

.b137 813 76.



University Free State



34300000177505

Universiteit Vrystaat

HIERDIE EKSEMPLAAR MAG ONDER  
GEEN OMSTANDIGHEDE UIT DIE  
BIBLIOTEK VERWYDER WORD NIE

# **THE *AMYLOSTEREUM* SYMBIONT OF *SIREX NOCTILIO* IN SOUTH AFRICA**

This thesis is presented in partial fulfilment of the requirements for the degree

***MAGISTER SCIENTIAE***

In the Faculty of Science, Department of Microbiology and Biochemistry,  
University of the Orange Free State

by

**BERNARD SLIPPERS**

December 1998

PROMOTOR: Prof. M.J. Wingfield

CO-PROMOTORS: Dr. T.A. Coutinho

Prof. B.D. Wingfield

## DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Magister Scientiae* to the University of the Orange Free State, contains my own independent work and has hitherto not been submitted for any degree at any other University.

A handwritten signature in black ink, appearing to read 'B. Slippers', written in a cursive style.

Bernard Slippers

December 1998

I dedicate this thesis to my loving father and mother.

Your supporting hands, loving hearts and guiding words will always carry me.



## TABLE OF CONTENTS

Acknowledgements	I
Preface	II
<b>CHAPTER 1</b>	<b>1</b>
<b>The <i>Sirex-Amylostereum</i> complex, with special reference to its influence on pine silviculture in the Southern Hemisphere</b>	
1.0 Introduction	3
2.0 Distribution and pest status	5
3.0 The insect-fungus-tree association	7
4.0 Management in the Southern Hemisphere	21
5.0 Conclusions	29
6.0 References	31
<b>CHAPTER 2</b>	<b>54</b>
<b>Population structure and possible origin of <i>Amylostereum areolatum</i> in South Africa</b>	
Abstract	55
Introduction	56
Materials & Methods	58
Results	60
Discussion	62
References	66

<b>CHAPTER 3</b>	80
<b>Phylogeny and taxonomy of the genus <i>Amylostereum</i> inferred from mitochondrial ribosomal DNA sequence</b>	
Abstract	81
Introduction	82
Materials & Methods	84
Results	87
Discussion	89
References	93
<b>CHAPTER 4</b>	126
<b>Taxonomy of <i>Amylostereum</i>, with special reference to <i>A. areolatum</i> associated with the wood wasps <i>Sirex noctilio</i> and <i>S. juvencus</i></b>	
Abstract	127
Introduction	128
Materials & Methods	130
Results	132
Discussion	136
References	140
<b>CHAPTER 5</b>	164
<b>The genus <i>Amylostereum</i> and its association with woodwasps : A contemporary review</b>	
Abstract	165
Introduction	166
Taxonomy and Phylogeny	167
Population Structure	170
Conclusions	173
References	174
<b>Summary</b>	IV
<b>Opsomming</b>	VI

## ACKNOWLEDGEMENTS

This work would not have been possible without the help of Jesus. Never have I needed to go on my knees more, and never did He fail to provide.

During the whole duration of this study, Jana, has been by my side. It has taken as much from her as it has from me. My gratitude comes from the bottom of my heart for all her love, support and the sacrifices she made to help me reach this goal. My only wish is that I to can be that same pillar of strength to her.

Last year I lost my father, but his love and teachings still carry me every day. To the same degree my mother still fills that role. You give meaning to unconditional love. Therefore, I offer you all of my love. To the rest of my family and family-in law I owe no less. Thank you for every thing on the long list of what you mean to me.

I have also been privileged to complete this study in a wonderful group, formerly the TPCP and now the TPCP under FABI. Mike has been and is one of the most amazing people I have ever met and continues to be my inspiration and a true model to look up too. So too, have Teresa and Brenda added much more than just insight to this project. Furthermore, FABI consists of a number of people, who have all contributed, to greater and smaller degrees, to my learning experience and my life.

I also want to thank Dr. Iben Thomsen, Dr. Rimvis Vasiliauskas, Dr. Geoff Tribe, Johan Cille and Judy Moore for supplying cultures and very valuable assistance during this study. Dr. Geoff Tribe also supplied the photographs in Figure 1a & b and 2b & d, Chapter 1.

The Foundation for Research Development (FRD), TPCP and the Department of Microbiology and Biochemistry, University of the Orange Free State, contributed funds and provided the facilities and opportunities needed to complete this project. A very big word of gratitude for what you all made possible.

## PREFACE

*Sirex noctilio* and its symbiotic fungus, *Amylostereum areolatum*, were first reported from New Zealand at the turn of the century. Since then it has spread to most Southern Hemisphere areas where pine is grown commercially. Unlike the situation in the Northern Hemisphere, where it is native, this insect-fungus complex has been responsible for great economic losses to the softwood industries of the Southern Hemisphere. These losses have prompted considerable research into controlling this pest complex, especially through the use of biological agents. The fungal symbiont has, however, received less attention, despite its important role in the symbiosis. The aim of this study was to investigate more closely, the fungal symbiont of a recently introduced population of *S. noctilio* in South Africa.

The literature concerning the *Sirex-Amylostereum* complex is reviewed in Chapter 1. The review focuses on *Sirex* in the Southern Hemisphere, but compares this to the Northern Hemisphere where applicable. Firstly, the distribution and spread of *Sirex* is considered. Furthermore, various aspects of the taxonomy and biology of the insect and the fungus are analysed separately, after which these features are combined in the disease cycle. Similarly, different hosts and the factors that predispose them to attack by *Sirex*, are investigated. Lastly, the management of *Sirex* in the Southern Hemisphere is reviewed.

In April 1994, *S. noctilio* was reported for the first time from standing pine trees in South Africa. In Chapter 2, the population structure of isolates of *A. areolatum* associated with this recently introduced *Sirex* population, is investigated. The possible origin of *Sirex* in South Africa is also considered by comparing the fungal population associated with it, with isolates and populations of *A. areolatum* from other regions of the world.

In 1958, Boidin described the genus *Amylostereum* and included the species *A. chailletii*, *A. areolatum* and *A. laevigatum*. In 1984, Boidin and Lanquetin added a fourth species, namely *A. ferreum*. Although mating studies in this last study indicated possible relationships among the species of this genus, their relationships to each

other was not clearly defined. In Chapter 3, the phylogenetic relationship between the species of *Amylostereum* is investigated using sequence analysis of the mitochondrial small-sub-unit of the rDNA complex. The relationship of *Amylostereum* with other Basidiomycetes is also unclear. This question is also addressed in the chapter by comparing sequence of the mt-SSU-rDNA of *Amylostereum* spp. with that of various other genera and species of Basidiomycetes.

*Amylostereum areolatum* reproduces asexually in its life cycle associated with *Sirex*. This effectively separates populations of this fungus associated with the different wasp species. In Chapter 4, the relationship between isolates of *A. areolatum* from different parts of the world and associated with different wasp species is investigated. This is achieved using sequence analysis of variable part of the rDNA complex, namely the nuclear intergenic spacer region. The usefulness of RFLP analyses of this region to delineate the various species of *Amylostereum*, is also assessed.

Renewed interest in *Amylostereum* as a symbiont of woodwasps in recent years, has led to exciting progress in this field. A summary of the recent findings regarding the taxonomy, phylogeny and ecology of *Amylostereum* spp., is given in Chapter 5. New questions and the future direction of research on *Amylostereum* and its symbiosis with woodwasps, are also discussed.







THE *SIREX-AMYLOSTEREUM* COMPLEX, WITH  
SPECIAL REFERENCE TO ITS INFLUENCE ON PINE  
SILVICULTURE IN THE SOUTHERN HEMISPHERE

## 1.0 INTRODUCTION

*Sirex noctilio* Fabricius is a member the Siricidae, a family of insects with a woodboring larval state (Chamberlin, 1960; Morgan, 1968). A common characteristic of these insects is a highly specialised mutualism with members of wood rotting Basidiomycetes (Cartwright, 1929; Francke-Grosmann, 1939; Morgan, 1968). In combination, through a phytotoxic mucus secreted by the wasp and the subsequent rotting of the wood by the fungus, these insect-fungal associations have the ability to kill a living tree (Coutts, 1969a & b). *S. noctilio* and its symbiotic fungus, *Amylostereum areolatum* Boidin, are, however, the only members of the Siricidae that regularly attack living trees (Hanson, 1939; Spradbery & Kirk, 1978).

In the Northern Hemisphere, where the *Sirex noctilio* – *Amylostereum areolatum* complex is native, it is considered to be a secondary pest of little economic importance (Hall, 1978; Hanson, 1939; Spradbery & Kirk, 1978). In these regions, trees are rarely killed and a tree may support a colony of woodwasps for more than one season (Spradbery, 1973; Spradbery & Kirk, 1978). This is in contrast to the Southern Hemisphere, where the *Sirex*-*Amylostereum* complex has caused extensive damage in the exotic pine plantations (Neumann & Marks, 1990; Chou, 1991)

*Sirex noctilio* was first reported from the Southern Hemisphere at the turn of the century from New Zealand and it subsequently spread to the other countries of Australasia (Tasmania and Australia) in the 1950's and 1960's (Madden, 1988; Neumann & Marks, 1990; Chou, 1991). More recently in the 1980's and 1990's *Sirex* was also reported from South America (Uruguay, Brazil and Argentina) and South Africa (Tribe, 1995a; Reardon, Eav, & Wetterberg, 1995). Damage in the exotic softwood plantations of these regions of the Southern Hemisphere has generally been at low levels, except for some major outbreaks where millions of trees were killed (Neumann, Morey & McKimm, 1987; Madden, 1988 & 1998b; Haugen, 1990; Neumann & Marks, 1990; Chou, 1991). These outbreaks were always associated with stress conditions on the trees that were attributable to either environmental stress (e.g. droughts) or stress caused by poor silvicultural management (Neumann & Marks, 1990; Chou, 1991). Outbreaks of *Sirex* have the potential of causing



losses of A\$1-4 billion to the softwood industries of Australasia over a 30 year rotation period (Bedding, 1995). The *Sirex-Amylostereum* complex is, therefore, of considerable economic importance in the Southern Hemisphere.

Through a national *Sirex* control programme in Australia, millions of A\$ have been spent on research and survey programmes to combat this pest (Neumann *et al.*, 1987; Madden, 1988). Mainly through this programme, control strategies have been devised that are used throughout affected countries of the Southern Hemisphere (Neumann *et al.*, 1987; Anonymous, 1991; Bedding, 1995). Today, control of *Sirex* is through a combination of silvicultural practises to ensure forest health and the use of a series of biocontrol agents (Neumann *et al.*, 1987; Anonymous, 1991). Where implicated thoroughly, these control strategies have been used to great effect. The importance of these principles of sound silviculture, thorough surveys and timely, adequate implementation of biocontrol programmes has, however, been obvious in areas where they were not enforced properly. In such regions, *Sirex* numbers steadily increased and eventually serious outbreaks followed (Neumann *et al.*, 1987; Madden, 1988 & 1998b; Haugen, 1990; Neumann & Marks, 1990; Chou, 1991).

The largest majority of the agents used in the biological control programmes of *S. noctilio* are parasitic wasps that attack the larvae of *Sirex* (Neumann *et al.*, 1987). The most effective and widely used biocontrol agent is, however, the nematode *Deladenus siricidicola* Bedding (Neumann *et al.*, 1987; Anonymous, 1991; Bedding, 1995). This nematode has a bicyclic life-cycle, that during the one stage, feeds and reproduces on the fungus, *A. areolatum*. During another phase it infects the larvae of *S. noctilio* and eventually sterilise the adult females of the wasp (Bedding, 1995).

Despite the amount of research that the *Sirex-Amylostereum* association has stimulated in the past many misconceptions have been and are still sometimes encountered, as pointed out by Thomsen (1996) in a review of the literature. In the light of the importance of this pest complex to the softwood industries in the Southern Hemisphere and the prevailing misconceptions regarding this pest complex, the aim of this review was to summarise all relevant information about the above mentioned fungus and insect. Specific attention is

given to the occurrence and ecology of the *Sirex-Amylostereum* complex in the Southern Hemisphere. The review also considers the influences that this pest complex has had, especially in the Southern Hemisphere, and the control strategies that have been used to combat it.

## **2.0 DISTRIBUTION AND PEST STATUS**

### **2.1 SIREX IN THE NORTHERN HEMISPHERE**

*Sirex noctilio* is found throughout the temperate regions of the Northern Hemisphere and is thought to be native to Eurasia (Benson, 1943; Morgan, 1968; Spradbery & Kirk, 1978). In its native range *S. noctilio* is considered a secondary pest that mainly affects felled or severely stressed and damaged trees (Chrystal, 1928; Hanson, 1939; Hall, 1978). Levels of infestation by *Sirex* are in general kept low by natural parasites of the wasp, except in cases where other factors severely damage and stress the trees (Hall, 1978). Of all the siricid species in Europe and North Africa, only *S. noctilio* regularly attacks living trees and then only severely physiologically stressed, damaged or over mature dying trees (Hanson, 1939; Spradbery & Kirk, 1978). These trees often recover and may support a colony of woodwasps for an extended period of time (Spradbery & Kirk, 1978). The few severe attacks recorded from Germany and Italy, followed serious primary epidemics of defoliating insects (Spradbery & Kirk, 1978). In their native range woodwasps are, thus, viewed as natural thinning agents and as the indicators of pathological conditions, rather than primary factors in causing them (Chrystal, 1928; Cartwright, 1929).

### **2.2 SIREX IN THE SOUTHERN HEMISPHERE**

#### **2.2.1 New Zealand and Australia**

Different species of wood wasps have occasionally been recorded from the Southern Hemisphere on imported timber or timber products (Chrystal, 1928; Morgan, 1968). Of these, only *Sirex noctilio* has successfully established itself in Australasia where it has become an economically serious pest (Neumann & Marks, 1990; Chou, 1991). *Sirex noctilio* was first reported from standing trees in New Zealand around 1900. It was found

in Tasmania in the early 1950's and on the mainland of Australia in 1961 (Neumann *et al.*, 1987, Madden, 1988).

*Sirex noctilio* did not cause serious losses in New Zealand until the drought of 1946 - 1948 (Hanson, 1939; Chou, 1991). During this period, it was responsible for the devastation of many stands of *Pinus radiata* D. Don, killing approximately 30% of the trees before 1951 (Chou, 1991). Apart from this outbreak, however, New Zealand escaped further serious outbreaks.

Despite considerable investment in research, the steady spread and occasional serious outbreaks of *S. noctilio* have not been curtailed in Australia (Neumann *et al.*, 1987; Madden, 1988 & 1998b; Haugen, 1990). The ever present, but moderate damage in Australia, has frequently been interspersed with serious to very severe outbreaks. The latest of these occurred in the Green Triangle (mainland Australia), despite an established control programme. This outbreak resulted in the death of approximately 4.8 million trees before 1990 (Haugen & Underdown, 1990a; Haugen, 1990). According to Bedding (1995) *Sirex* has the potential to cause a loss A\$1-4 billion over a 30-year rotation period in Australia.

### 2.2.2 South America and South Africa

More recently, the softwood industries of South America and South Africa have also fallen victim to *S. noctilio* (Tribe & Cillié, 1994; Tribe, 1995a; Reardon *et al.*, 1995). Despite attempts to control the pest, *S. noctilio* spread steadily through South America from the initially point of introduction in Uruguay in 1980 (Maderni, 1998) to Argentina in 1985 (Klasmer *et al.*, 1998) and Brazil in the late 1980s (Iede, Do Rocio Chiarello Penteadó & Schaitza, 1998; Reardon *et al.*, 1995). Although *S. noctilio* has not been detected in other pine growing countries in South America, it is considered the greatest threat facing the millions of hectares of exotic pine plantations, throughout this region. Countries such as Chile that grow large stands of *P. radiata*, have already implemented thorough detection and exclusion procedures (Aguilar, 1998; Poisson, 1998).

*Sirex noctilio* was first reported in imported wood in South Africa in 1962 (Taylor, 1962; Morgan, 1968). At this time the wasp apparently did not escape or become established in

pine plantations. In April 1994, *S. noctilio* and its fungal associate, *A. areolatum*, were reported from *P. radiata* trees in the Cape Province in South Africa (Tribe & Cillié, 1994, Tribe, 1995a; Baxter, Rong, & Schutte, 1995). It is supposed that the wasp entered the country in infected wood crates. Based on the number of exit holes in infected trees, it has been estimated that the initial introduction was at least 2 years before this report (Tribe, 1995a). During the first three seasons after the initial report, the wasp spread in a 90 km arc through the pine plantations of this region (Hinze, 1998; Tribe, 1996). Early biological control and the mountainous surroundings seemed to constrain *Sirex* to this region. Surveys in the 1997/1998 season, however, established that the woodwasp had spread to plantations more than 200km from the initial site of occurrence from where it was reported, along both the west and south coast of the Cape province (Tribe, 1997 & 1998). Currently the damage caused by the wasp is moderate. It is, however, evident that the *Sirex* woodwasp has become firmly established in South Africa. Furthermore, it is spreading at a steady rate, threatening the pine plantations of the country as a whole (Tribe, 1998).

### 3.0 THE INSECT-FUNGUS-TREE ASSOCIATION

*Sirex noctilio* and *Amylostereum areolatum* form an inseparable relationship in their quest for survival, which is also true for their association with *Pinus* spp. Their separate discussion in this review is thus artificial, but necessary to fully understand the biology of the system.

#### 3.1 TAXONOMY AND MORPHOLOGY

##### 3.1.1 The woodwasp

The taxonomic classification of *S. noctilio* as given by Neumann *et al.* (1987) is as follows:

Order:	Hymenoptera
Sub-order:	Sympata
Family:	Siricidae
Sub-family:	Siricinae
Genus:	<i>Sirex</i> Linnaeus, 1761
Species:	<i>Sirex noctilio</i> Fabricius, 1793

The order Hymenoptera includes amongst others, bees, wasps, ants and horntails (Chamberlin, 1960). These insects are characterised by having four membranous wings (clipped together in flight); various forms of an ovipositor (borer, saw, sting, etc.); biting, rasping or sucking mouthparts; as well as having a complete metamorphic life cycle. Included in this order is the family Siricidae under which the genera *Sirex*, *Xeris*, *Urocerus* and *Tremex* are grouped (Benson, 1943).

Certain members of the family Siricidae are easily distinguished by the distinct colour of their legs, antennae and thorax, as well as the differences in size ratios of certain body parts (Chrystal, 1928; Benson, 1943; Chamberlin, 1960). Others, like *S. noctilio*, *S. cyaneus* Fabr. and *S. juvencus* Linn. are, however, very similar in general appearance and are often mistaken for one another (Benson, 1943; Talbot, 1977; Thomsen, 1996). Differentiation due to geographical separation has also contributed to the confusion in classifying some of the members of the Siricidae (Benson, 1943; Cameron, 1967). The characteristics of *S. noctilio* are given in Table 1 and Figure 1.

### 3.1.2 The fungus

The taxonomic classification of *A. areolatum* as taken from Breitenbach & Kränzlin (1986) and Kendrick (1992) is as follows:

Kingdom:	Eumycota
Phylum:	Dikaryomycota
Sub-phylum:	Basidiomycotina
Class:	Holobasidiomycetes
Sub-class:	Hymenomycetae
Order:	Aphylliphorales
Family:	Corticaceae
Sub-family:	Stereaceae
Genus:	<i>Amylostereum</i> Boidin 1958
Species:	<i>Amylostereum areolatum</i> (Fr.) Boidin 1958

The genus *Amylostereum* includes four species. Boidin described the first three, *A. chailletii* (Pers. ex Fr.) Boid., *A. areolatum* (Fr.) Boid. and *A. laevigatum* (Fr.) Boid. and transferred them from *Stereum* species in 1958 (Boidin, 1958). A fourth species thought to be associated with Podocarpaceae, *A. ferreum* (Berk. ex Curt.) Boid., was only described in 1984 from a collection of *S. ferreum* isolates (Boidin & Lanquetin, 1984) from South America.

The work of Boidin and Lanquetin (1984) is the only phylogenetic study on the genus *Amylostereum*. In the mating studies used by Boidin and Lanquetin (1984), *A. ferreum* formed hybrid mycelia with *A. chailletii* and *A. laevigatum*, but not with *A. areolatum*. No mating was observed in any of the other crosses. This indicates an older evolutionary delineation between *A. areolatum* and the *A. chailletii/A. laevigatum* group, with *A. ferreum* forming a link between the two last species, that would presumably have speciated later.

As with the Siricidae, the taxonomy of the fungi associated with these insects has been controversial. This is evident from numerous incorrect classifications in the past (Talbot, 1977; Thomsen, 1996). The fungal symbiont of *S. noctilio* in the Southern Hemisphere was first thought to be *Stereum sanguinolentum* (Albertini & Schwein.:Fr.) Fr. or at least a species of *Stereum* (Cartwright, 1929; Parkin, 1941). Later Talbot (1964) showed that the symbiont of *Sirex* in Australia is a species of *Amylostereum*, but thought that it was *A. chailletii* or a strain of this species, as did King (1966). Gaut (1969) showed that *A. chailletii* does not produce arthrospores in culture, which the *S. noctilio* fungus constantly does. By using the biological species concept and the formation of clamps in successful matings (as described by Buller, 1931) in combination with polymorphisms in protein banding patterns, Gaut (1969) could show that the fungus associated with *S. noctilio* in Australia was *A. areolatum*.

Natural fructifications of *A. areolatum* are rare and do not occur naturally in Australia or New Zealand (Talbot, 1964). In those countries the fungus has had to be studied entirely in culture and from intersegmental sacs of the wasp, that contains arthrospores (Talbot, 1964; King, 1966). Reasonably mature fructifications could, however, be obtained by using the

wood block culture method of Tamblin and Da Costa (1958). This wood block method is also used by other researchers to overcome the problem of the rarity of the fruit bodies in combination with cultural studies of *Amylostereum* (Siepmann & Zycha, 1968). Studies of fructifications from wood blocks and natural fructifications, should, however, be approached with great caution as considerable differences exist between immature and mature stages (Talbot, 1964). Furthermore, the morphological characteristics of *A. areolatum* and *A. chailletii* mycelium and fruiting bodies are very similar and subject to some variation (Breitenbach & Kränzlin, 1986). The morphological characteristics of *A. areolatum* are presented in Table 2 and Figure 2, while the principal differences between *A. areolatum* and *A. chailletii* are summarised in Table 3.

Studies on the taxonomy and phylogeny of the fungal symbionts of siricids and the genus *Amylostereum* as a whole, rely on morphological characters, the biological species concept and polymorphisms in protein patterns (Boidin, 1958; Talbot 1964; King, 1966; Gaut, 1969; Gaut, 1970; Boidin & Lanquetin, 1984). It is curious, however, that despite numerous novel and powerful molecular techniques that have become available recently, no attempt has been made to substantiate the hypotheses raised by these researchers. Techniques such as PCR based sequencing and RFLP analysis have, for example, been useful in providing quick, reliable means of distinguishing different species of Basidiomycetes, as well as clarifying the phylogenetic relationships between them (Hibbett & Vilgalys, 1991; Harrington & Wingfield, 1995; Hibbett *et al.*, 1997).

## 3.2 ECOLOGY AND BIOLOGY

### 3.2.1 The woodwasp vector

Members of the family Siricidae are all characterised by a larval state which either lives in the stems of plants or are woodborers (Chamberlin, 1960; Morgan, 1968). Members of the genera *Sirex*, *Urocerus* and *Xeris* attack softwoods (especially conifers), while *Tremex* spp. attack hardwoods. *Tremex* is considered to be more highly evolved, due to its greater morphological reduction and the fact that it exhibits a higher form of specialisation by boring through the harder bark (Benson, 1943).

Common among the Siricidae are associations with specific wood destroying Basidiomycetes (Cartwright, 1929; Francke-Grosmann, 1939; Stillwell, 1964; Morgan, 1968). The only exception is the genus *Xeris* which is thought to parasitise wood already infected by other wasps and their associated fungi (Francke-Grosmann, 1939; Morgan, 1968; Spradbery, 1977). Conidia (oidia) or bundles of fungal mycelium are carried in a pair of special intersegmental pouches (mycangia) near the base of the ovipositor of the adult female and in external hypopleural organs by the female larvae (Buchner, 1928; Francke-Grosmann, 1939; Parkin, 1941). The fungus is then inoculated into the wood together with the eggs during oviposition. It is hypothesised that this fungal inoculation is instrumental in the subsequent development of the larvae (Madden & Coutts, 1979; Madden, 1981).

Gaut (1970) showed conclusively that the symbiosis between certain siricid and fungal species are always species specific. Certain fungal species are, however, carried by more than one siricid species. He found *A. areolatum* to be the symbiont of *S. noctilio*, *S. juvencus* and *S. nitobei*, while *A. chailletii* is carried by *S. cyaneus*, *S. imperiales* Kirby, *S. areolatus* Cress., *S. californicus* Ashmead, *Urocerus gigas* Linn., *U. augur augur* Klug. and *U. augur sah* Mocs. With the difficulties in the taxonomy of the Siricidae, this specific symbiosis can serve as a useful taxonomic character (Talbot, 1977).

Life cycles of siricid woodwasps vary from one to three years in length. In the cooler Northern Hemisphere, life cycles commonly span over two to three years (Hanson, 1939; Morgan, 1968; Spradbery & Kirk, 1978). In Australasia, however, the majority of *S. noctilio* wasps complete their life cycle in one year (Taylor, 1978; Neumann & Minko, 1981). Some even emerge after two and a half to three months, while it is reported that less than 10% undergo two-year cycles (Taylor, 1978; Neumann & Minko, 1981).

In the Southern Hemisphere adult *S. noctilio* wasps emerge between mid-summer and mid-autumn after which mating takes place, trees are attacked and eggs are laid (Neumann *et al.*, 1987; Neumann & Minko, 1981). It is reported that males tend to emerge before females and to also outnumber them in natural populations (Morgan, 1968). The life span of adult wasps vary with climatic conditions, being shorter in summer and longer ( $\pm$  two weeks) in the cooler autumn (Neumann *et al.*, 1987). The average life span for a female is



five days and approximately 12 days for male wasps (Neumann *et al.*, 1987). Emerging adults are sexually mature and mating directly follows emergence in the upper branches of trees after a mating ritual (Chrystal, 1928; Morgan, 1968).

Mating appears to be enhanced by sunny conditions, temperatures above 21°C and the greater ratio of males to females (Neumann *et al.*, 1987). The *S. noctilio* female is also facultatively parthenogenic and capable of ovipositing before mating. The eggs produced in this manner will develop into haploid male wasps. This phenomenon was first observed by Peacock & Gresson (1931) for *S. cyaneus* and for *S. noctilio* by Rawlings (1953) and is thought to be common among Siricidae (Morgan, 1968). It is possible that this may in part explain the predominance of males in the natural populations.

Siricids have a very complex oviposition behaviour that is specific to each genus (Spradbery, 1977). *Sirex noctilio* has a short ovipositor (average of 12.4 mm) compared to other Siricids and spends a shorter time drilling (average 9.4 min), which accounts for the shallower drill that just reaches the xylem (Spradbery, 1977). The number of eggs carried by a female depends on her size and varies from 30 to 450 (Madden, 1974). This will influence the amount of oviposition drills made by the female wasp (Madden, 1974). Neumann *et al.* (1987) calculated that the average annual reproductive potential of one fertilised female is  $\pm 53$  females.

A number of drills are made by a female as she moves up the stem from base to top and back down again. Each drill is evaluated on an individual basis for its potential of sustaining the eggs and larvae (Madden, 1974). In regions of high osmotic pressure only the first hole is made. This is filled with mucus and oidia of the fungus, possibly conditioning the tree for later attack (Madden, 1974; Madden & Coutts, 1979). As the osmotic pressure in the phloem decreases, more holes are drilled through the same entry (up to five), each containing fungal material and eggs, except the last hole of the site that contains only mucus and fungal spores (Coutts & Dolezal, 1969 cited in Madden, 1974).

The eggs that have been laid will generally hatch within two weeks of oviposition, but this period could be longer in cooler weather and shorter in warmer weather (Madden, 1981).

Given suitable conditions (intimately related to successful establishment of its fungal symbiont) the larvae develop and start burrowing principally along the grain of the wood, later turning inwards towards the heartwood (Hanson, 1939, Madden, 1981). During this time (in a one year life cycle) the larvae go through six or seven instar phases, turning to the outer sapwood, just under the bark before pupation in late Spring or Summer (Neumann *et al.*, 1987). The total length of the burrows from where the eggs were laid to the pupation site, could eventually range from five to 26 cm in length.

The female larvae from the second instar onwards acquires the fungus by scraping against it on the walls of its borrow (Parkin, 1941). The fungal arthrospores are stored in a waxy matrix in deep skin folds (hypopleural organs) on both sides, between the first and second abdominal segments (Parkin, 1941). The waxy pockets will preserve the fungal spores, should the fungus in the wood die prematurely (Gilmour, 1965). These hypopleural organs are shed during molting to the pupal form which, therefore, does not contain any fungal spores. Francke-Grosman (1957) hypothesised that abdominal movements made while boring out of the wood, moves the waxy pockets containing fungal spores up the outside of the ovipositor and through the vaginal openings to the internal mycangia. Here, proliferation of the fungus is stimulated by special glandular secretions within the mycangial walls, until the mycangium is filled with short hyphal filaments and arthrospores (Gilmour, 1965).

### 3.2.2 The fungal symbiont

In a review by Gilmour (1965) the life cycle of *A. areolatum* and its association with *S. noctilio* has been divided into three sections and will be discussed as such here.

**Adult female - tree stage** The main means of dissemination of this fungus and its infection of the host tree is by inoculation by *S. noctilio* females (Gilmour, 1965). As noted earlier, oidia or small mycelial fragments are carried in paired intersegmental sacs at the base of the ovipositor (Buchner, 1928; Francke-Grosman, 1939; Parkin, 1941). These mycangia are connected via a duct to the tube down which the egg moves during oviposition. Eggs are never laid without also introducing these fungal cells, while arthrospores and mucus are introduced alone in unsuitable hosts (Madden, 1974; Madden & Coutts, 1979). The reason for this becomes evident from the fact that the larvae of the wasp do not develop if the

fungus does not become established in the wood (Francke-Grosmann, 1939; Gilmour, 1965). Resistance of the tree to attack, which is correlated to resinosis, fungistatic volatiles and moisture content of the wood, therefore, centres around inhibition of fungal growth (Madden & Coutts, 1979). The benefit to the fungus is evident in that it is placed deep in the wood of suitable hosts where these factors are impaired (Madden, 1974). It is also speculated that growth of the fungus in the wood might also be stimulated by the glandular secretions of the insects (Talbot, 1977).

*Amylostereum areolatum* mainly reproduces by asexually formed arthrospores that are spread by the woodwasp vector. This results in large clones or vegetative compatibility groups that are maintained in the fungal population (Vasiliauskas, Stenlid & Thomsen, 1998; Slippers, 1998; Thomsen & Koch 1999; Vasiliauskas & Stenlid, 1999) (Chapter 2). These clonal lines have been shown to be spread over considerable distances and to be preserved over long time spans (Slippers, 1998; Thomsen & Koch, 1999; Vasiliauskas & Stenlid, 1999). This property could be very useful in tracing the origin and spread of *S. noctilio* throughout the world by comparing the VCG's of its associated fungus from different geographical regions.

**Tree - larval stage** It is at this stage that the fungus plays its most critical role in the mutualism. Larvae of *S. cyaneus* have been shown to live for three months on fungal cultures (Cartwright, 1929). This showed that they are at least partly mycetophagous, with the mycelium presumably being digested extra-intestinally (Morgan, 1968). The sparseness of the mycelium in the wood, however, suggests that both the fungus and the wood (after decomposition) contribute essential nutrients to the larval diet (Francke-Grosmann, 1939; Cooke, 1977; Madden & Coutts, 1979). Indigestible cellulose and lignin is converted into more readily digestible forms for the insect. The rotting of the wood at the same time facilitates burrowing of the larvae (Madden & Coutts, 1979). Kukor and Martin (1983) and Martin (1987) showed that the larvae of *S. cyaneus* acquire essential digestive enzymes, such as cellulases and xylanases, by ingesting the fungal mycelium. The activity of the fungus is also essential in creating a suitable developmental microniche for the eggs by drying out the sapwood, thus reducing the intensity of the tree's response to attack (Madden & Coutts, 1979).

The female larval stage of *S. noctilio* possess fungus-carrying organs in the form of paired hypopleural sacs (Parkin, 1941). These organs are situated on the outside of the body and are quickly filled with scrapes of mycelium and arthrospores. Here the fungal spores are coated in a protective wax like material (Gilmour, 1965). This serves to preserve the fungus, and is important should the fungal mycelium in the wood die due to desiccation or other factors.

**The larval - pupal - adult stage** The pupae do not appear to have any special organ for carrying the fungus (Parkin, 1941). Francke-Grosmann (1957) suggested that the adult only acquired the fungus when the pupal skin is shed and the female starts to leave the wood. The shrivelled larval and pupal skins remain attached to the ovipositor of the adult until emergence. The ritual movement of the abdomen as the wasp starts boring out, breaks up the wax pockets containing the fungal spores. The alternating movement of the two halves of the ovipositor causes these sticky wax pockets to move up the outside of the ovipositor, through the genital opening and into the intersegmental sacs. Here, glandular secretions stimulate growth of the fungal cells (Gilmour, 1965; Talbot, 1977). The mycangia subsequently become packed with arthrospores as the growing cells become pressed for space and nutrients (Gilmour, 1965).

As a saprophyte remaining in the tree after emergence of the wasp, *A. areolatum* can fruit and theoretically also spread by means of basidiospores. In the Southern Hemisphere this has, however, not been observed and despite some persistence, it is eventually replaced by other colonisers and decomposers (Vaartaja & King, 1964b). In the Northern Hemisphere fruiting does occur, although (especially in the case of *A. areolatum*) not very frequently (Thomsen 1993; Thomsen & Koch, 1993; Thomsen, 1998). *Amylostereum chailletii* (referred to as *S. chailletii*) has also been reported as an early colonist of cut or damaged ends of stumps or wood blocks (Basham, 1959; Etheridge & Morin, 1963). The influence of insects in these cases is, however, difficult to rule out entirely.

### 3.3 PATHOGENICITY

As stated earlier *Sirex* is considered to be a secondary pest of little economic importance in the Northern Hemisphere, while it has caused extensive tree mortality and economic loss in the Southern Hemisphere. This higher aggressiveness, and reason why only *S. noctilio* has become established, has been ascribed to a combination of factors. Firstly, *S. noctilio* is almost always confined to *Pinus* spp. that are present in millions of hectares of exotic monocultured plantations in the Southern Hemisphere, while other siricids rarely infest pines (Spradbery & Kirk, 1978 & 1981). Furthermore the annual life cycle and bioclimatic preferences of *S. noctilio* provide a greater potential for population increases in the Southern Hemisphere, than other siricid species (Hanson, 1939; Kirk, 1974). *Sirex noctilio* is, for example, most commonly found as a native in Mediterranean climates that are also common in the Southern Hemisphere (Kirk, 1974). In the Southern Hemisphere, periods of prolonged drought are also much more frequent than in the Northern Hemisphere. This provides more drought-stressed trees that are especially susceptible to attack by *S. noctilio* (Kirk, 1974; Spradbery & Kirk, 1978).

In Europe, *S. noctilio* emerges latest of all the siricid species (during autumn and early winter) (Spradbery, 1973; Spradbery & Kirk, 1978). In Australia, emergence peaks are from January - March (summer months) and also over a shorter period than in the Northern Hemisphere (Neumann *et al.*, 1987). This results in a higher number of adult wasps, and thus the number of attacks, per time section. Furthermore, it has been shown that the susceptibility of *P. radiata* is subject to seasonal variation, being greatest in summer and least in autumn and winter (Spradbery, 1973; Kile *et al.*, 1974) (see 1.3.4.2). These factors, together with the general absence of natural parasites of *Sirex* probably enabled *S. noctilio* to adapt to its new environment more effectively.

Of all the siricid species, only *S. noctilio* regularly attacks living trees (Spradbery, 1973; Spradbery & Kirk, 1978). The *S. noctilio* mucus also induces a drastic physiological change in the stem and foliage of trees. The mucus of other siricids, however, only induces a mild or absent reaction in the plants (Spradbery, 1973). The mucus reservoir, which is

connected via a duct to the ovipositor, is also markedly larger in *S. noctilio* than those found in other siricid species (Spradbery, 1977).

Inoculation of *S. noctilio* mucus into *P. radiata*, induces drastic change in the physiology of the stem and foliage within weeks and thus accounts for the early symptoms of attack. This includes a dramatic reduction in radial growth, serious chlorosis and loss of needles, increased respiration and a reduction in leaf pressure (Coutts, 1969b; Spradbery, 1973; Fong & Crowden, 1973 & 1976) (Figure 3). This activity of the mucus conditions trees for fungal growth, while presumably at the same time stimulating the fungal growth in the wood (Gilmour, 1965, Talbot, 1977). No differences were found in physiological activity of mucus from female wasps from Europe and Australia (Spradbery & Kirk, 1978). When the mucus was inoculated in trees together with *A. areolatum*, however, only one tree died in the trial in England, whereas all the trees in a similar experiment in Australia died (Coutts, 1969a & b; Spradbery, 1973). The only difference between these experiments was that the seasons differed.

*Amylostereum areolatum* is considered to be a weak facultative pathogen unable to establish itself and kill living trees. It is probably best described as a saprophyte and wood rot fungus (Vaartaja & King, 1964a & b; Gilmour, 1965; Coutts, 1969a). However, once the resistance of trees is broken down by the action of the mucus, *A. areolatum* grows slowly through the wood, in the end killing and drying out the sapwood (by causing a firm dry white rot) enough locally as to render it non-conducting (King, 1966; Coutts, 1969a). Multiple attack all around the stem thus amounts to many overlapping dry sapwood zones, which cause a girdling effect (restricting sap supply to the crown) that will eventually kill the tree (Coutts, 1969a) (Figure 3). This is similar to the way that certain blue-stain fungi (*Ophiostoma* spp. and *Ceratocystis* spp.) carried by bark beetles are thought to kill trees (Caird, 1935). Trees often die within two months of attack, but this period is determined by the season and the number of attacks recorded (King, 1966). In such a killed tree the combination of the advancing dry white rot and the boring activity of the larvae could then render the wood worthless within six months (Neumann & Marks, 1990).

### 3.4 THE HOSTS

#### 3.4.1 Different hosts

The hosts of *S. noctilio* and *A. areolatum* include all species of *Pinus*, as well as species of *Abies*, *Larix*, *Picea* and *Pseudotsuga menziesii* (Mirb.) Franco (Browne, 1968; Spradbery & Kirk, 1978; Gibson, 1979). In its native range the wasp prefers *Pinus* spp., and also standing rather than felled timber (Spradbery & Kirk, 1978 & 1981). *Pinus pinaster* Aiton appears to be the most attacked host tree in the native range of the wasp, but in New Zealand and Australia *S. noctilio* has successfully colonised *P. radiata* as the main host (Spradbery & Kirk, 1978). This is probably due to the overwhelming abundance of stands of this pine species in Australasia (Ray *et al.*, 1979; Anonymous, 1991; Chou, 1991).

South America (except Chile) and South Africa both have large *P. radiata* plantations, but this species is planted less commonly as main softwood source than in Australasia. In South America and South Africa other species of pine planted are also affected by the pest complex (Poynten, 1977; Anonymous, 1993; Aguilar, 1998; Iede *et al.*, 1998, Maderni, 1998). In South America *P. taeda* appears most affected by *S. noctilio*, while *P. pinaster*, *P. patula* Schlechtend. & Cham., *P. radiata*, *P. elliottii* Engelm., *P. echinata* Mill., *P. palustris* Mill., *P. halepensis* Mill. are also attacked, albeit at various levels of intensity (Iede *et al.*, 1998; Maderni, 1998). Species such as *P. elliottii*, for example, appears more resistant to attack (Maderni, 1998). In South Africa, *P. canariensis* Chr. Sweet ex Spreng, *P. elliottii*, *P. patula*, *P. pinaster*, *P. pinea* L. and *P. radiata* have all been confirmed as hosts of *S. noctilio* (Tribe, 1995a & 1996).

#### 3.4.2 Host selection and susceptibility

There is evidence that *S. noctilio* is, as is the case with other phytophagous insects, attracted to suppressed, drought-stressed or nutritionally deprived trees. (Chrystal, 1928; Stillwell, 1960; Thorsteinson, 1960; Madden, 1968b & 1971; Spradbery, 1973; Hall, 1978; Spradbery & Kirk, 1978 & 1981; Moeck, Wood & Lindahl, 1981). The timing and duration of attractiveness of the trees largely depends on the extent and persistence of the stress condition (Madden, 1968b & 1971; Spradbery & Kirk, 1978 & 1981).

Damage due to man-made forces, for example volatiles from freshly felled logs or wounds, encourage attack (Madden, 1968b & 1971; Simpson & McQuilkin, 1976a & b). Biological damage to the tree often plays a role in attracting *S. noctilio* wasps to a specific tree (Chrystal, 1928; Hanson, 1939; Stillwell, 1960; Hall, 1978; Spradbery & Kirk, 1978). It has also been reported that trees killed by other pathogenic fungi such as *Armillaria* are often attacked (Spradbery & Kirk, 1978). Trees previously attacked by bark beetles (notably *Ips* species), defoliating insects or other wasps were also found to be of accentuated interest to siricid females (Spradbery & Kirk, 1978). Attack by some fungal species (such as *Trichoderma* and blue stain fungi) seem to inhibit oviposition (Spradbery & Kirk, 1978). This is possibly due to the fact that the wasps' fungal symbiont would not be able to compete under these circumstances. The age of a tree appears to be less important than its condition for making it attractive for attack by *Sirex* females (Neumann *et al.*, 1987). However, older moribund trees are often very prone to attack (Spradbery & Kirk, 1978).

Climatic conditions play an important role in severity of attack by the wasps. In the Southern Hemisphere, adult wasps tend to emerge over a shorter period of time during the warm, sunny seasons than in the Northern Hemisphere, where emergence is in the cooler autumn weather (Spradbery & Kirk, 1978). This brings about a higher number of wasps, and thus number of attacks, in a given time period in the Southern Hemisphere than is the case in the Northern Hemisphere. This coincides with a reduction in growth and an increase in resistance in the pine trees in Europe and the opposite scenario in Australasia (Spradbery, 1973; Spradbery & Kirk, 1978). This could in part explain the increased incidence of death of trees in the Southern Hemisphere due to *Sirex* attack, compared to the Northern Hemisphere.

The physiological condition of trees plays an important role in their resistance or attractiveness to siricids. Moisture and lipid content of trees are significant factors that are involved in the interaction. *Sirex noctilio* has, for example, been shown to be attracted to trees with lower moisture content and generally oviposits in parts of the tree with a low osmotic pressure in the phloem (Madden, 1974; Spradbery, 1977). Many volatiles such as monoterpenes, ketones and alcohols, that are components of the bark oils produced by the phloem-cambium tissues of pine material, also serve as attractants to siricid wasps



(Simpson & McQuilkin, 1976a & b; Madden, 1977). Damage to the tree leads to higher transpiration and phloem respiration, which results in lower osmotic pressure in the phloem, as well as larger amounts of bark-oil volatiles (Madden, 1977). These changes both attract and benefit the wasp and its fungal symbiont. Resin streaming from wounds or oviposition punctures is also regarded as an attractant for siricid females, but contrary to this, resinosis is also considered to be an important resistance mechanism (Madden, 1968b; Spradbery, 1973; Coutts & Dolezal (1966) cited in Talbot, 1977).

Resistance is related to the ability of the tree to maintain high moisture content in its phloem; produce less of certain lipids and exude enough resin in and around the oviposition hole as to inhibit fungal growth (Madden, 1968b & 1974; Vaartaja & King, 1964a; Spradbery, 1973; Spradbery & Kirk, 1978). In resistant trees a layer of polyphenols is laid down around the oviposition drill to inhibit fungal growth chemically (through  $\alpha$ - and  $\beta$ -pinenes) (Spradbery, 1973). Resin then accumulates around this layer of polyphenols and further inhibits the fungus physically by engulfing the mycelium (Spradbery, 1973). Fong and Crowden (1973) also showed that a hypersensitive reaction in the foliage in response to the mucus could be correlated to resistance. The tree presumably rids itself of the mucal effects by discarding the affected needles.

Ethylene production is higher in resistant trees, and although it is not thought to be directly involved in resistance, it might be a useful parameter in breeding for disease resistance (Shain & Hillis, 1972). Despite signs of genetic resistance in certain pine trees to *S. noctilio*, attempts to breed for this trait have, however, not been successful (Simpson & Ades, 1990). This is mainly due to the long process involved in the breeding process (attack is uncommon in trees less than 9 years old) and the loss of initial material due to a forest fire (Simpson & Ades, 1990). Simpson and Ades (1990), however, call for renewed attempts to identify and breed for *P. radiata* trees resistant to attack by the *Sirex*-*Amylostereum* complex.

