Mutualism and asexual reproduction influence recognition genes in a fungal symbiont

Magriet A. VAN DER NESTa, Emma T. STEENKAMPb, Markus P. WILKENa, Jan STENLIDc, Mike J. WINGFIELDa, Brenda D. WINGFIELDa, Bernard SLIPPERSa,*
aDepartment of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
bDepartment of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
cDepartment of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

Abstract

Mutualism between microbes and insects is common and alignment of the reproductive interests of microbial symbionts with this lifestyle typically involves clonal reproduction and vertical transmission by insect partners. Here the Amylostereum fungus–Sirex wood-wasp mutualism was used to consider whether their prolonged association and predominance of asexuality have affected the mating system of the fungal partner. Nucleotide information for the pheromone receptor gene rab1, as well as the translation elongation factor 1a gene and ribosomal RNA internal transcribed spacer region were utilized. The identification of rab1 alleles in Amylostereum chailletii and Amylostereum areolatum populations revealed that this gene is more polymorphic than the other two regions, although the diversity of all three regions was lower than what has been observed in free-living Agaricomycetes. Our data suggest that suppressed recombination might be implicated in the diversification of rab1, while no evidence of balancing selection was detected. We also detected positive selection at only two codons, suggesting that purifying selection is important for the evolution of rab1. The symbiotic relationship with their insect partners has therefore influenced the diversity of this gene and influenced the manner in which selection drives and maintains this diversity in A. areolatum and A. chailletii.

© 2013 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Introduction

Most eukaryotic organisms reproduce sexually to generate offspring even though it is more costly than asexual reproduction (e.g., Barton & Charlesworth 1998; Otto 2003). This costly reproductive strategy could be maintained because recombination acts to provide advantageous genotypes necessary for adaptation to changing environments or because recombination acts to eliminate deleterious mutations (e.g., Zeyl & Bell 1997; Taylor et al. 1999; Neiman et al. 2010). Both of these hypotheses are consistent with the fact that the absence of sexual reproduction decreases the overall fitness of an organism and could ultimately lead to extinction (Butlin 2006; Paland & Lynch 2006; Howe & Denver 2008). An observation in fungi is that most asexually reproducing populations retain some level of sexual reproduction, despite its cost, thus generally favouring a system of mixed modes of reproduction (Taylor et al. 1999; Hsueh & Heitman 2008).
Notwithstanding the advantages, mixed modes of sexual and asexual reproduction appear not to be feasible in all organisms. For example, the fungal symbionts of insects mainly reproduce asexually and are transmitted from mother to offspring in a vertical fashion (Chapela et al. 1994; Judson & Normark 1996). These modes of reproduction and transmission ensure coevolution between the symbiotic partners, but could lead to a reduction in genetic diversity (Rispe & Moran 2000; Mira & Moran 2002). The absence of sexual recombination could also result in an accumulation of mildly deleterious mutations, increased genetic drift, more rapid sequence evolution (i.e., excess of amino acid substitutions), a shift in nucleotide base composition due to mutational bias, and genome erosion (Rispe & Moran 2000; Kaltenpoth et al. 2010). Nevertheless, several ancient lineages of asexually reproducing organisms still exist in successful symbiotic relationships (Welch & Meselson 2001; Jany & Pawłowska 2010). In these relationships, it is thought that selection by the host might limit the accumulation of deleterious mutations in the symbiont (Kaltenpoth et al. 2010).

In this study, we considered the ancient and obligate symbiotic relationship between the wood-rotting fungus Amylostereum areolatum and its hymenopteran Siricid insect partner, Sirex noctilio. In this relationship, the fungus is necessary for the development of the larvae, while the woodwasp spreads the asexual spores and/or mycelium of the fungus (Vasiliauskas et al. 1998; Thomsen & Koch 1999), thereby facilitating vertical transmission of A. areolatum (Madden 1981).

Like other fungal symbionts of insects (Chapela et al. 1994; Judson & Normark 1996), A. areolatum can also reproduce sexually and has a tetrapolar mating system, i.e., the genes governing sexual recognition in the fungus are present on two unlinked mating type loci (mat-A and mat-B) (Boidin & Lanquentin 1984; van der Nest et al. 2008, 2009). In fungi with a tetrapolar system, the mat-A locus harbours genes that encode homeodomain proteins (functional transcriptional factors), while the mat-B locus harbours genes that encode peptide pheromones and pheromone receptors (e.g., Brown & Casselton 2001; Heitman et al. 2007). The sexual spores of these fungi are, however, rarely found in nature and usually only in the native range of the insect and fungus (Vasiliauskas & Stenlid 1999; Slippers et al. 2003; Nielsen et al. 2009). The population biology of these fungi also suggests that, like other insect symbionts, they rely on the woodwasp for the effective spread of asexual spores (Vasiliauskas et al. 1998; Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999).

