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RESEARCH ARTICLE

Characterization of the systems governing sexual and self-recognition in the white rot homobasidiomycete *Amylostereum areolatum*

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Abstract This study considered the systems controlling sexual and self-recognition in *Amylostereum areolatum*, a homobasidiomycetous symbiont of the *Sirex* woodwasp. To investigate the structure and organization of these systems in *A. areolatum*, we identified a portion of a putative homologue (RAB1) of the pheromone receptor genes of *Schizophyllum commune* and *Coprinus cinereus*, and a portion of a putative homologue of the *S. commune* mitochondrial intermediate peptidase (*mip*) gene. Diagnostic DNA-based assays for mating-type were developed and their application confirmed that the fungus has a heterothallic tetrapolar mating system. Segregation analysis showed that *RAB1* is linked to mating-type B, while *mip* is linked to mating-type A. The results of sexual and vegetative compatibility tests suggest that sexual recognition in *A. areolatum* is controlled by two multiallelic *mat* loci, while self-recognition is controlled by at least two multiallelic *het* loci. Therefore, despite the association of *A. areolatum* with the woodwasp and the unique mixture of sexual and clonal reproduction of the fungus, both recognition systems of the fungus appear to be similar in structure and function to those of other homobasidiomycetes. This is the first report regarding the genes controlling recognition of a homobasidiomycete involved in an obligate mutualistic relationship with an insect.

Keywords Pheromone receptor · Mitochondrial intermediate peptidase · Mating-type · Vegetative incompatibility · *Het* loci

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Introduction

A typical homobasidiomycete life-cycle consists of a short homokaryotic phase during which hyphal cells contain a single type of nucleus, followed by a predominantly fertile heterokaryotic phase during which hyphal cells harbor more than one type of nucleus. The sexual recognition system, under the control of the mating-type (*mat*) loci, determines hyphal fusion or mating between homokaryotic hyphae (Kronstad and Staben 1997; Casselton and Olesnicky 1998). The vegetative incompatibility system, under the control of the heterokaryon incompatibility (*het*) loci, determines hyphal fusion between mostly heterokaryotic hyphae (i.e. self-recognition) (Worrall 1997). So far, these systems have been characterized in a limited number of homobasidiomycetes. This study represents the first report regarding the genes controlling sexual and self-recognition in *Amylostereum areolatum*, a homobasidiomycete involved in an obligate mutualistic relationship with Siricid woodwasps.

Genetic studies in homobasidiomycetes are hampered by the fact that some species are unculturable or their spores fail to germinate, while others (including *A. areolatum*) rarely fruit in nature and do not readily produce fruiting bodies in the laboratory (Martin 1992; James et al. 2004a). Furthermore, the variable nature of the genes encoded at the *mat* loci makes their genetic identification problematic,

because these genes do not cross-hybridize in Southern analysis and it is difficult to design degenerate primers (Badrane and May 1999; Halsall et al. 2000; James et al. 2004a). At the DNA-level, characterization of mating systems have almost exclusively been limited to the model homobasidiomycetes *Schizophyllum commune* and *Coprinus cinereus* (Kronstad and Staben 1997; Casselton and Olesnicky 1998). Even less information is available for the self-recognition systems, except that the homobasidiomycetes and other basidiomycetes harbor apparently fewer *het* loci than their ascomycete relatives (Worrall et al. 1997). Research regarding these systems will therefore provide valuable insight into the structure, organization, function and evolution of the recognition loci, as well as the role these loci play in the development and evolution of homobasidiomycetes and other fungi.

The white rot mushroom, *A. areolatum*, lives in an obligate symbiosis with various woodwasp species, including *Sirex noctilio* (Gaut 1969; Talbot 1977; Slippers et al. 2003). The symbiosis is a highly evolved mutualism in which the asexual spores of the fungus are spread by the wasp, while wood decay by the fungus is necessary for the development of the larvae (Gilmour 1965; King 1966; Madden and Coutts 1979). As a result, the life-cycle of *A. areolatum* involves a unique interplay between asexual and sexual reproduction (Gilmour 1965; King 1966; Madden and Coutts 1979). The importance of asexual reproduction in the life-cycle of this fungus is reflected in its overall low genetic diversity based on vegetative incompatibility and DNA-based studies (Vasiliauskas et al. 1998; Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999; Slippers et al. 2001). Furthermore, *A. areolatum* fruiting bodies have never been found in the southern hemisphere and are only rarely found in the northern hemisphere, except in some areas in central Europe (Thomsen 1998; Slippers and Vasaitis unpublished). The close association between the fungus and the woodwasp is the inferred explanation for this low heterogeneity, because the genetic homogeneity of the fungal partner is promoted through vertical transmission of asexual spores by the *Sirex* woodwasp, as has been shown for other insect–fungus symbioses (Frank 1996; Douglas 1998; Korb and Aanen 2003). This form of reproduction is thought to ensure maintenance of genotypes that are better adapted for symbiosis in *A. areolatum* (Herre et al. 1999).

The recognition loci of *A. areolatum* have not yet been characterized. It is, however, known that fusion of homokaryotic hyphae of this fungus is controlled by a tetrapolar mating system where sexual compatibility is governed by two unlinked *mat* loci (locus A and B) (Boidin and Lanquentin 1984). Only genetically distinct homokaryons with different allelic specificities at both their *mat* loci are sexually compatible and will allow reciprocal nuclear migration to occur after cell fusion to form a heterokaryon (Kronstad and Staben 1997; Casselton and Olesnicky 1998). The heterokaryon can produce fruiting bodies under favorable conditions with karyogamy and meiosis occurring within specialized cells (Kües et al. 2002). This sexual process, albeit rare in *A. areolatum* (Boidin and Lanquentin 1984; Thomsen 1998; Slippers et al. 2001), might be important to allow adaptation to changing environments. In terms of the self-recognition system it may be expected to operate the same as in other fungi, where hyphal fusion is only permitted between genetically similar heterokaryons sharing the same allele specificities at all of their *het* loci (Rayner 1991; Worrall 1997; Glass and Kaneko 2003). When two interacting heterokaryons are genetically dissimilar with different allelic specificities at some or all of their *het* loci, cell death of the interacting hyphae prevents unlike individuals from anastomosing (Rayner 1991; Worrall 1997). As vegetative incompatibility essentially represents a self/nonself recognition system that preserves genetic identity (Worrall 1997; Glass and Kaneko 2003), it is possible that it may help to ensure maintenance of *A. areolatum* genotypes that are better adapted for symbiosis.

In this study we characterized the loci controlling sexual and self-recognition in a set of *A. areolatum* homokaryons and their mated heterokaryons obtained from field-collected basidiocarps. Furthermore, a portion of a putative homologue of the pheromone receptors encoded at the *mat-B* locus of *S. commune* and *C. cinereus* were characterized and genetic linkage between this gene and mating-type B was established. Similarly, a portion of a putative homologue of the mitochondrial intermediate peptidase (*mip*) gene in *A. areolatum* was characterized and linkage between the putative *mip* gene and mating-type-A was demonstrated. These putative pheromone receptor and *mip* sequences were subsequently used to developed DNA-based assays for diagnosing mating-type in *A. areolatum*. These data allowed us to compare the recognition systems of *A. areolatum*, a homobasidiomycete closely associated with an insect symbiont, to those of other homobasidiomycetes.

