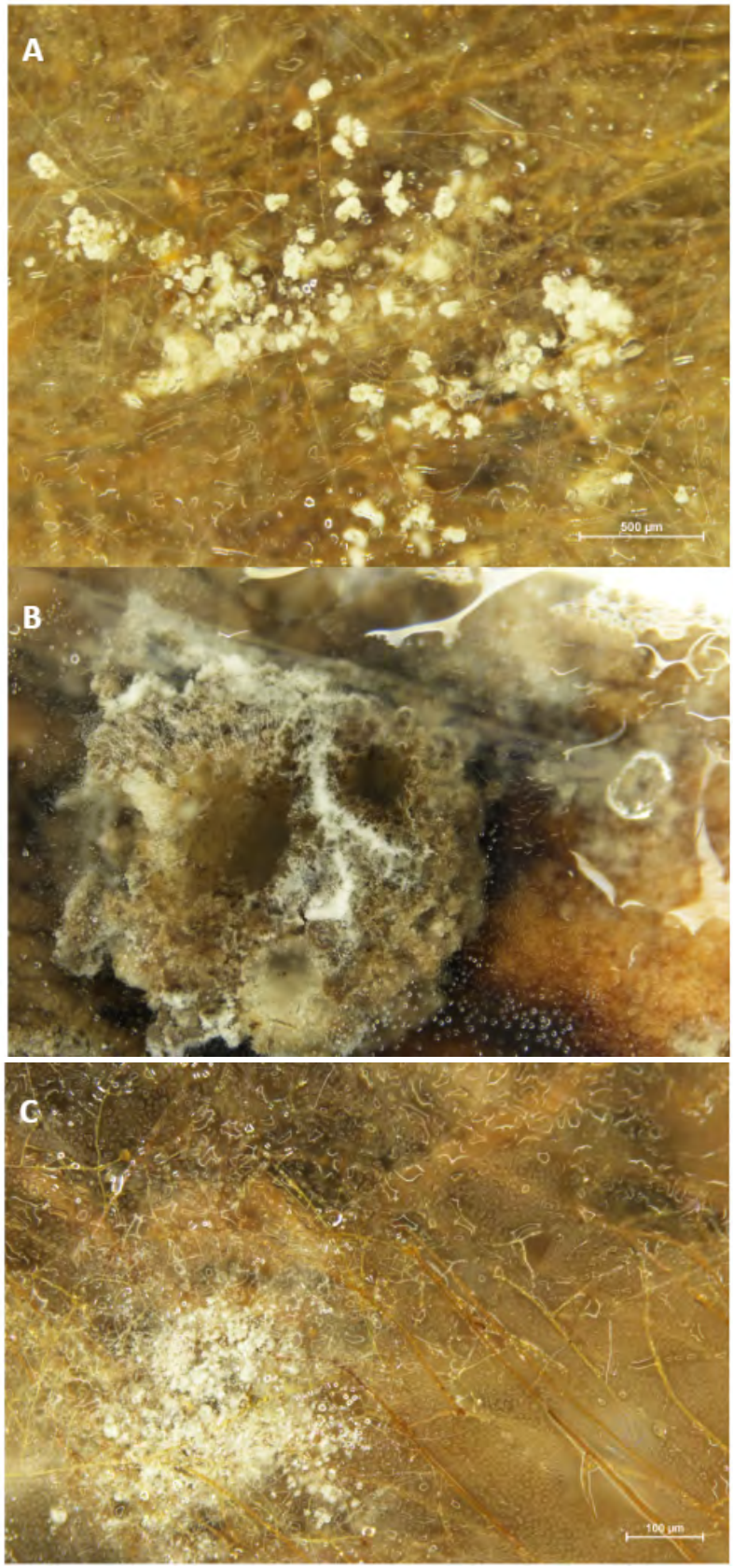

Figure 25: *Images taken under a stereomicroscope and showing conidia formed at the surface of the soil-like substrate in co-cultures in glass tubes. A= M21-38 (confronted with M21-16). B= M21-38 (confronted with M21-38). C= M21-38 (confronted with M21-89). Pictures were taken 35 days post-inoculation.*

Selection and cultivation of a Swiss morel cultivar

Ten mycelial isolates derived from morel specimens collected in Switzerland in 2019 and 2021 were selected to be grown in a soil-like substrate. Sclerotia production and mycelial growth were assessed, since both of them are two important features to eventually cultivate morels (Personal communication from a morel farmer. These isolates are named: M19-13, M19-23, M19-28, M19-39, M19-40, M21-20, M21-24, M21-38, M21-43, M21-82 (Figure 26A). The main criterion to select these isolates was because they could grow easily in a chemically defined media. Four isolates grew more rapidly than the others: M19-28, M19-39, M21-43, and M21-82. Four others produced important quantities of sclerotia: M19-23, M19-39, M21-20, and M21-24. Based on these features, the morel farmer with whom we collaborate used three of our specimens to produce a mycelial spawn (M19-28, M21-20, and M21-82; Figure 26B, C and D) that were then used as primary inoculants for actual crop cultures (Figure 26E). These "Swiss cultivars" were inoculated in the field in autumn 2021. *Morchella* sp. M19-28 was inoculated in Le Mont-sur-Lausanne, VD (20 m^2); *Morchella* sp. M21-82 in Font, FR (100 m^2); and *Morchella* sp. M21-20 in Siviriez, FR (55 m^2 and 40 m^2), Bonnefontaine, FR (2 m^2), and Courtelary, BE (2 m^2). The Chinese *M. sextelata* PYL was inoculated over 500m^2. At the end of May 2022, none of the cultures (including the Chinese cultivar) produced any ascocarps. Late sowing, early snow and/or soil contaminants could be in cause.

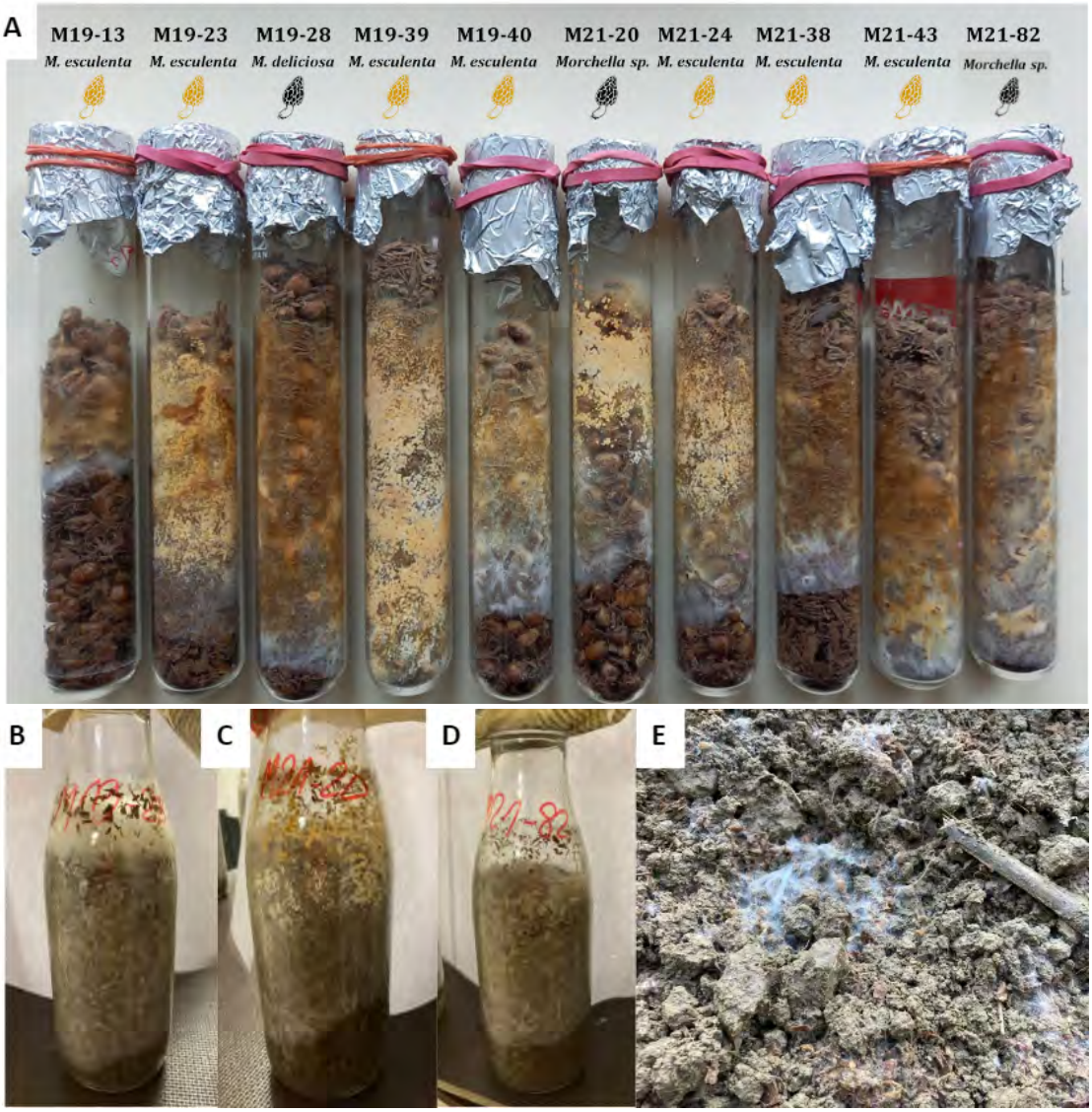

Figure 26: *Cultures of ten morel specimens in a soil-like substrate used for spawn production for actual morel cropping (A). Specimens M19-28, M21-20 and M21-82 were used for spawn production (B-D) prior to be inoculated in crops. An example of morel mycelium in a Swiss crop (E).*

3.4 Conclusion

The aim of this module was to produce sexual and asexual structures from morels. Asexual spores (conidia) were successfully produced in two soil-like systems (climatic chamber and glass tubes) and in co-cultures of single-ascospore isolates in malt agar (**Milestone M1-7**). The latter could be repeated, indicating the conditions to produce morel conidia (i.e., confrontation in malt agar without parafilm to allow the medium to dry, in the dark and at 22°C). Sexual spores (ascospores) were successfully collected from wild ascocarp and used to generate haploid isolates that were thoroughly investigate. Three isolates from specimens that were collected in Neuchâtel (M19-28 and M21-20) and Fribourg (M21-82) were used to produce spawn that was inoculated in six different crops by a morel farmer in autumn 2021 (**Milestone M1-8**). Ascocarps were unfortunately never generated and could thus not be genotyped (**Milestone M1-9**). Nonetheless, thanks to the help of this farmer, a precise procedure to grow morels indoors in a climatic chamber was developed and will be tested in our laboratory during 2022. Indeed, being able to produce morel ascocarps in controlled conditions without depending on the seasons will open priceless opportunities in the light of morel research.

