Molecular Ecology (2002) **11**, 1845–1854

DNA sequence and RFLP data reflect geographical spread and relationships of *Amylostereum areolatum* and its insect vectors

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Abstract

The white rot fungus, *Amylostereum areolatum* (Basidiomycetes), is best known for its symbiotic relationship with various siricid wood wasp species. In this study, the relationship between isolates of *A. areolatum* associated with two wood wasp species, *Sirex noctilio* and *S. juvencus*, are considered to identify possible intraspecific groups. Isolates from the northern (native) and southern (exotic) hemispheres are included to determine patterns of geographical spread and origin of introductions into the southern hemisphere. The phylogenetic relationships of these isolates to authentic isolates of *A. chailletii*, *A. laevigatum* and *A. ferreum* were also investigated. Sequence and restriction fragment length polymorphism (RFLP) analyses of the variable nuc-IGS-rDNA region provided markers to distinguish intraspecific groups within *A. areolatum*. Isolates of *A. areolatum* associated with *S. noctilio* and *S. juvencus* contained four heterogenic sequences in the DNA region analysed. These sequences occurred in one of five combinations in each isolate. Some of these sequences were unique to isolates of *A. areolatum* from either wasp species, while others were present in both groups. This shows the ancient and specialized evolutionary relationship that exists between these insects and fungi. Isolates from the southern hemisphere all share the same sequence group. This supports previous hypotheses that *S. noctilio* has spread between countries and continents of this region. At the interspecific level, the IGS-rDNA sequence analysis showed that *A. ferreum* and *A. laevigatum* are closely related to each other, and they in turn are related to *A. chailletii*. *Amylostereum areolatum* was the most distinctly defined species in the genus. This can be attributed to the obligate relationship between *A. areolatum* and its insect vectors. Polymerase chain reaction-RFLP analysis was also shown to be an effective tool to distinguish between the different species of *Amylostereum*.

Keywords: Basidiomycetes, clonal reproduction, IGS, PCR-RFLP fingerprinting, rRNA operon, symbiosis

Received 9 January 2002; revision received 22 May 2002; accepted 22 May 2002

Introduction

Members of the wood wasp family Siricidae have larval states that either live in the stems of plants or are woodborers (Benson 1943; Chamberlin 1960; Morgan 1968). The genera *Sirex* and *Urocerus*, for example, infest branches and trunks of softwoods (especially conifers), in which they spend the largest part of their life cycles as burrowing larvae. Adult wasps only emerge for a few weeks every one to three years, to mate, spread and lay eggs in other trees (Benson 1943; Morgan 1968).

A common characteristic of *Sirex* and *Urocerus* species is the highly evolved symbiotic association with specific wood-rotting basidiomycetes of the genus *Amylostereum* (Cartwright 1929; Francke-Grosmann 1939; Morgan 1968). These fungi produce asexual spores (oidia) or bundles of fungal mycelium that are carried in a pair of specialized

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intersegmental organs (mycangia) near the base of the ovipositor of the adult female wasps and in external organs of the female larvae (Buchner 1928; Francke-Grosmann 1939; Parkin 1941). The fungus is inoculated into the wood together with the eggs during oviposition, as the eventual decay of the wood by the fungus is necessary for the subsequent development of the larvae (Madden & Coutts 1979; Madden 1981).

Specific siricid species always carry the same *Amylostereum* species (Gaut 1970). One fungal species can, however, be carried by more than one wasp species. Gaut (1970) thus showed that *Amylostereum areolatum* (Fr.) Boid. is the symbiont of *Sirex noctilio* Fabr., *S. juvencus* Linn. and *S. nitobei* Mats., while *Amylostereum chailletii* (Pers. Fr.) Boid. is carried by *S. cyaneus* Fabr., *S. imperiales* Kirby, *S. areolatus* Cress., *S. californicus* Nort., *Urocerus gigas* Linn., *U. augur augur* Klug. and *U. augur sah* Mocs. The genetic relationship among isolates of one fungal species associated with different wasp species is, however, not known. Gaut (1970) reported intraspecific variation of isozymes among isolates of *A. areolatum*, but this phenomenon has not been studied further.

Recent studies, using vegetative compatibility and random amplified polymorphic DNA data, have revealed extensive clonal lineages in *A. areolatum* (Vasiliauskas *et al*. 1998; Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999; Slippers *et al*. 2001). This is unusual, as this fungus is heterothallic and it is expected that the heterokaryotic individual isolates from basidiospores (the most common method of dispersal in Basidiomycetes) would represent distinct genetic entities (Boidin & Lanquetin 1984). In *A. areolatum* these clonal lineages are, however, produced as a result of the symbiosis with the wasp species, which spread the asexually produced arthrospores of the fungus. This is the chief means of spread of *A. areolatum*, as basidiomata and basidiospores are also extremely rare in nature in the northern hemisphere and have never been found in the southern hemisphere (Thomsen 1996, 1998).

Siricidae and their fungal symbionts are native to the northern hemisphere. In these regions, these insect–fungal complexes are considered to be secondary pests that kill and remove sick or already dying trees. *Sirex noctilio* and its fungal symbiont, *A. areolatum*, have, however, been introduced into the southern hemisphere during the last century and now represent a serious threat to pine plantations in Australasia (recorded around 1900 in New Zealand and the 1950s in Australia for the first time) (Neumann *et al*. 1987; Madden 1988; Chou 1991). Recently, the *Sirex*–*Amylostereum* complex has been reported from South America (1980s) and South Africa (1994) and it now poses a threat to the softwood industries of these countries (Baxter *et al*. 1995; Reardon *et al*. 1995; Tribe 1995). Slippers *et al*. (2001), using vegetative compatibility, showed that the isolates from South America and South Africa are from the same compatibility group and thus share the same origin. The relationships of these isolates to isolates from Australasia and Europe could not, however, be determined with certainty.