Materials and methods

Fungal isolates

Totally 19 single-spored, homokaryotic isolates were obtained from a single basidiocarp of *A. areolatum* and a heterokaryotic culture (CMW16828) was made from the structure to serve as a genetic record of the parent. A second basidiocarp of *A. areolatum* was collected and yielded 80 single-spored, homokaryotic isolates and a culture (CMW16848) was taken from the parent structure. Both basidiocarps were collected in Austria by R. Vasaitis and

B. Slippers (Table 1). The parent strains are hereafter referred to as CMW16828 and CMW16848 and their respective homokaryotic progeny are referred to as $CMW16828_{[1–19]}$ and $CMW16848_{[1–80]}$. The parent strains, CMW16828 and CMW16848, are vegetatively incompatible and represent separate genets (see below). The single-spored isolates were obtained by placing a portion of the hymenophore on the underside of a Petri dish lid over 1% malt yeast agar (MYA) (20 g L^{-1} malt extract, 2 g L^{-1} yeast extract and 15 g L^{-1} agar) (Biolab, Johannesburg, South Africa) or by placing a piece of hymenophore on a piece of wax paper. Following a spore shower onto the paper, the collected spores were suspended in sterile water and dilutions of these spore suspensions were plated onto 1% MYA and incubated for 24 h at 25°C. Individual germinating spores were identified using a dissection microscope and transferred to separate MYA plates. For comparative purposes, representative heterokaryons from the southern and northern hemisphere were also included in the study (Table 1). All of the cultures were maintained on MYA made with pine extract (PE-MYA). Pine extract was prepared by twice autoclaving 200 g pine wood chips in 1 L of distilled water and then passing the liquid through a cotton cloth. All isolates of *A. areolatum* used in this study are stored and maintained at 4°C in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

AFLP analysis

To ensure that each of the respective homokaryotic progeny used in this study originated from either CMW16828 or CMW16848, AFLP (amplified fragment length polymorphism, Vos et al. 1995) analysis was used. It was necessary to verify the origin of the homokaryons, because samples were collected from the field, and it is possible that spores from another basidiocarp could have been sampled. Also, hyphae from the basidiocarp could have been sampled instead of germinating spores. For this purpose DNA was isolated from all of the homokaryons and the parent heterokaryons using the method described by Zhou et al. (2004) and stored at −20°C. AFLP analyses was performed as previously described (Vos et al. 1995) using three primer combinations (E-ac + M-02, E-ac + M-04 and E-tc + M-03) where both *Eco*RI (E) and *Mse*I (M) primers included two selective nucleotides at their 3′-ends. The *Eco*RI (E) primers were 5′-end labeled with IRDye™ 700 or IRDye™ 800 (LI-COR, Lincoln, NE) and the *Mse*I (M) primers (Inqaba Biotechnologies, RSA) were unlabelled. The resulting fragments were separated using the 4200 LI-COR® automated DNA sequencer (LI-COR) and analysed with QUANTAR Version 1.0 (KeyGene Products B.V., The Netherlands) during which fragments were scored as present or absent.

Table 1 Origin of *Amylostereum areolatum* heterokaryons used in this study

Culture no.[a]	Origin	Collector	*RAB1* allele/s[b]
CMW16828	Austria	B. Slippers	*RAB1.1*
CMW16848	Austria	B. Slippers	*RAB1.1*; *RAB1.2*
CMW8900	South Africa	B. Slippers	*RAB1.2*; *RAB1.3*
CMW8898	Brazil	B. Slippers	*RAB1.2*; *RAB1.3*
CMW3300	New Zealand	G.B. Rawlings	*RAB1.2*; *RAB1.3*
CMW28217	Lithuania	R. Vasaitis	*RAB1.1*; *RAB1.2*
CMW28219	Lithuania	R. Vasaitis	*RAB1.1*
CMW28225	Denmark	I.M. Thomsen	*RAB1.1*; *RAB1.3*
CMW28221	Norway	H. Solheim	*RAB1.1*; *RAB1.3*
CMW28223	Switzerland	O. Holdenrieder	*RAB1.2*
CMW28224	Switzerland	O. Holdenrieder	*RAB1.2*; *RAB1.3*

[a] CMW (Culture collection of the Tree Pathology Co-operative Programme, University of Pretoria, South Africa)

[b] *RAB1* alleles in each isolate was identified using DNA-based assays (Fig. 2) and sequence analysis

Morphological diagnosis of mating-types

Hyphal morphology was used to determine the mating-types of the homokaryons isolated from basidiocarps CMW16828 and CMW16848 by pairing homokaryotic isolates on PE-MYA and incubating these cultures at ±25°C in darkness. Mycelial plugs (±5 mm diameter) taken from the homokaryotic isolates were placed 1 cm apart and after 4 weeks an agar plug (±5 mm diameter) containing the intermingling mycelium was transferred to a new plate. After 2 weeks of growth, hyphal morphology was examined using a Zeiss Axiocam light microscope. The absence of clamp connections indicated incompatible mating interactions, their presence indicated compatible interactions, while the presence of curly and irregular hyphal structures and hyper branching, as well as the presence of clamp cells that are unable to fuse indicated partially successful sexual interactions. The mating-types of the 19 homokaryons for family CMW16828 were determined by pairing all of the homokaryons in this family in all possible combinations and studying the hyphal morphology of the interacting individuals. To determine the mating-types of the 80 homokaryons for family CMW16848, four tester strains were first identified by pairing eight homokaryons in all possible combinations. After assigning mating-types to the four tester homokaryons, they were used in pairings to determine the mating-types of the remaining homokaryons in family CMW16848.

PCR-based diagnoses of mating-types

PCR-based diagnostic procedures were developed to determine the mating-types of the homokaryons isolated from

basidiocarps CMW16828 and CMW16848. For mating-type A, we used segregation analysis to determine if the putative *mip* gene in *A. areolatum* is also linked to the *mat-A* locus, as was found in other homobasidiomycetes (James et al. 2004a). If the two genes are indeed linked, it will be possible to identify the alleles segregating at the *mat*-A locus based on segregation of *mip* alleles. For this purpose, a portion of *mip* was amplified from the parent heterokaryon CMW16848 using previously designed degenerate primers MIP1F and MIP2R (James et al. 2004a). PCR was performed on an Eppendorf thermocycler (Eppendorf AG, Germany) using a reaction mixture 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 0.05 U μL^{-1} FastStart Taq (Roche Diagnostics, Mannheim). Thermal cycling conditions included 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. DNA was separated by electrophoresis on 1% agarose gels (wt/v) (Roche Diagnostics) (Sambrook et al. 1989). Sizes of the amplicons were determined by comparison against a 100 base pair (bp) molecular weight marker (O'RangeRuler™ 100 bp DNA ladder, Fermentas Life Sciences). The resulting PCR products were purified using polyethylene glycol (PEG) precipitation (Steenkamp et al. 2006). The purified PCR products were resuspended in 20 µL sterile distilled water and cloned using pGEM-T Easy vector System I (Promega Corporation, Madison, USA) following the manufacturer's instructions.

The cloned products were amplified from individual colonies using plasmid specific primers, after which the PCR products were purified using PEG precipitation and sequenced using a Big Dye Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, USA) and an ABI3700 DNA analyzer (Applied Biosystems). Sequence analyses were performed with Chromas Lite 2.0 (Technelysium) and BioEdit version 7.0.2.5 (Hall 1999). The resulting DNA sequences were compared to those in the protein database of the National Centre for Biotechnology Information (http://www.ncbi.nih.nlm.gov) using *blastx* to confirm gene identity. From these sequences, allele-specific PCR primers MIPAro1F (5′-gtcctttcactcttcggtac-3′) and MIPAro1R (5′-caaataactggcgccatacc-3′) were designed using the programme Primer 3 (v. 0.4.0) (http://frodo.wi.mit.edu/) and applied to homokaryons $CMW16848_{[1–80]}$.

