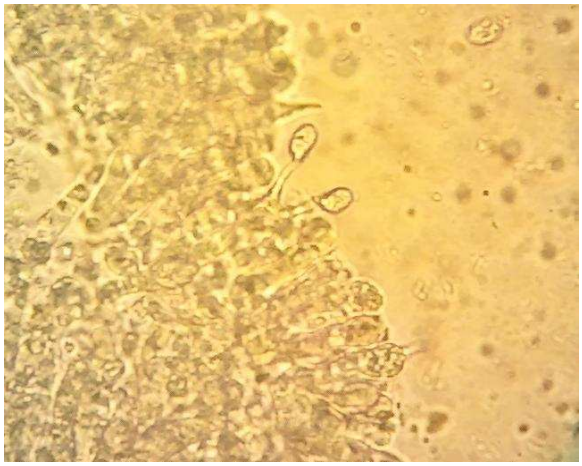
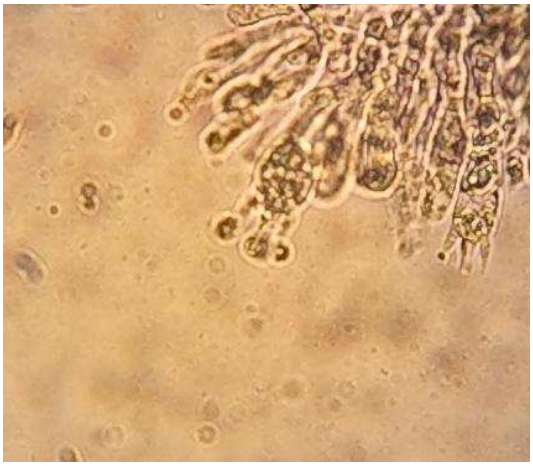
Evolution of asexual and sexual reproduction systems in a Basidiomycete: *Mycena galericulata*

Tim Möhlmann

Master thesis, May 2011





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Evolution of asexual and sexual reproduction systems in a Basidiomycete: *Mycena galericulata*

Tim W. R. Möhlmann
861 119 576 070
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Under supervision of Duur K. Aanen and Thom W. Kuyper
Laboratory of Genetics, Wageningen University and Research Center, the Netherlands

Abstract

Sexual reproduction has become the dominant reproductive system throughout many higher organisms. Although asexual reproduction seems to have disappeared where sexual reproduction evolved, in several species both reproduction cycles occur together. Sexual reproduction is widespread in fungi and is the force behind recombination and the rising of new genotypes. Basidiomycetes however, also have the opportunity to reproduce asexually. Within the Basidiomycetes, asexual reproduction is a rather common phenomenon, especially in the genus *Mycena*. In *Mycena galericulata* (Scop.) S.F. Gray 1821 both reproduction cycles still seem to exist together within the same species which is expressed in basidiocarps with either 2- or 4-spored basidia. However, it remains unclear what the relation is between the two forms of *M. galericulata*.

To get a better understanding of the relation between the two forms, fruiting bodies have been collected in the field, crossings were performed, a growth experiment has been conducted, fluorescence pictures were taken from different strains and sequences have been obtained using the primers ITS, RPB2, EF1α and B36F. The sequences from the ITS locus were analyzed by constructing a phylogenetic tree.

Firstly, the results from the field collection show that the ratio in which the 4-spored type occurs increases at the end of the growing season. Secondly, only successful crosses with clamp connections were obtained from 4-spored crossed with other 4-spored strains. Furthermore, the phylogenetic analysis, based on ITS sequences, did not show any difference between the 2- and 4-spored and suggests that it is still a mixed population. Furthermore, several sequences from the 2-spored type showed double peaks. This was found for all four primer combinations which strongly underpins the conclusion that the 2-spored form is heterozygous. The occurrence of heterozygous 2-spored is however, for the ITS locus, less common than in the 4-spored form. The other primer combinations do not show this pattern. In addition, 2-spored single spore cultures gave a single signal, while the sequences obtained from the same basidiocarp showed a double signal.

Secondary homothallism and diploidy are suggested as possible explanations for these results, both hypotheses however, can be rejected using counter arguments underpinned with results obtained during this research. The relationship between the 2- and 4-spored forms of *M. galericulata* is still not completely understood. More research is needed to truly understand the reproductive systems used by *M. galericulata*.

Table of Contents

1. Introduction

There is still no consensus how sexual reproduction has evolved. The first fossilized evidence for a sexual reproductive system originates from the Stenian period roughly 1 to 1.2 billion years ago (Butterfield 2000). In many higher organisms, including almost all animals and plants, sexual reproduction developed to become the dominant reproductive system. Although asexual reproduction seems to have disappeared, there are still species where both reproduction cycles occur together. Parthenogenesis has been found in for example plants and fungi but also in invertebrates like aphids, nematodes, bees, stick insects, scorpions and parasitic wasps, and even in some vertebrate species including reptiles, fish, birds and sharks (Chapman et al. 2008 and references therein).

There seem to be quite some disadvantages for sexual reproduction. First, it demands more time and energy than asexual reproduction. An example to illustrate this would be the time spent on finding a mate. Furthermore, the production of male structures in a sexual reproductive system is costly and therefore gives asexual reproduction an advantage (Stahl and Esser, 1976; Casselton and Olesnicky 1998; Brown and Casselton 2001; Aanen and Hoekstra 2007).

Nevertheless, sexual reproduction has evolved throughout many higher organisms. This means there should be an advantage to this reproductive system. A suggested explanation is the speed of adaptive evolution, since recombination occurring during sexual reproduction gives the offspring a higher chance of survival in an ever changing environment. Furthermore, favorable mutations are infrequent and the accumulation of beneficial mutations will take a long time when reproducing asexually (Stahl and Esser 1976; Carlile 1987). In addition to this, sexual reproduction helps to eliminate bad mutations (de Visser and Elena 2007).

Fungi are one of the most important organism groups in the world. As mutualists they are responsible for over 90 percent of the growth of vascular plants and play an important role in the ecosystem services depending on soil processes. The yearly total contribution of fungi via ecosystem services is estimated at 90 trillion dollars worldwide (Falconer et al. 2005 and references therein). Furthermore it is estimated that there are about 1.5 million fungal species. Considering these numbers and the fact that fungi have their own kingdom within the classification of organisms, it is surprising that we know so little about them (Falconer et al. 2005 and references therein). Together with the fact that getting to know more about fungi seems a logical step, fungi are very useful for studying more complicated processes in a fairly simple organism. Fungi can be used for questions involving higher organisms which are difficult to study and expand the findings of research done with fungi to the way higher organisms function.

1.1 Basidiomycetes

Within the subkingdom Dikarya, asexual reproduction is rather common in both the phyla Ascomycota as Basidiomycota. Asexual reproduction often happens through mycelial fragmentation and less common through sporogenesis. A number of asexually reproducing Basidiomycetes have been described (Stahl and Esser 1976 and references therein). When reproducing asexually through sporogenesis, the spores are often produced in reduced fruiting bodies. Other possibilities are the formation of conidia or monokaryotic fruiting bodies which produce the spores (Kuyper, personal communication).

Most sexual basidiomycetes either have a bipolar (unifactorial) or a tetrapolar mating system (bifactorial) (Kühner 1977; Casselton and Economou 1985; Brown and Casselton 2001). In bipolar

species, a single locus determines the mating type, whereas in tetrapolar species, two independent loci determine the mating type. Because mycelia are only compatible when the alleles at both loci are different, in the tetrapolar mating system only 25% of the spores are compatible with their sister basidiospores where 50% of the bipolar spores can cross with their sister spores. However, because of many different alleles within the population, over 90% of the monokaryons are compatible with each other (Kühner 1977; Casselton and Economou 1985; Carlile 1987; Casselton and Olesnicky 1998).

A typical Basidiomycete life cycle starts with two haploid mycelia (monokaryons) that fuse via plasmogamy (Fig. 1). When the mycelia are compatible the nuclei will migrate into both mycelia. A dikaryon is formed because karyogamy is delayed and every cell thus contains two nuclei. To ensure that both nuclei are transmitted to a new cell, the dikaryon forms clamp connections (Kühner 1977; Lamoure 1989). With the formation of a new cell, one of the nuclei is trapped in the clamp connection and later released into the new cell. However, not in all basidiomycetes clamp connections are formed by a dikaryon. The cultivated mushroom (*Agaricus*) for example, never forms clamp connections in a dikaryon. From a dikaryon fruiting bodies (basidiocarps) are formed. Within the cap of a basidiocarp gills with basidia are found (Fig. 2). Karyogamy takes place in the basidium and is immediately followed by meiosis. The four nuclei formed during meiosis migrate separately into one of the four spores formed on the basidium resulting in a haploid spore that after germination grows out to a monokaryon and thereby completing the cycle.