## 4.0 MANAGEMENT IN THE SOUTHERN HEMISPHERE

### 4.1 CHEMICAL CONTROL

Insecticides have been shown to be ineffective in *Sirex* control (Talbot, 1977; Spradbery & Kirk, 1978; Murphy, 1998a). According to these researchers this is due to the fact that *Sirex* adults are short-lived and do not feed. Furthermore, the larvae burrow deep in the wood of host trees. The only useful possibility is using insecticides on salvaged logs to prevent breeding in this attractive niche.

The dependence of the larva on *A. areolatum* also raises the possibility of using fungicides in the control programmes. Dispersal of these chemicals through the wood, however, proved to be insufficient (Talbot, 1977). Furthermore, volatiles produced by *Amylostereum* (possibly acetaldehyde), as well as by *Saccharomyces* sp. found in the oviposition holes, serve as attractants for some of the parasitoids of *Sirex* (Madden, 1968a & 1975a; Spradbery, 1970 & 1974). Killing these fungi would thus reduce the efficacy of these natural parasites. Programmes to breed for resistance in the trees have also not been successful. Biological control with natural enemies of *Sirex* thus presents the most viable method of managing this pest complex.

### 4.2 BIOLOGICAL CONTROL

#### 4.2.1 Parasites of *Sirex*

Much research has been done on natural enemies of siricids in an attempt to control *Sirex* in areas where it has become established without them. Birds are important predators in some areas (Marshall, 1967), but their effect in large-scale control is doubtful (Chrystal, 1928; Hanson, 1939). Insect parasitoids (notably parasitic wasp species) hold much more promise. *Rhyssa persuasoria* Linnaeus was brought to New Zealand as early as 1928 and this was followed by the introduction of *Ibalia leucospoides* Hochenwarth (Hanson, 1939; Gourlay, 1951). These parasitoids did not pose a sufficient threat to *Sirex*, as was shown by the outbreak of 1946-1948. Other insect parasitoids were introduced over time and today seven are reared and released annually in Australasia (Taylor, 1967; Neumann *et al.*, 1987).

These include *Ibalia ensiger* Norton, *I. leucospoides*, *Megarhyssa nortoni nortoni* Cresson, *M. nortoni quebencesis* Provancher, *Rhyssa hoferi* Rohwer, *R. persuasoria* and *Schlettererius cinctipes* Cresson.

Most of the parasitic wasp species released as biocontrol agents have been shown to respond positively to the volatiles produced by the associated fungi, which would thus help them locate larvae of *Sirex* (Madden, 1968a & 1975a; Spradbery, 1970 & 1974). Species of *Megarhyssa*, *Rhyssa* and *Schlettererius* are lethal to late instar larva of *S. noctilio* (Hanson, 1939; Neumann *et al.*, 1987). After location, these insects bore down to the *Sirex* larvae, paralyse them, lay their eggs on them and subsequently develop first as endoparasitic and later as ectoparasitic larvae. In contrast, *Ibalia* species lay their eggs down the original oviposition hole of *Sirex* where its larvae then parasitise on the early instar larvae of *Sirex* before killing the more mature larvae.

The nematode, *Deladenus siricidicola* Bedding, found in 1962 by Zondag in New Zealand has proved to be the most successful biological control agent (Zondag, 1969). Of the seven species of *Deladenus* that parasitise siricids, *D. siricidicola* was found to be the only one that would feed on *A. areolatum* and not also parasitise *Rhyssa* spp. (Bedding, 1968 & 1995). The action of this nematode is through sterilisation of the female wasps, but without impairing their natural fitness and oviposition behaviour (Bedding, 1967 & 1972). This also counts in favour of its effectiveness as it facilitates the spread of the nematode to other trees possibly containing still uninfected wasps.

#### 4.2.2 Biological control in Australasia

Use of different parasites, and especially *D. siricidicola*, in New Zealand, Tasmania and Australia, as biocontrol agents against *Sirex* has been the subject of extensive study. Much is to be learnt from the mistakes that were made in the control strategies implemented in these countries. Extensive releases of various biocontrol agents have characterised recent control programmes in Australia. Reviews of their use and effectivity in Australasia are given by Taylor (1967 & 1978), Neumann *et al.* (1987), Haugen (1990), Haugen and Underdown (1990a & b), the National *Sirex* Control Strategy – Operations worksheets of

Australia (Anonymous, 1991), Madden (1998b) and Murphy (1998b & c). Information is summarised in this section.

Among the insect parasitoids, *Ibalia* spp. have been the most successful in establishing and spreading after introductions, as well as recording high incidence of attack on *Sirex*. *Megarhyssa* spp. has also established well, but rates of parasitism were lower. The two *Rhyssa* spp. that have been used, did not establish well. Despite repeated introductions, parasitism has been disappointing. *Schlettererius cinctipes* and *Certonotus tasmaniensis* (an insect parasitoid native to Australasia) proved difficult to breed in captivity and accounts for a much smaller part in the biocontrol programmes. In combination the named parasitoids usually do not kill more than 40% of a *Sirex* population and are, therefore, not considered sufficient to control *Sirex* on their own.

By far the most effective biocontrol agent has been *D. siricidicola*. Since its discovery in the North and South Islands of New Zealand, much research has been conducted on its control (Zondag, 1969, 1971 & 1979). Since then methods have been improved to an extent where identified build-ups of the wasp can be brought under control in a few years (Neumann *et al.*, 1987; Bedding, 1995).

Various methods have been attempted in an effort to introduce *D. siricidicola* in the field. Zondag (1971) found introduction of already infected logs throughout the affected plantation to be the most effective. Emerging wasps would then spread the nematode to other trees. The use of trap trees, made attractive to *Sirex* wasps by physical damage, was the next step (Madden & Irvine, 1971). This was an improvement, but proved very labour intensive. Strategically placed groups of trap trees treated with Dicamba herbicide proved to be more effective (Neumann *et al.*, 1982; Neumann & Morey, 1984). It was further shown that maintaining the trap tree system over two seasons improved parasitism even further. Presently, a combination of random inoculation of trees, naturally infested with *Sirex*, and the trap tree system is used in Australia. As a medium for inoculating the nematodes into the chosen trees, gelatin solutions are the most successful (Bedding & Akhurst, 1974). This method results in good survival and establishment of the nematodes

as this overcome problems of starvation due to desiccation, the lack of aeration and migration from the medium.

By using the techniques described above, *Sirex* infections by *Deladenus* were brought to rates of almost 100% in inoculated trees (Bedding & Akhurst, 1974). Due to the development of a non-infective strain and incomplete release strategies, these rates were drastically reduced in later years (Bedding, 1995). Mistakes made in planning the releases in the Green Triangle had disastrous effects. Continuous monitoring of *Sirex* spread and population build-up, as well as parasitoid and *D. siricidicola* establishment in areas of release, is essential in the control programme. Inoculations need to be done promptly at the advancing front of the *Sirex* infestations and then through adequately proportioned, carefully planned and well monitored programmes. In areas where these measures were followed strictly and good silvicultural practices implemented, *S. noctilio* numbers were successfully reduced to very low and insignificant economic levels.

#### 4.2.3 Biological control in South America and South Africa

Biological control of *Sirex* in South America and South Africa is still in its infancy. The similarity to the Australasian situation and the depth of experience pertaining to this field has, however, contributed to establishment of biological control programmes rapidly after the detection of the pest complex in these regions.

From the reviews given by Filho, Iede & Do Rocio Chiarello Penteadó (1998), Iede *et al.* (1998), Klasmer *et al.* (1998) and Maderni (1998) the following summary can be given for the state of biocontrol in South America up to 1996. The parasitoid *Ibalia leucospoides* was apparently introduced to South America with *Sirex* and subsequently spread with the pest complex through Uruguay, Argentina and Brazil. Natural parasitism rates of *Ibalia* on *Sirex* in Brazil are reported to be between 20% and 40% depending on the season, which correlates well with the parasitism rates obtained in Australasia. In an attempt to support the spread and parasitism by the *Ibalia* wasp they are also reared in captivity and released in areas where the parasitoid is absent or its population levels are low. This will, however, not be sufficient to control *Sirex* population build-up and eventually, serious occurrences, as have been seen in Australasia will occur (Anonymous, 1991).

The parasitic nematode, *D. siricidicola*, was imported to South America from Australia in the late 1980's and although initial parasitism rates were low, high parasitism levels of up to 70% have since been recorded in Brazil. *Rhyssa persuaria* and *Megarhyssa nortoni* were also imported and released in 1996 and 1997, respectively, in Brazil. A further component of management of *Sirex* in South America is extensive surveys to monitor the spread of *Sirex* (especially through the use of herbicide treated trap trees) throughout this region and assessment of the damage caused by it. The fear of *Sirex* reaching the exclusive stands of exotic *P. radiata* in Chile, has also lead to extensive programmes for its exclusion (Aguilar, 1998; Poisson, 1998). Regular surveys are, for example, made in areas where introductions are likely to take place, such as at ports and boarder roads, as well as areas of arrival or movement of imported goods within wooden containers.

*Deladenus siricidicola* was imported into South Africa from Australia immediately after detection of the wasp, and the first releases were made as early as 1995 (Tribe, 1995a & b). Parasitism rates by the nematode were low during the first season after release, but steadily increased (23% - 1995/1996; 54% - 1996/1997; 94% - 1997/1998) (Tribe, 1996; Dr. G.D. Tribe, personal communication). These rates, however, only represent results from the initial plantation where the nematode was released, and results from other plantations where *Sirex* now occurs (even <5km away) show low or no parasitism (Dr. G.D. Tribe, personal communication). Control through natural spread of the nematode will thus not be sufficient to control *Sirex* throughout its distribution range and continual monitoring, evaluation and release of the parasite in new areas will thus be necessary. *Ibalia leucospoides* were imported from Uruguay in 1998 and is ear-marked to be released early in 1999 (Tribe, 1998). According to Dr. G. Tribe, the parasites *Megarhyssa nortoni* and *Rhyssa persuasoria* will also be imported (Tribe, 1998). Surveys to monitor the spread of *Sirex* are also undertaken regularly (Tribe, 1996, 1997 & 1998).

#### **4.2.4 Biology and culture of *Deladenus siricidicola***

*Deladenus siricidicola* has been shown to possess a bicyclic life cycle (Bedding, 1967 & 1972) (Figure 1). Extensive reviews of the biology of *D. siricidicola* are given in Bedding 1984, 1993 and 1995 and are summarised here as follows:

A free-living cycle in the tree, where the nematode feeds on *Amylostereum* sp., is in the mycetophagous form. This is also the cycle where oviparous reproduction of the nematode occurs. Mating takes place between mycetophagous females and males that contain large amoeboid macrospermatozoa. The female lays 50-500 eggs in the tracheids, resin canals and *Sirex* galleries. The eggs may hatch within hours of being laid, depending on the moisture content of the wood (optimal being 50 % moisture content). Juvenile nematodes feed on the fungus and may develop in either of the two phases, depending on environmental stimuli. The free-living cycle can prevail indefinitely and ensures that millions of nematodes develop in the host trees. This is very important in the effectiveness of the nematode considering the large area of a tree to be efficiently invaded in order to locate a host insect. This cycle is also extensively used in mass rearing of the nematode for field liberations.

The parasitic cycle includes the phase of the life cycle of the nematode where larvae of *S. noctilio* are parasitised. This cycle may originate either from eggs laid by the mycetophagous female or from the progeny of infective females. Development of young nematodes arising from either of the two forms is governed by environmental conditions. It is thought that high CO<sub>2</sub> levels and a low pH induces the change from mycetophagous to infective forms. This change occurs early in the development of the juvenile and the resulting two forms differ sufficiently in morphology and ecology to be placed in separate families.

Infective nematode females are fertilised by male nematodes similar to those mating with mycetophagous nematode females, except that they possess microspermatozoa. The fertilised adult nematodes bore into the *Sirex* larvae, leaving small black dot-like entry scars. Here the nematodes passively feed through microvilli on its surface, growing tremendously in this time. Towards pupation of the *Sirex* larva, thousands of juvenile nematodes are formed and released viviparously into the haemocoel of the pupa, from where they migrate to the ovaries or testes. In female hosts, egg production is reduced and those that are produced are filled with 100-200 nematodes. Even though the testes of male insects become filled with nematodes, they are not sterilised. These nematodes are not transmitted during copulation and die together with the adult male wasp. Other than for

sterilisation, the insects are unaffected by the invasion of nematodes and oviposition commences as usual. Eggs of the wasp containing nematodes are thus placed in trees, where free-living nematode cycles may again follow and develop into infective forms which, once again attack the young larvae of uninfected wasps.

Culturing *D. siricidicola* is done using the methods provided by Bedding and Akhurst (1974). With these methods, the nematodes are reared on PDA agar plates containing a culture of *A. areolatum*. The nematodes are also stored on these plates at 5 – 6 °C. For mass rearing the PDA plate cultures are then used to inoculate flasks containing wheat. It is possible to maintain the nematode on *A. areolatum* cultures indefinitely and this was the method used earlier (Bedding & Akhurst, 1974). This method, however, necessitates continual sub-culturing which forces the nematode through repeated mycetophagous generations and eventually selects for strains that rarely form the infective stage (Bedding, 1995). This became a problem in Australia and re-isolation of wild strains had to be done from the field sites where the original nematodes were released (Bedding, 1995). One alternative method of storing is in liquid nitrogen that would make indefinite preservation of the genetic integrity of the infective strains possible (Bedding, 1995).

#### 4.3 THE INTEGRATED MANAGEMENT APPROACH

From past outbreaks of *Sirex*, and damage assessments, it is evident that good silvicultural practices and knowledge of the pest, are essential in control (Ray *et al.*, 1981; Neumann *et al.*, 1987; Madden, 1975b & 1988; Haugen, 1990). In previous sections of this review, it has been shown that *Sirex* has a definite preference for suppressed and unhealthy trees. Attack levels have been shown to be higher in overstocked and unhealthy plantations and mortality is higher in damaged, malformed or dominated trees with a smaller diameter at breast height, than surrounding trees (Neumann *et al.*, 1987; Maderni, 1998). In healthy, well managed plantations the impact of *S. noctilio* is usually reduced to a secondary problem, and costly eradication campaigns and losses in production are prevented (Neumann *et al.*, 1987; Chou, 1991). In these cases, *S. noctilio* aids in the natural propagation and spread of biocontrol agents, as well as in removing unwanted trees. A good example of the opposite situation can be found in the Green Triangle of Australia



established itself in many regions of the Southern Hemisphere. It is highly probable that it will spread to unaffected areas. Better understanding of the pest complex as a whole will also lead to more effective control of *Sirex*.

In the Southern Hemisphere less research has, however, been done to understand the *Amylostereum* symbiont of *S. noctilio* than was done for the insect itself. The studies contained in this thesis thus contribute important information to enhance our understanding of the pest complex as a whole, which is important to ensure the health of pine plantations for the future.

## 6.0 REFERENCES

- Anonymous (1991). National *Sirex* control strategy. Operations worksheets. National Sirex Co-ordination Committee. August 1991.
- Anonymous (1993). Forest Owners Association South Africa, 23rd Annual Report for Period April 1992 to March 1993.
- Aguilar, A. M. (1998). Current situation of insects associated with *Pinus radiata* D. Don in Chile and a strategy developed for *Sirex noctilio* Fabr.: An insect which may get introduced. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids", 6-9 November 1996*. USDA Forest Service. (In preparation).
- Baxter, A. P., Rong, I. H. & Schutte, A. L. (1995). *Amylostereum areolatum* (Aphylophorales: Stereaceae) in South Africa. *South African Journal of Botany* **61**, 352-354.
- Basham, J. T. (1959). Studies in forest pathology. XX. Investigations of the pathological deterioration in killed Balsam Fir. *Canadian Journal of Botany* **37**, 291-326.
- Bedding, R. A. (1967). Parasite and free-living cycles in entomogenous nematodes of the genus *Deladenus*. *Nature* **214**, 174-175.
- Bedding, R. A. (1968). *Deladenus wilsoni* n. sp. and *D. siricidicola* n. sp. (Neotylenchidae), entomophagous-mycetophagous nematodes parasitic in siricid woodwasps. *Nematologica* **14**, 515-525.
- Bedding, R. A. (1972). Biology of *Deladenus siricidicola* (Neotylenchidae) an entomophagous-mycetophagous nematode parasitic in siricid woodwasps. *Nematologica* **18**, 482-493.
- Bedding, R. A. (1984). Nematode parasites of the Hymenoptera. In *Plant and Insect Nematodes* (ed. Nickle, W.R.), pp. 755-795. Marcel Dekker Inc., NY.
- Bedding, R. A. (1993). Biological control of the woodwasp *Sirex noctilio* in Australia. Paper by Bedding (Conferencia Regional da Vespa da Madeira, *Sirex noctilio*, na America de Sud, Brazil 1993).
- Bedding, R. A. (1995). Biological control of *Sirex noctilio* using the nematode *Deladenus siricidicola*. In *Nematodes and biological control of insect pests* (ed. R. A. Bedding, R. J. Akhurst, H. Kaya), pp. 11-20. CSIRO: Melbourne, Australia.

- Bedding, R. A. & Akhurst, R. J. (1974). Use of nematode *Deladenus siricidicola* in the biological control of *Sirex noctilio* in Australia. *Journal Australian Entomological Society* **13**, 129-135.
- Benson, R. B. (1943). Studies in Siricidae, especially of Europe and southern Asia (Hymenoptera, Symphyta). *Bulletin of Entomological Research* **34**, 27-51.
- Boidin, J. (1958). Heterobasidiomycetes saprophytes et Homobasidiomycetes resupines: V.- Essai sur le genre *Stereum* Pers. ex S. F. Gray. *Revue de Mycologie* **23**, 318-346.
- Boidin, J. & Lanquentin, P. (1984). Le genre *Amylostereum* (Basidiomycetes) intercompatibilités partielles entre espèces allopartriques. *Bulletin de la Société Mycologique de France* **100**, 211-236.
- Breitenbach, J. & Kränzlin, F. (1986). *Fungi of Switzerland Volume 2 (Non-gilled fungi)*. Mengis & Sticher A.G.: Lucerne.
- Browne, F. G. (1968). *Pests of Forest Plantation Trees*. Clarendon Press: Oxford.
- Buchner, P. (1928). Holznahrung und Symbiose. Vortrag gehalten auf dem X internationalen Zoologentag zu Budapest am 8 September 1927. Springer, Berlin, 1928, 13-16.
- Buller, A. H. R. (1931). *Researches on Fungi. Volume IV*. Longmans, Green and Co.: London, U.K.
- Caird, R. W. (1935). Physiology of pines infested with bark beetles. *Botanical Gazette* **46**, 709-733.
- Cameron, E. A. (1967). Notes on *Sirex juvencus californicus* (Hymenoptera: Siricidae), with a description of the male and a key to the California species of *Sirex*. *The Canadian Entomologist* **99**, 18-24.
- Cartwright, K. St. G. (1929). Notes on fungus associated with *Sirex cyaneus*. *Annals of Applied Biology* **16**, 182-187.
- Chrystal, R. N. (1928). The *Sirex* wood-wasps and their importance in forestry. *Bulletin of Entomological Research* **19**, 219-247.
- Chamberlin, W. J. (1960). *Insects Affecting Forest Products and Materials*. O.S.C. Cooperative Association: Oregon.

- Chou, C. K. S. (1991). Perspectives of disease threat in large-scale *Pinus radiata* monoculture - the New Zealand experience. *European Journal of Forest Pathology* **21**, 71-81.
- Ciociola, I. A. (1998). International cooperation regarding quarantine procedures. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Cooke, R. (1977). *The Biology of Symbiotic Fungi*. John Wiley & Sons: London, U.K.
- Coutts, M. P. (1969a). The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata*. I. Effects of the symbiotic fungus *Amylostereum* sp. (Thelophoraceae). *Australian Journal of Biological Sciences* **22**, 915-924.
- Coutts, M. P. (1969b). The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata*. II. Effects of *S. noctilio* mucus. *Australian Journal of Biological Sciences* **22**, 1153-1161.
- Etheridge, D. E. & Morin, L. A. (1963). Colonization by decay fungi of living and dead stems of Balsam Fir following artificial injury. *Canadian Journal of Botany* **41**, 1532-1534.
- Filho, W. R., Iede, E. T. & Do Rocio Chiarello Penteadó, S. (1998). Biological aspects of *Sirex noctilio* F. (Hymenoptera, Siricidae) and its parasitoid *Ibalia leucospoides* (Hymenoptera, Ibalidae). In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Fong, L. K. & Crowden, R. K. (1973). Physiological effects of mucus from the wood wasp, *Sirex noctilio* F., on the foliage of *Pinus radiata* D. Don. *Australian Journal of Biological Sciences* **26**, 365-378.
- Fong, L. K. & Crowden, R. K. (1976). Preliminary studies on the mucus secretion of the wood wasp, *Sirex noctilio* F. I. Physiochemical and biochemical properties. *Australian Journal of Biological Sciences* **29**, 21-32.
- Francke-Grosmann, H. (1939). Über das zusammenleben von holzwespen (Siricinae) mit pilzen. *Zeitschrift für angewandte Entomologie* **25**, 647-680.
- Francke-Grosmann, H. (1957). Über das schicksal der siricidenpilze während der metamorphose. *Bericht 8. Wanderversammlung deutscher Entomologen* **11**, 37-43.

- Gaut, I. P. C. (1969). Identity of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Biological Sciences* **22**, 905-914.
- Gaut, I. P. C. (1970). Studies of siricids and their fungal symbionts. Ph.D. thesis. University of Adelaide, Australia.
- Gibson, I. A. S. (1979). *Diseases of forest trees widely planted as exotics in the tropics and southern hemisphere*. Commonwealth Forestry Institute: University of Oxford.
- Gilmour, J. W. (1965). The life cycle of the fungal symbiont of *Sirex noctilio*. *New Zealand Journal of Forestry* **10**, 80-89.
- Gourlay, E. S. (1951). Notes on insects associated with *Pinus radiata* in New Zealand. *Bulletin of Entomological Research* **42**, 21-22.
- Graham, K. (1967). Fungal-insect mutualism in trees and timber. *Annual Review of Entomology* **12**, 155-162.
- Hall, M. J. (1978). A survey of siricid attack on radiata pine in Europe. *Australian Forestry* **32**, 155-162.
- Hanson, A. S. (1939). Ecological notes on the *Sirex* wood wasps and their parasites. *Bulletin of Entomological Research* **30**, 27-65.
- Harrington, T. C. & Wingfield, B. D. (1995). A PCR-based identification method for species of *Armillaria*. *Mycologia* **87**, 280-288.
- Haugen, D. A. (1990). Control procedures for *Sirex noctilio* in the Green Triangle: Review from detection to severe outbreak (1977-1987). *Australian Forestry* **53**, 24-32.
- Haugen, D. A. & Underdown, M.G. (1990a). *Sirex noctilio* control programme in response to the 1987 Green Triangle outbreak. *Australian Forestry* **53**, 33-40.
- Haugen, D. A. & Underdown, M.G. (1990b). Release of parasitoids for *Sirex noctilio* control by transporting infested logs. *Australian Forestry* **53**, 266-270.
- Hibbett, D. S., Pine, E. M., Langer, E., Langer, G. & Donoghue, M. J. (1997). Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *Proceedings of the National Academy of Sciences USA* **94**, 12002-12006.
- Hibbett, D. S. & Vilgalys, R. (1991). Evolutionary relationships of *Lentinus* to the Polyporaceae: Evidence from the restriction analysis of enzymatically amplified ribosomal DNA. *Mycologia* **83**, 425-439.

- Hinze, W. (1998) The distribution of *Sirex noctilio* in South Africa. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Iede, E. T., Do Rocio Chiarello Penteadó, S. & Schaitza, E. (1998). *Sirex noctilio* problem in Brazil – Detection, evaluation and control. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Kendrick, B. (1992). *The Fifth Kingdom*. Mycologue Publications: Ontario, Canada.
- Kile G.A., Bowling P.J., Dolezal J.E., Bird T. (1974). The reaction of *Pinus radiata* twigs to the mucus of *Sirex noctilio* in relation to resistance to *Sirex* attack. *Australian Forest Research* 6, 25-34.
- King, J. M. (1966). Some aspects of the biology of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Botany* 14, 25-30.
- Kirk, A. A. (1974). Bioclimates of Australian *Pinus radiata* areas and *Sirex noctilio* localities in the northern hemisphere. *Australian Forestry* 37, 126-131.
- Klasmer, P., Fritz, G., Corley, J. & Botto, J. (1998). Current status of research on *Sirex noctilio* F in the Andean – patagonian region in Argentina. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Kukor, J. J. & Martin, M. M. (1983). Acquisition of digestive enzymes by the Siricid woodwasps from their fungal symbiont. *Science* 220, 1161-1163.
- Madden, J. L. (1968a). Behavioral responses of parasites to the symbiotic fungus associated with *Sirex noctilio* F. *Nature* 218, 189-190.
- Madden, J. L. (1968b). Physiological aspects of host tree favourability for the woodwasp, *Sirex noctilio* F. *Proceedings of the Ecological Society of Australia* 3, 147-149.
- Madden, J. L. (1971). Some treatments which render Monterey pine (*Pinus radiata*) attractive to the wood wasp *Sirex noctilio* F. *Bulletin of Entomological Research* 60, 467-472.
- Madden, J. L. (1974). Oviposition behavior of the woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology* 22, 341-351.
- Madden, J. L. (1975a). Bacteria and yeasts associated with *Sirex noctilio*. *Journal of Invertebrate Pathology* 25, 283-287.

- Madden, J. L. (1975b). An analysis of an outbreak of the woodwasp, *Sirex noctilio* F. (Hymenoptera, Siricidae), in *Pinus radiata*. *Bulletin of Entomological Research* **65**, 491-500.
- Madden, J. L. (1977). Physiological reactions of *Pinus radiata* to attack by woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae). *Bulletin of Entomological Research* **67**, 405-426.
- Madden, J. L. (1981). Egg and larval development in the woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology* **29**, 493-506.
- Madden, J. L. (1988). *Sirex* in Australasia. In *Dynamics of Forest Insect Populations. Patterns, Causes, Implications*. (ed. A. A. Berryman), pp. 407-429. Plenum Press, New York.
- Madden, J. (1998a). *Sirex* management – silviculture, monitoring and biological control (an introduction). In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids", 6-9 November 1996*. USDA Forest Service. (In preparation).
- Madden, J. (1998b). Overview of *Sirex* control and development of management strategies in Australia. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids", 6-9 November 1996*. USDA Forest Service. (In preparation).
- Madden, J. L. & Coutts, M. P. (1979). The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). In *Insect-Fungus Symbiosis* (ed. L. R. Batra), pp. 165-174. John Wiley & Sons, New York.
- Madden, J. L. & Irvine, C. J. (1971). The use of lure trees for the detection of *Sirex noctilio* in the field. *Australian Forestry* **35**, 164-166.
- Maderni, J. F. (1998) The sirex wood wasp (*Sirex noctilio* F.) - present status in Uruguay. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids", 6-9 November 1996*. USDA Forest Service. (In preparation).
- Marshall, H. G. W. (1967). The effect of woodpecker predation on woodboring larvae of families Siricidae (Hymenoptera) and Melandryidae (Coleoptera). *The Canadian Entomologist* **99**, 978-985.

- Martin, M. M. (1987). Acquired enzymes in the siricid woodwasp *Sirex cyaneus*. In *Invertebrate-microbial interactions. Ingested fungal enzymes in arthropod biology*. pp.37-48. Cornell University Press.
- Moeck, H. A., Wood, D. L. & Lindahl, K. Q. (Jr.) (1981). Host selection behavior of bark beetles (Coleoptera: Scolytidae) attacking *Pinus ponderosa*, with special emphasis on the western pine beetle, *Dendroctonus brevicomis*. *Journal of Chemical Ecology* 7, 49-83.
- Morgan, F. (1968). Bionomics of Siricidae. *Annual Review of Entomology* 13, 239-256.
- Murphy, S. T. (1998a). Biological control of tropical forestry and agroforestry insect pests: A review. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Murphy, S. T. (1998b). Indigenous woodwasp (siricid spp.) parasitoid communities and principal biological control agents of *Sirex noctilio* in Australasia: A review. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Murphy, S. T. (1998c). The release and evaluation of parasitoids in classical biological control projects: A brief review. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Neumann, F. G., Harris, J. A., Kassaby, F. Y. & Minko, G. (1982). An improved technique for the early detection and control of the *Sirex* wood wasp in radiata pine plantations. *Australian Forestry* 45, 117-124.
- Neumann, F. G. & Marks, G. C. (1990). Status and management of insect pests and diseases in Victorian softwood plantations. *Australian Forestry* 53, 131-144.
- Neumann, F. G. & Minko, G. (1981). The sirex wood wasp in Australian radiata pine plantations. *Australian Forestry* 44, 46 - 63.
- Neumann, F. G. & Morey, J. L. (1984). Influence of natural enemies on the *Sirex noctilio* wasp in herbicide-treated trap trees of radiata pine in north-eastern Victoria. *Australian Forestry* 47, 218-224.
- Neumann, F. G., Morey, J. L. & McKimm, R. J. (1987). The *Sirex* woodwasp in Victoria. Department of Conservation, Forest and Lands, Victoria, Bulletin No. 29, 41pp.



- Parkin, E. A. (1941). Symbiosis in larval Siricidae (Hymenoptera). *Nature* **147**, 329.
- Peacock, A. D. & Gresson, R. A. R. (1931). Male haploidy and female diploidy in *Sirex cyaneus* F. (Hymen.). Proceedings of the Royal Society of Edinburgh **51**, 97-102.
- Poisson, M. A. (1998). Activities for *Sirex noctilio* detection in Chile. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Poynton, R. J. (1977). *Tree Planting in Southern Africa, Volume 1, The Pines*. S.A. Forestry Research Institute, Department of Forestry: Republic of South Africa.
- Rawlings, G. B. (1953). Rearing of *Sirex noctilio* and its parasite *Ibalia leucospoides*. New Zealand Forest Research Notes **1**, pp. 20-34.
- Ray, J. W., Nuttall, M. J., Gadgil, P. D., Edwards, D. W. & Neumann, F. G. (1979). Integrated plant protection in *Pinus radiata* in Australia and New Zealand. In *Proceedings of Symposium, IX International Congress of Plant Protection, 5-11 August 1979, Washington, D.C.*, (ed. T. Kommsdahl), pp.607-610.
- Reardon, R., Eav, B. & Wetterberg, G. (1995). The European woodwasp, *Sirex noctilio* (Hymenoptera: Siricidae) threat to conifer plantations in South America. In *Poster Abstracts, UIFRO XX World Congress, 6-12 August 1995, Tampere*, (ed. E. Korpilahti, T. Salonen & S. Oja), p. 95. Gummerus, Jyväskylä, Finland.
- Schaitza, E. (1998). Organization of information on *Sirex noctilio* – A simple, practical and cheap project. In *Proceedings of the course "Biological control of Sirex noctilio with the use of parasitoids"*, 6-9 November 1996. USDA Forest Service. (In preparation).
- Shain, L. & Hillis, W. E. (1972). Ethylene production in *Pinus radiata* in response to *Sirex-Amylostereum* attack. *Phytopathology* **53**, 529-531.
- Siepmann, R. & Zycha, H. (1968). Artdiagnose einiger holzerstörender Hymenomyceten an hand von reinkulturen. *Nova Hedwigia* **15**, 559-569.
- Simpson, J. A. & Ades, P. K. (1990). Screening *Pinus radiata* families and clones for disease and pest insect resistance. *Australian Forestry* **53**, 194-199.
- Simpson, R. F. & McQuilkin, R. M. (1976a). Terpenes of the bark oil of *Pinus radiata*. *Phytochemistry* **62**, 1407-1409.

- Simpson, R. F. & McQuilkin, R. M. (1976b). Identification of volatiles from felled *Pinus radiata* and the electroantennograms they elicit from *Sirex noctilio*. *Entomologiae Experimentalis et Applicata* **19**, 205-213.
- Slippers, B., Wingfield, M.J., Coutinho, T.A. & Wingfield, B.D. (1998). The identity and possible origin of the *Amylostereum* symbiont of *Sirex noctilio* in South Africa. In *Proceedings of the 7<sup>th</sup> International Congress of Plant Pathology, Edinburgh, 9-16 August 1998*. Volume 2, 3.7.55.
- Spradbery, J. P. (1970). Host finding by *Rhyssa persuasoria* (L.), an ichneumonid parasite of siricid woodwasps. *Animal Behavior* **18**, 103-114.
- Spradbery, J. P. (1973). A comparative study of the phytotoxic effects of woodwasps on conifers. *Annals of Applied Biology* **75**, 309-320.
- Spradbery, J. P. (1974). The responses of *Ibalia* species (Hymenoptera: Ibalidae) to the fungal symbionts of siricid woodwasp hosts. *Journal of Entomology Series A* **48**, 217-222.
- Spradbery, J. P. (1977). The oviposition biology of siricid woodwasps in Europe. *Ecological Entomology* **2**, 225-230.
- Spradbery, J. P. & Kirk, A. A. (1978). Aspects of the ecology of siricid woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with special reference to the biological control of *Sirex noctilio* F. in Australia. *Bulletin of Entomological Research* **68**, 341-359.
- Spradbery, J. P. & Kirk, A. A. (1981). Experimental studies on the responses of European siricid woodwasps to the host trees. *Annals of Applied Biology* **98**, 179-185.
- Stillwell, M. A. (1960). Decay associated with woodwasps in balsam fir weakened by insect attack. *Forest Science* **6**, 225-231.
- Stillwell, M. A. (1964). The fungus associated with woodwasps occurring in Beech in New Brunswick. *Canadian Journal of Botany* **42**, 495-496.
- Talbot, P. H. B. (1964). Taxonomy of the fungus associated with *Sirex noctilio*. *Australian Journal of Botany* **12**, 46-52.
- Talbot, P. H. B. (1977). The *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* **15**, 41-54.
- Tamblyn, N. & Da Costa, E. N. B. (1958). A simple technique for producing fruit bodies of wood-destroying basidiomycetes. *Nature* **181**, 578-579.

- Taylor, J. S. (1962). *Sirex noctilio* F., a recent introduction in South Africa. *Entomologist's Record* **74**, 273-274.
- Taylor, K. L. (1967). The introduction, culture, liberation and recovery of parasites of *Sirex noctilio* in Tasmania, 1962-1967. *Division of Entomology Technical Paper No. 8*. Commonwealth Scientific and Industrial Research Organization, Australia, Melbourne, 1967.
- Taylor, K. L. (1978). Evaluation of the insect parasitoids of *Sirex noctilio* (Hymenoptera: Siricidae) in Tasmania. *Oecologia* **32**, 1-10.
- Thomsen, I. M. (1993). Træhvepse og svampe – en skadelig kombination. *Skoven* **6/7**, 266-269.
- Thomsen, I. M. (1996). *Amylostereum areolatum* and *Amylostereum chailletii*, symbiotic fungi of woodwasps (*Sirex* sp. and *Urocerus* sp.). Ph.D. thesis. Danish Forest and Landscape Research Institute, Horsholm, Denmark.
- Thomsen, I.M. (1998) Fruitbody characters and cultural characteristics useful for recognizing *Amylostereum areolatum* and *A. chailletii*. *Mycotaxon* **69**, 419-428.
- Thomsen, I. M. & Koch, J. (1993). *Amylostereum areolatum* og *A. chailletii* – to ejendommelige rådsvampe på nåletræe i Danmark. *Svampe* **28**, 23-25.
- Thomsen, I. M. & Koch, J. (1999). Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research* **103**, (in press).
- Thorsteinson, A. J. (1960). Host selection in phytophagous insects. *Annual Review of Entomology* **5**, 193-218.
- Tribe, G. (1995a). The woodwasp *Sirex noctilio* Fabricius (Hymenoptera; Siricidae), a pest of *Pinus* species, now established in South Africa. *African Entomology* **3**, 215-217.
- Tribe, G. D. (1995b). Biological control of *Sirex noctilio* - First report: June 1995. Agricultural Research Council, Rosebank, Internal Report, 2 pp.
- Tribe, G. D. (1996). *Sirex noctilio*: report on its occurrence within South Africa and the progress with biological control up until June 1996. Agricultural Research Council, Rosebank, Internal Report, 9 pp.
- Tribe, G. D. (1997). *Sirex* distribution survey, November 1997. Agricultural Research Council, Rosebank, Internal Report, 1 pp.

- Tribe, G. D. (1998). The biological control of the *Sirex noctilio* woodwasp. Agricultural Research Council, Rosebank, Progress Report to Forest Owners Association, 6 pp.
- Tribe, G. & Cillié, J. J. (1994). *Sirex* woodwasps in South Africa. SAFCOL: Pretoria, South Africa, 2 pp.
- Vaartaja, O. & King, J. (1964a). Inoculation experiments with *Amylostereum* sp. from a wood wasp. *Plant Disease Reporter* **48**, 438-440.
- Vaartaja, O. & King, J. (1964b). Fungi associated with a wood wasp in dying pines in Tasmania. *Phytopathology* **54**, 1031-1032.
- Vasiliauskas, R. & Stenlid, J. (1999). Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. *Mycological Research* **103**, (in press).
- Vasiliauskas, R., Stenlid, J. & Thomsen, I. M. (1998). Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from Northern Europe. *New Phytologist* **139**, (in press).
- Zondag, R. (1969). A nematode infection of *Sirex noctilio* (F.) in New Zealand. *New Zealand Journal of Science* **12**, 732-747.
- Zondag, R. (1971). Control of *Sirex noctilio* (F.) with *Deladenus siricidicola* Bedding. Part I - 1967 Field Trial. *New Zealand Journal of Forestry Science* **1**, 5-14.
- Zondag, R. (1979). Control of *Sirex noctilio* F. with *Deladenus siricidicola* Bedding. Part II. Introductions and establishments in the South Island. 1968-1975. *New Zealand Journal of Forestry Science* **9**, 68-76.

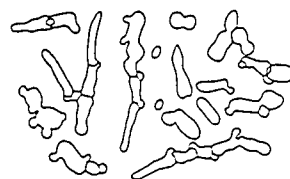
**Table 1:** Morphological characteristic of *Sirex noctilio* as described by Benson (1943) and Neumann *et al.* (1987).

<b>ADULTS</b>		
	<b>MALES</b>	<b>FEMALES</b>
<b>General colouration</b>	Metallic dark-blue	Metallic dark-blue
<b>Ovipositor</b>	None	Saw-tipped, needle-like ovipositor Straight, heavy and with average length of 13.5 mm
<b>Sawsheath</b>	None	Encasing ovipositor Projects 2-3 mm behind female abdomen
<b>Antennae</b>	20 flagellar black coloured segments Average length of 6.8 mm	21 flagellar black coloured segments Average of 7.8 mm in length
	Hair-like and slightly pubescent in colour Sensory patches on all segments	
<b>Legs</b>	Hind legs thick and powerful and metallic dark-blue colour Front and mid-legs thin and chestnut brown	All legs relatively thin and amber coloured
<b>Wings</b>	Chestnut brown	Amber coloured
	4 membranous wings, clipped together in flight	
<b>Abdomen</b>	Metallic dark-blue, except segment III and IV that are chestnut brown	All segments metallic dark-blue
	Terminates in prominent spine (vercus)	
<b>General</b>	Neumann <i>et al.</i> (1987), found the male and female to be on average about the same size, but males are often seen smaller Males usually occur in a higher ratio to females	
<b>EGGS</b>		
<b>General</b>	White, soft, smooth and elongate	
<b>Size</b>	Mean length of 1-1.5 mm and width of 0.2-0.3 mm Egg length increases with female size	
<b>LARVAE</b>		
<b>Colour</b>	Creamy white	
<b>General</b>	Deeply segmented, distinctly S-shaped and near-uniform diameter	
<b>Antennae</b>	One-segmented	
<b>Abdomen</b>	Terminates in dark brown to black sclerotic spine	
<b>Legs</b>	Thoracic and short	
<b>Sexes</b>	Males, on the first abdominal segment, contain 3 small brown sclerites on the ventral surface, while females only have two	
<b>PUPAE</b>		
<b>General</b>	Creamy white, later assuming colour of adult	

**Table 2:** The morphological characteristics of *Amylostereum areolatum* as described by Talbot (1964) and summarised by Breitenbach & Kränzlin (1986).