4. Module 4: Evaluating the germination and dispersal potential

4.1 Rationale

Dispersal capabilities and germination are important, respectively, for the range expansion of fungi and effective colonization of new environments. This can involve both the production and release of sexual (ascospores) and asexual (conidia) structures. In ascocarp development, asci are formed more than twenty days after primordial formation. Each ascus contains eight ascospores which, in morels, are then released from the mature ascocarp in the disintegration stage (He et al. 2017). Morel ascospores were identified as effective dispersal agents over distances of 100-900m (Dalgleish and Jacobson 2005). Interestingly, morels are not believed to disperse by natural means such as air and water, which could explain their important endemism levels (O'Donnell et al. 2011). Conidial production in the wild however remains poorly studied, contrary to crop cultures where conidia are systematically occurring before ascocarp formation (Liu et al. 2018). In our experiments, a time-lapse video of a germinating ascospore could be recorded. Ascospores and conidia were stained with DAPI in order to study their DNA content, in particular nuclei number.

4.2 Methods

Ascospores collection

Ascospores were collected as explained in § 3.2 "Single-ascospore cultures" from Module 3. The time-lapse movies were recorded under an inverted light microscope using ascospore suspended in DI water supplemented with 12 g/L malt in a cell-culture treated Petri dish.

DAPI staining

Ascospores and conidiospores were immersed in 2 µg/mL DAPI (4'-6-diamidino-2-phenylindole) in DI water, directly on a microscope slide, and incubated in darkness for 10 minutes. The slides were then observed under a fluorescent light microscope.

4.3 Results and discussion

Germination and nuclear content of ascospores and conidia

Both conidia and ascospores started to germinate in less than 12 hours. Ascospore germination and hyphal germ tube were more thoroughly investigated with a 24h time lapse obtained from a germinating ascospore of a black morel specimen (M21-48 ; Figure 27). The video (available in Supplementary files) indicated that the ascospore first enlarges within the first hours before becoming more ovoid after seven hours. The germination occurred after 7h-7h15 at a first extremity, and after 7h15-7h30 at the second end of the ascospore. Hence, two germ tubes emerge from a single ascospore. The first secondary branching was observed on the initial germ tube (i.e. hypha),

after 10h45. This was followed by multiple branching at both ends. The first septa appeared after 17h45. The same experiment was done with DAPI staining, in order to be able to track nuclei formation and distribution in the forming hyphal network. Unfortunately, the result was not conclusive as DAPI is light-sensitive and faded away after four hours already. However, microscopic pictures of multiple ascospores were taken and allowed to clearly distinguish the presence of multiple nuclei in each ascospore (Figure 28). Based on these pictures, we could conclude that ascospores from *Morchella* spp. contained between 13 and 17 nuclei. Based on the classification from Du et al. (2020), our specimens all belong to group 2 ("ascospores with more than ten nuclei" but less than twenty) similarly to *Morchella* sp. Mes-10, *Morchella* sp. Mes-15, *Morchella* sp. Mes-21, *Morchella* sp. Mes-25, *Morchella* sp. Mes-26, *Morchella americana*, *Morchella palazonnii*, *Morchella yishuica*, and *Morchella clivicola* (Du et al. 2020). Interestingly, *M. esculenta* from Du et al. (2020) contained less than ten nuclei, contrarily to our M19-42 that contained 13 nuclei. This could indicate that the Swiss specimen belongs to another species or sub-species. Morels are believed to be homokaryotic (i.e. genetically identical nuclei). However, this was claimed based on DAPI staining only (Du et al. 2017), which in our view, does not provide a strong evidence about the genetic diversity of the multiple nuclei contained within a single ascospore. However, cytological studies from He et al. (2017) on meiosis during ascosporogenesis showed that the multiple nuclei present in the ascospores came from a single initial nucleus, advocating the homokaryon hypothesis. Conidia were also stained with DAPI but no nucleus could be observed. Instead, the whole compartment was stained light blue as if it homogeneously contained DNA.

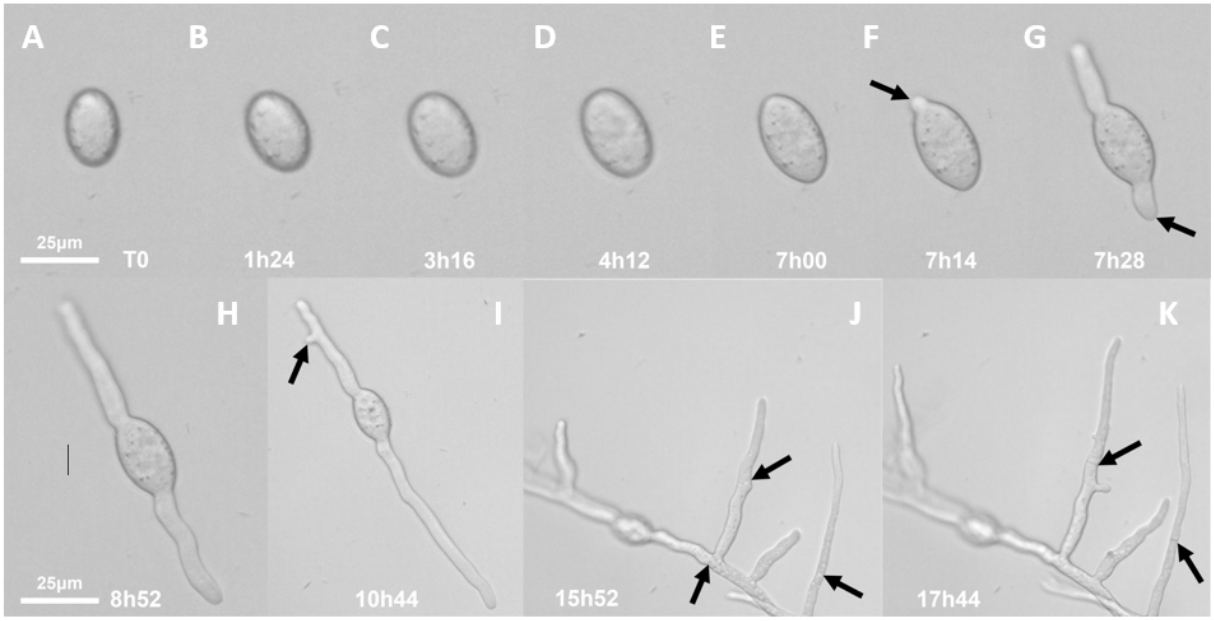

Figure 27: *time-lapse pictures of a germinating ascospore of* Morchella *sp. M21-48 in a DI water droplet supplemented with 12g/L malt. The time post inoculation is shown under each picture. A= time zero; B= the ascospore starts changing shape, indicating that germination will resume soon; C= the ascospore is still changing shape; D= intracellular movements; E= the ascospore becomes ovoid; F= formation of a germ tube at a first extremity; G= formation of another germ tube at the second extremity; H= elongation of both germ tubes, eventually resembling hyphae; I: first branching; J= apparition of numerous vesicles inside the hyphae; K= apparition of septa.*

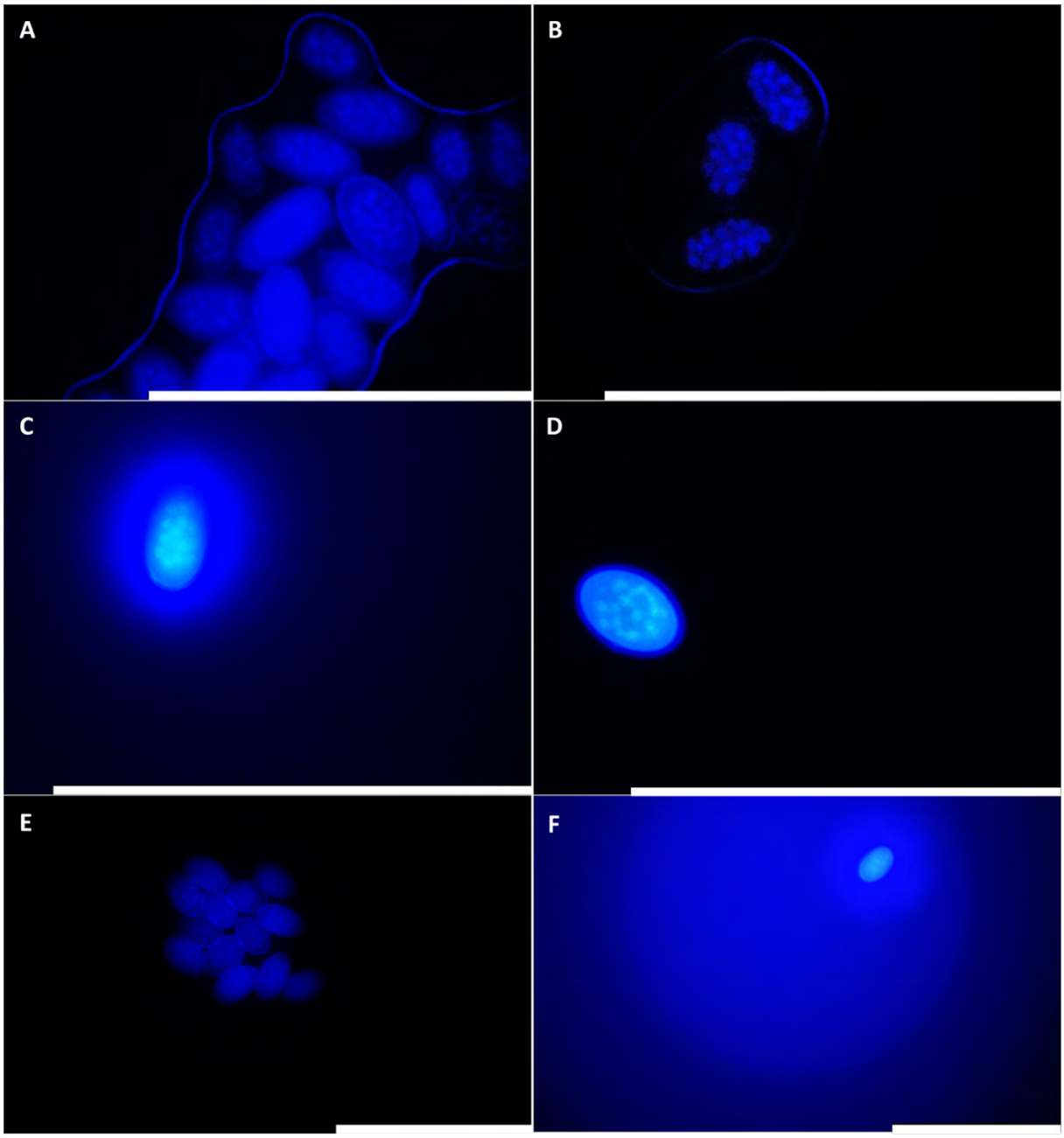

Figure 28: Ascospores of Morchella *spp. stained with DAPI to highlight their multinuclearity. A and B=* M. angusticeps/eximoides *M19-43; C=* Morchella *sp. M21-2; D and E=* M. esculenta *M21-42; F=* Morchella *sp. M21-48. Pictures were taken with a light microscope. Scale bars represent 100 µm.*