The evolutionary forces acting on the mat loci are thought to drive divergence between mat alleles, thus ensuring compatibility between individuals in a population (e.g., May et al. 1999; Devier et al. 2009). A form of balancing selection, known as negative frequency-dependent selection, probably acts to preserve the characteristically high allelic and nucleotide diversities at these loci (May et al. 1999). This mechanism involves the selection for rare alleles, because individuals carrying rare alleles will be sexually compatible with a larger proportion of other individuals in the population (May et al. 1999; Ruggiero et al. 2008). Diversity at the mat loci of eukaryotes is also promoted by accelerated evolutionary rates, which is evident in the increased frequency of nonsynonymous substitutions located in these regions (Vicoso et al. 2008; Devier et al. 2009). These high rates of substitution could be ascribed to suppressed recombination and/or positive selection, where the former prevents the loss of mutations and the latter acts to maintain beneficial amino acid substitutions (Uyenoyama 2005; Menkis et al. 2008; Vicoso et al. 2008).

The overall aim of this study was to determine whether the symbiotic relationship between A. areolatum and S. noctilio, together with a predominantly asexual mode of reproduction in the fungus, has influenced the evolution of the genes determining sexual recognition in the fungal partner. Two specific questions were addressed: (i) How does the pattern and extent of polymorphism at a mat locus compare to those in other regions of the genome? (ii) Which evolutionary forces most likely influence the patterns and rates of polymorphism at the mat loci? To answer these questions we utilized DNA sequence information for the pheromone receptor gene (rab1) encoded at the mat-B locus, as well as the eukaryotic translation elongation factor 1α (Tef-1α) gene and the ribosomal RNA (rRNA) internal transcribed spacer (ITS) region, which includes the spacers ITS1, ITS2, and the 5.8S rRNA gene. The rab1 gene has been demonstrated previously to be involved in sexual recognition in A. areolatum (van der Nest et al. 2008). For comparison, we included data from the closely related species Amylostereum chailletii that more frequently reproduces sexually (Vasiliauskas et al. 1998; Vasiliauskas & Stenlid 1999; Slippers et al. 2001). Finally, these systems were also compared with those of other free-living Agaricomycetes.

Materials and methods

Fungal strains

Heterokaryotic isolates of Amylostereum areolatum (CMW16848) and Amylostereum chailletii (NAC3) were included for the identification and characterization of pheromone receptor genes. Additionally, 25 isolates each of A. areolatum and A. chailletii obtained from various culture collections were included to investigate the allelic variation and diversity of pheromone receptor genes in naturally occurring isolates (Supplementary Tables 1 and 2). These isolates were selected to capture the known diversity of the fungi and were collected from South Africa, Brazil, Argentina, Australia, New Zealand, United States of America, Canada, France, Sweden, United Kingdom, Switzerland, Denmark, Norway, Austria, Italy, Greece, and Lithuania. Working cultures of these heterokaryons were maintained on potato dextrose agar (PDA) (24 g L⁻¹ of PDA, 1 g L⁻¹ glucose, and 1 g L⁻¹ yeast extract) (Biolab, Johannesburg, South Africa). All of the isolates used in this study are also maintained at 4 °C in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Genomic DNA was isolated from the isolates using the method described by Zhou et al. (2004).

PCR, cloning, and nucleotide sequencing of the rab1 gene

A large portion of the rab1 gene sequence for Amylostereum areolatum (CMW16848) was available from a previous study (van der Nest et al. 2008), while that for Amylostereum chailletii was
identified using degenerate PCR primers (br1-F and br1-R; Supplementary Table 3) designed by James et al. (2004b). All PCRs were performed on an Eppendorf thermocycler (Eppendorf AG, Germany) using reaction mixtures containing 1 ng µl⁻¹ DNA, 0.2 mM of each of the four dNTPs, 1.5 mM MgCl₂, 0.5 µM of each primer, and 2.5 U FastStart Taq (Roche Diagnostics, Mannheim). Thermal cycling conditions consisted of an initial denaturation step at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The resulting PCR products were purified using polyethylene glycol (PEG) precipitation (Steenkamp et al. 2006) and the purified PCR products were cloned using the pGEM-T Easy vector System I (Promega Corporation, Madison, USA). PCR products were cloned in order to obtain haplotype phases of all sequences derived from the heterokaryotic isolates. The cloned products were amplified from individual colonies with plasmid-specific primers (Steenkamp et al. 2006), after which the PCR products were purified using PEG precipitation. The purified products were then sequenced with the plasmid-specific primers, Big Dye Cycle Sequencing kit version 3.1 (Perkin-Elmer, Warrington, UK) using primer set ITS1 + ITS4 (Supplementary Table 3; White et al. 1990) was used for amplification of the ITS region of both species. Primer set ITS1 + ITS4 (Supplementary Table 3) for A. chailletii and primer set TEFaa1 + TEFaa2 (Supplementary Table 3) for A. areolatum. All the Tef-1α and ITS PCR products, as well as both of the 186- and 682-bp fragments of rab1, except those used for PCR–RFLPs, were purified, cloned and at least five clones per individual were sequenced, as described above.

