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Frequent diploidisation of haploid *Armillaria ostoyae* strains in an outdoor inoculation experiment

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Abstract

Very little is known about the biology and ecology of haploid *Armillaria* strains in nature and it is still a matter of speculation where exactly the basidiospores, giving rise to the haploid mycelia, germinate. In this outdoor inoculation experiment, we aimed to assess the virulence of six haploid *A. ostoyae* strains along with their diploid parent towards 2-year-old seedlings and 4-year-old saplings of Norway spruce (*Picea abies*), and to determine their ability to colonise freshly cut stumps. As inoculum source an *Armillaria*-colonised hazelnut (*Corylus avellana*) stem segment was inserted into the soil substrate. Systematic re-isolations from mycelial fans found at the root collar of infected trees or stumps were made to check the identity of the invading strains. Surprisingly, not a single haploid re-isolate could be recovered. Microsatellite genotyping of 133 re-isolates strongly suggests that the inoculated haploid strains were diploidised either by mating propagules (basidiospores or haploid mycelia) already present in the soil substrate or naturally disseminated in the course of the experiment from nearby forests. Consequently, no conclusion about the infectious ability of haploid *Armillaria* mycelia under natural conditions can be drawn. Nonetheless, the diploid half-sib families resulting from the diploidisation showed varying degrees of virulence, with a

high correlation between the experiment with 2-year-old seedlings and 4-year-old saplings, pointing to a genetic influence of the original haploid strain on virulence expression of the newly formed diploids. Despite extensive genotyping of re-isolates with microsatellite markers, no evidence for somatic recombination between haploid mating propagules and diploidised mycelia was detected, suggesting that this is an uncommon phenomenon in *A. ostoyae*.

Key words: pathogenicity, seedlings, microsatellite genotyping, *Picea abies*, *Armillaria ostoyae*

Introduction

The vegetative phase of fungi in the class Agaricomycetes is in general heterokaryotic, and typically dikaryotic. The homokaryotic (typically monokaryotic) phase is restricted to the unmated mycelium growing out from basidiospores and is seen as transient and short lived. In certain cases, the homokaryotic phase might, however, be prolonged, especially in protected substrata, such as inside the wood of logs, stumps or living trees (Coates and Rayner, 1985; Garbelotto et al., 1999; Redfern et al., 2001; Stenlid, 1994).

In the Agaricomycetes genus *Armillaria*, which includes important root rot pathogens of woody plants, haploid (monokaryotic) mycelia are rarely found in nature (Guillaumin, 1986). Basidiospores of *Armillaria* germinate easily in the laboratory and haploid cultures obtained from single spores can be maintained over years. Most *Armillaria* species are heterothallic with a tetrapolar (bifactorial) mating system (Baumgartner et al., 2011). To be sexually compatible, two haploid mycelia need to carry different alleles at the two unlinked mating

type loci (*MAT-A* and *MAT-B*). At both loci, numerous allelic variants exist (Guillaumin, 2005). Characteristically, in *Armillaria* the mycelium resulting from a compatible mating is diploid and not dikaryotic (heterokaryotic) as in the other members of the Agaricomycetes (Franklin et al., 1983; Korhonen, 1980; Korhonen and Hintikka, 1974; Ullrich and Anderson, 1978). On agar medium, haploid and diploid strains of *Armillaria* can be distinguished according to their culture morphology. Specifically, haploid strains produce a whitish or pale yellowish-brown, fluffy aerial mycelium, whereas the mycelium of diploid strains is usually crustose and dark brown, with sparse aerial hyphae (Hintikka, 1973). Both diploid and haploid strains may produce rhizomorphs in culture (Hintikka, 1973, R. Heinzelmann, *unpublished observation*).

The substrate colonised by basidiospores of *Armillaria* in nature is still unidentified (Baumgartner et al., 2011). It is assumed that the haploid mycelia may become established in bark fissures, small woody fragments, litter remnants on the soil surface, or directly on living roots (Baumgartner et al., 2011; Guillaumin and Legrand, 2005). Little evidence was found that the basidiospores germinate on the cut surface of fresh stumps. Artificial inoculations of stumps with *Armillaria* basidiospores were only successful under specific experimental conditions (Hood et al., 2008; Rishbeth, 1964, 1970). The occurrence of many small-sized and discrete, hence possibly recently established, *Armillaria* clones in a natural regenerating forest site where all stumps had been removed also suggests that the basidiospores more likely germinate on a different substrate than stump surfaces (Legrand et al., 1996).

Although in nature the haploid phase of *Armillaria* is restricted to the short period between spore germination and mating, in an experimental context haploid *Armillaria* strains can be very useful. First, haploid strains are crucial for the delimitation of biological species within the genus (e.g. Anderson and Ullrich, 1979; Hood and Ramsfield, 2016; Korhonen, 1978; Ota et al., 1998; Qin et al., 2007). Second, for haploid strains genomic analyses are more straightforward than for diploid strains because they carry only one genome. Third,

79 germinating basidiospores of *Armillaria* can be successfully genetically manipulated by
80 transformation (Baumgartner et al., 2010a; Ford et al., 2016). Nonetheless, very little is
81 known about the infectious and competitive ability of haploid *Armillaria* strains in nature.
82 The same also applies to the haploid phase in numerous other Agaricomycetes (Crockatt et
83 al., 2008; Hiscox et al., 2010; Nieuwenhuis et al., 2013).

84 A few decades ago, Shaw III and Loopstra (1988) and Raabe (1969, 1972) conducted
85 virulence experiments with haploid *Armillaria* strains. Surprisingly, the virulence of the tested
86 single-spore isolates was in most cases comparable to that of the diploid parental strains.
87 More recently, Heinzelmann and Rigling (2016) showed that haploid *A. ostoyae* strains are
88 able to invade the cambium of autoclaved stem sections of Norway spruce (*Picea abies*) and
89 to form mycelial fans within the cambial region. However, when instead of the autoclaved
90 stem sections fresh stem sections were used, diploidisation of the inoculated haploid strains,
91 presumably by basidiospores introduced with the stem segments, was observed (Heinzelmann
92 and Rigling, 2016). With this observation rose the question of whether the haploid strains in
93 the previous experiments by Shaw III and Loopstra (1988) and Raabe (1969, 1972) were also
94 accidentally diploidised. Unfortunately, in those studies no attempts to re-isolate *Armillaria*
95 from infected plants were done and this question remains unanswered.