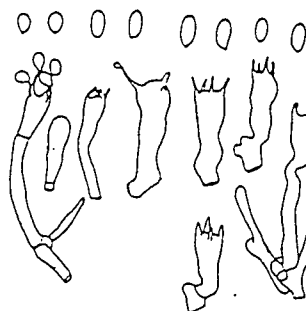
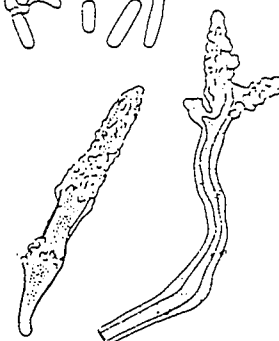
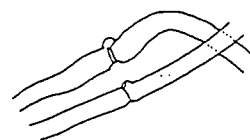
### SPORES IN MYCANGIUM OF *SIREX NOCTILIO*

- Fragmented mycelium in irregular shaped segments
- Septa separating oidia are clamped
- Clamps remain on end after break-up



### AGAR CULTURE

- Generative hyphae  
Branched, thin walled and hyaline  
Abundant simple clamp connections  
Binucleate cells  
form arthrospores and skeletal hyphae
- Arthrospores  
Oblong to cylindrical  
Narrower and more regular in shape than from mycangia  
Lack clamp connections, except the last arthrospore in the chain that was attached to rest of mycelium  
1-3 per chain
- Skeletal hyphae  
Brownish with thicker walls  
Modified into cystidia towards apices  
Cystidia at first thin walled with wide lumen, becoming densely encrusted with hyaline mineral material later
- Basidia and basidiospores  
No basidiocarp or definite hymenium in agar cultures  
Groups of homobasidiate, irregular clavate basidia, with 4 straight or slightly curved sterigmata
- Basidiospores  
Hyaline, smooth and amyloid when mature  
Broadly ellipsoid and attenuated to a small apiculus or subcylindrical with flattened side  
Uninucleate

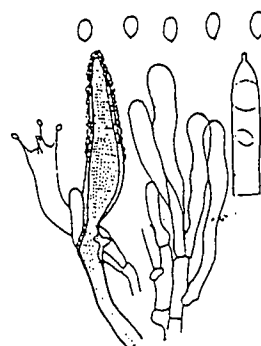
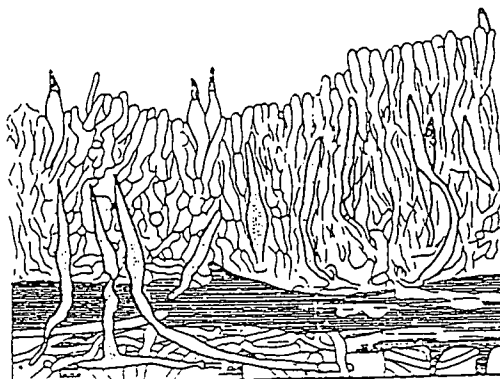


continued on page 44

Table 2: continued from page 43

## FRUCTIFICATION ON WOOD BLOCK CULTURE

- General
  - Resupinate
  - Smooth, undulating or tuberculate hymenium
  - Varies in colour from light dale brown to deep umber
  - Margin lighter coloured and appressed
- Underlying wood
  - Clamped generative hyphae and skeletal hyphae
  - Skeletal hyphae form cystidia in trama
- Trama
  - Gradually thickens and become stereoid in tissue distribution
  - Poorly defined cuticle and tomentum of skeletal and generative hyphae
  - Medullary layer with horizontally arranged skeletal and generative hyphae
  - Subhymenial and hymenial layer with vertically arranged hyphae
- Cystidia
  - Formed in or near hymenium; short, erect and of tramal origin
  - Formed throughout growth and are found in every stage, including in or beyond level of basidia
  - Stain deep and quick with safranin
- Basidia and basidiospores
  - Essentially as described from agar cultures
- Basidioles
  - Same dimensions as basidia
  - Terminate in apical constriction bearing small globose nipple
  - Vacuoles or oil globules of appreciable size

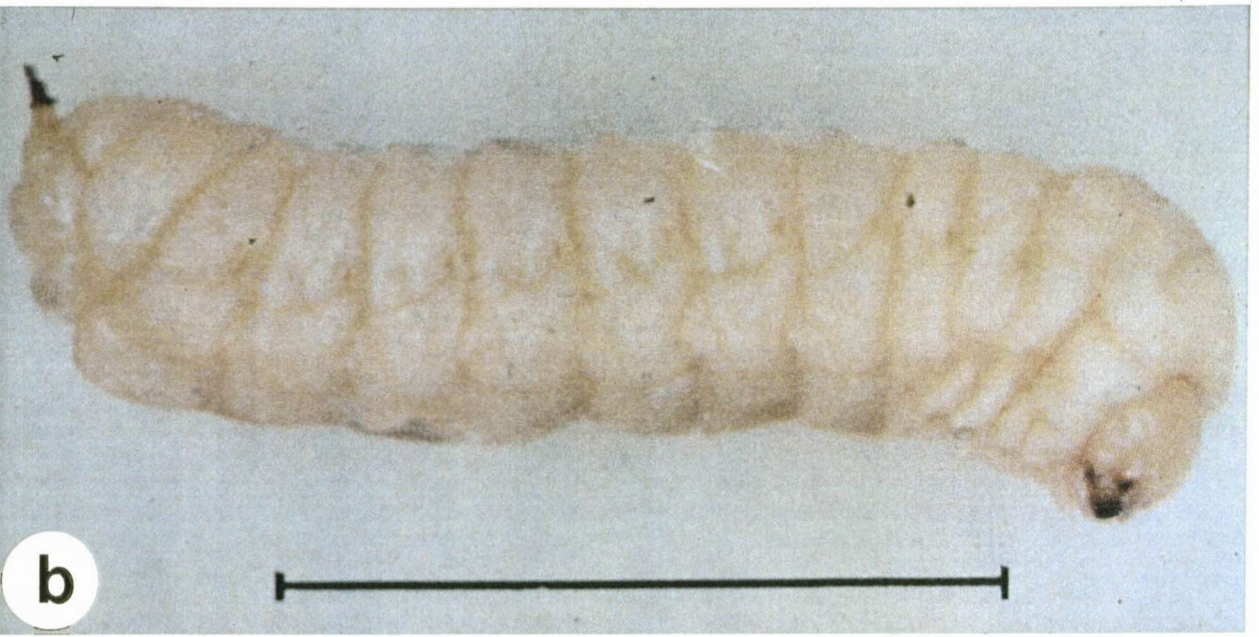
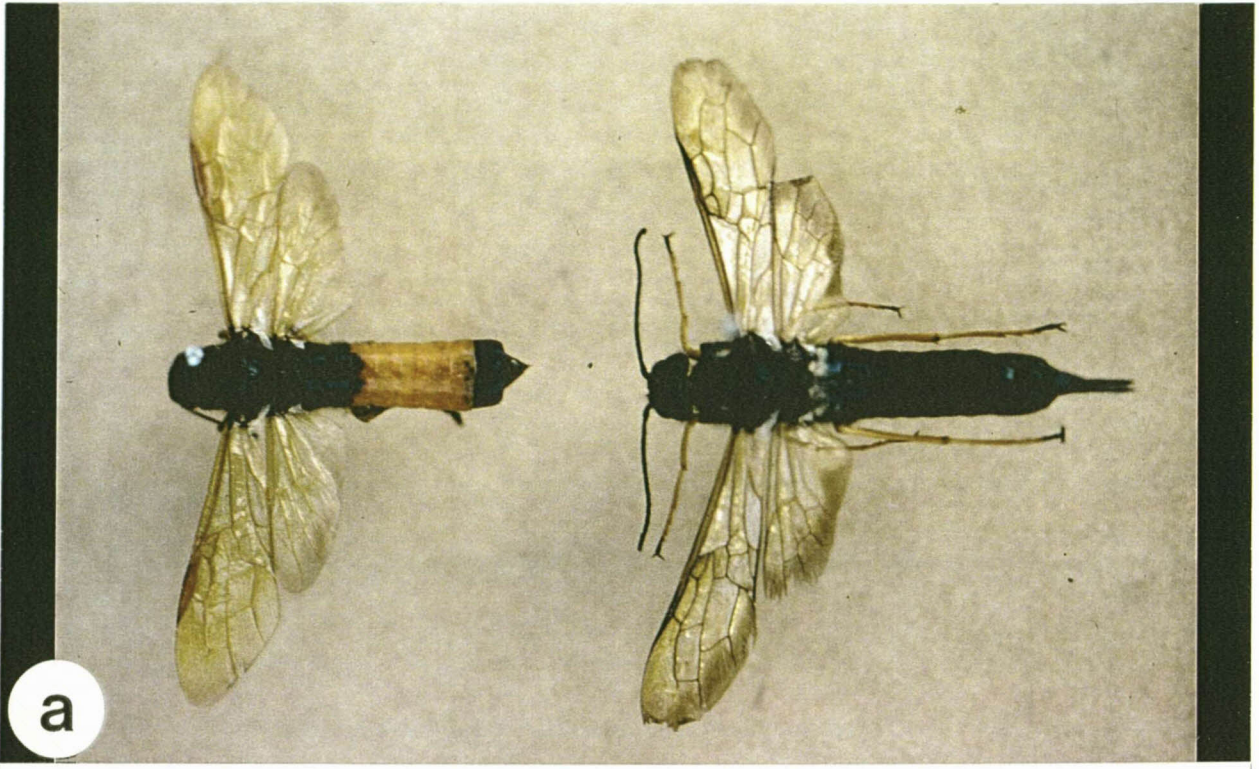


**Table 3:** A comparison of the morphological characteristics of *Amylostereum areolatum* and *A. chailletii* as described by Breitenbach & Kränzlin (1986) and Thomsen (1998).

	<i>A. areolatum</i>	<i>A. chailletii</i>
<b>•Cultural characteristics</b>	<p>Mycelial mat is yellowish-, rust- to leather-brown on MEA and PDA</p> <p>Reverse side of medium darkens within 3 weeks</p> <p>Regularly forms arthrospores</p>	<p>Cream coloured to pale-yellow or yellowish white on MEA and PDA</p> <p>Reverse side of medium darkens slowly and in patches</p> <p>Never forms arthrospores</p>
<b>•Macroscopic features of fruiting bodies</b>	<p>Brown- to brown-violet coloured with</p> <p>Undulating lighter margin that is wider than that of <i>A. chailletii</i></p> <p>Resupinate to effuso-reflexed, 1-2 mm thick</p> <p>Dark line of demarcation between tomentum and trama</p>	<p>Variable from cream, grey, to leather- or reddish-brown coloured</p> <p>Margin <math>\pm</math> demarked, darker brown to light but narrow</p> <p>Resupinate to effuso-reflexed <math>\frac{1}{2}</math> mm thick</p> <p>Thin demarcation line between hymenium and cortex</p>
<b>•Microscopic features of fruiting bodies</b>	<p>Basidiospores are elliptical and measures 4 - 6 <math>\mu\text{m}</math> x 2.4 - 3.2 <math>\mu\text{m}</math>.</p> <p>Basidia are 18-25 x 3-4 <math>\mu\text{m}</math> in size</p> <p>Skeletocystidia are 40-60 x 6-9 <math>\mu\text{m}</math> in size and some extend beyond the hymenium</p>	<p>Basidiospores are cylindrical to slightly allantoid and measures 5<math>\frac{1}{2}</math>-8 <math>\mu\text{m}</math> x 2.4 - 4 <math>\mu\text{m}</math></p> <p>Basidia are 33-45 x 4-5 <math>\mu\text{m}</math> in size</p> <p>Skeletocystidia are 40-50 x 3-6 <math>\mu\text{m}</math> in size</p>

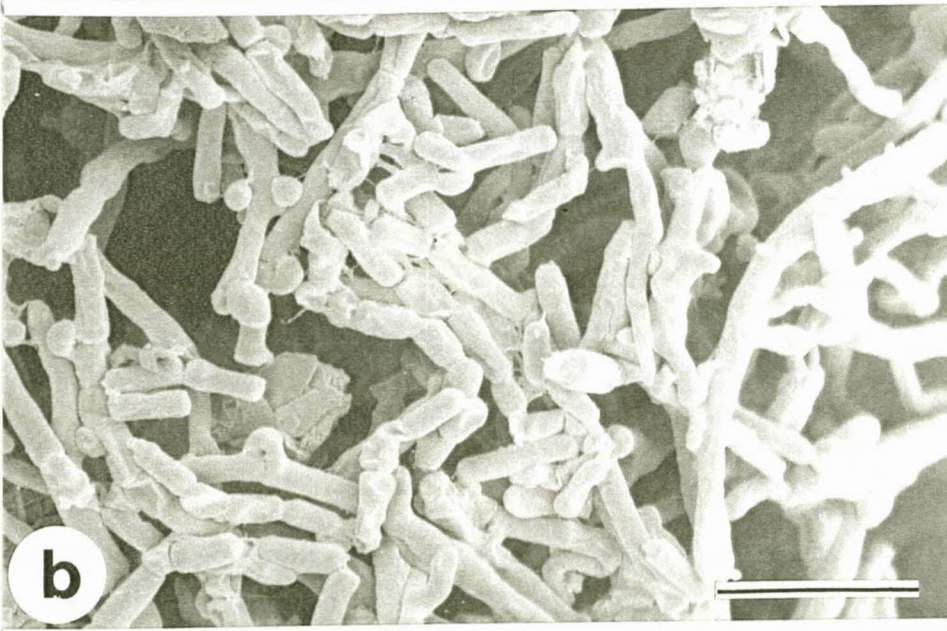
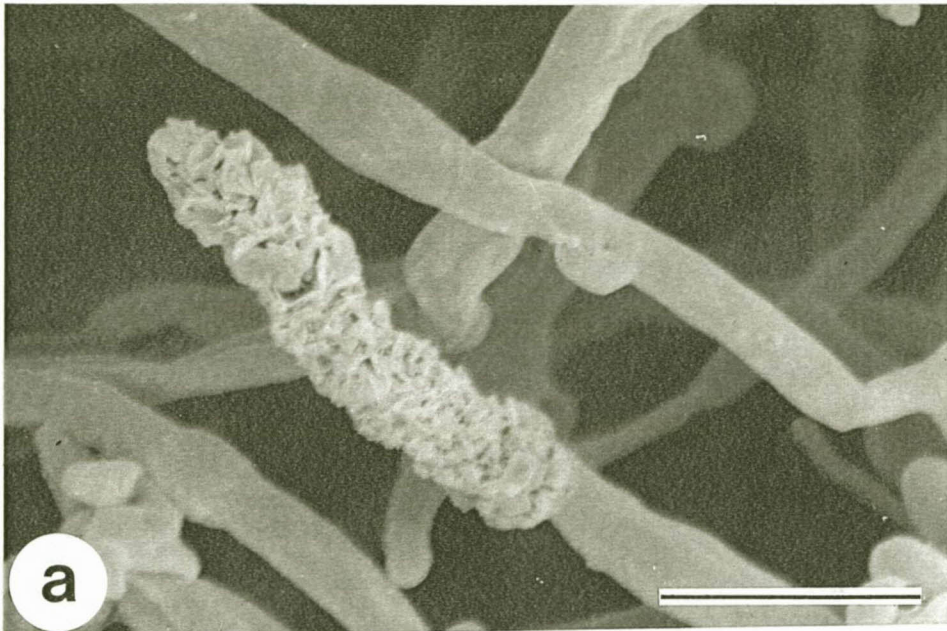


**Figure 1:** Mature and immature stages of the *S. noctilio* woodwasp. During the adult stage **(a)** male wasps (left) are characterised by the chestnut-brown coloured segments of the abdomen, while female wasps (right) are completely metallic dark-blue. Pupae **(b)** are creamy white, characteristically S-shaped and the abdomen terminates in a dark brown to black sclerotypic spine.



**Figure 2:** Micrographs of the morphological characteristics of *A. areolatum* in culture. **(a)** Hyaline encrusted cystidia and regular simple clamps at septa. *A. areolatum* also produce arthrospores in culture **(b)**, unlike other *Amylostereum* spp. Arthrospores found in the mycangia of woodwasps **(c)** are regularly clamped and irregularly shaped, unlike the arthrospores found in culture. (Bars = 10  $\mu\text{m}$ )





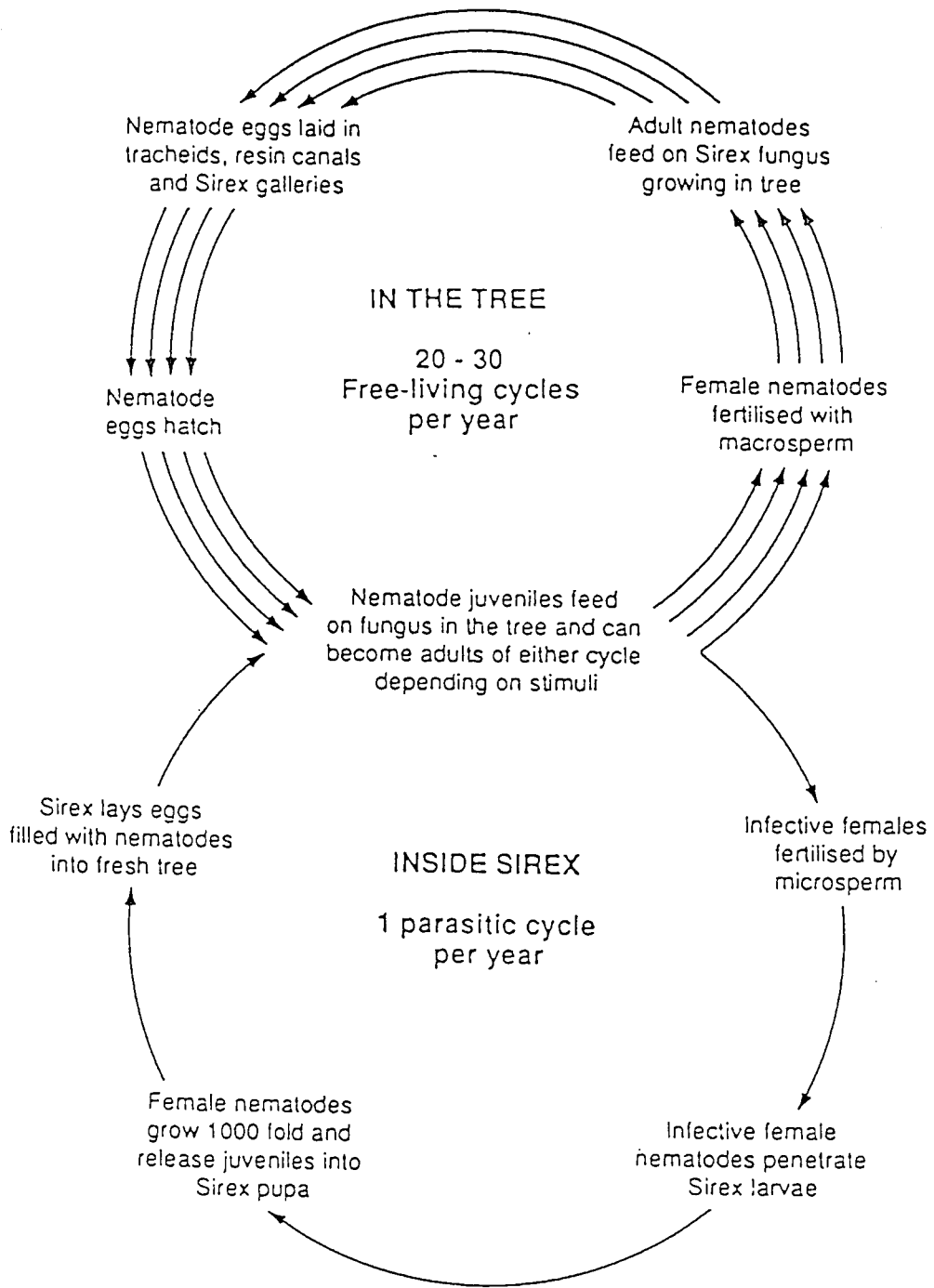
**Figure 3:** Symptoms of attack by *S. noctilio* on pine trees in the Southern Hemisphere include early symptoms, such as chlorosis and loss of needles (**a**), and are caused by the activity of the phytotoxic *Sirex* mucus. Attack is followed by the response of the tree in the form of resinosis and dribbles of resin can be seen running from oviposition holes (**b**). The tree is attacked all around the stem (**c**) and the overlapping dry zoned, as a result of the dry rot caused by the fungus, will cut off sap-flow to the crown and kill the tree. Characteristic round exit holes (**d**) of the adult wasps, can be seen after one year in the Southern Hemisphere in the wood and bark of attacked trees.





**Figure 4:** The bicyclic life cycle of *Deladenus siricidicola* that includes a mycetophagous and parasitic cycle (adapted from Bedding, 1995).







## POPULATION STRUCTURE AND POSSIBLE ORIGIN OF *AMYLOSTEREUM AREOLATUM* IN SOUTH AFRICA

### ABSTRACT

The woodwasp, *Sirex noctilio*, and its symbiotic fungus, *Amylostereum areolatum*, have been responsible for extensive damage to softwood plantations in the Southern Hemisphere. In 1994 *S. noctilio* was reported for the first time from standing pine trees in South Africa. The aim of this study was to assess the population diversity in this recently introduced population of *A. areolatum*, as well as to determine the possible origin of the pest complex. Vegetative incompatibility was tested by pairing the heterokaryotic isolates in order to determine vegetative compatibility groups (VCG), that serve as a character to delineate different genetic entities. Using this method, the South African population of *A. areolatum* was shown to constitute a single genetic entity. A collection of isolates from South America also represented only one VCG. Furthermore, this South American VCG was shown to represent the same genet as that occurring in SA. Isolates from Tasmania and New Zealand represented one VCG. This VCG produced a weaker incompatibility reaction when paired against isolates from the VCG found in SA and South America, than did any other isolate. Isolates associated with the biocontrol nematode, *Deladenus siricidicola*, from Australia, however, constituted a distinct genetic entity, different from field isolates in SA, South America, New Zealand and Tasmania. No compatibility was found between the isolates from the Southern Hemisphere and a collection of isolates from the Northern Hemisphere. We have, therefore, shown a close relationship between field isolates from the Southern Hemisphere, with the SA and South America sharing the same origin. The fact that isolates associated with the biocontrol nematode were distinct from those from SA and Brazil implies that this genetic entity has been accidentally introduced into these countries as part of a biological control initiative. It might also imply that biological control, using a nematode that was reared on a different genetic entity of *A. areolatum*, might be less effective.

## INTRODUCTION

*Sirex noctilio* Fabricius and its symbiotic fungal associate, *Amylostereum areolatum* (Fr.) Boidin, are indigenous in the temperate regions of the Northern Hemisphere and are thought to be of Eurasian origin (Benson, 1943; Morgan, 1968; Spradbery & Kirk, 1978). In these regions this insect-fungus complex is considered a secondary problem of little economic importance (Chrystal, 1928; Hanson, 1939; Hall, 1978; Spradbery & Kirk, 1978). During this century *S. noctilio* and *A. areolatum* have, however, been introduced to Australasia, South America and South Africa (Madden, 1988; Baxter, Rong & Schutte, 1995; Reardon, Eav & Wetterberg, 1995; Tribe, 1995). In these Southern Hemisphere regions, *Sirex* has been responsible for great economic losses in softwood industries. The large monocultured stands of pine, favourable bioclimatic conditions and general absence of natural enemies of *Sirex*, have all contributed to elevating this pest complex to a primary status (Spradbery & Kirk, 1978; Madden, 1988; Haugen, 1990; Neumann & Marks, 1990; Chou, 1991).

Gaut (1969) showed conclusively that the fungus associated with *Sirex noctilio* in Australasia is *Amylostereum areolatum*. This was accomplished using the biological species tests and protein gel electrophoresis. Moreover, homologous protein and enzyme patterns of isolates of *Amylostereum* from the same geographical origin were also found (Gaut, 1970). The greatest similarity was found between electrophoretic protein patterns of isolates from Australia and those originating from Belgium and Switzerland. Talbot (1977) viewed electrophoretic protein patterns as a useful tool in tracing origins of introduced fungi, and in the case of *Amylostereum*, also of the symbiotic woodwasp.

An simple method of recognising genotypes in fungi is through the use of the natural phenomenon of non-self rejection or vegetative incompatibility (Rayner, 1991; Worrall, 1997). This technique has been widely used in population studies of Basidiomycetes (Barrett & Uscuplic, 1971; Rayner & Todd, 1977 & 1978; Adams, Todd & Rayner, 1981; Adaskaveg & Gilbertson, 1987; Chamuris & Falk, 1987; Kay & Vilgalys, 1992). These studies are, however, often on a smaller spatial scale as dispersive clones (Anderson &

Kohn, 1995) are rare in these fungi. Clones of Basidiomycetes fungi are usually territorial and arise through vegetative growth (Thompson & Rayner, 1982; Stenlid, 1985; Rizzo, Blanchette & May, 1995; Worrall, 1994). This is because the normal spread of Basidiomycetes via basidiospores will result in mating of primary mycelia and the formation of heterokaryotic secondary mycelia that are genetically unique, except in non-outcrossing populations (Coates, Rayner & Todd, 1981; Ainsworth & Rayner, 1990; Ainsworth *et al.*, 1990).

Vasiliauskas, Stenlid & Thomsen (1998), Thomsen & Koch (1999) and Vasiliauskas & Stenlid, 1999 reported the existence of extensive clonal lineages or vegetative compatibility groups (VCG's) in *A. areolatum*. *Amylostereum areolatum* is a heterothallic fungus with a tetrapolar nuclear state (Boidin & Lanquetin, 1984). Thus, heterokaryotic isolates arising through pairing of primary mycelium from different basidiospores would represent separate genetic entities. These fungi are, however, spread through asexually produced arthrospores that are carried in a very specific association with siricid woodwasps (Talbot, 1977). This seems to be the predominant means of reproduction of the fungus in most cases. The sporocarps are rare in the Northern Hemisphere and have never been reported from the Southern Hemisphere (Thomsen, 1998). Through this process, clonal lines are spread over large areas and are preserved for long periods of time (Vasiliauskas *et al.*, 1998; Thomsen & Koch, 1999; Vasiliauskas & Stenlid, 1999). The origins of introduced isolates of *A. areolatum* could thus be determined by tracing these clonal lines.

Much research has been directed towards controlling *S. noctilio* in the Southern Hemisphere. In contrast, the symbiotic fungus has received little attention, despite the important role that it plays in the mutualism. No thorough population studies of *A. areolatum* in the Southern Hemisphere have, for example, been conducted. Thus, the possibility of using such knowledge to trace the spread of *Sirex* has not been exploited. Such information could also be important for biological control programmes, as the biological control agents are as intimately involved in the complex as are the fungus and woodwasp. In the present study we used vegetative or mycelial compatibility as character for investigating the population structure of the *Amylostereum* symbiont of *S. noctilio* in South Africa and South America. These populations are also compared to all other

available isolates of *A. areolatum* to determine the possible spread of *S. noctilio* in the Southern Hemisphere and the origin of the initial introductions.

## MATERIALS AND METHODS

### Collection and isolation of fungal isolates

Isolates of the fungal symbiont of *S. noctilio* in South African and South America were made from mycangia of female wasps or from wood around tunnels of the larvae of the wasp (Figure 1). *Sirex noctilio* wasps from South Africa were supplied by Dr. G. Tribe (Plant Protection Research Institute, Agricultural Research Council, Rosebank). These wasps were collected during each of the flight seasons from 1994/1995 to 1997/1998 from plantations in and around Cape Town or were raised in insectaries from stumps collected in the same area (Table 1). Wasps (*S. noctilio*) from various regions in Brazil were supplied by Mr. E. Schaitza (Table 1). One wasp from Uruguay was collected by Judy Moore (Plant Protection Research Institute, Agricultural Research Council, Rosebank).

To isolate the fungus from the mycangia of the wasps, the abdomens of a female wasps were cut from the rest of the body approximately two to three segments preceding the point of attachment of the ovipositor. A section was made either along the sides or the bottom of the abdomens and the abdomens spread open by pinning them onto a solid paraffin wax base. The mycangia were then exposed at the base of the ovipositor and the arthrospores contained in these structures were removed with a syringe needle or tweezers (Figure 1). The arthrospores were then plated onto a selective medium for Basidiomycetes (1 % Malt Extract, 1,5 % Agar, 2 ppm benomyl powder and 100 ppm streptomycin) (Hsiau, 1996), MYA (2% Malt Extract, 0,2% Yeast Extract and 1,5 % Agar) or pine extract MYA (PMYA) (150 - 200 g pine wood per liter of water was double autoclaved and poured through cheese cloth before adding the ingredients of MYA in same concentrations as described for normal MYA).

Isolates of *A. areolatum* were obtained from cultures of the nematode *Deladenus siricidicola* that are exported from Australia to various Southern Hemisphere countries as a

biological control agent. This nematode feeds and is maintained on isolates of *A. areolatum*. Isolations were made directly from nematode cultures imported to South Africa during 1995 (Table 1).

Dr. I.M. Thomsen and Dr. R. Vasiliauskas supplied isolates of *A. areolatum* from Europe (Table 1). Further isolates from different parts of the world were obtained from CBS (Centraal Bureau voor Schimmelcultures) and DAOM (Centre for Land and Biological Resources Research, Canada) culture collections (Table 1). All isolates were maintained on 9 cm diameter Petri dishes containing MYA or PMYA at 25 °C. The cultures were stored on MYA slants at 4 °C.

### **Heterokaryon compatibility**

Pairings of heterokaryons were done by placing two  $\pm 0.5$  cm mycelial plugs, cut from the edges of actively growing cultures, one centimeter apart at the side of a Petri dish. Isolates were sometimes also paired by placing plugs from more than two isolates on a plate. This method was, however, not preferred, as full confrontation between all isolates did not always occur. Pairings were done on MYA and PMYA. No differences were observed between reactions of a given set of isolates paired on MYA or PMYA. As the growth rates on MYA was slower than those on PMYA, the latter medium was preferred as it shortened the time needed for reactions to develop. Reactions were scored after two to three weeks, but cultures were also incubated for longer periods in order to confirm that the reaction did not change over time. Pairings were always repeated to confirm initial results.

Rayner (1991) stresses the need for controlled self pairings to distinguish between true incompatibility and "mutual staling or nutrient depletion". Reactions between paired isolates were, therefore, always scored alongside control reactions where isolates were paired against themselves (positive controls) and against isolates known to be incompatible from initial trial experiments (negative controls). Reactions were normally scored as compatible or incompatible, but an intermediate reaction was also observed. In such intermediate reactions, opposing cultures formed a zone of sparse mycelium growth in the

interaction zone, but did not show the brown discoloration characteristic of the interactions of incompatible reactions.

Pairings to determine VCG's occurred in different phases:

**South African population** South African (SA) isolates collected during each season (six isolates - 1994/1995; 56 isolates - 1995/1996; 45 isolates 1996/1997) were paired in all possible combinations, for each season. As isolates from each season formed one VCG, a smaller number of isolates collected during each of the different seasons (three from 1994/1995, more than 10 from 1995/1996 and 1996/1997, respectively, and one from 1997/1998) were then paired against each other.

**South American population** The 25 Brazilian isolates were first paired in all possible combinations with each other. Later five of these were paired against the single Uruguayan isolate.

**South African vs South American population** The South American isolates (ten from Brazil and one from Uruguay) were then paired against SA isolates representing each of the different seasons. In all the above experiments, Australian isolates were paired against the SA and South American isolates. As these pairings were known from initial trial experiments to be incompatible, they served as negative controls.

**South Africa & South America vs rest** SA isolates (at least two for each collection season from 1994/1995 - 1996/1997 and one for 1997/1998) and South American isolates (four from Brazil) were also paired with isolates from other culture collections. These included isolates from Central Europe (CBS305.82 and CBS334.66), Northern Europe (L204, L236, DK782, DK37, S225 and S227), New Zealand (DAOM 21785) and Tasmania (WaiteInst.6195). The isolates from Central Europe, Northern Europe, New Zealand and Tasmania and Australia were also paired with each other in all possible combinations.

## RESULTS

All isolates (108) collected within South Africa during the course of four seasons (1994/1995 - 1997/1998) were somatically compatible (Figure 2). Colony morphologies

and interactions sometimes appeared to be less uniform when compared to controls, but the mycelium always intermingled freely. Similarly all South American isolates (25 from Brazil and one from Uruguay) were compatible with each other and grew as a single entity when paired in various combinations. All South African and South American isolates were also vegetatively compatible with each other (Figure 3). Colony morphologies of the South African and South American cultures showed minor variations, but incompatible reactions between isolates were never observed.

The isolates from New Zealand (DAOM 21785) and Tasmania (Waite Inst. 6195), were fully compatible with each other (Figure 3). These isolates showed an intermediate reaction when paired against isolates from South Africa and South America (Figure 3 & 4). These intermediate interactions were much less intense than reactions between these isolates (from New Zealand, Tasmania, South Africa and South America) and any of the other isolates used in the study (Figure 4). The discoloration of the demarcation line at the interaction zone between isolates was very slight to almost undetectable, but the cultures did not grow as an entity as was seen in the positive controls. Colony morphologies of isolates DAOM 21785 and Waite Inst. 6195 were similar to those of isolates from South America and South Africa.

All pairings between South African and South American isolates and Australian isolates were strongly incompatible (Figure 3 & 4). Pairings between representative isolates from the single South African/South American VCG and isolates from the CBS culture collection (from Central Europe) and from the culture collections of Dr. Thomson and Dr. Vasiliauskas (Northern Europe) were strongly incompatible (Figure 4). None of the isolates from the CBS and DAOM culture collections were compatible, except isolates DAOM 21785 and Waite Inst. 6195 (as described above). The VCG's of isolates from Denmark, Lithuania and Sweden used in this study were the same as was determined for these isolates by Thomsen & Koch, 1999. These isolates were, however, not compatible with any of the other isolates used in this study.

## DISCUSSION

In this study we have shown that *A. areolatum* isolates collected over four seasons in South Africa, and soon after its introduction together with *S. noctilio* into the country, represent a single vegetatively compatible genetic entity. This suggests that the introduction of *S. noctilio* was through a limited number of wasps and probably from a single area. Thomsen & Koch (1999) showed that the wasps from the same tree usually carry isolates of *Amylostereum* representing the same VCG. Neumann, Morey & McKimm (1987) suggested that up to a few hundred wasps can emerge from a single tree. The introduction of *Sirex* into SA, therefore, did not have to be by a single female to account for the single VCG representing the population of *A. areolatum*. Furthermore, this finding indicates that the introduction of *S. noctilio* and *A. areolatum* could either have been a single event or could have taken place more than once, but then from the same source.

The single genetic entity of *A. areolatum* in South Africa has been preserved and spread over the last four seasons (1994/1995 – 1997/1998). This indicates that vegetative reproduction through arthrospores and spread through symbiosis with *S. noctilio*, is the predominant or only form of propagation of this fungus in South Africa. This might explain why no basidiocarps of *A. areolatum* have yet been found in South Africa.

Similar to the South African situation, a reasonably large collection of isolates of *A. areolatum* from South America also represented a single VCG. Here a small introduction, or alternatively spread of *S. noctilio* from surrounding countries, could account for the compatible genetic pool of the isolates collected. *Sirex noctilio* has been known from this region for more than 10 years, which means that the genetic entity has been preserved over an even longer period in South America, than is the case in South Africa.

The fact that the genet from South Africa and South America is the same was of considerable interest. This indicates that *S. noctilio* in these two countries either share a common origin, or that this foreign pest was introduced into South Africa from South America where it has been known for a longer time period. New introductions of *S. noctilio* into the Southern Hemisphere have, thus, not necessarily been separate



introductions from the Northern Hemisphere, but have more likely been between countries in the Southern Hemisphere.

Although incompatible reactions were never observed between isolates from South Africa and South America, interactions were sometimes not as uniform as seen in the positive controls. Thomsen & Koch (1999) and Vasiliauskas *et al.* (1998) also noted the variability in the degree of the interactions and incorporate the results of molecular screening to support their results obtained from vegetative compatibility tests. This phenomenon has also been reported by various other researchers when pairing either monokaryotic, synthesised heterokaryons or heterokaryotic field isolates from a population (Adams & Roth, 1967; Coates *et al.*, 1981; Boddy & Rayner, 1982; Rayner & Turton, 1982; Stenlid, 1985). General conclusions were that the rejection reaction was weaker between more closely related isolates than between unrelated isolates or isolates separated by larger geographical distances. These conclusions were incorporated in the interpretation of the results of the current study.

Isolates of *A. areolatum* from New Zealand and Tasmania (DAOM 21785 and Waite Inst. 6195) were vegetatively compatible with each other and similar to the South African – South American VCG. As *Sirex* was first reported in New Zealand, these results suggest that the introduction of *Sirex* into Tasmania was probably from New Zealand or that they share a common origin. Isolates from South Africa and Brazil were not fully compatible with the isolates from New Zealand and Tasmania, but were evidently more closely related to them than to any of the other isolates used in the study. These two isolates from New Zealand and Tasmania have been in culture for many years (1962 in the case of isolate Waite Inst. 6195) and might be expected to have lost vigour. Genetic change during sub-culturing could also explain the slight differentiation seen here. The possibility, therefore, exists that the introductions of *Sirex* to South America and South Africa originated in New Zealand or Tasmania.

The *D. siricidicola* nematode used in biocontrol programmes, are reared on cultures of *A. areolatum* in Australia, from where it is imported by other countries throughout the Southern Hemisphere. Considering the close relationship between other isolates from the

Southern Hemisphere (from New Zealand, Tasmania, South Africa and South America), the strong antagonism of all the isolates from the Southern Hemisphere towards the strain of *A. areolatum* from Australia on which the nematode is reared, was surprising. These isolates from Australia were not necessarily collected from the field in Australasia, but possibly originated from earlier isolations of the nematode in Europe, and might even have originated from *S. juvenicus* (R.A. Bedding, CSIRO, Australia; personal communication).

The strong antagonism between the isolates from the nematode cultures and isolates from other countries in the Southern Hemisphere, that import this isolate with cultures of the biological control nematode, has two possible consequences. Firstly, it might influence the feeding and reproduction of the biocontrol nematode, when introduced to new countries or areas. This would be consistent with suggestions that certain isolates of *A. areolatum* from the field in Australia are better than others for rearing the nematode (R.A. Bedding, personal communication). Secondly, it implies that a different genetic entity of *A. areolatum* has been introduced into South Africa and South America with the nematode and it will be interesting to see how this influences the population structure of *A. areolatum* in these countries in the future.

Although it was possible to show that *A. areolatum*, and, therefore, also *S. noctilio*, in South Africa and South America probably had the same origin, it was not possible to show where this might have been. All pairings between isolates from South Africa, South America, Tasmania and New Zealand suggest that these cultures are genetically related, but their pairings with isolates from all other sources available were always strongly incompatible. Isolates from Switzerland and Belgium could not be obtained, which would have made it possible to test Gaut's (Gaut, 1970) findings that isolates from Australia are most similar to isolates from these two countries.

In this study, we have shown that vegetative compatibility tests can be useful in determining the original source of introduction of *Sirex* to the Southern Hemisphere, should isolates from a wide enough range be obtained. This technique has also been used by Vasiliauskas *et al.* (1998), Thomsen & Koch (1999) and Vasiliauskas & Stenlid (1999) to exploit the formation of dispersive clones (where clonal genets are spatially separated) in

*A. areolatum* and *A. chailletii*. One advantage of this technique is that it allows for relatively easy and inexpensive screening of a large number of isolates for genetic similarities and dissimilarities (Stenlid, 1985). Some disadvantages, however, include the fact that while mycelial incompatibility clearly constitutes genetic difference, mycelial compatibility does not necessarily constitute clonality or somatic compatibility (Worrall, 1997). Worrall (1997), therefore, urges the use of molecular techniques to detect nuclear identity in addition to vegetative pairings. Although indications from work of Vasiliauskas *et al.* (1998) and Thomsen & Koch (1999) are that the genetic entities represented by a VCG in *A. areolatum* are clonal, the true clonality of the VCG found in South Africa and South America cannot be implied from the present results.

## REFERENCES

- Adams, D. H. & Roth, L. F. (1967). Demarcation lines in paired cultures of *Fomes cajanderi* as a basis for detecting genetically distinct mycelia. *Canadian Journal of Botany* **45**, 1583-1589.
- Adams, T. J. H., Todd, N. K. & Rayner, A. D. M. (1981). Antagonism between dikaryons of *Piptoporus betulinus*. *Transactions of the British Mycological Society* **76**, 510-513.
- Adaskaveg, R. L. & Gilbertson, R. L. (1987). Vegetative incompatibility between intraspecific dikaryotic pairings of *Ganoderma lucidum* and *G. tsugae*. *Mycologia* **79**, 603-613.
- Ainsworth, A. M. & Rayner, A. D. M. (1990). Mycelial interactions and outcrossing in the *Coniophora puteane* complex. *Mycological Research* **94**, 627-634.
- Ainsworth, A. M., Rayner, A. D. M., Broxholme, S. J. & Beeching, J. R. (1990). Occurrence of the unilateral genetic transfer and genomic replacement between strains of *Stereum hirsutum* from non-outcrossing and outcrossing populations. *New Phytologist* **115**, 119-128.
- Anderson, J. B. & Kohn, L. M. (1995). Clonality in soilborne, plant-pathogenic fungi. *Annual Review of Phytopathology* **33**, 369-391.
- Barrett, D. K. & Uscuplic, M. (1971). The field distribution of interacting strains of *Polyporus schweinitzii* and their origin. *New Phytologist* **70**, 581-598.
- Baxter, A. P., Rong, I. H. & Schutte, A. L. (1995). *Amylostereum areolatum* (Aphyllophorales: Stereaceae) in South Africa. *South African Journal of Botany* **61**, 352-354.
- Benson, R. B. (1943). Studies in Siricidae, especially of Europe and southern Asia (Hymenoptera, Symphyta). *Bulletin of Entomological Research* **34**, 27-51.
- Boddy, L. & Rayner, A. D. M. (1982). Population structure, inter-mycelial interactions and infection biology of *Stereum gausapatum*. *Transactions of the British Mycological Society* **78**, 337-351.
- Boidin, J. & Lanquentin, P. (1984). Le genre *Amylostereum* (Basidiomycetes) intercompatibilités partielles entre espèces allopartriques. *Bulletin de la Société Mycologique de France* **100**, 211-236.

- Chamuris, G. P. & Falk, S. P. (1987). The population structure of *Peniophora rufa* in an aspen plantation. *Mycologia* **79**, 451-457.
- Chou, C. K. S. (1991). Perspectives of disease threat in large-scale *Pinus radiata* monoculture - the New Zealand experience. *European Journal of Forest Pathology* **21**, 71-81.
- Chrystal, R. N. (1928). The *Sirex* wood-wasps and their importance in forestry. *Bulletin of Entomological Research* **19**, 219-247.
- Coates, D., Rayner, A. D. M. & Todd, N. K. (1981). Mating behavior, mycelial antagonism and the establishment of individuals in *Stereum hirsutum*. *Transactions of the British Mycological Society* **76**, 41-51.
- Gaut, I. P. C. (1969). Identity of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Biological Sciences* **22**, 905-914.
- Gaut, I. P. C. (1970). Studies of siricis and their fungal symbionts. Ph.D. thesis. University of Adelaide, Australia.
- Hall, M. J. (1978). A survey of siricid attack on radiata pine in Europe. *Australian Forestry* **32**, 155-162.
- Hanson, A. S. (1939). Ecological notes on the *Sirex* woodwasps and their parasites. *Bulletin of Entomological Research* **30**, 27-65.
- Haugen, D. A. (1990). Control procedures for *Sirex noctilio* in the Green Triangle: Review from detection to severe outbreak (1977-1987). *Australian Forestry* **53**, 24-32.
- Kay, E. & Vigalys, R. (1992). Spatial distribution and genetic relationships among individuals in a natural population of the oyster mushroom *Pleurotus ostreatus*. *Mycologia* **84**, 173-182.
- Madden, J. L. (1988). *Sirex* in Australasia. In *Dynamics of Forest Insect Populations. Patterns, Causes, Implications*. (ed. A. A. Berryman), pp. 407-429. Plenum Press, New York.
- Morgan, F. (1968). Bionomics of Siricidae. *Annual Review of Entomology* **13**, 239-256.
- Neumann, F. G. & Marks, G. C. (1990). Status and management of insect pests and diseases in Victorian softwood plantations. *Australian Forestry* **53**, 131-144.
- Neumann, F. G., Morey, J. L. & McKimm, R. J. (1987). The *Sirex* woodwasp in Victoria. Department of Conservation, Forest and Lands, Victoria, Bulletin No. **29**, 41pp.

- Rayner, A. D. M. (1991). The challenge of the individualistic mycelium. *Mycologia* **83**, 48-71.
- Rayner, A. D. M. & Todd, N. K. (1977). Intraspecific antagonism in natural populations of wood-decaying basidiomycetes. *Journal of general Microbiology* **103**, 85-90.
- Rayner, A. D. M. & Todd, N. K. (1978). Polymorphism in *Coriolus versicolor* and its relation to interfertility and intraspecific antagonism. *Transactions of the British Mycological Society* **71**, 99-106.
- Rayner, A. D. M. & Turton, M. N. (1982). Mycelial interactions and population structure in the genus *Stereum*: *S. rugosum*, *S. sanguinolentum* and *S. rameale*. *Transactions of the British Mycological Society* **78**, 483-493.
- Reardon, R., Eav, B. & Wetterberg, G. (1995). The European woodwasp, *Sirex noctilio* (Hymenoptera: Siricidae) threat to conifer plantations in South America. In *Poster Abstracts, UIFRO XX World Congress, 6-12 August 1995, Tampere*, (ed. E. Korpilahti, T. Salonen & S. Oja), p. 95. Gummerus, Jyväskylä, Finland.
- Rizzo, D. M., Blanchette, R. A. & May, G. (1995). Distribution of *Armillaria ostoyae* genets in a *Pinus resinosa* - *Pinus banksiana* forest. *Canadian Journal of Botany* **73**, 776-787.
- Spradbery, J. P. & Kirk, A. A. (1978). Aspects of the ecology of siricid woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with special reference to the biological control of *Sirex noctilio* F. in Australia. *Bulletin of Entomological Research* **68**, 341-359.
- Stenlid, J. (1985). Population structure of *Heterobasidion annosum* as determined by somatic incompatibility, sexual incompatibility, and isoenzyme patterns. *Canadian Journal of Botany* **63**, 2268-2273.
- Talbot, P. H. B. (1977). The *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* **15**, 41-54.
- Thompson, W. & Rayner, A. D. M. (1982). Spatial structure of a population of *Tricholomopsis platyphylla* in a woodland site. *New Phytologist* **92**, 103-114.
- Thomsen, I. M. (1996). *Amylostereum areolatum* and *Amylostereum chailletii*, symbiotic fungi of woodwasps (*Sirex* sp. and *Urocerus* sp.). Ph.D. thesis. Danish Forest and Landscape Research Institute, Horsholm, Denmark.

- Thomsen, I.M. (1998) Fruitbody characters and cultural characteristics useful for recognizing *Amylostereum areolatum* and *A. chailletii*. *Mycotaxon* **69**, 419-428.
- Thomsen, I. M. & Koch, J. (1999). Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research* **103**, (in press).
- 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. (1999). Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. *Mycological Research* **103**, (in press).
- Vasiliauskas, R., Stenlid, J. & Thomsen, I. M. (1998). Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from Northern Europe. *New Phytologist* **139**, (in press).
- Worrall, J. J. (1994). Population structure of *Armillaria* species in several forest types. *Mycologia* **86**, 401-407.
- Worrall, J. J. (1997). Somatic compatibility in basidiomycetes. *Mycologia* **89**, 24-36.

