Fungal Genetics and Biology 87 (2016) 54–63

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

MAT – gene structure and mating behavior of *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus*

Tea Wey, Markus Schlegel, Sophie Stroheker, Andrin Gross *

Forest Pathology and Dendrology, Institute of Integrative Biology (IBZ), ETH Zurich, Universitätsstrasse 16, 8092 Zurich, Switzerland

ARTICLE INFO

Article history:
Received 20 October 2015
Revised 22 December 2015
Accepted 23 December 2015
Available online 24 December 2015

Keywords:
Homothallism
Haploid-selfing
Hybridization
Emerging diseases
Reproductive mode
Mating system
Ash dieback

ABSTRACT

Recently, different reproductive modes were proposed between the emerging forest pathogen *Hymenoscyphus fraxineus* and its closely related avirulent sister species, *Hymenoscyphus albidus*. In the present study, inter- and intraspecific crosses were performed to experimentally assess the reproduction barriers between the two species and to *verify H. albidus*' putative haploid-selfing reproductive mode. By means of *H. fraxineus*-specific microsatellite markers, no hybridization was observed in 29 apothecia that emerged from inter-specific crosses, suggesting reproduction barriers are well-established. In a similar experimental setup, we used two newly developed polymorphic *H. albidus*-specific microsatellites to show that haploid-selfing is *H. albidus*' only reproductive mode ($N = 17$ apothecia). Further to this, the reproductive modes of both species were investigated under natural conditions. Microsatellite allele-segregation studies of *H. fraxineus*' single-spore progeny of apothecia ($N = 31$) from field samples suggest that often more than two paternal nuclei are involved in mating. In contrast, analysis of single-spore progeny of field-collected *H. albidus* apothecia ($N = 21$) confirmed the solely haploid-selfing reproductive mode detected *in vitro*. Furthermore, we present the complete mating type 1-1 locus of *H. fraxineus* and report the finding of three additional genes within this region; the as yet unobserved typical mating type gene *MAT1-1-1*, a *DNA polymerase zeta catalytic subunit*-like gene and a *pre-mRNA-splicing factor SLU7*-like gene. The same genes were also detected in the homothallic mating type locus of *H. albidus*. Further analysis confirmed the expression of all typical mating type genes (*MAT1-2-1*, *MAT1-1-3*, *MAT1-1-1*) in both species. Interestingly, the *MAT1-1-3* gene of homothallic *H. albidus* is expressed despite three stop codons interrupting the coding sequence. Overall, our findings highlight vital differences in the reproduction systems of the two species and suggest that interspecific hybridization is not possible.

1. Introduction

The reproductive mode (i.e. inbreeding vs. outbreeding) and mating system (i.e. sexual vs. asexual vs. mixed) of fungal crop pathogens are important determinants of the endurance of plant resistance. Outcrossing pathogens with mixed reproduction (asexual and sexual) are thought to have a higher evolutionary potential and are thus more likely to "break down" a plant's resistance gene than inbred or asexual pathogens (McDonald and Linde, 2002). Other factors influencing the evolutionary potential of pathogens are mutation frequency, effective population size and gene/genotype flow, as well as the efficacy of directional selection (McDonald and Linde, 2002). The validity of this concept within forest pathology has never been assessed systematically. Nevertheless, the concept carries interesting implications for invasive forest pathogens. Most invasive species reveal a severe genetic founder effect in the introduced range due to the introduction of a non-representative number of strains from the native population (Dlugosch and Parker, 2008). The lack of genetic diversity in the new range should consequently result in reduced evolutionary potential, regardless of the reproductive mode and mating system of the invasive organism. This, in return, should benefit hosts and bring them at least a short-term advantage in the evolutionary arms race against the invasive pathogen. However, recent examples of rapid evolutionary changes in invasive forest pathogens teach us that the advantage can diminish swiftly, an example being the introgression of new genetic variants from the native range or hybridization incidents with closely related species (Brasier, 2000; Olson and Stenlid, 2002). For instance, *Ophiostoma novo-ulmi*, the causal agent of Dutch elm disease, went through several evolutionary changes (possibly via interspecific hybridization with *O. ulmi*)