96 For experimental investigations on haploid *Armillaria* strains, basidiospores are needed
97 from which single spore cultures can be obtained. One of the few *Armillaria* species which
98 produces reliably basidiocarps *in vitro* is *A. ostoyae* (Guillaumin et al., 1985). This *Armillaria*
99 species is widely distributed in the northern hemisphere and considered as an important
100 pathogen infecting a broad range of conifers in forests and plantations (Guillaumin et al.,
101 2005). Secondary spread of the persistent diploid mycelium in *A. ostoyae*, like in the other
102 *Armillaria* species, occurs either via rhizomorphs, i.e. root like fungal structures which grow
103 through the soil, or by direct mycelial transfer from infected tissue to healthy roots (Rishbeth,
104 1985). Because of its pathogenic potential and widespread occurrence, *A. ostoyae* has been

the target organism of numerous studies (Labbé et al., 2017; Legrand et al., 1996; Prospero et al., 2004; Prospero et al., 2008). Genomic and transcriptomic data are also available for *A. ostoyae* (Ross-Davis et al., 2013; Sipos et al., 2017).

The aim of the present study was to investigate the infectious abilities of haploid *Armillaria* strains under semi-natural conditions. Specifically, we aimed to assess (i) the virulence of haploid *A. ostoyae* strains towards Norway spruce seedlings and saplings, (ii) their ability to colonise freshly cut Norway spruce stumps, and (iii) the occurrence and frequency of diploidisation of the inoculated haploid strains. For this, an inoculation experiment with potted Norway spruce seedlings and saplings and six haploid single spore progeny of a virulent, diploid *A. ostoyae* strain was set up. Fresh mini-stumps were produced by cutting half of the saplings during the experiment. *Armillaria* was systematically re-isolated from infected plants and stumps and checked for diploidisation using microsatellite markers.

Material and methods

Armillaria *strains*

In this study, six haploid, single spore progeny of *A. ostoyae* and their diploid parental strain were investigated (Table 1). The single basidiospore isolates (SBIs) were obtained from spores collected in the laboratory from a basidiocarp, which developed in an inoculation experiment conducted in 2003 (D. Rigling, *unpublished data*). The spores were suspended in sterile water and stored at -80 °C until use. The single spore isolate SBI C18/9 was obtained in 2004 and the other five single spore isolates in 2012. The parental strain C18 was isolated

in 1995 from an approximately 90-year-old Norway spruce tree damaged by lightening, which was located in a forest stand in Switzerland (Steinmaur, 8.4435 ° E, 47.5071 ° N). A previous inoculation experiment showed that this strain is highly virulent towards Norway spruce seedlings (Prospero et al., 2004).

The haploidy of the SBIs was tested by characterizing their mating types. For this, the SBIs were paired with each other in all possible combinations on 2% malt extract agar (20 g Diamalt (Hefe Schweiz AG, Stettfurt, Switzerland), 15 g Plant Propagation Agar, 1 L deionized water). Mating type A_1B_1 was arbitrarily assigned to strain SBI C18/9. Compatible strains were considered to be of mating type A_2B_2 and hemicompatible strains, i.e. strains which formed a broad barrage zone when paired with SBI C18/9, of mating type A_2B_1, following Darmono and Burdsall (1992). Strains compatible with A_2B_1 strains, were assigned to mating type A_1B_2. In addition, all strains were genotyped at 11 microsatellite loci (see below) to verify their identity and ploidy.

Host plants, inoculations and stump creation

The inoculation experiment was conducted with potted seedlings (2-year-old) and saplings (4-year-old) of Norway spruce (Heinzelmann et al., 2017). The seedlings were grown from seeds in the forest nursery of the Swiss Federal Research Institute WSL in Birmensdorf, Switzerland. The seeds originated from two seed orchards in the Swiss Plateau (Birmenstorf AG, 8.2853° E, 47.4462° N, 400 m a.s.l. and Bremgarten, 8.3083° E, 47.3607° N, 425 m a.s.l.). In April 2013, the field grown plants were transferred into pots. The potting substrate was composed of 33% wood fiber material (Ökohum GmbH, Herrenhof, CH), 67% potting soil (42% bark humus, 42% peat, 12% wood fibers, 4% clay, NPK-fertilizer with trace elements, Ökohum GmbH) supplemented with 2 g/L of a pattern release fertilizer (Osmocote

plus Standard 8-9 months, Everris International B.V.P.O., Geldermalsen, NL), 1 g/L "Unikorn I" (Hauert HBG Dünger AG, Grossaffoltern, CH), and 2 g/L horn meal. The seedlings were planted in five to 3.5 L pots, whereas the saplings were planted individually in 10 L pots. The *Armillaria* inoculum was produced as described by Heinzelmann et al. (2017) using autoclaved stem segments (10 cm length × 3 - 4 cm in diameter) of common hazel (*Corylus avellana*).

In June 2013, i.e. six weeks after planting, the pots were inoculated with *Armillaria*. In each pot one *Armillaria* colonised stem segment was buried in the rooting zone of the seedlings/sapling. For each *Armillaria* strain, eight pots with seedlings and 20 pots with saplings were inoculated. Control pots were inoculated with autoclaved stem segments. The inoculated pots with the seedlings and saplings were kept outdoors in the forest nursery of the Swiss Federal Research Institute WSL and watered as required.