The rarity, and often absence, of sporocarps of *Amylostereum* spp. in nature has resulted in significant problems in the taxonomy of the fungal associates of Siricidae, because identification of these fungi is chiefly based on sporocarp morphology. The result is that these fungi are often studied entirely in culture or based on spores in the mycangia of the insect vectors, neither of which is clearly definitive (Talbot 1964; King 1966). For example, the fungal symbiont of *S. noctilio* in the southern hemisphere proved difficult to identify since its introduction around 1900, and it was only correctly identified as *A. areolatum* by Gaut (1969). Characters such as the production of arthrospores in culture and the *in vitro* production of fruiting structures have been used successfully to distinguish some of the *Amylostereum* spp. (Tamblyn & Da Costa 1958; Talbot 1964; Thomsen 1998). However, this process is time consuming, characters are variable and the results are not always conclusive. There is therefore a need for a quick, yet reliable, tool to identify the different *Amylostereum* spp.

In this study variation among *A. areolatum* isolates associated with *S. noctilio* and *S. juvencus* from the northern (native) and southern (introduced) hemispheres was considered using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) fingerprinting and analysis of nuclear intergenic spacer (IGS) sequence data. These data have also been correlated with hypothesized geographical patterns of spread of *A. areolatum* in the southern hemisphere. The data were also used to determine phylogenetic relationships and differences between the four different species of *Amylostereum* associated with *Sirex* wood wasps.

Materials and methods

Isolates studied, DNA isolation, PCR amplification

Fungal cultures (Table 1) used in this study were isolated and maintained as described by Slippers *et al*. (2000, 2001). DNA extractions were also performed as described in Slippers *et al*. (2000). The IGS region between the nuclear large subunit (LSU) and the 5S gene of the ribosomal RNA (rRNA) operon was amplified using PCR primers specific for basidiomycetes, P-1 (5′ TTG CAG ACG ACT TGA ATG G 3′) (Hsiau 1996) and 5S-2B (5′ CAC CGC ATC CCG TCT GAT CTG CG 3′) (T. Harrington, personal communication). IGS PCR fragments were generated using the Expand™ High Fidelity PCR System on a Hybaid TouchDown (Hybaid Ltd, UK) PCR unit. The PCR reaction conditions were as previously reported (Slippers *et al*. 2000). PCR products were subjected to electrophoresis on a 1% (w/v) ethidium-bromide-stained agarose gel and

Table 1 Isolates of *Amylostereum* used in this study

CMW no.	Culture no.	Identity	Host or source of isolate	Origin	Date isolated	Collector
CBS cultures						
3309	305.82	*Amylostereum areolatum*	Unknown	France	1964	J. Boiden
3310	334.66	*A. areolatum*	*Picea abies*	Germany	1967	Dimitri
2905	483.83	*A. chailletii*	Mycangium of *Urocerus gigas*	Scotland, UK	1981	D.B. Redfern
3311	624.84	*A. laevigatum*	*Juniperus nana*	France	1978	P. Lanquetin
3045	633.84	*A. ferreum*	*Podocarpus lambertii*	Brazil	1978	R.T. Guerrero
CLBRR cultures						
3295	DAOM 21327	*A. chailletii*	Fruiting body on *Abies balsamea*	Ontario, Canada	1948	R.F. Cain
3302	54–95	*A. chailletii*	Fruiting body on wood in stand of hemlock conifers	Ontario, Canada	1954	A. Hill & S. Gibson
3296	Stillwell 309(3)	*Amylostereum* sp.	Mycangium of *S. areolatus*	California, USA	Unknown	Stillwell
3298	Waite Inst. 6195	*A. areolatum*	Mycangium of *S. noctilio*	Tasmania	1962	Unknown
3301	DAOM 21785	*A. areolatum*	*P. radiata* wood infested with *S. noctilio*	New Zealand	Unknown	G.B. Rawlings
Other European isolates						
4632	L204	*A. areolatum*	Wood of wounded *P. abies*	Lithuania	1994	R. Vasiliauskas
8897	L236	*A. areolatum*	Wood of wounded *P. abies*	Lithuania	1995	R. Vasiliauskas
	DK37	*A. areolatum*	Fruiting body on *P. abies*	Denmark	1993	I.M. Thomson
4636	DK782	*A. areolatum*	Fruiting body on *P. abies*	Denmark	1987	J. Koch
4641	S225	*A. areolatum*	Wood of wounded *P. abies*	Sweden	1994	R. Vasiliauskas
8896	S227	*A. areolatum*	Wood of wounded *P. abies*	Sweden	1994	R. Vasiliauskas
Australian isolates						
4644	*A3, A4, A11	*A. areolatum*	From nematode cultures from CSIRO	Australia	1995	B. Slippers
South American isolates						
8898	*Br17, Br60	*A. areolatum*	Mycangia of *S. noctilio*	Brazil	1997	B. Slippers
South African isolates						
4658	*M5W	*A. areolatum*	*P. radiata* wood infested by *S. noctili*	South Africa	1994	M.J. Wingfield
4659	*SN19A	*A. areolatum*	Mycangia of *S. noctilio*	South Africa	1996	B. Slippers

CMW (Culture collection of the Tree Pathology Co-operative Programme, University of Pretoria, Pretoria, South Africa); CBS (Centraal Bureau voor Schimmelcultures, Baarn, Netherlands); CLBRR (Centre for Land and Biological Resources Research, Canada).

visualized under ultraviolet illumination. Size estimates of the PCR fragments were made using a 100-base pair (bp) ladder (Promega) as a molecular weight marker.

PCR amplification for some isolates of *Amylostereum areolatum* resulted in two fragments of different size (Fig. 1). To separate these bands for analysis, DNA represented by the bands on agarose gels was used as template DNA for secondary PCR, for isolates A8 and DK 37. A sterile disposable tip of a Gilson Pipetman was pushed into the band on the gel and stirred in the reaction mixture. The reaction mixture and reaction cycle used were the same as those used for the initial PCR.