Following sequence analysis with Chromas Lite and BioEdit, sequence alignments for each locus were produced using MAFFT version 5.85 (http://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2002). For each of the datasets, nucleotide diversity (π; Nei & Li 1979) was determined using the software package DnaSP version 5.10 (Librado & Rozas 2009), while allelic frequencies were calculated using GENEPOP software version 4.2.1. Because a limited number of clones were sequenced per individual, the possibility of underestimating allelic diversity could not be excluded.

**Molecular evolution of rab1**

To identify the evolutionary forces acting on the pheromone receptor genes of Amylostereum areolatum and Amylostereum chailletii, all the unique rab1 alleles identified in this study were examined. The A. areolatum rab1 alleles were amplified and sequenced using primer set RAB9 + RAB10 (Supplementary Table 3) for isolates CMW8900 and CMW2822, while the identified A. chailletii rab1 alleles were amplified and sequenced using primer set RAB11 + RAB12 (Supplementary Table 3) for isolates LIIAc116, DAC2, US2, and It1.8.

The CODEML program in the PAML version 3.14 package (Yang & Nielson 2002) was used to determine patterns of selective pressure acting on the pheromone receptor alleles identified in the two fungi. The phylogenetic tree required by CODEML was generated by subjecting an Multiple Alignment using Fast Fourier Transform (MAFFT)-generated nucleotide alignment of rab1 to a maximum likelihood (ML) analyses using PhyML version 3.0 software (Guindon & Gascuel 2003). This ML analysis employed gamma correction (G) to account for among site rate variation, a proportion of invariable sites (I) and the HKY (Hasegawa et al. 1985) nucleotide substitution model as indicated by jModeltest version 0.1.1, and the Akaike Information Criterion (Posada 2008).

Positive selection was evaluated by computing ω across all the sites for each of the loci (Yang et al. 2000; Devier et al. 2009), where ω reflects the nonsynonymous (dN)/synonymous (dS) substitution rate ratio (Yang & Nielson 1998). To test for
variation of selective pressures across the codons, goodness of fit was calculated for the different site-specific models proposed by Yang et al. (2000). Statistical significance was calculated with likelihood ratio tests (LRT), which entailed analysis of the $\chi^2$ distribution of $2\Delta \ln$ (i.e., twice the log likelihood difference between the two models) values for the different models (Yang & Nielson 1998), where the degrees of freedom were equal to the differences in the number of parameters between the two models (Yang et al. 2000).

To determine whether balancing selection acts on rab1 to maintain rare alleles over long evolutionary times (Vieira et al. 2008), a phylogenetic tree based on the amino acid sequences of the pheromone receptors present in A. areolatum and A. chailletii and sequences from other Basidiomycetes and Ascomycetes available in GenBank was constructed (See Fig 3 for accession numbers for the pheromone receptors of other fungi). The amino acid sequences were aligned using MAFFT and an ML phylogeny inferred with PhyML, which utilized the LG (Le & Gascuel 2008) model of amino acid substitution, I, and the observed amino acid frequencies, as indicated by ProtTest 2.4 (Abascal et al. 2005). Branch support was determined using PhyML with the same best-fit model and 1000 bootstrap replicates. The tree and dataset have been submitted to TreeBASE (http://www.treebase.org/treebase/index.html) and the Study Accession URL is: http://purl.org/phylo/treebase/phylows/study/TB2:S12966.

DnaSP was used to study the extent of recombination within the rab1, ITS, and Tef-1x regions examined. The recombination parameter R was calculated (Hudson et al. 1987), while the minimum number of recombination ($R_m$) events during the history of the species (Hudson & Kaplan 1985) was estimated using neutral coalescence simulations, based on the number of segregating sites, intermediate levels of recombination, and 10 000 replications (Librado & Rozas 2009). The extent of recombination within each species was also compared by examining single-locus phylogenies for incompatibility. The latter analyses were based on separate trees inferred from the DNA sequence information for the three loci of the 13 selected isolates of each of A. areolatum and A. chailletii. MAFFT-generated datasets were subjected to PhyML analyses using best-fit model parameters, as described before. ML analysis of the rab1 dataset employed the HKY with I and G, the ITS dataset employed the TrNeF model (Tamura & Nei 1993; Posada 2008), and the Tef-1x dataset employed the TrN model (Tamura & Nei 1993; Posada 2008). For each dataset, branch support was determined using the respective best-fit models and 1000 bootstrap replicates. To assess congruencies between the resulting gene trees, a strict consensus tree was computed using Mega software version 4.0.2 (Kumar et al. 2008). The partition homogeneity test using PAUP version 4.0b10 (Swofford 2000) was used to examine the null hypothesis of recombination in A. areolatum and A. chailletii (Houbraken et al. 2008). Significance was assigned by comparing the summed tree length from the actual data to those from 100 artificial datasets.