The 29th of April 2015 (≈ 23 months after inoculation), half of the surviving saplings, at that time point about 6-year-old, were cut 15 cm above the soil level to simulate "mini-stumps" (Prospero et al., 2006). Between 7 and 10 stumps were produced for each *A. ostoyae* strain depending on the mortality observed until the time point of cutting.

The entire inoculation experiment was conducted in parallel with another inoculation experiment which included several diploid strains of *A. ostoyae*, *A. borealis* and *A. cepistipes* (Heinzelmann et al., 2017).

Assessment of seedling mortality and stump colonisation

The seedlings were regularly assessed for symptoms of Armillaria root disease (i.e. wilting, discoloration of needles, defoliation, and mortality) over three growing seasons (June 2013 to

November 2015). Dead seedlings/saplings were examined for the presence of mycelial fans in the root collar region directly in the field.

In November 2015 (≈ 29 months after inoculation and 6 months after stump creation), at the end of the experiment, the root collar of all remaining seedlings and saplings and of all stumps was carefully inspected for *Armillaria* lesions (i.e. presence of resinosis and/or mycelial fans in the cambial region). Seedlings and saplings were classified either as healthy (absence of *Armillaria* lesions and mycelial fans), as infected (presence of *Armillaria* lesions at the root collar, less than 100% of the root collar girdled with mycelial fans) or dead (dead, 100% girdled root collar). Stumps were categorized as colonised (mycelial fans present) or not colonised (mycelial fans absent).

Rhizomorph production and viability of mycelium in inoculum segments

The abundance of rhizomorphs present in the pots with seedlings was estimated using a 0 (rhizomorphs absent) to 3 (rhizomorphs widely spread) scale as described in Heinzelmann et al. (2017). In the sapling experiment, only the presence or absence of rhizomorphs was recorded. If rhizomorphs were absent in a pot, the presence of living *Armillaria* mycelium in the inoculum segment was verified. For this, the inoculum segment was split with an axe and the halves were incubated in a moist chamber at room temperature. The outgrowth of the typical brushes of aerial hyphae after about one week incubation indicated the presence of viable *Armillaria* inoculum.

Re-isolations

207 *Armillaria* was systematically re-isolated from all but five of the killed or infected
208 seedlings/saplings (n = 149) and from all colonised stumps (n = 44) inoculated with a haploid
209 *A. ostoyae* strain. In the seedling experiment, re-isolations were also performed from
210 rhizomorphs which developed in pots without infected or killed plants. Finally, for control
211 purposes, re-isolations were also done from a subset of plants and stumps infected with the
212 diploid, parental strain C18. All *Armillaria* re-isolations were done as described in
213 Heinzelmann et al. (2017).

214 *Armillaria* was also re-isolated from the interior of eight "haploid" inoculum segments
215 and from two segments containing the parental strain C18. Prior to isolation, the inoculum
216 segments were washed under running tap water, dried with paper towels, and split
217 longitudinally in two sections using an axe previously sterilized with 70% ethanol. When
218 distinct sectors separated by a demarcation line or a few millimetre-wide dark zone (Figure 1)
219 were visible on the split wood surface, a re-isolation was attempted from each sector. For this,
220 small pieces of wood were removed with a sterile forceps and placed without surface
221 sterilization on Maloy's medium (12 g Bacto Malt Extract, 15 g Plant Propagation Agar, 1 L
222 deionized water supplemented with 2 mg benomyl and 100 mg streptomycin, modified from
223 Maloy (1974)). After about a week of incubation at room temperature, the outgrowing
224 mycelium was transferred to 2% malt extract agar.

225

226

227 ***Microsatellite genotyping***

228

229 The original *A. ostoyae* strains and the re-isolates were genotyped at eleven microsatellite loci
230 (Arm02, Arm05, Arm09, Arm13, Arm11, Arm15, Arm16, Arm17, Am109, Am111, Am124)
231 as described in Heinzelmann and Rigling (2016). With this specific set of microsatellite loci
232 all five annulated *Armillaria* species in Europe can be distinguished (Prospero et al., 2010, R.

Heinzelmann, *unpublished data*). In *A. ostoyae* eight loci (Arm05, Arm09, Arm11, Arm15, Arm16, Am109, Am111, Am124) are amplified, whereof six are polymorphic. To increase the resolution among *A. ostoyae* genotypes, one additional locus, AC18, which was developed in *A. cepistipes* and is also polymorphic in *A. ostoyae* (Tsykun et al., 2017), was also included in the microsatellite analyses.

Statistical analysis

Statistical analyses were performed with R (R Development Core Team, 2016). The association of rhizomorph production and the percentage of killed plus infected seedlings in the seedlings experiment was assessed by calculation of the Pearson's product-moment correlation coefficient. The percentage of killed plus infected seedlings and saplings, respectively among haploid strains was compared among the seedling and sapling experiment using the same coefficient.

Results

We used colonised hazelnut segments to inoculate six haploid, single spore progeny of *A. ostoyae* and their diploid parental strain into pots containing 2-year-old Norway spruce seedlings or 4-year-old saplings. The disease development in the inoculated pots was followed over three growing seasons. In addition, stumps created during the experiment were assessed for colonisation by the inoculated strains. Finally, we systematically re-isolated *Armillaria*

from infected plants, stumps and inoculum segments to verify their identity using microsatellite markers.

Infection and mortality of plants and colonisation of stumps

By the end of the experiment, 91.4% (43 out of 47) of the originally haploid inoculum segments had produced rhizomorphs in the seedling experiment and 85.0% (102 out of 120) in the sapling/stump experiment. Inoculum segments which did not form rhizomorphs, either did not contain any viable *Armillaria* mycelium or this was confined to a small spot. These segments were, therefore, considered as non-viable. All inocula of the parental diploid strain C18 were viable and produced rhizomorphs.