4.4 Conclusion

Both ascospores and conidia germinate overnight (**Milestone M1-10**). The time-lapse video showed that the ascospores initially germinate at one extremity and then at the other. It would be interesting to be able to follow nuclei to investigate their dispersal and multiplication from the ascospore through the forming hyphal network. DAPI staining on both sexual and asexual spores showed that ascospores from three different morel species contained from 13 to 17 nuclei, that are probably homokaryotic based on studies from He et al. (2017). On the contrary, we were not able to visualize nuclei in conidia.

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Single-ascospore isolates in confrontation in malt agar (35 dpi).

Features of the confrontations are provided: the mating type of each isolate (MAT1-1, MAT1-2 or both); the presence of sclerotia (Yes or No, and where they were located); the presence or brown pigments (Yes or no, and where they were located); the aspect of the confrontation zone; the presence of conidia. In bold the isolate with the best mycelial growth. N.A.: not available.

Strains (M21-48-1 top and X)	M21-48-1 vs **M19-43-1**	M21-48-1 vs **M19-43-2**	M21-48-1 vs **M19-43-3**
Mating type	MAT1-1 / N.A.	MAT1-1 / N.A.	MAT1-1 / N.A.
Sclerotia	Yes / No	No / Yes	Yes / No
Location of the sclerotia	Spreading from inoculum / N.A.	N.A. / One spot, periphery	Spread / N.A.
Melanization	Yes / Yes	Yes / Yes	Yes / Yes
Location of the melanization	Spread / Periphery	Spread / Periphery	Spread / Periphery
Conidia	No / No	No / No	No / No
Confrontation zone	Tissue-like structure	Exclusion	Exclusion
Strains (M21-48-2 top and X)	M21-48-2 vs **M19-43-1**	M21-48-2 vs **M19-43-2**	M21-48-2 vs **M19-43-3**
Mating type	MAT1-2 / N.A.	MAT1-2 / N.A.	MAT1-2 / N.A.
Sclerotia	No / No	No / No	No / No
Location of the sclerotia	N.A. / N.A.	N.A. / N.A.	N.A. / N.A.
Melanization	Yes / Yes	Yes / Yes	Yes / Yes
Location of the melanization	Spread / Periphery	Spread / Periphery	Spread / Spread
Conidia	No / No	No / No	No / No
Confrontation zone	Merged	Exclusion	Exclusion
Strains (M21-48-1 top and X)	M21-48-1 vs M19-43-4	M21-48-1 vs **M19-43-6**	M21-48-1 vs **M21-2-1**
Mating type	MAT1-1 / N.A.	MAT1-1 / N.A.	MAT1-1 / MAT1-2
Sclerotia	N.A. / N.A.	Yes / No	No / Yes
Location of the sclerotia	N.A. / N.A.	Inoculum / N.A.	N.A. / Spreading from inoculum
Melanization	N.A. / N.A.	Yes / Yes	Yes / Yes
Location of the melanization	N.A. / N.A.	Spread / Spread	Spread / Sclerotia
Conidia	No / Yes	No / No	Yes / No
Confrontation zone	Exclusion	Exclusion	Exclusion
Strains (M21-48-2 top and X)	M21-48-2 vs **M19-43-6**	M21-48-2 vs **M21-2-1**	**M21-48-2** vs M21-2-2
Mating type	MAT1-2 / N.A.	MAT1-2 / MAT1-2	MAT1-2 / MAT1-1
Sclerotia	No / Yes	No / Yes	Yes / No
Location of the sclerotia	N.A. / Spread	N.A. / Spreading from inoculum	Spreading fro / N.A.
Melanization	Yes / Yes	Yes / Yes	Yes / No
Location of the melanization	Spread / Spread	Spread / Sclerotia	Spreading fro / N.A.
Conidia	No / No	No / No	No / No
Confrontation zone	Exclusion	Merged	Merged
Strains (M21-48-1 top and X)	**M21-48-1** vs M21-2-2	M21-48-1 vs **M21-2-5**	M21-48-1 vs **M21-43-2**
Mating type	MAT1-1 / MAT1-1	MAT1-1 / MAT1-2	MAT1-1 / N.A.
Sclerotia	Yes / No	Yes / No	No / No
Location of the sclerotia	Spreading from inoculum / N.A.	One spot / N.A.	N.A. / N.A.

Melanization	Yes		No	Yes		No	Yes	Yes
Location of the melanization	Spreading from inoculum		N.A.	Spread		N.A.	Spread	Periphery
Conidia	No		No	No		No	No	No
Confrontation zone	Merged			Merged			Exclusion	
Strains (M21-48-2 top and X)	M21-48-2	vs	**M21-2-5**	M21-48-2	vs	**M21-43-2**	M21-48-2	vs **M21-43-2**
Mating type	MAT1-2		MAT1-2	MAT1-2		N.A.	MAT1-2	N.A.
Sclerotia	Yes		No	No		No	No	No
Location of the sclerotia	Spread		N.A.	N.A.		N.A.	N.A.	N.A.
Melanization	Yes		Yes	Yes		Yes	Yes	Yes
Location of the melanization	Spread		Spots	Spread		Spread	Spread	Spread
Conidia	No		No	No		No	No	No
Confrontation zone	Delimitation			Exclusion			Exclusion	
Strains (M21-48-1 top and X)	**M21-48-1**	vs	M21-48-1	M21-48-1	vs	**M21-48-2**	M21-48-1	vs **M21-48-5**
Mating type	MAT1-1		MAT1-1	MAT1-1		MAT1-2	MAT1-1	MAT1-2
Sclerotia	No		No	Yes		Yes	Yes	Yes
Location of the sclerotia	N.A.		N.A.	Spread		Spread	Inoculum	Spread
Melanization	Yes		Yes	Yes		Yes	Yes	Yes
Location of the melanization	Periphery		Spread	Sclerotia		Spread	Sclerotia	Spots
Conidia	No		No	No		No	No	Yes
Confrontation zone	Exclusion			Exclusion			Exclusion	
Strains (M21-48-2 top and X)	M21-48-2	vs	**M21-43-3**	M21-48-2	vs	**M21-43-4**	M21-48-2	vs **M21-43-5**
Mating type	MAT1-2		N.A.	MAT1-2		N.A.	MAT1-2	N.A.
Sclerotia	No		No	No		No	No	No
Location of the sclerotia	N.A.		N.A.	N.A.		N.A.	N.A.	N.A.
Melanization	Yes		Yes	Yes		Yes	Yes	Yes
Location of the melanization	Spread		Spread	Spread		Spread	Spread	Spread
Conidia	No		No	No		No	No	No
Confrontation zone	Exclusion			Exclusion			Exclusion	
Strains (M21-48-1 top and X)	M21-48-1	vs	**M21-48-8**	M21-48-1	vs	**M21-48-11**	M21-48-1	vs **M21-48-12**
Mating type	MAT1-1		MAT1-2	MAT1-1		MAT1-2	MAT1-1	MAT1-2
Sclerotia	Yes		Yes	Yes		Yes	Yes	No
Location of the sclerotia	One spot		Center	Spreading fro		Center	Spread	N.A.
Melanization	No		Yes	Yes		Yes	Yes	Yes
Location of the melanization	N.A.		Spots	Spreading fro		Periphery	Spread	Spots
Conidia	No		No	No		No	No	No
Confrontation zone	Exclusion			Exclusion			Exclusion	
Strains (M21-48-2 top and X)	M21-48-2	vs	M21-48-1	M21-48-2	vs	**M21-48-2 new**	M21-48-2	vs **M21-48-4**
Mating type	MAT1-2		MAT1-1	MAT1-2		MAT1-2	MAT1-2	MAT1-2
Sclerotia	Yes		Yes	Yes		No	Yes	Yes
Location of the sclerotia	Spreading from inoculum		Center	Huge spot at i		N.A.	Spread	Spread
Melanization	Yes		Yes	Yes		Yes	Yes	No