**Table 1** Isolates of *Amylostereum areolatum* used in this study in heterokaryon pairings.

Culture nr.	I.D.	Host or source of isolation	Origin	Date isolated	Isolator
<b>CBS cultures</b>					
305.82	<i>Amylostereum areolatum</i>	Unknown	France	1964	J. Boiden
334.66	<i>A. areolatum</i>	From <i>Picea abies</i>	Germany	1967	Dimitri
<b>CLBRR cultures</b>					
Waite Inst. 6195	<i>Amylostereum</i> sp.	Mycangium of <i>S. noctilio</i>	Tasmania	1962	Unknown
DAOM 21785	<i>Amylostereum</i> sp.	Wood of <i>P. radiata</i> around oviposition bores of <i>S. noctilio</i>	New Zealand	Unknown	G.B. Rawlings
<b>Other European isolates</b>					
L204	<i>A. areolatum</i> ( $\pm$ Clone S)*	Wood of wounded <i>P. abies</i>	Lithuania	1994	R. Vasiliauskas
L236	<i>A. areolatum</i> (Clone A)*	Wood of wounded <i>P. abies</i>	Lithuania	1995	R. Vasiliauskas
DK37	<i>A. areolatum</i> (Clone A)*	Fruiting body on <i>P. abies</i>	Denmark	1993	I.M. Thomson
DK782	<i>A. areolatum</i> (Clone B)*	Fruiting body on <i>P. abies</i>	Denmark	1987	J. Koch
S225	<i>A. areolatum</i> (Clone S)*	Wood of wounded <i>P. abies</i>	Sweden	1994	R. Vasiliauskas
S227	<i>A. areolatum</i> (Clone S)*	Wood of wounded <i>P. abies</i>	Sweden	1994	R. Vasiliauskas
<b>Australian isolates</b>					
A3, A4, A6, A7, A8, A9, A10, A11	<i>Amylostereum</i> sp.	Isolates from nematode cultures from CSIRO	Australia	1995	B. Slippers
<b>South American isolates</b>					
25 Isolates numbered Br1 -Br94	<i>Amylostereum</i> sp.	Mycangia of <i>S. noctilio</i> wasps	Brazil	1997	B. Slippers
U1	<i>Amylostereum</i> sp.	Mycangia of <i>S. noctilio</i> wasps	Uruguay	1998	B. Slipper

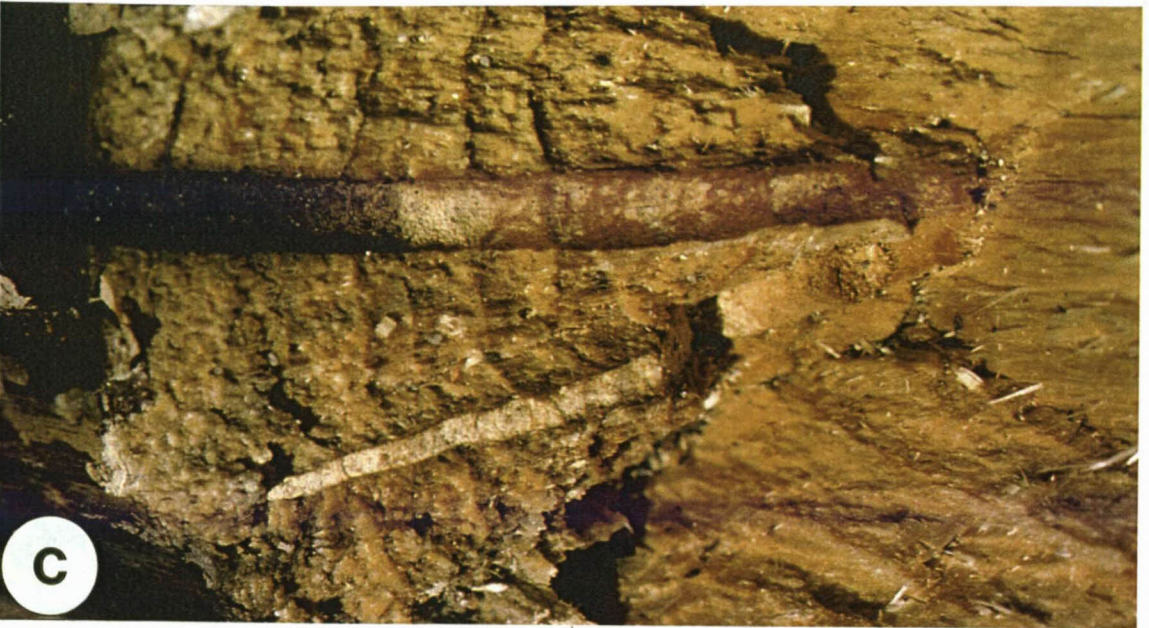
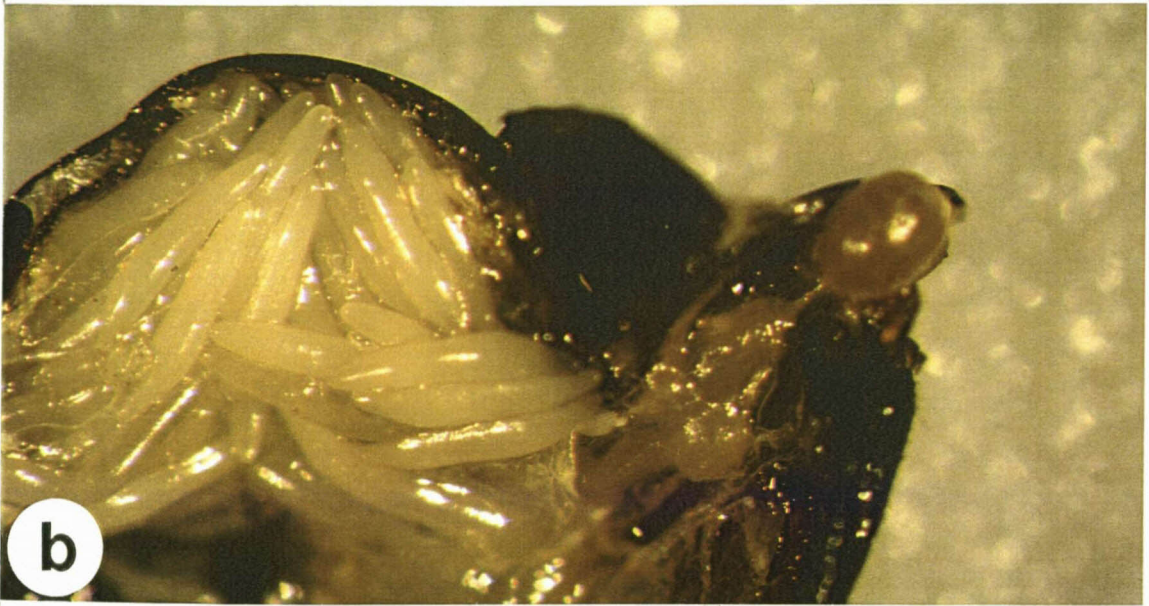
\* Clones as reported by Thomson (1996).



**Table 1** (continued)

Culture nr.	I.D.	Host or source of isolation	Origin	Date isolated	Isolator
<b>South African isolates</b>					
<b>1994/1995</b>					
M1W, M5W, M6W, M7W	<i>Amylostereum</i> sp.	<i>P. radiata</i> wood after attack by <i>S. noctilio</i>	South Africa	1994	M.J. Wingfield
B3S, B5S	<i>Amylostereum</i> sp.	Mycangia of <i>S. noctilio</i> wasps	South Africa	1995	B. Slippers
<b>1995/1996</b>					
56 Isolates numbered SN1 – SN 49 (A/B)	<i>Amylostereum</i> sp.	Mycangia of <i>S. noctilio</i> wasps	South Africa	1996	B. Slippers
<b>1996/1997</b>					
45 Isolates numbered B1, B2 and K1.1 – K77	<i>Amylostereum</i> sp.	Mycangia of <i>S. noctilio</i> wasps	South Africa	1997	B. Slippers
<b>1997/1998</b>					
C1	<i>Amylostereum</i> sp.	Mycangium of <i>S. noctilio</i> wasp	South Africa	1998	B. Slippers

**Figure 1:** Isolations of *A. areolatum* were made from female *S. noctilio* woodwasps (a). In female woodwasp the abdomen was removed to expose the mycangium (b), that contain the arthrospores of *A. areolatum*, at the base of the ovipositor. Isolations of *A. areolatum* were also made from wood of attacked pine trees around tunnels (c) of *S. noctilio* larva.



**Figure 2:** Top (a) and bottom (b) view of South African isolates (A1, A2, B1 and B2 – isolates M7W, B5S, SN31, K34.2, and C1) of *A. areolatum* paired in various combinations, as well as a positive (B3 - isolate K34.2) and negative control (A3 - isolates L279 and K7.2).



1

2

3

A

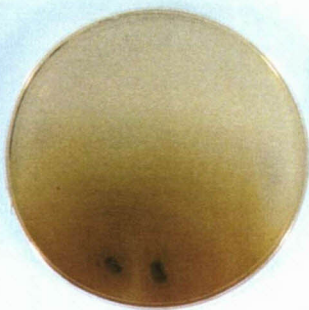


B

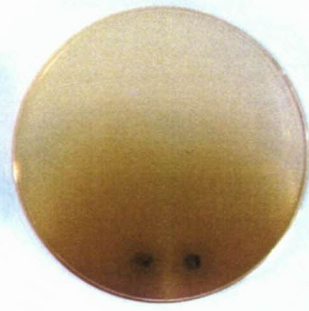
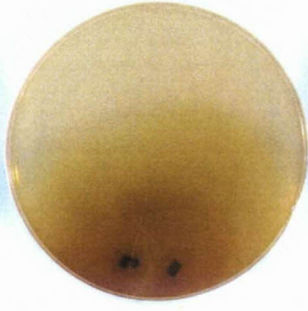


a

A



B



b

**Figure 3:** Reactions between heterokaryotic isolates from the Southern Hemisphere, as viewed from the top (**a**) and the bottom (**b**), when paired on Petri dishes containing PMYA. Lines and columns one and two represent field isolates from Tasmania (WaiteInst.6195) and New Zealand (DAOM21785) respectively, while numbers four and five represent field isolates from South Africa (B5S) and Brazil (Br.7), respectively. Line 3 and column 3 represents isolates of *A. areolatum* collected from cultures of the biocontrol nematode, *D. siricidicola*, that were imported into South Africa from Australia.



1

2

3

4

5

1

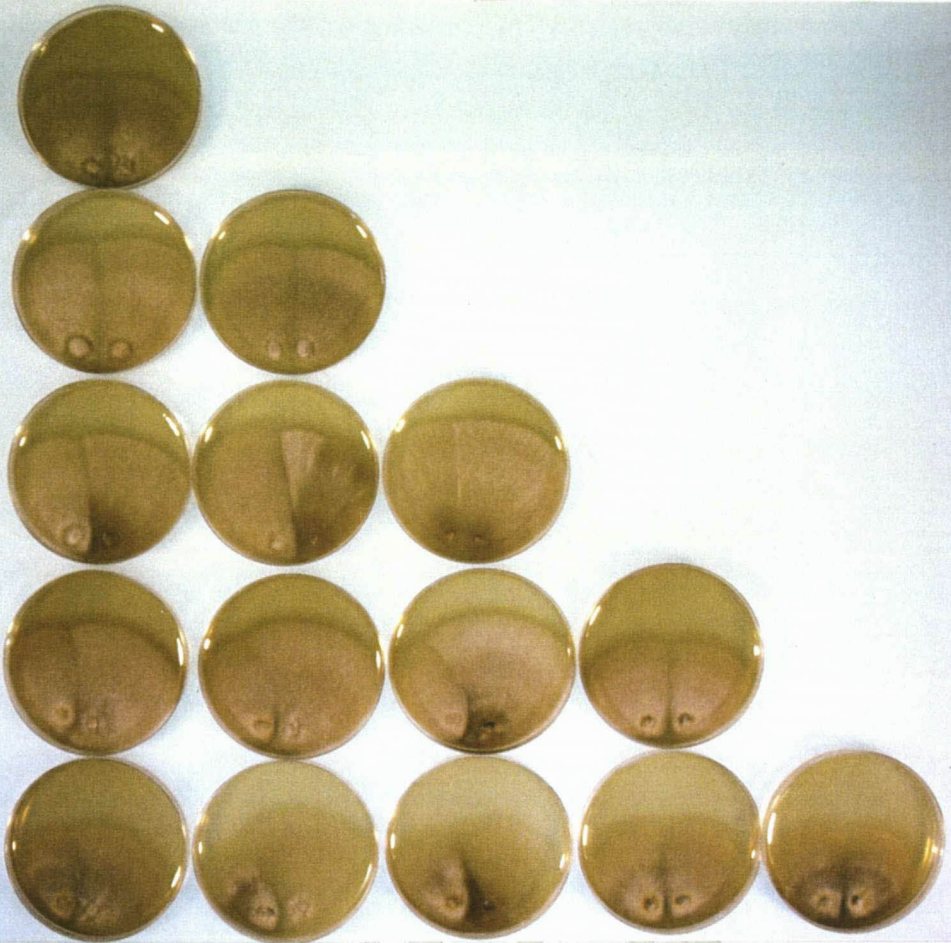
2

3

4

5

a



1

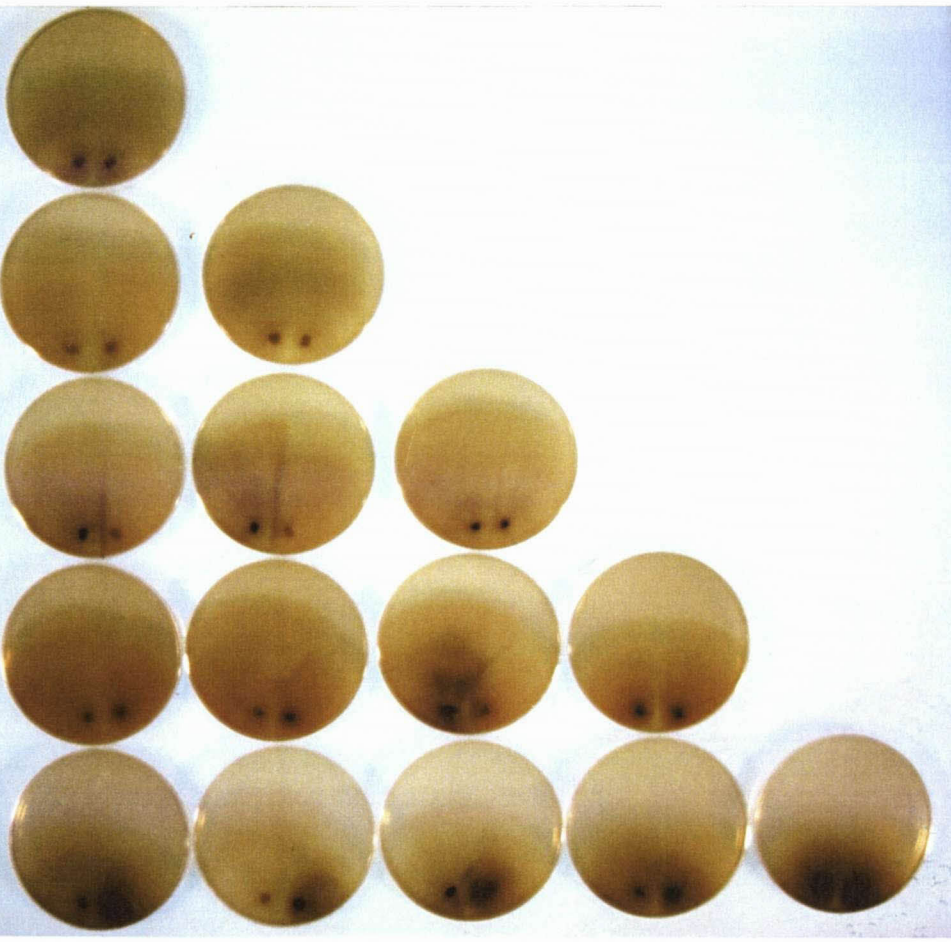
2

3

4

5

b



**Figure 4:** Vegetative incompatibility reactions between isolates of *A. areolatum* representing the South African VCG (B5S and K7.2) and isolates from Tasmania (A1 – isolate Waite Inst.6195), New Zealand (A2 – isolate DAOM 21785), Australia (A3 – isolate A9), Brazil (B1 – isolate Br.7) and Europe (B2 – isolate CBS305.82), as viewed from above (a) and below (b). Pairing B3 represents a positive control self pairing (K7.2).

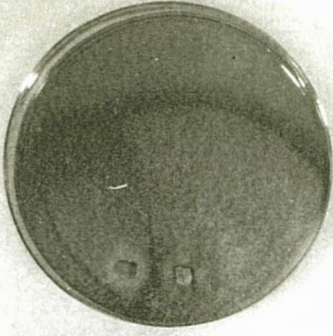


1

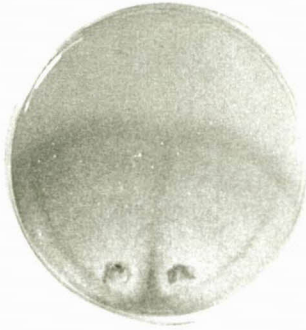
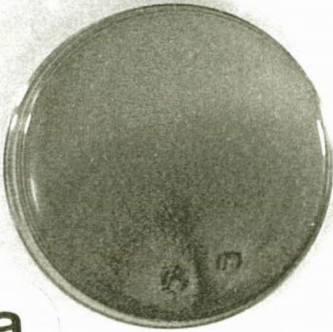
2

3

A

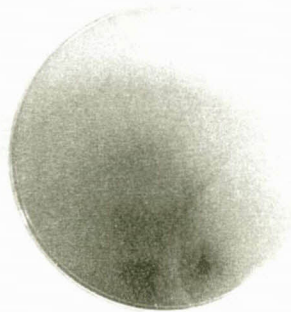


B

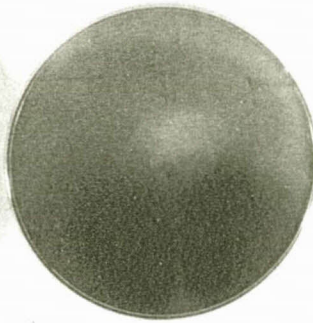


a

A



B



b







# PHYLOGENY AND TAXONOMY OF THE GENUS *AMYLOSTEREUM* INFERRED FROM MITOCHONDRIAL RIBOSOMAL DNA SEQUENCE

## ABSTRACT

The genus *Amylostereum* currently includes four species, namely *A. areolatum*, *A. chailletii*, *A. laevigatum* and *A. ferreum*. Two of these species, *A. areolatum* and *A. chailletii*, are well known for their association with siricid woodwasps. Despite much interest in these fungus-woodwasp symbioses, the taxonomy and phylogeny of this genus received little attention in the past. The aim of this study was to investigate the phylogenetic relationship between the four species of *Amylostereum*. The placement of *Amylostereum* spp. among the Basidiomycetes was also investigated based on mt-SSU-rDNA sequence analyses. These data also clarify the taxonomic status of previously unidentified isolates. In this study, we have shown that *A. areolatum* is more distantly related to the three other species of *Amylostereum*, than they are to each other. Of the remaining three species, *A. ferreum* and *A. laevigatum* are more closely related to each other. One isolate that was collected from *Sirex areolatus*, and, therefore, expected to be *A. chailletii*, was more closely related to *A. laevigatum* and *A. ferreum*. As neither of the latter species have been implicated in associations with woodwasps, this finding warrants further investigation. Our data show that *Amylostereum* spp. groups with neither *Stereum* nor *Peniophora*, as has been previously hypothesised, but rather with *Echinodontium tinctorium*. From this and other studies there was also an obvious relationship between *Amylostereum*/*Echinodontium* and *Russula*.

## INTRODUCTION

Members of the genus *Amylostereum* are best known for their mutualistic association with Siricidae, a family of woodwasps with a woodboring larval state (Talbot, 1977). These woodwasps and their associated fungi have the potential to cause serious damage and mortality to various species of conifers such as *Pinus*, *Abies*, *Picea*, *Pseudotsuga* (Spradbery & Kirk, 1978 & 1981). In the Northern Hemisphere where woodwasps originate, a natural balance exists between them, their natural parasites and their host trees, such that they are generally considered as secondary invaders (Hall, 1978; Spradbery & Kirk, 1978).

The *Sirex noctilio*-*Amylostereum areolatum* complex has been introduced into a number of countries in the Southern Hemisphere where it causes severe damage to exotic pine plantations (Neumann & Marks, 1990; Chou, 1991; Bedding, 1995). In these regions this pest complex is considered a primary problem. A combination of the environmental stresses on pine trees, the genetic uniformity of these plantations and the absence of natural enemies of *Sirex* have all contributed to the increase in pathogenicity of this wasp-fungus association in the Southern Hemisphere (Spradbery, 1973; Spradbery & Kirk, 1978).

Boidin (1958) first described the genus *Amylostereum* as distinct from species of *Stereum* and *Peniophora*. General morphological characters include smooth amyloid basidiospores, brown encrusted cystidia and regular simple clamps. *Amylostereum chailletii* (Pers.:Fr.) Boid., the type species, and *A. areolatum* (Fr.) Boid. are the only two species of *Amylostereum* implicated in associations with woodwasps (Gaut, 1970; Boidin & Lanquetin, 1984). Both species were initially included in the genus *Stereum* as *S. chailletii* (Pers.:Fr. as *Thelephora*) Fr. and *S. areolatum* (Fr.:Fr. as *Thelephora*) Fr. respectively (Boidin, 1958). *Amylostereum chailletii* and *A. areolatum* are morphologically very similar, but can be distinguished in culture based on the fact that only *A. areolatum* forms arthrospores in culture (Thomson, 1998).

The third species described by Boidin (1958) in the genus *Amylostereum*, *A. laevigatum* (Fr.) Boid., was known as *Peniophora laevigata* (Fr. as *Thelephora*) Karst. and later as *S.*

*juniperi* (Karst.) Boid. *Amylostereum laevigatum* is also found in softwood trees, predominantly species of *Juniperus*. This species differs from *A. chailletii* and *A. areolatum* in the absence of horizontal hyphae in the fruiting structures, as well as in the fact that it has a monomytic hyphal system (Breitenbach & Kränzlin, 1986).

Boidin and Lanquetin (1984) described *A. ferreum* (Berk. & Curt.) Boid. & Lanq. (= *Stereum ferreum*) as a fourth species in the genus *Amylostereum*. A major difference between *A. ferreum* and the three other species of *Amylostereum* is its occurrence exclusively in *Podocarpus* species. Unlike the other three species that are known from the Northern Hemisphere, *A. ferreum*, has been found only in South America (Boidin & Lanquetin, 1984).

Boidin and Lanquetin (1984) evaluated the genus *Amylostereum* based on mating studies and the Buller phenomenon (Buller 1931). They concluded that *A. chailletii*, *A. laevigatum* and *A. ferreum* are more closely related to each other than they are to *A. areolatum*. No positive mating reactions were observed between *A. areolatum* and the other three species. No compatible mating was observed between *A. chailletii* and *A. laevigatum*, but *A. ferreum* gave partially compatible crosses with both these species. Boidin and Lanquetin (1984) also hypothesised that, based on morphological evidence, the genus *Amylostereum* could be more closely related to *Peniophora* than to *Stereum*.

Morphological studies of the Basidiomycetes are complicated by the limited number of available characters, as well as the influence of convergent and parallel evolution (Hibbett *et al.*, 1997). For example, in a study of 89 Basidiomycete species, using sequence data from the nuclear and mitochondrial small subunit rRNA operon, Hibbett *et al.* (1997) showed that a major character such as gills might have evolved six times. Similarly various researchers have used the combined features of conserved and less conserved regions in the rRNA genes to resolve problematic phylogenetic and taxonomic questions in the Basidiomycetes, often in conjunction with morphological data (Hibbett & Vilgalys, 1991 & 1993; Hibbett, 1992; Swann & Taylor, 1993 & 1995; Zambino & Szabo, 1993; Hibbett & Donoghue, 1995; Hsiau, 1996).

The aim of this study was to test the hypotheses of Boidin and Lanquetin (1984) as well as other researchers regarding the phylogenetic relationships between the different species of *Amylostereum*, based on part of the mitochondrial ribosomal gene complex. In addition, relationships between species of *Amylostereum* and other Basidiomycetes are also considered. The taxonomic status of isolates of unknown or uncertain identity is also investigated using these data.

## MATERIALS & METHODS

### Isolates used in the study

Isolates used in this study were obtained from a variety of sources (Table 1). These include those made from *S. noctilio* collected in South Africa and Brazil, those from cultures of the parasitic nematode *Deladenus siricidicola*; isolates from Europe supplied by Dr. I.M. Thomson (Danish Forest and Landscape Research Institute, Hoersholm, Denmark), Dr. R. Vasiliauskas (Swedish University of Agricultural Sciences, Uppsala, Sweden), those from culture collections CBS (Centraal Bureau voor Schimmelcultures, Baarn, Netherlands) and DAOM (Centre for Land and Biological Resources Research, Canada). Isolates were maintained on MYA (2 % Malt Extract, 0.2 % Yeast Extract and 1.5 % Agar) at 25 °C and stored in McCartney bottles containing MYA at 4 °C.

### DNA isolation

Mycelium from actively growing cultures on MYA was used to inoculate liquid MY (2% Malt Extract and 0.2% Yeast Extract) medium (100 ml in 250 ml Erlenmeyer flasks). These were incubated at 25 °C on a shaker for approximately two weeks. A modification of the method of Raeder and Broda (1985) was used for isolating DNA from mycelium. Unlike the Raeder and Broda (1985) method, each sample was divided into two equal amounts for the whole extraction procedure, after cell debris had been removed. Furthermore, the phenol chloroform extraction (1:1 phenol to chloroform) step was repeated several times until the interphase between the aqueous and upper phases was clean from contaminating proteins and cell debris. Precipitation of the nucleic acids was done

using 3M NaAc (0,1 v/v) and isopropanol (0,6 v/v) and was incubated overnight at  $-20^{\circ}\text{C}$ . After centrifugation, to harvest the nucleic acids, and washing with 70 % EtOH, the pellet was resuspended in 200  $\mu\text{l}$  sterile SABAX water. The two samples of each isolate were then combined. One  $\mu\text{l}$  RNaseA (10mg/ml) was added to the resuspended sample and left at  $37^{\circ}\text{C}$  overnight to degrade all RNA in the sample. DNA concentrations were subsequently determined using an UV spectrophotometer (Beckman Du Series 7500) (Maniatis *et al.*, 1982).

### PCR amplification and purification

A portion of the mitochondrial small sub-unit ribosomal RNA gene (mt-SSU-rDNA) was amplified with the primers MS1 and MS2 (White *et al.*, 1990) using the Polymerase Chain Reaction (PCR). PCR was performed using the Expand™ High Fidelity Polymerase System. Total volumes of the reaction mixtures varied from 50  $\mu\text{l}$ , 75  $\mu\text{l}$  and 100  $\mu\text{l}$ . The reaction mixture consisted of a final concentration of 2,65 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  of each of the four dNTP's, Expand High Fidelity buffer with MgCl<sub>2</sub>, 0,375  $\mu\text{M}$  of each of the two primers and 2,6 U Expand™ High Fidelity Taq polymerase mixture (Boehringer Mannheim, South Africa). Extracted genomic DNA (50 ng to 80 ng) was used as template for the amplification reactions.

PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid limited, U.K.). Reaction conditions included an initial denaturation step of 3 minutes at  $94^{\circ}\text{C}$  followed by 10 cycles of denaturation at  $94^{\circ}\text{C}$  for 15 seconds, primer annealing at  $55^{\circ}\text{C}$  for 45 seconds and elongation at  $72^{\circ}\text{C}$  for 1 minute. This was followed by 20 cycles using the same reaction conditions, but with an increase of 20 seconds elongation time per cycle. A final elongation step at  $72^{\circ}\text{C}$  for 7 minutes ensured complete elongation of the amplification product. PCR products were subjected to electrophoresis on an 1 % (wt/v) ethidium bromide stained agarose (Molecular biology grade, Techcomp Ltd., Hong Kong) gel and visualised under UV illumination. Size estimates of the PCR fragments were done using a 100 bp ladder (Promega, Madison, Wisconsin, U.S.A.) as a molecular weight marker.

## DNA sequencing and sequence data analysis

DNA sequencing of the amplified mt-SSU-rDNA was performed on an ABI PRISM™ 377 automated DNA sequencer. PCR products were purified prior to sequencing, using a Nucleon™QC PCR/OLIGO clean up kit (Amersham Life Science Inc.). Thermo Sequenase™ dye terminator cycle sequencing pre-mix kit (Amersham Life Science Inc.) with Thermo Sequenase™ DNA polymerase was used for all sequencing reactions. The primers MS1 and MS2 were used to sequence both DNA strands.

To determine the phylogenetic relationships amongst *Amylostereum* species, mt-SSU-rDNA sequences of all isolates (Table 1) were manually aligned by inserting gaps. All characters were given equal weight and gaps were coded as newstate (fifth character). Analysis of the data was done using PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1 (Swofford, 1993). Heuristic searches using TBR (Tree Bisection Reconstruction) branch swapping and MULPAR on, were done to determine the most parsimonious relationships between the taxa. Strict and semi-strict consensus trees were obtained in PAUP for all equally parsimonious trees saved. Trees were not rooted to an outgroup taxon, but rather by midpoint rooting. Branch supports and confidence intervals were determined using BOOTSTRAP analysis (1000 replicates) (Felsenstein, 1993).

In order to consider the relationship of *Amylostereum* spp. with other Basidiomycetes, sequence data of the mt-ssu-rDNA for 89 species of Basidiomycetes (Hibbett and Donoghue, 1995; Hibbett *et al.*, 1997) were obtained from Genbank and TREEBASE. Sequence data of *A. chailletii* was initially compared to all 89 species using PAUP to resolve a clade of maximum relationship. Sequence data from the most closely related taxa determined using this analysis, were then compared to DNA sequence data of all four described species of *Amylostereum*. Sequence analysis was done using PAUP, as described above, except that all resulting trees were rooted to an outgroup taxon. Here, *Laxitextum bicolor* (Fr.) Lentz. was chosen as an outgroup because of its basal relationship to the taxa selected as closely related to *Amylostereum* in the analysis of Hibbett *et al.* (1997).



## RESULTS

### PCR amplification

The region of the mt-SSU-rRNA gene targeted with the MS1 and MS2 primers was highly conserved in all the species of *Amylostereum*, based on the size of the amplified PCR fragments. Fragments of approximately 570 bp were observed for all but three isolates used in this study (Figure 1). The three exceptions, isolates Stillwell 309(3), CBS 624.84 (*A. laevigatum*) and CBS 633.84 (*A. ferreum*), produced PCR amplification fragments of approximately 590 bp in length (Figure 1).

### Sequence data

Manual alignment of sequences representing the amplified region of the mt-SSU-rDNA of the different species of *Amylostereum* resulted in the total alignment of 538 characters (Figure 2). Absolute lengths of the sequences ranged from 518 bp to 537 bp. Sequences of the above-mentioned region were highly conserved for all the species of *Amylostereum*. One variable region was observed between 190 and 226 bp (aligned length) of the fragment.

Heuristic searches using PAUP of these sequences resulted in 18 equally parsimonious trees (CI = 0.968; HI = 0.032; RI = 0.986) of 31 steps each (Figure 3). The topology of these trees was similar and differences were due to variations in branch length and the arrangement among isolates CBS624.84 (*A. laevigatum*), CBS633.84 (*A. ferreum*) and Stillwell 309(3) (isolated from *S. areolatus*).

The main feature of the trees obtained from heuristic searches of sequence data of the different *Amylostereum* spp., was the appearance of two major clades supported by a 100 % confidence interval at the branching point. The one clade contained representative isolates of *A. areolatum*. Within the *A. areolatum* clade only one branch was retained in consensus trees that was weakly supported by bootstrap analysis (65 %). The second main clade was comprised of representative isolates of *A. chailletii*, *A. laevigatum*, *A. ferreum*

and isolate Stillwell 309(3). *A. chailletii* grouped on a separate branch (93 % confidence interval) within this second clade from *A. laevigatum*, *A. ferreum* and isolate Stillwell 309(3). *A. ferreum*, Stillwell 309(3) and *A. laevigatum* were grouped together and basal to *A. chailletii* in strict and semi-strict consensus trees, as well as by bootstrap analysis. Therefore, a revised form of the evolutionary tree of decent reported by Boidin and Lanquetin (1984) (Figure 4(a)), is proposed (Figure 4(b)).

Manual alignment of sequence data of 16 selected species from the data set of Hibbett *et al.* (1997) and the four species of *Amylostereum* (Figure 5) resulted in a total aligned data set of 771 characters. Absolute values varied from 513 bp for *Russula compacta* Frost to 674 bp for *Peniophora muda* (Fr.) Bres. Sequences could be divided into four relatively conserved regions, interspersed with three hypervariable regions, as was reported by other researchers (Hibbett and Donoghue, 1995; Hsiao, 1996; Hibbett *et al.*, 1997). The three hypervariable regions were located between bases 55 and 128, bases 266 and 400 and bases 623 and 671 (based on aligned values).

Alignment in these hypervariable regions was difficult and often impossible. This resulted in a large amount of ambiguity in their alignment. Analysis of the data was thus performed with and without these hypervariable regions. In the latter case, this resulted in the exclusion of 258 bp (aligned values). The general topology of the trees showed some variation compared to the trees resulting from analysis of the full sequence, but most of the species groupings were not affected.

Heuristic searches of the full sequence data set resulted in three equally parsimonious trees of 1495 steps (CI = 0.601; HI = 0.399; RI = 0.522) (Figure 6). The topology of the trees were the same except for variations in branch lengths and whether *A. laevigatum* and *A. ferreum* were put on separate branches or not. Seven most parsimonious trees of 639 steps (CI = 0.604; HI = 0.396; RI = 0.595) was obtained when analysis were conducted on the DNA sequences with the variable regions excluded (Figure 7). Differences in the seven trees could again be ascribed to variation in branch lengths.

The four species of *Amylostereum* formed a monophyletic clade basal to *Echinodontium tinctorium* Ell. & Ev. The branch separating the *Amylostereum* spp. from *Echinodontium* was supported by a 98 % bootstrap value irrespective of the inclusion or exclusion of the hypervariable regions. The bootstrap branch support for the *Echinodontium* – *Amylostereum* grouping was 70 % when the hypervariable regions were included and 94 % when they were excluded. *Heterobasidion annosum* (Fr.) Bref. and *R. compacta* grouped together and neighbouring the group that contained *Echinodontium* and *Amylostereum* spp. *Lentinellus omphalodes* (Fr.) Kar. and *L. ursinus* (Fr.) Küh. , *Auriscalpium vulgare* S. F. Gray, *Clavicornia pyxidata* (Fr.) Doty and *Hericium ramosum* (Bull. ex Mér) Let. were also grouped close to *Echinodontium*, *Amylostereum*, *Heterobasidion* and *Russula* in analysis of the data set without exclusion of the hypervariable regions. In analyses ignoring the sequence of the hypervariable regions, *Hericium* and *Clavicornia* were removed from this group. *Heterobasidion* and *Russula* also grouped closer to *Lentinellus* and *Auriscalpium* spp. than to the *Echinodontium* – *Amylostereum* group in this analysis. Neither *Stereum* nor *Peniophora* spp. grouped in the above-mentioned groups, in any of the analyses. Instead, *Stereum* spp. were grouped with *Gloeocystidiellum leucoxantha* (Bres.) Boid. and *P. nuda* with *Scitinostroma alutum* Lanq. in all trees.

## DISCUSSION

In this study the phylogenetic relationships of the four species of *Amylostereum* could be resolved using sequence data of the mt-ssu-rDNA. Isolates representing *A. areolatum* clustered on a well supported branch, separate from all the other species in the genus. This confirms the hypothesis of Boidin and Lanquetin (1984) that *A. areolatum* is the most clearly defined species in the genus. In their study, no mating compatibility was observed between isolates of *A. areolatum* and any of the other *Amylostereum* species in this group, whereas partial compatibility was observed between some of the other species of *Amylostereum*.

Boidin and Lanquetin (1984) could not clearly define the relationship between *A. chailletii*, *A. laevigatum* and *A. ferreum*. In their study European isolates of *A. chailletii* and *A. laevigatum* showed no mating compatibility, but both these species showed partial mating

compatibility with *A. ferreum*. Our analysis showed that *A. chailletii*, *A. laevigatum* and *A. ferreum* formed a cluster together and separate from *A. areolatum*, which is in agreement with their mating studies. Isolates of *A. chailletii* formed a separate group within the latter clade, while *A. laevigatum* and *A. ferreum* could not be separated in strict analyses of the data. These results suggest a closer relationship between *A. ferreum* and *A. laevigatum* than between either of these species and *A. chailletii*.

In this study, it was possible to confirm the identity of isolates of *Amylostereum* that could previously not be assigned species names. The two CLBBR cultures identified only as *Amylostereum* sp. (Waite Inst 6195 and DAOM 21785) from Tasmania and New Zealand, clearly resided in the clade containing identified isolates of *A. areolatum* (CBS 305.82, CBS 334.66 and isolates from Europe that were identified by Drs. Thomsen and Vasiliauskas). Also represented in this group are isolates from South Africa, Brazil and isolates obtained from nematode (*Deladenus siricidicola*) cultures imported to South Africa from Australia. Furthermore, the two Canadian isolates of *A. chailletii* (DAOM 21327 and 54-95) clearly clustered with other identified isolates of *A. chailletii* (L234, Sc62.8 and CBS 483.83). Boidin and Lanquiten (1984) found partial mating compatibility between two Canadian *Amylostereum* isolates and authentic isolates of *A. chailletii*, *A. laevigatum* and *A. ferreum*. According to our data this mating behaviour, therefore, only supports the close relationship between the three last named species.

Isolate Stillwell 309(3) is reported to have been isolated by Dr. Stillwell from the mycangium of *S. areolatus*. We would, therefore, expect it to be *A. chailletii* as was suggested by Gaut (1970). This isolate was deposited in DAOM as an *Amylostereum* sp. Results of this study show that the isolate is most closely related to *A. laevigatum* and *A. ferreum*. Neither of these species have previously been implicated in associations with woodwasps. If this isolate is, therefore, an actual sub-culture of the isolate collected from *S. areolatus*, it might represent a link between the species associated with woodwasps (*A. areolatum* and *A. chailletii*) and the other two species (*A. laevigatum* and *A. ferreum*). It might also represent an undescribed species of *Amylostereum*. Further study of this isolate is clearly warranted.

Various hypotheses have been proposed for the placement of *Amylostereum* amongst the Basidiomycetes (Boidin & Lanquetin, 1984; Parmasto, 1995; Hsiau, 1996), but in general *Amylostereum* is considered most closely related to *Stereum* and *Peniophora* (in which *Amylostereum* spp. were originally described). Boidin and Lanquetin (1984) speculated that *Amylostereum* might be more closely related to *Peniophora* based on the presence of gloeocystidia positive in sulfuric-aldehyde, normal nuclear behaviour and the tetrapolarity in all four species. Parmasto (1995) in a cladistic study using 86 morphological characters to test the phylogeny amongst the genera of the Corticoid fungi reduced the Stereaceae to synonymy with the Peniophoraceae. In his analysis, *A. chailletii* groups sister to *Stereum* and *Xylobolus* (P. Karst.) and the former three genera form a group basal to the group that contains the genus *Peniophora*. In a study by Hsiau (1996) using mt-ssu-rDNA, *A. chailletii* grouped sister to *Stereum* and further away from *Peniophora*. In the present study the four *Amylostereum* spp. formed a monophyletic group that was sister to neither *Stereum* nor *Peniophora*, but rather to *E. tinctorium*. This observation was supported by strong bootstrap values for this grouping in all analyses (70 % when the hypervariable regions were included in the analysis and 94 % when they were excluded). It is interesting to note that *E. tinctorium* is also characterised by amyloid basidiospores and encrusted cystidia, such as is formed by *Amylostereum* spp.

*Echinodontium tinctorium* has been described as closely related to *Stereum* (Gross, 1964; Stalpers, 1978). Hibbett *et al.* (1997), however, found that *E. tinctorium* is more closely related to *Peniophora nuda* than to any of the *Stereum* spp. included in their analysis. In the present study, the *Amylostereum-Echinodontium* group was, however, more closely related to *Russula*, *Heterobasidion*, *Lentinellus* and *Aurisclapium* in all analyses, than to either *Stereum* or *Peniophora*.

Topologies of the trees derived in this study differed from those reported by Hibbett *et al.* (1997), from which some of the sequences were obtained. The major differences in tree topologies are in the arrangements of the species groupings, while the species contained in each group were less affected. Some of these differences are in accordance with findings in other studies. For example, the *Heterobasidion-Russula* grouping indicated by this study, that was closely linked to the group containing *Amylostereum*, is consistent with the data of

Hsiau (1996), while Hibbett *et al.* (1997) group *Echinodontium* and *Russula* together. Despite these differences, it is obvious from this study that there is a closer relationship between *Amylostereum* and *Echinodontium* than was previously recognised. There is also an obvious relationship between *Amylostereum/Echinodontium* and *Russula*

## REFERENCES

- Bedding, R. A. (1995). Biological control of *Sirex noctilio* using the nematode *Deladenus siricidicola*. In *Nematodes and biological control of insect pests* (ed. R. A. Bedding, R. J. Akhurst, H. Kaya), pp. 11-20. CSIRO: Melbourne, Australia.
- Boidin, J. (1958). Heterobasidiomycetes saprophytes et Homobasidiomycetes resupines: V.- Essai sur le genre *Stereum* Pers. ex S. F. Gray. *Revue de Mycologie* **23**, 318-346.
- Boidin, J. & Lanquentin (1984). Le genre *Amylostereum* (Basidiomycetes) intercompatibilités partielles entre espèces allopatriques. *Bulletin de la Société Mycologique de France* **100**, 211-236.
- Breitenbach, J. & Kränzlin, F. (1986). *Fungi of Switzerland Volume 2 (Non-gilled fungi)*. Mengis & Sticher A.G.: Lucerne.
- Buller, A. H. R. (1931). *Researches on Fungi. Volume IV*. Longmans, Green and Co.: London, U.K.
- Chou, C. K. S. (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 Inferenec Package), Version 3.5. University of Washington.
- Gross, H. L. (1964). The Echinodontiaceae. *Mycopathologia et Mycologia Applicata* **24**, 1-26.
- Gaut, I. P. C. (1970). Studies of siricids and their fungal symbionts. Ph.D. thesis. University of Adelaide, Australia.
- Hall, M. J. (1978). A survey of siricid attack on radiata pine in Europe. *Australian Forestry* **32**, 155-162.
- Hibbett, D. S. (1992). Towards a phylogenetic classification for *Shiitake*: Taxonomic history and molecular perspectives. *Reports of the Mycological Institute* **30**, 30-42.
- Hibbett, D. S. & Donoghue, M. J. (1995). Progress toward a phylogenetic classification of the Polyporaceae through parsimony analysis of mitochondrial ribosomal DNA sequence. *Canadian Journal of Botany* (Suppl. 1), 853-861.

- Hibbett, D. S., Pine, E. M., Langer, E., Langer, G. & Donoghue, M. J. (1997). Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *Proceedings of the National Academy of Sciences USA* **94**, 12002-12006.
- Hibbett, D. S. & Vilgalys, R. (1991). Evolutionary relationships of *Lentinus* to the Polyporaceae: Evidence from the restriction analysis of enzymatically amplified ribosomal DNA. *Mycologia* **83**, 425-439.
- Hibbett, D. S. & Vilgalys, R. (1993). Phylogenetic relationships of *Lentinus* (Basidiomycotina) inferred from molecular and morphological characters. *Systematic Botany* **18**, 409-433.
- Hsiau, P. T-W. (1996). The taxonomy and phylogeny of the mycangial fungi from *Dendroctonus brevicomis* and *D. frontalis* (Coleoptera : Scolytidae). D.phil thesis. Iowa State University. Ames, Iowa.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbor: New York.
- Neumann, F. G. & Marks, G. C. (1990). Status and management of insect pests and diseases in Victorian softwood plantations. *Australian Forestry* **53**, 131-144.
- Parmasto, E. (1995). Corticoid fungi: a cladistic study of a paraphyletic group. *Canadian Journal of Botany* (Suppl. 1), 843-852.
- Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Applied Microbiology* **1**, 17-20.
- Spradbery, J. P. (1973). A comparative study of the phytotoxic effects of woodwasps on conifers. *Annals of Applied Biology* **75**, 309-320.
- Spradbery, J. P. & Kirk, A. A. (1978). Aspects of the ecology of siricid woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with special reference to the biological control of *Sirex noctilio* F. in Australia. *Bulletin of Entomological Research* **68**, 341-359.
- Spradbery, J. P. & Kirk, A. A. (1981). Experimental studies on the responses of European siricid woodwasps to the host trees. *Annals of Applied Biology* **98**, 179-185.
- Stalpers, J. A. (1978). Identification of wood-inhabiting Aphylophorales in pure culture. *Studies in Mycology* **16**, 1-248.
- Swann, E. C. & Taylor, J. W. (1993). Higher taxa of Basidiomycetes: an 18s rRNA gene perspective. *Mycologia* **85**, 923-936.