In both experiments, very little plant mortality occurred until the second growing season (Figure 2). Within the first eleven months after inoculation only two out of 122 exposed saplings (i.e. saplings in pots with rhizomorphs) were killed. From the end of the second growing season (August to October 2014) onwards, seedling/sapling mortality caused by both the originally haploid strains and the parental strain C18 increased steadily. At the end of the experiment in November 2015 (i.e. 29 months after inoculation), 53.0 % of the exposed seedlings contained in pots inoculated with an originally haploid strain had been killed (Supplementary Table 1). By the same time, the parental strain C18 killed 60% of the exposed seedlings. The incidence of infected, but not yet killed seedlings, was very low (originally haploid strains: 1.4%, parental strain C18: 0.0%). In the sapling experiment, the incidence of killed plants was 18.8% for the originally haploid strains, and 45.0% for the parental strain C18. The portion of infected but not yet killed plants in the sapling experiment was considerably higher than that in the seedling experiment and reached 30.6% for the originally haploid strains, and 20.0% for the parental strain C18.

283 In both experiments, the percentage of killed plus infected seedlings varied considerably
284 among originally haploid strains (Figs. 2 and 3). In the seedling experiment, the percentages
285 of killed/infected plants ranged from 12.0 to 77.5% and in the sapling experiment from 11.1%
286 to 80.0%. Noteworthy, there was a strong positive correlation of the percentage of
287 killed/infected plants between the two experiments (r = 0.936, *P* = 0.006) in respect to the
288 results of the originally haploid strains. The parental strain killed or infected 60.0% of the
289 exposed seedlings and 65.0% of the exposed saplings. In the seedling experiment, where
290 rhizomorph production was quantified, a correlation of plant mortality/infection and
291 rhizomorph production was observed for pots inoculated with the haploid strains (r = 0.885, *P*
292 = 0.019).

293 Within six months after creation, almost all exposed stumps were colonised by *Armillaria*
294 (Figure 3, Supplementary Table 2). In total, the originally haploid strains had colonised 95.7%
295 of the exposed stumps and the parental strain C18 87.5% of them. Among the originally
296 haploid strains, only the strain SBI C18/9 had colonised less than 100% of the exposed
297 stumps.

298

299

300 ***Re-isolations from plants and stumps***

301

302 Considering only the pots inoculated with a haploid strain, one re-isolate from each of the 117
303 killed or infected seedlings was recovered (Table 2). In the case of the saplings, re-isolates
304 were obtained from 84.4% (27 out of 32) of the killed or infected saplings and from 100%
305 (44) of the colonised stumps. In addition, a re-isolate was obtained from rhizomorphs from
306 each of the six pots without mortality or infection in the seedling experiment.

307 In the pots with an originally haploid inoculum, none of the re-isolates obtained from
308 seedlings, saplings, stumps or rhizomorphs showed the fluffy and whitish appearance, which

is typical for haploid *Armillaria* isolates. Instead, the mycelium was in all cases crustose and brownish, suggesting that the re-isolates were rather diploid than haploid. This hypothesis was confirmed by microsatellite genotyping of a subset of 110 re-isolates. In each genotype, at least one of seven polymorphic loci (on average 3.3 loci) was heterozygous.

Although the genotypes of the analysed re-isolates were diploid, the original haplotype of the inoculated haploid strain was present in all cases (Table 2, Supplementary Table 3). Based on the allelic composition at the microsatellite loci, the occurrence of mating events among the inoculated haploid strains can be excluded. Furthermore, all genotypes of the re-isolates were different from the genotype of the parental strain C18 and from any other diploid *A. ostoyae* strain used in the parallel experiment (Heinzelmann et al., 2017). In the seedling experiment, none of the genotypes of the re-isolates was shared among pots, whereas in the sapling experiment two re-isolates from different pots had an identical genotype. Microsatellite analysis of multiple re-isolates from eight pots revealed that individual seedlings within a pot were not necessarily infected by the same *A. ostoyae* genotype (Table 3, Supplementary Table 3). In fact, from the different seedlings in a pot up to three distinct *A. ostoyae* genotypes were isolated. Based on the above findings, each originally haploid strain in our experiment was most likely represented among the re-isolates by a group of diploid siblings, sharing the genome of the inoculated haploid strain.

Re-isolations from inoculum segments

Because of advanced decay and bacterial contaminations, *Armillaria* was more difficult to re-isolate from the inoculum segments than from the plants/stumps. Only 20 out of 26 isolation attempts were successful. However, in five of the six cases where isolation failed, *Armillaria* DNA could be extracted in sufficient amounts for genetic analyses directly from wood tissues.

All 18 re-isolates from the originally haploid inoculum segments were characterized by a diploid culture morphology. Microsatellite genotyping supported this assumption. All re-isolates revealed heterozygous loci, suggesting they were actually diploid. The investigated inoculum segments showed a zonation pattern when split (Figure 1). In most cases those macroscopically defined zones harboured distinct genotypes (Table 3, Supplementary Table 3). From an individual inoculum segment, between two and four different diploid *A. ostoyae* genotypes were isolated. Some of those genotypes were also isolated from the infected seedlings in the same pot. All diploid re-isolates contained the haplotype of the originally inoculated haploid strain. None of the genotypes was identical with the parental strain C18 or any other diploid *A. ostoyae* strain from the parallel experiment.

For comparison, two inoculum segments inoculated with the parental strain C18 were investigated. The interior of these segments was homogenous and showed no zonation. The microsatellite genotype of the re-isolates was identical with the genotype of the inoculated strain C18.

