

Fungal symbionts of siricid woodwasps: isolation techniques and identification

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Summary

European woodwasps of the genera *Sirex* and *Urocerus* have symbiotic relationships with basidiomycetes, *Amylostereum chailletii* (Pers. : Fr.) Boid. and *Amylostereum areolatum* (Fr.) Boid. The fungal symbionts of siricid woodwasps are contained in a pair of mycangia located at the top of the ovipositor. A technique used for isolating the fungal symbionts from mycangia is described and illustrated using *Urocerus gigas* L. and *Sirex juvenus* L., and isolation of the fungi from wood and fruitbodies is described briefly. Some characteristics are given of the involved fungi, *A. chailletii* and *A. areolatum*.

1 Introduction

In their natural areas of distribution, siricid woodwasps are secondary pests attacking only wounded, stressed or newly felled trees (Spradbery and Kirk 1978, 1981; Basham 1984). This may, however, change when woodwasps are introduced into ecosystems outside their native range. The European woodwasp *Sirex noctilio* F. has been accidentally introduced to several countries in the southern hemisphere, and as an invasive species this woodwasp has created extensive problems in plantations of introduced pines, where it is able to kill healthy trees (Hurley et al. 2007). Recently, *S. noctilio* has been discovered in eastern North America on both sides of the border between Canada and United States of America (Hoebeke et al. 2005; NAPPO Pest Alerts 2005, 2007; de Groot 2007; Schneeberger 2007; USDA APHIS 2008). This has led to worry that *S. noctilio* will cause extensive damage on pines in North America in the future (Ciesla 2003; Yemshanov et al. 2009).

The ability of siricid woodwasp larvae to develop in wood and the potential of *S. noctilio* as primary pest are connected to the obligatory symbiosis with basidiomycetes and to exudation of phytotoxic substances during oviposition. The female woodwasps have specialized structures, mycangia, for carrying spores of the symbiotic fungi, which are deposited during egg laying in host trees. Another special structure is a reservoir containing a phytotoxic mucus. The toxic nature of the mucus has been proven for *S. noctilio*; the mucus is injected by the woodwasp during egg laying to help create favourable conditions for the symbiotic fungus (Coutts 1969; Fong and Crowden 1973).

Buchner (1928) was the first to describe the mycangia, which he called intersegmental sacs, and that they were filled with the arthrospores (oidia) of a basidiomycete. Since then, these organs have received considerable attention (e.g. Gilmour 1965; Stillwell 1966; Francke-Grosmann 1967), and isolations of the symbiotic fungi have been made for the purpose of identification, comparisons, or experiments (most recently Vasiliauskas et al. 1998; Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999; Nielsen et al. 2009). However, isolation methods have not been described in detail, and there is a dearth of papers describing both the anatomy of the woodwasp in relation to the structures containing spores of the symbiotic fungus, as well as the isolation and identification of the symbionts. The main purpose of this paper is to remedy this lack.

The main symbionts of the genera *Sirex* and *Urocerus* are the basidiomycetes *Amylostereum areolatum* (Fr.) Boid. and *Amylostereum chailletii* (Pers. : Fr.) Boid. The relationship is considered to be very strict in that all members of a woodwasp species carry the same fungal species (Talbot 1977). Until recently, the only woodwasp species known to have *A. areolatum* as symbiont were *Sirex noctilio*, the European *Sirex juvenus* (*juvenus*) L., and the Japanese species *Sirex nitobei* Matsumura (Gaut 1969, 1970; Talbot 1977). However, Nielsen et al. (2009) found *A. areolatum* to be associated with the native *Sirex edwardsii* Brulle and *Sirex* sp. '*nitidus*' in North America. Two Asian *Urocerus* species have a symbiotic relationship with *Amylostereum laevigatum* (Fr.) Boid. (Tabata and Abe 1997, 1999). All other European and North American *Sirex* and *Urocerus* species have *A. chailletii* as symbiont, according to available literature (e.g. Gaut 1970; Talbot 1977; Gilbertson 1984). However, not all woodwasp/*Amylostereum* complexes have been properly identified, especially in North America.