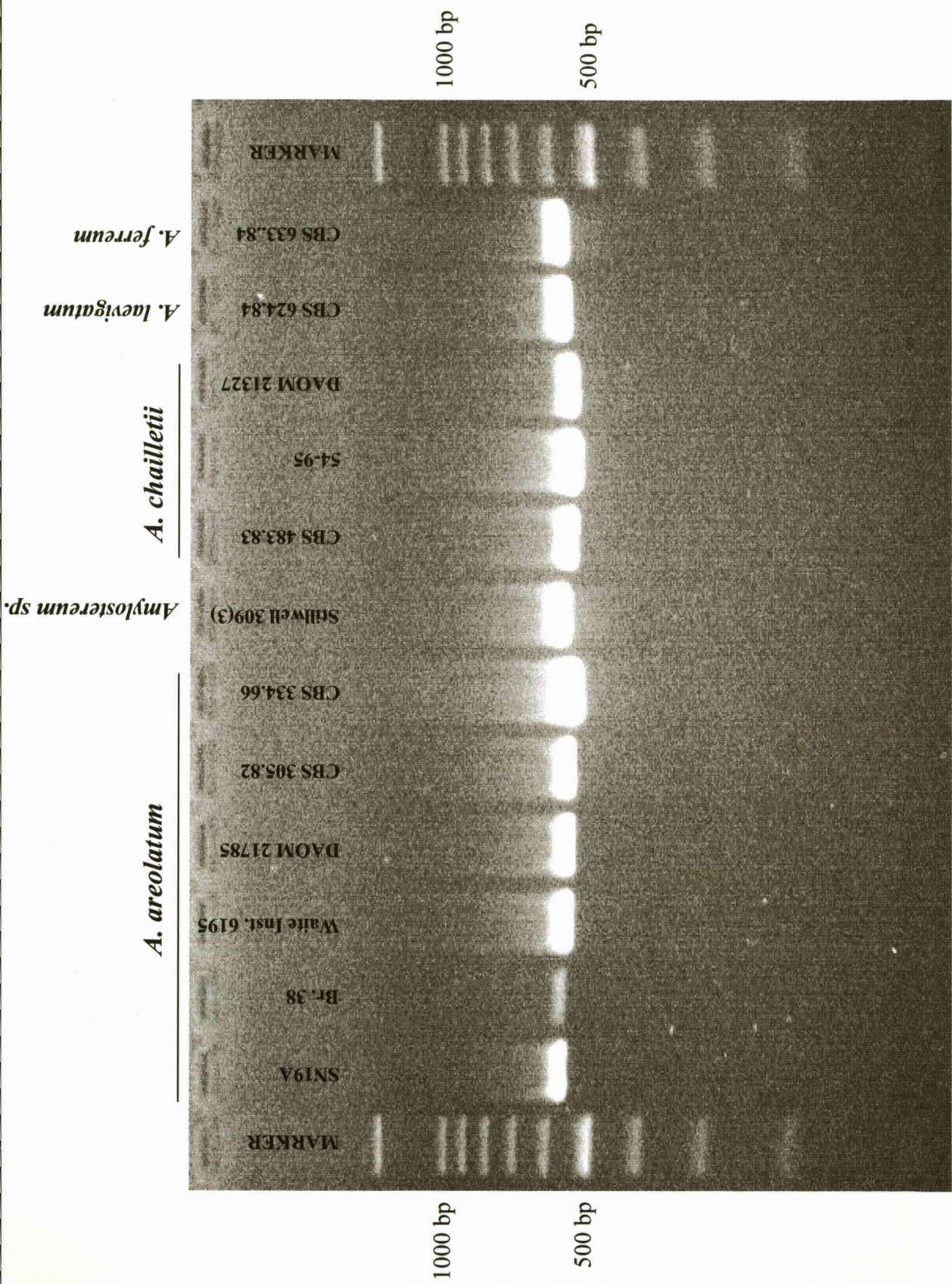


- Swann, E. C. & Taylor, J. W. (1995). Phylogenetic perspectives on basidiomycete systematics: evidence from the 18S rRNA gene. *Canadian Journal of Botany* (Suppl. 1), 862-868.
- Swofford, D. L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by Illinois Natural History Survey, Champaign, Illinois.
- Talbot, P. H. B. (1977). The *Sirex-Amylostereum-Pinus* association. *Annual Review of Phytopathology* 15, 41-54.
- Thomsen, I.M. (1998) Fruitbody characters and cultural characteristics useful for recognizing *Amylostereum areolatum* and *A. chailletii*. *Mycotaxon* 69, 419-428.
- White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A Guide to Methods and Applications* (ed. M. A. Innis, D. H. Gelfand, J.J. Sninsky and T. J. White) pp. 315-322. Academic Press: San Diego, U.S.A.
- Zambino, P. J. & Szabo, L. J. (1993). Phylogenetic relationships of selected cereal and grass rusts based on rDNA sequence analysis. *Mycologia* 85, 401-414.

**Table 1:** Isolates of different species of *Amylostereum* used in this study.

Culture Number	Identity.	Host or source of isolation	Origin	Date isolated	Isolator
<b>CBS cultures</b>					
305.82	<i>Amylostereum areolatum</i>	Unknown	France	1964	J. Boiden
334.66	<i>A. areolatum</i>	From <i>Picea abies</i>	Germany	1967	Dimitri
483.83	<i>A. chailletii</i>	Mycangium of woodwasp <i>Urocerus gigas</i>	Scotland, UK	1981	D.B. Redfern
624.84	<i>A. laevigatum</i>	<i>Juniperus nana</i>	France	1978	P. Lanquetin
633.84	<i>A. ferreum</i>	<i>Podocarpus lambertii</i>	Brazil	1978	R.T. Guerrero
<b>CLBRR cultures</b>					
DAOM 21327	<i>A. chailletii</i>	Sporophore on <i>Abies balsamea</i>	Ontario, Canada	1948	R.F. Cain
54-95	<i>A. chailletii</i>	Sporophore on fallen log in stand of hemlock conifers	Ontario, Canada	1954	A. Hill & S. Gibson
Stillwell 309(3)	<i>Amylostereum sp.</i>	Mycangium of <i>Sirex areolatus</i>	California, U.S.A.	Unknown	Stillwell
Waite Inst. 6195	<i>Amylostereum sp.</i>	Mycangium of <i>S. noctilio</i>	Tasmania	1962	Unknown
DAOM 21785	<i>Amylostereum sp.</i>	Wood of <i>P. radiata</i> around oviposition bores of <i>S. noctilio</i>	New Zealand	Unknown	G.B. Rawlings
<b>Other European isolates</b>					
Sc 62.8	<i>A. chailletii</i>	Fruiting body on <i>Picea sitchensis</i>	Scotland, U.K.	1981	D.B. Redfern
L234	<i>A. chailletii</i>	Wood of wounded <i>P. abies</i>	Lithuania	1995	R. Vasiliauskas
L204	<i>A. areolatum</i>	Wood of wounded <i>P. abies</i>	Lithuania	1994	R. Vasiliauskas
DK37	<i>A. areolatum</i>	Fruiting body on <i>P. abies</i>	Denmark	1993	I.M. Thomson
S225	<i>A. areolatum</i>	Wood of wounded <i>P. abies</i>	Sweden	1994	R. Vasiliauskas
<b>Australian isolates</b>					
A3	<i>Amylostereum sp.</i>	Isolates from nematode cultures from CSIRO	Australia	1995	B. Slippers
<b>South American isolates</b>					
Br 38	<i>Amylostereum sp.</i>	Mycangia of <i>S. noctilio</i>	Brazil	1997	B. Slippers
<b>South African isolates</b>					
M5W	<i>Amylostereum sp.</i>	Wood around <i>S. noctilio</i> in <i>P. radiata</i>	South Africa	1994	M.J. Wingfield
SN19A	<i>Amylostereum sp.</i>	Mycangia of <i>S. noctilio</i>	South Africa	1996	B. Slippers

**Figure 1:** PCR fragments of the mt-SSU-rDNA of the different species of *Amylostereum*, visualised on a 1.5 % agarose gel stained with ethidium bromide. A 100 bp molecular size marker was run in the outer lanes of the gel.



**Figure 2:** Aligned DNA sequence data of part of the mt-SSU-rDNA of *Amylostereum* spp., obtained using the primers MS1 and MS2. Gaps that were inserted due to alignment are indicated by a dash (-). Isolates SN19A, M5W, Br 38, A3, Waite Inst. 6195, DAOM 21785, CBS 305.82, CBS 334.66, L204, DK37 and S225 represent isolates of *A. areolatum* or expected to be *A. areolatum* (Table 1). Isolates CBS483.83, 54-95, DAOM 21327, L234 and Sc62.4 represent *A. chailletii*, isolate CBS 624.84 *A. laevigatum* and isolate CBS 633.84 *A. ferreum* (Table 1). Isolate Stillwell 309(3) represent an *Amylostereum* sp. (Table 1).

	10	20	30	40	50	60	70	80
DAOM21785	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
CBS305.82	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
SN19A	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
M5W	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
A3	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
CBS334.66	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
Br38	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
L204	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
DK37	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
S225	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
WaiteInst.6195	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATAATGA	TATTACCTTA
CBS483.83	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
54_95	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
DAOM21327	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
L234	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
Sc62.8	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
CBS624.84	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
Stillwell309_3	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA
CBS633.84	AAGAGTGAAC	TAGCTAACTG	AAATCAATCT	ACATATAAAAT	ATTGATTGAT	AACGTAAGGG	AAAATTATGA	TATTACCTTA

	90	100	110	120	130	140	150	160
DAOM21785	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
CBS305.82	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
SN19A	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
M5W	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
A3	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
CBS334.66	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
Br38	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
L204	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
DK37	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
S225	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
WaiteInst.6195	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
CBS483.83	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
54_95	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
DAOM21327	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
L234	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
Sc62.8	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
CBS624.84	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
Stillwell1309_3	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA
CBS633.84	CTATTAGTGT	CGTCCAAATC	TGGTGCCAGA	AGACTCGGTA	AGGCCAGAGA	CGCAAACGTT	AATCGTCTTA	AACAGGCGTA

	170	180	190	200	210	220	230	240
DAOM21785	AAGGGTTTGT	AGGCTGCTTT	AAATTTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
CBS305.82	AAGGGTTTGT	AGGCTGCTTT	AAATTTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
SN19A	AAGGGTTTGT	AGGCTGCTTT	AAATTTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
M5W	AAGGGTTTGT	AGGCTGCTTT	AAATTTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
A3	AAGGGTTTGT	AGGCTGCTTT	AAAATTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
CBS334.66	AAGGGTTTGT	AGGCTGCTTT	AAAATTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
Br38	AAGGGTTTGT	AGGCTGCTTT	AAATTTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
L204	AAGGGTTTGT	AGGCTGCTTT	AAAATTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
DK37	AAGGGTTTGT	AGGCTGCTTT	AAAATTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
S225	AAGGGTTTGT	AGGCTGCTTT	AAAATTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
WaiteInst.6195	AAGGGTTTGT	AGGCTGCTTT	AAATTTTATT	T-----	-----	-GTGAAGATA	GCCCAAC-AG	ATAATTAATT
CBS483.83	AAGGGTTTGT	AGGCAGCTTT	GAATTTTCTC	T-----T	TT-CC----	----AAG-T-	----AATTAG	ATAATTAATC
54_95	AAGGGTTTGT	AGGCAGCTTT	GAATTTTCTC	T-----T	TT-CC----	----AAG-T-	----AATTAG	ATAATTAATC
DAOM21327	AAGGGTTTGT	AGGCAGCTTT	GAATTTTCTC	T-----T	TT-CC----	----AAG-T-	----AATTAG	ATAATTAATC
L234	AAGGGTTTGT	AGGCAGCTTT	GAATTTTCTC	T-----T	TT-CC----	----AAG-T-	----AATTAG	ATAATTAATC
Sc62.8	AAGGGTTTGT	AGGCAGCTTT	GAATTTTCTC	T-----T	TT-CC----	----AAG-T-	----AATTAG	ATAATTAATC
CBS624.84	AAGGGTTTGT	AGGCTGCTTT	GAATATTATC	TTTTAA---T	TTACC-TTAT	GGTAAA-ATA	GCGC-ATTAG	ATAATTAATC
Stillwell1309_3	AAGGGTTTGT	AGGCTGCTTT	TAATTTTATC	TATAACAAA-	TTACCCCTCC	GGTAAACATA	GCACAAT-AG	ATACTTAATA
CBS633.84	AAGGGTTTGT	AGGCTGCTTT	GAATTTTATT	TAATAGAC-T	TTACCCC-AA	GGTAAA-ATA	GCAC-ATTAG	ATACTTAATC

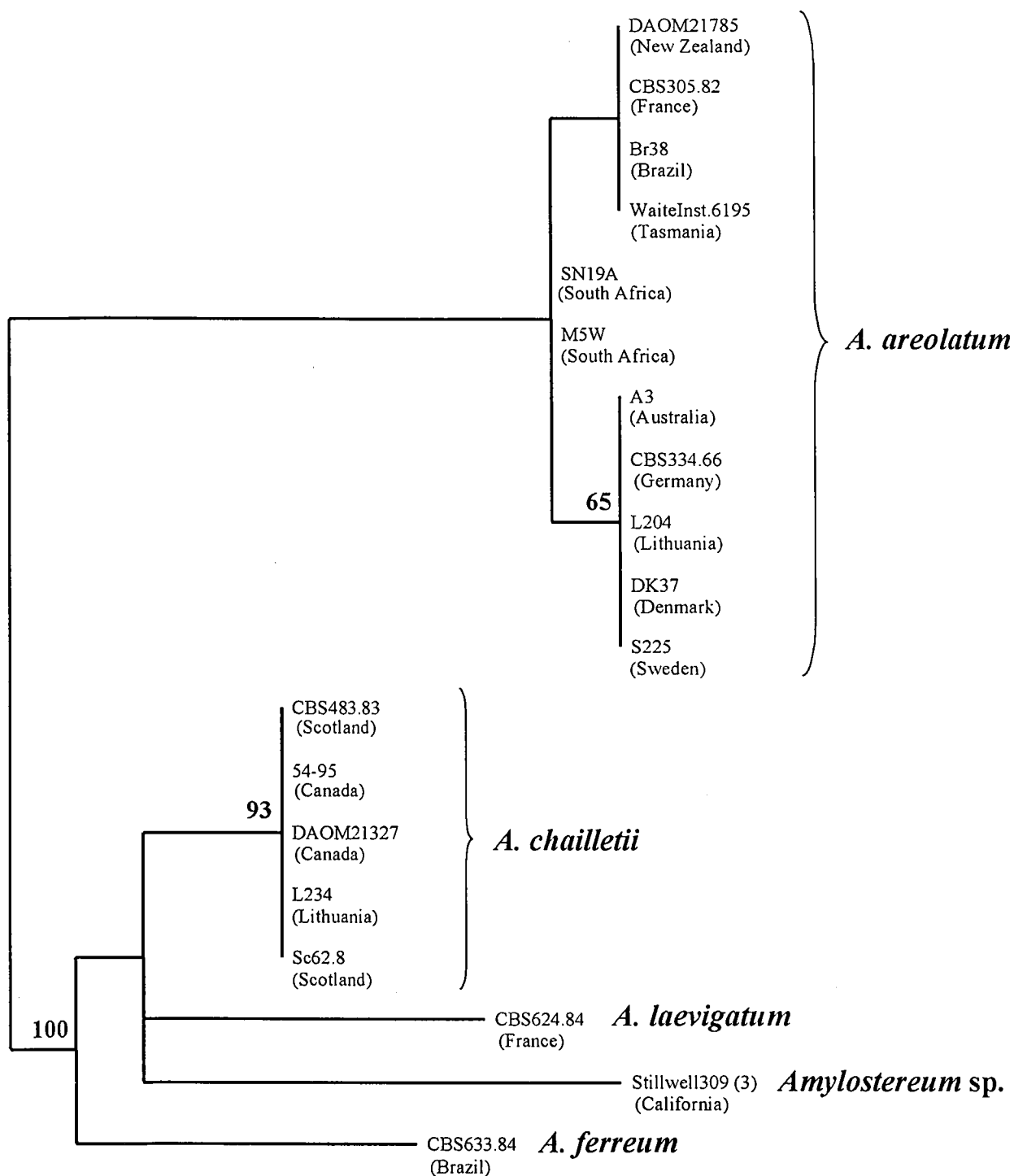


	250	260	270	280	290	300	310	320
DAOM21785	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
CBS305.82	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
SN19A	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
M5W	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
A3	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
CBS334.66	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
Br38	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
L204	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
DK37	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
S225	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
WaiteInst.6195	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CATATTAAGT	GGAATATTAA
CBS483.83	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
54_95	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
DAOM21327	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
L234	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
Sc62.8	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
CBS624.84	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
Stillwell309_3	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA
CBS633.84	AAAGCTAGAA	TCAAATAGAG	GTTATATTAT	ATAATGCTTA	AAGTAGGTCT	AATATCCTAA	CAGATTAAGT	GGAATATTAA

	330	340	350	360	370	380	390	400
DAOM21785	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
CBS305.82	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
SN19A	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
M5W	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
A3	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
CBS334.66	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
Br38	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
L204	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
DK37	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
S225	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
WaiteInst.6195	AAGCGAAGGC	TTATTTTCCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
CBS483.83	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
54_95	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
DAOM21327	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
L234	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
Sc62.8	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
CBS624.84	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
Stillwell309_3	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT
CBS633.84	AAGCGAAGGC	TTATTTACCA	TAAATGATTG	ACGCTGAGAA	ACGAAGGTGA	GGATAGGAAA	TAGGATTAGA	TACCCAAAAT

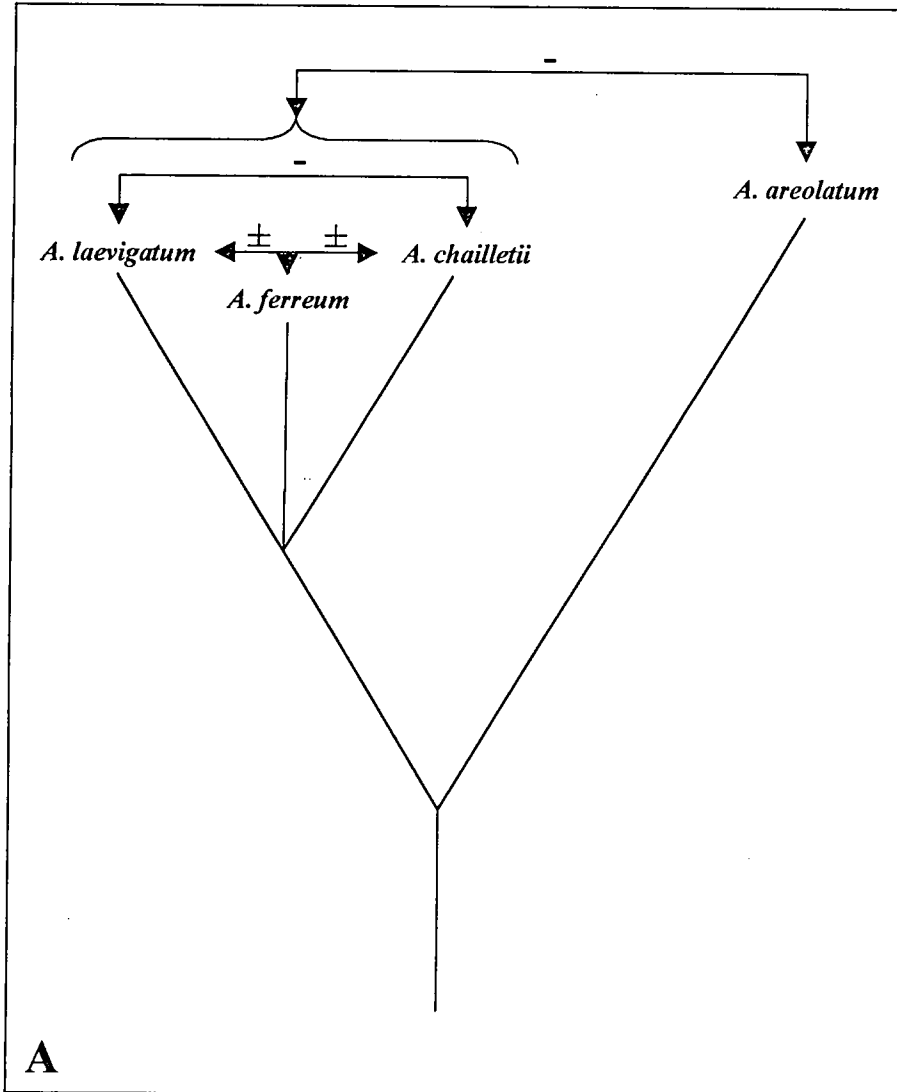
	410	420	430	440	450	460	470	480
DAOM21785	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	CAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
CBS305.82	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	CAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
SN19A	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
M5W	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
A3	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
CBS334.66	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
Br38	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	CAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
L204	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
DK37	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
S225	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	GAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
WaiteInst.6195	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	CAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
CBS483.83	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
54_95	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
DAOM21327	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
L234	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
Sc62.8	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
CBS624.84	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
Stillwell309_3	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG
CBS633.84	ACCCCTCTCT	GTCAACGATG	AATGGTGGTT	GCTAATTATA	AAATTAGTAG	CTATGTTAAC	ACGATAACCA	TTCCGCCTTG

**Figure 3:** One of the most parsimonious trees obtained by heuristic searches of the sequence data of the mt-SSU-rDNA for isolates representing the different species of *Amylostereum* (Table 1). The length of the tree = 31 steps, CI = 0.968, HI = 0.032 and RI = 0.986. Bootstrap values (1000 replicates) are given at the branching points.

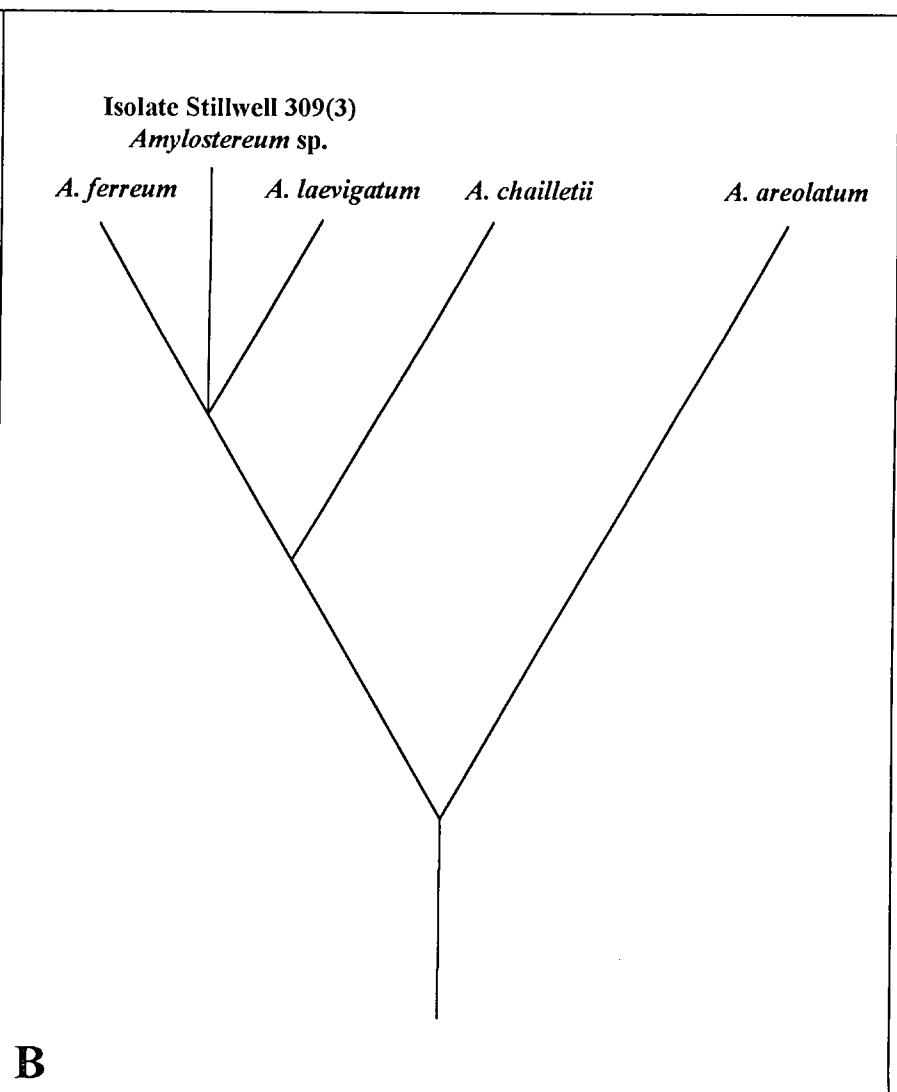


**Figure 4:** (a) The tree of descent of *Amylostereum* spp. reported by Boidin and Lanquetin (1984). This tree is based on mating behaviour between the four *Amylostereum* spp. The results of their mating studies between the *Amylostereum* spp. are indicated here as sexually incompatible (-) or partially compatible ( $\pm$ ).

(b) The tree of descent indicated by the results from the present study based on mt-SSU-rDNA sequence data analysis.



**A**



**B**

**Figure 5:** Aligned DNA sequence data of part of the mt-SSU-rDNA of the four *Amylostereum* spp., as well as 15 other Basidiomycetes. All sequences, except those of the *Amylostereum* spp. were obtained from TREEBASE and originate from Hibbett *et al.* (1997). Gaps that were inserted due to alignment are indicated by a dash (-). *Amylostereum areolatum* is represented by isolate SN19A, *A. chailletii* by isolate CBS483.83, *A. laevigatum* by isolate CBS 624.84 and *A. ferreum* by isolate CBS 633.84.



	10	20	30	40	50	60	70	80
<i>Bondarzewia berkleyi</i>	--TCGC--	GAGTGA-	CTAGCTAACTGAAATCCTCGC-	GAGTGA-	CTAGCTAACTGAAATCAATGTAA----	TATTAGAT		
<i>Echinodontium tinctorium</i>	GCTCGGGAAGAGTGA	ACTAGCTAACTGAAATCCAA--	TCTACATA-----	TAAA--	TATT-GAT			
<i>Amylostereum areolatum</i>	-----AAGAGTGA	ACTAGCTAACTGAAATC-AA--	TCTACATA-----	TAAA--	TATT-GAT			
<i>Amylostereum chailletii</i>	-----AAGAGTGA	ACTAGCTAACTGAAATC-AA--	TCTACATA-----	TAAA--	TATT-GAT			
<i>Amylostereum laevigatum</i>	-----AAGAGTGA	ACTAGCTAACTGAAATC-AA--	TCTACATA-----	TAAA--	TATT-GAT			
<i>Amylostereum ferreum</i>	-----AAGAGTGA	ACTAGCTAACTGAAATC-AA--	TCTACATA-----	TAAA--	TATT-GAT			
<i>Clavicornia pyxidata</i>	-----AANGNACTAGNTCCCTGAAATCC-----	TACAT-----	TAAATAATATT-GAT					
<i>Heterobasidium annosum</i>	-CTCGA--	AGAGTGA	ACTAGCTAACTGAAATCCTCGAAGAGTGA	ACTAGCTAACTGAAATCAATGAAAATAAT---	AGAT			
<i>Russula compacta</i>	--TCGC-	CAGAGTGA-	CTAGCTACCTGAAATCCAAT-----	TAAAA-----	TA-AT			
<i>Hericium ramosum</i>	-CTCG-	GAAGAGTGA	ACTAGCTAACTGAAATCCAA-----	TAAAA--	TATT----			
<i>Auriscalpium vulgare</i>	GCTCGG--	AGAGTGA	ACTAGCTAACTGAAATCCAA-----	TGTAAATT-	TA-TAGAT			
<i>Lentinellus omphalodes</i>	--TCGC-	ACGAGTGA-	CTAGCTAACTGAAATCCAA--	TCTA-----	TTTTA-----	TAGAT		
<i>Lentinellus ursinus</i>	-CTCGA--	CGAGTGA	ACTAGCTAACTGAAATCCAA-TCTCTA-----	TTTTAC-----	AGAT			
<i>Scytinostroma alutum</i>	-----TGACCTAGCTANCTGAAATCTATATGTTTTTTTTNCAA-	TAATTCTTTTTTATTAAGTAGTTAATATTTA						
<i>Peniophora nuda</i>	----GC-	AAGAGTGA	ACTAGCTAACTGAAATCCAT-TCTTTTTTAA----	GTTTTATTTCTATAAAGTTACAAGAATTAG				
<i>Stereum annosum</i>	----AC---	GAGTGA-	CTAGCTAACTGAAATCCAGATAAAGCGAAACTT-GTTTGG---	CTTTATCA-----				
<i>Stereum hirsutum</i>	-----GAGTGG	ACTAGCTAACTGAAATCCGAATCTAGCAAAACTTTGTTTGG---	CTAGATTC-----					
<i>Gloeocystidiellum leucoxantha</i>	-----CTAGCTAACTGAAATCAGA-	TATAGAAAATAAGA-TTTC---	CTATATCA-----					
<i>Laxitextum bicolor</i>	-----GA-TNA-	CTAGCTAACTGAAATCAAAA-AAAG-----	GTTGANC--	CTATTTNT-----				

90

100

110

120

130

140

150

160

*Bondarzewia berkleyi*  
*Echinodontium tinctorium*  
*Amylostereum areolatum*  
*Amylostereum chailletii*  
*Amylostereum laevigatum*  
*Amylostereum ferreum*  
*Clavicornia pyxidata*  
*Heterobasidion annosum*  
*Russula compacta*  
*Hericium ramosum*  
*Auriscalpium vulgare*  
*Lentinellus omphalodes*  
*Lentinellus ursinus*  
*Scytinostroma alutum*  
*Peniophora nuda*  
*Stereum annosum*  
*Stereum hirsutum*  
*Gloeocystidiellum leucoxantha*  
*Laxitextum bicolor*

T-----GATAACATAAGGGAGAA-TAATGATGT  
T-----GATAACGTAAGGGAAAA-TAATGATAT  
T-----GATAACGTAAGGGAAAA-TAATGATAT  
T-----GATAACGTAAGGGAAAA-TTATGATAT  
T-----GATAACGTAAGGGAAAA-TTATGATAT  
T-----GATAACGTAAGGGAAAA-TTATGATAT  
T-----GATAACGTAAGGGAAAA-TAATGATAT  
T-----GATAACGTAAGGGAAAA-TAATGATAT  
T-----GATAACGTAAGGGAAAA-TAATGATGG  
T-----GATAACGTAAGGGAAAA-TAATGATGT  
T-----GATAACGTAGGGGAAAA-TAATGATAT  
T-----GATAACGTAGGGGAAAA-TAATGATAT  
TTAAGAA---AA---ATT--ATAAA---AAACAAAATAAA-----GATAAGGTAAGGGAAAA-TAATGATAT  
TTTACTAGTTTAAGTGATTTAATAAAAATAAACAAAATAAAATAAAAAAGTAAGATAAGGTAAGGATAA-TTATGATAC  
-----GATAT-GTAAGGCCAAAAATAATGATGA  
-----GATAT-GTAAGGCCAAAAATAATGATGA  
-----GATAT-GTNAGGCCAAAAATAATGATAA  
-----GATAACGTAAGGGAAAA-TAATGATAT

*Bondarzewia\_berkleyi* TACCTTATTAATAGTGTGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAGTCGTCCTAAT  
*Echinodontium\_tinctorium* TACCTTACTATTAGTGTGCGCCAAAATCTGGTGCCAGAAGACTCGGTCAAGGCCAGAGACGCAAACGTTAATCGCCATAAT  
*Amylostereum\_areolatum* TACCTTACTATTAGTGTGCGTCCAAATCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCGTCTTAAA  
*Amylostereum\_chailletii* TACCTTACTATTAGTGTGCGTCCAAATCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCGTCTTAAA  
*Amylostereum\_laevigatum* TACCTTACTATTAGTGTGCGTCCAAATCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCATCTTAAA  
*Amylostereum\_ferreum* TACCTTACTATTAGTGTGCGTCCAAATCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCGTCTTAAA  
*Clavicornia\_pyxidata* TACCTTACTAATAGTGTGTCGTCGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAAAACGCAAACGTTAGTCGTCCTAAT  
*Heterobasidion\_annosum* TACCTTACTATTAGTGTGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCGTCTTAAAT  
*Russula\_compacta* TACCTTATTATTAGTGTGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCGTCTTAAAT  
*Hericium\_amosum* TACCTTACTATAAGTGTGCGCCAAAATCTGGTGCCAGAAGACTCGGT-AAGGCCAGATACGCGAACGTTAATCGCCTTAAAT  
*Auriscalpium\_vulgare* TACCTTACTAATAGTGTGTCGTCGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAAAACGCAAACGTTAGTCGTCCTAAT  
*Lentinellus\_omphalodes* TACCTTACTATTAGTGTGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAGTCGTCCTAAT  
*Lentinellus\_ursinus* TACCTTACTATTAGTGTGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAGTCGTCCTAAT  
*Scytinostroma\_alutatum* TACCTTACTA-TAGTGTGTCGTCGCGCCAAAATTTGGTGCCAGAAGACTCGGT-AAGGCCAAAACGCAAACGTTAGNCATCTTTAT  
*Peniophora\_nuda* TACTTTACTA-TAGTGTGTCGTCGCGCCAAAATTTGGTGCCAGAAGACTCGGT-AAGGCCAAAACGCAAACGTTAGTCATCTTTAT  
*Stereum\_annosum* AACCTTACTATTAGTGTGCGCCAAAAGCTAGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAGTCGTCATTAT  
*Stereum\_hirsutum* AACCTTACTATTAGTGTGCGCCAAAATCTAGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCGAACGTTAGTCGTCATTAT  
*Gloeocystidiellum\_leucoxantha* ATCCTTACTATGAGTGTGCGTCGTCGCGCCAAAAGCTAGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCGAACGTTAGTCGTCATTAT  
*Laxitextum\_bicolor* TACCTTACTAATAGTGTGCGCCAAAAGCTGGTGCCAGAAGACTCGGT-AAGGCCAGAGACGCAAACGTTAATCGTCTTAAAT

*Bondarzewia\_berkeleyi*  
*Echinodontium\_tinctorium*  
*Amylostereum\_areolatum*  
*Amylostereum\_chailletii*  
*Amylostereum\_laevigatum*  
*Amylostereum\_ferreum*  
*Clavicornia\_pyxidata*  
*Heterobasidion\_annosum*  
*Russula\_compacta*  
*Hericium\_amosum*  
*Auriscalpium\_vulgare*  
*Lentinellus\_omphalodes*  
*Lentinellus\_ursinus*  
*Scytinostroma\_alutum*  
*Peniophora\_nuda*  
*Stereum\_annosum*  
*Stereum\_hirsutum*  
*Gloeocystidiellum\_leucoxantha*  
*Laxitextum\_bicolor*

CAGGCGTAAAGGGTTTGTAGGCAGCTT-GAAATTT---CTTTCTTAT-ATAGCACTAACTTAAAATACAACTATTATAG  
CAGGCGTAAAGGGTTTGTAGGCTGCTT-GAAATTT-ATCTT-CTAGCAATGCCCTCCTATTCTCTAAGAAAGGAGTCATT  
CAGGCGTAAAGGGTTTGTAGGCTGCTTTAAA-TTTTATTT-----GTGAAGATAGCCCAACA-----  
CAGGCGTAAAGGGTTTGTAGGCAGCTTTGAA-TTTTC-CT-----TTT-CCAA---GT-AA-----  
CAGGCGTAAAGGGTTTGTAGGCTGCTTTGAA-TATTATCTTTTA-A-TTTACCTTATGGTAAAAT-AGCGCA-----  
CAGGCGTAAAGGGTTTGTAGGCTGCTTTGAA-TTTTATTTAATAGACTTTACCCCAAGGTAAAAT-AGCACA-----  
CAGGCGTAAAGGGTGTGTAGGCGGCTTAAAAATTTT-CCTTTTCT-ATATTGCTCTAATTNGAAATAAGTAAAGTATAAA  
CAGGCGTAAAGGGTTTGTAGGCTGCTTTAA-----CTTAGAATATAA-----  
CAGGCGTAAAGGGTTTGTAGGCGGTTTTAAA-TTTA-CTTACAAAAAATTAATAAGTTAAGAA-----  
CAGGCGTAAAGGGTGTGTAGGCAGCTT-GACACTAT-CAATTTTCAAT-TAGCACAATATAAAAATTAATAAGCTTTTTAA  
CAGGCGTAAAGGGTTTGTAGGCTGCTT-CAAATTCT-CCTTATT--ATATAGCTTCCGCTAATATGAATAATAAGGAGG  
CAGGCGTAAAGGGTTTGTAGGCAGCTT-CAAATTT-ACCTTTTTT-ATATAGCTCCTTACTTTAAGAGTATTAAGAGAA  
CAGGCGTAAAGGGTTTGTAGGCTGCTT-CAAATTT-ACCTTTTTT-ATATAGCTCCATAGAATAAGAGTATTAAGAGAA  
CAGGCGTAAAGGGTTTGTAGGCGGCCTTAATAG-----CTTTTAAATTTTATTATTTAAACAAAAGAAAATTTACTTAAA  
CAGGCGTAAAGGGTTTGTAGGCGGCCT-AATT----ACCTGTCACAGTAGATTTTCTACTAACAA-----TTACT-ACA  
CAGGCGTAAAGGGTTTCGTAGGCGGCTT-GAAATCT---CTTTCTTT--AAAAATAGCACTCTTCTATTTTAA-----  
CAGGCGTAAAGGGTTTCGTAGGCGGCTT-AAGATTT---CTTTTTTT-AAAAAATAGCACTCTTCTCATTTAAATTTGGTT  
CAGGNGTAAAGGGTTTCGTAGGCGGCTTTGAGAT-----CTTTTTCTTTAAAAAATAGCACTCTTCTTAGAGTTAAGGAAAG  
CAGGCGTAAAGGGTGTGTAGGCGGCTTTGAAATTTTA-CTTTTTTTAATAGCA-----CTTTTTAATTTTTTCATTAATT

```

Bondarzewia_berkeleyi      TTATTTAAAGGAGTTA-----
Echinodontium_tinctorium  TAAGGT--AGGC-TTAAAAAAGATAAAAT-----
Amylostereum_areolatum    -----GATAATT-----
Amylostereum_chailletii   -----TTA----GATAATT-----
Amylostereum_laevigatum   -----TTA----GATAATT-----
Amylostereum_ferreum      -----TTA----GATACTT-----
Clavicornona_pyxidata     AAAAAANTAAGG-----TAAATTTAGTTTAAAGG-----GGAAG
Heterobasidion_annosum   -----
Russula_compacta         -----
Hericium_amosum          CATTTTTTTTTAAAAATAAATTAATTTAATTAAGAAATGTTTAATAAAAAAAGAATAATTAATTTAATATAAAGGAAG
Auriscalpium_vulgare     AAACGATTGAACTTA-AATTAATTTT-AAGTAACATCTTATAGATAT-----
Lentinellus_omphalodes   AAACCTAATTATCCATCATTAATAAAGT-----
Lentinellus_ursinus      AAAC--AATTCT-----
Scytinostroma_alutum     ATTAAGTAAAC--TAA----GGCT-AAAT-----
Peniophora_nuda          ATTACAACCATTGTAAGACAGGCTTAACT-----
Stereum_annosum         -----GA--GTTAAGGAAAGA-----
Stereum_hirsutum         TAAATTAATAAAAAATTATATAAAAAATAAGAAGGTTAAAGAGAGA-----
Gloeocystidiellum_leucoxantha
AG-----
Laxitextum_bicolor      AANAATAAAAAAAGTGAAG-----

```

*Bondarzewia\_berkleyi* -----AACAAATTAAGCTAGAAATCAAATAGAGGTTATATTAATAATACTTAGTG---TAGGGCCGATATCCTTAGAT  
*Echinodontium\_tinctorium* -----AATAAAAGCTAGAAATCAAATAGAG-TTATATTATATAATGCTTAAAG---TAGGGCTAATATCCTAACAG  
*Amylostereum\_areolatum* -----AATTAAGCTAGAAATCAAATAGAGGTTATATTATATAATGCTTAAAG---TAGGTCTAATATCCTAACAT  
*Amylostereum\_chailletii* -----AATCAAAGCTAGAAATCAAATAGAGGTTATATTATATAATGCTTAAAG---TAGGTCTAATATCCTAACAG  
*Amylostereum\_laevigatum* -----AATCAAAGCTAGAAATCAAATAGAGGTTATATTATATAATGCTTAAAG---TAGGTCTAATATCCTAACAG  
*Amylostereum\_ferreum* -----AATCAAAGCTAGAAATCAAATAGAGGTTATATTATATAATGCTTAAAG---TAGGTCTAATATCCTAACAG  
*Clavicornia\_pyxidata* -----AATTTAAGCTAGAAATCAAATAGAGGATATATTAATAATAATGCTTAAAG---TAGGTCTGATATCCTTAGAT  
*Heterobasidion\_annosum* -----AAC--TAAAAGCTAGAAATCAAATAGAGGTTATATTACATAATACTTAGAG---TAGGTCTGATATCCTTAGAT  
*Russula\_compacta* -----TAAAAGCTAGAAATCAAATAGAGGATATATCAAATAATGCTTAGAG---TAGGTCTGATATCCTTAAAT  
*Hericium\_amosum* AATGGT-AACAATTAAGCTAGAAATCAAATAGAGGTTATATTAAAGAATGCTTGGAG---TAGGGCTGATATCCTTAGAT  
*Auriscalpium\_vulgare* -----TAAAAGCTAGAAATCAAATAGAGGATATATTAATAATACTTAGAG---GAGGTCTGATATCCTTAAAT  
*Lentinellus\_omphalodes* -----AATAAAAGCTAGAAATCAAATAGAGGATATATTAATAATACTTAGAT---CAGGTCTGATATCCATAGAT  
*Lentinellus\_ursinus* -----AC--TAAAAGCTAGAAATCAAATAGAGGATATATTAATAATACTTAGAG---TAGGTCTGATATCCTTAAAT  
*Scytinostroma\_alutum* -----TAAGTGCTAGAGTCAATTAGAGGTTATATTATAGAATGCTTAAAG---TAGGGCCAATATCCACAAG  
*Peniophora\_nuda* -----TAAGTGCTAGAGTCAATTAGAGGTTAGATTAAATAATACTTAAAC---AAGGGGTGATATCCTGAAAG  
*Stereum\_annosum* -----AACAAATCAAAGCTAGAAATCATAAAGAGGTCATATTGAATAATTCTAAGACTATAAGGGGTGAAATCTTTAGAT  
*Stereum\_hirsutum* -----AACAAATTAAGCTAGAAATCATAAAGAGGTCATATTGAATAATTCTAAGACTATAAGGGGTGATATCTTAAAGAT  
*Gloeocystidiellum\_leucoxantha* -----ACAATTAAGCTAGAAATCATNAAGAGGTCATATTAAATAATTCTAAGGCAATAAGGGGTATAATCTTAAAT  
*Laxitextum\_bicolor* -----CAATCAAAGCTAGAAATCAAATAGAGGATATATTAATAATACTTAGAGTA-TAGGTCTGATATCCATAGAT

*Bondarzewia berkleyi* ACTA-AGGGGAATATT-AAGG-GCGAAAGCTTTTTT-CC-----ACTAATG-ATT-----GACTCTGAGAAACGA-GG  
*Echinodontium tinctorium* ATTA-AGTGGGAATATT-AAAA-GCGAAGGCTTATTTTCC-----ATAAATG-ATT-----GACGCTGAGAAACGAAGG  
*Amylostereum areolatum* ATTA-AGTGGGAATATT-AAAA-GCGAAGGCTTATTTTCC-----ATAAATG-ATT-----GACGCTGAGAAACGAAGG  
*Amylostereum chailletii* ATTA-AGTGGGAATATT-AAAA-GCGAAGGCTTATTTTACC-----ATAAATG-ATT-----GACGCTGAGAAACGAAGG  
*Amylostereum laevigatum* ATTA-AGTGGGAATATT-AAAA-GCGAAGGCTTATTTTACC-----ATAAATG-ATT-----GACGCTGAGAAACGAAGG  
*Amylostereum ferreum* ATTA-AGTGGGAATATT-AAAA-GCGAAGGCTTATTTTACC-----ATAAATG-ATT-----GACGCTGAGAAACGAAGG  
*Clavicornia pyxidata* ATTA-AGTAGAATATT-AAAG-GCGAAGGCTTTTTTCC-----NNAATG-ATT-----GACGCTGAGAAACGAAGG  
*Heterobasidion annosum* ACTA-AGGGGAATATT-AAGG-GCGAAAGCTTTTTTACC-----ATTAATG-ATT-----GACGCTGAGAAACGAAGG  
*Russula compacta* ACTA-AGTAGAATATT-AAAG-GCGAAAGCTTTTTT-CC-----ATTAATG-ATT-----GACGCTCAGAAACGAAGG  
*Hericium ramosum* ACCA-GGTAGAATATT-AAGA-GCGAAGGCTTTTTT-CC-----ATTGATG-ATT-----GACGCTGAGACACGAAGG  
*Auriscalpium vulgare* ACTA-AGTGGGAATATT-AACG-GCGAAGGCTTTTTTCC-----ATTAATG-ATT-----GACGCTGAGAAACGAAGG  
*Lentinellus omphalodes* ACTA-AGTGGGAATATT-AACA-GCGAAGGCTTTTTTCC-----AATAATG-ATT-----GACGCTGAGAAACGAAGG  
*Lentinellus ursinus* ACTA-AGTGGGAATATT-AACA-GCGAAGGCTTTTTTCC-----AATAATG-ATT-----GAC-CTGAGAAACGA-GG  
*Scytinostroma alutum* ATTA-AGCAGAATATT-AAGG-GCGAAGGCTTTTTCTCCACTACGGAAAATATAAT--AACTGACGCTGAGAAACGAAGG  
*Peniophora nuda* ATTA-AGTAGAATACT-AAGA-GCGA-GGCTTTTTTCC---ACTACCGAAC-AATAAACTGACGCTGAGAAACTAAGG  
*Stereum annosum* ACTT-GGAGGAATATTAAAGG-GCGAAGGCTTTTTTCC-----ATAAATG-ATT-----GACGCTGAGGAACGAAGG  
*Stereum hirsutum* ACTT-GGAGGAATATTAAAGG-GCGAAGGCTTTTTTCC-----ATAAATG-ATT-----GAC-CTGAGGACCGAAGG  
*Gloeocystidiellum leucoxantha* TTCTTAGAGGAATATTAAAGG-GCGAAGGCTTTTTTCC-----ATAAATG-ATT-----GACGCTGAGGAACGAAGG  
*Laxitextum bicolor* ACTA-AGGGGAATATT-AATA-GTGAAGCTTTTTTCT---AC--TAA-TG-ATT-----GACGCTGAGAAACGAAGG