Discussion

The main aim of this study was to investigate the infectious ability of haploid *A. ostoyae* strains by conducting an inoculation experiment with potted Norway spruce seedlings and saplings, whereof half of the latter were cut during the experiment to simulate fresh mini-stumps. To verify that the strains which invaded the exposed plants and stumps were those initially inoculated (i.e. C18 and its haploid progeny), systematic re-isolations from mycelial fans at the root collar were made. Surprisingly, none of the inoculated haploid strains could be recovered in its original haploid state, i.e. all re-isolates were diploid. The same applied to re-isolates from rhizomorphs and inoculum segments. Consequently, all phenotypic traits

assessed in this study (i.e. plant mortality and infection, stump colonisation, rhizomorph production) were expressed by diploidised strains. It is, therefore, not possible to draw any definitive conclusion about the persistence and infectious ability of haploid *Armillaria* mycelia under natural or semi-natural conditions. Nevertheless, plant mortality and infection caused by the originally haploid strains was strongly positively correlated between the two experiments of this study, suggesting a strong genetic influence of the original haploid strain on virulence expression of the newly formed diploids. In addition, the gradual variation observed among the originally haploid strains in the ability to infect and kill plants also demonstrates that virulence in *A. ostoyae* is a quantitative trait with a potentially polygenic origin. This result is in agreement with previous studies on pathogenicity and virulence in *Armillaria* species (Morrison and Pellow, 2002; Omdal et al., 1995; Prospero et al., 2004).

Microsatellite genotyping of a large number of re-isolates allowed us to unravel the identity of the re-isolated diploid genotypes. Based on the allelic composition at the genotyped microsatellite loci, we can exclude that the new genotypes were the result of sibling matings, a contamination with the parental strain C18, or a diploid *A. ostoyae* strain from the parallel experiment. The presence of the haplotype of the inoculated haploid strain in each diploid re-isolate and the large diversity of new genotypes rather suggest that the inoculated haploid strains were diploidised in the field by basidiospores or haploid mycelia present in the soil substrate. Diploidisation of the haploid strains in the laboratory prior to the field inoculations seems very unlikely. In a different experiment with autoclaved stem segments conducted under sterile laboratory conditions, the inoculated original haplotypes could always successfully be re-isolated (Heinzelmann and Rigling, 2016).

Noteworthy, in the eight pots from the seedling experiment, where re-isolates from multiple infected seedlings, as well as from the inoculum segment were genotyped, between two and five different diploid *A. ostoyae* genotypes were detected. All investigated inoculum segments were zoned in the interior, and with a few exceptions those zones harboured

different genotypes. These findings strongly suggest that the inoculated haploid mycelium frequently had mated with several different basidiospores or haploid mycelia, and that those mating events became visible as somatic incompatibility zones in the inoculum segment. The recovery of at least some of the diploid *A. ostoyae* genotypes from both the inoculum segment and from infected seedlings within an individual pot suggests that the diploidisation of the haploid mycelium had occurred in/on the inoculum segment. Subsequently, the seedlings were infected by secondary spread of the diploid strain, either by rhizomorphs or direct mycelial transfer from the inoculum segment to the roots of the seedlings. Frequent diploidisation of haploid mycelium on artificially colonised woody substrates have also been reported for other basidiomycete species (e.g. Edman and Gustafsson, 2003). However, we cannot completely exclude the possibility that the haploid mycelium was diploidised outside of the inoculum, i.e. in the soil substrate or on the seedlings or saplings.

Given the high frequency at which diploidisation occurred in our experiment, the "contaminating" mating propagules (basidiospores or haploid mycelia) presumably have not only encountered haploid but also diploidised or diploid mycelia. In the laboratory, such conspecific haploid-diploid pairings of *Armillaria* (so-called Buller phenomenon, Guillaumin et al., 1991) typically result in the replacement of the haploid nucleus by the diploid nucleus (Rizzo and May, 1994). In a few cases, a diploid mycelium with a recombinant genotype developed or a stable triploid stage was formed (Carvalho et al., 1995). Population genetic studies of *A. ostoyae* and *A. mellea* provide indirect evidence that such somatic recombination might also occur in nature (Baumgartner et al., 2010b; Prospero et al., 2008). However, in our experiment we never observed any potentially recombinant or triploid genotype across all the 145 re-isolates genotyped. The re-isolates always carried the complete haplotype of the haploid strain originally inoculated into the pot. Likewise, all re-isolates from pots inoculated with the parental strain C18 had the identical genotype as the original strain used for

412 inoculation. Based on these results, we hypothesize that somatic recombination between
413 haploid and diploid mycelia may be rather a rare phenomenon in *A. ostoyae* in nature.
414 Although the microsatellite genotyping of the re-isolates convincingly demonstrates the
415 diploidisation of inoculated haploid strains, it gives no hint about the origin of the
416 "contaminating" mating propagules. We may speculate that basidiospores or tiny haploid
417 mycelia of *A. ostoyae* were already present in the non-sterile soil substrate at the time point of
418 inoculation, or airborne basidiospores were deposited later on the plant and soil surface and
419 washed down into the substrate by rain. Based on the findings of Shaw III (1981),
420 basidiospores of *Armillaria* may remain dormant for several months on tree bark and survive
421 harsh winter conditions, which is supported by the study by Heinzelmann and Rigling (2016).
422 Possibly, dormant basidiospores, or alternatively tiny haploid mycelia, might have reached the
423 soil substrate via the contained bark humus and wood fibre material. Such spores could have
424 then interacted immediately with the haploid aerial mycelium, which was abundant on the
425 surface of the inoculum segments. Based on the microsatellite data we cannot determine
426 whether the diploidisation of the haploid strains occurred at the beginning of the experiment
427 or throughout a longer time period. However, the fact that plant mortality induced by the
428 originally haploid strains was not or just little delayed compared to that induced by the
429 parental strain C18, might suggest a rather early diploidisation.
430 Our experimental sites were only about 200 m distant from a mixed coniferous forest,
431 where basidiocarps of *A. ostoyae* were observed about four months after inoculum insertion in
432 the pots. The presence of airborne basidiospores of *Armillaria* at least 150 m away from a
433 forest edge has been demonstrated by Power et al. (2008). Spore trapping experiments in
434 other wood-inhabiting Agaricomycetes showed high deposition rates of airborne
435 basidiospores at local scale and confirmed occasional long-distance spore dispersal (Edman
436 and Gustafsson, 2003; Edman et al., 2004; Hallenberg and Kuffer, 2001). It is thus likely that
437 at least some airborne spores of *A. ostoyae* reached the experimental site. To find the true

origin of the "contaminating" mating propagules, however, further experiments would be required. For example, by using inoculum segments colonised by haploid *Armillaria* mycelium as baits, the presence or absence of basidiospores or haploid *Armillaria* mycelia in sterilized and unsterilized soil substrate could be tested. Similarly, by exposing such inoculum segments to the air, the presence of airborne *Armillaria* spores could be detected, as demonstrated in other Agaricomycetes (e.g. Edman and Gustafsson, 2003).