The invasion ecology and hence the prediction of the ecological and economical impact of the introduced siricid woodwasps is connected to the nature of the woodwasp/basidiomycete complex and to the interactions with native species. Further, the identity of the fungal symbiont can help identify the origin(s) of introduction(s) and tracking the spread of the invader.

Detailed descriptions of isolation methods for the symbiotic fungi from woodwasps as well as from other substrates are difficult to find or non-existent. The present paper contains the first comprehensive and illustrated description of a successfully tested technique for isolation of fungal symbionts from siricid woodwasps, covering the exposure of mycangia as well as the subsequent extraction of oidia for incubation, culturing and identification. In addition, isolation of *Amylostereum areolatum* and *A. chailletii* from wood and fruitbodies is described briefly. Finally, some important characteristics of the symbiotic fungi are given.

2 Materials and methods

2.1 Materials

The main tree species used for collecting woodwasps and fungi was Norway spruce [*Picea abies* (L.) H. Karst.], but Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], Nordmann fir [*Abies nordmanniana* (Steven) Spach], European Silver fir (*Abies alba* Mill.), Grand fir [*Abies grandis* (Douglas ex D. Don) Lindl], and larch (*Larix* sp.) were included. Stems selected had been felled at least 2 years before collection and had either fruitbodies of the symbiotic fungi or exit holes of woodwasps. The material was collected from various parts of Denmark over 5–10 years (Thomsen and Koch 1999), however most of the woodwasps originated from forests in North Zealand within a 2-year span. Stems with bark beetle infestations were avoided for isolations from wood, due to the risk of contamination with the blue stain fungi associated with them.

Stem pieces with woodwasp emergence holes were placed in upright tubes of thick plastic (diameter 30 cm, length 1 m). Each tube was sealed with a tight plastic lid at the bottom, and fine, white netting was tied over the top. The tubes were kept under warm conditions, often above 30°C, and inspected each day for emerged woodwasps. In a few cases, ovipositing females were captured in nature. The 49 reared or captured woodwasps were all specimens of either *U. gigas* or *S. juvencus*.

The implements used for isolation of fungal symbionts from woodwasps were eye surgery instruments and consisted of micro scissors and scoop from the German company HEBUmedical (Catalog 2009 version p 761 and 748 respectively, available at <http://www.hebumedical.de/flipbook/flipviewerxpress.html>). The micro scissors (Secondary Cataract Scissors Vannas HB 7381) had curved cutting blades with a length of 5 mm, and total length of the scissors was 8 cm. The micro scoop (Exenteration Scoop or Chalazion Curette, Meyhoefer HB 7300 sharp) had a diameter of 0.5 mm, and the length was 13 cm. Other implements were lens paper for wiping the instruments, a small brush, and 96% alcohol.

Isolation from wood was done with a chisel, a hammer, two gouges (diameters 12 and 6 mm), and a set of tweezers. Isolation from fruitbodies was done with a sharp razor blade. The isolations were made on Potato Dextrose Agar (PDA) in 9 cm Petri dishes with 5- to 6-mm thick agar layer and a fairly dry and firm surface. A suitable surface could be achieved by storing the Petri dishes for 3 weeks in open plastic sleeves or drying the agar surface by leaving the lidded Petri dishes in sterile air flow for 6–12 h. For microscopy, spores from mycangia or mycelium from cultures were placed in water or a drop of lactophenol on microscope slides.

2.2 Method for isolating fungal symbionts from female woodwasps

The woodwasps were kept in 125 ml glass containers with tight lids. The insects were killed by applying 2–5 drops of ethyl acetate with a Pasteur capillary pipette directly to the head of the wasp. If they were very active, a small wad of tissue paper wetted in ethyl acetate was put into the glass container first. As woodwasps are quite difficult to kill, they were left for at least 10 min in the container with the lid on after the direct application of ethyl acetate.

A dead wasp was held steady by hand or with tweezers under a stereo microscope at 15–20 times magnification. The last sternum of the abdomen was carefully wiped with a small brush wetted in 96% alcohol. The micro scissors were dipped in alcohol and shaken gently to remove excess moisture. No heating was used, as this would dull the sharp edge. For the same reason non-abrasive lens paper was used to wipe the instruments when necessary.