Results

PCR, cloning, and nucleotide sequencing of the rab1 gene

It was possible to identify and sequence the complete pheromone receptor gene rab1 (1539 bp) in Amylostereum areolatum (GenBank accession number HQ864712), as well as a large portion of the gene in Amylostereum chailletii (1265 bp) (GenBank accession number HQ864716). Typical of pheromone receptors, the inferred amino acid sequences for the Amylostereum Rab1 (Fig 1) harboured the seven transmembrane-spanning helices that are characteristic of the rhodopsin-like superfamily of G protein-linked receptors, as well as extracellular and cytoplasmic loop domains and a long cytoplasmic tail (e.g., James et al. 2004a; Raudaskoski & Kothe 2010). The rab1 gene also contained five introns (Fig 1), which are comparable with those reported for the pheromone receptors of Coprinopsis cinerea that has four or five, Coprinellus disseminatus that has five, and Schizophyllum commune that has three introns (Vaillancourt et al. 1997; James et al. 2006). The position of introns 2–5 closely corresponded to those in previously identified receptor sequence introns, while intron 1 in the rab1 N-terminal appears to represent a novel position. The position and size of the introns present in the rab1 genes in A. chailletii and A. areolatum were similar, except that the last intron in A. chailletii was larger than in A. areolatum.

Allelic variation and diversity of rab1, Tef-1α, and ITS

A total of eight and six polymorphisms were observed within the 186-bp alignment of rab1 for the 25 representative isolates of each of Amylostereum areolatum and Amylostereum chailletii, respectively (Supplementary Tables 1 and 2). For 13 of the 25 representative isolates of A. areolatum and A. chailletii, a larger portion of the rab1 gene, as well as a portion of Tef-1x and ITS was also analyzed. Following cloning of each PCR product, sequencing, and alignment, the datasets for ITS, Tef-1x, and rab1 in these representative isolates consisted of 606, 474, and 682 nucleotides, respectively. Based on the sequence alignments, two types of Tef-1x were identified. Because the one type contained a premature stop codon, it was considered a pseudogene and was not included in subsequent analyses.

Among the Tef-1x, ITS, and rab1 sequences of the 13 representatives of A. areolatum and of A. chailletii, a total of 19 (Supplementary Table 4) and 24 (Supplementary Table 5) nucleotide polymorphisms, respectively, were identified. However, in both species, about half of the polymorphisms were located in the rab1 dataset. As a result, the nucleotide diversity or $\pi$-values (0.004 and 0.02, respectively) for the rab1 regions in A. areolatum and A. chailletii were generally higher than the $\pi$-values for the ITS and Tef-1x regions (Table 1). The GenBank accession numbers for the ITS sequences of A. areolatum and A. chailletii are respectively HQ864715 and HQ864718, while

Fig 1 – The predicted amino acid sequences for the various alleles of the putative pheromone receptor rab1 in A. areolatum and A. chailletii. Protein domains were predicted with TOPCON (http://topcons.cbr.su.se) and codons under positive selections are indicated in the shaded boxes.
the accession number for Tef-1α in A. chailletii is HQ864717 and for the two types of Tef-1α identified in A. areolatum are HQ864714 and HQ864713, respectively.

Among the 13 representatives for each species, the number of alleles identified in the three genes (rab1, ITS, and Tef-1α) investigated ranged from two to six for A. areolatum and three to five for A. chailletii (Fig 2A). With regards to rab1, the same three alleles that were identified within the set of 13 A. areolatum isolates were also detected in the set of 25 A. areolatum isolates collected in different regions of the world (Supplementary Table 4). In contrast, an additional rab1 allele was detected in the global collection of 25 A. chailletii isolates. The A. chailletii alleles isolated in this study thus harboured at least four pheromone receptor alleles (Supplementary Tables 2 and 5), although some apparently occur at very low frequencies (Fig 2A). These findings were also supported by the PCR–RFLP analysis with EcoRV.