Under the prevailing conditions of our experiment, "contaminating" mating propagules were obviously abundant. If this also applies to the natural habitat of *Armillaria*, i.e. forest sites, would need to be clarified in further studies. Some first evidence for the presence of *Armillaria* in forest soils even in absence of any by eye visible mycelia or rhizomorphs is given by a study conducted by Lochman et al. (2004) who detected *Armillaria* in soil samples using genus-specific primers. Therefore, we hypothesize that it may not be the spore availability, but rather the lack of suitable conditions for spore germination, mating and establishment that limits primary spread by basidiospores in *Armillaria*. Further research in this direction would not only provide a better understanding of the biology and ecology of basidiospores and haploid mycelia in *Armillaria*, but also potentially help to prevent new *Armillaria* infections.

Overall, the findings of this study show that conducting inoculation experiments with haploid *Armillaria* strains under natural or semi-natural conditions may be difficult, as the risk of diploidisation by conspecific mating propagules (basidiospores or haploid mycelia) is rather high. In cases where phenotypic traits of specific haplotypes have to be assessed, e.g. for genetic mapping, working in a sterile environment may be a good alternative. Otherwise, we recommend at least to check the soil substratum for *Armillaria* contaminations prior to the experiment and to perform systematic re-isolations at different stages of the experiment, to control for possible diploidisation.

464

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466

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474 Appendix A. Supplementary data

475

476 Supplementary data related to this article can be found at …

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606
607

608 **Table 1.** Strains of *Armillaria ostoyae* used in this study.
609

Strain	Ploidy	Mating type	Source[b)]	Year of isolation
Parent				
C18[a)]	2n	A_1B_1/A_2B_2	M	1995
Haploid progeny				
SBI C18/9	n	A_1B_1	SBI	2004
SBI C18/40	n	A_2B_2	SBI	2012
SBI C18/54	n	A_2B_1	SBI	2012
SBI C18/56	n	A_1B_2	SBI	2012
SBI C18/66	n	A_2B_2	SBI	2012
SBI C18/78	n	A_1B_2	SBI	2012

610
611 [a)] Reference for the parental strain C18: Prospero et al. 2004.
612 [b)] M: mycelial fan, SBI: single basidiospore isolate.
613

614

615 **Table 2.** Re-isolations of *Armillaria ostoyae* from seedlings, stumps or rhizomorphs and the results of the
616 microsatellite genotyping.
617

	Plants		Stumps		Rhizomorphs	
Inoculated strain	Re-isolates[a)]	Microsatellite genotypes[b)]	Re-isolates[a)]	Microsatellite genotypes[b)]	Re-isolates[a)]	Microsatellite genotypes[b)]
Experiment with 2-year-old seedlings						
Parent						
C18	4/24	4/0/4	–	–	–	–
Haploid strains						
SBI C18/40	26/26	0/14/14	–	–	0/0	0/0/0
SBI C18/54	15/15	0/8/8	–	–	2/2	0/2/2
SBI C18/56	31/31	0/15/15	–	–	0/0	0/0/0
SBI C18/66	23/23	0/9/9	–	–	0/0	0/0/0
SBI C18/78	19/19	0/8/8	–	–	0/0	0/0/0
SBI C18/9	3/3	0/3/3	–	–	4/4	0/4/4
Total	117/117	0/57/57	–	–	6/6	0/6/6
Experiment with 4-year-old saplings						
Parent						
C18	6/8	4/0/4	5/7	4/0/4	–	–
Haploid strains						
SBI C18/40	5/6	0/5/5	9/9	0/4/4	0/4	–
SBI C18/54	2/3	0/2/2	8/8	0/3/3	0/6	–
SBI C18/56	9/11	0/8/8	7/7	0/3/3	0/2	–
SBI C18/66	3/4	0/3/3	8/8	0/4/4	0/5	–
SBI C18/78	7/7	0/6/6	7/7	0/3/3	0/6	–
SBI C18/9	1/1	0/1/1	5/5	0/5/5	0/3	–
Total	27/32	0/25/25	44/44	0/22/22	0/26	–

618
619 [a)] No. of re-isolates/No. of infected plants.
620 [b)] Genotypes identical with inoculated strain/Genotypes containing the original haplotype/Number of re-isolates
621 genotyped.
622

623 **Table 3.** Diversity of microsatellite genotypes in eight pots with multiple re-isolates of *Armillaria ostoyae*.
624

Inoculated haploid strain	Pot[a)]	Re-isolated genotype	Isolation of genotype from	
			Seedlings[b)]	Inoculum segment[c)]
SBI C18/9	P5	AO1	X	X
		AO2	X	–
		AO3	X	X
SBI C18/40	P4	AO4	X (4)	X
		AO5	–	X
	P7	AO6	X (2)	–
		AO7	X (2)	X (2)
		AO8	–	X
SBI C18/54	P6	AO9	X (3)	X
		AO10	–	X (2)
		AO11	–	X
		AO12	–	X
SBI C18/56	P2	AO13	X (3)	–
		AO14	–	X
		AO15	–	X
		AO16	–	X
		AO17	–	X
	P5	AO18	X (2)	X
		AO19	X (2)	X
		AO20	X	X
SBI C18/66	P1	AO21	X (2)	–
		AO22	X	X
		AO23	–	X
SBI C18/78	P3	AO24	X	–
		AO25	X	X
		AO26	–	X