For mating-type B, we targeted the *A. areolatum* homologue of the pheromone receptor present at the *mat-B* locus of *S. commune* and *C. cinereus* (Kronstad and Staben 1997; Casselton and Olesnicky 1998). This gene was amplified in the heterokaryotic parental isolate CMW16848 using degenerate primers br1-F and br1-R designed by James et al. (2004b). The fragment was cloned and sequenced as described above. After confirming the identity of the sequenced portion using *blastx* as described above, PCR-based genome walking (Siebert et al. 1995) was used to obtain the upstream and downstream sequences of this fragment. Based on the resulting information, sequence-specific primers RAB1-470F (5′-tcttgggctgacttttcc-3′) and RAB1-1800R (5′-ggcaggtagatcgaggttga-3′) were designed using Primer 3. These primers were used to amplify a portion of the putative pheromone receptor gene in the parental heterokaryon CMW16848, as well as representative isolates from the southern and northern hemisphere. The resulting fragments were purified, cloned and at least five clones per heterokaryotic isolate were sequenced. These sequences were compared to identify unique restriction sites to use in a PCR–RFLP (restriction fragment length polymorphism) procedure that will allow identification of different alleles of the putative pheromone receptor in the parental heterokaryon CMW16848 and its progeny. The PCR–RFLP entailed amplification of the putative *A. areolatum* pheromone receptor with primers RAB1-470F and RAB1-1800R, followed by digestion with the enzyme *Eco*RV (Roche Diagnostics) at 37°C for 2 h. In all cases, χ^2 tests were used to evaluate the goodness of fit of observed allelic distributions to expected Mendelian segregation ratios, as well as to test co-segregation of alleles identified using microscopy and those identified using the DNA-based assays (Steel et al. 1997).

Generation of synthetic heterokaryons

Sibling heterokaryons were generated by pairing sexually compatible homokaryons originating from parental strain CMW16828, in all possible combinations. For the homokaryons derived from CMW16848, sibling heterokaryons were generated in the same way. To generate sib-related heterokaryons, homokaryons from the same basidiocarp were paired with a sexually compatible but unrelated homokaryon. The sib-related heterokaryons were thus produced by pairing the unrelated homokaryon $CMW16848_{[57]}$ with sexually compatible homokaryotic progeny of strain CMW16828. Homokaryon $CMW16828_{[1]}$ was also paired with homokaryotic progeny of strain CMW16848, to generate a second set of sib-related heterokaryons. All the sib-related heterokaryons, therefore, had a nucleus in common (i.e. that of either homokaryon $CMW16848_{[57]}$ or $CMW16828_{[1]}$) with vegetative incompatibility being determined only by the related nucleus. Synthetic heterokaryons were generated by placing mycelial plugs (±5 mm diameter) containing homokaryotic hyphae 1 cm apart on PE-MYA, followed by incubation at ±25°C in the dark. After 4 weeks, agar plugs containing the intermingling heterokaryotic mycelium were transferred to new plates. Successful interaction and heterokaryon formation was verified

by the presence or absence of clamp connections, observed using a Zeiss Axiocam light microscope (Carl Zeiss Ltd., München, Germany) as described before.

Vegetative compatibility

To assay compatibility all of the pairings were done by placing mycelial plugs (±5 mm diameter) 1 cm apart from each other on PE-MYA, followed by incubation at ±25°C in the dark. Hyphal interactions were scored after 4 weeks. Compatible reactions were scored as 0 and showed no visible reaction, with the interacting hyphae intermingling. Weakly incompatible reactions were scored as 1, with very light pigmentation of the interaction zone. Strongly incompatible reactions were scored as 2, based on relative sparseness of aerial hyphae and brown discoloration of interacting hyphae. All pairings were done in duplicate and a self-pairing was always included as a compatible control.

The vegetative compatibility groups (VCGs) for all of the *A. areolatum* isolates used in this study were determined. The parental heterokaryotic isolates (CMW16828 and CMW16848) were paired with each other to verify that the two basidiocarps from which the respective progenies were derived, represent different VCGs. This also provided verification that a homokaryon belonging to one family could be used as an unrelated homokaryon to generate sib-related heterokaryons with the other family. The vegetative incompatibility of the sib-related heterokaryons for both the families were determined by pairing 16 selected sib-related heterokaryons from each family with each other in all possible combinations. Thus, sib-related heterokaryons derived from compatible homokaryons from one basidiocarp (CMW16828) were paired with each other and likewise, sib-related heterokaryons derived from compatible homokaryons from the other basidiocarp (CMW16848) were also paired with each other. To confirm the presence of individual VCGs in family CMW16848, four sib-related heterokaryons that belong to the identified VCGs were paired with the rest of the sib-related heterokaryons isolated from that basidiocarp. The results of these pairings were confirmed with sibling pairings, where 25 selected sibling heterokaryons from basidiocarp CMW16828 were paired with each other in all possible combinations, and likewise, 25 sibling heterokaryons of basidiocarp CMW16848 were paired with each other in all possible combinations.

Results

AFLP analysis

We confirmed the parent–progeny relationship between heterokaryons CMW16828 and CMW16848 and their respective homokaryotic offspring using AFLPs. The homokaryons in each family shared all of their AFLP fragments with the heterokaryon from which it originated. Each of the individual homokaryons also was recombinant as they displayed unique fingerprints.

Morphological diagnosis of mating-type

The four possible mating-types of the homokaryons isolated from basidiocarps CMW16828 and CMW16848 were determined using microscopy. Based on these findings alleles A1B1, A1B2, A2B1 and A2B2 segregating at the *mat-A* and *mat-B* loci, representing the four mating-types, were identified for the progeny of parental heterokaryon CMW16848. Similarly, alleles A3B3, A3B4, A4B3 and A4B4 segregating at the *mat-A* and *mat-B* loci, representing the four mating-types, were identified for the progeny of parental heterokaryon CMW16828. Sexually incompatible homokaryons were identified based on the absence of clamp connections, which indicated that homokaryons belong to the same mating-type and share the same alleles at both their *mat* loci. In contrast, sexually compatible homokaryons were identified based on the presence of clamp connections, which indicated that the homokaryons belong to opposite mating-types and have different alleles at both their *mat* loci (e.g. A1B1 vs. A2B2 and A1B2 vs. A2B1). Homokaryons displaying partial sexual compatibility were identified based on their inability to produce a heterokaryon with clamp connections, but displayed abnormal hyphal morphology. Interacting homokaryons that have different alleles at their *mat-B* locus, but the same allele at their *mat-A* locus (e.g. A1B1 vs. A1B2; A2B2 vs. A2B1), were identified by the presence of curly and irregular hyphal structures and hyper-branching growth. Interacting homokaryons with different alleles at their *mat-A* locus, but with the same allele at their *mat-B* locus (e.g. A1B1 vs. A2B1; A2B2 vs. A1B2), were identified by the presence of false clamp connections. Representative homokaryons of the four mating-types in one family were all sexually compatible with those of the other family, while the ratio of compatible to incompatible crosses between homokaryons of the same progeny or basidiocarp was 1:3.

PCR-based diagnosis of mating-types

The alleles (A1B1, A1B2, A2B1 and A2B2) assigned to the four possible mating-types of the homokaryons isolated from basidiocarp CMW16848 were also identified using PCR-based assays. The *mip* gene that has been shown to co-segregate with the *mat-A* locus in other homobasidiomycetes (James et al. 2004a) was successfully amplified using MIP1F and MIP2R in the parental heterokaryon CMW16848. Based on the *blastx* analyses, the sequence

(GenBank accession number EU380311) of the 415 bp fragment of *A. areolatum* was 67% similar at the amino acid level to the *mip* gene of *S. commune* (GenBank accession number AAB01371). Specific primers that amplify the one *mip* allele of *A. areolatum* based on this sequence, generated a 332 bp PCR product in 44 of the 80 homokaryons of family CMW16848 (Fig. 1). It was thus possible to distinguish between the *mip* alleles present in the progeny with the two *mip* alleles segregating 1:1 in the progeny ($P < 0.05$). As predicted, the two *mip* alleles also co-segregated ($P < 0.05$) with the microscopically identified *mat-A* alleles (A1 and A2), confirming that the putative *mip* gene of *A. areolatum* is indeed closely linked to the *mat-A* locus.