Heterozygous and homozygous genotypes were detected in both A. chailletii and A. areolatum for all three gene regions investigated (Fig 2B). Nine, eight, and five unique ITS, Tef-1α, and rab1 genotypes were identified for the A. chailletii individuals. Among the A. areolatum isolates respectively three, seven, and three ITS, Tef-1α, and rab1 genotypes were identified. Compared to A. areolatum, the A. chailletii individuals thus harboured considerably more unique ITS (nine vs. three) and rab1 (five vs. three) genotypes. Amylostereum chailletii also included many more genotypes represented by a single individual (i.e., six vs. one ITS genotypes and two vs. zero rab1 genotypes) in comparison with A. areolatum. Some of the identified genotypes were overrepresented. For example, 76.9% of the A. areolatum individuals shared the same ITS genotype (Fig 2B). These overrepresented genotypes were also homozygotic, with both nuclei of the heterokaryon having the same allelic state.

**Molecular evolution of rab1**

A large proportion of the three Amylostereum areolatum rab1 (1579 bp) alleles, as well as the five Amylostereum chailletii rab1 (1448 bp) alleles were sequenced (Fig 1). In A. areolatum, alleles shared 99.0–99.9% nucleotide sequence identity with 15 polymorphic sites (Supplementary Table 6). The alleles in A. chailletii shared more than 98.0% nucleotide sequence identity with each other (Supplementary Table 6), while they shared only 83% nucleotide sequence identity with the A. areolatum rab1 alleles. This suggests that all the alleles belong to the same sublocus, as the pheromone receptors in Coprinopsis cinerea belonging to the same sublocus are ~60% similar, while pheromone receptors from different subloci are <32–35% similar (Riquelme et al. 2005).

Comparison of the rab1.1 allele with the rab1.2 and the rab1.3 alleles in A. areolatum revealed two polymorphic sites that result in nonsynonymous amino acid substitutions (present in the long cytoplasm carboxy-terminal intracellular tail) (Fig 1). The single polymorphic site identified in the rab1.2 and rab1.3 alleles represented a silent substitution, which suggests that the rab1.2 and rab1.3 alleles are functionally equivalent in sharing the same mating type specificity. Several nonsynonymous substitutions were identified among the rab1 alleles present in A. chailletii (Fig 1), including one in transmembrane region 1, one in the transmembrane region 5, two in the intracellular loop 3, and two in the long cytoplasm carboxy-terminal intracellular tail.

Calculations using model M3 with variable selective pressures acting on the codons and models that assume no selection (M0) provided a better fit for rab1 (Table 2). The log likelihood difference between M3 and M0 was 4.57 with a significant χ² distribution test result (P < 0.05), while the log likelihood differences were not significant for M2 and M1, M5 and M1, M6 and M1 as well as for M8 and M7. Therefore, the positive-selection models (M2, M5, M6, and M8) did not provide a better fit in comparison to models that assume no positive selection (M1 and M7). Even though the models that assume positive selection did not provide a better fit, all of these models identified two positively selected codons (codons 256 and 373) respectively located in the intracellular loop EL3 region and in the long cytoplasm carboxy-terminal intracellular tail. However, at the 95% level, only models M5 and M6 identified these codons as being subject to positive selection (Table 2).

The rab1 alignment (consisting of 337 amino acids) and ML phylogeny included sequences of the A. areolatum and A. chailletii rab1 alleles identified in this study, as well as sequences from the pheromone receptors present in other Basidiomycetes and Ascomycetes (Fig 3). All of the Amylostereum pheromone receptors group in the same major cluster. The putative pheromone receptors of A. areolatum and A. chailletii appeared to be each other’s closest neighbours and they grouped together.
with proteins from *C. cinerea* (including CcSTE3.3, Rcb3B43, AAQ96346) and *Laccaria bicolor* (including LbSTE3.3; 192000).

No evidence of recombination within *rab1*, ITS or Tef-1α for *A. areolatum* and *A. chailletii* was detected. This is because of the low values for the recombination parameters (R and RM) (Table 3) that were observed for all three loci in both species, suggesting that meiotic recombination between segregating sites is limited (Garg et al. 2007). However, evidence of recombination among individuals was detected in this study. Two distinct clades were identified for all the three genes, with one clade containing only *A. areolatum* isolates and the other only *A. chailletii* isolates (Supplementary Fig 1A, B, and C). Clonal lineages were observed between the three trees for both the species. For example, the alleles of the *A. areolatum* alleles in *A. areolatum* and *A. chailletii* clustered in a species-specific manner, closely with the pheromone receptor genes CcSTE3.3 in *C. cinerea* and LbSte3.3 in *L. bicolor* (Niculita-Hirzel et al. 2008; Martinez et al. 2009).

