FUNGAL BIOLOGY 117 (2013) 439–450

British Mycological Society promoting fungal science

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

Mutualism and asexual reproduction influence recognition genes in a fungal symbiont

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ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form
23 April 2013
Accepted 1 May 2013
Available online 16 May 2013
Corresponding Editor:
Conrad Lamoraal Schoch

Keywords:
Amylostereum areolatum
Pheromone receptor and evolution
Sirex noctilio
Symbiosis

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* woodwasp 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 1α 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*.

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).

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1878-6146/$ – see front matter
http://dx.doi.org/10.1016/j.funbio.2013.05.001

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 codependence 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 & Pawlowska 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 sporocarps 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 $MgCl_2$, 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) and an ABI3700 DNA analyzer (Applied Biosystems, Foster City, USA).

To obtain the sequences upstream and downstream of these fragments in *A. areolatum* and *A. chailletii*, nested PCR primers designed with Primer 3 (cgi v0.2) (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) and PCR-based genome-walking (Siebert *et al.* 1995) were used. The nested primers used for genome-walking included RAB1–4 (Supplementary Table 3) for *A. areolatum* and RAB5 (Supplementary Table 3) for *A. chailletii*. The remaining portion of the pheromone receptor gene for *A. chailletii* was obtained using a primer (RAB6; Supplementary Table 3) based on the sequence of *A. areolatum*. These PCR products were amplified, cloned, and sequenced as described above.

All sequence files were analyzed with Chromas Lite 2.0 (Technelysium) and BioEdit version 7.0.2.5 (Hall 1999). They were also compared to those in the protein database of the National Centre for Biotechnology Information (www.ncbi.nih.nlm.gov) using BlastX. To predict the features in the secondary structure of the Rab1 protein, we used TOPCON (http://topcons.cbr.su.se/), which calculates consensus predictions using a Hidden Markov Model and inputs five commonly used topology prediction methods (Bernsel *et al.* 2009).

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

To identify unique *rab1* alleles in *Amylostereum areolatum* and *Amylostereum chailletii*, we used two approaches to discover polymorphisms among a diverse set of 25 isolates of each species. The one approach entailed sequence analysis of a 186-base pair (bp) region of *rab1*, which was previously shown to be polymorphic (van der Nest *et al.* 2008). This fragment was amplified and sequenced using primer set RABF + RABR (Supplementary Table 3). The second approach involved PCR–RFLP (restriction fragment length polymorphism) analysis of a 682-bp fragment of the *rab1* gene. For this purpose, PCR products were generated with primers RAB1-470F and RAB1-1800R (Supplementary Table 3) for the 25 isolates of *A. areolatum*. The amplicons were then digested with the enzyme *Eco*RV (Roche Diagnostics) and visualised with agarose gel (Roche Diagnostics) electrophoresis (Sambrook *et al.* 1989; van der Nest *et al.* 2008).

Based on the polymorphisms observed, a set of 13 isolates for each of *A. areolatum* and *A. chailletii*, were selected. For these isolates, the 682-bp portion of the *rab1* gene was sequenced for *A. areolatum*, as well as for *A. chailletii* using primer set RAB7 + RAB8 (Supplementary Table 3). For comparative purposes, portions of the two housekeeping loci, ITS and Tef-1α, were also amplified and sequenced for the 13 isolates of each species. Primer set ITS1 + ITS4 (Supplementary Table 3; White *et al.* 1990) was used for amplification of the ITS region of both species. For the Tef-1α region, the primer set TEFac1 + TEFac2 (Supplementary Table 3) was used 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.