* Corresponding author.
E-mail addresses: tea_wey@hotmail.com (T. Wey), markus.schlegel@usys.ethz.ch (M. Schlegel), sophie.stroheker@usys.ethz.ch (S. Stroheker), andrin.gross@usys.ethz.ch (A. Gross).

http://dx.doi.org/10.1016/j.fgb.2015.12.013
1087-1845/

during the pandemic of the second half of the 20th century. These changes allowed the fungus to switch its reproductive mode from clonal to sexual and led to an increase in its genetic diversity. The subsequent increase in the number of vegetative compatibility groups in the pathogen had a negative effect on the propagation of antagonistic RNA viruses that used to decrease the aggressiveness of the pathogen (Brasier et al., 2004a). As a result, the American elm *Ulmus americana* was driven close to extinction in North America. Another example is provided by *Phytophthora alni*, which has been causing the decline of riparian alder trees since the late 1990s. Research studies suggested that the highly aggressive subspecies, *P. alni* subsp. *alni*, emerged through multiple hybridization events (Brasier et al., 1999, 2004b; Ioos et al., 2006). Therefore, thorough knowledge of the risk of pathogen evolution, as assessed by determining mating system, reproductive mode and the hybridization potential, is an important criterion for elaborating on efficient management strategies.

Hymenoscyphus albidus and *Hymenoscyphus fraxineus* are two closely related ascomycete species; the former is a harmless leaf colonizer with a presumed distribution in the entire range of common ash *Fraxinus excelsior* in Europe before the epidemic (see Fig. 23 in Baral and Bemmann, 2014) and the latter a devastating invasive forest pathogen causing ash dieback on *F. excelsior* in Europe (Gross et al., 2014a). Since the early 1990s the disease spread presumably starting from Poland, where it was reported for the first time, to almost the whole distribution range of *F. excelsior*, where it has caused massive tree mortality and has left behind heavily damaged ash stands (Pautasso et al., 2013). Only a low percentage of ash trees seems to bear a high level of tolerance toward the disease and therefore the future of ash in Europe is still at risk (McKinney et al., 2014). Moreover, a further spread of *H. fraxineus* to other susceptible host species such as the North American ash species *F. nigra* and *F. pennsylvanica* is possible (Drenkhan and Hanso, 2010; Gross and Sieber, in press; Kowalski et al., 2015). As expected for an invasive species, *H. fraxineus* populations in Europe revealed a strong founder effect compared to native populations (Gross et al., 2014b). Analysis of the mating type (*MAT*) locus suggested a structurally homothallic system for *H. albidus* and a structurally heterothallic system for *H. fraxineus* (Gross et al., 2012b). The heterothallic mating system was confirmed in a crossing assay (Gross et al., 2012b). Both species exclusively propagate via means of sexual reproduction. However, only *H. fraxineus* seems to produce the *Chalara*-anamorph (Kirisits et al., 2013), whose spores do not germinate on various substrates (Hosoya et al., 1993; Kirisits et al., 2009) and likely only function as spermatia (Gross et al., 2012b).