*Bondarzewia\_berkeleyi* TGAGGATAGGAAATAGGATTAGATACCCAAATTACCCCTCTCTGTCAACGATGAATGGTGGCT--ACTAGTGAATCA---  
*Echinodontium\_tinctorium* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTAAACGATGAATGGTGGTT--GCTAATTAGTAAA--  
*Amylostereum\_areolatum* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--GCTAATTATAGAA--  
*Amylostereum\_chailletii* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--GCTAATTATAAAA--  
*Amylostereum\_laevigatum* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--GCTAATTATAAAA--  
*Amylostereum\_ferreum* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--GCTAATTATAAAA--  
*Clavicornia\_pyxidata* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTAGTT--ACTAGTAAATGAA--  
*Heterobasidion\_annosum* TGAGGAAAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTAACTA--TTAGTAATAAAA--  
*Russula\_compacta* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTAGTTA--TTAGTAATAAAT--  
*Hericium\_ramosum* GGAGGAAAGGAAATAGGATTAGATACCCAAATACCCCTCCCTGTCAACGATGAATGGTGGTT--ACTAGTTATAATAA--  
*Auriscalpium\_vulgare* TGAGGAAAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--GCTAGT-ATAAAA---  
*Lentinellus\_omphalodes* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--ACTAGTAAAAAACC--  
*Lentinellus\_ursinus* TGAGGAAAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTT--ACTAGTAAATAACAC--  
*Scytinostroma\_alutum* GGAGGATAGGAAATAGGATTAGATACCCAGAATACCCCTCTCTGTCAACGATGAATGGTAATTT--CTAGTAAGTTATAA--  
*Peniophora\_nuda* GGAGGATAGGAAAAGGATTAGATACCC-AATTACCCCTCTCTGTCAACGATGAATGGTAATTT--CTAGTGATTTGTAA--  
*Stereum\_annosum* GGAGGAGCGGAATTAGGATTAGATACCC-AACTACCCCTCTCTGTCAACGATGAATGGTAGTTAACCTGTAGACAAAA--  
*Stereum\_hirsutum* GGAGGAGAGGAATTAGGATTAGATACCC-AACTACCCCTCCCTGTCAACGATGAATGGTAGTTAACCTGTAGACAAAAA--  
*Gloeocystidiellum\_leucoxantha* GGAGGATAGGAATAAGGATTAGATANCC-AACTACCCCTCTCTGTCAACGATGAATGGTAGTTAACCTGT-AATAA----  
*Laxitextum\_bicolor* TGAGGATAGGAAATAGGATTAGATACCCAAATACCCCTCTCTGTCAACGATGAATGGTGGTCA--CTAGTCAA-----

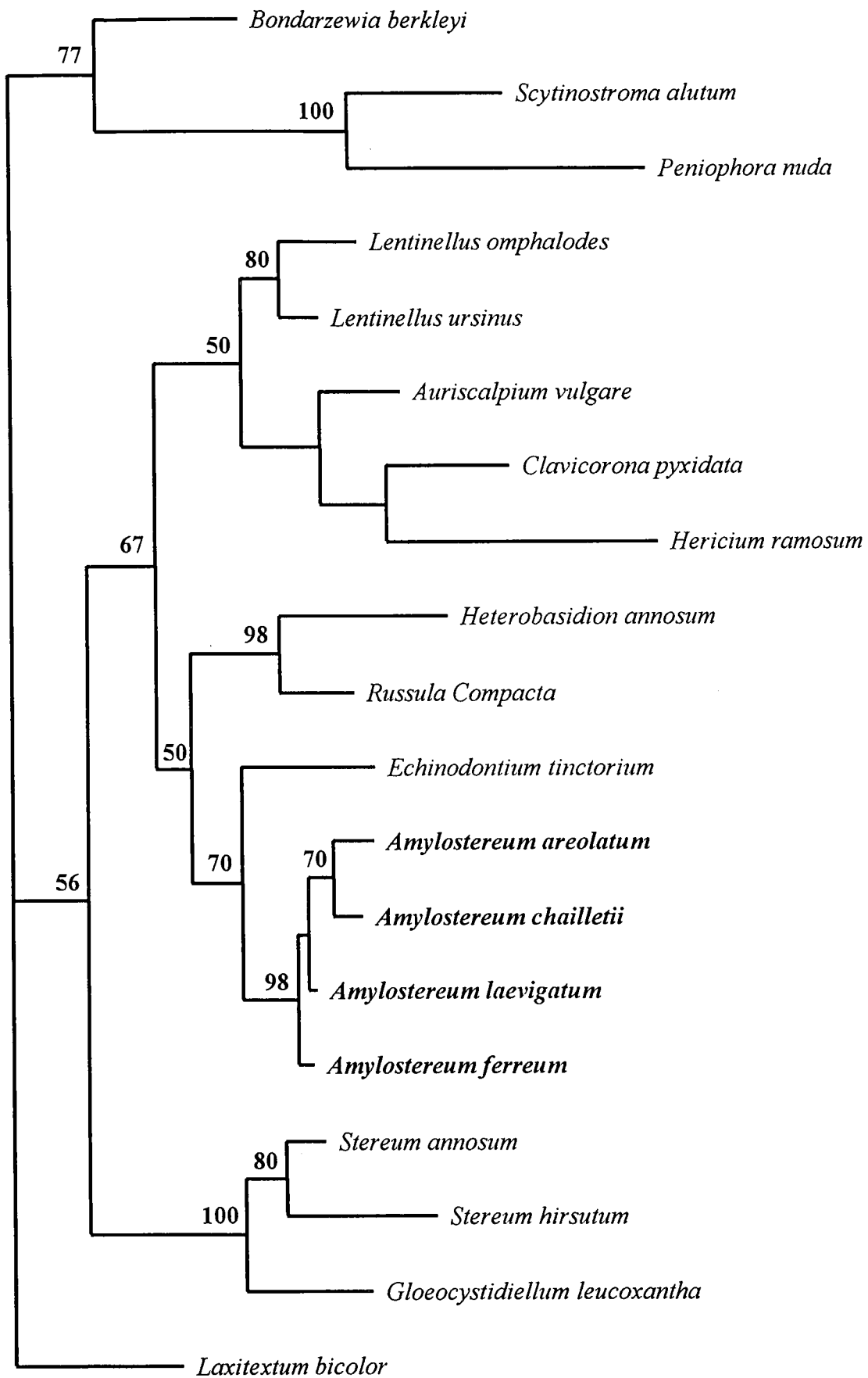


<i>Bondarzewia_berkeleyi</i>	-----TTG-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG
<i>Echinodontium_tinctorium</i>	-----TTG-GTAGCTATGTTAACACGATAACCATTCCGCCTTGTG
<i>Amylostereum_areolatum</i>	-----TTA-GTAGCTATGTTAACACGATAACCATTCCGCCTTGTG
<i>Amylostereum_chailletii</i>	-----TTA-GTAGCTATGTTAACACGATAACCATTCCGCCTTGTG
<i>Amylostereum_laevigatum</i>	-----TTA-GTAGCTATGTTAACACGATAACCATTCCGCCTTGTG
<i>Amylostereum_ferreum</i>	-----TTA-GTAGCTATGTTAACACGATAACCATTCCGCCTTGTG
<i>Clavicornia_pyxidata</i>	-----TTATTG-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG
<i>Heterobasidion_annosum</i>	-----TTA-GTAGTAATGTTAACACAATAACCATTCCGCCTTGTG
<i>Russula_compacta</i>	-----TTATTAA-TGGCAATGTTAACACGATAACCATTCCGCCTTGTG
<i>Hericium_amosum</i>	-----TTA-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG
<i>Auriscalpium_vulgare</i>	-----TTATTA-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG
<i>Lentinellus_omphalodes</i>	-----TTATTA-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG
<i>Lentinellus_ursinus</i>	-----TATTA-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG
<i>Scytinostroma_alutum</i>	AAAATAACCAATA-----TTA-GTTTTAATGTTAACACGTTAACCATTCCGCCTTGTG
<i>Peniophora_nuda</i>	GAATATTACTAAATAGTTAGTTTTATAATTACAATACTTATTA-GTTTTAATGTTAACACGTTAACCATTCCGCCTTGTG
<i>Stereum_annosum</i>	-----TAGGTGGCAAAGCTAACGCGATAACCATTCCGCCATGCG
<i>Stereum_hirsutum</i>	-----CAGGAGGCAAAGCTAACGCGATAACCATTCCGCCATGCG
<i>Gloeocystidiellum_leucoxantha</i>	-----TAGGTAGCAATGCTAACGCTATAACCATTCCGCCATGCG
<i>Laxitextum_bicolor</i>	-----TTA-GTGGCGATGTTAACACGATAACCATTCCGCCTTGTG

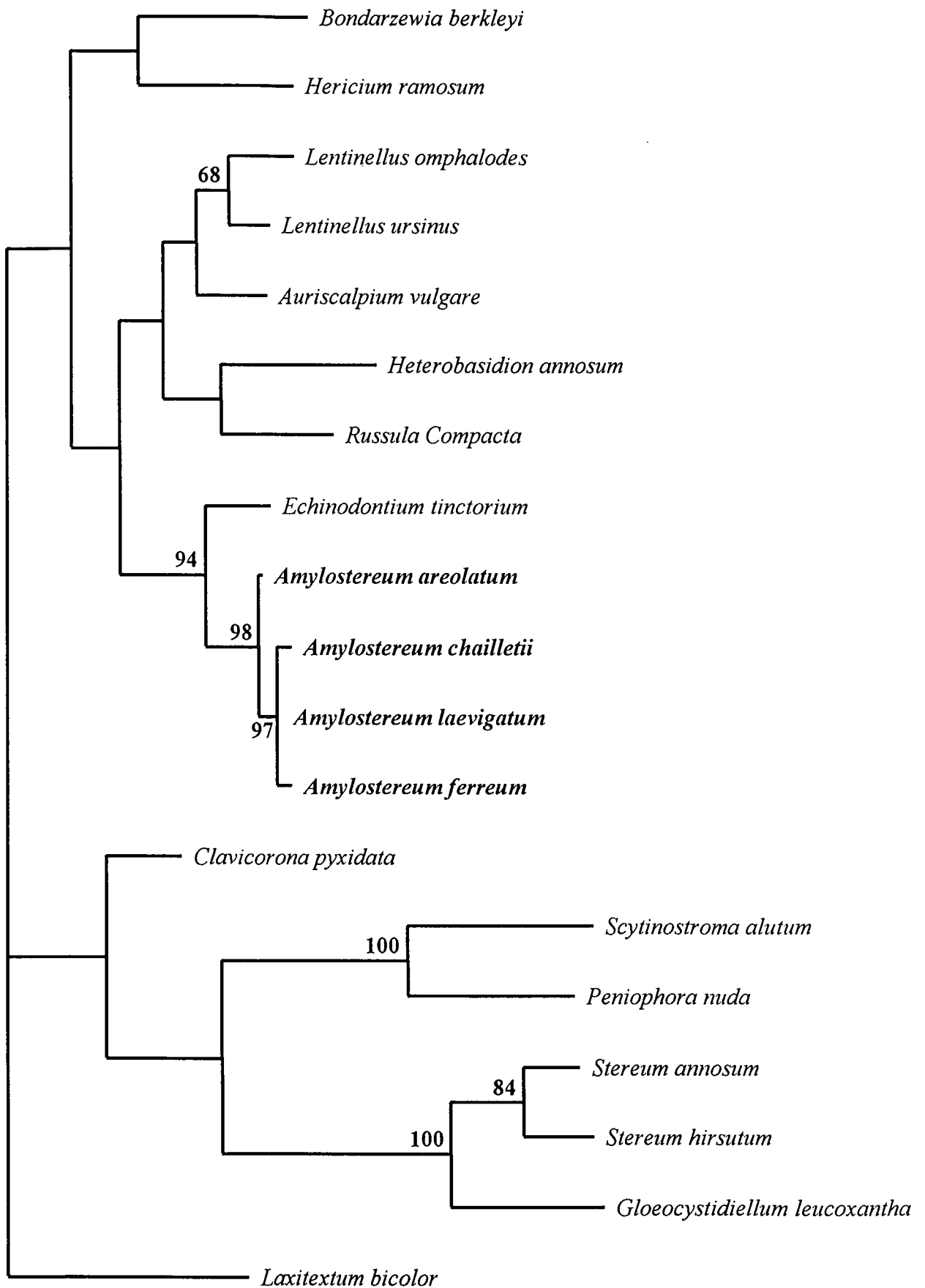
*Bondarzewia\_berkleyi*  
*Echinodontium\_tinctorium*  
*Amylostereum\_areolatum*  
*Amylostereum\_chailletii*  
*Amylostereum\_laevigatum*  
*Amylostereum\_ferreum*  
*Clavicornia\_pyxidata*  
*Heterobasidium\_annosum*  
*Russula\_compacta*  
*Hericium\_amosum*  
*Auriscalpium\_vulgare*  
*Lentinellus\_omphalodes*  
*Lentinellus\_ursinus*  
*Scytinostroma\_alutum*  
*Peniophora\_nuda*  
*Stereum\_annosum*  
*Stereum\_hirsutum*  
*Gloeocystidiellum\_leucoxantha*  
*Laxitextum\_bicolor*

AGTACGACTGC-AAAGTTGAAAA-CCAAAAAATTAGTCGGTCTCGAA--CAAA-CGAAGC-  
 AGTACGACTGC-AAAGTTGAAA--CCAAAAAATTAGTCGGTTTCGGAG-CAC--CGAA-TG  
 AGTACGACTGC-AAAGTTGAAAA-CAAAAAAATTAGTCGGTTTCGGAG-CAAA-CGAAGTG  
 AGTACGACTGC-AAAGTTGAAAAACAAAAA-TTAGTCGGTTTCGGAG-CAAA-CGAAGTG  
 AGTACGACTGC-AAAGTTGAAAA-CAAAAAAATTAGTCGGTTTCGGAG-CAAA-CGAAGTG  
 AGTACGACTGC-CAAGTTGAAAA-CAAAAAAATTAGTCGGTTTCGGAG-CAAA-CGAAATG  
 AGTACGACTNC-AAAGTTGAAAACCCAAAAAATTATTCGGTCTCGAAN-CNTAACGGANTG  
 ACTACGACTGC-AAAGTTAAAAACCCAAAAAATTAGTCGGTCTCGAAG-CAC---GAAAAC  
 AGTACAACCTGC-AAAGTTGAAAACCCAAAAAATTAGTCGGTTTCGGAG-CACT-CGAAGTG  
 AGTACGACTGC-AAAGTTAAAAA-CCAAAAAATTAGTCGGTTTCGAAG-CAAA-CGAAGTC  
 AGTACGACTGC-AAAGTTGAAAA-CCAAAAAATTAGTCG-TCTCGAAG-CAAA-CGAA-TC  
 AGTACGACTGC-AAAGTTGAAAACCCAAAAAATTAGTCGGTTTCGAAG-CACA-CGAAGTG  
 AGTACGACTGC-AAAGTTGAAAA-CCAAAAAATTAGTCGGTCTCGAAG-C----CGAAGTG  
 AGTACGATTGN-NAAATTGAAAA-NNAAAAAATTAGTCGGTTTCGG-AGC-GANCGAA---  
 AGTACGATTGCCAAA-TTGAAAA-CCAAAAAATTAGTCGGTTTCGGAG-CG-ATCGAAGTG  
 AGTACGACTGC-AAAGTTGAAA--CCAAAAAATTAGTCGGTTTCGAAG-CA-TACGAAGTG  
 AGTACGACTGC-AAAGTTGAAA--CCAAAAAATTAGTCGGTTTCGAAG-CACTACGAAGTG  
 AGTACGACTGC-AAAGTTGAAAG-NCAAAAAAATTAGTCGGTTTCGAAGC--AAACGAA-TG  
 AGTACGACTGC-AAAGTTGAAAA-NNAAAAAATTAGTCGGNNTCGA-AGCA-NNCGGATCG

**Figure 6:** One of the most parsimonious trees obtained by heuristic searches of the full sequence data set (including the hypervariable regions) of the mt-SSU-rDNA for 19 different Basidiomycetes spp, including the four *Amylostereum* spp. The tree length = 1495 steps, CI = 0.601, HI = 0.399 and RI = 0.522. Bootstrap values (1000 replicates) are given at the branching points.



**Figure 7:** One of the most parsimonious trees obtained by heuristic searches of the mt-SSU-rDNA for 19 different Basidiomycetes spp, including the four *Amylostereum* spp., where the hypervariable regions were excluded. Bootstrap values (1000 replicates) are given at the branching points. Tree length = 639 steps, CI = 0.604, HI = 0.396 and RI = 0.595.





## TAXONOMY OF *AMYLOSTEREUM*, WITH SPECIAL REFERENCE TO *A. AREOLATUM* ASSOCIATED WITH THE WOOD WASPS *SIREX NOCTILIO* AND *S. JUVENCUS*

### ABSTRACT

Members of the genus *Amylostereum* (Basidiomycetes) are best known for their symbiotic relationship with siricid wood wasps. In this study, the phylogenetic relationship between *Amylostereum areolatum*, *A. chailletii*, *A. laevigatum* and *A. ferreum* was investigated using sequence and RFLP analyses of the variable nuc-IGS-rDNA region. Special attention was given to isolates of *A. areolatum* associated with *S. noctilio* and *S. juvencus*, as the specificity of the associations separates populations of the fungus. RFLP analysis of this DNA region is also evaluated as a potential diagnostic tool to delineate *Amylostereum* spp. The DNA sequence analysis showed that *A. ferreum* and *A. laevigatum* are most closely related to each other. Of the two other species in the genus, *A. chailletii* was more closely related to these two species than to *A. areolatum*, which was the most clearly defined species in the genus. One isolate that was obtained from *S. areolatus* and was expected to be *A. chailletii*, grouped more closely to *A. laevigatum*. This isolate might represent a new species or a distinct group in of one of the species. Isolates of *A. areolatum* associated with both *S. noctilio* and *S. juvencus* contained four heterogenic sequences in the DNA region analysed. These heterogenic sequences were contained in each isolate of the fungus in one of five combinations. Neither the heterogenic sequences contained in the fungal isolates, nor the different combinations of these sequences, were consistent in their association with *S. noctilio* or *S. juvencus*. The isolates of *A. areolatum* associated with these two species of wood wasps could thus not be defined in two separate groups using these methods. Despite the heterogenic nature of this DNA region in some isolates, RFLP analysis could effectively be used to distinguish between the different species of *Amylostereum*.



## INTRODUCTION

Members of the wood wasp family Siricidae have larval states that either live in the stems of plants or are woodborers. For example the genera *Sirex* and *Urocerus*, infest softwoods and especially conifers (Benson, 1943; Chamberlin, 1960; Morgan, 1968). A further common characteristic of these two genera is the highly evolved association with specific wood-infecting basidiomycetes (Cartwright, 1929; Francke-Grosmann, 1939; Morgan, 1968). Conidia (oidia) or bundles of fungal mycelium are carried in a pair of specialised intersegmental mycangia near the base of the ovipositor of the adult female wasps and in external hypopleural organs of the female larvae (Buchner, 1928; Francke-Grosmann, 1939; Parkin, 1941). The fungus is then inoculated into the wood together with the eggs during oviposition, and it has been hypothesised that this infection is necessary for the subsequent development of the larvae (Madden & Coutts, 1979; Madden, 1981).

Using polymorphisms in protein banding patterns and mating studies, Gaut (1970) showed that the relationship between Siricidae and fungi is species specific. One fungal species can, however, be carried by more than one species of Siricidae. Gaut (1970) also found that *Amylostereum areolatum* (Fr.) Boid. is the symbiont of *S. noctilio* Fabr., *S. juvenicus* 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 morphological differences between the different woodwasp species are often subtle and their identities have frequently been confused, as have the identities of their fungal associates (Benson, 1943; Thomsen, 1996). The specificity of the symbiosis can thus serve as a useful taxonomic character when attempting to identify a woodwasp or its fungal symbiont.

During the last century, *S. noctilio* and its fungal symbiont have become established and is a serious pest in pine plantations of Australasia (Neumann, Morey & McKimm, 1987; Madden, 1988; Chou, 1991). The fungal symbiont associated with this wood wasp proved difficult to identify and it was not until 1969 that it was correctly identified as *A. areolatum* (Gaut, 1969). Recently the *Sirex-Amylostereum* complex has been reported from South America (1980) and South Africa (1994) and now pose a threat to the

softwood industries of these countries (Baxter, Rong & Schutte, 1995; Reardon, Eav & Wetterberg, 1995; Tribe, 1995).

The rarity and absence of sporocarps in nature has resulted in significant problems in the taxonomy of the fungal associates of Siricidae. The result is that these fungi often had to be studied entirely in culture or based on structures in the mycangia of the insect vectors (Talbot, 1964; King, 1966). Fruiting structures can be obtained artificially by using the wood-block culture method described by Tamblyn & Da Costa (1958), but Talbot (1964) reported that differences exist between the different stages of maturity using this method. He, therefore, suggested caution when using this method. A further complicating factor is that *A. areolatum* and *A. chailletii* are very similar in culture and fruiting body morphology. The clearest difference between these two species is that *A. areolatum* produces arthrospores in culture and these structures are absent in *A. chailletii*.

The phylogenetic relationship between isolates of one fungal species associated with different siricid species has not been addressed previously. In this study the evolutionary distance between *A. areolatum* isolates associated with *S. noctilio* and *S. juvencus* was considered using PCR based RFLP fingerprinting, as well as analysis of nuclear IGS sequence. Sequence data and RFLP data of this region was also used to evaluate the phylogenetic relationships between the four different species of *Amylostereum*.

The taxonomy of the Siricidae as well as that of their fungal symbionts is complicated. It would, therefore, be useful to find an easy and reliable technique to distinguish both these parties. Harrington & Wingfield (1995) showed the value of restriction fragment length polymorphism's (RFLP's) of PCR fragments, in the delineation of Basidiomycetes in culture. A second aim of this chapter was, therefore, to use PCR based RFLP analysis of the intergenic spacer (IGS) region of the nuclear ribosomal DNA (nuc rDNA) operon to distinguish between *A. areolatum*, *A. chailletii*, *A. laevigatum* (Fr.) Boid. and *A. ferreum* (Berk. & Curt.) Boid. & Lanq.

## MATERIALS AND METHODS

### Isolates studied

Isolates (Table 1) were maintained on Petri dishes (9 cm diameter) containing MYA (2% malt extract, 0,2% yeast extract and 1,5% agar) or pine extract MYA (pine extract prepared by autoclaving pieces of pine wood in the water to be added to the medium) at 25 °C. The cultures were stored in McCartney bottles on MYA at 4 °C. South African isolates of the fungal symbiont of *S. noctilio* were all made from mycangia of female *S. noctilio* wasps. A detailed description of the technique used in these isolations is given in Chapter 2. Isolates from Brazil were also obtained from the mycangia of female *S. noctilio* wasps (supplied by Mr. E. Schaitza, Embrapa, Colombo, Brazil). Isolates from Australia were purified from cultures of the nematode *Deladenus siricidicola*, imported into South Africa from Australia as part of a biological control programme for *S. noctilio*. These nematodes are maintained on cultures of *A. areolatum* and nematode inoculum *de facto* carries the fungus. Isolates of *A. areolatum* and *A. chailletii* from Europe were supplied by Dr. I. M. Thomsen (Danish Forest and Landscape Research Institute, Hoersholm, Denmark) and Dr. R. Vasiliauskas (Swedish University of Agricultural Sciences, Uppsala, Sweden). Other authenticated isolates of the four *Amylostereum* spp. from different parts of the world were obtained from CBS (Centraal Bureau voor Schimmelcultures, Baarn, Netherlands) and DAOM (Centre for Land and Biological Resources Research, Canada).

### DNA isolation, PCR amplification

DNA was extracted from cultures using a modified version of the method described by Raeder & Broda (1985). The extracted genomic DNA (50 - 80 ng) was used directly as template for PCR. The intergenic spacer region (IGS) between the nuclear large subunit (LSU) and the 5S gene of the ribosomal RNA (rRNA) operon) was amplified using PCR. New 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') (Coetzee, 1997) were tested. IGS PCR fragments were generated using the Expand™ High Fidelity PCR System on a Hybaid TouchDown (Hybaid limited, U.K.) PCR unit. PCR reaction mixes and reaction conditions were the same as those described previously (Chapter 3).

PCR amplification for some isolates of *A. areolatum* resulted in two fragments of different size. To separate these bands for analysis, DNA represented by the bands on agarose gels were 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 for were the same as those used for the initial PCR.

### DNA sequencing and sequence data analysis

DNA sequence of the IGS region of 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 *S. 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 pre-mix 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.

To interpret the double product amplified for some isolates of *A. areolatum* and to obtain sequence for the whole amplified fragment, 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, U.S.A.). Screening for positive colonies containing the insert were done by PCR using the M13U and M13R primers. Cloned products were precipitated and purified as described above and sequenced using primers M13R and M13U. Sequence obtained from the cloned products was compared to sequence of *Armillaria* species from South Africa (Coetzee, 1997), to identify the flanking regions of the large subunit (28S) and 5S genes to which the P-1 and 5S-2B primers bind respectively.

DNA sequences were manually aligned by inserting gaps, which were treated as a fifth character (newstate). PAUP version 3.1.1 (Phylogenetic Analysis Using Parsimony) (Swofford, 1993) was used to analyse the sequence data by executing heuristic searches with TBR (Tree Bisection Reconstruction) branch swapping and MULPAR activated. Bootstrapping (1000 replicates) (Felsenstein, 1993) was used to determine 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 *AluI* and *CfoI*.

### Restriction analysis

Restriction analysis of the amplified IGS fragment was done using the restriction endonucleases *AluI* and *CfoI* (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. Resulting restriction fragments were separated by electrophoresis on ethidium bromide stained 2 % (wt/v) agarose gels and visualised under UV illumination. Size estimates of RFLP fingerprints were made using a 100 bp ladder run as a molecular size marker. RFLP fingerprint fragments smaller than 100 bp were not included in the analysis, due to their weak visibility and the absence of comparative bands smaller than 100 bp in molecular marker. Exact restriction fragment sizes were, however, deduced from sequence data.

## RESULTS

### PCR amplification

Strong amplification products of the IGS region were obtained using the primers P-1 and 5S-2B (Figure 1). PCR fragment sizes were calculated to be 552 bp from sequence data for isolates of *A. 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 (Figure 2). In the first 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 results, however, showed that this apparent single band was heterologous and represented two fragments of 622 and 638 bp. An exception was isolate CBS 305.82 for which a single fragment of 618 bp was amplified. In the second form, a single PCR fragment of 570 bp was amplified (CBS 334.66). In the third 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. These double bands were successfully reamplified using each DNA band from the gel as template in a PCR reaction for isolates A4 and DK 37. Two single bands were thus obtained with fragment sizes (638 and 570 bp) identical to each of the original DNA fragments obtained with the initial PCR.

#### **DNA sequencing and sequence data analysis**

Full sequence of the PCR product including the primer binding sites was obtained for the each of the separately cloned double products of isolate DK 37. There was a greater than 90% homology between the first 100 bp from the 5' end and last 94 bp on the 3' end sequences of this isolate, and the corresponding flanking sequence obtained from the IGS region of different isolates of *Armillaria* from South Africa. Sequences obtained using the primers P-1 and 5S-2B for other isolates of *Amylostereum* extended into these highly conserved 28S (5' end) and 5S (3' end) genic regions. They were, however, between 16 and 54 bp short of the full sequence of the PCR fragment, that would include the primer binding sites. Isolate DAOM 21785 (*A. areolatum*), however, lacked 95 bases on the 3' end of the fragment. Due to the highly conserved nature of these regions, it was assumed that these absent sequences were identical to the sequences obtained for these regions from isolate DK37. Sequence from isolate DK 37 was, therefore, used to complete the flanking sequences of other isolates, in order to calculate total sizes for the restriction map. These full sequences amounted to a total of 694 characters after alignment (Figure 3).

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* (Figure 3). Sequences B, C and D were the same, other than in two sites where major indels were observed. Most isolates of *A. areolatum* contained two of these four sequences in any of five combinations (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 D, while isolates L236, DK37, A3, A4 and A11 contained sequences B and D. Sequence for the IGS region was homologous in isolates CBS 305.82 (sequence C) and CBS 334.66 (sequence D). 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, however, 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 (CI = 0.912; HI = 0.088; RI = 0.900) (Figure 4). 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. *A. laevigatum* and *A. ferreum* together formed a sister group to *A. chailletii*. Isolate Stillwell 309(3) grouped closest to *A. laevigatum*, with a high confidence interval at the branching point of 79 %.

### Restriction analysis

Restriction maps for both the restriction endonucleases *AluI* and *CfoI* were determined from sequence data (Figure 5a & b). PCR products of all *Amylostereum* spp. gave different restriction patterns when digested with the restriction endonuclease *AluI* (Figure 6a). Isolates representing or thought to represent *A. areolatum* showed different restriction patterns using this endonuclease (Figure 6a & 7a). The restriction fragment pattern that was produced for isolate CBS 305.82, had three fragments of 98, 159 and 361 bp in size, while that for isolate CBS 334.66 had two bands of 159 and 381 bp. Another pattern, comprised of 98, 159 / 163, 361 and 381 bp bands, was derived for isolates Waite Inst.

6195, DAOM 21785, Br17, Br60, M5W, SN19A and DK 782. For isolates S225, S227 and L204 a restriction pattern of 159 / 163, 361 and 381 bp bands was produced, while a pattern of 98, 159 and 381 bp bands was produced for isolates L236, DK37, A3, A4 and A11. These RFLP fingerprints obtained using *AluI* thus support the heterogenic nature of the IGS region as indicated by the sequence data.

Restriction fragment sizes obtained with *AluI* were 187 and 329 bp for isolates of *A. chailletii* (CBS 483.83; DAOM 21327; 54-95), 188 and 350 bp for *A. laevigatum* (CBS 624.84) and 176 and 381 bp for *A. ferreum* (CBS 633.84) (Figure 6a). The restriction fingerprints for isolate Stillwell 309(3) (218 and 351 bp) differed from RFLP fingerprints obtained for all isolates used in this study representing the different species of *Amylostereum* (Figure 6a).

The restriction fingerprints obtained using the restriction endonuclease *CfoI* for isolates of *A. areolatum* varied between different isolates (Figure 6b & 7b). Isolates with PCR fragments of 622 and 638 bp (Waite Inst. 6195; DAOM 21785; Br17; Br60; M5W; SN19A; DK 782), gave restriction fragments of 315 / 310 (indistinguishable on agarose gels), 187, 120 and 99 bp. The restriction fingerprint obtained for isolate CBS 305.82 (PCR fragment size of 618 bp) had fragments of 310, 187 and 100 bp in size. For isolate CBS 334.66, that had a PCR product of 570 bp, fragments of 242, 187 and 120 bp were produced. For isolates L204, S225 and S227 (PCR fragments of 622 and 570 bp) restriction fragments of 315, 242, 187 and 120 bp were obtained, while fragments of 310, 242, 187 and 120 bp were obtained for isolates L236, DK37, A3, A4 and A11 (PCR fragments of 638 and 570 bp). RFLP fingerprints obtained using the endonuclease *CfoI* thus also support the heterogenic nature of the IGS region as indicated by the sequence data. Together with the *AluI* fingerprints all five combinations of sequences, as seen during sequence analysis, could be distinguished.

All isolates of *A. areolatum* could be distinguished from isolates of *A. chailletii*, *A. laevigatum* and *A. ferreum* by RFLP fingerprints obtained using the restriction endonuclease *CfoI* (Figure 6b). Restriction fingerprints using the restriction endonuclease *CfoI* (Figure 6b) also differentiated isolates of *A. chailletii* from isolates of *A. laevigatum* (CBS 624.84) and *A. ferreum* (CBS 633.84). The isolates of *A. laevigatum* (CBS 624.84)



and *A. ferreum* (CBS 633.84), however, produced identical fingerprints when the PCR products were digested with *Cfo*I. The PCR product of isolate Stillwell 309(3) produced restriction fragments of similar size to *A. laevigatum* and *A. ferreum* when cleaved with *Cfo*I. Restriction fragment size patterns were 139 and 351 bp for *A. laevigatum*, 139 and 353 for *A. ferreum* and 139 and 337 for isolate Stillwell 309(3). PCR products from isolates of *A. chailletii* resulted in restriction fragments with sizes of 261 and 139 bp.

## DISCUSSION

In this study we have shown that isolates of *A. areolatum* used, contained one or two of four different sequences (groups A, B, C and D) in any of five combinations (A/B, A/D, B/D, C and D). Two different sized PCR products indicated the existence of 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. Such heterogenic sequences are known to occur in some Basidiomycetes and could be contained in different ways in the genome of the fungus. Hibbett & Vilgalys (1991) reported on 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 nuclei contained in dikaryons or as divergent paralogs. The existence of such paralogs in some isolates of *A. areolatum* is not unexpected considering the heterokaryotic nuclear state that dominates its life cycle. The predominantly vegetative form of reproduction of the fungus, in association with the wood wasp vector, would help to sustain such heterogeneity. This association is highly specific, with a clonal line of the fungus carried by a specific wasp and its descendants (Vasiliauskas, Stenlid, & Thomsen, 1998; Thomsen, 1999; Chapter 2). 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, Stenlid, & Thomsen, 1998; Thomsen, 1999; Chapter 2) would still prevent these sexually derived fungal descendants from recombining with isolates of the fungus carried by wood wasps.

Isolates, presumably from *S. noctilio*, always contained the group A sequence, but group A sequence was also found in isolates presumed to be from *S. juvencus*. The same

situation applied to sequence group B that was present in isolates of the fungus from *S. noctilio* and *S. juvencus*. Sequence group D was always associated with isolates believed to be from *S. juvencus*, but sequence D was always associated with either sequence A or B. Both groups A and B were found associated with both *S. noctilio* and *S. juvencus*. Neither the heterogenic sequences contained in some isolates of *A. areolatum*, nor the different combinations of these sequences, were thus always consistent in their association with *S. noctilio* or *S. juvencus*. This observation can be explained in two 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 allow the different species of wasp to come in contact with the different DNA sequence groups of *A. areolatum*.

Gaut (1969) reported differences in protein banding patterns between the isolate of *A. areolatum* from France that he uses as reference culture, and his isolate from *S. noctilio*-infested wood in Tasmania. He ascribes these differences, that are quite substantial (6 bands out of 20 were variable), as normal variation within the species. Isolate CBS 305.82 is a sub-culture of the isolate that Gaut (1969) used as reference culture, and which from our data is homogenic for sequence C. 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) could be explained by the different groupings within *A. areolatum* that are highlighted by this 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. This is consistent with the results from a previous study (Chapter 2) where isolates of the fungus from these regions showed a high degree of similarity. Isolates A3, A4 and A11 (isolated from cultures of the biocontrol nematode, *Deladenus siricidicola*) contained sequences B and D, while isolates from South Africa and Brazil contained sequences A and B. These results confirm those of somatic compatibility studies (Chapter 2) that showed that these are distinct genetic entities. It is also in accordance with the conclusion that a different genetic entity of *A. areolatum* has been inadvertently

introduced into to the countries of the Southern Hemisphere that have imported *D. siricidicola* as biocontrol agent from Australia.

DNA sequence analysis of the region from all four species of *Amylostereum* showed that *A. ferreum* and *A. laevigatum* are the most closely related species within the genus. These two species are also the only two species of *Amylostereum* not associated with wood wasps. Together, *A. ferreum* and *A. laevigatum* were more closely related to *A. chailletii* than to *A. areolatum*. *A. areolatum* was the most clearly defined species within the genus. These results confirm the findings and conclusions of a previous study (Chapter 3) that were based on analysis of 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 *AluI* and *CfoI* restriction endonucleases. *A. laevigatum* and *A. ferreum* gave similar restriction patterns using the endonuclease *CfoI*, but could be delineated using the endonuclease *AluI*. The sum of RFLP fragment sizes resulting in a total size that is smaller than the size of the original PCR product were attributable to the existence of unresolved fragments smaller than 100 bp. Hibbett & Vilgalys (1991) also attributed such smaller sums to small unresolved fragments or fragments of similar size, migrating together. Including these smaller fragments, that were inferred from sequence data, in the analysis, defied the objective of developing an easy method for identifying species specific RFLP fingerprints for identifying the different species of *Amylostereum*. Such fragments were thus excluded for the purposes of this study. These results now provide a means to easily distinguish between species, such as has been achieved with other fungi (Harrington & Wingfield, 1995).

Isolate Stillwell 309(3) collected from the mycangium of *S. areolatus* (thus expected to be *A. chailletii* according to Gaut, 1970), gave a unique RFLP banding pattern (with the endonuclease *AluI*) when compared to all other isolates included in the study. With the endonuclease *CfoI* the restriction fragment pattern of isolate Stillwell 309(3) was very

similar (except for a small size difference in one fragment) to that of *A. laevigatum* and *A. ferreum*. Sequence analysis of the region also showed that isolate Stillwell 309(3) is different to all the known species of *Amylostereum*, although it is most closely related to *A. laevigatum*. This finding supports the view (Chapter 3) that isolate Stillwell 309(3) is closely related to, but different from *A. laevigatum* and *A. ferreum*. This isolate might represent a species of doubtful authenticity, a distinct species or a sub-group of *A. laevigatum* and *A. ferreum*. As neither *A. laevigatum* nor *A. ferreum* has been implicated in associations with wood wasps, this finding warrants further investigation.

## REFERENCES

- Baxter, A. P., Rong, I. H. & Schutte, A. L. (1995). *Amylostereum areolatum* (Aphylliphorales: Stereaceae) in South Africa. *South African Journal of Botany* **61**, 352-354.
- Benson, R. B. (1943). Studies in Siricidae, especially of Europe and southern Asia (Hymenoptera, Symphyta). *Bulletin of Entomological Research* **34**, 27-51.
- Buchner, P. (1928). Holznahrung und Symbiose. Vortrag gehalten auf dem X internationalen Zoologentag zu Budapest am 8 September 1927. Springer, Berlin, 1928, 13 - 16.
- Cartwright, K. St. G. (1929). Notes on fungus associated with *Sirex cyaneus*. *Annals of Applied Biology* **16**, 182-187.
- Coetzee, M. P. A. (1997). Characterisation of *Armillaria* in South Africa. M.Sc. thesis. University of the Orange Free State, Bloemfontein, South Africa.
- Chamberlin, W. J. (1960). *Insects Affecting Forest Products and Materials*. O.S.C. Cooperative Association: Oregon.
- Chou, C. K. S. (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 Inferenec Package), Version 3.5. University of Washington.
- Francke-Grosman, H. (1939). Über das zusammenleben von holzwespen (Siricinae) mit pilzen. *Zeitschrift für angewandte Entomologie* **25**, 647-680.
- Gaut, I. P. C. (1969). Identity of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Biological Sciences* **22**, 905-914.
- Gaut, I. P. C. (1970). Studies of siricids and their fungal symbionts. Ph.D. thesis. University of Adelaide, Australia.
- Gieser & Rizzo (1998). Identification and isolation of nuclear haplotypes from dikaryotic specimens of *Phellinus* for molecular population and phylogenetic analyses. Abstracts of papers and posters presented at the annual meeting of the Mycological Society of America, 11-16 June 1998, San Juan, Puerto Rico, USA. *Inoculum (supplement to Mycologia)* **49**, 21.

- Harrington, T. C. & Wingfield, B. D. (1995). A PCR-based identification method for species of *Armillaria*. *Mycologia* **87**, 280-288.
- Hibbett, D. S. & 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, P. T-W. (1996). The taxonomy and phylogeny of the mycangial fungi from *Dendroctonus brevicomis* and *D. frontalis* (Coleoptera : Scolytidae). D.phil thesis. Iowa State University. Ames, Iowa.
- King, J. M. (1966). Some aspects of the biology of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Botany* **14**, 25-30.
- Madden, J. L. (1981). Egg and larval development in the woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology* **29**, 493-506.
- Madden, J. L. (1988). *Sirex* in Australasia. In *Dynamics of Forest Insect Populations. Patterns, Causes, Implications*. (ed. A. A. Berryman), pp. 407-429. Plenum Press, New York.
- Madden, J. L. & Coutts, M. P. (1979). The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). In *Insect-Fungus Symbiosis* (ed. L. R. Batra), pp. 165-174. John Wiley & Sons, New York.
- Morgan, F. (1968). Bionomics of Siricidae. *Annual Review of Entomology* **13**, 239-256.
- Neumann, F. G., Morey, J. L. & McKimm, R. J. (1987). The *Sirex* woodwasp in Victoria. Department of Conservation, Forest and Lands, Victoria, Bulletin No. **29**, 41pp.
- Parkin, E. A. (1941). Symbiosis in larval Siricidae (Hymenoptera). *Nature* **147**, 329.
- Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Applied Microbiology* **1**, 17-20.
- 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*, (ed. E. Korpilahti, T. Salonen & S. Oja), p. 95. Gummerus, Jyväskylä, Finland.
- Swofford, D. L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by Illinois Natural History Survey, Champaign, Illinois.

- Talbot, P. H. B. (1964). Taxonomy of the fungus associated with *Sirex noctilio*. *Australian Journal of Botany* **12**, 46-52.
- Tamblyn, N. & Da Costa, E. N. B. (1958). A simple technique for producing fruit bodies of wood-destroying basidiomycetes. *Nature* **181**, 578-579.
- Thomsen, I. M. (1996). *Amylostereum areolatum* and *Amylostereum chailletii*, symbiotic fungi of woodwasps (*Sirex* sp. and *Urocerus* sp.). Ph.D. thesis. Danish Forest and Landscape Research Institute, Horsholm, Denmark.
- Thomsen, I.M. & Koch, J. (1999). Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research* **103**, (in press).
- 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, I. M. (1998). Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from Northern Europe. *New Phytologist* **139**, 751-758.

**Table 1:** Isolates of *Amylostereum* used in this study.

Culture nr.	Identity	Host or source of isolate	Origin	Date isolated	Collector
<b>CBS cultures</b>					
305.82	<i>Amylostereum areolatum</i>	Unknown	France	1964	J. Boiden
334.66	<i>A. areolatum</i>	<i>Picea abies</i>	Germany	1967	Dimitri
483.83	<i>A. chailletii</i>	Mycangium of <i>Urocerus gigas</i>	Scotland, UK	1981	D.B. Redfern
624.84	<i>A. laevigatum</i>	<i>Juniperus nana</i>	France	1978	P. Lanquetin
633.84	<i>A. ferreum</i>	<i>Podocarpus lambertii</i>	Brazil	1978	R.T. Guerrero
<b>CLBRR cultures</b>					
DAOM 21327	<i>A. chailletii</i>	Sporophore on <i>Abies balsamea</i>	Ontario, Canada	1948	R.F. Cain
54-95	<i>A. chailletii</i>	Sporophore on fallen log in stand of hemlock	Ontario, Canada	1954	A. Hill & S. Gibson
Stillwell 309(3)	<i>Amylostereum</i> sp.	Mycangium of <i>S. areolatus</i>	California, U.S.A.	Unknown	Stillwell
Waite Inst. 6195	<i>A. areolatum</i> <sup>1</sup>	Mycangium of <i>S. noctilio</i>	Tasmania	1962	Unknown
DAOM 21785	" " 1	Oviposition sites of <i>S. noctilio</i> in <i>P. radiata</i> wood	New Zealand	Unknown	G.B. Rawlings
<b>Other European isolates</b>					
L204	<i>A. areolatum</i> (± Clone S) <sup>2</sup>	Wood of wounded <i>P. abies</i>	Lithuania	1994	R. Vasiliauskas
L236	" " (Clone A) <sup>2</sup>	Wood of wounded <i>P. abies</i>	Lithuania	1995	R. Vasiliauskas
DK37	" " (Clone A) <sup>2</sup>	Fruiting body on <i>P. abies</i>	Denmark	1993	I.M. Thomson
DK782	" " (Clone B) <sup>2</sup>	Fruiting body on <i>P. abies</i>	Denmark	1987	J. Koch
S225	" " (Clone S) <sup>2</sup>	Wood of wounded <i>P. abies</i>	Sweden	1994	R. Vasiliauskas
S227	" " (Clone S) <sup>2</sup>	Wood of wounded <i>P. abies</i>	Sweden	1994	R. Vasiliauskas
<b>Australian isolates</b>					
*A3, A4, A11	<i>A. areolatum</i> <sup>1</sup>	Isolates from nematode cultures from CSIRO	Australia	1995	B. Slippers
<b>South American isolates</b>					
*Br17, Br60	<i>A. areolatum</i> <sup>1</sup>	Mycangia of <i>S. noctilio</i> wasps	Brazil	1997	B. Slippers
<b>South African isolates</b>					
*M5W	<i>A. areolatum</i> <sup>1</sup>	Wood around <i>S. noctilio</i> in <i>P. radiata</i>	South Africa	1994	M.J. Wingfield
*SN19A	" " 1	Mycangia of <i>S. noctilio</i> wasps	South Africa	1996	B. Slippers

<sup>1</sup>/ Identity as determined in a previous study (Chapter 3).