Fig 3 – An amino acid-based ML phylogeny of the pheromone receptors present in *A. areolatum*, *A. chailletii*, and other *Agaricomycetes*. Percentage bootstrap (100 replicates) values greater than 50% are shown below the tree branches. All of the *rab1* alleles in *A. areolatum* and *A. chailletii* clustered in a species-specific manner, closely with the pheromone receptor genes CcSTE3.3 in *C. cinerea* and LbSte3.3 in *L. bicolor* (Niculita-Hirzel et al. 2008; Martinez et al. 2009).
Heterozygosity tests

Likelihood scores and parameter estimates for the site-specific models of the three genes present in A. areolatum and A. chailletii.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Species</th>
<th>(A^b)</th>
<th>(\Pi^c)</th>
<th>(\pi_{\text{n}}/\pi_{\text{S}}^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>A. areolatum</td>
<td>2</td>
<td>0.0004</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>A. chailletii</td>
<td>5</td>
<td>0.0020</td>
<td>–</td>
</tr>
<tr>
<td>EF</td>
<td>A. areolatum</td>
<td>5</td>
<td>0.0050</td>
<td>0.00*</td>
</tr>
<tr>
<td></td>
<td>A. chailletii</td>
<td>5</td>
<td>0.0060</td>
<td>0.00*</td>
</tr>
<tr>
<td>rab1</td>
<td>A. areolatum</td>
<td>3</td>
<td>0.0042</td>
<td>0.00*</td>
</tr>
<tr>
<td></td>
<td>A. chailletii</td>
<td>4</td>
<td>0.0200</td>
<td>0.00*</td>
</tr>
</tbody>
</table>

Significantly different values are indicated with an asterisk (\(P < 0.05\)).

a All estimates were determined with GENEPOP version 4.
b Model likelihoods used for calculating statistical significance with LRT, which entailed analysis of the \(\chi^2\) distribution of 2\(\Delta\ln\) (i.e., twice the log likelihood difference between the two models) for the different models (Yang & Nielsen 1998).
c The nonsynonymous (\(dN\))/synonymous (\(dS\)) substitution rate ratio (Yang & Nielsen 1998).
d Parameters estimated for each model according to those proposed by Yang (2007).
e NEB = Naive empirical Bayes.

Discussion

This study represents the first attempt to investigate the evolution of a mating type locus of a fungus involved in an obligate mutualism with an insect. The Amylostereum–Sirex association has impacted significantly on the biology and evolution of the fungal partner, similar to that observed in other symbionts. This is reflected in the predominantly asexual mode of reproduction of Amylostereum areolatum and Amylostereum chailletii and their overall low genetic diversity. The data presented here clearly demonstrate higher levels of diversity in the rab1 pheromone receptor in A. areolatum and A. chailletii compared to ITS and Tef-1x. However, the diversity of all three genes was lower than the corresponding genes in other free-living Agaricomycetes. Rather than positive or balancing selection, purifying selection represents an important driving force in the evolution of rab1 in A. chailletii and A. areolatum. Our study thus suggests that the long-term symbiotic relationship with their insect partners has not only affected the diversity at this locus, but it has also impacted on the manner in which selection drives and maintains this diversity in A. areolatum and A. chailletii.

Comparison of the inferred amino acid sequences of the Amylostereum rab1 with those in other Agaricomycetes revealed that they share the same structure and conserved domains (e.g., James et al. 2004a; Raudaskoski & Kothe 2010). The Rab1 protein is thus likely to function in the same way as pheromone receptors of other fungi. In yeast, a conformational change in the receptor causes the release of the G protein bound to the pheromone receptor protein when the pheromone binds to the pheromone receptor protein. This results in the activation of the Mitogen-activated protein (MAP) kinase signalling that activates specific transcriptional factors involved in mating and heterokaryon formation (Marsh et al. 1991). To confirm that this occurs in Amylostereum, it will be necessary to do transcript profiling and functional analyses.

Identification of naturally occurring pheromone receptor rab1 alleles in A. chailletii and A. areolatum revealed that these genes are multiallelic and polymorphic. This is consistent with what is known for other fungi with tetrapolar mating systems (e.g., May et al. 1999; Kothe et al. 2003; Riquelme et al. 2005; Devier et al. 2009). Both A. areolatum and A. chailletii displayed nucleotide diversities for rab1 that are higher than the values observed for their corresponding ITS and Tef-1x sequences. This is similar to what has previously been found for other eukaryotes (e.g., May et al. 1999; James et al. 2001; Devier et al. 2009) where the genes controlling sexual recognition are more polymorphic and diverse than the rest of the genome.

Table 1 – Heterozygosity tests\(^*\) of the three genes present in the 13 heterokaryons each of A. areolatum and A. chailletii.

Table 2 – Likelihood scores and parameter estimates for the site-specific models (Yang et al. 2000) evaluated in this study.