Although much has been learned about the reproductive mode and mating system of the two species, several knowledge gaps still exist, resulting in an incomplete picture of the reproduction system and the risk of pathogen evolution: (i) Thanks to genome sequencing, the missing fragment of the mating type locus of *H. fraxineus MAT1-1* (see Gross et al., 2012b) was uncovered (Saunders, 2013) but the *MAT* locus of *H. albidus* still contains a gap and the *MAT1-1-1* gene, typically found at the *MAT* locus (Zaffarano et al., 2010), is as yet unknown for both species. Is this gene absent in the genus *Hymenoscyphus* and if so, is there another gene that could compensate its function? Moreover, are the *MAT* genes expressed in both species? (ii) Although the *MAT* locus organization differs, homologous *MAT* genes are strongly conserved between the two species and only exhibit few amino acid substitutions (Gross et al., 2012b). In addition, morphological and genetic evidence point to a very close relationship (Baral and Bemmann, 2014; Gross et al., 2015; Queloz et al., 2011). Thus, the question arises whether the two species are clearly separated or still capable of hybridization? (iii) Homothallism is a rarely observed and investigated breeding system. Theoretically, a homothallic mating system would allow each haploid individual to mate with itself and with any other individual of the same species, thus allowing for outcrossing (Giraud et al., 2008). However, no conclusive evidence exists as to whether there are also homothallic species that only reproduce via haploid-selfing (e.g. same clone mating) in nature (Billiard et al., 2012; Gioti et al., 2013). Accordingly, is the structurally homothallic *H. albidus* able to reproduce through haploid-selfing and what is its preferred mating system in nature? (iv) Allele segregation studies using single-spore progeny isolates of *H. fraxineus* suggest multiple males might be involved in the fertilization of a single apothecium, which implies a hyper-outcrossing reproductive mode (Gross et al., 2012a). Is this extreme form of outcrossing frequently found in nature?

In the present study we aimed to fill these knowledge gaps. Specifically, we intended to complete the mating type loci of *H. fraxineus* and *H. albidus* and to verify the expression of the mating type genes. Subsequently, we investigated the mating systems of *H. fraxineus* and *H. albidus* in the laboratory by performing intra- and interspecific crosses. Finally, allele segregation studies were carried out to characterize the mating systems of the two species in nature.

2. Materials and methods

2.1. Sequencing of the missing MAT fragments of H. fraxineus and H. albidus

The entire *MAT1-1* locus of *H. fraxineus* (found by Saunders (2013)) was extracted from the KW1 assembled genome (contig: Cf746869_TGAC_s1_v1_contig_1182) available from the open ash dieback repository (MacLean et al., 2013). Primers to amplify the missing *MAT1-1* fragment of the previously sequenced *H. fraxineus* strain Abts_22 (see Gross et al., 2012b) were designed with Primer3 v0.4.0. (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Table S1). Conventional and long-range PCRs were performed as previously described (Gross et al., 2012b). Products were purified using an ExoSap protocol (Werle et al., 1994) and sequenced by Microsynth (Balgach, Switzerland). Sequences were aligned with the extracted reference sequence and with the previously sequenced *MAT* fragments of strain Abts_22 using Geneious v6.1.3 (Kearse et al. (2012), Biomatters Ltd., Auckland, New Zealand). The same procedure was followed to complete the mating type sequence of *H. albidus* strain Maga_04 (see Gross et al., 2012b), but different primers were used (Table S2). The gene prediction tool FGENESH (http://linux1.softberry.com/berry.phtml) was used for the *Botryotinia fuckeliana* or *Sclerotinia sclerotiorum*-specific gene-finding parameters to identify genes on the additional sequence fragment. Subsequently BLASTp searches (http://blast.ncbi.nlm.nih.gov) were performed with predicted mRNAs from FGENESH. The updated *MAT* reference sequence of *H. fraxineus* Abts_22 and Maga_04 are deposited in GenBank under the accessions JX169799 and JX305312, respectively.

2.2. Expression of MAT genes of H. fraxineus and H. albidus

The expression of the *MAT1-1-1*, *MAT1-1-3* (both *MAT1-1* idiomorph) and the *MAT1-2-1* (*MAT1-2* idiomorph) genes was confirmed in *H. fraxineus* strains Abts_22 (*MAT1-1*) and Abts_33 (*MAT1-2*), and in the *H. albidus* strain Maga_04. Liquid cultures were prepared in a 50 ml 2% (w/v) malt extract (Difco Laboratories, Detroit, USA) broth kept constantly shaking (110 rpm) at 20 °C in the dark for 14 days. Mycelium was harvested via filtration and immediately frozen in liquid nitrogen. Total RNA was extracted from approximately 50 mg of mycelium per strain using the RNeasy Plant mini Kit (Qiagen, Hombrechtikon, Switzerland),