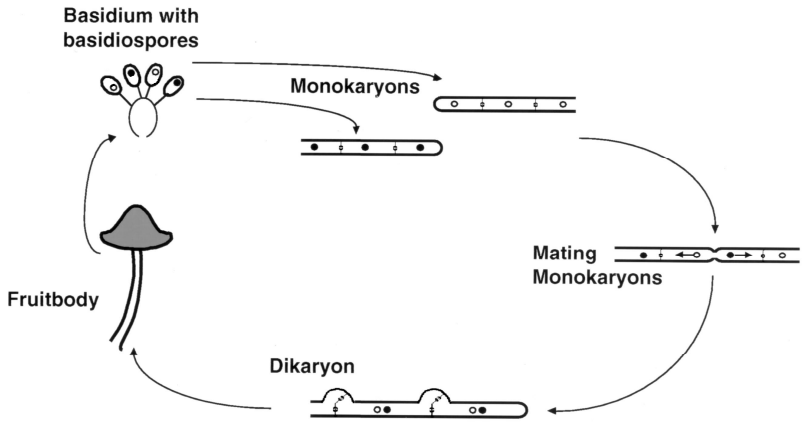

Fig. 1. Sexual reproduction cycle of a basidiomycete with clamp connections in dikaryon, meiosis in the fruiting body and thus four spores at the basidium.

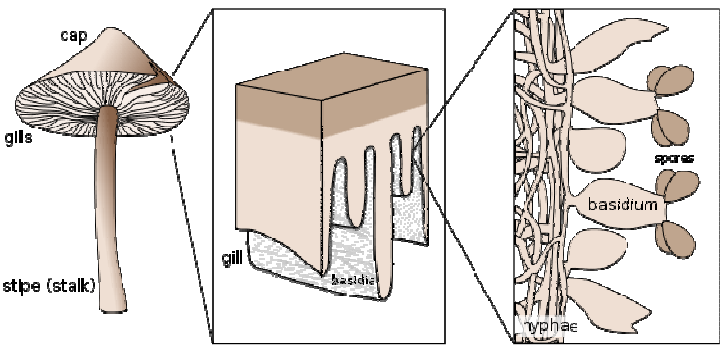

Fig. 2. A zoom in on a basidiocarp, the gills within the cap contain basidia. On the basidium four haploid spores are formed after karyogamy in the basidium and migration of the nuclei into the spores. Source: Wikipedia

Sexual reproduction is widespread in fungi and is the force behind recombination and the rising of new genotypes (Carlile 1987). Basidiomycetes however, also have the opportunity to reproduce asexually. Asexual reproduction in basidiomycetes was already described 110 years ago in *Hygrocybe*, and more fungi with monokaryotic fruiting have been described thereupon (Stahl and Esser 1976). It seems to be a rather common phenomenon in basidiomycetes that besides dikaryotic fruiting (sexual) also monokaryotic fruiting (asexual) is possible. Different terms have been used in literature for monokaryotic fruiting including, homokaryotic fruiting, parthenogenetic reproduction, apogamous, apomictic or haploid fruiting (Stahl and Esser 1976; Kühner 1977).

Aanen and Hoekstra (2007) hypothesized that a mutation in a fungus that causes asexual reproduction but would keep its ability to donate a nucleus to another monokaryotic mycelium would have a 1.5-fold advantage over the sexual reproducer. The reason is that it can reproduce 100% by cloning itself asexually and still donate a nucleus for 50% reproductive success to another mycelium, resulting in a 150% reproductive success relative to sexual reproduction. In theory such a mutation should therefore increase quickly within the population and eventually eliminate the sexual reproduction cycle.

1.2. Mycena

Within the Basidiomycetes, it has been described for the genus *Mycena* that asexual reproduction occurs (Smith 1934; Lamoure, 1960). In *Mycena galericulata* (Scop.) S.F. Gray 1821 both reproduction cycles still seem to exist together within the same species. Either the asexual reproductive system started recently, there is another (environmental) selection pressure acting upon the reproductive system or the different reproductive systems belong to separate species. Because the variation in the reproductive system of *M. galericulata* was already described 50 years ago by Lamoure (1960), the option of recent mutation is not a very likely possibility, unless the phenomenon of asexual reproduction occurs more often in evolution. Asexual reproduction could have a short-term advantage, giving it the opportunity to evolve and a long-term disadvantage keeping it in balance with the sexual reproductive system.

So there is either a balance between the short-term advantage and some unknown disadvantage, keeping the polymorphism, or the two reproductive systems are no longer interbreeding. The forms of *M. galericulata* seem to have the same morphological fruiting body in both monokaryotic and dikaryotic fruiting, although the 2-spored form has some more distinct striated lamellae at the base of the stipe and the pileus. Furthermore, it has been concluded from earlier studies that the 2-spored type and the monokaryon of the 4-spored type of *M. galericulata* are uninucleate (Kühner 1927; Lamoure 1989)

Crossing tests performed by Lamoure (1960) showed that monokaryons of the 2-spored type of *M. galericulata* are incompatible. It is possible to identify whether a mycelium is a monokaryon or dikaryon by searching for clamp connections under the microscope. A dikaryotic mycelium has clamp connections while the monokaryon has no clamps. If the two types used for crossing are incompatible they sometimes show a barrage (Lamoure 1960; Carlile 1987). From previous literature it is known that *M. galericulata* makes clamps if it is a dikaryon (Kühner 1977).

Because the strains used by Lamoure (1960) came from locations far apart, she assumed that it would be very strange if they were incompatible as a result of sharing (one of) the same alleles of the sexual compatibility genes. Therefore she concluded that the 2-spored type of *M. galericulata* has lost its sexuality and is reproducing parthenogenetic. Furthermore she found that different strains of *M. galericulata* showed a lot of variability in the way of growing, so she suggested there is

some polymorphism. Apparently Lamoure also conducted crossings between the normal 4-spored form and the uninucleate mycelium of the parthenogenetic 2-spored form (Kühner 1977). From this no secondary mycelium was obtained, however, those results have never been published.

Therefore it remains unclear what the relation is between the 2-spored and the 4-spored forms of *M. galericulata.* It was expected that the ratio in which the two forms would be present in the field would differ over time, where the 2-spored form can be found the whole growing season while the 4-spored form only occurs around October and increase in number over time (Maas Geesteranus 1977). To confirm this observation fruiting bodies were collected in the field. Furthermore, it was expected that the 2-spored was reproducing asexually and that it could no longer cross with a 4-spored strain (Lamoure 1960, Kühner 1977). Still the crosses between 4-spored and 4-spored strains should show clamp connections after crossing. To investigate this, the collected cultures were used for crosses to get insight in their compatibility. This would help to understand the relation between the 2- and 4-spored forms since compatibility is a good indication for the relatedness of the two types (Stahl and Esser 1976).

If indeed crosses show that the two forms are incompatible as suggested by Kühner (1977), several following steps are possible. A hypothesis would then be that both forms diverged during evolution and that they now belong to separate species. When this difference occurred for the first time, how often it occurred in the evolutionary history at what time they diverged and whether there is still some exchange between the populations will be questions to be answered. Hopefully, with the investigation of *M. galericulata,* it will be possible to get a better insight in the complex reproduction system of fungi. It might give a better insight on what levels selection takes place and provide better knowledge about the evolution of sexual reproduction and the trade-offs between asexual and sexual reproduction. Fungi can create a first understanding about reproductive systems and with this general insight can be obtained for asexual and sexual reproduction in higher organisms. In addition, it will be possible to explore the phylogeny of the genus *Mycena* with new detailed genetic information.

2. Material and Methods

2.1. Field collection

Mycena galericulata (Scop.) S.F. Gray 1821, Class: Basidiomycetes, Family: Mycenaceae, Genus: *Mycena*. This fungus species is also known as the Common Bonnet, it grows on decaying wood from spring to autumn. It is a very common and widely distributed species. Two forms can be distinguished, the 2- and 4-spored type (Fig. 3, Left and Middle). The 2-spored type seems to have an asexual reproduction cycle (Fig. 3, Right) as described by Lamoure (1960).

For many *Mycena* species it is difficult to identify them. However *M. galericulata* has some more distinct characteristics than most *Mycenae*. *M. galericulata* has a cap (Ø 2-6 cm) which is clearly striated, particularly at the margin. Also at the base of the plates a striated pattern is visible, which is more distinct for the 2-spored type. The cap's colour varies from greyish brown to dark brown and the shape is bell-like with an umbo, often with a mealy odour and taste. The stipe (2-10 cm x 2-8 mm) is hollow, white, tough and thin, without a veil and with a root-like base. The gills are linked to the stem by the whole depth of the lamina. The spore print is white and the gills pink at maturity (Aanen and Kuyper, personal communication; Wikipedia).

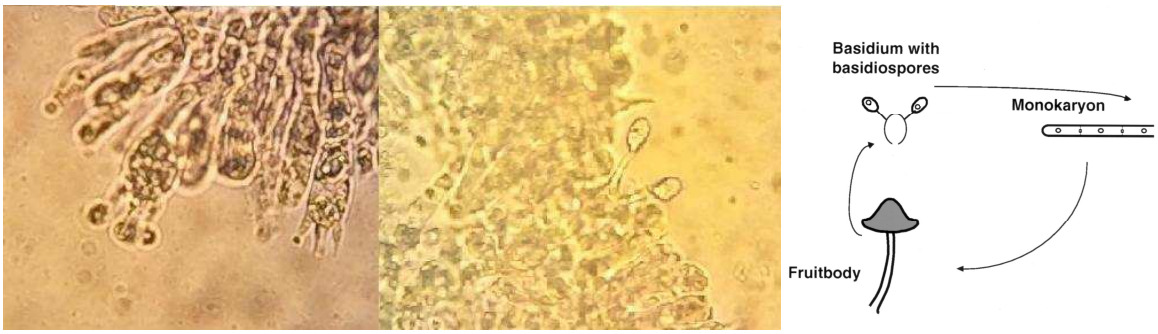

Fig. 3. Left: Basidium with 4 spores, Middle: Basidium with 2 spores, Right: Asexual reproduction cycle of *M. galericulata* with no clamp connections, mitosis in the fruiting body and thus two spores at the basidium.