The point of the scissors was inserted at one side between the last tergal plate and the sternal plate surrounding the ovipositor sheath (Fig. 1). The part containing the ovipositor was parted from the rest of the abdomen by carefully cutting all the way around the last two sterni, that is, both the ventral plate around the ovipositor and the small plate just above the base of the ovipositor (Fig. 2). The mycangia are right behind the top of the ovipositor, and cutting above the second last sternum minimized the risk of puncturing them.

The part containing ovipositor, mycangia (intersegmental sacs), mucus reservoir, and various other organs was held with the exposed interior upwards (Fig. 3). The mycangia are located at the very top of the back of the ovipositor and are clearly visible as two small grey balls with a net of whitish 'veins' (Figs 3 and 4). The other body contents were cut and scraped away with the

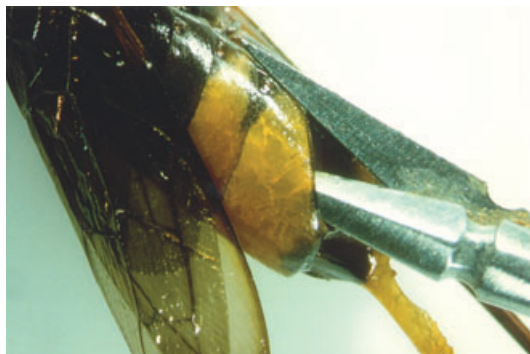


Fig. 1. Dissection of female *Urocerus gigas* woodwasp. Microscissors are inserted along the tergal plates to sever the abdomen by creating an incision along the ventral portion of the last segment.

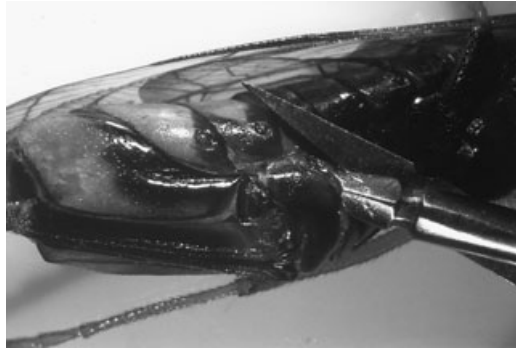


Fig. 2. A horizontal incision is made along the sternal plate above the base of the ovipositor sheath, and the last abdominal segments are cut free. Upon opening of the abdomen, the mycangia are situated right behind the sternal plate. Making the incision just above the sternal plate minimizes the risk of puncturing the mycangia.

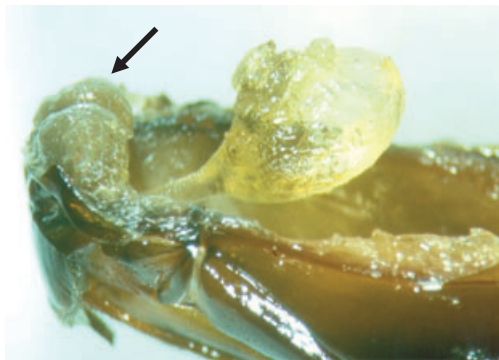


Fig. 3. In the dissected part of the abdomen, the mycangia are recognized as greyish balls with whitish veins (left, arrow). The large sac (right) is the mucus reservoir.

scissors. The brush was dipped in alcohol, and the area around the mycangia carefully cleaned. Any tissue residue on the mycangia themselves was very lightly brushed away.

The scoop was dipped in 96% alcohol and gently shaken. The round tip was dipped with the back (outward curving) side down in PDA in an unused Petri dish. This was done to avoid negative impact of ethanol residues on the fungal spores. The scoop was inspected in the microscope to ensure that no agar stuck to the tip (Fig. 4). The scoop was then inserted in either of the mycangia by pressing one edge of the 'spoon' lightly against the wall of the sac. The sharp edge of the scoop easily opened the mycangium wall. If too much pressure was used, the spore mass exuded from the sac. However, it could easily be caught by the scoop, as it was quite viscous and stuck to the sac wall.