Location of the melanization	Spread		One spot	Sclerotia	Spreading from inoculum		Spread	N.A.
Conidia	No		No	No	No		No	No
Confrontation zone	Exclusion			Exclusion + Tissue-like structure			Tissue-like structure	
Strains (M21-48-1 top and X)	M21-48-1	vs	**M21-48-13**	M21-48-1	vs	**M21-48-14**	M21-48-1	vs	**M21-48-17**
Mating type	MAT1-1		MAT1-2	MAT1-1		MAT1-1	MAT1-1		MAT1-1+MAT1-2
Sclerotia	Yes		Yes	Yes		No	Yes		Yes
Location of the sclerotia	Spreading from inoculum		Inoculum	Spreading fro		N.A.	Spots		Spread
Melanization	Yes		Yes	Yes		No	Yes		Yes
Location of the melanization	Spreading from inoculum		Spots	Spreading fro		N.A.	Spread		Spread
Conidia	No		No	No		No	Yes		No
Confrontation zone	Exclusion			Exclusion			Exclusion		
Strains (M21-48-2 top and X)	M21-48-2	vs	**M21-48-5**	M21-48-2	vs	**M21-48-8**	M21-48-2	vs	**M21-48-11**
Mating type	MAT1-2		MAT1-2	MAT1-2		MAT1-2	MAT1-2		MAT1-2
Sclerotia	Yes		Yes	Yes		Yes	Yes		Yes
Location of the sclerotia	Inoculum		Spread	Inoculum		Spread	Inoculum		Inoculum
Melanization	Yes		Yes	Yes		Yes	No		No
Location of the melanization	Spread		Spread, spots	Spread		Spread	N.A.		N.A.
Conidia	No		No	No		No	No		No
Confrontation zone	Exclusion			Tissue-like structure			Exclusion		
Strains (M21-48-1 top and X)	M21-48-1	vs	**M21-48-18**	M21-48-1	vs	**M21-48-19**	M21-48-1	vs	**M21-48-20**
Mating type	MAT1-1		MAT1-1+MAT1	MAT1-1		MAT1-2	MAT1-1		MAT1-1+MAT1-2
Sclerotia	Yes		Yes	Yes		Yes	Yes		Yes
Location of the sclerotia	Spreading from inoculum		Inoculum	Spreading fro		Inoculum	Inoculum		Periphery and inoculum
Melanization	Yes		No	Yes		No	No		Yes
Location of the melanization	Spreading from inoculum		N.A.	Spreading fro		N.A.	N.A.		Spots
Conidia	No		No	No		No	No		No
Confrontation zone	Exclusion			Exclusion + Tissue-like structure			Delimitation		
Strains (M21-48-2 top and X)	M21-48-2	vs	**M21-48-12**	M21-48-2	vs	**M21-48-13**	M21-48-2	vs	**M21-48-14**
Mating type	MAT1-2		MAT1-2	MAT1-2		MAT1-2	MAT1-2		MAT1-1
Sclerotia	Yes		No	Yes		Yes	Yes		Yes
Location of the sclerotia	Inoculum		N.A.	Spread		Inoculum	Inoculum		Inoculum
Melanization	Yes		Yes	Yes		Yes	Yes		No
Location of the melanization	Spread		Spread	Inoculum		Inoculum	Spots		N.A.
Conidia	No		No	No		No	No		No
Confrontation zone	Exclusion			Exclusion			Exclusion		
Strains (M21-48-2 top and X)	M21-48-2	vs	**M21-48-15**	M21-48-2	vs	**M21-48-16**	M21-48-2	vs	**M21-48-17**
Mating type	MAT1-2		MAT1-2	MAT1-2		MAT1-2	MAT1-2		MAT1-1+MAT1-2
Sclerotia	Yes		Yes	Yes		Yes	Yes		Yes
Location of the sclerotia	Inoculum		Spread	Inoculum		Spread	Spread		Inoculum
Melanization	No		No	Yes		Yes	Yes		Yes
Location of the melanization	N.A.		N.A.	Inoculum		Spread	Inoculum		Spot

Conidia	No				Yes		Yes		No		No	No
Confrontation zone	Exclusion						Exclusion				Tissue-like structure	
Strains (M21-48-2 top and X)	M21-48-2		vs	**M21-48-18**			M21-48-2	vs	**M21-48-19**		M21-48-2	vs **M21-48-20**
Mating type	MAT1-2			MAT1-1+MAT1			MAT1-2		MAT1-2		MAT1-2	MAT1-1+MAT1-2
Sclerotia	Yes			No			Yes		Yes		Yes	Yes
Location of the sclerotia	Spread			N.A.			Inoculum		Spread		Inoculum	Spread
Melanization	Yes			No			Yes		Yes		No	Yes
Location of the melanization	Spread			N.A.			Spread		Spread, spots		N.A.	Spots
Conidia	Yes				Yes		No		No		No	No
Confrontation zone	Exclusion						Tissue-like structure				Exclusion	

Assessing the performance of mating type primers in non-Asian black and yellow morel populations

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Abstract

Morels are highly prized edible fungi where sexual reproduction is essential for fruiting-body production. As a result, a more comprehensive understanding of their sexual reproduction is of great interest for their artificial cultivation. Central to this is the understanding of the reproductive strategies used by morels. Sexual reproduction in fungi is controlled by mating-type (*MAT*) genes and morels are thought to be heterothallic with two idiomorphs, MAT1-1 and MAT1-2. Genomic sequencing of black (Elata clade) and yellow (Esculenta clade) morel species led to the development of PCR primers designed to amplify genes from the two mating-type idiomorphs for rapid genotyping of *Morchella* isolates from these two clades. These primers were developed to genotype *Morchella* isolates obtained from Asian populations, but their performance in populations from other regions has not yet been assessed. In this study, wild populations from Switzerland with representatives of both black and yellow morels were used to evaluate the performance of these previously published *MAT*-specific primers. A PCR product for either *MAT1-1-1* or *MAT1-2-1* was obtained in 82.63% samples using the PCR primers designed for black morels. This was independent of the clade to which the sample initially belonged to. Verification by sequencing confirmed that the primers can successfully amplify mating-type genes, but in the case of *MAT1-1-1*, unspecific amplification was common. With the Esculenta MAT-specific primers, an amplification was possible in 77.5% of the samples, but after verification by sequencing, none of the products generated actually corresponded to mating-type genes. More worrisomely, the products obtained with one of the primer pairs (EMAT1-2L/R) corresponded instead to the RNA polymerase II subunit (*RPB2*) gene. *In-silico* PCR confirmed the results obtained, that is that black morel specific primer pairs (MAT11L/R and MAT22L/R) indeed target the mating-type region but that MAT11L/R are likely to lead to off-target products. In contrast, the published primers specific for MAT in yellow morels appear not to be appropriate to assess any specific reproductive strategy in this clade. In conclusion, the black morel MAT-specific primer pairs seem reliable to amplify mating type genes, even though sequencing of MAT1-1-1 products sometimes failed due to the presence of off-target products. Conversely, our results show that the yellow morel MAT-specific primers cannot be recommended to assess or claim the identification of reproductive strategies in morel species.

Keywords: PCR, MAT idiomorphs, *Morchella*, heterothallic, homothallic, sexual reproduction, fruiting bodies, morels.

Introduction

True morels, belonging to the genus *Morchella*, are highly prized ascomycete fungi due to the exceptional organoleptic properties of their ascocarps. While morels can form ascocarps under adequate environmental conditions in natural systems, production of these sexual structures through artificial cultivation remains a challenge (Q. Liu et al. 2018). This has motivated efforts to better understand the biological mechanisms that permit sexual reproduction in these fungi,

with the aim of facilitating the cultivation of morel fruiting bodies. In Ascomycota, sexual reproduction is generally controlled by a bipolar mating system, consisting in one genetic locus having two modalities, each one carrying one to multiple mating-type genes (Wilken et al. 2017) encoding entirely unrelated proteins (Casselton 2002). Given that the genes at this locus are not homologous, they are referred to as idiomorphs rather than alleles (Arie et al. 1997; Zheng et al. 2013; Chai et al. 2017). In heterothallic species sexual reproduction and the production of sexual spores (ascospores) requires the fusion of two haploid mycelia of opposite mating types, that is *MAT1-1* or *MAT1-2* idiomorphs (Coppin et al. 1997). In contrast, homothallic and secondary homothallic species carry both idiomorphs in a single ascospore and are thus considered self-fertile (Wilson et al. 2015). An often-defining characteristic of the two mating-type idiomorphs are genes that contain high mobility group (HMG) domains. The *MAT1-1-1* gene encodes an α1 protein belonging to the MATα_HMG family, while *MAT1-2-1* encodes a protein belonging to the MATA_HMG family (Arie et al. 1997; Zheng et al. 2013; Zou et al. 2019; Robinson and Natvig 2019). In addition of their importance in determining the mode of reproduction of ascomycete fungi, mating-type genes can be a useful tool to improve species determination and aid in phylogenetic analyses (M. Du et al. 2005). This is due to the observation that *MAT1-1-1* and *MAT1-2-1* have a high interspecific and low intraspecific variability in several examined fungal lineages (Coppin et al. 1997).

Morchella spp. are considered to primarily be heterothallic, but secondary homothallic strategies have also been observed. Furthermore, the mating strategies can be mixed within a single species. For instance, the black morel *Morchella importuna* can reproduce by three different mating systems: heterothallism, homothallism, and pseudohomothallism (X.-H. Du et Yang 2021). The design of compatible mating assays to permit ascocarp formation relies on the ability to detect the mating-type genes. Since no obvious morphological differences have been observed in mycelial isolates containing either idiomorph, genetic tools are currently required to characterize fungal isolates of interest. The design of primers that specifically amplify relevant mating-type genes is an important tool to enable rapid and cost-effective identification of *MAT* idiomorphs using polymerase chain reaction (PCR) followed by gel agarose electrophoresis (X.-H. Du et al. 2017; 2020; Chai et al. 2017; 2019).

Primers designed to amplify partial regions of two *MAT* genes in *Morchella* spp. of the Elata (black) clade have been previously published in order to assess the presence of one or both idiomorphs in isolates belonging to this clade. Given that black and yellow morels differ in their ecology and morphology (Pilz et al. 2007) and are phylogenetically divergent (O'Donnell et al. 2011), the loci contributing to sexual mating could also differ. This was the motivation for the development of specific primers to examine species of the Esculenta clade as well (X.-H. Du et al. 2020). Subsequently, differences in the genetic structure of the partial *MAT1-1-1* and *MAT1-2-1* sequences obtained using these primers were observed between the black and yellow clades. The length of the genes differed between both clades: in black morels, the size of *MAT* genes varied between 729-736 bp (*MAT1-1-1*) and 398-408 bp (*MAT1-2-1*) while in yellow morels, the length varied between 708 bp (*MAT1-1-1*) and 869-880 bp (*MAT1-2-1*) (X.-H. Du et al. 2017; 2020). In addition, a notable difference in the mating system of black and yellow morels was highlighted by Chai et al. (2019); these authors discovered three different genes (*MAT1-1-1, MAT1-1-10* and *MAT1-1-11*) within the mating region in *M. importuna* (Elata clade), but only two genes (*MAT1-1-1* and *MAT1-1-10*) in *Morchella* sp. Mes-20 (Esculenta clade).