Fig. 4. Map of sampling locations of *Mycena galericulata* in the Netherlands. All samples were collected in the period from September-November 2010. Green pushpins are sample sites visited one time, the sites at the red pushpins have been sampled more than once.

During the period from September 15th until November 25th 2010, basidiocarps (n=327) from *Mycena galericulata* where collected in the surroundings of Wageningen and Eindhoven in the Netherlands (Fig. 4 and Appendix I). At least every week some samples were collected at a different site as well as a repetition at the same site every week. The samples were transported to the lab in plastic containers. *M. galericulata* is a very common and widely distributed species that mainly grows on decaying wood from deciduous trees, sometimes on Douglas fir trees and can only be found on coniferous wood in area's with a high nitrogen deposition (Kuyper, personal communication). If more

basidiocarps were present on a tree trunk only one was taken as sample. For all individuals it was determined whether they where 2- or 4-spored. A small piece of the lamellae was put on a microscope slide with a coverslip on top. With a magnification of 10x it was possible to distinguish either four or two spores on the basidia (Fig.3). From every sample collected in the field a piece of the basidiocarp was stored at -20 ^{o}C in a 1.5 mL Eppendorf tube with 96% alcohol.

Single spore cultures were isolated by placing the cap of a mature basidiocarp on the inside of the cover of a small (Ø 51mm) Petri dish containing a 5mm layer of malt-yeast agar (MYA; 20 g/L malt extract, 2g/L yeast extract and 15 g/L agar). After 20-30 min of sporulation the cover was removed and a fresh cover was placed over the Petri Dish. After 14-21 days the germinated spores were cut out of the agar and inoculated on a fresh Petri Dish with MYA. All cultures were incubated at room temperature (19 ^{o}C) and taped with parafilm to prevent the cultures from drying out (Appendix V for more information on the obtained cultures).

2.2. Crossings

Crosses were performed in small Petri dishes (Ø 51mm). From the mycelium of a single spore culture that grew on MYA a small piece of approximately 2x2mm was cut out of the agar. The two pieces from different single spore cultures were inoculated approximately 0.3cm apart in the centre of a new Petri dish with MYA.

After 7-14 days a small piece of 2x2mm was cut from the contact zone of each tested pair and transferred to fresh Petri dishes with cellophane on top of a thin layer of MYA to increase the visibility of the clamp connections. After 4-10 days the mycelium was examined for clamp connections by putting the whole Petri dish upside down under the microscope. Crosses were performed by inoculating 2-spored with 2-spored (n=10), 4-spored with 4-spored (n=45) and 2-spored with 4-spored (n=114) single spore cultures.

2.3. Sequence analysis

DNA was extracted from the basidiocarps stored at the -20 ^{o}C or from single spores cultured on cellophane. For the DNA extraction from basidiocarps approximately 0.5cm^2 from the lamellae was cut off and air dried on filter paper. For single spores cultures, the mycelium was grown on cellophane. After 6-14 days of growth the mycelium was scraped from the cellophane. The dried piece from the lamellae, or the scraped material from the cellophane was put into an Eppendorf tube in which 5-10 glass balls were added. The tube was frozen in liquid nitrogen and subsequently shaken for 10 seconds using Silomat S5, after this, the freezing and shaking was repeated. To the Eppendorf tube 100 µl 5% Chelex and 10 µl proteinase K was added and afterwards incubated for 30 min at 56 ^{o}C and 10 min at 95 ^{o}C subsequently. Afterwards the tube was centrifuged at 12,000rpm, the DNA could then be collected from the supernatant.

Gene fragments were used from: the internal transcribed spacer (ITS) region, partial sequences of the large nuclear RNA polymerase subunit RPB2, sequences of the nuclear Elongation Factor 1 alpha (EF1α) and primer sequences relative to the β-tubulin sequence B36F. The following primer combinations were used: ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3'), bRPB2-6F (5'-TGGGGYATGGTNTGYCCYGC-3') and bRPB2-7.1R (5'-CCCATRGCYTGYTTMCCCATDGC-3') and EF595F (5'-CGTGACTTCATCAAGAACATG-3') in combination with EF1160R (5'-CCGATCTTGTAGACGTCCTG-3') (De Fine Light et. al. 2006) and finally B36F (5'-CACCCACTCCCTCGGTGGTG-3') in combination with B12R (5'-CATGAAGAAGTGAAGACGCGGGAA-3') (Thon and Royse 1999).

PRCs were run on a BioRad iCycler or on a BioRad MyCycler (thermal cycler) PCR was performed in 25-µl reactions containing 5 µl 5x GoTaq Buffer, 1 µl 10nHdNTP, 1 µl of each primer, 0.1 µl GoTaq, 2 µl magnesium, 2 µl DNA (1:10) and 12,9 µl MQH_2O. PCR program for ITS primers (ITS1-F and ITS4-R) consisted of an initial denaturing step of 5 min at 94 °C followed by 35 cycles (60 s at 94 °C, 60 s at 53 °C and 60 s at 72 °C) finished by a final elongation step of 10 min at 72 °C and a dwell temperature of 4 °C. The program for B36F and B12R primers consisted of an initial denaturing step of 2 min at 95 °C followed by 35 cycles (45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C) finished by a final elongation step of 7 min at 72 °C and a dwell temperature of 15 °C. For the rRPB2 primers (bRPB2-6F and bRPB2-7.1R) another program was used. Here the initial denaturing step of 4 min at 94 °C was followed by 35 cycles (60 s at 95 °C, 60 s at 58 °C and 60 s at 72 °C) finished by a final elongation step of 10 min at 72 °C and a dwell temperature of 7 °C. Finally the EF595F and EF1160R primers were used, the program for consisted of an initial denaturing step of 5 min at 94 °C followed by 35 cycles (60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C) finished by a final elongation step of 10 min at 72 °C and a dwell temperature of 4 °C. The PCR products were checked on a 1.0% agarose gel with GelRed and a GeneRuler 100bp ready-to-use (1:4) DNA ladder, before purification with a GenElute PCR Clean-Up Kit from SIGMA Life Science. Sequencing was performed by Eurofins MWG operon. For all gene fragments the purified PCR product was sequenced.

Double peaks with similar height were present in the sequence chromatogram at multiple positions. To retrieve the haplotype of those sequences the single spore cultures were sequenced as described above. A Chi-square test was performed to see if there was a difference between the observed and expected number of sequences with double peaks (heterozygotes), in the 2-spored and 4-spored (Appendix III).

2.4. Phylogenetic analysis

DNA sequences were checked and corrected using ChromasPro version 1.5 (Technelysium Pty Ltd, 2009, Free trial). An alignment was performed using MAFFT (online version) using standard settings. A neighbor-joining analysis was performed on the aligned sequences using MEGA. Gaps were included as difference and clade stability was tested with 100 bootstrap replications with default settings from MAFFT online version. For the analysis the haplotypes of the ITS1-F sequences were used from both 2-spored (n=55) and 4-spored (n=20) (Appendix IV).

2.5. Growth experiment

The 4-spored single spore cultures 58:1, 91:2, 119:2, 113:2, 123:2, 130:2 and 255:1 (n=7) were used for this experiment. From the 2-spored single spore cultures strains 62:2, 63:2, 64:2, 92:1, 96:2, 97:2 and 99:2 (n=7) were used (Appendix II and V). Finally the dikaryons formed by crosses between 58:1&107:1, 115:1&58:1 and 115:1&107:1 (n=3) were used for this experiment. For all samples used in this experiment a small piece of 2x2mm was cut from a plate with mycelium and inoculated on a fresh Petri Dish with MYA on the 21st of December. All strains were inoculated in duplicate and one of each sample was incubated at 6 °C and the other at 25 °C. The diameter was measured (in mm) after 13 days, 17 days, 27 days and 34 days. In addition the growth of the 6 °C treatment was measured one more time after 64 days (Appendix II).

2.6. Fluorescence pictures

Nuclei of different cultures where counterstained with DAPI (4',6-diamidine-2-phenylindole) before pictures were taken under a fluorescence microscope (Axioplan-2, Carl-Zeiss GmbH). After staining the genetic material was visible as fluorescent light blue under the microscope. The pictures were merged with MetaMorph software (MetaMorph Imaging System, Universal Imaging Corp., PA). The contrast and brightness were optimized using the same computer program.

3. Results

3.1. Field collection

A total of 327 basidiocarps were collected from which 280 could be identified as either 2- or 4-spored type (Appendix I). The remaining 47 collected basidiocarps were either identified as the wrong species, no basidia with sterigmata or spores could be found, or they seemed to have 3-spored basidia as well. The percentage of 4-spored basidiocarps found in the field increases during the autumn months from 3% in September (n=38) to 24% in October (n=169) and 26% in November (n=73) as can be seen in Fig. 5. In December no basidiocarps were collected because of frost and snow.