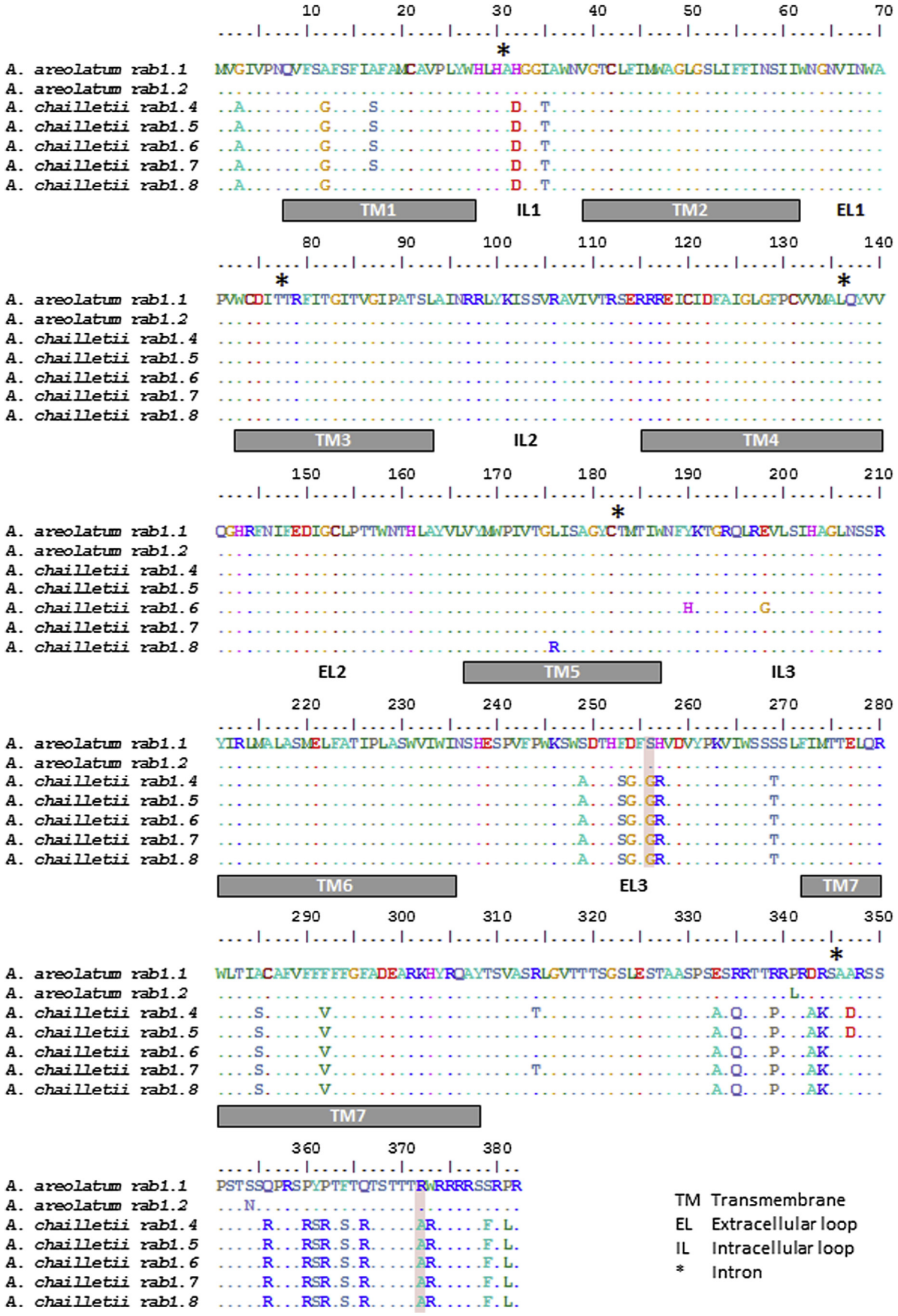
Following sequence analysis with Chromas Lite and BioEdit, sequence alignments for each locus were produced using MAFFT version 5.85 (http://mafft.cbrc.jp/alignment/server/) (Katoh *et al.* 2002). For each of the datasets, nucleotide diversity (π; Nei & Li 1979) was determined using the software package DnaSP version 5.10 (Librado & Rozas 2009), while allelic frequencies were calculated using GENEPOP software version 1.2. 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