DNA sequencing and sequence data analysis

DNA sequence of the IGS region was determined for isolates CBS 483.83 (*A. chailletii*), CBS 624.84 (*A. laevigatum*) and CBS 633.84 (*A. ferreum*), Stillwell 309(3) (*Amylostereum* sp. isolated from *Sirex areolatus*) and all isolates of *A. areolatum* (Table 1). DNA sequencing of PCR products was performed using an ABI PRISM™ 377 automated DNA sequencer. PCR products were purified using a Nucleon™ QC PCR/OLIGO clean-up kit (Amersham Life Science Inc.) prior to sequencing. Thermo Sequenase™ dye terminator cycle sequencing premix kit (Amersham Life Science Inc.) with Thermo Sequenase™ DNA polymerase was used in all sequencing reaction mixtures. Sequencing of both strands of amplified PCR products was achieved using the primers P-1 and 5S-2B.

Secondary PCR products of isolate DK 37 were cloned for sequencing. PCR fragments were purified with the Nucleon™ QC PCR/OLIGO clean-up kit (Amersham Life Science Inc.) and cloned using the pGEM®-T Easy Vector System as described in the pGEM®-T and pGEM®-T Easy Vector Systems technical manual (Promega Corporation). Screening for positive colonies containing the insert was

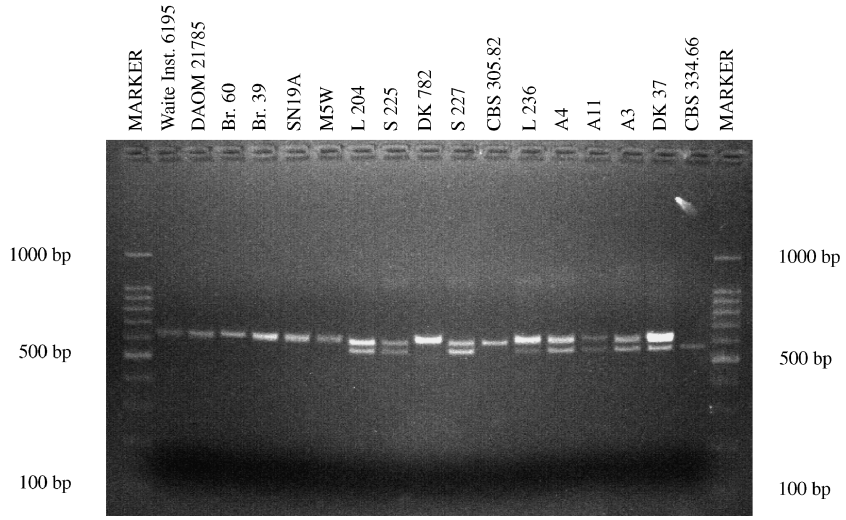

Fig. 1 (a) IGS PCR fragments of isolates of *Amylostereum areolatum* visualized on a 1.5% agarose gel stained with ethidium bromide. The 100 bp size markers were included as size standards in marker lanes. Isolates are those identified in Table 1.

carried out by PCR using the M13U and M13R primers. Cloned products were precipitated and purified as described above and sequenced using primers M13R and M13U.

DNA sequences were manually aligned by inserting gaps, which were treated as a fifth character (newstate). BLAST searches were performed to identify related sequences. No alignable sequences were, however, found in public databases. PAUP version 3.1.1 (Phylogenetic Analysis Using Parsimony) (Swofford 1993) was used to analyse the sequence data by executing heuristic searches with tree bisection reconstruction (TBR) branch swapping and MULPAR activated. Trees were rooted to the midpoint. Bootstrapping (1000 replicates) (Felsenstein 1993) was used to determine the confidence intervals of branching points on the shortest tree. Sequence data were used to determine exact sizes of PCR products, as well as the restriction sites of the restriction endonucleases *Alu*I and *Cfo*I.

Restriction analysis

Restriction analysis of the amplified IGS fragment was done using the restriction endonucleases *Alu*I and *Cfo*I (Boehringer Mannheim, Germany). Both enzymes (10 units) were added to 20 µL of the unpurified PCR reaction mix containing the amplified products and digested overnight at 37 °C. Amplification products containing two PCR products of different size were digested in the same way, without separating the two products. The resulting restriction fragments were separated by electrophoresis on ethidium-bromide-stained 2% (w/v) agarose gels and visualized under ultraviolet illumination. Size estimates of RFLP fingerprints were made using a 100-bp ladder run as a molecular size marker. Exact restriction fragment sizes were, however, deduced from sequence data.

Results

PCR amplification

PCR fragment sizes were calculated to be 552 bp from sequence data for isolates of *Amylostereum chailletii* (CBS 483.83; DAOM 21327; 54–95), 583 bp for *A. laevigatum* (CBS 624.84), 558 bp for *A. ferreum* (CBS 633.84) and 569 bp for the isolate Stillwell 309(3).

PCR fragment sizes for isolates of *A. areolatum* or isolates thought to represent *A. areolatum* could be divided into three forms that were consistently present and reproducible in each isolate (Fig. 1). In one form, a single PCR fragment of 570 bp was amplified (CBS 334.66). In a second form, two PCR fragments with sizes of 638 and 570 bp or 622 and 570 bp were observed for each of isolates L204, L236, S225, S227, DK37, A3, A4 and A11. The double bands could be re-amplified using each DNA band from the gel as template in a PCR reaction, yielding fragments that were identical to each of the original DNA fragments obtained with the initial PCR. In the fourth form, a single fragment of approximately 630 bp for isolates Waite Inst. 6195, DAOM 21785, Br17, Br60, M5W, SN19A, DK 782 and CBS 305.82, was observed on the agarose gels. Sequencing and RFLP results, however, showed that this apparent single band was heterologous and represented two fragments of 622 and 638 bp. As a fourth form, a single fragment of 618 bp was amplified for isolate CBS 305.82.