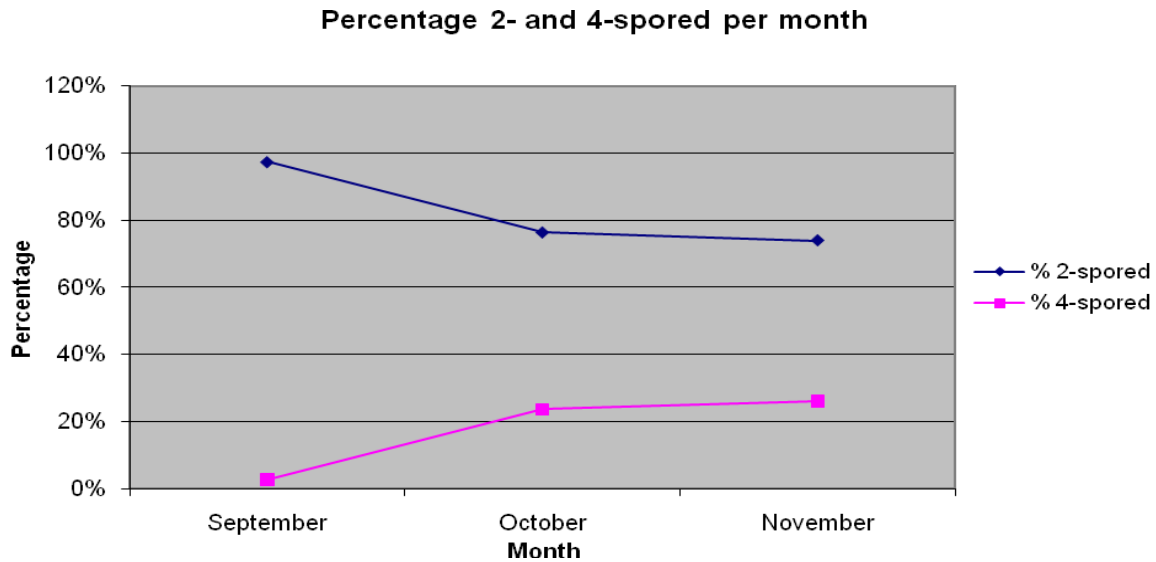

Fig. 5. Percentage of 2-spored (diamonds) and 4-spored (squares) fruiting bodies found per month in the period September 15th until November 25th 2010, basidiocarps (n=280).

3.2. Crossings

The compatibility tests show that single spore cultures from a 4-spored basidiocarp almost always show clamp connections when crossed with another single spore culture from a 4-spored basidiocarp (Fig. 7). In total 39 from the 45 crosses between 4-spored showed clamp connections. Furthermore, all reciprocal 2-spored crossings give a negative result. In total 10 crossings from which zero showed clamps (Fig. 6). More importantly crossings between 2-spored single spore cultures and 4-spored single spore cultures never showed clamp connections (Fig. 7). Those results are based on 114 crossings where zero showed clamps in the contact zone.

	61:1 (2spr)	A:1 (2spr)	1:1 (2spr)	121:1 (2spr)
61:1 (2spr)		-	-	-
A:1 (2spr)	-		-	-
1:1 (2spr)	-	-		-
121:1 (2spr)	-	-	-	

Fig. 6. Crossing between single spore cultures from 2-spored basidiocarps. The minus symbol indicates that no clamp connections have been found in the contact zone.

(4spr)	107:1 (4spr)	119:2 (4spr)	61:2 (2spr)	171:1 (2spr)	163:2 (2spr)	222 (2spr)
58:1	+	+	-	-	-	-
91:2	+	+	-	-	-	-
113:2	+	+	-	-	-	-
115:2	+	+	-	-	-	-
119:2	+		-	-	-	-
123:1	+	+	-	-		
123:2	+	+	-	-	-	-
130:1	+	+	-	-	-	-
130:2	+	+	-	-		
130:3	+	+	-	-		
131:2	+	+	-	-	-	-
131:3	+	+	-	-		
131:4	+	+/-	-	-	-	-
170	+	+	-	-	-	-
217:1	+	+	-	-	-	-
239:1	+	-	-	-	-	-
249:1	+	+	-	-	-	-
296:1		+	-	-	-	-

Fig. 7. Crosses between single spore cultures from 2-spored and 4-spored basidiocarps and crosses where 4-spored and 4-spored were crossed. The minus symbol indicates that no clamp connections have been found in the contact zone. The plus symbol indicates that clearly visible clamp connections have been found in the mycelium picked up from the contact zone. A plus/minus symbol means it was not clear whether the bulges where true clamp connections. The empty grey boxes represent crosses that have not been performed.

3.3. Growth experiment

All cultures have a slower growth rate at 6 °C (growth rates between 0.24-0.43 mm/day) than at 25 °C (growth rates between 0.79-1.38 mm/day). For both temperature treatments the dikaryon seems to grow fastest, followed by the 2-spored single spore culture mycelium. The single spore culture from the 4-spored basidiocarps always has the slowest growth at both temperatures (Fig. 8 and Appendix II).

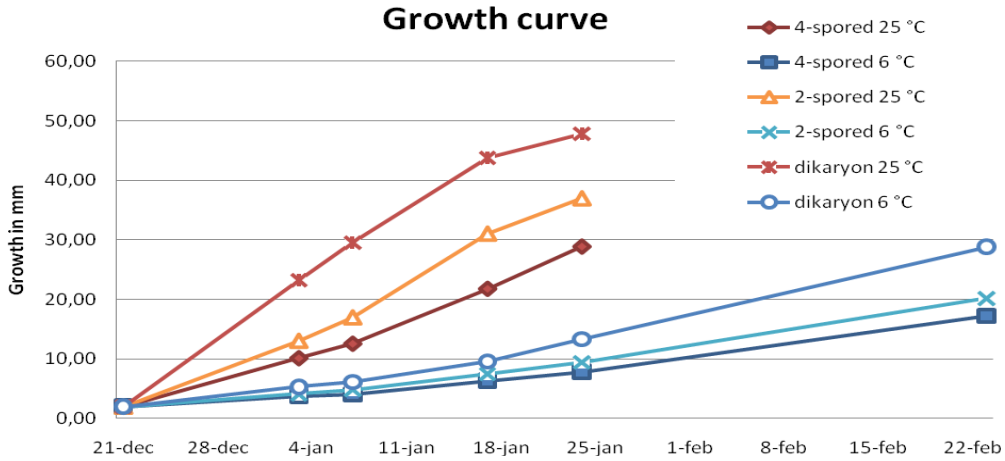

Fig. 8. Growth rate (in mm) at either 6 °C or 25 °C for 4-spored single spore cultures (n=7), 2-spored single spore cultures (n=7) and dikaryons from 4-spored crosses (n=3). The growth was measured after 13 days, 17 days, 27 days and 34 days. In addition the growth of the 6 °C treatment was measured one more time after 64 days.

3.4. Sequences

The phylogenetic analysis shows that the 2- and 4-spored basidiocarps form a mixed population. The results show that no distinct clades can be found only with 2-spored (n=55) or only with 4-spored (n=20) individuals (Fig. 9). Furthermore, the haplotypes generated from one genotype are also spread over the tree, indicating that the whole population is still mixed (Fig. 9 and Appendix IV).

In all four primers, some of the 2-spored samples obtained from a basidiocarp showed a double signal (Appendix III). Single spore cultures from 2-spored samples were selected, for those strains that had showed to give a double signal in the ITS sequence obtained from the basidiocarp (191:1, 315:1 and 327:2). The ITS sequences from all three single spore cultures showed a single signal. Furthermore, several single spore cultures from the 4-spored strains were sequenced. Out of 19 single spore cultures, three of them (239:1, 217:1 and 298:1) showed a double signal.