Until now, these previously published primers were mainly used to investigate mating types in morel strains from an Asian origin (X.-H. Du et al. 2017; 2020). However, due to the diverging evolutionary histories of morel species from different geographical regions (O'Donnell et al. 2011), we tested whether these primers are also effective to characterize the mating type genes in European species isolated in Switzerland. Given the existence of conserved regions within Ascomycete mating-type genes (Coppin et al. 1997; Martin et al. 2010), it is expected that the existing primers could work in other lineages despite the important continental endemism and provincialism of some *Morchella* species (O'Donnell et al. 2011). To test this, PCR amplification using these previously published MAT-specific primers was performed on three Chinese strains as reference, and in samples (fruiting bodies, *in vitro* propagated

sclerotia and mycelia) originating from 40 Swiss morel specimens collected in the field, including representatives from both the Elata and Esculenta clades. Amplicon sequences were obtained with the Elata-specific primers for 89 samples representing several species from both clades. Conversely, the Esculenta-specific primers did not amplify the targeted mating-type genes. To further evaluate these primers and support our experimental findings, *in*-silico investigations were also performed. Custom hidden Markov model (HMM) profiles were designed to identify *in-silico* putative *MAT1-1-1* and *MAT1-2-1* genes in a selection of *Morchella* genomes. These investigations provided further support that the Elata-specific *MAT1-2-1* primer pair worked as intended by amplifying the correct MAT target in the Elata clade, but also amplified this marker in the Esculenta clade of yellow morels. On the other hand, all other examined primers would amplify incorrect targets in diverse *Morchella* genomes. The complementary experimental and *in-silico* analyses preformed herein indicate that the primers previously published to identify mating-type idiomorphs in *Morchella* isolates belonging to either the Elata clade or Esculenta clade generally do not function as intended. This work highlights important considerations when designing and evaluating primers for the characterization of diverse fungi and provides insights as to how mating-type specific primers can be properly developed using available genetic resources.

Methods

Fungal material collection and cultivation

Morel fruiting bodies were wild foraged across different locations in Switzerland between March and May 2019. After receiving an ascocarp, a brief description of its color and shape was made, and morphological traits such as shape and color of caps and stipes were used to ascribe field identifications and clade designations. Round-shaped yellow and light brown fruiting bodies were considered as belonging to the Esculenta clade. Elongate and dark brown caps were considered as belonging to Elata species. In this study, each fruiting body collected in the wild was considered a biological specimen and assigned an identifier starting by the prefix "M19" followed by an Arabic number.

Tissue from fruiting bodies (specimens M19-1 to M19-40) was vegetatively propagated to obtain pure mycelial isolates in culture (later referred to as isolate). To do so, a piece of fresh hymenium (1 x 1 x 0.5 cm^3) was dissected with a sterile scalpel and placed on potato dextrose agar (PDA, 39 g/L; Sigma-Aldrich). After one day, growing mycelium was transferred to fresh PDA in order to prevent potential contaminants originating from the fruiting bodies. Growing morel mycelium was transparent-white at an early stage and often turned brown after 7-12 days. Mycelia with other morphology and bacteria were considered as contaminants. Once pure, mycelial cultures were maintained on PDA at 21-24°C in darkness until further use. From these cultures, 17 mycelia and 4 sclerotia were analyzed with MAT-specific primer pairs. Alternatively, to obtain ascospore-derived isolates, fruiting bodies from specimens M19-41 to M19-43 were used to collect the sexual ascospores. Briefly, ascocarps were incubated in plastic jars in a dark oven at 22°C for 2-3 days to trigger natural sporulation. Spores were diluted in sterile water and spread on PDA for germination. To obtain spore-derived mycelial cultures, individual microcolonies were picked and transplanted into fresh medium right after germination (1 day). Eventually, this allowed obtaining and analyzing 25 ascospore-derived isolates with seven isolates obtained from specimen M19-41, eleven from specimen M19-42, and seven from specimen M19-43. Mycelial cultures were maintained on PDA at 21-24°C in darkness until further use.

In addition to the Swiss isolates, strains NEU142, NEU143 and PYL were isolated from imported inocula provided as mycelial spawn used for outdoor morel cultivation in China. These isolates were maintained identically as described for environmental isolates. A summary of all the biological samples considered in this study is presented in Supplementary File S1.

DNA extraction

DNA extractions were performed with the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the manufacturer protocol. DNA was obtained directly from fresh pieces of hymenium (about 1 cm^3) for specimens M19-1 to M19-40 (38 samples; File S1) and from mycelia scrapped from the surface of 7-days old cultures with a dissecting needle (42 samples; File S1). DNA was also extracted from sclerotia (about 2 cm^3 each; 4 samples; File S1) harvested from mycelial cultures on malt-extract and agar medium (MA; 12 g/L malt extract, Fluka, and 15 g/L agar, Merck). Eluted DNA was quantified with a Qubit kit (Invitrogen, USA) with the Broad Range buffer and reagents to obtain 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 and stored at 4°C until use.

Taxonomic identification

The region encompassing the internal transcribed spacer ITS1, the 5.8S ribosomal RNA gene, and the ITS2 was amplified by PCR with the primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) and Sanger sequenced to identify *Morchella* isolates to the species level. In this study, ITS was used as a quick barcode method to taxonomically distinguish the different samples analyzed. However, we are aware that for an accurate phylogenetic analysis, additional molecular markers should be used. For each DNA sample, the PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix (HighQu, Germany), 0.2 µM forward (ITS1-F) and 0.2 µM reverse (ITS4) primer. Finally, 1 µL of the 2 ng/µL diluted DNA extract was used as a template. Amplifications were performed in a Thermo Scientific Arktik thermal cycler. The following parameters were used for ITS amplification: 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 2min. 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. Positive PCR products (i.e., single band at the expected size of 750-1200 bp) 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 appear dry; then, 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained on the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were fluorometrically quantified with a Qubit® 2.0 Fluorometer (Invitrogen, USA). Final concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing. The forward and reverse sequences obtained were manually trimmed and assembled using BioEdit 7.2. They were analyzed with the pairwise alignment tool of the Westerdijk Fungal Biodiversity Institute (https://wi.knaw.nl/page/Pairwise_alignment) (X.-H. Du, Zhao, et Yang 2015). Queries of species indicating the highest percentages of query cover and similarity were used to identify the species corresponding to each DNA sample (File S1). All the sequences are available in Supplementary File S2 and were deposited in GenBank under accession number XXX.

Detection of the mating types by PCR amplification and sequencing

To identify the presence of the mating type genes *MAT1-1-1* and *MAT1-2-1* in all samples described in table S1, partial PCR amplification was performed using the primer pairs listed in Table 1. After amplification, 39 amplicon products generated with the MAT11L/R or MAT22L/R primer pairs and 12 amplicon products generated with the EMAT1-1L/R or EMAT1-2L/R primer pairs were randomly chosen for sequencing (File S1).
The PCR mix was adapted from Du et al. (2017) and contained: PCR-grade water, 1.2 mg/mL bovine serum albumin (BSA), 1X polymerase buffer (1.5 mM MgCl2), 0.4 µM forward (MAT1-1L or MAT1-2L) and 0.4 µM reverse (MAT1-1R or MAT1-2R) primer, 0.2 mM TaKaRa dNTPs and 1.5 U of TaKaRa TaqTM DNA Polymerase (Takara Bio Inc., Japan). The DNA template was 1µL of the same 2 ng/µL dilution used for species identification by ITS sequencing. A gradient PCR ranging from 50°C to 60°C was used to optimize annealing temperatures for

both primer pairs. The thermocycling parameters were then adapted from Du et al. (2017) as follows: denaturation at 94°C for 3min, 35 cycles of denaturation, annealing and elongation (94°C for 1 min, 54°C [MAT11L/R and EMAT1-1L/R] or 51.5°C [MAT22L/R and EMAT1-2L/R] for 30 sec, 72°C for 1 min), final elongation at 72°C for 10 min. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator. Positive PCR products (i.e., single band at the expected size; Table 1) were then purified, quantified and sequenced as described in the previous section. Forward and reverse sequences were manually trimmed and assembled with BioEdit 7.2. They were then compared to sequences in the NCBI database using the nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) set to search for highly similar sequences (> 98% ID and > 96% query coverage) (megablast algorithm) (Altschul et al. 1990). The 28 amplicon sequences of *MAT1-1-1* and 37 amplicon sequences of *MAT1-2-1* that were generated can be found in Supplementary File S3.

Analysis of published mating-type specific primers for Morchella

A total of 9 *Morchella* genome assemblies retrieved from NCBI and representing 7 species were searched using ThermonucleotideBLAST (tntBLAST) (Gans et Wolinsky 2008) (Supplementary File S4) and previously published primer pairs (see Table 1) as the query. tntBLAST was run with the following settings: the assay format (-A) was set to 'PCR', the minimum allowed binding temperature (-e) was set to 50°C and the maximum allowed binding temperature (-x) was 65°C. Two sets of Hidden Markov model (HMM) profiles were created for both *MAT1-1-1* and *MAT1-2-1*. One set was created using protein sequences from several *Tuber* and *Morchella* species, and the other set was created using non-Pezizomycetes sequences from diverse ascomycetes. Details of these HMM profiles and their summary statistics can be found in Supplementary File S5. Amino-acid alignments for the creation of the HMM profiles were generated using Clustal Omega (Sievers et al. 2011). The non-Pezizomycetes HMM profile included sequences from highly studied taxa that helped establish canonical models for mating-type genes, such as *Neurospora* and *Magnaporthe*, making them generally more trustworthy than largely unverified sequences from *Tuber* or *Morchella*. Additionally, published sequences for *Morchella* and *Tuber* were quite limited in number. Searches of *Morchella* genomes performed with these HMM models identified similar top candidates for mating-type genes. Putative mating-type genes identified by these HMM profiles were further analyzed by comparing their position relative to the *APN2* and *SLA2* genes within the genome for each isolate examined. The *APN2* and *SLA2* genes were identified in *Morchella* genomes through homology-based searches using genes from *Neurospora* as a reference (NCBI accessions: XP_964240.1 and ESA43843.1). Putative mating-type genes identified in the HMM searches that were also located between APN2 and SLA2 loci were considered to have the strongest support and used for genotyping each genome.