The spore mass was transferred to PDA in a Petri dish by scraping the scoop on the surface of the agar. Usually, several Petri dishes were made from each woodwasp, mostly two or three per mycangia. Germination and growth of the fungus were best, if

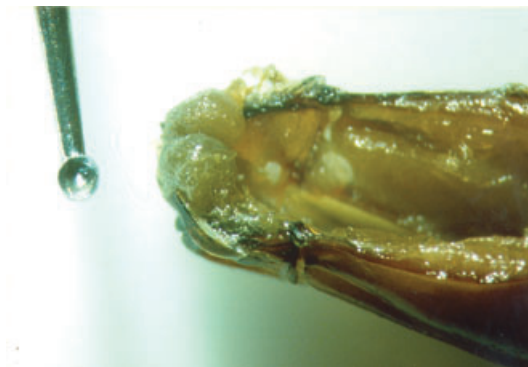


Fig. 4. The mycangia have been exposed by scraping away surrounding tissue, hereby making it easier to remove the spore mass from the sacs using a micro scoop (left).

the agar surface was fairly dry, and the spores were placed on the surface of the agar instead of being pushed into it. Incubation of the dishes took place in natural daylight and darkness at room temperature (20–22°C).

2.3 Method for isolating the fungi from wood

Longitudinal sections were made with a band saw, circular saw or just by splitting stem pieces with an axe or a chisel and hammer. This exposed the extent of wood colonized by the fungus and thus facilitated the choosing of isolation points (Fig. 5). If larval tunnels were visible on sections, isolations were done within 5 cm of these. If there were fruitbodies, isolations were done as close to the surface under the fruitbody as possible.

The gouges used for isolation were dipped in 96% ethanol and quickly flame sterilized. Two horizontal grooves were cut into the wood with the 12-mm gouge, next to each other and overlapping by one-fourth of the groove width. A third groove was then cut in the middle. Between each cut the gouge was sterilized as described above, in order to ensure that the newly exposed surface was not contaminated. The 6-mm gouge was then sterilized, and an almost vertical cut was made with the gouge and hammer, but a few degrees off to one side. A similar cut was made from the other side, so that a small V shaped chunk of wood came loose (about 6 mm on each side). An ethanol-and-flame sterilized pair of tweezers was used to transfer each small piece of wood to a sterile, empty Petri dish. Subsequent transfer of the wood pieces to PDA (2–3 chunks per Petri dish) then took place with sterilized tweezers in a horizontal laminar flow bench. In all isolations, Petri dishes were incubated for 2–4 weeks at room temperature and checked regularly for growth of mycelium. Reisolation onto fresh agar was done, if there were more than one fungal isolate growing from the wood pieces or in case of contaminations with bacteria or mould fungi, and also for the purpose of vegetative compatibility tests (Fig. 6).



Fig. 5. Longitudinal section through larch stem with wood wasp larval tunnels and rot. Red arrows: wound from branch pruning, which probably attracted the wood wasps. Blue arrows: frass packed wood wasp larval tunnels. Black arrows: rot column of *Amylostereum* sp. distinguished from the reddish heartwood by a light brown color.