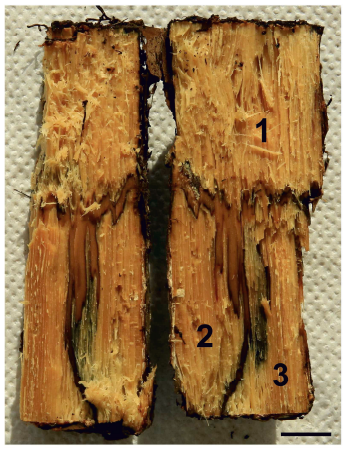
625
626 [a)] For each haploid strain, the *A. ostoyae* genotypes present in one or two selected pots (out of the eight
627 inoculated, P1 to P8) were analysed.
628 [b)] X, genotype present; – genotype not present. If a genotype was isolated from more than one seedling, the
629 number of isolates obtained is indicated in parentheses. An individual seedling was always colonised by a
630 single *A. ostoyae* genotype.
631 [c)] Re-isolates were recovered from defined zones within the inoculum segment, see Figure 1. If a genotype was
632 isolated from more than one zone, the number of isolates obtained is indicated in parentheses.
633

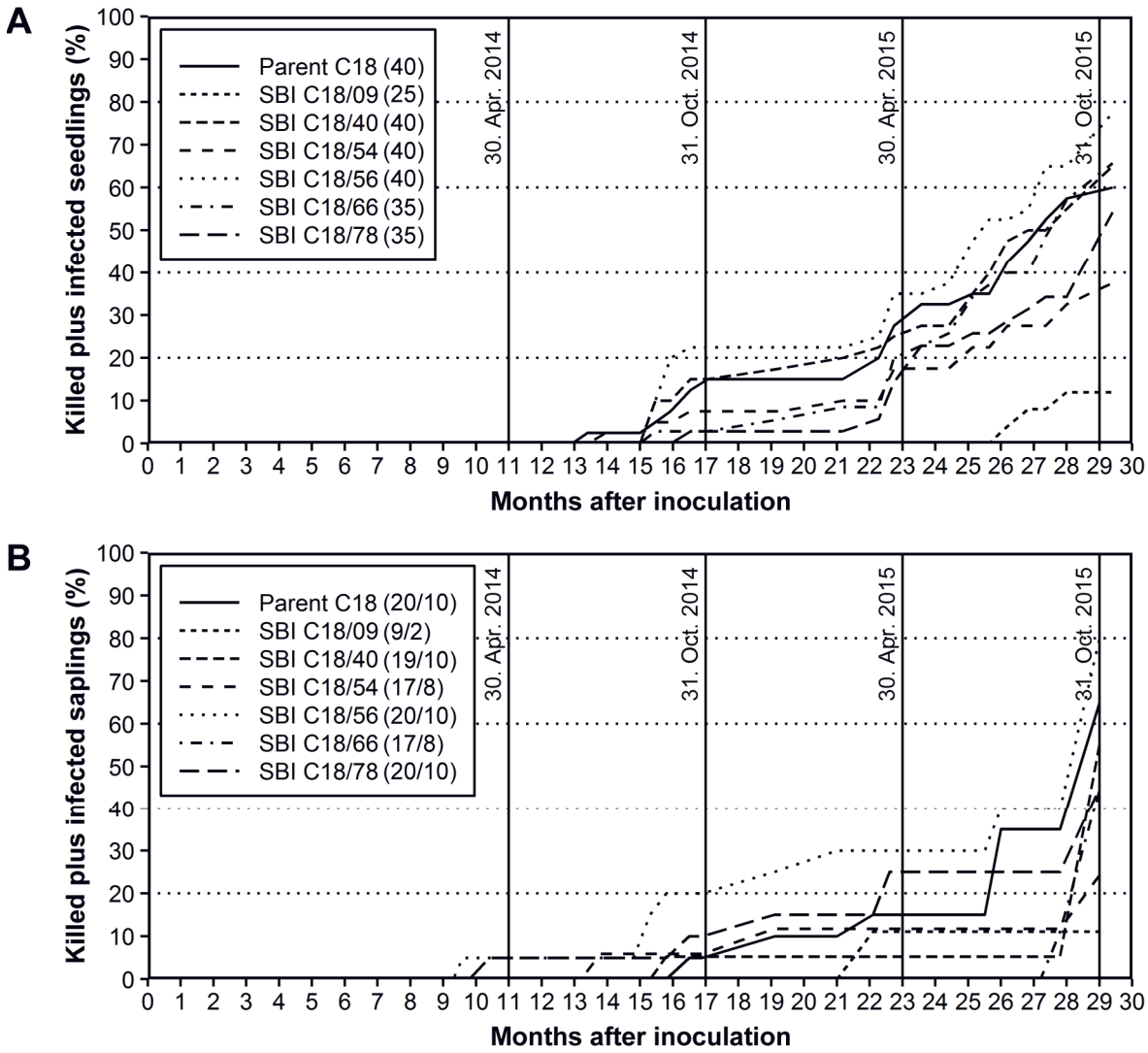
634 **Figure 1.** Interior of a split inoculum segment originally inoculated with the haploid
635 *Armillaria ostoyae* strain SBI C18/56. The three zones (1 to 3) separated by dark demarcation
636 lines were occupied by a different diploid *A. ostoyae* genotype. As all three diploid genotypes
637 contained the haplotype of the originally inoculated haploid strain, we assume that the new
638 genotypes were produced by independent mating events of the inoculated strain with
639 basidiospores or haploid mycelia present in the substrate. Scale bar = 1 cm.
640
641
642 **Figure 2.** Cumulative mortality of 2-year-old seedlings (A) and 4-year-old saplings (B) of
643 Norway spruce (*Picea abies*) during 29 months after inoculation with the diploid *Armillaria*
644 *ostoyae* strain C18 and six of its haploid progeny. The last assessment also included infected
645 but not yet killed seedlings/saplings. Because of diploidisation of the inoculated haploid
646 strains, each haploid strain is represented by a group of siblings, which share the genome of
647 the inoculated haploid strain. The number of seedlings/saplings corresponding to 100% is
648 given in parentheses after the strain name in the legend. In panel B, the number of exposed
649 saplings before and after stump creation (i.e. after 23 months) is given.
650
651
652 **Figure 3.** Percentage of 2-year-old seedlings (A), 4-year-old saplings (B), and stumps (C) of
653 Norway spruce (*Picea abies*) colonised 29 months after inoculation with the diploid
654 *Armillaria ostoyae* strain C18 and six of its haploid progenies. Panels A and B: black = killed
655 seedlings/saplings, grey = infected seedlings/saplings, white: = uncolonised
656 seedlings/saplings; Panel C: black = colonised stumps, white = uncolonised stumps. Because
657 of diploidisation of the inoculated haploid strains, each haploid strain is represented by a
658 group of siblings, which share the genome of the inoculated haploid strain. The number of
659 seedlings, saplings or stumps corresponding to 100% is given on the top of each bar. Stumps