Results

Species identification

Pure *Morchella* mycelia were obtained for each specimen sampled in the wild by culturing directly hymenia fragments (M19-1 to M19-40), dry ascospores (M19-41, M19-42, M19-43), or by using mycelial spawn as inocula (NEU142, NEU143, PYL; File S1). A few examples of mycelial cultures maintained *in vitro* are provided in Figure 1. The internal transcribed spacer (ITS) region, including the 5.8S rDNA, was used to ascribe each sample to a given species. Based on the ITS only, the fourty morel fruiting-bodies (wild specimens), the corresponding mycelial isolates, and the three mycelial spawn examined corresponded to five different species including: *M. esculenta* (21 biological specimens, 23 mycelial isolates, and the 4 sclerotia), *M. sextelata* (2 mycelial isolates), *M. importuna* (1 mycelial isolate), *M. angusticeps* (12 biological specimens, 7 mycelial isolates) and *M. deliciosa* (4 biological specimens, 1

mycelial isolate). All the *M. crassipes* were considered as *M. esculenta* as the first is probably nested within *M. esculenta* (Richard et al. 2015). Three black morels (M19-30, M19-31, and M19-41) could not be assigned to a species using the ITS marker. At the end, the collection corresponded to an almost equal number of specimens belonging to the Elata clade (19 Swiss specimens; Table 2) and to the Esculenta clade (21 specimens; Table 3).

Detection of the mating types by PCR amplification

To amplify the mating type genes without amplifying unspecific genetic regions, the PCR parameters had to be optimized as follows: annealing temperature was changed from 50°C to 54°C (MAT11L/R); 50°C to 51.5°C (MAT22L/R); 53°C to 54°C (EMAT1-1L/R); 53°C to 51.5°C (EMAT1-2L/R). After optimization, PCR products close to the expected length of 729-736 bp (*MAT1-1-1*) and 398-408 bp (*MAT1-2-1*) (X.-H. Du et al. 2017) were obtained in 138/167 examined samples (82.63%) using the Elata primer pairs (MAT11L/R/MAT22L/R). For these 138 samples, both MAT were amplified. Conversely, no amplification products were obtained in 19 samples with MAT11L/R and 10 samples with MAT22L/R (Supplementary table S1). Amplification failed at equal proportions in samples from black (13/72=18.06%) and yellow (17/95=16.89%) morels. Examples of the PCR products obtained are shown in Figure 2. In many samples, bands for both *MAT1-1-1* and *MAT1-2-1* gene products were observed. *MAT1-1-1* amplicons were within the expected size range (Figure 2A), while *MAT1-2-1* amplicons were always larger than the expected size, as they were above the 500 bp band from the ladder after gel electrophoresis (Figure 2B). In addition, unspecific amplification was also observed for the MAT11L/R (Supplementary File S6).

The same analysis was conducted with the Esculenta-specific (EMAT1-1L/R and EMAT1-2L/R) primer pairs with 40 samples including both black and yellow morel isolates (Supplementary File S1). Unspecific PCR products represented by the appearance of multiple bands were observed in most samples following amplification with EMAT1-1L/R, the *MAT1-1-1* Esculenta MAT-specific primer pair (Figure 3A). In those samples in which a single PCR band was obtained, the signal was located around the expected 708 bp size (X.-H. Du et al. 2020). The amplicon products generated with EMAT1-2L/R, the *MAT1-2-1* Esculenta MAT-specific primer pair, were always shorter than the expected size of 869-880 bp (except for *M. esculenta* M19-21; 870 bp), as exemplified by their location below the 750 bp band from the ladder after gel electrophoresis (Figure 3B). The remaining samples were not examined since the majority of the initial samples did not produce the expected products (see below).

Sequencing of the mating type genes

A total of 81 PCR amplicons (28 MAT11L/R, 37 MAT22L/R, 8 EMAT1-1L/R, and 8 EMAT1-2L/R amplicons) were sequenced. Amplicons generated with the Elata-specific primer pairs (MAT11L/R and MAT22L/R) corresponded to *Morchella* spp. mating type genes reported in GenBank. Some of the *MAT1-1-1* sequences corresponded to the expected length of 729-736 bp. However, the length varied between 800 bp (largest) and 630 bp (shortest), with the mean length of the *MAT1-1-1* amplicon sequences was 724 bp. Comparing the *MAT1-1-1* amplicon sequences of yellow morels revealed larger amplicons than black morels, on average, in black (478 bp; min. 430 bp and max. 650 bp) than in yellow (577 bp; min 700 bp and max. 800 bp) species. The quality of the sequences was generally lower (i.e., greater quantity of "N" nucleotides) for the *MAT1-1-1* than for the *MAT1-2-1*, and the trimming that was necessary might explain the shorter size. For the *MAT1-2-1* amplicon sequences, only one sample (M19-34-1, 406 bp) was in the range of the expected length of 398-408 bp (X.-H. Du et al. 2017). After trimming, the *MAT1-2-1* amplicon sequences ranged between 420-650 bp, with a mean length of 512 bp. Comparing the *MAT1-2-1* amplicon sequences of black and yellow morels revealed larger amplicons, on average, in black (538 bp; min. 430 bp and max. 650 bp) than in yellow (492 bp; min 420 bp and max. 550 bp) species.

Concerning the EMAT primers, none of the 16 sequences obtained corresponded to either *MAT1-1-1* or *MAT1-2-1* when compared to the NCBI nucleotide database using BLAST. Rather, top BLAST matches (75% query cover, 60-70% similarity) for the sequences amplified with EMAT1-1L/R corresponded to DNA fragments located on different chromosomes of *Fusarium oxysporum*. These amplicon sequences ranged between 606-671 bp. The 8 amplicon sequences generated from the EMAT1-2L/R primers most closely resembled the second largest subunit of RNA polymerase II (*RPB2*) gene (725-734 bp) of *Morchella esculenta* (best match with 99-100% query cover and similarity in NCBI nucleotide database). A summary of the amplicon products and sequencing results described here can be found in Supplementary File S7.

Bioinformatic analyses of the published mating-type specific primers

To complement the experimental results obtained, *in-silico* analysis of the published PCR primers designed to amplify *MAT1-1-1* and *MAT1-2-1* genes in *Morchella* were performed. First, we genotyped the *M. eximia* (GCA_003314645.1) genome assembly *in silico*, which was used to generate the Elata-specific primers (X.-H. Du et al. 2017) with custom designed HMM profiles. The results from this HMM search indicated that this genome has a putative *MAT1-2-1* gene, but not a putative *MAT1-1-1* (Table 4). Moreover, we did not obtain any predicted PCR product with either primer pair (MAT11L/R and MAT22L/R) using ThermonucleotideBLAST (tntBLAST) (Table 4 and Supplementary File S8). Using the same HMM profiles, we were able to determine that one of the *Morchella* genome assemblies examined in our primer screen with tntBLAST (*M. importuna* CCBAS932) contained a probable *MAT1-1-1* gene. This putative *MAT1-1-1* gene was also flanked by *SLA2* and *APN2* genes further supporting the likelihood that it is a mating type gene. *M. importuna* is a black morel, yet the published MAT11 primer set for this clade (X.-H. Du et al. 2017) failed to produce any predicted amplicon products in our analysis with tntBLAST. A genome from *M. sextelata*, another black morel, had a predicted amplicon product from the MAT22 primers published for this clade (Table 4) that overlapped with the putative *MAT1-2-1* gene identified by the HMM profile. This putative mating type region identified by tntBLAST and the HMM profile was also flanked by the *SLA2* and *APN2* genes.

The EMAT1-1 primer set (X.-H. Du et al. 2020) had predicted amplicon products in 7 of the 9 examined genomes (predicted product in *M. conica*, *M. eximia*, two *M. importuna* genomes, *M. sextelata*, and *M. septimelata*) corresponding to both black and yellow morels (Table 4). However, all the predicted products were longer (905 bp) than the 708 bp length reported by the authors. When these predicted EMAT1-1 products were aligned to the NCBI nucleotide database using BLASTN, the top hits were consistently sequences annotated as the second largest subunit of the RNA polymerase II (*RPB2*) gene. In addition, the genome from which this primer set was derived could not be located using the GenBank submission identifier provided by the authors (SUB6606995) for further investigations. However, comparisons with other *MAT1-1-1* sequences published as part of the NCBI PopSet (1809496744) for this publication using BLASTN revealed no alignment was found between the predicted EMAT1-1 amplicon product and the published *MAT1-1-1* sequences. Analysis with tntBLAST also predicted an amplicon product from the EMAT1-2 primers designed for yellow morels (X.-H. Du et al. 2020) in an examined *M. crassipes* and a *Morchella* sp. genome (Table 4). The predicted amplicon product from these genomes was smaller (796 bp) than the range reported in the publication (869-880 bp). When this predicted MAT1-2 amplicon product from *M. crassipes* was aligned to the NCBI nucleotide database using BLASTN, the top hits were once again annotated *RPB2* sequences. The alignments between published MAT1-2 sequences from this work (NCBI PopSet 1809496908) and our predicted amplicon product using BLASTN revealed no alignment between the sequences.

Discussion

The importance of sexual structures in the life-cycle of numerous species used for human consumption (e.g., *Morchella* spp.) (Pilz et al. 2007), medicine, or as insect pests biocontrol agents (e.g., *Cordyceps* sp.) (Zheng et al. 2013; Zou et al. 2019) makes a general understanding of fungal sexual reproduction relevant to many fields. This knowledge is particularly essential in conservation biology, where the potential for species invasiveness and genetic recombination between geographically isolated populations depends strongly on the possibility of genetic exchange and the formation of viable hybrids. In addition, the study of mating types also contributes to studies in phylogeny and evolution of fungi (X.-H. Du et al. 2017; Zou et al. 2019), as mating genes evolve more rapidly than non-mating ones, and they delimit species (Chai et al. 2019). In the specific case of morels, even though it has been possible to cultivate morels, the exact mechanisms underlying fruiting body formation are still not completely understood. It is generally assumed that two aspects are essential for the formation of fruiting-bodies: the intricate ecological requirements of the fungus, which include climatic, edaphic and biotic factors; as well as the possibility to achieve a sexual cycle through the appropriate encounter of compatible participants (Q. Liu et al. 2018). Morels are considered as mostly heterothallic and thus, encounters of mycelia carrying the two opposite idiomorphs is necessary to produce fruiting bodies. Some species such as *M. importuna* can produce ascospores containing both mating types. These pseudohomothallic sexual spores give rise to fertile ascocarps and are easier to cultivate in comparison to heterothallic individuals (X.-H. Du et Yang 2021). Therefore, a reliable way to identify mating types is necessary. For this purpose, several primer pairs were designed to rapidly and readily amplify by PCR mating-type genes *MAT1-1-1* and *MAT1-2-1* in black (X.-H. Du et al. 2017) and yellow morels (X.-H. Du et al. 2020). However, our analyses indicated that three out of four of those primer pairs are not reliable. Despite multiple trials to replicate the results published in two peer-reviewed articles (X.-H. Du et al. 2017; 2020), we did not manage to obtain the expected results. This led us to further investigate the amplicons generated by sequencing, followed by an in-depth bioinformatic analysis of the sequences we generated, and the genomic sequences used to design the examined primers.