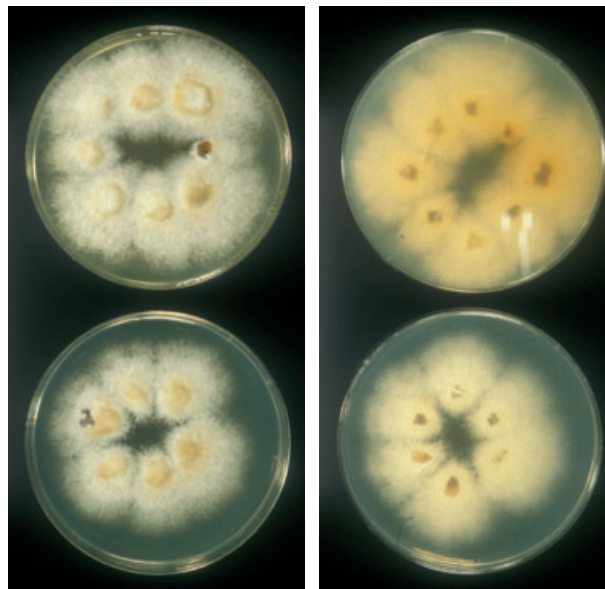


Fig. 6. Cultures of *A. chailletii* from the mycangia of two female *Urocerus gigas* wood wasps reared from the same log. The isolations were placed alternately in a circle and have grown together completely, thus proving that both wood wasps carried the same genet of the fungus. Left: Petri dish from above, right: the same Petri dish from below.

2.4 Method for isolating from fruitbodies

The fruitbodies of *Amylostereum chailletii* and *A. areolatum* are mostly resupinate and rather thin, which is not optimal for isolation from the hat trama. However, using a sharp razor blade or a scalpel it was possible to remove small pieces. The razor blade was ethanol-and-flame sterilized, and the surface of the fruitbody was removed to expose a non-contaminated part of the underlying trama. The razor blade was re-sterilized, and a triangular piece (1 mm on each side) was cut out of the fruitbody. *A. areolatum* has thicker fruitbodies than *A. chailletii*, and isolation was thus easier.

The small fruitbody pieces were transferred to PDA with a sterile needle. The needle was dipped in ethanol, flame sterilized and then dipped into agar in a Petri dish. The last step was important for two reasons. First, it cooled the needle, so that the heat would not damage the fruitbody piece. Second, the agar melting from the heat made the needle a little sticky, which made it easier to capture the fruitbody cut-out. The fruitbody pieces were deposited on the surface of the agar, taking care not to insert it into the agar.

3 Results and discussion

3.1 Collecting woodwasps

Of 19 *S. juvencus* woodwasps reared or captured, 13 were females, and of 30 *U. gigas* reared or captured, 20 were females. The woodwasps, which emerged from logs kept in the plastic tubes, could easily be captured by hand or with a small plastic cup slipped below the netting. Although woodwasps can bore through tough material, they did not do so, once they had left the logs, but usually sat on the lower side of the net across the top of the tube. No effort was made to trap woodwasps with lures, because the method of collecting stems with emergence holes was successful. However, lures of α - and β -pinene have been successfully used (Nielsen et al. 2009), and non-mineral turpentine on freshly felled conifer stems or wooden boards will similarly attract female woodwasps during the flight season (J. O. Martin pers. comm.)

Selecting conifer stems with woodwasp emergence holes ascertains colonization and, due to the varying development time (1–3 years) of woodwasp in Europe, also emergence, although relatively low. Each collected stem mostly yielded 1–3 woodwasps, occasionally 10 woodwasps. This is sufficient for species determination and for obtaining cultures of the symbiont. However, this method may be less useful, if the life cycle of the woodwasp studied is only 1 year, as is generally the case with *S. noctilio* in the southern hemisphere (Neumann and Minko 1981; Slippers 1998), and which may also be the case in North America (Haugen 2007). In such cases, the stems should be collected before or at the beginning of the flight season, as soon as the first woodwasps have emerged.

3.2 Isolations from mycangia

At the time of isolation, all female wasps were either newly killed or had been dead and stored no longer than 5 days. Success in isolation was highest for newly killed woodwasps, as the mycangia dried out fairly quickly. Isolation of the symbiont could still succeed, but there were fewer viable colonies and higher rates of contamination. Isolation of the symbiotic fungus was successful in 11 out of 13 cases with *S. juvencus* females and with 19 out of 20 *U. gigas* females. Isolation failed with three specimens, where the mycangia had become dry and hard during storage under too dry conditions.