DNA sequencing and sequence data analysis

The sequences amounted to a total of 694 characters after alignment. Four different sequences (hereafter referred to as sequence A, B, C and D) for the nuc-IGS-rDNA region were observed for isolates of *A. areolatum*. Sequences B, C and D were the same, other than in two

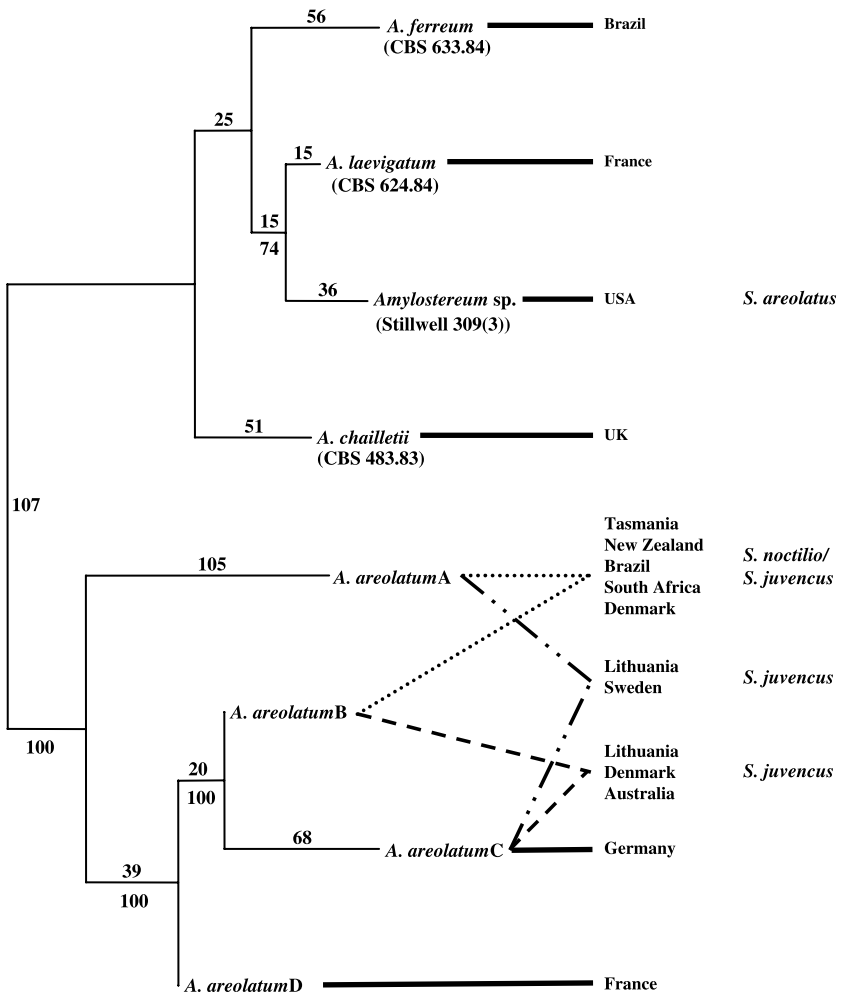

Fig. 2 The most parsimonious phylogenetic tree of 537 steps (CI = 0.912; RI = 0.900) generated after a heuristic search with TBR (Tree Bisection Reconstruction) of the manually aligned IGS sequence data of the different species of *Amylostereum*. The midpoint is used to root the tree. Branch-lengths are indicated above the branches and bootstrap values (1000 replicates) are shown below the branches. Different combinations of heterogenic sequences (A/B/C/D) contained in isolates of *A. areolatum* and their origin and wasp association of the isolates containing each combination, are indicated (see Table 2).

sites where major indels were observed. The heterogenic sequences contained in some isolates of *A. areolatum* were all included in the analyses. The heterogeneity in sequences of the IGS region observed in isolates of *A. areolatum*, was not observed in any of the other species of *Amylostereum*. However, fewer isolates were used for the other species than for *A. areolatum*.

Heuristic search analysis of the sequence data from all isolates used in this study resulted in one tree of 537 steps [Consistency Index (CI) = 0.912; Homoplasy Index (HI) = 0.088; Retention Index (RI) = 0.900] (Fig. 2). The separation of sequences A, B, C and D from isolates of *A. areolatum* was supported by high confidence intervals of 99% at the branching points. The different sequences within *A. areolatum* were separated from the other species of *Amylostereum* with a 100% confidence interval at the branching point. *Amylostereum laevigatum* and *A. ferreum* together formed a sister group to *A. chailletii*. Isolate Stillwell 309(3) was expected to be *A. chailletii*, but grouped closest to *A. laevigatum*, with a high (79%) confidence interval at the branching point.

Restriction analysis

Restriction maps for both the restriction endonucleases *Alu*I and *Cfo*I were determined from sequence data (Fig. 3). PCR products of all *Amylostereum* spp., as well as the unknown *Amylostereum* sp. [isolate Stillwell 309(3)], gave unique restriction patterns when digested with the restriction endonuclease *Alu*I (Fig. 4). Restriction fingerprints using the restriction endonuclease *Cfo*I, differentiated isolates of *A. areolatum* and *A. chailletii* from each other and from isolates of *A. laevigatum* (CBS 624.84) and *A. ferreum* (CBS 633.84). The isolates of *A. laevigatum* (CBS 624.84), *A. ferreum* (CBS 633.84) and isolate Stillwell 309(3), however, produced identical fingerprints when the PCR products were digested with *Cfo*I.