Elata MAT-specific primers

Primers designed to amplify regions of the mating-type genes *MAT1-1-1* and *MAT1-2-1* in black morels (MAT1-1L/R) (X.-H. Du et al. 2017) were used in the present study on 167 DNA samples extracted from sclerotia, mycelia and fruiting bodies of black (*Morchella* sp., *M. sextelata, M. importuna, M. angusticeps, M. deliciosa*) and yellow morels (*M. esculenta*). Interestingly, mating types could be amplified in both yellow and black species, although the primers were designed on a black morel genome (*M. eximia*) as template. Among the 138 samples from which *MAT* genes were amplified with the MAT11L/R and MAT22L/R primer pairs, 59 belonged to morel samples from the Elata clade and 79 to those from the Esculenta clade. The amplification was not possible in 13 black and 17 yellow morel samples, indicating the MAT11L/R and MAT22L/R primer pairs were equally efficient to identify mating types in morel species from both clades. However, multiple bands were generally detected after gel electrophoresis of *MAT1-1-1* amplicons, indicating that non-specific off target regions are potentially amplified (Supplementary File S6). These experimental findings were supported by the *in-silico* analysis of the primers. Analysis of the *M. eximia* genome used to generate the primers indicated that this strain is likely heterothallic, containing only a putative *MAT1-2-1* gene (Table 4). Therefore, it is surprising that both primer pairs are claimed to have been designed based on this single genome. In addition, our analysis indicated that this region likely served as the template for primer design, thereby resulting in the production of reliable primers for the *MAT1-2-1* gene in this clade. However, it appears that the *MAT1-1-1* primers for this clade were likely not produced based on the genomic sequence, as no putative *MAT1-1-1* gene could be confidently identified. One possible explanation is that an HMG domain from a region other than the mating-type region was mistakenly identified as the MATα_HMG sequence and used as a template for primer design. This could explain why a PCR product is still obtained, but also the existence of multiple bands resulting from off target amplification

products corresponding to other HMG domain genomic regions. However, detailed information on how the mating-type regions were identified or how the primers were designed is not presented in the methods from the original paper (X.-H. Du et al. 2017), making it difficult to investigate possible shortcomings in the design of this primer set. Noteworthy, the BLAST analysis revealed that MAT11L/R sequences did correspond to *MAT1-1-1* genes published by another group (Chai et al. 2017) indicating the MAT11L/R primers, when they were able to generate an amplicon, could be reliable to target the *MAT1-1-1* gene. However, the verification of this still requires the publication of a genome in which the genomic context of the target region can be confidently assessed.

Esculenta MAT-specific primers

The amplification of actual mating type genes was never possible with the yellow morel MAT-specific primers (EMAT1-1L/R and EMAT1-2L/R), in either black or yellow morels. Based on the sequence analysis of the amplicons, the primers supposed to amplify *MAT1-1-1* and *MAT1-2-1* amplified a region corresponding to *Fusarium* sp. genes and to *RPB2* regions in yellow morel species, respectively. The *in-silico* analysis clearly showed that the two primer sets should not amplify mating-type genes. Further, after a verification of the primer sequence, it was observed that the exact same primer sequences were already published previously by the same group. In an article from 2012, the primers were designed to amplify the *RPB2* loci in black (RPB2B-F/R) and yellow morels (RPB2Y-F/R), respectively (X.-H. Du et al. 2012), but the same sequences were republished in 2020 and claimed to target mating-type genes. Moreover, *RPB2* regions are known to be conserved among the Ascomycetes (Y. J. Liu, Whelen, et Hall 1999), and thus it is surprising that the primers were designed to amplify this genetic region in very specific groups of organisms, namely *Morchella* spp. from the Elata and the Esculenta clades.

Conclusion

Overall, the experimental results demonstrate that the previously published black morel primers (MAT11L/R and MAT22L/R) can amplify both MAT1-1-1 and MAT1-2-1 in black and yellow morel from Swiss populations, whereas the *in-silico* analysis demonstrated that only the MAT22L/R was trustworthy as it did not lead to off-targets. In contrast, the EMAT1-1L/R and EMAT1-2L/R primer pairs appear as inappropriate for assessing mating genotypes in Morchella since they target other genetic regions. The primary value in these primers would be the quick and efficient identification of mating type in *Morchella* isolates, but the potential for those primer pairs to produce off-target products (MAT11L/R) or target incorrect genes altogether (EMAT1-1L/R and EMAT1-2L/R) eliminates this application. The primer pair (MAT22L/R) to amplify *MAT1-2-1* in Elata morels was trustworthy, however multiple optimization tests were necessary to obtain single-band after PCR. For future studies the *in silico* and experimental validation of other primer sets (Chai et al. 2017; 2019), or the design of new primer sets based on genomes from single-ascospore cultures genome is still needed to identify putative MAT idiomorphs and sexual reproduction strategies in morels.

Declarations

Abbreviations
HMM: Hidden Markov Model
ITS: Nuclear rDNA internal transcribed spacer region
MAT: Mating type
RPB2: RNA polymerase II subunit
tntBLAST: Thermonucleotide BLAST

Ethics approval and consent to participate

Not applicable.

Adherence to national and international regulations
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files S2 and S3. In addition, the ITS sequences were submitted to GenBank under accession number XXX.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
MC conducted the laboratory work, analyzed the data and wrote the manuscript. AJR performed the bioinformatic analyses and wrote the manuscript. PH helped processing the morel collection. PSC reviewed the manuscript. PJ and SB designed the study, analyzed the data and reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Primer sequences used to amplify partial mating type genes *MAT1-1-1* and *MAT1-2-1*.

Target species	Target idiomorph	Primer	Sequence (5'-3')	Expected length of the amplified fragment	Reference
Elata clade	MAT1-1			729-736 bp	
		MAT11L	CCACCTTCTGAGTCCATTAT		X.-H. Du et al. (2017)
		MAT11R	GTTATTCTCGACAAGGTGTG		X.-H. Du et al. (2017)
Elata clade	MAT1-2			398-408 bp	
		MAT22L	TTATTAGACCATGTTCCTCG		X.-H. Du et al. (2017)
		MAT22R	CAGTATTATCACCAACCGTA		X.-H. Du et al. (2017)
Esculenta clade	MAT1-1			708 bp	
		EMAT1-1L	TAGGTAGGTCCCAAGAACACC		X.-H. Du et al. (2020)
		EMAT1-1R	GATACCATGGCGAACATTCTG		X.-H. Du et al. (2020)
Esculenta clade	MAT1-2			869-880 bp	
		EMAT1-2L	CTTGCCACTACGCGGTCTAT		X.-H. Du et al. (2020)
		EMAT1-2R	CACGGCTCTGGTATCCATTC		X.-H. Du et al. (2020)

Table 2. Black morel (Elata clade) fruiting-bodies (Switzerland) and mycelial spawn (China) investigated in this study. The putative species and the origin are indicated. For M19-41 and M19-43 the species was inferred from ascospore-derived mycelial cultures (indicated as A in Supplementary File S1).

Biological specimen	ITS-determined species	Origin
M19-1	*Morchella angusticeps*	Switzerland
M19-2	*Morchella angusticeps*	Switzerland
M19-3	*Morchella angusticeps*	Switzerland
M19-4	*Morchella angusticeps*	Switzerland
M19-5	*Morchella angusticeps*	Switzerland
M19-6	*Morchella angusticeps*	Switzerland
M19-7	*Morchella angusticeps*	Switzerland
M19-8	*Morchella angusticeps*	Switzerland
M19-9	*Morchella angusticeps*	Switzerland
M19-10	*Morchella angusticeps*	Switzerland
M19-11	*Morchella angusticeps*	Switzerland
M19-12	*Morchella angusticeps*	Switzerland
M19-28	*Morchella deliciosa*	Switzerland
M19-29	*Morchella deliciosa*	Switzerland
M19-30	*Morchella* sp.	Switzerland
M19-31	*Morchella* sp.	Switzerland
M19-32	*Morchella deliciosa*	Switzerland
M19-41	*Morchella* sp.	Switzerland
M19-43	*Morchella angusticeps*	Switzerland
NEU142	*Morchella importuna*	China
NEU143	*Morchella sextelata*	China
PYL	*Morchella sextelata*	China

Table 3. Yellow morel (Esculenta clade) fruiting-bodies investigated in this study. The putative species and the origin are indicated.

Biological specimen	**ITS-determined species**	**Origin**
M19-13	*Morchella esculenta*	Switzerland
M19-14	*Morchella esculenta*	Switzerland
M19-15	*Morchella esculenta*	Switzerland
M19-16	*Morchella esculenta*	Switzerland
M19-17	*Morchella esculenta*	Switzerland
M19-18	*Morchella esculenta*	Switzerland
M19-19	*Morchella esculenta*	Switzerland
M19-20	*Morchella esculenta*	Switzerland
M19-21	*Morchella esculenta*	Switzerland
M19-22	*Morchella esculenta*	Switzerland
M19-23	*Morchella esculenta*	Switzerland
M19-24	*Morchella esculenta*	Switzerland
M19-25	*Morchella esculenta*	Switzerland
M19-27	*Morchella esculenta*	Switzerland
M19-33	*Morchella esculenta*	Switzerland
M19-34	*Morchella esculenta*	Switzerland
M19-36	*Morchella esculenta*	Switzerland
M19-37	*Morchella esculenta*	Switzerland
M19-39	*Morchella esculenta*	Switzerland
M19-40	*Morchella esculenta*	Switzerland
M19-42	*Morchella esculenta*	Switzerland

Table 4. *In silico* detection of mating type genes and PCR in genomes of various *Morchella* spp. HMM= results of the customized HMM search with specific profiles. tntBLAST= predicted amplicon. RPB2 is indicated for the amplicons corresponded to the RNA polymerase II subunit 2 (*RPB2*) gene. N.A.= not applicable.