The length of the females from head to tip of ovipositor varied from 1.9 to 4.0 cm. Larger woodwasps were easier to handle, and they had slightly bigger mycangia, that is, more spore mass could be obtained. In the successful isolations, the fungi grew out within 2–3 weeks. Most of the failed isolations were due to lack of growth of the symbiotic fungus and not to contaminations of the agar in the separate Petri dishes. However, in these cases the agar used was probably slightly too soft and wet, so that the spore mass was inadvertently drowned. The importance of using firm and fairly dry agar and of placing the spore mass on the surface of the agar was illustrated by these failures. Isolations always succeeded in Petri dishes in which the agar was more than 3 weeks old, or when the agar surface had been dried by sterile air flow. The same effect might be achieved by using a thin layer of agar, but the slow growth of especially *A. areolatum* means that the isolates can be kept in the original Petri dishes for months, if the agar does not dry out below the surface.

Bedding and Akhurst (1974) described another method of isolating the symbiont of *S. noctilio*. The female woodwasps were dipped in ethanol, ignited and plunged into sterile water, in which the woodwasps were then dissected to remove the mycangia (which they called 'glands'). Under sterile conditions, the mycangia were then burst and streaked over PDA. In another study (Baxter et al. 1995) obtained the symbiont from female *S. noctilio* by floating the 'reproductive organs' in sterile water overnight and then incubating them on 1.5% malt extract agar. However, it was not described how the mycangia were extracted from the wasps. In any case, using sterile water seems to be an unnecessary complication, as few difficulties or contaminations were encountered in the 'dry' technique described in this paper.

3.3 Isolations from wood and fruitbodies

The methods for isolation of *Amylostereum chailletii* and *A. areolatum* from wood and fruitbodies were very successful, as cultures were obtained in almost 80% of the cases, and problems with contamination were low. Both fungi grew out willingly even on non-selective media such as PDA, but other studies have used selective agar or included extract of pine to promote growth of *Amylostereum* (Vasiliauskas et al. 1996; Slippers et al. 2001). This can be recommended if working with *A. areolatum*, as growth of this fungus is slow and may even cease completely after 5–6 weeks.

In longitudinal sections, the presence of the fungus was often visible as a red discolouration of the wood (Fig. 5), similar to the phenomenon called 'Rotstreifigkeit' in German literature (Pechmann et al. 1967). An inoculation experiment with *A. areolatum*, *A. chailletii*, and *Stereum sanguinolentum* (A. & S. : Fr) Fr. in standing Norway spruce showed that the reddish discoloration extended far lengthwise in the sap wood (Thomsen, unpublished data). At the end of the stain column, the discoloration usually preceded the presence of all three fungi by up to 30 cm. Studies of decay of *A. areolatum* and *A. chailletii* are rare, but the fungi have been found to be active in standing trees, even after the woodwasps have departed (Vasiliauskas 1999). Thus, confirmation of the presence of *A. areolatum* is possible several years after infestation of trees by for example, *S. noctilio*, which could be useful when checking for the presence of this species in non-native habitats. Isolating *Amylostereum* or other decay fungi from standing trees is most simply done with an increment borer (Stenlid 1985; Vasiliauskas et al. 1996). In these studies samples were taken with an 8-mm ethanol-sterilized increment borer from under healthy bark 1–3 cm from wounds. Such cores can be transported to the lab in sterile tubes or zipper bags and incubated on agar. This method would be less labour consuming than the collection of stems and isolation in the laboratory used in this study, but at the cost of smaller chances of obtaining isolates, as shown for *Heterobasidion annosum* (Stenlid and Wästerlund 1986).

3.4 Identity of the symbionts

Identification of both species of symbiotic fungi, based on morphological characteristics, could usually be made within 6 weeks of isolation from woodwasps, wood or fruitbodies. Both fungi produce numerous clamp connections on their hyphae, and after 3–4 weeks older parts of the mycelium have developed cystidia, that is, hyphal ends that are pointed and encrusted (Stalpers 1978). In some cases, the colour of the mycelium can be used to differentiate *A. areolatum* and *A. chailletii* (Thomsen 1998), see Fig. 7.