<sup>2</sup>/ Vegetative compatibility group as determined by Thomsen (1996).



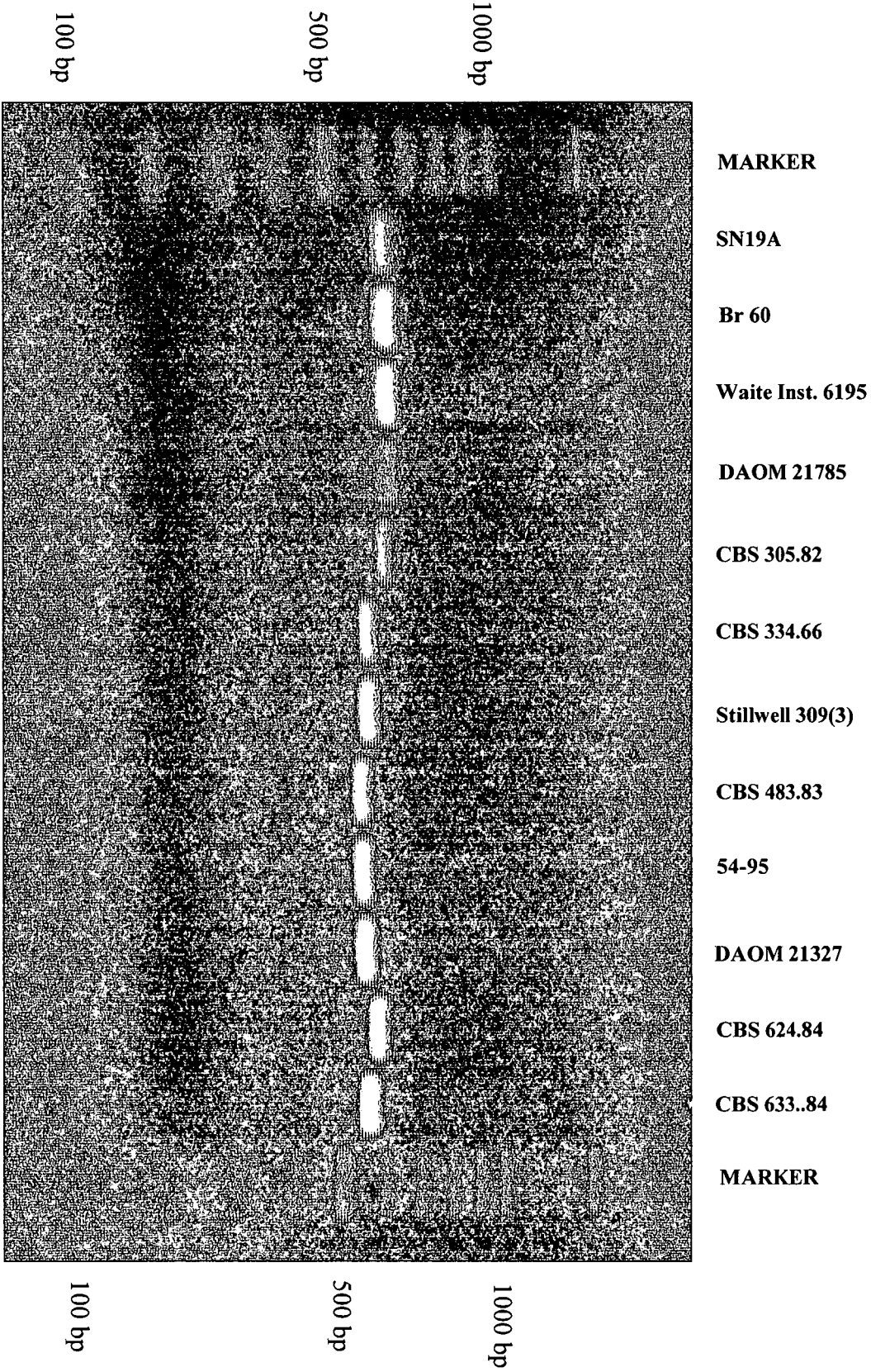
**Table 2:** Combinations of heterogenic sequences<sup>1</sup> of the nuc-IGS-rDNA region of isolates of *A. areolatum*.

Sequence <sup>1</sup>	Isolate nr.	Origin	Associated wasp species	VCG <sup>2</sup>
A/B	DAOM 21785	New Zealand	<i>Sirex noctilio</i>	SH (±)
	WaiteInst. 6195	Tasmania	<i>S. noctilio</i>	SH (±)
	Br17	Brazil	<i>S. noctilio</i>	SH
	Br60	Brazil	<i>S. noctilio</i>	SH
	M5W	South Africa	<i>S. noctilio</i>	SH
	SN19A	South Africa	<i>S. noctilio</i>	SH
	DK782	Denmark	<i>S. juvencus</i>	B
A/D	L204	Lithuania	<i>S. juvencus</i>	S (±)
	S225	Sweden	<i>S. juvencus</i>	S
	S227	Sweden	<i>S. juvencus</i>	S
B/D	L236	Lithuania	<i>S. juvencus</i>	A
	DK37	Denmark	<i>S. juvencus</i>	A
	A3	Australia	Unknown	?
	A4	Australia	Unknown	?
	A11	Australia	Unknown	?
C	CBS 305.82	France	Unknown	?
D	CBS 334.66	Germany	Unknown	?

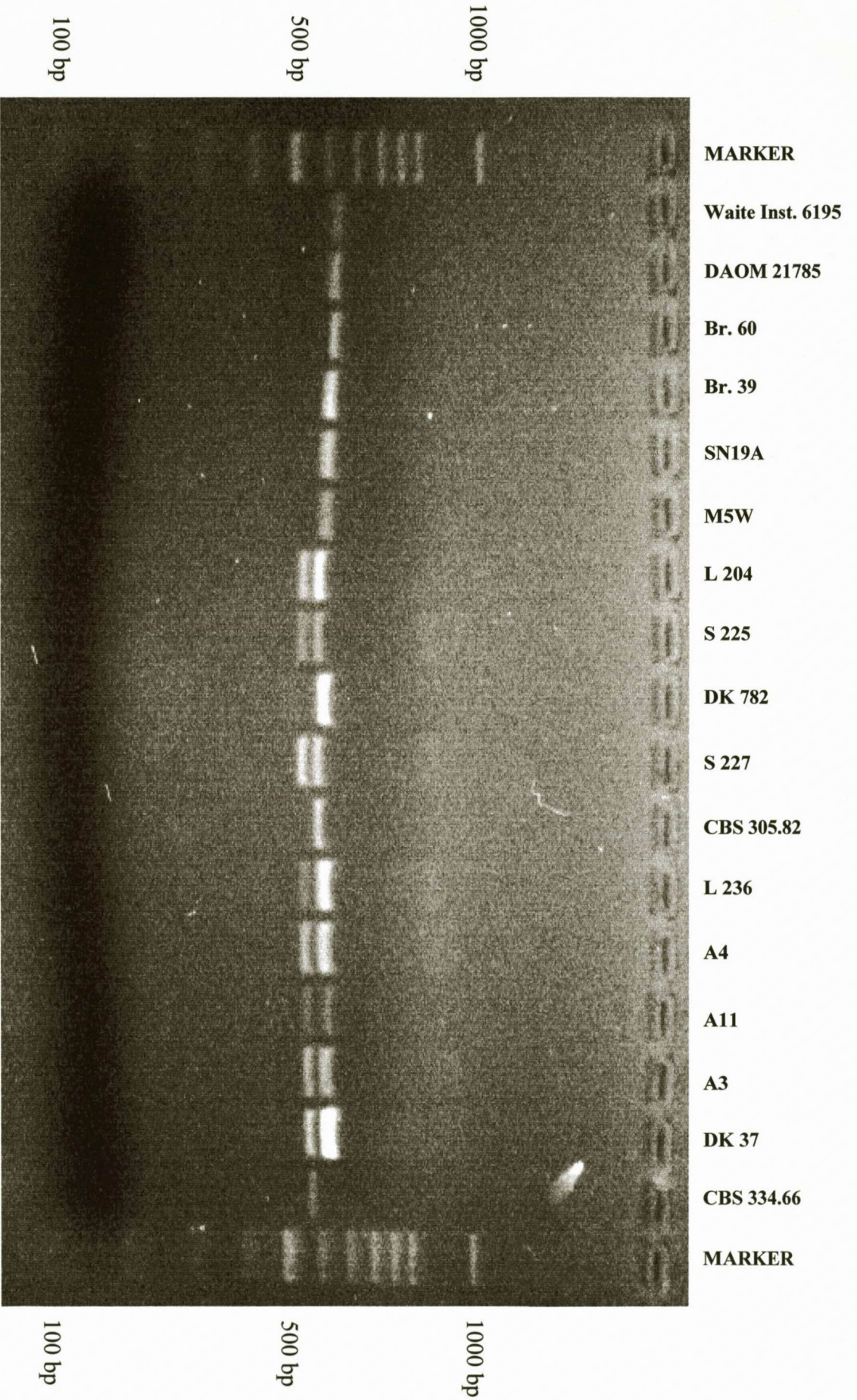
<sup>1</sup>/ These sequences were observed during sequencing and their presence confirmed using RFLP analysis of the PCR fragments. Their simultaneous presence in sequencing reactions, however, made it impossible to obtain their full sequence without cloning, as was done for isolate DK 37.

<sup>2</sup>/ VCG's as determined in a previous study (Chapter 2) and by Thomsen (1996).

**Figure 1:** A 1.5 % agarose gel stained with ethidium bromide, showing the IGS PCR fragments of the different species of *Amylostereum*. A 100 bp size marker was run on either side of the set of PCR fragments. Isolates S19A, Br60, Waite Inst. 6195, DAOM 21785, CBS305.82 and CBS334.66 represent *A. areolatum*. Isolate Stillwell 309(3) represent an *Amylostereum* sp. Isolates CBS483.83, 54-95 and DAOM 21327 represent *A. chailletii*, isolate CBS624.84 *A. laevigatum* and isolate CBS633.84 *A. ferreum*.



**Figure 2:** IGS PCR fragments of isolates of *A. areolatum* visualised on a 1.5 % agarose gel stained with ethidium bromide. 100 bp size marker were included as size standards in marker lanes. Isolates are those identified in Table 1.



**Figure 3:** Aligned DNA sequence data for the IGS of the rDNA operon for isolates representing the species of *Amylostereum*. Unknown sequence on the flanking regions of the fragment are indicated by lettering in italics. These sequences were inferred from the matching sequence of the highly conserved 5S (3') and 28S (5') rDNA genes between isolate DK37 (full sequence were determined after cloning) and various *Armillaria* species (Coetzee, 1997). Restriction sites for the restriction endonuclease *AluI* are shaded (▒) and underlined (⏟) for *CfoI*. Gaps inserted due to alignment are indicated by a dash (-).

*A. ferreum* is represented by isolate CBS 633.84, *A. laevigatum* by isolate CBS 624.84, *A. chailletii* by isolate CBS 483.83 and the *Amylostereum* sp. by isolate Stilwell 309(3). Sequence for *A. areolatum* (A) was obtained from isolate DAOM 21785, *A. areolatum* (B) from cloned PCR products of isolate DK 37, *A. areolatum* (C) from isolate CBS 305.82 and *A. areolatum* (D) also from cloned PCR products of isolate DK 37. These sequences were also observed for other isolates of *A. areolatum* (Table 2).

	10	20	30	40	50	60	70	80
<i>A. ferreum</i>	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>A. laevigatum</i>	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>Amylostereum</i> .sp.	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>A. chaillietii</i>	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>A. areolatum</i> (A)	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>A. areolatum</i> (B)	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>A. areolatum</i> (C)	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
<i>A. areolatum</i> (D)	TTGCAGACGA	CTTGAATGGA	ACGGGGTACT	GTAAGCGGTA	GAGTAGCCTT	GTTGCTACGA	TCCGCTGAGG	TTAAGCCCTT
	90	100	110	120	130	140	150	160
<i>A. ferreum</i>	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	TCTTCCGCGC	GGGGGCTTTA	GGGCAGGGCT
<i>A. laevigatum</i>	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	TCTTCCGCGC	GGGGGCTTAA	GGGCAGGTCT
<i>Amylostereum</i> .sp.	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	TCTTCCGCGC	GGGGGCTTAA	GGGCAGGTCT
<i>A. chaillietii</i>	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	TCTTCCGCGC	GGGGGCTTAA	GGGCAGGTCT
<i>A. areolatum</i> (A)	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	-CTTCCGCAC	GACGGCTTTA	GGGCAGGGC-
<i>A. areolatum</i> (B)	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	-CTTCCGCAC	GACGGCTTTA	GGGCAGGGC-
<i>A. areolatum</i> (C)	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	-CTTCCGCAC	GACGGCTTTA	GGGCAGGGC-
<i>A. areolatum</i> (D)	GTTTCGACAGA	TTTGTTCAAC	CTCGGTTGGA	CTTCTCTCTC	TTCTCTTTTT	-CTTCCGCAC	GACGGCTTTA	GGGCAGGGC-
	170	180	190	200	210	220	230	240
<i>A. ferreum</i>	TTT--CAGAC	TTGTGCTGTT	CCACGCGCGT	GTG-AGGAAA	-GGGAAGGGG	G---TTT---	--CGCAATTT	TGAAGGGTT-
<i>A. laevigatum</i>	TTT--CAGAC	TTGTGCTGTT	CCACGCGCGT	CTG-AGGAAA	-GGGAAGGGG	----ATT---	-GCGCAATTT	TGAAGGGTT-
<i>Amylostereum</i> .sp.	TTT--CAGAC	TTGTGCGGTT	CCACGTGCGT	GTG-AGGAAA	-GGGAAGGGG	G---GTT---	-GCGCAATTT	TGAAGGGCT-
<i>A. chaillietii</i>	TGT--CAGAC	TTGTGCTGTT	CCACGCGCGT	GTG-AGGAAA	-GGGAAGGGG	G---CTT---	--CGCAATTT	TGAAGGGTT-
<i>A. areolatum</i> (A)	TGTTCCAGAC	TTGTGCTGTT	CC----TCGT	GCGCAGGAAA	AGCGAAGGGG	GAAGGTTATG	TACGCA--TG	TCAAAGGT--
<i>A. areolatum</i> (B)	TGTTCCAGAC	TTGTGCTGTT	CC----TCGT	GCGCAGGAAA	AGCGAAGGGG	GAAGGTTATG	TACGCA--TG	TCAAAGGTTG
<i>A. areolatum</i> (C)	TGTTCCAGAC	TTGTGCTGTT	CC----TCGT	GCGCAGGAAA	AGCGAAGGGG	GAAGGTTATG	TACGCA--TG	TCAAAGGTT-
<i>A. areolatum</i> (D)	TGTTCCAGAC	TTGTGCTGTT	CC----TCGT	GCGCAGGAAA	AGCGAAGGGG	GAAGGTTATG	TACGCA--TG	TCAAAGGTTG

	250	260	270	280	290	300	310	320
<i>A. ferreum</i>	-----	-----	-----CCGTA	CA-A-CGA--	-----GTTG	-A-AGAAGGA	-----	-----
<i>A. laevigatum</i>	-----	-----	-----CTGTA	CAGAGCGAAG	GGGG-AGTTG	-A-AGGG-GA	-----	-----
<i>Amylostereum.sp.</i>	-----	-----	-----CTGTA	CAGAATGAAG	GGGGGAGTTG	-A-AGGG--	-----	-----
<i>A. chailletii</i>	-----	-----	-----CTG--	-----TGCAG	GGGG--TTG	-A-AGGGTGA	-----	-----
<i>A. areolatum</i> (A)	-----	-----G	AGGTTCTCGA	CAGAATGGTT	GAGGTACTCG	TCCGAGGTAA	AGTGGAACCTT	GTGCGCGGTA
<i>A. areolatum</i> (B)	GCATACAAAA	ACGAAGGTCG	AGGTTCTCGA	CGGAATGAAG	GCGGACCTTG	TACAGGGTGA	AGTGCGACAT	TTGCGCAAAA
<i>A. areolatum</i> (C)	-----	-----G	AGGTTCTCGA	CGGAATGAAG	GCGGACCTTG	TACAGGGTGA	AGTGCGACAT	TTGCGCAAAA
<i>A. areolatum</i> (D)	GCATACAAAA	ACGAAGGTCG	AGGTTCTCGA	CGGAATGAAG	GCGGACCTTG	TACAGGGTGA	AGTGCGACAT	TTGCGCAAAA

	330	340	350	360	370	380	390	400
<i>A. ferreum</i>	-----	--TTGGATGC	AAAATG----	-----	-----AA-	-A--GAGCAT	GCTTGAGGGA	AG---TTGAA
<i>A. laevigatum</i>	-----	GTTTGGGA--A	AAAATG----	-----	-----AA-	-AGGGAGCA-	-CTTGAAAGA	AA---TTGAA
<i>Amylostereum.sp.</i>	-----GC	GTTTGGGA--C	AAAATG----	-----	-----AA-	-AGGGTGA-	-CTTGAAAAA	AA---TTGAA
<i>A. chailletii</i>	-----	--TTGGA--	AAAATG----	-----	-----AA-	-AGGGGGC--	GCTTGAAGGG	GCGGTTTGAA
<i>A. areolatum</i> (A)	TGTTGGGAGT	TCTTCA---C	AAAATGTAGG	AGGTACCTGA	GAAGGATAAG	GATGGAACAA	AGATGTGGGA	GTTTCAAA
<i>A. areolatum</i> (B)	TGTAGGGGGT	GCTTGAA--G	AAAATG----	-----	-----AAG	-AGGGAACAA	GGA-GGGGA	G-GGTTTGAA
<i>A. areolatum</i> (C)	TGTAGGGGGT	GCTTGAA--G	AAAATG----	-----	-----AAG	-AGGGAACAA	GGA-GGGGA	G-GGTTTGAA
<i>A. areolatum</i> (D)	TGTAGGGGGT	GCTTGAA--G	AAAATG----	-----	-----AAG	-AGGGAACAA	GGA-GGGGA	G-GGTTTGAA

	410	420	430	440	450	460	470	480
<i>A. ferreum</i>	GGGGCGGTTT	GAACGAAACG	AGGGTGCAC	ATTCGTACGA	AACGTAGTCA	AACTTCAAAC	CGAAATAAAC	AAAGTGCTC-
<i>A. laevigatum</i>	GGGACGGTTT	GAACAAAACG	AAGGTGCAC	ATTTTTCGA	AACGTACTCG	TCAAAC	CAAAATGAAC	AAAGTGTTTG
<i>Amylostereum.sp.</i>	GGGACGGTTT	GAACAAAACA	AAGGTGCAC	ATTTGTACGT	AACGTAGTCG	TCAAAC	CAAAATGAAC	AA-GTGTGTTG
<i>A. chailletii</i>	CGAAATG----	-----	AAGGTGCAC	GTTTGTACGA	AACGTAGTCA	TCAAGC	CGGAATGAAC	AAAGTGTTTG
<i>A. areolatum</i> (A)	CAAGGTATAA	TCGATTC-	-----	-----TTCA	TTGCTG	CTTGAA-CAA	TGCCATATGC	-----G
<i>A. areolatum</i> (B)	CGAAGTGTAG	TGGATTC	AAAGGGAACA	AACAAAAGTG	TTCAAACCTG	CTTGAA-CAA	AGCCATATGC	-----G
<i>A. areolatum</i> (C)	CGAAGTGTAG	TGGATTC	AAAGGGAACA	AACAAAAGTG	TTCAAACCTG	CTTGAA-CAA	AGCCATATGC	-----G
<i>A. areolatum</i> (D)	CGAAGTGTAG	TGGATTC	AAAGGGAACA	AACA-----	-----	-----	-----	-----

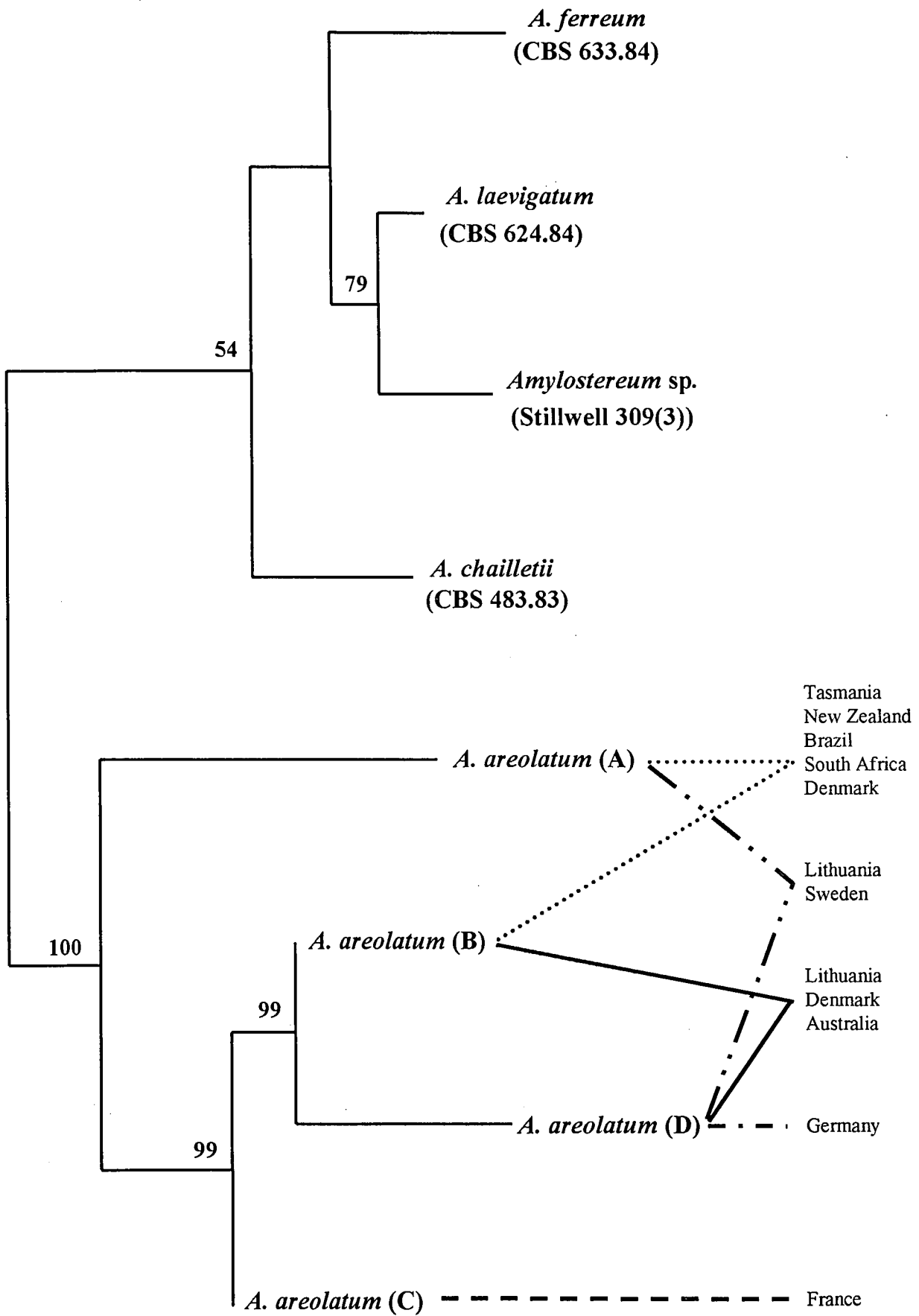


	490	500	510	520	530	540	550	560
<i>A. ferreum</i>	AA-CTG---G	A-TCG	TTGATCAAAG	TGAAGAGAGA	CTTA-C--GT	TTGGACTGTA	-GGGCAGGCC	TAAAA-AGTC
<i>A. laevigatum</i>	A	GACTG AAGCA	TTGATCAAAG	TGAAGGGAGA	--TATCAGGT	GTGGACTTCA	-GGGTGGGCC	TAAAA-A-TC
<i>Amylostereum.sp.</i>	ACGCCGAGTG	AAGCAGG--C	TTGATCAAAG	TGAAGGGATA	-TT--CAGG-	GTG-----	-----GGCC	TAAAA-AG-C
<i>A. chailletii</i>	AAGC-----	---A	TTGATCAAAA	TGAAGGGAGA	--T-TCAGGT	TTGGACTTCA	-GGGTGGGCC	TAAAA-AGTC
<i>A. areolatum</i> (A)	ACGGAT-CTG	AAGGCATATC	TC-ACGGATC	TGAAG-GGGA	-TTA-C	TTGGGCTTCA	CGGGCATTGC	TCCATGAGTC
<i>A. areolatum</i> (B)	AGGGAT-CTG	AAGGAAAA-C	TC-ACCAATG	TGAAGGGGGA	-TTA-C	TTGGGCTTCA	-GGGCAG-GC	TCAAA-AGTC
<i>A. areolatum</i> (C)	AGGGAT-CTG	AAGGAAAA-C	TC-ACCAATG	TGAAGGGGGA	-TTA-C	TTGGGCTTCA	-GGGCAG-GC	TCAAA-AGTC
<i>A. areolatum</i> (D)	-----	-----	-----	-----GGGA	-TTA-C	TTGGGCTTCA	-GGGCAG-GC	TCAAA-AGTC

	570	580	590	600	610	620	630	640
<i>A. ferreum</i>	GGG----TTT	-G-AC-----	-TTTGATTAA	AAT-GAATTA	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>A. laevigatum</i>	CAAAAATTTT	CG-ATTTTAT	TTTTAATTAA	AAT-GAATTA	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>Amylostereum.sp.</i>	CAAAAATTTT	CG-ATTTTAT	TTTTAATTAA	AAT-GAATTA	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>A. chailletii</i>	CAAAAAAAGT	CG-ATTTTAT	TTTTAATTAA	AAT-GAATTA	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>A. areolatum</i> (A)	CACAAGGACA	TGCACTT-AT	TTCTAATTAA	ATTGCAAT-A	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>A. areolatum</i> (B)	CAAAAAAAGT	CG-ATTTTAT	TTTTAATTAA	AAT-GAAT-A	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>A. areolatum</i> (C)	CAAAAAAAGT	CG-ATTTTAT	TTTTAATTAA	AAT-GAAT-A	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT
<i>A. areolatum</i> (D)	CAAAAAAAGT	CG-ATTTTAT	TTTTAATTAA	AAT-GAAT-A	AAACCACAGC	ACCCAGGATT	CCCGCGTGGT	CCCCCACCGT

	650	660	670	680	690
<i>A. ferreum</i>	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>A. laevigatum</i>	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>Amylostereum.sp.</i>	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>A. chailletii</i>	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>A. areolatum</i> (A)	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>A. areolatum</i> (B)	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>A. areolatum</i> (C)	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG
<i>A. areolatum</i> (D)	GGTACTAAGT	GGGCGGCACT	GTGGTAACT	<u>GCGCAGATCA</u>	GACGGGATGG GGTG

**Figure 4:** The most parsimonious phylogenetic tree of 537 steps (CI = 0.912; HI = 0.088; 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. Bootstrap values (1000 replicates) are given at the branching points. Different combinations of heterogenic sequences (A / B / C / D) contained in isolates of *A. areolatum* and the origin of the isolates containing each combination, are indicated (see Table 2).



**Figure 5:** Restriction maps for the restriction enzymes *AluI* (a) and *CfoI* (b) for the nuc-IGS-rDNA region of different species of *Amylostereum*. Specific restriction sites ( $\downarrow$ ) and fragment sizes (numbers indicate sizes in bp) were inferred from sequence data.

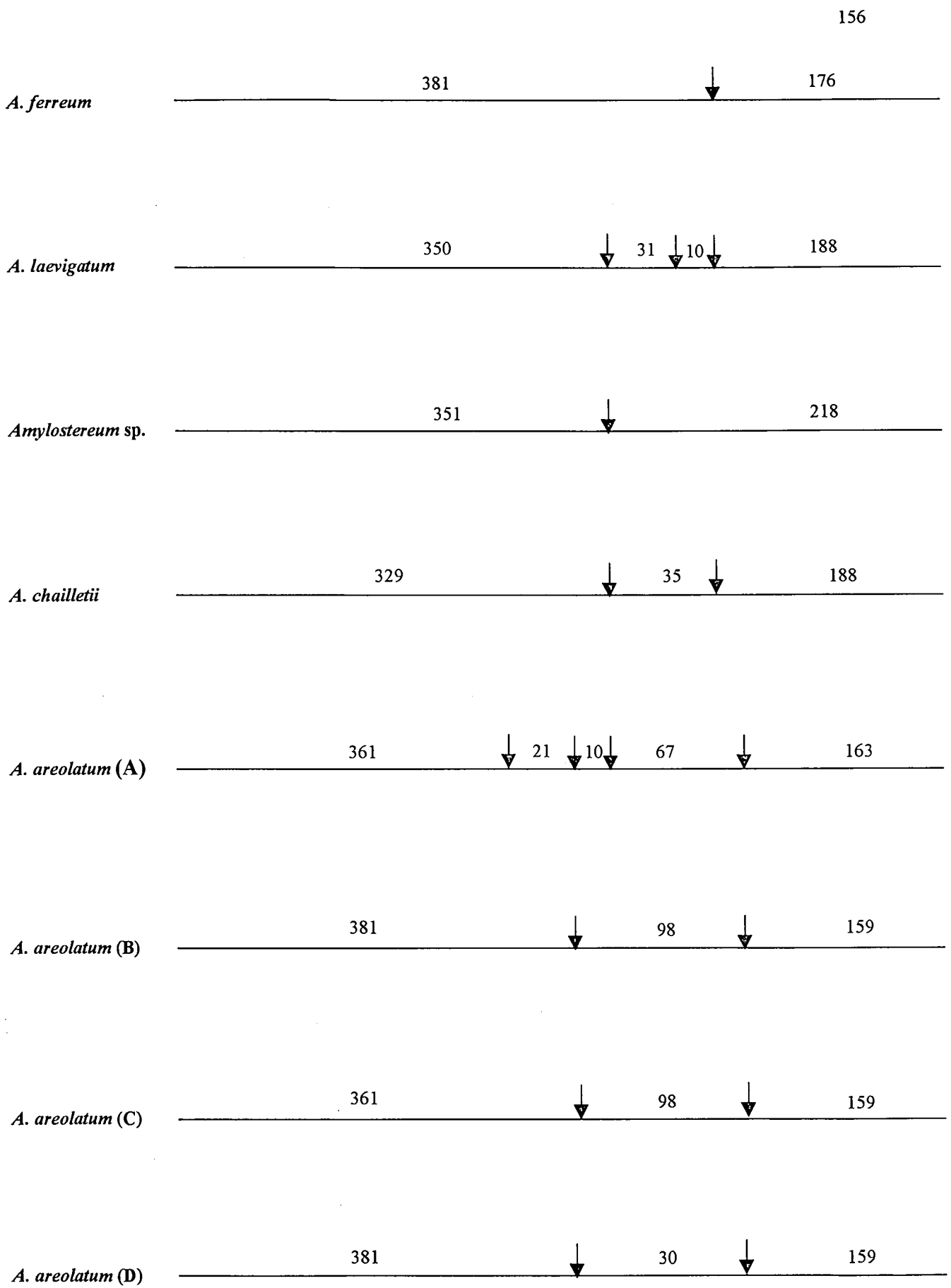


Figure 5(a)

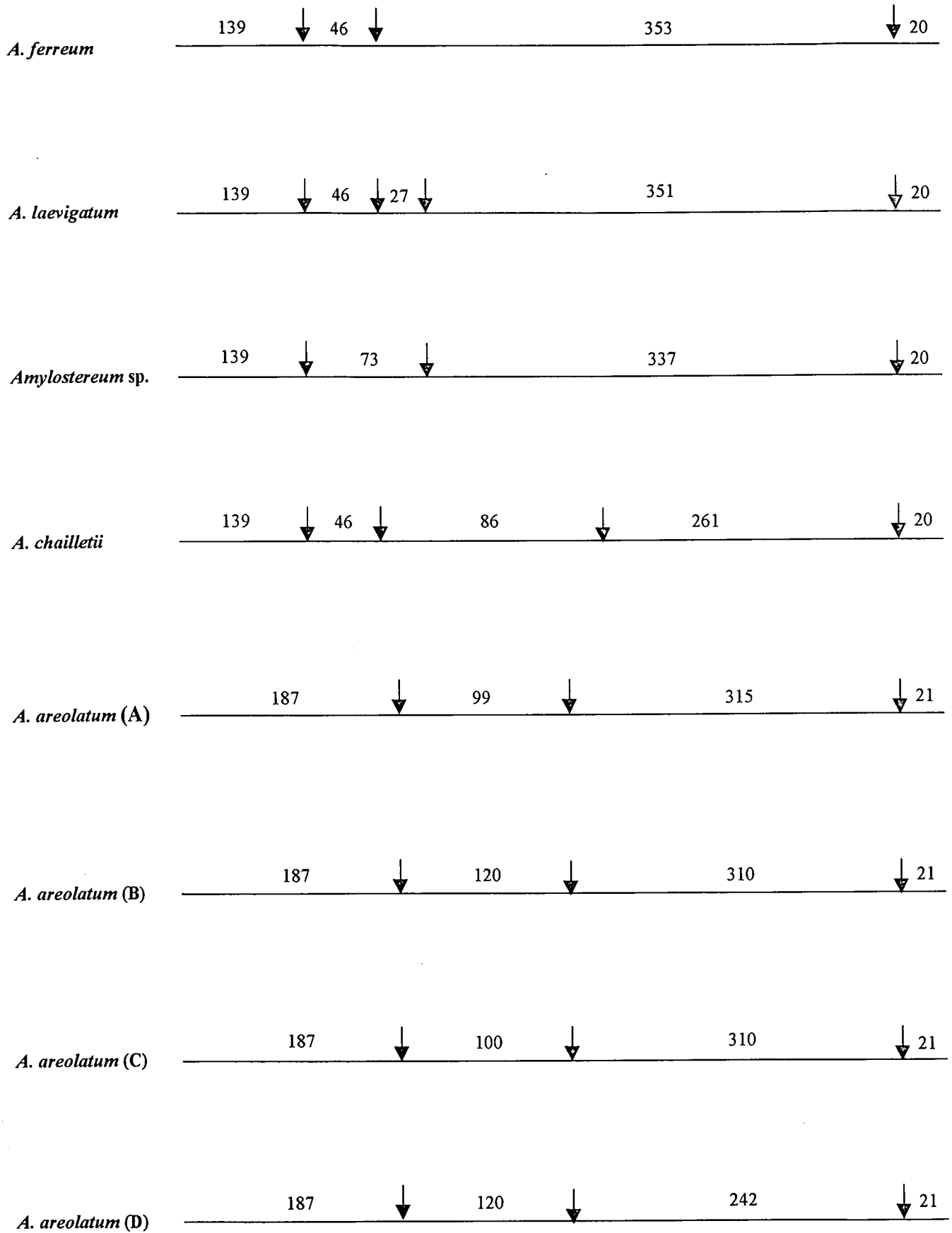


Figure 5(b)

**Figure 6:** A 2 % agarose gel stained with ethidium bromide showing *AluI* (a) and *CfoI* (b) restriction fragments of the IGS PCR products of different species of *Amylostereum*. Lanes marked a 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 *A. laevigatum* and isolate CBS633.84 *A. ferreum*.

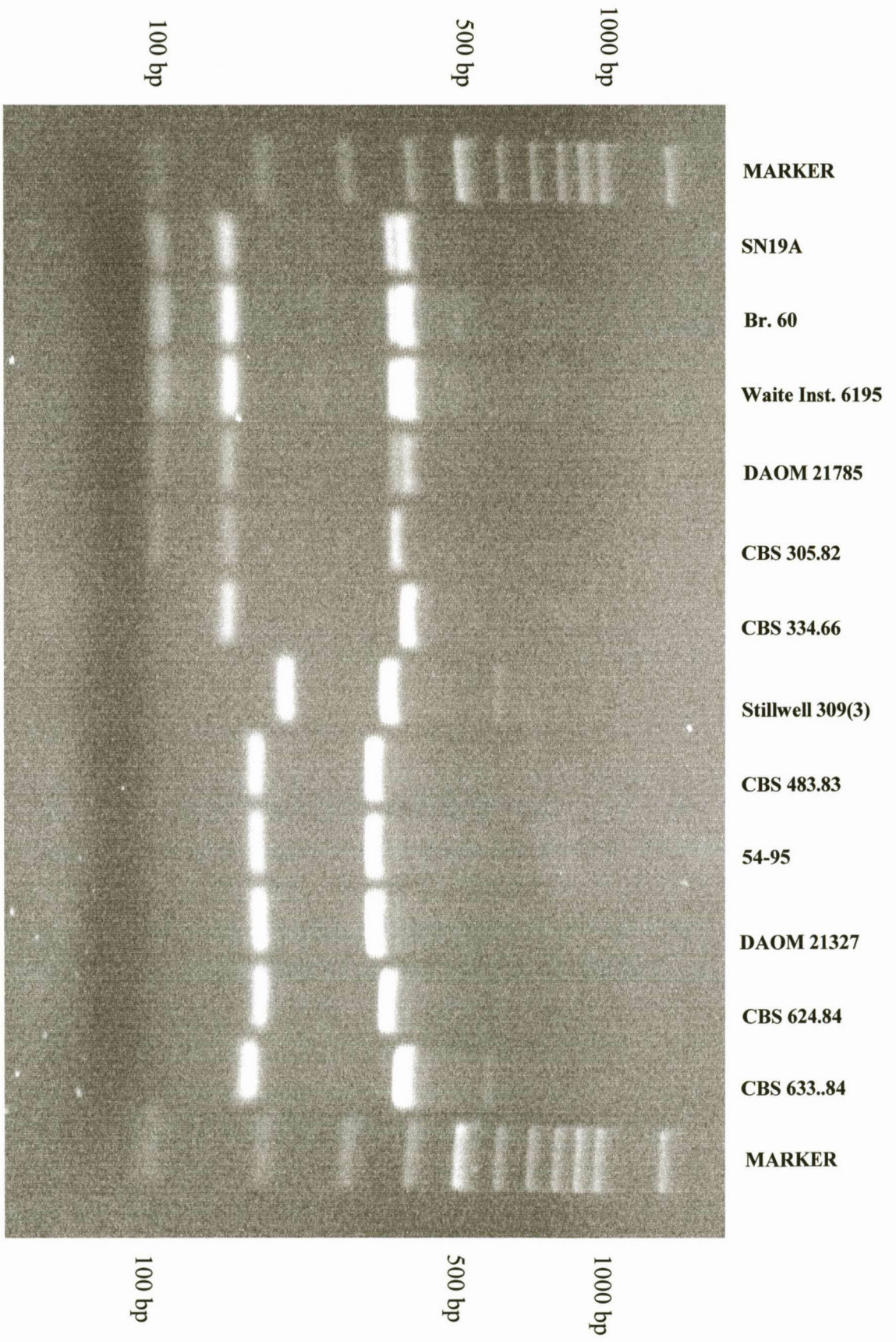


Figure 6(a)



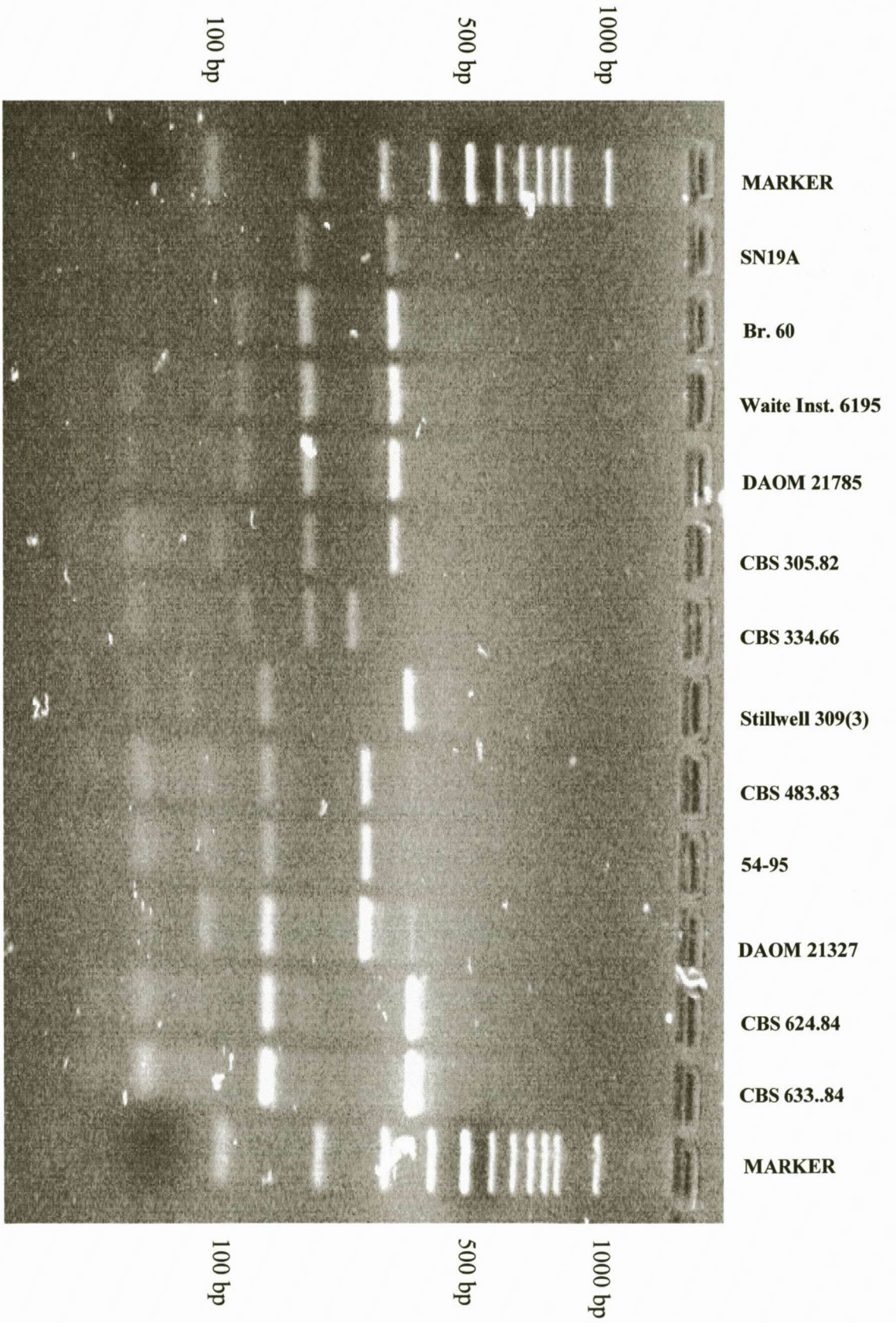
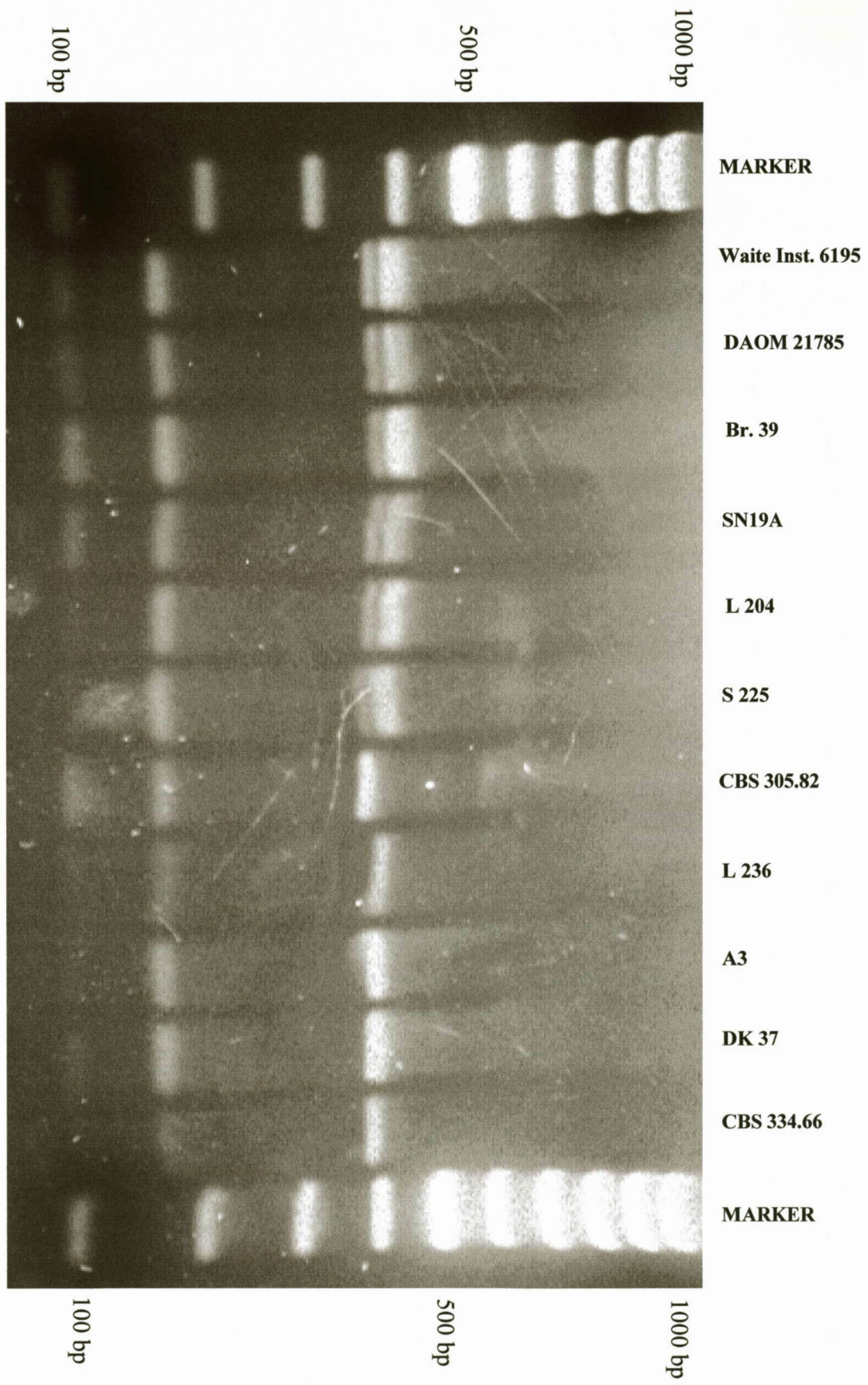


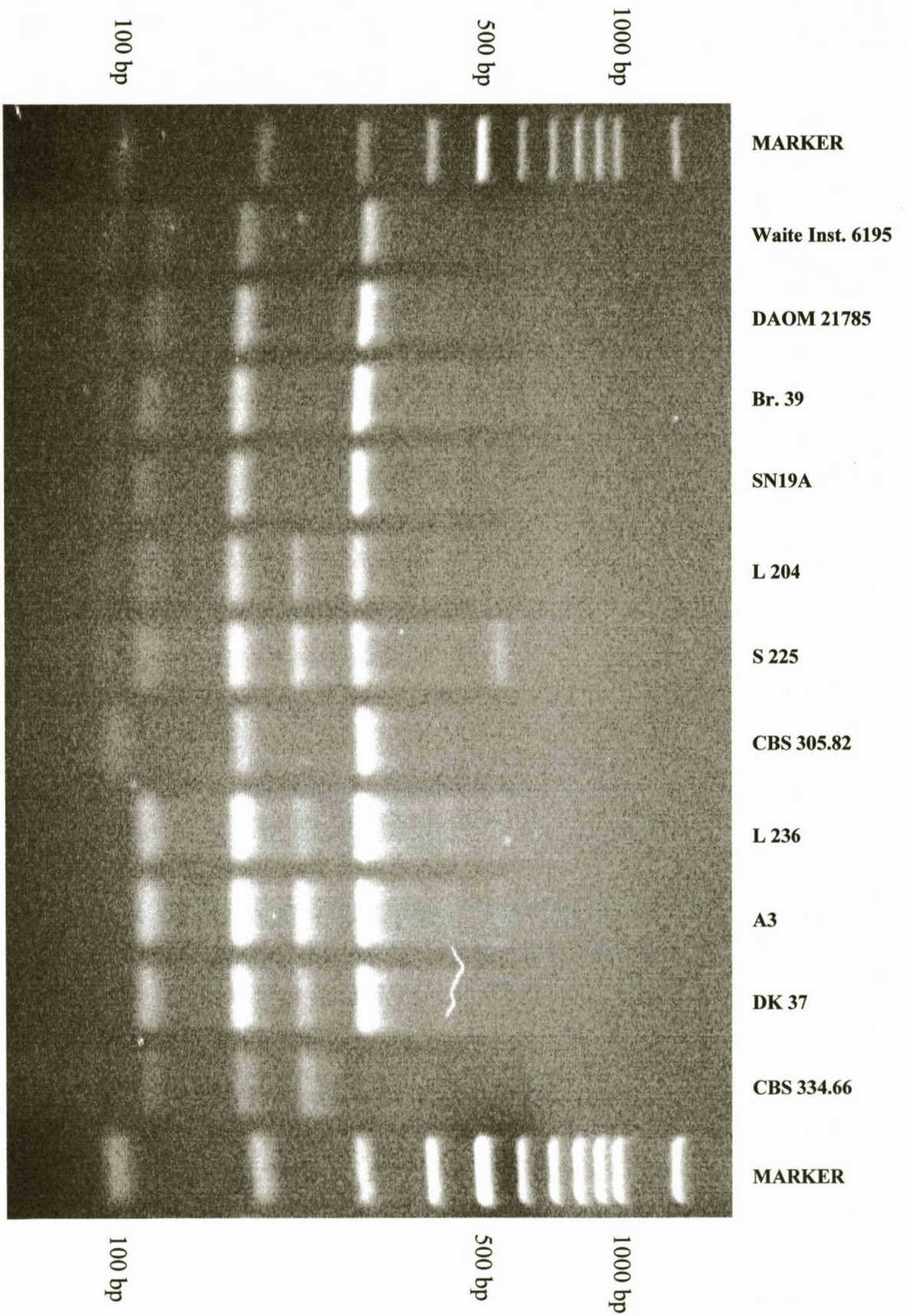
Figure 6(b)

**Figure 7:** *AluI* (a) and *CfoI* (b) restriction fragment patterns of the IGS PCR products of different isolates of *A. areolatum*, visualised on a 2 % agarose gel stained with ethidium bromide. Marker lanes contain a 100 bp size marker.