IGS PCR products of isolates representing or thought to represent *A. areolatum*, showed different restriction patterns using the endonucleases *Alu*I and *Cfo*I, which is explained by the heterogenic nature of the IGS region in this species. Using the restriction maps produced from sequence data, it was determined that most isolates of

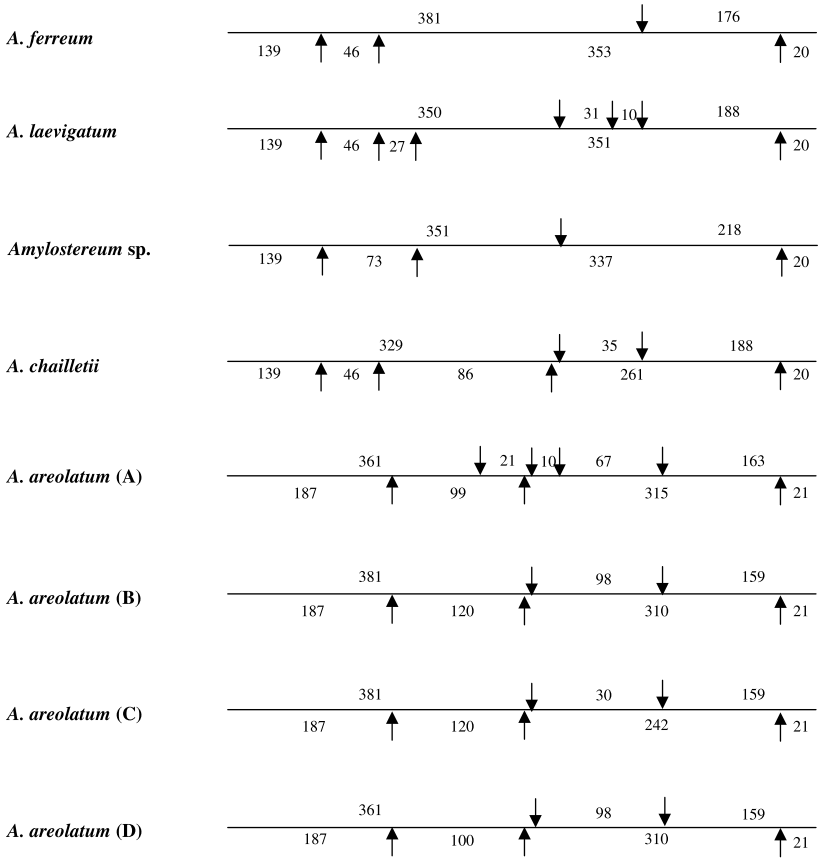

Fig. 3 Restriction maps for the restriction enzymes *Alu*I (above the line) and *Cfo*I (below the line) for the nuc-IGS-rDNA region of different species of *Amylostereum*. Fragment sizes (numbers indicate sizes in bp) were inferred from sequence data.

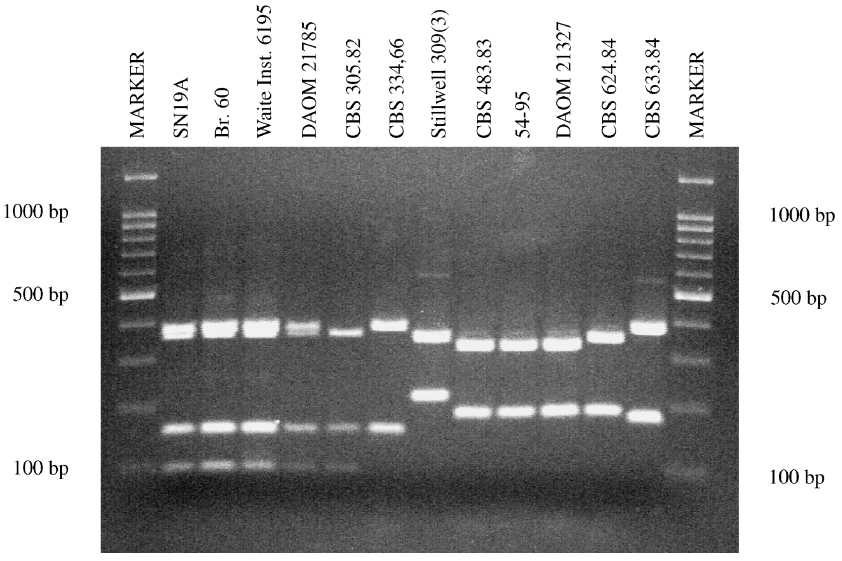

Fig. 4 A 2% agarose gel stained with ethidium bromide showing *Alu*I restriction fragments of the IGS PCR products of different species of *Amylostereum*. Lanes marked as marker contain a 100-bp size marker. Isolates S19A, Br60, Waite Inst. 6195, DAOM 21785, CBS305.82 and CBS334.66 represent *A. areolatum*. Isolate Stillwell 309(3) represents an *Amylostereum* sp. Isolates CBS483.83, 54–95 and DAOM 21327 represent *A. chailletii*, isolate CBS624.84 represents *A. laevigatum* and isolate CBS633.84 represents *A. ferreum*.

A. areolatum contained two of the four sequences (A, B, C and D) (Table 2). Isolates Waite Inst. 6195, DAOM 21785, Br17, Br60, M5W, SN19A, DK782 contained sequences A and B. Isolates L204, S225 and S227 contained sequences A and C, while isolates L236, DK37, A3, A4 and A11 contained sequences B and C. The sequence for the IGS region was homologous in isolates CBS 334.66 (sequence C) and CBS 305.82 (sequence D).