A. areolatum rab1.1
A. areolatum rab1.2
A. chailletii rab1.4
A. chailletii rab1.5
A. chailletii rab1.6
A. chailletii rab1.7
A. chailletii rab1.8
TM1
IL1
TM2
EL1
TM3
IL2
TM4
EL2
TM5
IL3
TM6
EL3
TM7
TM7
TM Transmembrane
EL Extracellular loop
IL Intracellular loop
* Intron

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 χ^2 distribution of 2Δln (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-1α 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-1α 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 of *A. chailletii*, a larger portion of the *rab1* gene, as well as a portion of Tef-1α and ITS was also analyzed. Following cloning of each PCR product, sequencing, and alignment, the datasets for ITS, Tef-1α, and *rab1* in these representative isolates consisted of 606, 474, and 682 nucleotides, respectively. Based on the sequence alignments, two types of Tef-1α 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-1α, 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 π-values (0.004 and 0.02, respectively) for the *rab1* regions in *A. areolatum* and *A. chailletii* were generally higher than the π-values for the ITS and Tef-1α 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.

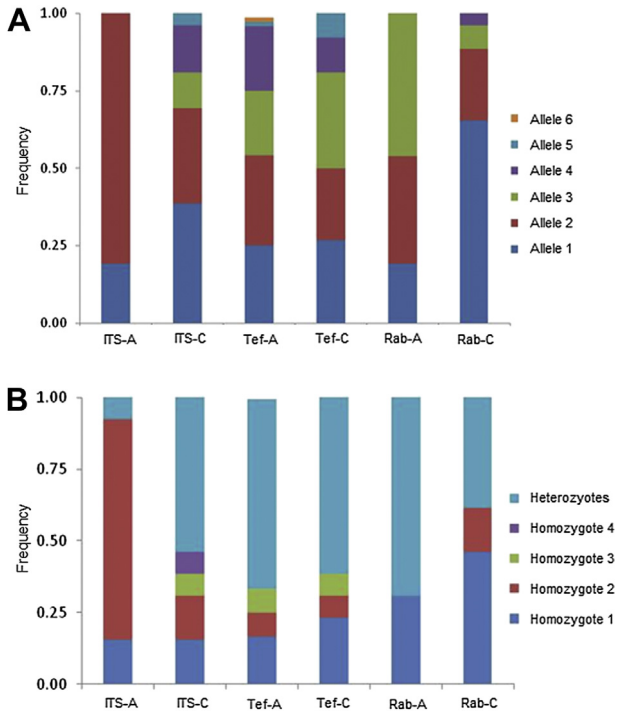


Fig 2 – Stack histograms of allele frequencies (A) and genotype frequencies (B) at individual loci. ITS-A, ITS of *A. areolatum*; ITS-C, ITS of *A. chailletii*; Tef-A, Tef-1α of *A. areolatum*; Tef-C, Tef-1α of *A. chailletii*; Rab-A, *rab1* of *A. areolatum*; Rab-C, *rab1* of *A. chailletii*.

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* isolates included 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 *Eco*RV.

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 χ^2 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