Genome	Accession	HMM	tntBLAST	*In silico* amplicon (bp)	Expected amplicon (bp)
Morchella sextelata	GCF_020137385.1	MA1-2-1	MAT22/EMAT11 (RPB2)	501	398-408
Morchella sextelata	GCA_009741755.1	Not annotated	N.A.	N.A.	N.A.
Morchella eximia	GCA_003314645.1	Not annotated	EMAT11 (RPB2)	905	708
Morchella importuna	JGI Morco1 CCBAS932	MAT1-1-1	EMAT11 (RPB2)	905	708
Morchella importuna	JGI Morimp1 SCYDJ1-A1	MAT1-2-1	EMAT11 (RPB2)	905	708
Morchella importuna	GCA_003444645.1	Not annotated	EMAT11 (RPB2)	905	708
Morchella conica	GCA_008079325.1	Not annotated	EMAT11 (RPB2)	905	708
Morchella septimelata	GCA_003313775.1	Not annotated	EMAT11 (RPB2)	905	708
Morchella crassipes	GCA_009192285.1	Not annotated	EMAT22 (RPB2)	796	869–880
Morchella sp.	GCA_013407065.1	Not annotated	EMAT22 (RPB2)	796	869–880

Figures

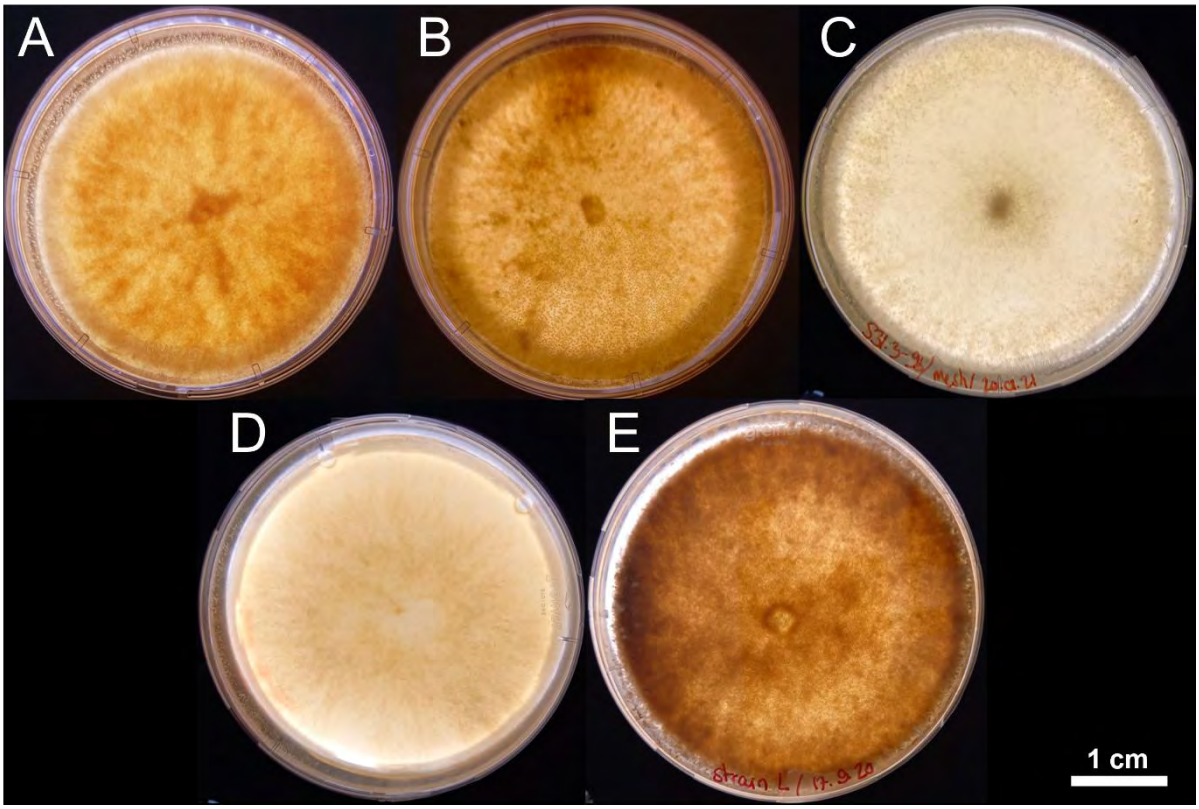

Figure 1. Mycelial cultures obtained from five specimens of *Morchella* spp. on potato dextrose agar. Pictures were taken one month post inoculation. A= *M. esculenta* M19-23 (CH); B= *M. deliciosa* M19-28 (CH); C= *Morchella* sp. Mes-9 M19-42 (CH); D= *M. angusticeps* M19-34 (CH); E= *M. sextelata* PYL (CN).

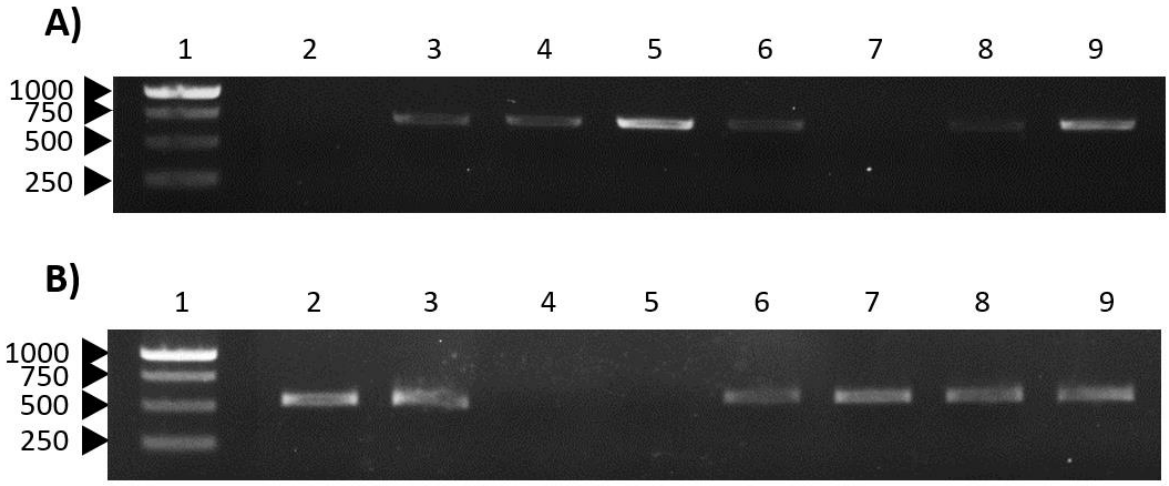

Figure 2. Agarose gel electrophoresis showing examples of putative *MAT1-1-1* (A) and *MAT1-2-1* (B) PCR products amplified with the Elata MAT-specific primers (MAT11L/R (A) and MAT22L/R (B)). Lane 1= 1 Kb BrightMAX™ DNA ladder (1'000, 750, 500 and 250 bp); Lanes 2-8= *M. angusticeps* M19-43 (six individual ascospore-derived cultures); Lane 9= *M. sextelata* PYL (China).

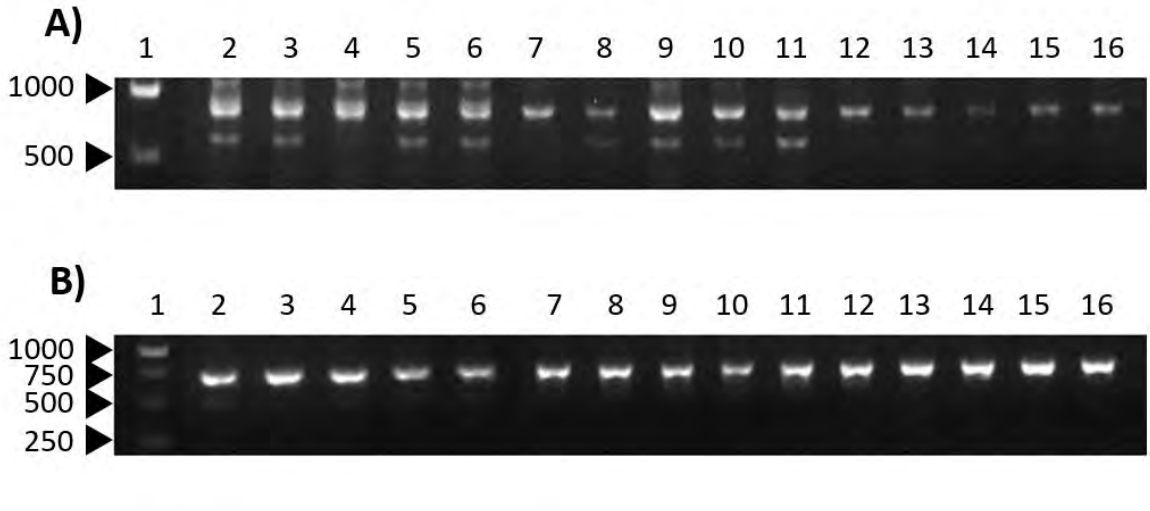

Figure 3. Agarose gel electrophoresis showing examples of putative *MAT1-1-1* (A) and *MAT1-2-1* (B) PCR products amplified with the Esculenta MAT-specific primers (EMAT1-1L/R (A) and EMAT1-2L/R (B)). Lane 1= 1 Kb BrightMAX™ DNA ladder (A: 1'000 and 500 bp; B: 1'000, 750, 500 and 250 bp); Lanes 2-16= *Morchella* sp. Mes-9 M19-42 (15 individual ascospore-derived cultures).

Supplementary Information

Supplementary File S1. Summary of the biological samples included in this study; H= Hymenium; M=hymenium-derived mycelial culture; A=ascospore-derived mycelial culture; S=sclerotia. For the mating type PCRs += PCR product obtained; -= no PCR product obtained; N.A.= not applicable. For the product sequenced, the matching sequence is indicated in brackets.

Supplementary File S2. Summary of ITS sequences in FASTA format.

Supplementary File S3. Putative *MAT* sequences generated with the four primer pairs: MAT1-1 (MAT1), MAT1-2 (MAT2), EMAT1-1 (EMAT1) and EMAT1-2 (EMAT2).

Supplementary File S4. *Morchella* genome assemblies used for the in isilico verification of mating type primers. N.A.=not applicable.

Supplementary File S5. Information in the sequences used for the generation of Hidden Markov model (HMM) profiles.

Supplementary File S6. Agarose gel electrophoresis showing examples of putative *MAT1-1-1* PCR products amplified with the Elata MAT-specific primers (MAT11L/R). Lane 1= 1 Kb BrightMAX™ DNA ladder (1'000, 750, 500 and 250 bp); The following lanes correspond to PCR products from different morel samples.

Supplementary File S7. Summary of the amplicon products and sequencing results.

Supplementary File S8. Results of the tntBLAST analysis.