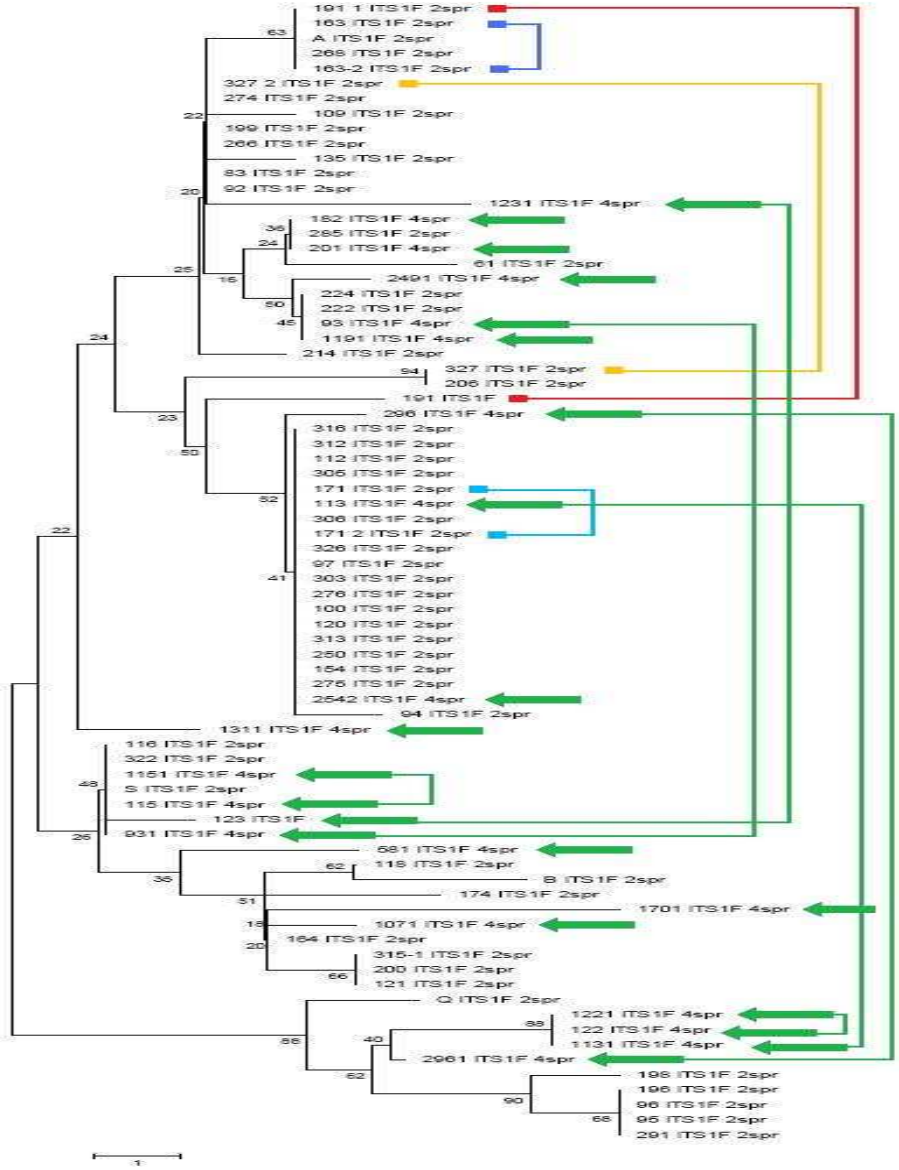

Fig. 9. Phylogenetic tree from ITS-sequences obtained from 2-spored (n=55) and 4-spored (n=20) individuals, indicated with a green arrow. All samples used in the analysis were haplotypes from the ITS locus, which acts as a single gene copy. From 9 individuals both haplotypes were known and used in the analysis. The haplotypes that belong to the same genotype are connected with lines. Bootstrap was used to give an indication for the credibility of the tree. The 1 step index equals 1 base difference in the sequence.

3.5. X^2-test

For all primers (ITS1-F, bRPB2-6F, EF595F and B36F) sequences with double signals were found in the 2-spored samples. A Chi-square test was performed to see whether the number of heterozygous (double signal) strains differs between the 2- and 4-spored (Appendix III). For the ITS sequences from the 2-spored, 12 heterozygous and 45 homozygous strains were counted, while for the 4-spored ITS sequences 16 heterozygous and 5 homozygous strains were found. The Chi-square test was significant ($p<0,005$). For the other three primers however, these results were not significant. In a total of 15 samples from which seven 2-spored and eight 4-spored, no difference could be found between the types in the amount of heterozygous and homozygous strains (Appendix III).

3.6. Fluorescence pictures

The pictures taken with the fluorescence microscope clearly show that a single spore culture from a 4-spored basidiocarp only has one nucleus per cell (Fig. 10), while the dikaryon, with the clamp connections, has two nuclei per cell (Fig. 11). Furthermore, the single spore culture from a 2-spored basidiocarp also possesses just one nucleus, but it seems to be larger than the nucleus in a 4-spored cell from the mycelium (Fig. 12). A spore from a 2-spored basidiocarp shows only one nucleus on the fluorescence picture (Fig. 13).

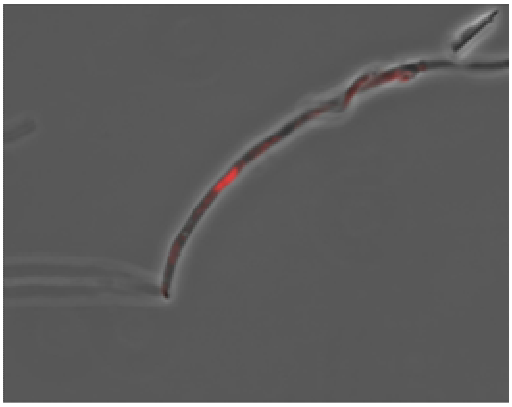

Fig. 10. Chromosomes from a 4-spored single spore culture (170) stained with DAPI.

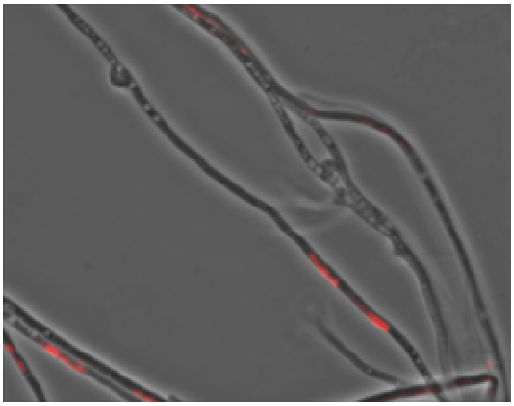

Fig. 11. Chromosomes from a dikaryon from a 4-spored culture that crossed with itself (58:7) stained with DAPI.

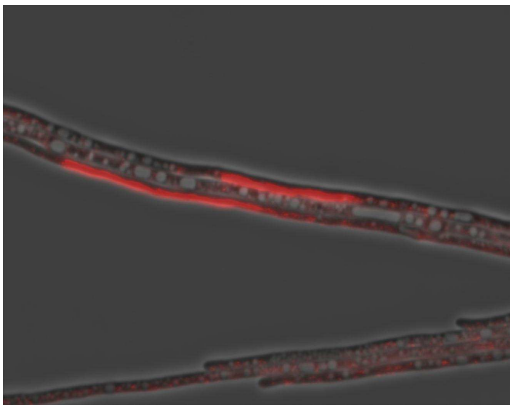

Fig. 12. Chromosomes from a 2-spored single spore culture (222) stained with DAPI.

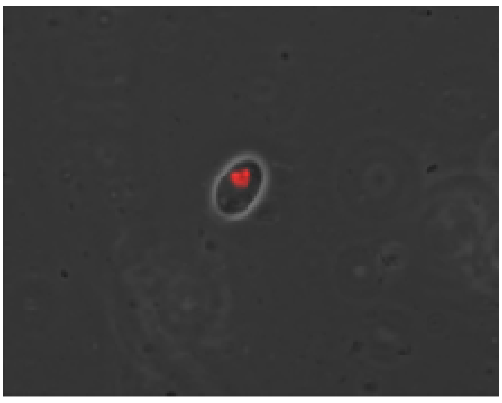

Fig. 13. Chromosomes from a spore originative from a 2-spored basidiocarp (61) stained with DAPI.

5. Discussion

5.1. Collection

The results obtained from the field collection differ from the suggested ratio between the 2- and 4-spored types from Maas Geesteranus (1977). He found a much stronger increase in 4-spored fruiting bodies, up to 46% in November and even 58% in December, while the results from this research only show an increase up to 26% in November. Unfortunately, no field collections could be done in December because of early frost and snowfall. There are two possible explanations for the difference in results. First, the collection Maas Geesteranus used for his research was only a small sample size (n=53) with a maximum of 13 samples in one month. Second, the samples were obtained from herbaria. As collectors are prone to keep exceptions rather than ordinary fruiting bodies, the suggested increase in the 4-spored type could be biased by which samples were kept for storage in the collection. Especially because the 4-spored type is less obvious recognizable as a typical *M. galericulata*. Therefore, although the major conclusion is the same for both studies, the results found in this research however, seem more reliable.

An interesting observation was made by Ronikier and Aronsen (2007). They found that in *M. clavata*, where also 2- and 4-spored forms are present, there is no correlation between the number of spores and time of collection. However, they did notice that the 4-spored form was found more frequently in central and southern Europe while it was almost absent in Scandinavian countries.

The sexual reproduction cycle, and thus recombination, increases the genetic variation and the survival chances in harsh conditions at the end of the growing season. Asexual reproduction often produces fast germinating spores so they can spread rapidly (Aanen and Hoekstra 2007). This phenomenon of switching from asexual to sexual reproduction is also found in other organisms like aphids (Treisman 1976, Ward et. al. 1984) and might be an explanation why, through some sort of induction, *M. galericulata* produces more 4-spored forms at the end of the growing season.

Finally, from the 327 basidiocarps that were collected for this research, only 280 could be identified as 2- or 4-spored. For some it was impossible to find the basidia with spores because they were too old. For some however it looked like they also had three spores besides the usual two or four spores.