Many misidentifications of *A. areolatum* and *A. chailletii* have been made in the past (Thomsen 1996). However, distinguishing *A. areolatum* from *A. chailletii* is most easily done by looking for oidia in culture (Stalpers 1978; Siepmann and Zycha 1968 using the codes given by Nobles (1965), which should be compared with Nobles' description in Basham (1959)). Although both fungi break up into hyphal fragments reminiscent of oidia in the mycangia of the woodwasps (Fig. 8a,b), only *A. areolatum* produces oidia in culture (Fig. 8c,d). The oidia produced in the mycelium of *A. areolatum* grown on agar are more regularly shaped than those found in the mycangia of *S. juvencus* (Fig. 8b) and *S. noctilio* (Slippers 1998).

The two woodwasp species studied were consistent in carrying either *Amylostereum areolatum* (*Sirex juvencus*) or *A. chailletii* (*Urocerus gigas*). As expected, all cultures isolated from the mycangia of the same woodwasp were always identical. In addition, different woodwasp individuals of the same species reared from the same log in most cases also yielded the same clone (vegetative compatibility group) of the relevant fungus (Fig. 6). Thus, it may be concluded that only a few samples are necessary per tree, either from woodwasps emerging from the same tree, from the wood or from fruitbodies on the substrate in order to obtain and identify the symbiont.

3.5 Conclusions and implications for studies of invasive siricid woodwasps

The methods described are very successful in obtaining cultures of the fungal symbionts. In the technique used for isolation from mycangia it is a major prerequisite for a high rate of isolation success that the woodwasps are newly killed. For culturing the symbionts on media, the most important factor is that the agar is firm and the surface not too wet. The two fungal symbionts, *A. areolatum* and *A. chailletii* are most easily distinguished by the presence of oidia in cultures of *A. areolatum*.

Isolation of *A. areolatum* and *A. chailletii* from wood or fruitbodies can be useful in those circumstances, where woodwasps are not available. Once a few successful isolations and identification have been made, no further samples are needed to determine the resident fungus and by implication the possible woodwasp species, which have inhabited the tree. The low sampling intensity needed per log makes it possible to include more trees and larger areas in a survey for woodwasp infestations or for studies of the population structure of the symbiotic fungi (Vasiliauskas et al. 1998; Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999).

However, *A. chailletii* produces fruitbodies regularly and can infect suitable substrates with basidiospores. *A. chailletii* may thus be found as a saprophyte on dead conifer stem wood without any association with woodwasps. In contrast, *A. areolatum*

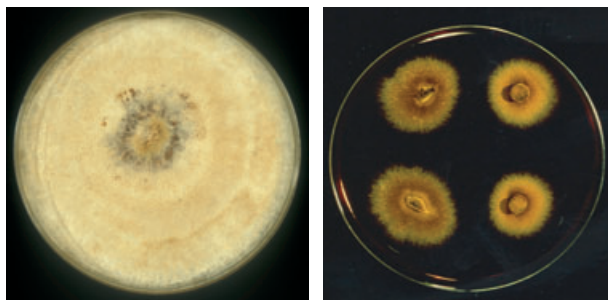


Fig. 7. *A. chailletii* culture (left) and two different cultures of *A. areolatum* (right) after re-isolation and growth for 10 weeks. Note the difference in colour and growth rate between the two *Amylostereum* species, and the difference in colour between the two *A. areolatum* isolates.