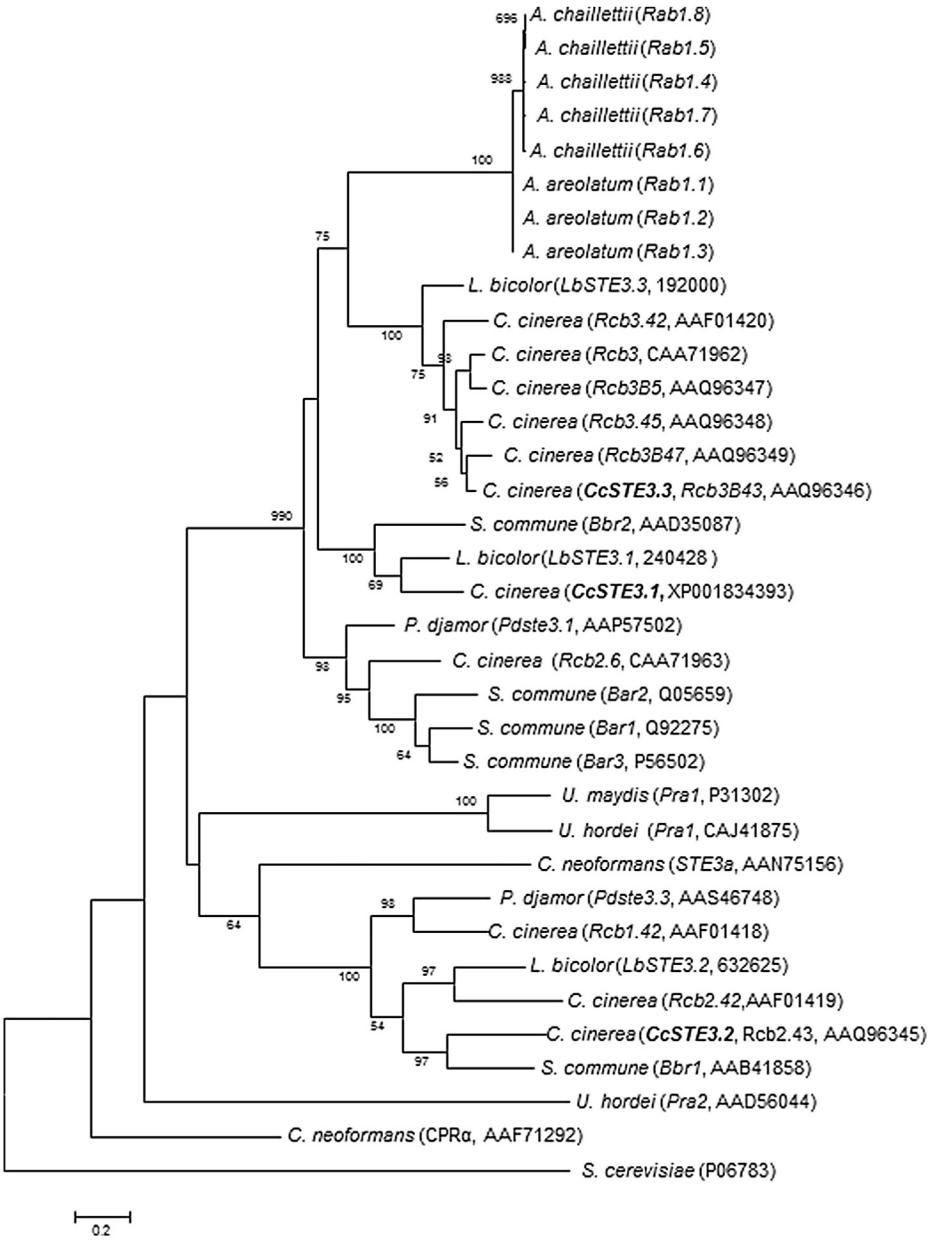


Fig 3 – An amino acid-based ML phylogeny of the pheromone receptors present in *A. areolatum*, *A. chailletii*, and other *Agaricomycetes*. Percentage bootstrap (100 replicates) values greater than 50 % are shown below the tree branches. All of the *rab1* alleles in *A. areolatum* and *A. chailletii* 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).

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 R_M) (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* isolates from the Southern Hemisphere grouped together for all the three genes (Supplementary Fig 1A, B, and C). However, consistent with the results of the partition homogeneity test ($P < 0.001$), incongruences were also observed among the *rab1*, Tef-1α, and ITS phylogenies (Supplementary Fig 1A, B, and C). For example, the alleles of the *A. areolatum* isolates obtained from Southern Hemisphere countries grouped together with different isolates for each of the gene trees. No structure was evident in the strict consensus tree for either *A. areolatum* or *A. chailletii*

Table 1 – Heterozygosity tests[a] of the three genes present in the 13 heterokaryons each of *A. areolatum* and *A. chailletii*.