(Supplementary Fig 1D), suggesting that recombination is restricted to within-species interactions.
The pheromone receptor encoded by rab1 in the fungi examined is thus subject to selection for diversification.

Previous work on sex-related genes has shown that their diversification may be driven by balancing selection and suppressed recombination (Meyer & Thomsen 2001; Uyenoyama 2005). Balancing selection is known to maintain fungal mating type alleles distributed throughout populations at roughly equal frequencies (James et al. 2001). However, we found no evidence for balancing selection operating on the rab1 alleles of Amylostereum. All of the rab1 alleles in A. areolatum and A. chailletii clustered in a species-specific manner, closely with the pheromone receptor genes CcSTE3.3 in Coprinopsis cinerea and LbSte3.3 in Laccaria bicolor (Niculita-Hirzel et al. 2008; Martinez et al. 2009). Neither species thus harboured traces of transspecies polymorphism, which is a hallmark of balancing selection (Vieira et al. 2008; Devier et al. 2009). Nevertheless, no evidence of recombination within the region of the rab1 analyzed was detected, which is in agreement with the notion that allelic diversity of eukaryotic recognition loci is influenced by suppressed recombination (e.g., Uyenoyama 2005; Menkis et al. 2008). Our results thus suggest that suppressed recombination and not balancing selection could explain the higher levels of diversity in the rab1 gene of A. chailletii and A. areolatum.

Positive selection is also known to maintain high levels of diversity of sex-related genes (e.g., Civetta & Singh 1998; Karlsson et al. 2008). As expected, we detected the effects of positive selection on the Amylostereum rab1, albeit only at two codons of the gene. One of the codons is situated in the third extracellular (loop EL3) of the inferred Rab1 receptor protein. In fact, a large proportion (27 %) of the nonsynonymous substitutions occurring between the A. areolatum and A. chailletii rab1 alleles were also located in this region. These results are consistent with previous reports that the first and third extracellular loops of the pheromone receptor are usually more variable, because these regions interact with the mating pheromones (Reneke et al. 1988; Niculita-Hirzel et al. 2008). The second codon potentially under positive selection is located in the long C-terminal cytoplasmic tail following the last transmembrane domain, a region that also harboured a large proportion of the nonsynonymous substitutions between A. areolatum and A. chailletii.

It is likely that one or more of the nonsynonymous substitutions identified in rab1 may determine mating type specificity. This could be true for either of the codons under positive selection, or any other of the nonsynonymous substitutions between A. areolatum and A. chailletii detected in this study. For example, the nonsynonymous substitutions located in the third extracellular loop region EL3 of the pheromone receptor that interacts with the mating pheromones (Reneke et al. 1988; Niculita-Hirzel et al. 2008) could alter the mating type specificity. Nonsynonymous substitutions located in the third intracellular region (loop IL3) and the C-termini cytoplasmic tail have also been implicated in mating type specificity as both these regions of the receptor interact with G proteins and affect G protein signalling (Marsh et al. 1991; Gola et al. 2000; Karlsson et al. 2008). In A. areolatum and A. chailletii, the possibility cannot be excluded that another pheromone receptor closely linked to rab1 may determine mating type specificity, although rab1.1 and rab1.2 have previously been shown to cosegregate with mating type specificity in A. areolatum (van der Nest et al. 2009). Future genome sequence-based studies will determine whether these substitutions or those located at other receptors confer mating type specificity in Amylostereum.

The results presented here suggest that the entire rab1 gene is under purifying selection and not positive selection (Table 2), which is different to what is expected (e.g., Civetta & Singh 1998; Karlsson et al. 2008). The ω-values for rab1 in these fungi were significantly below 1, indicating that purifying selection was responsible for eliminating most amino acid substitutions that might have arisen. Similar results have been obtained for Microbotryum spp. (Devier et al. 2009). These fungi also have a tetrapolar mating type system, but they predominantly represent selffertilizers that require only two pheromone receptor alleles and apparently do not need positive selection to generate and maintain additional alleles. A similar situation may exist in A. areolatum, which also appears to have only two functional rab1 alleles, based on the differences in the amino acid level, that could alter mating type specificity. The possibility that there are additional alleles at this locus that were missed in this study cannot be excluded, even though we sampled