Table 2 Combinations of heterogenic sequences[1] of the nuc-IGS-rDNA region of isolates of *A. areolatum*

Isolate no.	Origin	Associated wasp species	VCG†
A/B sequence*			
DAOM 21785	New Zealand	*Sirex noctilio*	± SH
WaiteInst. 6195	Tasmania	*S. noctilio*	± SH
Br17	Brazil	*S. noctilio*	SH
Br60	Brazil	*S. noctilio*	SH
M5W	South Africa	*S. noctilio*	SH
SN19A	South Africa	*S. noctilio*	SH
DK782	Denmark	*S. juvencus*	DK-B
A/C sequence			
L204	Lithuania	*S. juvencus*	± A2
S225	Sweden	*S. juvencus*	A2
S227	Sweden	*S. juvencus*	A2
B/C sequence			
L236	Lithuania	*S. juvencus*	DK-A
DK37	Denmark	*S. juvencus*	DK-A
A3	Australia	Unknown	Unknown
A4	Australia	Unknown	Unknown
A11	Australia	Unknown	Unknown
C sequence			
CBS 334.66	Germany	Unknown	Unknown
D sequence			
CBS 305.82	France	Unknown	Unknown

*These sequences were observed during sequencing and their presence confirmed using RFLP analysis of the PCR fragments.
†VCGs as determined in previous studies (Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999; Slippers *et al*. 2001).
± refers to unclear results during somatic compatibility tests.

Discussion

In this study we show that the highly specialized symbiotic relationship between the white rot fungus *Amylostereum areolatum*, and woodboring wasps of the family Siricidae has left polymorphic sequences within the nuc-IGS region of the fungal associate. These polymorphic markers from isolates of the fungus associated with *Sirex noctilio* correspond with recent geographical movements of the wasp. Some of these markers are also shared between isolates of *A. areolatum* associated with different wasp species (*S. noctilio* and *S. juvencus*). The fingerprints originating from PCR-RFLP analysis of the DNA region also provide a useful tool for identification and phylogenetic characterization of *Amylostereum* spp.

IGS heterogeneity and ecology of A. areolatum

Isolates of *A. areolatum* contain one or two of the four different sequence groups A, B, C and D. Two different-size PCR products indicate the existence of such a heterogenic sequence in the IGS of the nuclear rDNA locus in isolates of *A. areolatum*. This was confirmed by RFLP analysis, cloning and sequencing of these fragments. The source of this variation is, however, not known. One likely, although untested, explanation is that these divergent sequences represent orthologs or alleles contained on separate nuclei in each cell, as a heterokaryotic or dikaryotic nuclear state dominates the life cycle of *A. areolatum*. Such heterogenic sequences are also known to occur in some other Basidiomycetes. Hibbett & Vilgalys (1991) reported on divergent paralogs or heterogeneity between rDNA copies in the genome of *Lentinus*. Gieser & Rizzo (1998) also noted the possibility of amplification of multiple haplotypes that could be contained as heterozygous loci in different nuclei contained in dikaryons or in the same nucleus as divergent paralogs.

The predominantly vegetative mode of reproduction in *A. areolatum*, and its association with the wood wasp vector, would help to sustain the observed heterogeneity of the IGS region. This fungus–insect association is highly specific, with a clonal line of the fungus carried by a specific wasp and its descendants (Vasiliauskas *et al*. 1998; Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999; Slippers *et al*. 2001). This would certainly prevent the mixing of the different sequence groups described in this study. Sexual reproduction could allow for the recombination of such polymorphisms, but the specificity of the wasp–fungus association and somatic incompatibility (Vasiliauskas *et al*. 1998; Thomsen & Koch 1999; Vasiliauskas & Stenlid 1999; Slippers *et al*. 2001) would still prevent these sexually derived fungal descendants from recombining with isolates of the fungus carried by wood wasps.

Wood wasp associations and geographical spread of A. areolatum

Isolates of *A. areolatum* from *S. noctilio*, always contained the group A sequence but the group A sequence was also found in isolates from *S. juvencus*. The same situation applied to sequence group B, which was present in isolates of the fungus from *S. noctilio* and *S. juvencus*. Sequence group C was, however, unique to isolates from *S. juvencus*. There was therefore no clear distinction between the heterogenic sequences or combinations thereof in isolates of *A. areolatum* associated with either *S. noctilio* or *S. juvencus*. This observation can be explained in two possible ways. Firstly, the formation of the polymorphic loci and differentiation in the sequences could have occurred before the association of the fungus with the wasp. Alternatively the association of the wood wasp and the fungus could have been less specific in the initial stages of its development than is the case today. This would have allowed the different species of wasp to come in contact with the different DNA sequence groups of *A. areolatum*.

Our results, however, show clearly that gene flow has occurred between isolates of *A. areolatum* associated with *S. noctilio* and *S. juvencus*, at some point.

Gaut (1969) reported differences in protein banding patterns between the isolate of *A. areolatum* from France that he used as a reference culture, and his isolate from *S. noctilio*-infested wood in Tasmania. He ascribed these differences, which are quite substantial (six bands out of 20 were variable), as normal variation within the species. We have found isolate CBS 305.82, used by Gaut (1969) for reference purposes to be homogenic for sequence D. In contrast, the isolate from Tasmania used in our study contains sequence groups A and B. The differences in protein banding patterns in *A. areolatum* isolates reported by Gaut (1969), can thus be explained by the different groupings within *A. areolatum*, that are highlighted in the present study.

Isolates of *A. areolatum* from South Africa, Brazil, New Zealand and Tasmania contain the same heterogenic combination of sequences for the IGS region, namely sequence groups A and B. *Sirex noctilio* was introduced to the southern hemisphere around 1900 (Chou 1991). Our results indicate that subsequent movement of this pest complex into new areas and continents of the southern hemisphere has been between countries of this region, and not from the northern hemisphere. These results support the hypothesis that *S. noctilio* in South Africa and Brazil share the same origin (Slippers *et al*. 2001) and clarify uncertainties regarding the relationship between isolates of the fungal symbiont *A. areolatum* from these countries and Australasia.