An 884 bp fragment of a putative pheromone receptor was amplified in the parental heterokaryon of family CMW16848 using degenerate primers br1-F and br1-R (James et al. 2004b). Based on the *blastx* analyses the sequence of this fragment was 62% similar at the amino acid level to the Bbr2 pheromone receptor sequence of *S. commune* (GenBank accession number AAK58068) and 68% similar to the rcb1 pheromone receptor sequence of *C. cinereus* (GenBank accession number AAQ96348). This putative pheromone receptor in *A. areolatum* was designated the acronym RAB1 (pheromone **r**eceptor in ***A****mylostereum* present at *mat* locus **B**; GenBank accession numbers EU380312 and EU380313). A further ~500 bp of the upstream and downstream sequence was obtained using PCR genome-walking. Specific primers (RAB1-946F and RAB1-1800R) that amplify an 845 bp fragment of *RAB1* in *A. areolatum* were designed based on this sequence. Amplification, cloning and sequencing of *RAB1* in the parental heterokaryon CMW16848 revealed that one of the *RAB1* alleles, but not the other, harbors an *Eco*RV restriction site. The undigested RAB1 fragment (845 bp) was present in 39 of the 80 homokaryons of family CMW16848 and is designated *RAB1.1* (Fig. 2). The digested fragments (652 and 193 bp) were present in the remaining homokaryons and are designated *RAB1.2* (Fig. 2). The two *RAB1* alleles segregated 1:1 in the progeny ($P < 0.05$) of CMW16848 and they also co-segregated with the microscopically identified *mat-B* alleles (B1 and B2) in the homokaryons of family CMW16848 ($P < 0.05$). Since there is more than one pheromone receptor encoded at the *mat-B* locus of the homobasidiomycetes studied thus far (Halsall et al. 2000), it is possible that RAB1 or another pheromone receptor tightly linked to this putative pheromone receptor, may be involved in determining mating-type specificity in these crosses.

Application of the RAB1-specific primers designed in this study also generated the expected 845 bp fragment in heterokaryon CMW16828 and its homokaryotic offspring, as well as the representative southern and northern hemisphere heterokaryons (Table 1). Based on restriction digestion with *Eco*RV and sequence analyses of the cloned 845 bp products, heterokaryons CMW16828, and CMW28219 appeared to be homozygous for the *RAB1.1* allele while heterokaryon CMW28223 was homozygous for the *RAB1.2* allele. All of the remaining heterokayons appeared to be heterozygous, with heterokaryons CMW16848 and CMW28217 harboring *RAB1.1* and *RAB1.2*, heterokaryons CMW28225 and CMW28221 harboring *RAB1.1* and *RAB1.3*, and the southern hemisphere heterokaryons CMW8900, CMW8898 and CMW3300 and the Swiss heterokaryon CMW28224 harboring *RAB1.2* and *RAB1.3*. Comparison of the sequences of the three identified alleles to the corresponding region of the pheromone receptor genes of the model homobasidiomycetes revealed that *RAB1* harbors three putative introns at positions similar to those identified for Rcb3.42 of *C. cinereus* (Halsall et al. 2000). Of the 14 polymorphic sites, eight are located in the introns, while those in the putative exons represent synonymous substitutions not affecting the inferred amino acid sequences (Fig. 3). We did however, only sequence a portion of RAB1 (i.e. 845 bp) and only five clones per heterokaryon. It may therefore be possible that we might have missed additional alleles for the putative pheromone receptor gene of *A. areolatum*.

Vegetative incompatibility

The heterokaryon interactions, which included the sibling, sib-related and unrelated interactions, were scored after 4 weeks as 0, 1 or 2 (Fig. 4). All self-pairings included as

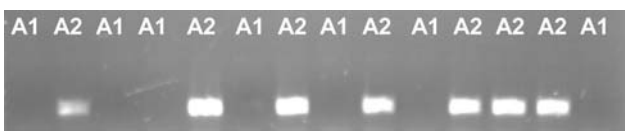

Fig. 1 Segregation of the putative mitochondrial intermediate peptidase (*mip*) gene fragment among CMW16828$_{[n]}$ and CMW16848$_{[n]}$ progeny. Application of primers MIPAro1F and MIPAro1R in PCR generates ~350 bp products only in those isolates designated as harboring the A1 allele of locus *mat-A*

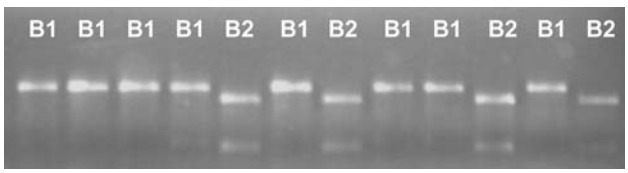

Fig. 2 Segregation of the putative pheromone receptor *RAB1* PCR–RFLP fragments among CMW16828$_{[n]}$ and CMW16848$_{[n]}$ progeny. The 845 bp amplified portion of the *RAB1* allele linked to mating-type B2 is digested into two fragments of sizes 652 and 193 bp, while the 845 bp amplified portion of the *RAB1* allele linked to mating-type B1 is undigested

	164	176	180	191	193	197	252	261	390	477	671	701	783	822
RAB1.1	c	t	g	t	g	t	a	a	c	c	g	t	t	a
RAB1.2	t	c	a	t	t	g	g	g	t	t	t	c	c	g
RAB1.3	c	t	g	c	g	t	a	a	t	t	t	c	c	g
	*	*	*	*	*	*					*	*		

Fig. 3 Polymorphic nucleotide sites associated with the 845 bp portion of the putative pheromone receptor RAB1 of *A. areolatum*. The respective *RAB1* alleles are indicated to the left of the nucleotides and their positions are indicated at the top. Eight of these polymorphisms are located in putative introns (indicated with *asterisk*), while those located in the exons represent synonymous substitutions. The diagnostic *Eco*RV restriction site that distinguishes *RAB1.1* from the other two alleles is located at position 193

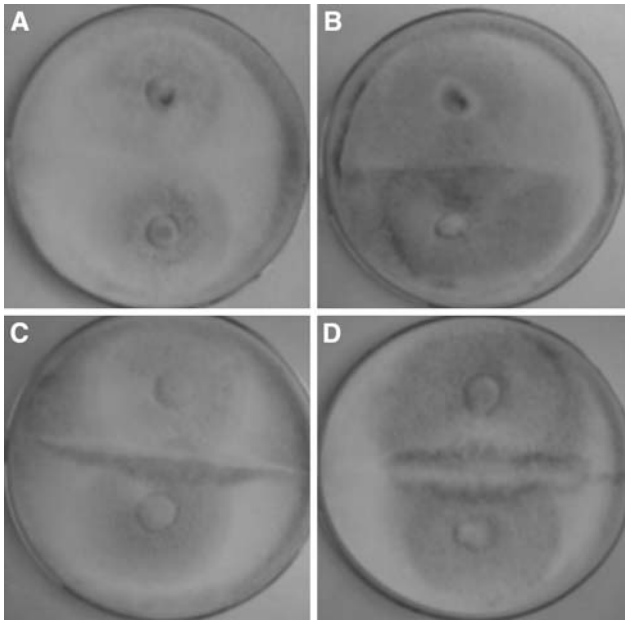

Fig. 4 The outcome of vegetative compatibility interactions between sib-related heterokaryons CMW16848$_{[n]}$ × CMW16828$_{[1]}$. **a** Compatible interactions was scored as 0 when the hyphae of the interacting heterokaryons behaved as one confluent mycelium. **b** Weakly incompatible interactions were scored as 1 when a thin demarcation line was visible between the interacting heterokaryons. **c** Strongly vegetative incompatible interactions were scored as 2 when thinning and pigmentation of the interacting mycelium occurred in either one of the heterokaryons. **d** Strongly vegetative incompatible interactions were also scored as 2 when the thinning and pigmentation of mycelium occurred in both of the heterokaryons

compatible controls (0 reactions) were characterized by intermingling hyphae. Pairing of the heterokaryons CMW16828 and CMW16848 with those collected in the southern hemisphere (South Africa, New Zealand and Brazil) served as strongly incompatible controls (2 reactions) and interacting hyphae were relatively sparse with brown discoloration.