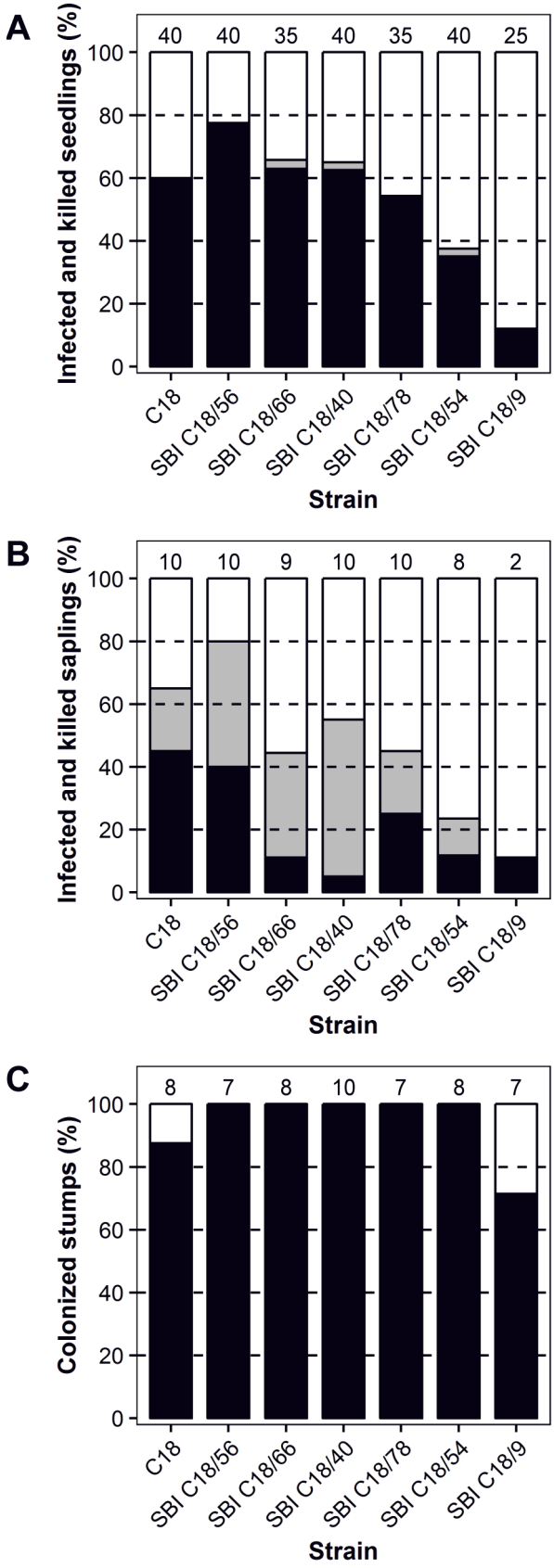
660 were produced by cutting half of the surviving saplings at about 10 cm above soil level ≈ 23

661 months after pot inoculation.

662




A
Killed plus infected seedlings (%)
Parent C18 (40)
SBI C18/09 (25)
SBI C18/40 (40)
SBI C18/54 (40)
SBI C18/56 (40)
SBI C18/66 (35)
SBI C18/78 (35)
30. Apr. 2014
31. Oct. 2014
30. Apr. 2015
31. Oct. 2015
Months after inoculation
B
Killed plus infected saplings (%)
Parent C18 (20/10)
SBI C18/09 (9/2)
SBI C18/40 (19/10)
SBI C18/54 (17/8)
SBI C18/56 (20/10)
SBI C18/66 (17/8)
SBI C18/78 (20/10)
30. Apr. 2014
31. Oct. 2014
30. Apr. 2015
31. Oct. 2015
Months after inoculation


A
Infected and killed seedlings (%)
40 40 35 40 35 40 25
0 20 40 60 80 100
C18
SBI C18/56
SBI C18/66
SBI C18/40
SBI C18/78
SBI C18/54
SBI C18/9
Strain
B
Infected and killed saplings (%)
10 10 9 10 10 8 2
0 20 40 60 80 100
C18
SBI C18/56
SBI C18/66
SBI C18/40
SBI C18/78
SBI C18/54
SBI C18/9
Strain
C
Colonized stumps (%)
8 7 8 10 7 8 7
0 20 40 60 80 100
C18
SBI C18/56
SBI C18/66
SBI C18/40
SBI C18/78
SBI C18/54
SBI C18/9
Strain

Highlights

- Potted Norway spruce seedlings were inoculated with haploid *A. ostoyae* strains.
- Unforeseen, all inoculated haploid *A. ostoyae* strains became diploidised.
- The mating propagules most likely originated from the environment.
- The absence of recombinant strains suggests that somatic recombination is rare in *A. ostoyae*.
- Virulence in *A. ostoyae* seems to be a quantitative polygenic trait.