5.2. Crossings

The fact that only clamp connections are found from crosses between 4-spored forms suggests that the 2- and 4-spored forms belong to separate populations that are no longer able to cross. The results from the phylogenetic analysis however, at first seem to contradict this hypothesis. Both the 2-spored and the 4-spored forms are completely scattered between the clades within the phylogenetic tree. In addition, the two haplotypes that were obtained from one genotype are also scattered over different clades within the tree (Fig. 9). An explanation for this contradicting result could be that crosses between 2- and 4-spored forms need to be induced (by for example; temperature, light availability, nutrients or moisture) before they are able to cross, that the in vitro experiments do not reflect the reality in nature, or that there is formation of polyploidy strains which occurs frequently. Both the first and second explanations seem very unlikely since it was easy to successfully obtain crosses between 4-spored cultures. However, it could be possible that the frequent occurrence of polyploidy prevents the different types from crossing while they are still

found to form a highly mixed phylogenetic tree. How this would explain the rest of the results will be explained in the next section.

Another possibility could be that the mycelia are still compatible and cross, but do not show clamp connections as is known for some other Basidiomycetes (Smith 1934; Boidin 1986; Petersen and Methven 1994). Then however, it remains difficult to understand why crosses between 4-spored cultures almost always show clamps.

5.3. Sequences

The bootstrap value for the whole phylogenetic tree is low, except for the bottom branch (88%). This was expected because there are only a few differences between the sequences resulting in low bootstrap values (Fig. 9).

As stated before, the 2- and 4-spored forms do not seem to be separated populations, based on the ITS sequences. Yet, no clamp connections could be found when crossing either 2- with 2-spored or 2- with 4-spored. Furthermore, in all four primers, some of the 2-spored samples obtained from a basidiocarp showed a double signal. Those basidiocarps must therefore be heterozygous.

Several single spore cultures from the 4-spored strains were sequenced. Remarkable was that out of 19 single spore cultures, three of them (239:1, 217:1 and 298:1) showed a double signal. It could be that these single spore cultures were actually no single spore cultures and already crossed with itself before isolated as such. However, none of them had clamp connections when examined under the microscope. Another explanation could be that the meiosis was incomplete. The nuclei where incomplete meiosis occurred could fuse inside one spore and become a diploid (2-spored) type. These cultures would have a larger nucleus and would no longer cross with monokaryons from the 4-spored type. This is however in conflict with the results from the crossings, where all three cultures showed clamp connections in the crossings.

All things considered, there seem to be two options that would explain the observations made so far. The 2-spored form of *M. galericulata* is either secondary homothallic, or it is a diploid. However, for both explanations there is also evidence against these hypotheses.

First, secondary homothallism could explain the two spores found in some basidiocarps, since two nuclei are transferred into one spore, resulting in only two spores as a result of a normal meiosis (Petersen 1995). Furthermore, this would also explain the difference found in the amount of heterozygous strains between the 2- and 4-spored. The process of secondary homothallism would bring together more of the similar nuclei, resulting in a higher ratio of homozygotes in the 2-spored strains. However, no difference between heterozygous strains was found for the other three primer combinations. This would, in the event of secondary homothallism, also result in a higher frequency of homozygotes. Secondly the fluorescent picture from the spore obtained from a 2-spored basidiocarp only showed one nucleus. This could in turn be explained by secondary homothallism followed by it becoming diploid, or because a cross resulted in a diploid mycelium, which was the other explanation.

Another explanation for a 4-spored type becoming diploid could be the following. In some cases it was found that also 3-spored basidia were present. In such 3-spored forms it is imaginable that one spore contains two nuclei and that these nuclei fuse in the spore to become a diploid. Since the observation of 3-spored basidia has been made regularly it would mean that the formation of diploid spores happens often. This would also explain why the phylogenetic tree shows a mixed population and why the two types do not cross anymore. The fact that they do not cross anymore, does however not mean that no exchange in genetic material can occur through for example the

Buller phenomenon or parasexuality (Aanen and Kuyper, personal communication). A cross between 2- and 4-spored types would result in the exchange of genetic material but would however not form a dikaryon.

If the 2-spored type would be a diploid, this would explain that it does not show clamp connections after crossing and it would be an explanation for the heterozygous strains that can be found in the 2-spored form. In addition the reduced number of heterozygotes found in the 2-spored form could in the event of diploidy be explained by concerted evolution within the nucleus (James et. al. 2001; Aanen et al., 2001). Furthermore, the enlarged nucleus visible under the fluorescent microscope in a 2-spored single spore culture (Fig. 12) could confirm that there is more genetic material present in the nucleus than in the 4-spored monokaryon, which would point towards a diploid. Also the fact that a 2-spored single spore culture seems to grow faster than a 4-spored monokaryon seems in line with the assumption of diploidy.

However, diploidy seems to be a very rare event in Basidiomycetes as it is only found in the honey fungus, *Armillaria* so far. This might be comparable to the situation of *M. galericulata*, although *Armillaria* has clamp connections on the basidia, while those cannot be found on the basidia of *M. galericulata* (Aanen and Kuyper, personal communication). Furthermore diploidy does not explain why crossings between 2- and 4-spored single spore cultures never show clamp connections, because it is known from *Armillaria* that a diploid can still donate a nucleus to another monokaryon via a Buller-interaction (Aanen, personal communication).

Since diploidy seemed to explain the relation between the 2- and 4-spored forms, single spore cultures from the 2-spored basidiocarps were sequenced to confirm that they would also give a double signal. Single spore cultures were selected, for those strains that had showed to be heterozygous for ITS sequence in the basidiocarp (191:1, 315:1 and 327:2). Surprisingly, all three the ITS sequences from the single spore cultures gave a single signal. This might be explained by very fast concerted evolution within the nucleus, but because such fast process is highly unlikely the explanation that the 2-spored type would be a diploid has to be rejected.

As last option, the combination of both secondary homothallism and diploidy could explain the homozygous strains obtained from a heterozygous basidiocarp. Similar nuclei could end up in the same spore, resulting in a homozygous diploid mycelium with more genetic material then a monokaryon. However, if this would be the existing system, than all the 2-spored forms should become homozygous for the sequences from all primer combinations. Summarizing, the hypothesis that the 2-spored form is a diploid seems to explain most of the results found so far. However, there is some evidence that contradicts this hypothesis. Furthermore it could be argued what the advantages of diploidy would be compared to being dikaryotic, since a dikaryon is still able to donate a nucleus, while the diploid could lose such advantage.

5.4. Future research

To completely understand the relation between the 2- and 4-spored forms more research is needed. A next step would be to see whether the single spore cultures from the 2-spored type also give a single signal with the primers EF1α, B36 and bRPB2. This would confirm that the single spore culture from a 2-spored type is not a diploid. If this conclusion seems to be correct more detailed information should be gathered about how the two genomes from the basidiocarp come together again after the single genome spores have been formed and what exactly happens within the basidia.

Furthermore, crossings between 2-spored cultures can be made to further investigate what happens at the contact zone. Fluorescence pictures could be taken from the contact zone to see whether a dikaryon is formed without clamp connections and in addition the contact zone could be sequenced to confirm or reject the hypothesis that a diploid is formed when the 2-spored cultures are crossed. In addition to this, the mycelium from the contact zone could be used in another growth experiment to see how this is related to a 4-spored dikaryon and a 2-spored single spore culture. If a stable diploid is formed, it is expected that this should have an advantage over the dikaryon. Thus it could be argued that the mycelium from the contact zone would grow even faster than the dikaryon from the 4-spored type. Or other traits could be looked at.

If the 2-spored is indeed a diploid, it is expected that a genetic difference can be found between the two forms. For this, the use of other primers than the ITS are useful. Using more markers can either confirm or reject the view based on the ITS results and might increase the accuracy with which conclusions can be drawn.

It is known that Mycenae is very polyphyletic and has to be subdivided (Prillinger 1982; Kuyper, personal communication). The appearance of both 2- and 4-spored forms is a rather common phenomenon in Mycenae and should therefore have a more fundamental evolutionary explanation. Further research should therefore not only look at *M. galericulata*, also other species of Mycenae should be investigated.

Furthermore, it would be nice if it is possible to fruit the fungus in the lab. It should than be possible to investigate which fruiting bodies originate from which mycelium and how they are related. For this Millichamp could be used, which is also used for the cultivation of mushrooms (Baars, personal communication).

Another component that can be looked to in further detail is the presence of more 4-spored forms later in the season. As stated before, the phenomenon of switching from asexual during the growing season to sexual reproduction before a survival period is present in other organisms. If the mycelium would have a hard time surviving in the winter period this could be a plausible explanation. Unfortunately, not much is known about the survival of the mycelium through the year and this could therefore be an interesting component to look at in further detail.

4. Conclusion

It is still not completely understood what the relation is between the 2- and 4-spored forms. But some conclusions can be drawn from the results. First, the ratio of the 4-spored form indeed increases at the end of the growing season. Secondly, in line with our expectation, the only successful cross with clamp connections was obtained from 4- with 4-spored strains. Therefore it was expected that the 2- and 4-spored forms would belong to separate populations. In contradiction to this expectation the phylogenetic analysis, based on ITS sequences, did not show any difference between the two forms and suggests that it is still a mixed population. Therefore, it remains unclear what the exact relation is between the 2- and 4-spored forms.