The proportion of strongly incompatible (2 reactions) interactions for sib-related heterokaryons for family CMW16828 (pairings CMW16828$_{[n]}$ × CMW16848$_{[57]}$) and for family CMW16848 (pairings CMW16848$_{[n]}$ × CMW16828$_{[1]}$), were both 19% (Tables 2, 3, 4). Fifty four percent of the pairings between the sib-related heterokaryons for family CMW16828 and 59% for family CMW16848 were weakly incompatible (1 reactions). Segregation of the compatibility trait in both sets of sib-related heterokaryons (22 and 27%) did not differ significantly from the 1:3 ratio ($P < 0.05$). A segregation pattern of 50, 25, 12.5, or 6.25% indicate that there are either one, two, three or four loci controlling vegetative incompatibility, respectively. Given that the percentage of compatible interactions in both sets of sib-related heterokaryons did not deviate significantly (at the 0.05 level) from 25%, vegetative incompatibility in *A. areolatum* is probably controlled by at least two *het* loci. Furthermore, for all of the sib-related pairings, it appeared that the mating-type genes do not to play a role in vegetative incompatibility, because the VCGs did not significantly (at the 0.05 level) correlate with the different mating-types (Tables 3, 4).

The proportion of strongly incompatible interactions (2 reactions) between the sibling heterokaryons for family CMW16848 (pairings CMW16848$_{[n]}$ × CMW16848$_{[n]}$) was 12% (Tables 2, 5) and 9% between sibling heterokaryons for family CMW16828 (pairings CMW16828$_{[n]}$ × CMW 16828$_{[n]}$) (Tables 2, 6). Fifty-five percent of the pairings between the sibling heterokaryons for family CMW16828

Table 2 Vegetative incompatibility between sib-related and siblings heterokaryons

Heterokaryons paired	Number of pairings[e]	Pairings rated as %[f]		
		0	1	2
Sib-related heterokaryons				
CMW16848$_{[n]}$ × CMW16828$_{[1]}$[a]	129	22	59	19
CMW16828$_{[n]}$ × CMW16848$_{[57]}$[b]	132	27	54	19
Sibling heterokaryons				
CMW16848$_{[n]}$ × CMW16848$_{[n]}$[c]	166	22	66	12
CMW16828$_{[n]}$ × CMW16828$_{[n]}$[d]	132	36	55	9

[a] Sib-related heterokaryons obtained by pairing homokaryons obtained from fruiting body CMW16848 with unrelated homokaryon CMW16828$_{[1]}$ obtained from fruiting body CMW16828

[b] Sib-related heterokaryons obtained by pairing homokaryons obtained from fruiting body CMW16828 with unrelated homokaryon CMW16848$_{[57]}$ obtained from fruiting body CMW16848

[c] Sibling heterokaryons obtained by pairing two homokaryons obtained from fruiting body CMW16848

[d] Sibling heterokaryons obtained by pairing two homokaryons obtained from fruiting body CMW16828

[e] Excluding self pairings

[f] *0* compatible reaction, *1* weak incompatible reaction and *2* strong incompatible reaction

Table 3 Vegetative incompatibility between sib-related heterokaryons CMW16848$_{[n]}$ × CMW16828$_{[1]}$

	Strain	*mat* alleles [a]	VCG[b]	*het* alleles [c]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	41	A1B1	G1	1a2a	–																	
2	30	A1B1	G1	1a2a	0	–																
3	55	N	G2	1a2b	0	0	–															
4	60	A2B2	G2	1a2b	0	1	0	–														
5	14	A2B1	G2	1a2b	0	0	1	0	–													
6	77	A1B2	G2	1a2b	0	0	1	1	1	–												
7	57	A1B1	G2	1a2b	0	1	1	0	1	0	–											
8	38	A2B2	G2	1a2b	1	0	1	1	1	N	1	–										
9	43	A2B1	G2	1a2b	N	N	1	N	1	1	1	1	–									
10	58	A2B1	G2	1a2b	1	1	1	1	1	1	1	1	1	–								
11	59	A2B2	G3	1b2b	2	1	1	1	1	1	1	1	1	1	–							
12	87	A1B2	G3	1b2b	2	2	1	1	1	1	1	1	1	1	1	–						
13	42	A2B2	G3	1b2b	2	1	1	1	0	1	0	1	1	1	1	1	–					
14	12	A1B2	G3	1b2b	2	2	1	1	0	1	1	1	1	1	1	1	1	–				
15	23	A1B1	G3	1b2b	2	2	1	0	1	1	1	1	1	1	1	0	1	1	–			
16	5	A1B1	G3	1b2b	2	1	2	0	1	0	1	2	1	1	N	1	1	0	1	–		
17	3	A2B1	G4	1b2a	0	N	2	2	2	2	1	N	1	2	1	1	1	0	0	0	–	
18	25	A1B2	G4	1b2a	1	2	2	2	2	2	2	2	2	2	1	1	1	0	0	1	0	–

Heterokaryons were obtained from a pairing between a homokaryon obtained from fruiting body of family CMW16848$_{[n]}$ with an unrelated homokaryon CMW16828$_{[1]}$ obtained from fruiting body of family CMW16828

[a] Sib-related heterokaryons of family CMW16848 could be separated into four mating-type groups based on microscopy and DNA-based assays (Figs. 1, 2). The mating-type groups represent the four different alleles of the two loci controlling mating in *A. areolatum*, with alleles A1 and A2 at locus *mat-A* and alleles B1 and B2 of locus *mat-B*. The *mat*-alleles for strain CMW16848$_{[55]}$ were not determined

[b] Sib-related heterokaryons of family CMW16828 could be separated into four VCGs based on strong incompatibility, sib-related heterokaryons in VCG 1 were strongly incompatible with sib-related dikaryons in VCG3 while those in VCG 2 were strongly incompatible with the heterokaryons in VCG 4. Interactions that could not be clearly assigned as 0, 1 or 2 (Table 2) are indicated with "N"

[c] The four different alleles (1a and 1b at locus *het-1* and alleles 2a and 2b of locus *het-2*) present at the two *het* loci in *A. areolatum* were arbitrary assigned to the four VCGs, with alleles 1a2a assigned to VCG 1, alleles 1b2b assigned to VCG 3, alleles 1a2b assigned to VCG 2 and alleles 1b2a assigned to VCG 4

and 66% for family CMW16848 were weakly incompatible (1 reactions). The proportion of compatible interactions between the sibling heterokaryons for family CMW16848 was 22 and 36% between sibling heterokaryons for family CMW16828. No obvious vegetative compatibility groupings were observed for the pairings between the siblings in either of the two families. The parental heterokaryons of the two families were vegetatively incompatible and the sibling heterokaryons of family CMW16848 were also never compatible with sibling heterokaryons of family CMW16828. It is, therefore, likely that both the parental heterokaryons are heterozygous at the two *het* loci and that the homokaryons representing the two families do not share alleles at either of these loci. An alternative hypothesis is that the parental heterokaryons are heterozygous at different *het* loci and that there are more than two *het* loci in *A. areolatum*.