The nematode *Deladenus siricidicola* Bedding infects and sterilizes the eggs of *S. noctilio* and is used as biological control agent against the wasp in the southern hemisphere (Bedding 1995). When not infecting the eggs of the wasp, the nematode feeds on *A. areolatum* and is therefore reared and maintained on cultures of the fungus (Bedding 1995). Isolates A3, A4 and A11 (isolated from cultures of the biocontrol nematode) contained sequences B and C, which is the same as that in isolates from *S. juvencus*, but differs from the fungus collected in southern hemisphere countries. A different genetic entity of *A. areolatum* has thus, inadvertently been introduced into the countries of the southern hemisphere that have imported *D. siricidicola* as biocontrol agent from Australia. These results confirm a previous supposition based on somatic compatibility groups (Slippers *et al*. 2001).

Phylogeny and PCR-RFLP identification of Amylostereum *spp.*

DNA sequence analysis of the nuc-IGS region from all four species of *Amylostereum*, showed that *A. ferreum* and *A. laevigatum* are the most closely related of the known species within the genus. Together, *A. ferreum* and *A. laevigatum* are more closely related to *A. chailletii* than to *A. areolatum*. *Amylostereum areolatum* is the most clearly defined species within the genus. These results confirm the findings and conclusions of previous studies (Vasiliauskas *et al*. 1999; Slippers *et al*. 2000; Tabata *et al*. 2000), that were based on analysis of the nuc-ITS-rDNA and the more conserved mt-SSU-rDNA region.

Despite the heterogenic sequences contained in the IGS region of some isolates of *A. areolatum*, RFLP analysis of the PCR fragment of this region successfully distinguished the different species of *Amylostereum*. The two species most often confused in the past, *A. areolatum* and *A. chailletii*, were easily and clearly distinguished from each other, as well as from the other species of *Amylostereum*, using both *Alu*I and *Cfo*I restriction endonucleases. *Amylostereum laevigatum* and *A. ferreum* gave similar restriction patterns using the endonuclease *Cfo*I, but could be delineated using the endonuclease *Alu*I. These results now provide a means to easily distinguish between species, such as has been achieved with other Basidiomycetes that are difficult to distinguish due to the common absence of sporocarps. Although ex-type and other authenticated isolates of the *Amylostereum* spp. have been used in this study, the variation observed within *A. areolatum* might also occur in other species for which fewer isolates were available.

Based on the view of Gaut (1970), we would have expected isolate Stillwell 309(3) collected from the mycangium of *S. areolatus* to be *A. chailletii*. However, this isolate gave a unique RFLP banding pattern with the endonuclease *Alu*I. With the endonuclease *Cfo*I, the restriction fragment pattern for this isolate was very similar to that of *A. laevigatum* and *A. ferreum*. *Amylostereum ferreum* has not been associated with wood wasps, while *A. laevigatum* has only recently been shown to be associated with the wasp species *Urocerus japonicus* and *U. antennatus* from Japan (Tabata & Abe 1997, 1999). Sequence analysis of the region showed that isolate Stillwell 309(3) is different from all the known species of *Amylostereum*, but is most closely related to *A. laevigatum*. These data confirm the view of Slippers *et al*. (2000), who suggested that this isolate probably represents an undescribed species of *Amylostereum*.

Analysis of the IGS region of the *Amylostereum* spp. allowed characterization at the taxonomic, phylogenetic and population levels. Most importantly it produced markers that efficiently and reliably identify the different species and reflect intraspecific groups of *A. areolatum*. Considering the significance of the *A. areolatum*/*S. noctilio* complex in exotic pine plantations of the southern hemisphere, this information will be important for studying the spread of *S. noctilio* and in planning quarantine measures to avoid new or repeated introductions. In future planning of biological control strategies against this pest

complex, these data can also be used to prevent accidental introductions of new genotypes of the fungus, as we have shown to have happened in the past.

Acknowledgements

The authors thank Dr G. Tribe, Mr J. Cille and Ms. J Moore (PPRI, Stellenbosch, South Africa), Mr E. Schaitza (Embrapa, Colombo, Brazil), Dr I.M. Thomsen (Danish Forest and Landscape Research Institute, Hoersholm, Denmark) and Dr R. Vasiliauskas (Swedish University of Agricultural Sciences, Uppsala, Sweden) who provided wasps and fungal cultures used in this study. Other isolates of the four *Amylostereum* spp. were obtained from CBS (Centraal Bureau voor Schimmelcultures, Utrecht, Netherlands) and DAOM (Centre for Land and Biological Resources Research, Canada).

References

Baxter AP, Rong IH, Schutte AL (1995) *Amylostereum areolatum* (Aphyllophorales: Stereaceae) in South Africa. *South African Journal of Botany*, **61**, 352–354.

Bedding RA (1995) Biological control of *Sirex noctilio* using the nematode *Deladenus siricidicola*. In: *Nematodes and Biological Control of Insect Pests* (eds Bedding RA, Akhurst RJ, Kaya H), pp. 11–20. CSIRO, Melbourne, Australia.

Benson RB (1943) Studies in Siricidae, especially of Europe and southern Asia (Hymenoptera, Symphyta). *Bulletin of Entomological Research*, **34**, 27–51.

Boidin J, Lanquetin P (1984) Le genre *Amylostereum* (Basidiomycetes) intercompatibilités partielles entre espèces allopatriques. *Bulletin de la Société Mycologique de France*, **100**, 211–236.

Buchner P (1928) Holznahrung und Symbiose. In: *Vortrag Gehalten Auf Dem X Internationalen Zoologentag Zu Budapest Am 8 September 1927*, pp. 13–16. Springer, Berlin.

Cartwright KStG (1929) Notes on fungus associated with *Sirex cyaneus*. *Annals of Applied Biology*, **16**, 182–187.

Chamberlin WJ (1960) *Insects Affecting Forest. Products and Materials*. O.S.C. Cooperative Association. Oregon.

Chou CKS (1991) Perspectives of disease threat in large-scale *Pinus radiata* monoculture — the New Zealand experience. *European Journal of Forest Pathology*, **21**, 71–81.

Felsenstein J (1993) PHYLIP *(Phylogeny Inference Package), Version 3.5*. University of Washington, Seattle.