In addition, several sequences from the 2-spored type showed double peaks. This was found in four different primers which strongly underpins the conclusion that the 2-spored form is heterozygous. Furthermore, for the ITS sequences the number of heterozygous strains that can be found in the 2- and 4-spored type differs, with a much higher percentage of 4-spored strains being heterozygous compared to the percentage heterozygous strains found in the 2-spored strains ($p<0,005$). This however, is not true for the other primer combinations used to investigate sequences from *M. galericulata*. Finally, the growing experiment suggests that a dikaryon from the 4-spored form has the fastest growth, followed by the single spore culture from the 2-spored, where the single spore culture from the 4-spored form has the slowest growth. This conclusion can be drawn for every temperature in the range from 6 °C to 25 ° C.

In conclusion, the relationship between the 2- and 4-spored forms of *M. galericulata* is still not completely understood. Although it is clear that only 4-spored show clamp connections after crossing, the sequencing data suggest that the population is still genetically mixed. Furthermore, in the 2-spored form heterozygous individuals occur, but they are less common than in the 4-spored form. Secondary homothallism and diploidy are suggested as possible explanations for these results, for both hypotheses however, counter arguments can be formulated underpinned with clear results obtained during this research.

It is obvious that more research has to be done to truly understand the reproductive system that is used by *M. galericulata*. Hopefully, in the future the research on *M. galericulata* will provide better insight in the complex reproduction system of fungi.

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Appendix I

Field collection

Date	2-spored	% 2-spored	4-spored	% 4-spored	Not sure	Total useable
15-sep	2	100%	0	0%	0	2
23-sep	18	100%	0	0%	3	18
28-sep	1	100%	0	0%	0	1
29-sep	8	100%	0	0%	2	8
30-sep	8	89%	1	11%	12	9
1-okt	7	100%	0	0%	8	7
4-okt	3	43%	4	57%	0	7
5-okt	6	75%	2	25%	0	8
8-okt	26	74%	9	26%	4	35
11-okt	2	40%	3	60%	0	5
12-okt	8	89%	1	11%	0	9
14-okt	9	53%	8	47%	0	17
18-okt	10	91%	1	9%	1	11
21-okt	37	90%	4	10%	1	41
28-okt	21	72%	8	28%	4	29
1-nov	0	0%	1	100%	0	1
5-nov	15	63%	9	38%	2	24
11-nov	16	89%	2	11%	2	18
18-nov	9	56%	7	44%	0	16
22-nov	3	100%	0	0%	1	3
25-nov	11	100%	0	0%	2	11
	220		**60**		**42**	280
Per week						
Date	**2-spored**	**% 2-spored**	**4-spored**	**% 4-spored**	**Not sure**	**Total useable**
37	2	100%	0	0%	0	2
38	17	100%	0	0%	3	17
39	25	96%	1	4%	22	26
40	35	70%	15	30%	4	50
41	19	61%	12	39%	0	31
42	47	90%	5	10%	2	52
43	21	72%	8	28%	4	29
44	15	60%	10	40%	2	25
45	16	89%	2	11%	2	18
46	9	56%	7	44%	0	16
47	14	100%	0	0%	3	14
48	**NO COLLECTIE BECAUSE OF SNOW AND FROST**					
49	**NO COLLECTIE BECAUSE OF SNOW AND FROST**					
50	**NO COLLECTIE BECAUSE OF SNOW AND FROST**					
	220		**60**		**42**	280
Per Month	**2-spored**	**% 2-spored**	**4-spored**	**% 4-spored**	**Not sure**	**Total useable**
September	37	97%	1	3%	17	38
October	129	76%	40	24%	18	169
November	54	74%	19	26%	7	73
December	**NO COLLECTIE BECAUSE OF SNOW AND FROST**					0

Appendix II

Growth experiment started at December 21st 2010

	3-jan		7-jan		17-jan		24-jan		23-feb	
	25 degrees	6 degrees	25 degrees	6 degrees	25 degrees	6 degrees	25 degrees	6 degrees	25 degrees	6 degrees
4 spored										
113:2	7,75	3,5	8,5	4,25	17,75	8	x	10		17
123:2	13	3,75	17	4,75	29,5	8,25	37,5	11		19,5
130:2	11	4,25	12,5	4,75	22,75	8,5	30	10,75		19,75
255:1	8	3	10,5	3	21	4,5	28,75	5,25		14,25
58:1	7,25	3	9,75	3	9,75	3	14,5	3		11,5
119:2	11	4,5	13	4,5	22,75	6,25	27,5	7		19,25
91:2	13	4	16,75	4	29	5,75	35	7,75		19,25
Average	**10,14**	**3,71**	**12,57**	**4,04**	**21,79**	**6,32**	**28,88**	**7,82**	**0,00**	**17,21**
2 spored										
92:1	6,5	3,25	7,75	3,25	13	4,5	15,5	6		11,5
64:2	19,25	5	24	7	44,5	11,25	52	14		30
63:2	18,25	4,5	24,25	5,25	44,5	7,25	52	10,5		27,5
62:2	13	3,5	16,75	4,5	31,5	7	40,5	9		19,5
99:2	15,5	4	22,75	4,75	42,75	7,5	46	10		21,75
97:2	9	4	10,5	4	15,25	6	17,5	6		12,5
96:2	10	4,75	13	5,25	26	8,75	35,5	10,5		18,5
Average	**13,07**	**4,14**	**17,00**	**4,86**	**31,07**	**7,46**	**37,00**	**9,43**	**0,00**	**20,18**
Dikaryon										
58:1+107:1	21,25	4,5	27,75	5,75	43,75	9,25	47,5	12,5		27
115:1+58:1	18	5	24,25	5,75	39	9,5	44	12,5		28,25
115:1+107:1	30,5	6,5	36,5	7	48,5	10	52	15		31,25
Average	**23,25**	**5,33**	**29,50**	**6,17**	**43,75**	**9,58**	**47,83**	**13,33**	**0,00**	**28,83**
Total Average	**15,49**	**4,40**	**19,69**	**5,02**	**32,20**	**7,79**	**37,90**	**10,19**	**0,00**	**22,08**

Appendix III

X^2-test

Observed ITS1-F			**Expected ITS1-F**				
	2spr	**4spr**		**2spr**	**4spr**	**X2**	**12,99678**
Double	12	16	**Double**	20,46	7,54	**df**	**1**
Single	45	5	**Single**	36,54	13,46	**p value**	**0,000312**
Total	57	21	**Total**	57,00	21,00		

Observed EF1160R			**Expected EF1160R**				
	2spr	**4spr**		**2spr**	**4spr**	**X2**	**0,005952**
Double	2	5	**Double**	1,91	5,09	**df**	**1**
Single	1	3	**Single**	1,09	2,91	**p-value**	**0,938503**
Total	3	8	**Total**	3,00	8,00		

Observed B36F			**Expected B36F**				
	2spr	**4spr**		**2spr**	**4spr**	**X2**	**0,081633**
Double	6	8	**Double**	6,53	7,47	**df**	**1**
Single	1	0	**Single**	0,47	0,53	**p-value**	**0,775097**
Total	7	8	**Total**	7,00	8,00		

Observed rRPB2F			**Expected rRPB2F**				
	2spr	**4spr**		**2spr**	**4spr**	**X2**	**0,040816**
Double	3	4	**Double**	3,27	3,73	**df**	**1**
Single	4	4	**Single**	3,73	4,27	**p-value**	**0,839893**
Total	7	8	**Total**	7,00	8,00		

Appendix IV

Overview sequences obtained

DNA Isolates (2spr)	ITS-1F	B36F	rRPB2F	EF595R	Haplotype sequences	Remarks
A	1				1	
B	1				1	
Q	1				1	
S	1				1	
61	1	1	1	1	Edited	
83	1	1	1	1	1	
92	1				1	
94	1				1	
95	1				1	
96	1				1	
97	1				1	
100	1				Edited	
108	1				Not useable	
109	1				Edited	
112	1				1	
114	1				Not useable	
116	1				1	
117	1				Not useable	
118	1				1	
120	1				1	
121	1				1	
124	1				Edited	
135	1	1	1	1	Edited	
154	1	1	1	1	1	
162	1				Not useable	
163	1	1	1	1	1	
164	1				1	
171	1	1	1	1	1	
174	1	1	1	1	Edited	
191	1				Edited	
196	1				1	
198	1				1	
199	1				1	
200	1				Edited	
206	1				1	
214	1				1	
222	1				1	
224	1				1	
241	1				Edited	
243	1				Not useable	
250	1				1	
266	1				Edited	
268	1				1	
274	1				1	
275	1				1	
276	1				1	
278	1					Blast: Rhodocallylia
279	1					Blast: M. galapus
284	1					Blast: Other Mycena
285	1				1	
291	1				1	
294	1					Blast: M. cinerella
303	1				1	
305	1				1	
306	1				1	
312	1				1	
313	1				1	
315	1				Not useable	
316	1				1	
322	1				1	