Sib-related heterokaryons of families CMW16828 and CMW16848 were separated into four groups based on strong incompatibility (2 reactions) (Tables 3, 4). For both sets of sib-related interactions, the heterokaryons in VCG 1 were strongly incompatible with sib-related heterokaryons in VCG 3, while those in VCG 2 were strongly incompatible with the heterokaryons in VCG 4. These groups may, therefore, reflect the four different alleles of the two loci controlling strong incompatibility in the isolates examined. These would thus have alleles *1a* and *1b* at the *het-1* locus and alleles *2a* and *2b* at the *het-2* locus for family CMW16848 and alleles *1c* and *1d* at the *het-1* locus and alleles *2c* and *2d* at the *het-2* locus for family CMW16828. It was, therefore, possible to assign alleles *1a2a* to VCG 1, *1a2b* to VCG 2, *1b2b* to VCG 3 and *1b2a* to VCG 4 for family CMW16848 or alleles *1c2c* to VCG 5, *1c2d* to VCG 6, *1d2d* to VCG 7 and *1d2c* to VCG 8 for family CMW16828.

The presence of the four VCGs in each family, identified with the sib-related pairings, was verified when sibling heterokaryons from homokaryons in VCG 2 were strongly incompatible with sibling heterokaryons of homokaryons in VCG 4 for family CMW16848 (Tables 2, 5). Likewise the sibling heterokaryons of family CMW16828 (Tables 2, 6)

Table 4 Vegetative incompatibility between sib-related heterokaryons CMW16828$_{[n]}$ × CMW16848$_{[57]}$

	Strain	*mat* alleles[a]	VCG[b]	*het* alleles[c]	1	2	3	4	5	6	7	9	8	10	11	12	13	14	15	16	17	18
1	10	A3B4	G5	1c2c	–																	
2	1	A4B4	G6	1c2d	1	–																
3	16	A3B3	G6	1c2d	1	1	–															
4	18	A3B4	G6	1c2d	N	1	1	–														
5	2	A4B4	G6	1c2d	1	1	1	1	–													
6	15	A4B4	G6	1c2d	1	1	1	1	1	–												
7	6	A3B3	G6	1c2d	1	1	1	1	1	1	–											
8	13	A4B4	G6	1c2d	0	1	0	1	1	1	1	–										
9	19	N	G7	1d2d	1	1	0	1	0	N	0	1	–									
10	8	A3B3	G7	1d2d	0	0	0	1	1	1	0	1	1	–								
11	7	A4B3	G7	1d2d	2	1	1	1	N	1	1	1	1	1	–							
12	11	A4B3	G7	1d2d	2	N	1	1	1	1	1	1	0	1	0	–						
13	17	A4B4	G7	1d2d	2	1	1	2	0	1	1	1	0	1	1	1	–					
14	12	A3B3	G7	1d2d	2	2	0	1	1	0	1	0	0	1	1	0	1	–				
15	4	A3B4	G7	1d2d	2	2	2	2	1	1	0	0	1	0	0	0	1	1	–			
16	5	N	G7	1d2d	2	2	2	2	2	1	1	1	1	1	0	1	0	1	1	–		
17	3	A4B4	G7	1d2d	1	1	2	2	2	0	1	1	1	1	1	0	1	1	0	1	–	
18	14	A3B3	G8	1d2c	2	2	2	2	2	2	2	1	1	0	0	0	0	0	0	0	0	–

Heterokaryons were obtained from a pairing between homokaryons obtained from fruiting body of family CMW16828$_{[n]}$ with an unrelated homokaryon CMW16848$_{[57]}$ obtained from fruiting body of family CMW16848

[a] Sib-related heterokaryons of family CMW16848 could be separated into four mating-type groups based on microscopy and DNA-based assays (Figs. 1, 2). The mating-type groups represent the four different alleles of the two loci controlling mating in *A. areolatum*, with alleles A3 and A4 at locus *mat-A* and alleles B3 and B4 of locus *mat-B*. The *mat*-alleles for strain CMW16828$_{[19]}$ and strain CMW16828$_{[5]}$ were not determined

[b] Sib-related heterokaryons of family CMW16828 could be separated into four VCGs based on strong incompatibility, sib-related heterokaryons in VCG 5 were strongly incompatible with sib-related dikayons in VCG 7 while those in VCG 6 were strongly incompatible with the heterokaryons in VCG 8. Interactions that could not be clearly assigned as 0, 1 or 2 (Table 2) are indicated with "N"

[c] The four different alleles (1c and 1d at locus *het-1* and alleles 2c and 2d of locus *het-2*) present at the two *het* loci in *A. areolatum* were arbitrary assigned to the four VCGs, with alleles 1c2c assigned to VCG 5, alleles 1d2d assigned to VCG 7, alleles 1c2d assigned to VCG 6 and alleles 1d2c assigned to VCG 8

consisting of homokaryons in VCG 6 were strongly incompatible with sibling heterokaryons derived from homokaryons in VCG 8. The presence of the four VCGs in each family was further confirmed when four selected sib-related heterokaryons representing the four VCGs of family CMW16848 were paired with all of the other sib-related heterokaryons of this family to give the predicted outcomes of compatible (0 reactions), weakly incompatible (1 reactions) and strongly incompatible reactions (2 reactions). Different from what was expected, some of the sib-related heterokaryons in VCG 5 (with alleles *1c* and *2c*) were strongly incompatible with sib-related heterokaryons in VCG 8 (with alleles *1d* and *2c*) of family CMW16828. Furthermore, some of the sib-related heterokaryons in VCG 6 (with alleles *1c* and *2d*) were strongly incompatible with some of the sib-related heterokaryons in VCG 7 (with alleles *1d* and *2d*). These sib-related heterokaryons have different alleles at the *het-1* locus, but they share the same allele at the *het-2* locus (Table 4). Also different from what was expected, sibling heterokaryons from homokaryons in VCG 2 of family CMW16848 were not only strongly incompatible with sibling heterokaryons from homokaryons in VCG4, but also with sibling heterokaryons from VCG 1 and VCG 3 (Table 5). This indicates that vegetative incompatibility in *A. areolatum* may be more complex than an allelic interaction of the two identified *het* loci.