---

**Table 3 — Results of the recombination tests as determined with DnaSP 5.10 (Librado & Rozas 2009).**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Species</th>
<th>R²</th>
<th>R_M²</th>
<th>Confidence interval</th>
<th>P (R_M ≤ observed R_M)</th>
<th>Avg. R_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>rab1</td>
<td>A. areolatum</td>
<td>0.0010</td>
<td>1.0000</td>
<td>0.0, 0.0</td>
<td>2.8190</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>A. chailletii</td>
<td>0.0007</td>
<td>0.9940</td>
<td>0.0, 1.0</td>
<td>0.9944</td>
<td>0.1709</td>
</tr>
<tr>
<td>ITS</td>
<td>A. areolatum</td>
<td>0.0100</td>
<td>0.001</td>
<td>0.0, 0.0</td>
<td>2.8068</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>A. chailletii</td>
<td>0.5000</td>
<td>0.9920</td>
<td>0.0, 1.0</td>
<td>4.2558</td>
<td>0.1728</td>
</tr>
<tr>
<td>Tef-1α</td>
<td>A. areolatum</td>
<td>7.0990</td>
<td>0.6417</td>
<td>0.0, 2.0</td>
<td>1.2937</td>
<td>0.3911</td>
</tr>
<tr>
<td></td>
<td>A. chailletii</td>
<td>0.0200</td>
<td>0.9995</td>
<td>0.0, 0.0</td>
<td>1.5337</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

a Estimate of the population recombination parameter R excluding alignment gaps and haplotype corrections.
b The observed minimum number of recombination events in the data.
c Neutral coalescence simulations given the number of segregating sites, with an intermediate level of recombination and 10 000 replications.
d The confidence interval (lower limit, upper limit) for R_M.
e The probability that R_M is less than or equal to the observed R_M.
f The average value of R_M.
from various regions of the world. Nevertheless, our results suggest that purifying selection represents an important driving force in the evolution of rab1 in A. chailletii and A. areolatum.

The nucleotide diversities for rab1, ITS, and Tef-1α in A. areolatum and A. chailletii were all much lower than those observed for the regions examined in other fungi (e.g., Ciampi et al. 2009; Engh et al. 2010). For example, the rab1 alleles of A. areolatum and A. chailletii shared high levels of sequence identity (between 97 % and 99 % nucleotide identity), while pheromone receptors from the same subgroup in Schizophyllum commune share only 90 % sequence identity (Wendland & Kothe 1996; Gola & Kothe 2003). Also, the A. areolatum and A. chailletii rab1 nucleotide diversity values were considerably lower than those reported for Coprinillus disseminatus (James et al. 2006) and Serpula lacrymans (Engh et al. 2010). Similarly, the observed π-values for the ITS and Tef-1α regions in A. areolatum and A. chailletii were significantly lower than the values observed for these genes in S. lacrymans (Engh et al. 2010). The low nucleotide diversities observed for the Amylostereum rab1 gene thus appear to be associated with limited genetic diversity at other unlinked loci. This is consistent with previous observations that the overall genetic diversity of A. areolatum and A. chailletii is generally very low (e.g., Vasiliauskas et al. 1998; Vasiliauskas & Sterlidi 1999). Compared to free-living Agaricomycetes, A. areolatum and A. chailletii have a low overall genetic diversity, as well as a low genetic variation of their mat-B locus. This low diversity could be due to the close association between the fungus and the woodwasp.

Collectively, the results of this study suggest that the patterns of polymorphism observed at rab1 in A. areolatum and A. chailletii are determined by the combined effects of the selection and demographic processes brought about by the unique lifestyle of these fungi. For example, inbreeding and/or asexuality in A. areolatum and A. chailletii could explain the low nucleotide diversity of the genes investigated in this study (Glémin et al. 2006; Haag & Roze 2007; Haudry et al. 2008). Similar observations have also been made in other microbial symbionts with reduced levels of recombination and effective population sizes (Rispe & Moran 2000; Mira & Moran 2002). However, recombination does not appear to be completely absent in A. areolatum and A. chailletii because the results showed that both species are characterized by unique multilocus genotypes among individuals (Supplementary Tables 4 and 5) and by incongruencies among the phylogenies inferred from the three loci studied (Supplementary Fig 1). Our results thus highlight the fact that, despite a predominant asexual mode of reproduction, A. areolatum and A. chailletii also reproduce sexually (Milgroom 1996; Otto 2003; Haag & Roze 2007; Houbraken et al. 2008). This is consistent with the notion that asexual reproduction allows for the maintenance of the symbiotic relationships of these fungi, while occasional sexual reproduction ensures their fitness and ability to adapt to change. During such sexual interactions, polymorphism at the mat loci ensures compatibility among partners, which is the consequence of suppressed recombination that drives diversification of these loci. However, the predominantly asexual lifestyle of these fungi has apparently relaxed the need for positive selection to maintain additional mat alleles.

Acknowledgements

We thank the National Research Foundation (NRF), members of the Tree Pathology Cooperative Programme (TPCP), and the THRIP initiative of the Department of Trade and Industry (DTI), South Africa for financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2013.05.001.

References