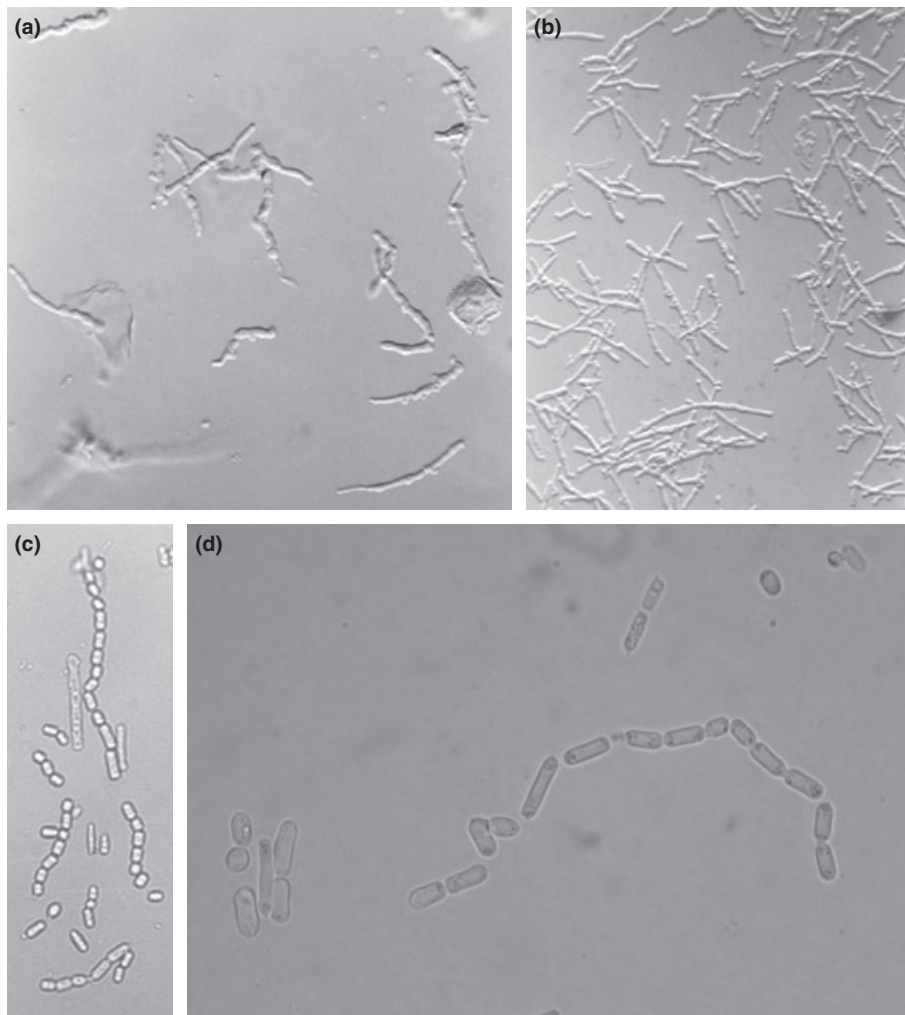


Fig. 8. Hyphal fragments (arthrospores), often called oidia, of: (a) *Amylostereum chailetii* from the mycangia of a female *Urocerus gigas*. *A. chailetii* only produces oidia in these circumstances and never in culture; (b) *Amylostereum areolatum* from the mycangia of a female *Sirex juvencus*. Note that oidia produced by *A. areolatum* in *S. juvencus* mycangia did not differ morphologically from *A. chailetii* oidia in the mycangia of *U. gigas* (a); (c) Oidia (arthrospores) of *Amylostereum areolatum* from culture on PDA made from *S. juvencus*; (d) Oidia (arthrospores) of *Amylostereum areolatum* from culture on PDA made from a fruitbody (number 20 and VCG A in Thomsen and Koch 1999). Notice the more regular shape of the spores in (c) and (d) than those seen in *S. juvencus* mycangia (b), this size and form is expected of typical oidia.

fruitbodies are rare in most of Europe, and the fungus is mainly spread via woodwasps. Fruitbodies of *A. areolatum* have never been recorded in North America, nor in the southern hemisphere, where the fungus seems to be spread exclusively by *Sirex noctilio*.

The identity of the basidiomycete in the woodwasp/symbiont complexes has proved to be important in tracing the origin of introduced siricid woodwasps in the southern hemisphere where no native siricid woodwasps are present (Slippers et al. 2001, 2002) and in North America where several indigenous species of the genus *Sirex* occur (Nielsen et al. 2009). Even when native woodwasps carry *A. areolatum* as symbiont, population genetics based on vegetative compatibility groups and ITS sequencing can be used to distinguish the local isolates from *S. noctilio* borne genets of *A. areolatum* (Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999; Slippers et al. 2002; Nielsen et al. 2009). Most surveys for *S. noctilio* as an invasive species focus on the rearing of the insects from symptomatic trees or capture of woodwasps in lures during the flight season, but isolation of *A. areolatum* from wood of standing trees or logs and subsequent comparison with isolates of known origin, that is, native or introduced, may be equally useful.

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