Discussion

In this study we characterized the sexual and self-recognition systems of *A. areolatum*. Self-recognition in this fungus appears to follow the general trend identified in basidiomycetes in that it is controlled by a small number of multiallelic *het* loci. Our results also confirmed that sexual recognition among individuals are determined by a tetrapolar mating system in which two multiallelic *mat* loci are involved. The basic function and genetic make-up of these loci seem to resemble those of the model homobasidiomycetes. We identified a homologue in *A. areolatum* of a pher-

Table 5 Vegetative incompatibility between the sibling heterokaryons of family CMW16848

	Strains	Homokaryon group[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	42 × 41	G3G1	–																		
2	59 × 30	G3G1	0	–																	
3	59 × 41	G3G1	0	1	–																
4	42 × 30	G3G1	0	0	1	–															
5	3 × 55	G4G2	1	1	1	0	–														
6	14 × 25	G2G4	1	1	1	0	1	–													
7	3 × 77	G4G2	1	1	1	0	0	1	–												
8	58 × 25	G2G4	1	0	1	1	0	0	1	–											
9	3 × 25	G4G4	1	1	1	1	1	1	1	0	–										
10	60 × 30	G2G1	1	0	1	0	0	1	0	1	1	–									
11	60 × 41	G2G1	1	1	0	1	1	1	1	1	1	1	–								
12	38 × 41	G2G1	1	N	1	1	1	1	1	1	1	0	1	–							
13	59 × 5	G2G3	0	0	1	1	1	0	1	0	1	0	0	1	–						
14	38 × 5	G2G3	1	1	0	1	1	1	1	1	1	1	1	1	1	–					
15	38 × 23	G2G3	1	1	1	1	1	1	0	1	1	1	1	1	1	1	–				
16	14 × 55	G2G2	0	0	0	1	1	N	1	1	1	1	0	1	0	1	1	–			
17	58 × 55	G2G2	0	0	0	N	2	2	1	1	2	0	1	1	1	1	1	1	–		
18	14 × 77	G2G2	2	2	2	2	2	2	2	2	N	1	1	1	0	1	1	1	N	–	
19	58 × 77	G2G2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0	–

Sibling heterokaryons obtained from pairing between two homokaryons from the CMW16848 basidiocarp

[a] Homokaryons isolated from basidiocarp CMW16848 separated into four VCGs based on strong incompatibility (Table 3). Interactions that could not be clearly assigned as 0, 1 or 2 (Table 2) are indicated with "N"

Table 6 Vegetative incompatibility between the sibling heterokaryons of family CMW16828

	Strains	Homokaryon group[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1 × 16	G6G6	–																	
2	2 × 16	G6G6	1	–																
3	12 × 17	G7G7	1	1	–															
4	3 × 12	G7G7	1	1	0	–														
5	4 × 7	G7G7	1	1	1	0	–													
6	16 × 3	G6G7	2	N	1	0	0	–												
7	2 × 12	G6G7	2	1	1	0	0	0	–											
8	2 × 8	G6G7	0	1	1	0	0	0	0	–										
9	16 × 17	G6G7	1	1	1	0	0	0	0	0	–									
10	8 × 13	G7G6	1	1	1	1	1	1	1	1	1	–								
11	6 × 17	G6G7	1	1	1	1	0	1	1	0	1	0	–							
12	1 × 8	G6G7	1	1	1	1	1	1	1	2	1	1	1	–						
13	8 × 17	G7G7	1	1	0	N	1	1	0	1	1	0	0	0	–					
14	3 × 8	G7G7	1	0	1	N	1	1	1	1	1	0	0	0	0	–				
15	3 × 14	G6G7	2	2	1	0	1	1	1	1	1	1	0	0	1	0	–			
16	14 × 17	G8G7	2	2	1	1	1	1	1	1	1	1	0	2	0	0	0	–		
17	14 × 15	G8G7	2	2	N	1	0	1	1	1	0	0	1	1	0	0	0	0	–	
18	2 × 14	G5G8	2	2	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	–

Sibling heterokaryons obtained from pairing between two homokaryons from the CMW16828 basidiocarp

[a] Homokaryons obtained from basidiocarp CMW16828 separated into four VCGs based on strong incompatibility (Table 4). Interactions that could not be clearly assigned as 0, 1 or 2 (Table 2) are indicated with "N"

omone receptor (RAB1) encoded at the *mat-B* locus in *S. commune* and *C. cinereus* that was linked to mating-type B. We also identified a putative *mip* gene in *A. areolatum* that co-segregated with mating-type A, which has been shown to be linked to the *mat-A* locus of other homobasidiomycetes. This study represents the first report regarding the genes implicated in sexual and self-recognition in a homobasidiomycetous obligate symbiont of an insect. The observed segregation of *mat* and *het* alleles in the progenies studied will facilitate construction of a genetic linkage map and subsequent characterization of the recognition loci in their entirety in *A. areolatum* and other *Amylostereum* species. Comparison of these regions among *Amylostereum* species, some of which are not associated with insects, will allow full appreciation of how this association influences the evolution of the recognition loci.

The observed 1:3 ratio of sexually compatible to incompatible crosses between homokaryons of *A. areolatum* from the same basidiocarp is consistent with a tetrapolar mating system that consists of two *mat* loci resulting in four possible mating-types (Boidin and Lanquentin 1984). The four mating-types of the homokaryons derived from two sets of progeny were identified using microscopy, following pairings, as well as through PCR-based diagnostic assays developed in this study. Our data show that, like the *mat* loci of some other homobasidiomycetes, the *mat* loci of *A. areolatum* are multiallelic (Kothe 1996). Three alleles (*RAB1.1*, *RAB1.2* and *RAB1.3*) were present in the small number of isolates tested, confirming the observed multiallelism. This is also illustrated by the fact that all of the homokaryons from one family were compatible with the tested homokaryons of the other family. Homokaryons from different families thus all differ at both *mat* loci, allowing the formation of heterokaryons with clamp connections. However, because there is potentially additional pheromone receptors located at the *mat-B* locus of *A. areolatum*, it may be possible that the two homokaryotic families are actually dissimilar at different pheromone receptors (Kothe 1996; Halsall et al. 2000).

Morphology-based diagnosis of mating-types in basidiomycetous fungi like *A. areolatum* is a tedious and time consuming process that does not always yield conclusive results. Mating-types can only be assigned after pairings between homokaryotic individuals have been incubated for extended periods of time, and even then morphological differences associated with the presence or absence of certain *mat* alleles are not always evident. DNA-based diagnoses of mating-types are therefore far less subjective, more efficient and can be used for both homo- and hetero-karyotic isolates. In this study, we used published information on the *mat* locus of basidomycetes (James et al. 2004a; James et al. 2004b) to develop DNA-based assays for identifying mating-types in *A. areolatum*. For locus *mat-A*, we designed specific primers that allow amplification of a portion of the *mip* gene, closely linked to the *mat-A* locus, that enable differentiation between the alleles of this locus. For locus *mat-B*, we developed a PCR–RFLP method based on putative pheromone receptor sequences for RAB1 for differentiating the alleles of this locus. Although similar approaches have been used extensively for differentiating MAT-1 and MAT-2 (Coppin et al. 1997; Turgeon and Yoder 2000) mating-types of ascomycetes (e.g. Mara and Milgroom 1999; Steenkamp et al. 2000; Yokoyama et al. 2004), multiallelism and the hyperdiverse nature of sequences encoded at the mating-type loci of homobasidiomycetes (James et al. 2004b), limit the use of DNA-based mating-type assays for non-model homobasidiomycetes. The fact that our PCR-based mating-type assays did not function on close relatives (i.e. *A. chialletii* and *A. laevigatum*, results not shown) of *A. areolatum* is, therefore, to be expected. Also, the DNA-based diagnostic procedures described here should be used with caution in broad population studies of the fungus as additional alleles which have as yet not been characterised might be missed.