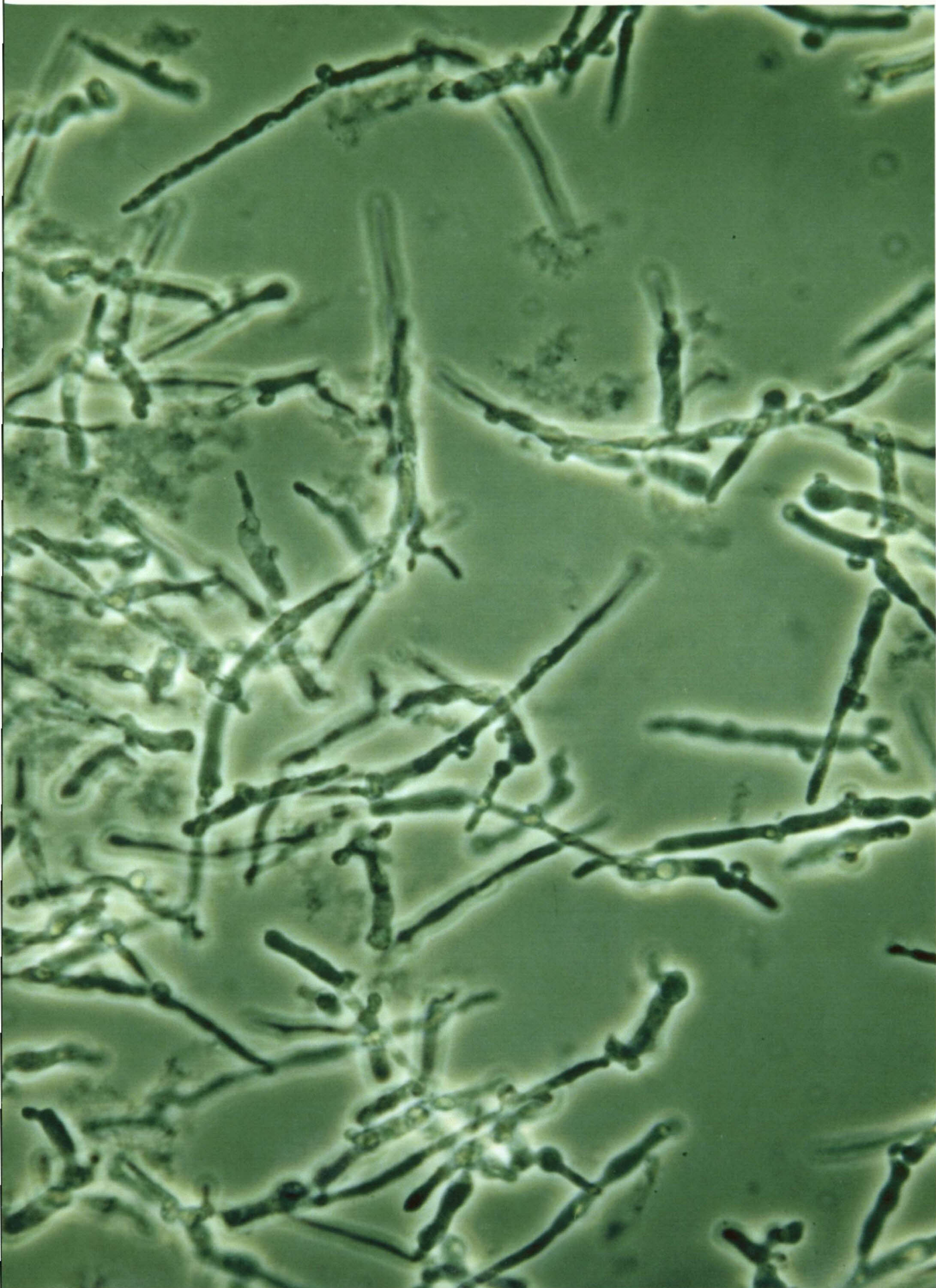
**Figure 7(a)**





**Figure 7(b)**





## THE GENUS *AMYLOSTEREUM* AND ITS ASSOCIATION WITH WOODWASPS : A CONTEMPORARY REVIEW

### ABSTRACT

A fascinating symbiosis exists between the fungi, *Amylostereum chailletii* and *A. areolatum*, and various species of Siricid woodwasps. These intrinsic symbioses and their importance to forestry have stimulated much research activity in the past. The fungi have, however, often been confused or misidentified. Similarly, the phylogenetic relationships of the *Amylostereum* spp. with each other, as well as with other Basidiomycetes, have been unclear for a long period. Recent studies based on molecular data have, however, given new insight into the taxonomy and phylogeny of the genus *Amylostereum*. Molecular sequence data have shown that *A. areolatum* is most distantly related to other *Amylostereum* spp. Of the three remaining species, *A. laevigatum* and *A. ferreum* are most closely related. Sequence data have also made it possible to develop PCR RFLP-fingerprints to delineate *Amylostereum* spp., which presents a solution to the difficulties that are generally experienced identifying these fungi using traditional methods. Furthermore, sequence data suggest that there is an evolutionary relationship between *Amylostereum* spp. and such divergent species as *Echinodontium tinctorium*, *Russula compacta*, *Heterobasidion annosum* and *Peniophora nuda*. Recent studies, investigating the population structure of *A. areolatum* and *A. chailletii*, have also substantially increased our understanding of the ecology of these species that are associated with woodwasps. Clonal lineages have been shown to occur in both *A. areolatum* and *A. chailletii*. These genetic lines, that arise as a result of the association with woodwasps, are spread over large distances and are preserved over time. This character of the populations, now presents an opportunity to trace the geographical origin of these fungi and their associated wasps. The occurrence of heterogenic sequences in the nuc-IGS-rDNA region of isolates of *A. areolatum* also gives insight into the structure and relationship between populations of this fungus that are isolated as a result of its association with different woodwasp species. In this review an overview is given of these recent developments and the opportunities for future research.

## INTRODUCTION

*Amylostereum* represents a fascinating genus of Basidiomycetes, of which two species live in a highly evolved symbiosis with Siricid woodwasps. Internal glands in some woodwasp species that contained fungal oidia were first reported in the 1920's (Buchner, 1928; Chrystal, 1928; Cartwright, 1929). The presence of clamp connections suggested that these fungi belonged to the Basidiomycetes, but their identities were uncertain. Subsequently, these fungi were identified as *Stereum sanguinolentum* (Alb. & Schw.:Fr.) Fr., *S. chailletii* (Pers.:Fr.) Fr., *Stereum* sp., *Peniophora* sp., among others (Thomsen, 1996). Eventually it was, however, conclusively shown that these fungi should reside in the genus *Amylostereum* (Talbot, 1964; Gaut, 1969 & 1970). Today it is known that two *Amylostereum* spp., *A. chailletii* (Pers.:Fr.) Boid. and *A. areolatum* (Fr.) Boid., are symbionts of a variety of woodwasp species (Gaut, 1970).

The relationship between *Amylostereum* spp. and woodwasps is highly evolved and has been shown to be always species specific (Gaut, 1970; Talbot, 1977). The principle advantage of the relationship for the fungus is that it is spread and effectively inoculated into new wood during wasp oviposition (Gilmour, 1965; King, 1966). In turn the fungus rots and dries the wood, so providing a suitable environment, nutrients and enzymes that are important for the survival and development of the insect larvae (Francke-Grosman, 1939; Morgan, 1968; Madden & Coutts, 1979; Kukor & Martin, 1983; Martin, 1987).

The burrowing activity of the Siricid larvae and rot of the wood by *Amylostereum* spp., makes this insect-fungus symbiosis potentially harmful to host trees, which include important commercial species. In the Northern Hemisphere, where the Siricidae are native, the insect is, however, of little economic importance (Hanson, 1939; Hall, 1978; Spradbery & Kirk, 1978). Here a natural balance exists between the insect-fungus complex, its natural parasites and trees. In contrast, *Sirex noctilio* Fabr. and *A. areolatum*, that have been introduced into various countries of the Southern Hemisphere, have caused extensive mortality in exotic pine plantations in this region (Neumann, Morey & McKimm, 1987; Madden, 1988; Haugen, 1990; Neumann & Marks, 1990; Chou, 1991).

The association between woodwasps and *Amylostereum* and their importance to forestry in the Southern Hemisphere, has stimulated much research in this field. This work has been reviewed a number of times in the past (Morgan, 1968; Talbot, 1977; Neumann & Minko, 1981; Madden, 1988; Bedding, 1995; Thomsen, 1996; Chapter 1). Much of this research has, however, concentrated on the woodwasps and especially their control in the Southern Hemisphere. Recent studies on the fungal symbionts of Siricidae, has given new insight into the taxonomy, phylogeny and ecology of these fungi. The aim of this review is, therefore, to provide a contemporary view of *Amylostereum* and its symbiosis with woodwasps.

## TAXONOMY AND PHYLOGENY

### **AMYLOSTEREUM SPP.**

The genus *Amylostereum* was established in 1958 by Boidin to accommodate species of *Stereum* that have, among other characteristics, smooth amyloid basidiospores, hyaline encrusted cystidia and resupinate to effuso-reflexed fruiting bodies (Boidin, 1958). The genus then included *A. chailletii* (Pers.:Fr.) Boid., the type species, *A. areolatum* (Fr.) Boid. and *A. laevigatum* (Fr.) Boid. Boidin and Lanquetin (1984) added a fourth species in the genus, namely *A. ferreum* (Berk. & Curt.) Boid. & Lanq. (= *Stereum ferreum*) (Boidin & Lanquetin, 1984).

In a recent review Thomsen (1996) notes many misidentifications of isolates of *Amylostereum*, especially the species associated with woodwasps, have been made in the past. This can in most cases be ascribed to the rarity or absence of the sporocarps of these fungi and the fact that the sporocarps of *A. chailletii* and *A. areolatum* are very similar (Thomsen, 1998). However, spore size and the colour and texture of the sporocarps can also be used to distinguish these species (Thomsen, 1998). The symbionts of woodwasps often had to be studied entirely in culture or from the mycangia of the wasps, where *A. chailletii* and *A. areolatum* are morphologically similar. These species can, however, be separated by the fact that only *A. areolatum* forms arthrospores in culture (Gaut, 1970; Boidin & Lanquetin, 1984; Thomsen, 1998). *A. laevigatum* and *A. ferreum* cause less confusion, as neither of these species are associated with woodwasps. Unlike the other species, *A. laevigatum* has a monomytic hyphal system and *A. ferreum* has been isolated



only from *Podocarpus* species (Boidin & Lanquetin, 1984; Breitenbach & Kränzlin, 1986).

Despite the fact that it is possible to distinguish the species of *Amylostereum* morphologically (Boidin & Lanquetin, 1984; Thomsen, 1998), the many misidentifications in the past show that the small differences that delineate species such as *A. chailletii* and *A. areolatum* still present difficulties to the non-specialised researcher. Another method that have been shown to successfully distinguish the species of *Amylostereum* is PCR RFLP fingerprinting of the nuc-IGS-rDNA region (Chapter 4). As has been shown for other Basidiomycetes fungi, this method has the potential to serve as a quick, yet precise, identification tool to distinguish these morphologically similar fungal species in culture (Harrington & Wingfield, 1995).

Boidin and Lanquetin (1984) used mating studies and the Buller phenomenon (Buller, 1931) to evaluate the phylogenetic relationships among the *Amylostereum* spp. The results from this study show an unusual triangular mating system. *A. chailletii* and *A. laevigatum* were completely incompatible, but both species were partially compatible with *A. ferreum*. *Amylostereum areolatum* was, however, not compatible with any of these species. The conclusion was that *A. areolatum*, which is morphologically close to *A. chailletii*, must have diverged earlier. Furthermore, the other three species are more closely related, although their specific relationship to each other is not clearly defined. Following these observations, Boidin and Lanquetin (1984) concluded that a more complete study, including a wider range of isolates that are associated with gymnosperms, would provide a more lucid view of the speciation process in lignicolous fungi.

Sequence data from the mt-SSU- and nuc-IGS-rDNA complexes support previous hypotheses regarding the phylogeny of *Amylostereum* spp. and give new insight into previously unclear relationships (Chapter 3 & 4). Both these studies support the hypotheses (Boidin & Lanquetin, 1984) that *A. areolatum* is the most clearly defined species. Furthermore, these studies show that, of the three remaining species, *A. laevigatum* and *A. ferreum* are the most closely related. This finding leads to more comprehensive understanding of the results of sexual matings (Boidin & Lanquetin,

1984), which showed that *A. ferreum* and *A. laevigatum* were partially compatible more often (60 %) than were *A. chailletii* and *A. ferreum* (44 %).

Only *A. chailletii* and *A. areolatum* have been associated in symbioses with woodwasps (Gaut, 1970). An isolate from the wasp *S. areolatus*, that is believed to carry *A. chailletii*, has, however, been shown to be more closely related to *A. laevigatum* and *A. ferreum* (Chapter 3 & 4). This isolate might represent a new species or alternatively a sub-group of one of the known species. It might also represent a link between the latter species and the species associated with woodwasps.

*Amylostereum areolatum* are carried by a number of different wasps species (Gaut, 1970). The association with different wasp species and predominance of asexual reproduction, separates populations of this fungus genetically. The occurrence and combination of heterogenic sequences of the nuc-IGS-rDNA in isolates of *A. areolatum*, makes it possible to determine the relationship between such separated populations of the fungus (Chapter 4). At least four such heterogenic sequences of the nuc-IGS-rDNA region have been shown to occur in five different combinations in isolates of *A. areolatum* (Chapter 4). A preliminary study using this data have shown that isolates of *A. areolatum* associated with *S. noctilio* and *S. juvencus* did not, however, form two distinct genetic groups (Chapter 4). These populations rather shared some of these heterogenic sequences.

## RELATEDNESS TO OTHER BASIDIOMYCETES

The relationship of *Amylostereum* to other Basidiomycetes has been uncertain in the past. Before Boidin (1958) described the genus *Amylostereum*, species resided in *Stereum*, as *S. chailletii* (Pers.:Fr.) Fr., *S. areolatum* (Fr.:Fr.) Fr., *S. juniperi* (Karst.) Boid. and *S. ferreum* Berk. & Kurt. *Amylostereum laevigatum* was, however, better known as *Peniophora laevigata* (Fr.) Karst. This classification and the general macromorphological similarities between some *Amylostereum* spp. and species of *Stereum* (e.g. *A. chailletii* and *S. sanguinolentum*), supported the view that *Amylostereum* is closely related to *Stereum*. Boidin and Lanquetin (1984), however, argue that the presence of gloeocystidia positive in sulfuric-aldehyde, normal nuclear behaviour and a tetrapolar mating system in all four *Amylostereum* spp. makes this genus more closely related to *Peniophora* than to the family Stereaceae. Parmasto (1995), using 86 morphological and physiological characters

in a cladistic study of the genera of Corticoid fungi, found that *A. chailletii* forms a sister group to *Stereum* and *Xylobolus*, while this clade groups basal to *Peniophora*.

In a study including 89 Basidiomycete species, Hibbett *et al.* (1997) showed that morphological characters can be misleading and that molecular data might give a clearer view of the true phylogeny of these fungi. From this study it became clear, for example, that a major character such as gills might have evolved as many as six times. In a study using mt-SSU-rDNA sequence data of a number of Basidiomycetes fungi, Hsiau (1996) found that *A. chailletii* grouped more closely to *Stereum*, than to *Peniophora*. When mt-SSU-rDNA sequence data of all four *Amylostereum* spp. were included in the extended database of Hibbett *et al.* (1997), these species grouped with neither *Stereum* nor *Peniophora*, but strongly with the wood decay fungus *Echinodontium tinctorium* Ell. & Ev. (Chapter 3).

Despite obvious macro-morphological differences between the *Amylostereum* spp. and *E. tinctorium*, all these species have amyloid basidiospores and form encrusted cystidia (Gross, 1964; Boidin & Lanquetin, 1984). Furthermore, it has been hypothesised that *E. tinctorium* is closely related to *Stereum* based on morphological data (Gross, 1964; Stalpers, 1978). Hibbett *et al.* (1997), however, groups *Russula compacta* Frost and *E. tinctorium* together and this clade more closely to *Peniophora* than to *Stereum*. Slippers (Chapter 3) groups *R. compacta* and *Heterobasidion annosum* (Fr.) Bref. together and more closely to the *Amylostereum/Echinodontium* group, than to *Stereum* and *Peniophora*. Hsiau (1996) also showed that *R. compacta* and *Heterobasidion annosum* group together and close to *A. chailletii*. Various studies (Hsiau, 1996; Hibbett *et al.*, 1997; Chapter 3), thus, support the view that there is a close relationship between *Amylostereum*, *Echinodontium*, *Russula* and *Heterobasidion*, and that this group might be more closely related to *Peniophora* than to *Stereum*.

## POPULATION STRUCTURE

*Amylostereum* spp. are heterothallic and have a tetrapolar nuclear state (Boidin & Lanquetin, 1984). The heterokaryotic isolates that are, thus, derived from the pairing of primary mycelia arising from basidiospores, will give rise to genetically different entities.

Both *A. areolatum* and *A. chailletii* are, however, also spread by woodwasps in the form of asexually produced oidia in a very strict symbiosis (Talbot, 1977).

In the Northern Hemisphere, it has been shown that, as a result of the spread of oidia of *A. areolatum* and *A. chailletii* by woodwasps, clonal lines of these fungi are preserved over time and are spread over large areas (Vasiliauskas, Stenlid & Thomsen, 1998; Thomsen & Koch, 1999; Vasiliauskas & Stenlid, 1999). These studies also showed that the presence of clones was very frequent among isolates of *A. areolatum*, but more rare among isolates of *A. chailletii*. It can be concluded from these studies that *A. areolatum* is predominately spread by woodwasps with which it is associated, while *A. chailletii* is regularly spread both via basidiospores and by woodwasps. This is in accordance with the fact that the sporocarps of *A. areolatum* are much less common than those of *A. chailletii* (Thomsen, 1998).

*Amylostereum areolatum* has been introduced, together with *S. noctilio*, into various pine growing regions of the Southern Hemisphere during this Century. It was reported in New Zealand around 1900, Tasmania in the early 1950's, on mainland Australia in 1961, in South America in the 1980's and more recently in South Africa in 1994 (Madden, 1988; Chou, 1991; Baxter, Rong, & Schutte, 1995; Reardon, Eav, & Wetterberg, 1995; Tribe, 1995). Large vegetative compatibility groups (VCG) have been shown to occur in isolates of *A. areolatum* associated with *S. noctilio* in the Southern Hemisphere (Chapter 2). Isolates from South Africa represent a single genetic entity. Furthermore, Brazil and Uruguay also share the same VCG, and this VCG is the same as the one in South Africa. Partial vegetative compatibility was also observed between isolates representing this VCG and isolates from New Zealand and Tasmania. This suggests that the spread of *Sirex* through the Southern Hemisphere during this century has taken place among the continents and countries of the Southern Hemisphere, rather than based on introductions from the Northern Hemisphere. The clonal nature of populations of *A. areolatum* in the Southern Hemisphere further indicates that the fungus mainly spreads asexually through its association with *S. noctilio* in this region. This is also confirmed by the fact that sporocarps of *A. areolatum* have never been found in the Southern Hemisphere.

Vegetative compatibility groups do not necessarily constitute clonality (Worrall, 1997). Vasiliauskas *et al.* (1998) have, however, shown that VCG's of *A. areolatum* found in Northern Europe, represent clonal lines. Molecular markers, such as those that have been used in the Northern Hemisphere (Vasiliauskas *et al.*, 1998), must be applied to studies investigating the population structure of *A. areolatum* from the Southern Hemisphere, in order to establish the true genetic structure of these isolates.

The nematode, *Deladenus siricidicola*, sterilises female *S. noctilio* wasps during a parasitic phase of its life cycle (Bedding, 1995). This nematode is used extensively in biological control programmes in the Southern Hemisphere (Bedding, 1995). For this purpose the mycetophagous phase of the life cycle of the nematode, during which it feeds on *A. areolatum*, is used to mass rear the nematode. Isolates of *A. areolatum* that are used to rear *D. siricidicola*, have, however, been shown to be genetically distinct from other field isolates from South Africa, Brazil, New Zealand and Tasmania (Chapter 2). This nematode has been imported and released in both South Africa and Brazil as part of a biological control initiative against *S. noctilio*. A different genetic entity of the fungus has thus been introduced into these countries along with the *D. siricidicola*. This has the potential to influence the population structure of *A. areolatum* in these countries. The efficacy of *D. siricidicola* as biocontrol agent might also be negatively influenced by a strain of the fungus in South Africa and Brazil, that is different to the one on which it was reared (Chapter 2).

The predominance of asexual reproduction and spread of *A. areolatum* in its symbiosis with woodwasps, has led to the preservation of heterogenic sequences of the nuc-IGS-rDNA region, in this fungus (Chapter 4). The distribution of such heterogenic sequences among isolates of the fungus can be useful in characterising populations of the fungus. Isolates from the Southern Hemisphere, for example, share the same combination of these sequences (Chapter 4). This supports the hypothesis, based on VCG studies (Chapter 2), that the isolates from different regions in the Southern Hemisphere are genetically related.

## CONCLUSIONS

1. Molecular techniques, particularly those based on DNA sequencing, have only recently been applied to questions pertaining to *Amylostereum*. These have clarified previously hypotheses, that were based on morphological and mating studies, regarding the relationships among *Amylostereum* spp. They have also raised new and challenging questions, such as the identity of the fungal isolates associated with woodwasps such as *S. areolatus*. Furthermore, these techniques can now be used to determine other phylogenetic relationships, such as the one between the two types of *A. laevigatum* thought to exist on different hosts in Europe.
2. PCR RFLP fingerprinting can be used to differentiate between the various *Amylostereum* spp. This technique provides a useful tool to overcome difficulties in identifying the morphologically similar *Amylostereum* symbionts of woodwasps.
3. There is a phylogenetic relationship between *Amylostereum* spp. and *E. tinctorium*, that has previously not been recognised using traditional methods. Furthermore, there is a relationship between these species and other Basidiomycetes such as *Russula*, *Heterobasidion* and *Peniophora*. Further studies, combining both molecular and morphological data, are needed to resolve the exact evolutionary relationship between these morphologically disparate fungi.
4. Extensive clonal lineages exist among isolates of *A. areolatum* and *A. chailletii* that are associated with Siricid woodwasps. A study of the population structure of these fungi, using both VCG's and molecular markers, from many parts of the world will give valuable insight into the geographical origin and spread of these fungi, as well as their associated Siricid wasps.
5. Heterogenic sequences in the nuc-IGS-rDNA region of isolates of *A. areolatum* make it possible to compare and characterise populations of these fungi that are associated with different wasp species. The occurrence and combination of these sequences provides insight into both the geographical distribution and evolutionary relationships of populations of the fungus.

## REFERENCES

- Baxter, A. P., Rong, I. H. & Schutte, A. L. (1995). *Amylostereum areolatum* (Aphylophorales: Stereaceae) in South Africa. *South African Journal of Botany* **61**, 352-354.
- Bedding, R. A. (1995). Biological control of *Sirex noctilio* using the nematode *Deladenus siricidicola*. In *Nematodes and biological control of insect pests* (ed. R. A. Bedding, R. J. Akhurst, H. Kaya), pp. 11-20. CSIRO: Melbourne, Australia.
- Boidin, J. (1958). Heterobasidiomycetes saprophytes et Homobasidiomycetes resupines: V.- Essai sur le genre *Stereum* Pers. ex S. F. Gray. *Revue de Mycologie* **23**, 318-346.
- Boidin, J. & Lanquentin, P. (1984). Le genre *Amylostereum* (Basidiomycetes) intercompatibilités partielles entre espèces allopatriques. *Bulletin de la Société Mycologique de France* **100**, 211-236.
- Breitenbach, J. & Kränzlin, F. (1986). *Fungi of Switzerland Volume 2 (Non-gilled fungi)*. Mengis & Sticher A.G.: Lucerne.
- Buchner, P. (1928). Holznahrung und Symbiose. Vortrag gehalten auf dem X internationalen Zoologentag zu Budapest am 8 September 1927. Springer, Berlin, 1928, 13-16.
- Buller, A. H. R. (1931). *Researches on Fungi. Volume IV*. Longmans, Green and Co.: London, U.K.
- Cartwright, K. St. G. (1929). Notes on fungus associated with *Sirex cyaneus*. *Annals of Applied Biology* **16**, 182-187.
- Chou, C. K. S. (1991). Perspectives of disease threat in large-scale *Pinus radiata* monoculture - the New Zealand experience. *European Journal of Forest Pathology* **21**, 71-81.
- Chrystal, R. N. (1928). The *Sirex* wood-wasps and their importance in forestry. *Bulletin of Entomological Research* **19**, 219-247.
- Francke-Grosman, H. (1939). Über das Zusammenleben von Holzwespen (Siricinae) mit Pilzen. *Zeitschrift für angewandte Entomologie* **25**, 647-680.
- Gaut, I. P. C. (1969). Identity of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Biological Sciences* **22**, 905-914.

- Gaut, I. P. C. (1970). Studies of siricis and their fungal symbionts. Ph.D. thesis. University of Adelaide, Australia.
- Gilmour, J. W. (1965). The life cycle of the fungal symbiont of *Sirex noctilio*. *New Zealand Journal of Forestry* **10**, 80-89.
- Gross, H. L. (1964). The Echinodontiaceae. *Mycopathologia et Mycologia Applicata* **24**, 1-26.
- Hall, M. J. (1978). A survey of siricid attack on radiata pine in Europe. *Australian Forestry* **32**, 155-162.
- Hanson, A. S. (1939). Ecological notes on the *Sirex* wood wasps and their parasites. *Bulletin of Entomological Research* **30**, 27-65.
- Harrington, T. C. & Wingfield, B. D. (1995). A PCR-based identification method for species of *Armillaria*. *Mycologia* **87**, 280-288.
- Haugen, D. A. (1990). Control procedures for *Sirex noctilio* in the Green Triangle: Review from detection to severe outbreak (1977-1987). *Australian Forestry* **53**, 24-32.
- Hibbett, D. S., Pine, E. M., Langer, E., Langer, G. & Donoghue, M. J. (1997). Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *Proceedings of the National Academy of Sciences USA* **94**, 12002-12006.
- Hsiau, P. T-W. (1996). The taxonomy and phylogeny of the mycangial fungi from *Dendroctonus brevicomis* and *D. frontalis* (Coleoptera : Scolytidae). D.phil thesis. Iowa State University. Ames, Iowa.
- King, J. M. (1966). Some aspects of the biology of the fungal symbiont of *Sirex noctilio*. *Australian Journal of Botany* **14**, 25-30.
- Kukor, J. J. & Martin, M. M. (1983). Acquisition of digestive enzymes by the Siricid woodwasps from their fungal symbiont. *Science* **220**, 1161-1163.
- Madden, J. L. (1988). *Sirex* in Australasia. In *Dynamics of Forest Insect Populations. Patterns, Causes, Implications*. (ed. A. A. Berryman), pp. 407-429. Plenum Press, New York.
- Madden, J. L. & Coutts, M. P. (1979). The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). In *Insect-Fungus Symbiosis* (ed. L. R. Batra), pp. 165-174. John Wiley & Sons, New York.



- Martin, M. M. (1987). Acquired enzymes in the siricid woodwasp *Sirex cyaneus*. In *Invertebrate-microbial interactions. Ingested fungal enzymes in arthropod biology*. pp.37-48. Cornell University Press.
- Morgan, F. (1968). Bionomics of Siricidae. *Annual Review of Entomology* **13**, 239-256.
- Neumann, F. G. & Marks, G. C. (1990). Status and management of insect pests and diseases in Victorian softwood plantations. *Australian Forestry* **53**, 131-144.
- Neumann, F. G. & Minko, G. (1981). The sirex wood wasp in Australian radiata pine plantations. *Australian Forestry* **44**, 46 – 63.
- Neumann, F. G., Morey, J. L. & McKimm, R. J. (1987). The *Sirex* woodwasp in Victoria. Department of Conservation, Forest and Lands, Victoria, Bulletin No. **29**, 41pp.
- Parmasto, E. (1995). Corticoid fungi: a cladistic study of a paraphyletic group. *Canadian Journal of Botany* (Suppl. 1), 843-852.
- Reardon, R., Eav, B. & Wetterberg, G. (1995). The European woodwasp, *Sirex noctilio* (Hymenoptera: Siricidae) threat to conifer plantations in South America. In *Poster Abstracts, UIFRO XX World Congress, 6-12 August 1995, Tampere*, (ed. E. Korpilahti, T. Salonen & S. Oja), p. 95. Gummerus, Jyväskylä, Finland.
- Spradbery, J. P. & Kirk, A. A. (1978). Aspects of the ecology of siricid woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with special reference to the biological control of *Sirex noctilio* F. in Australia. *Bulletin of Entomological Research* **68**, 341-359.
- Stalpers, J. A. (1978). Identification of wood-inhabiting Aphyllophorales in pure culture. *Studies in Mycology* **16**, 1-248.
- Talbot, P. H. B. (1964). Taxonomy of the fungus associated with *Sirex noctilio*. *Australian Journal of Botany* **12**, 46-52.
- Talbot, P. H. B. (1977). The *Sirex*-*Amylostereum*-*Pinus* association. *Annual Review of Phytopathology* **15**, 41-54.
- Thomsen, I. M. (1996). *Amylostereum areolatum* and *Amylostereum chailletii*, symbiotic fungi of woodwasps (*Sirex* sp. and *Urocerus* sp.). Ph.D. thesis. Danish Forest and Landscape Research Institute, Horsholm, Denmark.
- Thomsen, I. M. (1998) Fruitbody characters and cultural characteristics useful for recognizing *Amylostereum areolatum* and *A. chailletii*. *Mycotaxon* **69**, 419-428.

- Thomsen, I. M. & Koch, J. (1999). Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. *Mycological Research* **103**, (in press).
- 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. (1999). Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. *Mycological Research* **103**, (in press).
- Vasiliauskas, R., Stenlid, J. & Thomsen, I. M. (1998). Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from Northern Europe. *New Phytologist* **139**, 751-758.
- Worrall, J. J. (1997). Somatic compatibility in basidiomycetes. *Mycologia* **89**, 24-36.

## SUMMARY

In Chapter 1 of this thesis, the literature pertaining to the symbiosis between *Sirex noctilio* and *Amylostereum areolatum* in the Southern Hemisphere, is reviewed. It is evident from this review that *S. noctilio* and *A. areolatum* have become established throughout the pine growing regions of the Southern Hemisphere, despite measures to prevent its introduction. Unlike its relative unimportance as a pathogen in the Northern Hemisphere, this fungal-insect complex has resulted in great losses to softwood industries during a number of severe outbreaks in the Southern Hemisphere. The use of biological control agents in combination with preventative silvicultural practices, has been shown to be very effective in controlling *Sirex* in Australasia. It is, however, also evident from this review that despite the rather large collection of knowledge concerning the wasp and its control, information regarding the population structure and phylogenetic relationships of the fungal symbiont of *Sirex*, is scarce.

The recent introduction of *S. noctilio* into South Africa and its confinement to a rather small area in this country provided the opportunity to study the population of its fungal symbiont in detail. Results from Chapter 2 suggest that the fungus has a very narrow genetic base in South Africa and that the introduction of *Sirex* into this country was limited. The genetic base of *A. areolatum* in Brazil and Uruguay is similarly uniform. Of even greater interest is the fact that South Africa and Brazil share a common vegetative compatibility group and, thus, a common origin of *A. areolatum* and *S. noctilio*. Moreover, field isolates from the Southern Hemisphere appear to be closely related, which indicates that *Sirex* might have spread among countries of the Southern Hemisphere and were not necessarily new introductions from the Northern Hemisphere. Isolates of the fungus associated with the biocontrol nematode, *Deladenus siricidicola*, are, however, distinct from isolates from other Southern Hemisphere populations of the fungus. This could negatively influence the efficacy of the nematode as biocontrol agent in countries to which the nematode has been distributed.

Boidin and Lanquetin (1984) report triangular mating incompatibility between isolates from the different *Amylostereum* spp. Results of Chapter 3 support their

conclusions by clearly showing that *A. areolatum* is more distantly related to *A. chailletii*, *A. laevigatum* and *A. ferreum*, than these three species are to each other. The relationship between the latter three species is, however, more clearly defined in Chapter 3 where it is shown that *A. ferreum* and *A. laevigatum* are most closely related to each other. One isolate collected from *Sirex areolatus*, and, therefore, expected to be *A. chailletii*, was most closely related to *A. laevigatum* and *A. ferreum*. Neither of the latter species has, however, been implicated in associations with woodwasps. Furthermore, the data from this study show that *Amylostereum* spp. group with neither *Stereum* nor *Peniophora*, as has been previously hypothesised, but rather with *Echinodontium tinctorium*. This grouping was included in a larger clade that included species of *Russula*, *Heterobasidion*, *Lentinellus* and *Auriscalpium*.

Analysis of DNA sequence data derived from the nuc-IGS-rDNA in Chapter 4 supported the phylogenetic relationships of the *Amylostereum* spp. inferred in Chapter 3. Similarly, the isolate obtained from *S. areolatus*, did not group with any of the four species of *Amylostereum* and might represent a new species or a distinct group in of one of the current species. Isolates of *A. areolatum* associated with both *S. noctilio* and *S. juvencus* contained four heterogenic sequences in the DNA region analysed. These heterogenic sequences were contained in each isolate of the fungus in one of five combinations. Neither the heterogenic sequences included in the fungal isolates, nor the different combinations of these sequences, separated the populations of *A. areolatum* associated with different wasp species. Despite the heterogenic nature of this DNA region in some isolates, RFLP analysis was used effectively to distinguish between the different species of *Amylostereum*.

The work presented in this thesis represents the first molecular view of the phylogeny of the genus *Amylostereum*, as well as that of some of the *Amylostereum* spp. associated with woodwasp species. It is clear from Chapter 5 that these findings now provide a powerful tool to give a clearer picture of the taxonomy and evolution of these fungi, as well the ecology of their symbiosis with woodwasps. The study of the genetic structure of the fungal populations associated with woodwasps also gives new insight into the geographical origin and history of both the insects and their associated fungi.

## OPSOMMING

In Hoofstuk 1 word 'n oorsig gegee van die literatuur aangaande die simbiose tussen *Amylostereum areolatum* en *Sirex noctilio*. Uit hierdie oorsig blyk dit dat *S. noctilio* en *A. areolatum* deeglik gevestig is in dié areas van die suidelike halfgrond met kommersiële denneplantasies, ten spyte van pogings om dit te verhoed. In die noordelike halfgrond het hierdie wesp/swamkompleks min ekonomiese invloed, maar in die suidelike halfgrond was dit verantwoordelik vir groot finansiële verliese vir dennehout-industrieë. 'n Kombinasie van biologiese beheermaatreëls en voorkomende beheer deur deeglike bosboupraktyke is egter effektief in die bekamping van *Sirex* in Australasië. Verder is dit duidelik uit die oorsig dat, ten spyte van die groot databasis oor die wesp en sy beheer, min inligting beskikbaar is oor die populasiestruktuur en filogenetiese verwantskappe van die simbiot van *Sirex*.

Die onlangse aankoms van *S. noctilio* in Suid Afrika en die redelik beperkte verspreiding van die pes in die land, bied 'n geleentheid om die populasiestruktuur van die swam-simbiot deeglik te bestudeer. Die resultate in hoofstuk 2 toon aan dat die genetiese basis van *A. areolatum* in Suid Afrika baie klein is en dat *S. noctilio* die land dus in 'n beperkte getal binnegekom het. Net so het die swam ook 'n klein genetiese basis in Brasilië en Uruguay. Van meer belang is dat die isolate van die swam van Suid Afrika and Suid Amerika een vegetatiewe verenigbare groep vorm en dus 'n oorsprong van beide *A. areolatum* en *S. noctilio* het. Verder blyk die isolate van die suidelike halfgrond naby verwant te wees aan mekaar. Dit beteken dat die beweging van *Sirex* na nuwe areas in die suidelike halfgrond moontlik tussen die lande van die halfgrond is en nie noodwendig nuwe aankomelinge van die noordelike halfgrond nie. Isolate van die swam, afkomstig van kulture van die nematode (*Deladenus siricidicola*) wat gebruik word in biologiese beheer, was egter geneties baie verskillend van die isolate van ander suidelike halfgrond lande. Dit kan die effektiwiteit van die nematode as biologiese beheeragent beïnvloed, sowel as die populasies van die swam in lande waar die nematode bekend gestel is.

Die resultate in hoofstuk 3 toon duidelik dat *A. areolatum* minder verwant is aan *A. chailletii*, *A. laevigatum* en *A. ferreum*, as wat die laasgenoemde drie spesies aan

mekaar is. Van hierdie laaste drie spesies is *A. laevigatum* en *A. ferreum* die naaste verwant. Die verwagting was dat een isolaat wat afkomstig is van *S. areolatus* saam met ander isolate van *A. chailletii* sou groepeer. Dit het egter saam met *A. laevigatum* en *A. ferreum* gegropeer, alhoewel nie een van die laasgenoemde spesies al ooit in simbiose met wespes gevind is nie. Verder het die studie getoon dat *Amylostereum* spesies die naaste verwant is aan *Echinodontium tinctorium* van al die spesies wat by die analise ingesluit is, en nie aan *Peniophora* of *Stereum*, soos wat vroeër voorgestel is nie. Hierdie groepering was ingesluit in 'n groter groepering wat ook spesies van *Russula*, *Heterobasidion*, *Lentinellus* and *Auriscalpium* ingesluit het.

Basisopeenvolging analise van die nukleêre intergeen spasiëring van die ribosomale DNS kompleks (nuk-IGS-rDNS) in hoofstuk 4, ondersteun die bevindings in hoofstuk 3 aangaande die filogenetiese verwantskappe van die *Amylostereum* spesies. Die isolaat afkomstig van *S. areolatus* het apart van die bestaande *Amylostereum* spesies gegropeer en verteenwoordig moontlik 'n nuwe spesie of 'n afsonderlike groep binne een van die spesies. Vier heterogeniese DNS basisopeenvolgings is geïdentifiseer vir die nuk-IGS-rDNS gebied in isolate van *A. areolatum* afkomstig van beide *S. noctilio* en *S. juvencus*. Hierdie heterogeniese opeenvolgings het in een van vyf kombinasies voorgekom in elke isolaat. Nie die heterogeniese opeenvolgings, of die verskillende kombinasies daarvan kon egter die populasies van die swam skei volgens hul assosiasie met die verskillende wesp spesies nie. Desnieteenstaande hierdie variasie, kon die verskillende *Amylostereum* spesies suksesvol onderskei word met beperkingsfragment lengte-polimorfisme (RFLP) analise van die DNS gebied.

Die resultate van hierdie tesis verteenwoordig die eerste molekulêre analise van die genus *Amylostereum*, sowel as die filogenie en taksonomie van sekere *Amylostereum* spesies geassosieer met wespes. Uit hoofstuk 5 is dit duidelik dat hierdie ontwikkelinge nou 'n kragtige tegniek bied wat 'n duideliker beeld kan gee aangaande die taksonomie en evolusie van hierdie swamme, asook die ekologie van hul simbiose met wespes. Verder gee die bestudering van die genetiese stuktuur van die swampopulasies, geassosieer met wespes, nuwe insig oor die geografiese oorsprong en geskiedenis van beide die swam en die insek.