Locus	Species	A[b]	Π[c]	π_n/π_s[d]
ITS	*A. areolatum*	2	0.0004	–
	A. chailletii	5	0.0020	–
EF	*A. areolatum*	5	0.0050	0.00*
	A. chailletii	5	0.0060	0.00*
rab1	*A. areolatum*	3	0.0042	0.00*
	A. chailletii	4	0.0200	0.00*

Significantly different values are indicated with an asterisk ($P < 0.05$).
a All estimates were determined with GENEPOP version 4.
b Average number of alleles per locus.
c Mean number of pair-wise differences among sequences (Nei & Li 1979).
d Ratio of nonsynonymous nucleotide variation to synonymous nucleotide variation.

(Supplementary Fig 1D), suggesting that recombination is restricted to within-species interactions.

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-1α. 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-1α 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 2 – Likelihood scores and parameter estimates for the site-specific models (Yang *et al.* 2000) evaluated in this study.

Model[a]	ln Likelihood[b]	Sites with $P(\omega > 1) > 0.95$ NEB[e]	dN/dS[c]	Estimates of parameters[d]
M0 (one-ratio)	−2100.89	n.a.	0.0682	$\omega_1 = 0.0682$
M1 (neutral)	−2096.32	n.a.	0.1321	$\omega_0 = 0.01309\ p_0 = 0.89171\ \omega_1 = 1\ p_1 = 0.10829$
M2 (selection)	−2096.32	–	0.1200	$\omega_0 = 0.01308\ p_0 = 0.89171$ $\omega_1 = 1\ p_1 = 0.04405$
M3 (discrete)	−2096.14	n.a.	0.1283	$\omega_0 = 0.00000\ p_0 = 0.81906\ \omega_1 = 0.48426$ $p_1 = 0.16723\ \omega_2 = 3.44796\ p_2 = 0.01371$
M5 (gamma)	−2096.31	256 G[e] 372 A[e]	0.1194	$a = 0.06624\ b = 0.36890$
M6 (double gamma)	−2096.14	256 G[e] 372 A[e]	0.1283	$p_0 = 0.89259\ a_0 = 0.04008\ b_0 = 1.13382$ $p_1 = 0.10741\ a_1 = 2.73793\ b_1 = 2.73793$
M7 (β distribution)	−2096.30	n.a.	0.1183	$p = 0.01742\ q = 0.11677$
M8 (β + positive selection)	−2096.30		0.1260	$p_0 = 0.96972\ p = 0.05439\ q = 0.44414$ $\omega = 1.000$

a Site-specific models implemented in the CODEML program in PAML version 3.14 package (Yang & Nielson 2002).
b Model likelihoods used for calculating statistical significance with LRT, which entailed analysis of the χ^2 distribution of 2Δln (i.e., twice the log likelihood difference between the two models) for the different models (Yang & Nielson 1998).
c The nonsynonymous (dN)/synonymous (dS) substitution rate ratio (Yang & Nielson 1998).
d Parameters estimated for each model according to those proposed by Yang (2007).
e NEB = Naive empirical Bayes.

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

Genes	Species	R[a]	R_M[b]	Coalescence simulations[c]		
				Confidence interval[d]	P ($R_M \leq$ observed R_M)[e]	Avg. R_M[f]
rab1	*A. areolatum*	0.0010	1.0000	0.0, 0.0	2.8190	0.0002
	A. chailletii	0.0007	0.9940	0.0, 1.0	0.9944	0.1709
ITS	*A. areolatum*	0.0010	0.0001	0.0, 0.0	2.8068	0.0000
	A. chailletii	0.5000	0.9920	0.0, 1.0	4.2558	0.1728
Tef-1α	*A. areolatum*	7.0990	0.6417	0.0, 2.0	1.2937	0.3911
	A. chailletii	0.0200	0.9995	0.0, 0.0	1.5337	0.0005

a Estimate of the population recombination parameter R excluding alignment gaps and haploid 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.

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 at 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

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 *Coprinellus disseminates* (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 & Stenlid 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.

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