Like other homobasidiomycetes, *A. areolatum*, has fewer *het* loci than the ascomycetes that have been studied thus far (Worrall 1997). Our results suggest that vegetative incompatibility in *A. areolatum* is controlled by at least two *het* loci (loci A and B), since 25% of the vegetative interactions among the sib-related heterokaryons were compatible. Examples of homobasidiomycetes with relatively few *het* loci include *Phellinus weirii* with a single *het* locus, *Serpula lacrymans* with two *het* loci and *Heterobasidion annosum* that has three to four *het* loci (Hansen et al. 1994; Kauserud et al. 2006; Lind et al. in 2007). In contrast the ascomycetes such as *Neurospora crassa* has at least 11 *het* loci, and *Podospora anserina* has at least nine *het* loci (Perkins 1988; Bérgueret et al. 1994). The two *het* loci identified in *A. areolatum* appear to be multiallelic as is also the case for other homobasidiomycetes (Stenlid and Vasiliauskas 1998; Lind et al. 2007), but different to the situation in ascomycetes that mostly have biallelic *het* loci (Cortesi and Milgroom 1998; Muirhead et al. 2002). This is because sib-related heterokaryons of the one family were never compatible with sib-related heterokaryons of the other family. However this incompatibility between the families may be due to heterozygosity at additional *het* loci. Also, additional *het* loci may be present in *A. areolatum* that could not be detected in our study due to possible homozygosity in the parental heterokaryons tested. Nevertheless, our results conclusively show that *A. areolatum* harbors at least two *het* loci that appear to be multallelic. It may therefore be expected that *A. areolatum* has many different VCGs, just like the multiallelic nature of *mat* loci increase the number of expected number of mating-types (Kothe 1996).

Among the vegetative compatibility pairings examined in this study, two types of incompatible interactions were observed, i.e. strongly incompatible and weakly incompatible. It would be expected that sib-related dikaryons differing at both loci are strongly incompatible, whereas interacting sib-related heterokaryons differing at a single locus would display weak incompatibility. This was, however, not always the case, as some of the sib-related heterokaryons consisting of homokaryons derived from parent isolate CMW16828, presumably differing at only a single locus, were strongly incompatible. Furthermore, sibling heterokaryons consisting of homokaryons derived from parent CMW16828 differing at a single locus were also strongly incompatible. The same result was observed for sibling heterokaryons consisting of homokaryons derived from the parental isolate CMW16848. These findings suggest that in some cases, a single locus (*het-1* locus) controls strong incompatibility. This is similar to the situation in *Collybia fusipes*, where only one of the possible three *het* loci controls strong incompatibility (Marçais et al. 2000). This locus, however, clearly does not act in isolation. If a single locus exclusively determines vegetative incompatibility, as is the case for *Phellinus gilvus* (Rizzo et al. 1995), the sib-related heterokaryons of family CMW16828 should have separated into two and not four groups based on strong incompatibility. Also, not all of the sib-related heterokaryons differing at their *het-1* locus are strongly incompatible, as would be expected if the *het-1* locus alone controls strong incompatibility. It, therefore, appears that there may be other genes present in *A. areolatum* that interfere in determining the strength of vegetative incompatibility interactions.

Additional genes potentially involved in determining the outcome of vegetative incompatibility in *A. areolatum*, may represent genes that are not encoded at the *het* loci. This has been shown for the *tol* gene of *N. crassa* and the *mod* genes for *P. anserina*. The *tol* gene appears to influence the outcome of mating-type mediated vegetative incompatibility in *N. crassa*, although it is not a mating-type nor a *het* gene (Leslie and Yamashiro 1997). In *P. anserina*, the *mod* (for modifier) genes have been demonstrated to interfere with the signals mediating vegetative incompatibility, thereby repressing cell autolysis and preventing fusion of the interacting individuals (Bernet 1992; Barraeau et al. 1998). However, these examples refer specifically to ascomycetes and non-*het* genes involved in vegetative incompatibility have not yet been identified for basidiomycetes. In addition, the *mat* loci are not implicated in self-recognition of the basidiomycetes that have been examined (Hansen et al. 1993; Marçais et al. 2000) and they also do not appear to be involved in determining vegetative incompatibility in *A. areolatum*.

The presence of *het* loci, in addition to those identified, might offer an alternative explanation for the absence of strong incompatibility in some pairings between individuals differing at the *het-1* locus. Their presence would have been masked if it is closely linked to the *het-1* locus. This is also known in other fungi. For example, in the ascomycete *N. crassa*, the closely linked *pin-c* (for partner for incompatibility) and *het-c* loci interact in a non-allelic fashion to mediate vegetative incompatibility (Kaneko et al. 2006). Although the intensity of this non-allelic interaction is also influenced by allelic interactions at the *het-c* locus, allelic interactions at the *pin-c* locus has no effect. In *P. anserina*, it has been shown that not all of the various non-allelic interactions between the *het-c* and *het-e* loci will mediate vegetative incompatibility (Saupe et al. 1995). For example, only interactions between *het-c1* with *het-e2* and *het-e3*, and interactions between *het-c2* with *het-e1* mediate vegetative incompatibility. Following these examples, it may thus be possible that multiple *het* alleles in *A. areolatum* from tightly linked genes act together through an allelic and/or non-allelic mechanism. It is also possible that not all of these allelic interactions will mediate vegetative incompatibility, because not all of the loci would contribute equally to the interaction or not all the alleles present at these loci would interact with each other to mediate vegetative incompatibility.

The low genetic diversity combined with asexual reproduction in *A. areolatum* in some populations of the fungus (Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999; Slippers et al. 2001) may eventually result in inbreeding. This could happen as a result of sexual recombination between related individuals in specific regions where the diversity is low. Such an inbred population would be characterized by increased homozygosity, i.e. a reduction in the number of alleles at a specific locus (Charlesworth and Charlesworth 1987; Stenlid and Vasiliauskas 1998; Milgroom and Cortesi 1999). It was demonstrated that such an increase in homozygosity only happens when a large percentage of the population reproduce asexually (Nauta and Hoekstra 1996). The apparent lack of such an increase in homozygosity at the recognition loci in certain populations may be due to the fact that the forces governing evolution of the recognition loci differ from those acting on the rest of the genome (May et al. 1999; Takebayashi et al. 2004; Uyenoyama 2004). It is believed that the recognition loci are kept diverse by evolutionary forces, such as balancing selection, that maintain a large number of alleles that are evenly distributed in a population (Richman 2000, Muirhead et al. 2002, Takebayashi et al. 2004). Extensive population studies and detailed examination of the sexual and self-recognition systems and the loci governing them, will determine if the recognition loci in *A. areolatum* is also under selective pressure to remain diverse and if the frequency of asexual reproduction is indeed high enough to reduce the number of alleles present at the recognition loci.

The present study showed that vegetative incompatibility assays could not differentiate successfully between closely related *A. areolatum* individuals such as full siblings. This is clear from the fact that a large proportion of the *A. areolatum* siblings examined in this study (36% for family CMW16828) were vegetatively compatible. This is consistent with the situation in some other basidiomycetes, where the capacity of VCG assays to differentiate between close relatives (e.g. siblings) is limited. For example, Barret and Uscuplic (1971) showed compatibility between 98% of the *Phaeolus schweinitzii* siblings that they examined. The low levels of incompatibility observed between the *A. areolatum* siblings used in this study can be explained by the fact that only the two parental alleles at the *het* loci can segregate in the siblings. VCG assays allowed for the identification of a maximum of four VCGs among the offspring, even though their genomic AFLP fingerprints clearly showed that each individual is genetically unique. Application of VCG assays would therefore not be useful for determining the diversity of *A. areolatum* populations with a low genetic diversity, because it would merely reflect the limited heterozygosity of the *het* loci (Matsumoto et al. 1996; Stenlid and Vasiliauskas 1998; Kauserud 2004). This inability of VCGs to distinguish between genetically different but closely related, individuals may result in an underestimation of the genetic diversity as suggested by previous authors (Matsumoto et al. 1996; Stenlid and Vasiliauskas 1998; Kauserud 2004). However, the effectiveness of VCG assays is closely correlated with allelic diversity of the *het* loci which appears to be relatively high in *A. areolatum*, suggesting that VCG analysis remains a useful tool for population studies of this fungus on larger geographic scales. However, VCG assays are not selectively neutral and caution should therefore be taken when interpreting VCG data and should preferably be used in combination with selectively neutral genetic markers.

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