Appendix V

Edited collection

Strain	Single spore culture	Location	Date	2-spored	4-spored	Remarks
	in freezer -80 degrees					
1		Unknown	15-sep	1		
	1:1					
	1:2					
2		Unknown	15-sep	1		
	2:1					
	2:2					
3	3	Unknown				
4	4	Unknown				
5	5	Unknown				
A		De Born	**23-sep**	1		Sequenced
	A:1					
	A:2					
B		De Born	**23-sep**	1		Sequenced
	B:1					
	B:2					
F		De Born	**23-sep**	1		
	F:1					
	F:2					
G		De Born	**23-sep**	1		
	G:1					
	G:2					
H	H:1	De Born	**23-sep**	1		
J		De Born	**23-sep**	1		
	J:1					
	J:2					
K	K:1, K:2	De Born	**23-sep**			
L		De Born	**23-sep**	1		
	L:1					
	L:2					
	L:3					
N		De Born	**23-sep**	1		
	N:1					
	N:2					
P		De Born	**23-sep**	1		
	P:1					
	P:2					
Q		De Born	**23-sep**	1		Sequenced
	Q:1					
	Q:2					
R		De Born	**23-sep**	1		
	R:1					
	R:2					
S		De Born	**23-sep**	1		Sequenced
	S:1					
	S:2					
	S:3					
	S:4					
A		Unknown	28-sep	1		
	A:1					
	A:2					
58		De Born	**30-sep**		1	Sequenced
	58:1					
	58:2					
	58:3					
	58:4					
	58:5					
	58:6					
	58:7					
	58:8					
	58:9					
	58:10					
	58:11					
61		De Born	**30-sep**	1		Sequenced
	61:1					
	61:2					
	61:3					
62		De Born	**30-sep**	1		
	62:1					
	62:2					
	62:3					

63		De Born	**30-sep**	1		
	63:1					
	63:2					
	63:3					
64		De Born	**30-sep**	1		
	64:1					
	64:2					
	64:3					
91		De Born	8-okt		1	Sequenced
	91:1					
	91:2					
92		De Born	8-okt	1		Sequenced
	92:1					
	92:2					
93		De Born	8-okt		1	Sequenced
	93:1					
	93:2					
94		De Born	8-okt	1		Sequenced
95		De Born	8-okt	1		Sequenced
	95:1					
	95:2					
96		De Born	8-okt	1		Sequenced
	96:1					
	96:2					
97		De Born	8-okt	1		Sequenced
	97:1					
	97:2					
98		De Born	8-okt	1		
	98:1					
	98:2					
99	99:1, 99:2	De Born	8-okt	JA		Strange Lamellae
101		De Born	8-okt	1		
	101:1					
	101:2					
102		De Born	8-okt	1		
	102:1					
	102:2					
105	105	De Born	8-okt	1		
107	107	Dassenboslaan	8-okt		1	Sequenced
108		Dassenboslaan	8-okt	1		
109		Dassenboslaan	8-okt	1		Sequenced
	109:1					
	109:2					
112		Dassenboslaan	8-okt	1		Sequenced
	112:1					
	112:2					
	112:3					
	112:4					
113		Dassenboslaan	8-okt		1	Sequenced
	113:1					
	113:2					
114		Dassenboslaan	8-okt	1		Sequenced
	114:1					
	114:2					
115		Dassenboslaan	8-okt		1	Sequenced
	115:1					
	115:2					
116		Dassenboslaan	8-okt	1		Sequenced
	116:1					
	116:2					
117	117:1	Dassenboslaan	8-okt			
118		Dassenboslaan	8-okt	1		Sequenced
	118:1					
	118:2					
	118:3					
	118:4					
119		Dassenboslaan	8-okt		1	Sequenced
	119:1					
	119:2					
120		Dassenboslaan	8-okt	1		Sequenced
	120:1					
	120:2					

121		Dassenboslaan	8-okt	1		Sequenced
	121:1					
	121:2					
122		Dassenboslaan	8-okt		1	Sequenced
123		Dassenboslaan	8-okt		1	Sequenced
	123:1					
	123:2					
124	124	Dassenboslaan	8-okt			
125		Dassenboslaan	8-okt	1		
	125:1					
	125:2					
130	130:1, 130:2, 130:3	Onbekend	**11-okt**		JA	3 spores? Sequenced
131		Onbekend	**11-okt**		1	Sequenced
	131:1					
	131:2					
	131:3					
	131:4					
162	162					
163		Son en Breugel	18-okt	1		Sequenced
	163:1					
	163:2					
164		Son en Breugel	18-okt	1		Sequenced
	164:1					
	164:2					Strange growth
170	170	Son en Breugel	18-okt		1	Sequenced
171		Son en Breugel	18-okt	1		Sequenced
	171:1					
	171:2					
191		De Born	**21-okt**	1		Sequenced
	191:1					
	191:2					
196		De Born	**21-okt**	1		Sequenced
	196:1					
	196:2					
198		De Born	**21-okt**	1		Sequenced
	198:1					
	198:2					
199		De Born	**21-okt**	1		Sequenced
	199:1					
	199:2					
200		De Born	**21-okt**	1		Sequenced
	200:1					
	200:2					
204		De Born	**21-okt**	1		
	204:1					
	204:2					
205		De Born	**21-okt**	1		
	205:1					
	205:2					
206		De Born	**21-okt**	1		Sequenced
	206:1					
	206:2					
212	212	De Born	**21-okt**	1		
213		De Born	**21-okt**	1		Sequenced
	213:1					
	213:2					
214	214	De Born	**21-okt**	1		Sequenced
216		Heiland	28-okt		1	Sequenced
	216:1					
	216:2					
	216:3					
217		Heiland	28-okt		1	Sequenced
	217:1					
	217:2					
	217:3					Strange growth
	217:4					
222	222	Heiland	28-okt	1		Sequenced
224	224	Heiland	28-okt	1		Sequenced
226	226	De Born	28-okt	1		
229		De Born	28-okt	1		
	229:1					
	229:2					

235	235	De Born	28-okt	1		
237		De Born	28-okt	1		
	237:1					
	237:2					
239		De Born	28-okt		1	Sequenced
	239:1					
	239:2					
241	241:1, 241:2	De Born	28-okt			Both 2 and 4 spores? Sequenced
243		De Born	28-okt	1		Sequenced
	243:1					
	243:2					
249		Grebbenberg	4-nov		1	Sequenced
	249:1					
	249:2					
	249:3					
	249:4					
250	250	Grebbenberg	4-nov	1		Sequenced
252		Grebbenberg	4-nov		1	Blast: Other species
	252:1					
	252:2					
253		Grebbenberg	4-nov		1	Blast: Other species
	253:1					
	253:2					
	253:3					
254		Grebbenberg	4-nov		1	Sequenced
	254:1					
	254:2					
255		Grebbenberg	4-nov		1	Blast: Other species
	255:1					
	255:2					
	255:3					
	255:4					
	255:5					
	255:6					
	255:7					
	255:8					
258		Grebbenberg	4-nov		1	Blast: Other species
	258:1					
	258:2					
	258:3					
259		Grebbenberg	4-nov		1	Blast: Other species
	259:1					
	259:2					
260		Grebbenberg	4-nov	1		Dark stipe
	260:1					
	260:2					
262		De Born	4-nov	1		
	262:1					
	262:2					
265	265	De Born	4-nov		1	Blast: Other species
266		De Born	4-nov	1		Sequenced
	266:1					
	266:2					
	266:3					
267		De Born	4-nov	1		
	267:1					
	267:2					
268	268:1, 268:2	De Born	4-nov	JA		Strange growth, Sequenced
272		De Born	4-nov	1		
	272:1					
	272:2					
	272:3					
273	273:1	De Born	4-nov			Both 2 and 4 spores?
274		De Born	4-nov	1		Sequenced
	274:1					
	274:2					
275		Dikkenbergweg	**11-nov**	1		Sequenced
	275:1					
	275:2					
276		Dikkenbergweg	**11-nov**	1		Sequenced
	276:1					
	276:2					

	276:3					
278		Dikkenbergweg	**11-nov**	1		Blast: Other species
	278:1					
	278:2					
	278:3					
279	279	Dikkenbergweg	**11-nov**	1		Blast: Other species
284	284:1	Dikkenbergweg	**11-nov**	1		Blast: Other species
285		De Born	**11-nov**	1		Sequenced
	285:1					
	285:2					
	285:3					
	285:4					
288	288	De Born	**11-nov**		1	Blast: Other species
291		De Born	**11-nov**	1		Sequenced
	291:1					
	291:2					
	291:3					
294		De Born	**11-nov**	1		Blast: Other species
	294:1					
	294:2					
296		De Born	18-nov		1	Sequenced
	296:1					
	296:2					
298		De Born	18-nov		1	Sequenced
	298:1					
	298:2					
	298:3					
	298:4					
	298:5					
	298:6					
	298:7					
303		Arboretum Onderlangs	18-nov	1		Sequenced
	303:1					
	303:2					
	303:3					
	303:4					
305		Arboretum Onderlangs	18-nov	1		Sequenced
	305:1					
	305:2					
	305:3					
	305:4					
	305:5					
	305:6					
306		Arboretum Onderlangs	18-nov	1		Sequenced
	306:1					
	306:2					
312		Eindhoven	**22-nov**	1		Sequenced
	312:1					
	312:2					
313	313:1	Eindhoven	**22-nov**	1		Sequenced
315		De Born	25-nov	1		Sequenced
	315:1					
	315:2					
316	316:1	De Born	25-nov	1		Sequenced
322		De Born	25-nov	1		Sequenced
	322:1					
	322:2					
326	326:1	Nol in t Bosch	25-nov	1		Sequenced
327		Nol in t Bosch	25-nov	1		Sequenced
	327:1					
	327:2					
Totaal				**83**	**25**	