Francke-Grosmann H (1939) Über das zusammenleben von holzwespen (Siricinae) mit pilzen. *Zeitschrift für Angewandte Entomologie*, **25**, 647–680.

Gaut IPC (1969) Identity of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Biological Sciences*, **22**, 905–914.

Gaut IPC (1970) Studies of Siricids and their Fungal Symbionts. PhD Thesis. University of Adelaide, Adelaide, Australia.

Geiser DM, Rizzo DM (1998) Identification and isolation of nuclear haplotypes from dikaryotic specimens of *Phellinus* for molecular population and phylogenetic analyses. In: *Abstracts of the Annual Meeting of the Mycological Society of America. 11–16 June 1998, San Juan, Puerto Rico, USA. Inoculum*, **49**, 21.

Hibbett DS, Vilgalys R (1991) Evolutionary relationships of *Lentinus* to the Polyporaceae: Evidence from the restriction analysis of enzymatically amplified ribosomal DNA. *Mycologia*, **83**, 425–439.

Hsiau PT-W (1996) The taxonomy and phylogeny of the mycangial fungi from *Dendroctonus brevicomis* and *D. frontalis* (Coleoptera: Scolytidae). PhD Thesis. Iowa State University, Ames, Iowa.

King JM (1966) Some aspects of the biology of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Botany*, **14**, 25–30.

Madden JL (1981) Egg and larval development in the woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology*, **29**, 493–506.

Madden JL (1988) *Sirex* in Australasia. In: *Dynamics of Forest Insect Populations. Patterns, Causes, Implications*. (ed. Berryman AA), pp. 407–429. Plenum Press, New York.

Madden JL, Coutts MP (1979) The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). In: *Insect–Fungus Symbiosis* (ed. Batra LR), pp. 165–174. John Wiley & Sons, New York.

Morgan F (1968) Bionomics of Siricidae. *Annual Review of Entomology*, **13**, 239–256.

Neumann FG, Morey JL, McKimm RJ (1987) The *Sirex* woodwasp in Victoria. *Department of Conservation, Forest and Lands, Victoria Bulletin*, **29**, 41.

Parkin EA (1941) Symbiosis in larval Siricidae (Hymenoptera). *Nature*, **147**, 329.

Reardon R, Eav B, Wetterberg G (1995) The European woodwasp, *Sirex noctilio* (Hymenoptera: Siricidae) threat to conifer plantations in South America. In: *Poster Abstracts, IUFRO XX World Congress, 6–12 August 1995, Tampere*, (eds Korpilahti E, Salonen T), p. 95. Gummerus, Jyväskylä, Finland.

Slippers B, Wingfield MJ, Wingfield BD, Coutinho TA (2000) Relationships among *Amylostereum* species associated with Siricid woodwasps inferred from mitochondrial ribosomal DNA sequences. *Mycologia*, **92**, 955–963.

Slippers B, Wingfield MJ, Wingfield BD, Coutinho TA (2001) Population structure and possible origin of *Amylostereum areolatum* in South Africa. *Plant Pathology*, **50**, 206–210.

Swofford DL (1993) PAUP*: Phylogenetic Analysis Using Parsimony*, Version 3.1.1. Computer program distributed by Illinois Natural History Survey, Champaign, Illinois.

Tabata M, Abe Y (1997) *Amylostereum laevigatum* associated with the Japanese horntail, *Urocerus japonicus*. *Mycoscience*, **38**, 421–427.

Tabata M, Abe Y (1999) *Amylostereum laevigatum* associated with a horntail, *Urocerus antennatus*. *Mycoscience*, **40**, 535–539.

Tabata M, Harrington TC, Chen W, Abe Y (2000) Molecular phylogeny of species in the genera *Amylostereum* and *Echinodontium*. *Mycoscience*, **41**, 585–593.

Talbot PHB (1964) Taxonomy of the fungus associated with *Sirex noctilio*. *Australian Journal of Botany*, **12**, 46–52.

Tamblyn N, Da Costa ENB (1958) A simple technique for producing fruit bodies of wood-destroying basidiomycetes. *Nature*, **181**, 578–579.

Thomsen IM (1996) *Amylostereum areolatum* and *Amylostereum chailletii*, symbiotic fungi of woodwasps (*Sirex* sp. and *Urocerus* sp.). PhD Thesis. Danish Forest and Landscape Research Institute, Horsholm, Denmark.

Thomsen IM (1998) Characters of fruitbodies, basidiospores and cultures useful for recognizing *Amylostereum areolatum* and *A. chailletii*. *Mycotaxon*, **69**, 419–428.

Thomsen IM, Koch J (1999) Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research*, **103**, 817–823.

Tribe G (1995) The woodwasp *Sirex noctilio* Fabricius (Hymenoptera; Siricidae), a pest of *Pinus* species, now established in South Africa. *African Entomology*, **3**, 215–217.

Vasiliauskas R, Stenlid J, Thomsen IM (1998) Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from Northern Europe. *New Phytologist*, **139**, 751–758.

Vasiliauskas R, Stenlid J (1999) Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. *Mycological Research*, **103**, 824–829.

Vasiliauskas R, Johannesson H, Stenlid J (1999) Molecular relationships within the genus *Amylostereum* as determined by internal transcribed spacer sequences of the ribosomal DNA. *Mycotaxon*, **71**, 155–161.

This work formed part of a graduate project of Bernard Slippers conducted under the supervision of Professors Mike Wingfield, Brenda Wingfield and Teresa Coutinho. The project forms part of a larger programme (Tree Pathology Co-operative Programme) on tree diseases. One of the aims of the programme is to characterize the taxonomy and population structure of various tree pathogens in exotic tree plantations in the tropics and subtropics. This knowledge is used to plan and implement control strategies and quarantine measures to protect natural and agricultural resources in these areas. Bernard Slippers is continuing with a PhD project on the taxonomy, phylogeny and ecology of pathogenic fungi.