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Detection and Identification of the Invasive *Sirex noctilio* (Hymenoptera: Siricidae) Fungal Symbiont, *Amylostereum areolatum* (Russulales:Amylostereacea), in China and the Stimulating Effect of Insect Venom on Laccase Production by *A. areolatum* YQL03

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ABSTRACT The Eurasian woodwasp *Sirex noctilio* F. was first detected in Daqing, Heilongjiang Province, in the northeast region of China in 2013. Here, we investigated the *S. noctilio*'s fungal symbiont, *Amylostereum areolatum*, and insect venom produced in its acid (venom) gland. Overall, seven out of 10 fungal isolates obtained from the mycangia of 10 adult *S. noctilio* females in this study were identified as *A. areolatum*. The remaining three isolates were identified as *Trichoderma viride*, *Verticillium dahlia*, and *Geosmithia pallida*, which were probably contaminants that entered during the mycangia-spore extraction process. The enzyme activity bioassay showed that the level of laccase activity of *A. areolatum* YQL03 in liquid medium is prominently enhanced by insect venom, but was relatively low when venom was not available as an inducer. This study confirms the presence of *A. areolatum* in *S. noctilio* specimens from China. The putative heat-stable factors identified in *S. noctilio* venom may contribute novel information about the pathogenic mechanism of the *S. noctilio*–*A. areolatum* pine-killing pest complex on host trees.

KEY WORDS *Sirex noctilio*, *Amylostereum areolatum*, laccase, venom, mutualism

The Eurasian woodwasp, *Sirex noctilio* F., attacks dead and dying *Pinus* species in its native ranges of Eurasia and North Africa. In these regions, the woodwasp is generally considered to be a secondary pest of pine (Spradbery and Kirk 1978, Haugen 2007), because it has negligible economic impact (Coutts and Dolezal 1966, Borchert et al. 2007). However, *S. noctilio* was accidentally introduced to several countries in the Southern Hemisphere during the twentieth century. In these countries, *S. noctilio* causes severe damage to introduced North American pine plantations, resulting in up to 70% tree mortality (Hurley et al. 2007). Pines are not native to the Southern Hemisphere, but have been extensively planted to bolster the forestry industry (Ciesla 2003). For example, *S. noctilio* was first reported in New Zealand around 1900 followed by Tasmania (Australia) in the 1950s, Uruguay in1980, Argentina in 1985, Brazil in 1988, South Africa in 1994, and Chile in 2001 (Rawlings 1948, Iede et al. 1998, Madden 1988, Tribe 1995).

A very strict mutualistic relationship exists between *S. noctilio* and the white-rot fungus *Amylostereum areolatum* (Fr.) Boidin (Taylor 1981). *S. noctilio* attacks on pine are, in fact, the act of oviposition. Female insects drill one to five small holes in the host tree through the bark to the cambium, injecting phytotoxic venom together with the arthrospores of *A. areolatum*, and sometimes an egg, into the holes (Coutts and Dolezal 1966, Coutts 1969c). Together, the fungus and venom act to kill the tree. Subsequent wood decay caused by the fungus provides *Sirex* larvae with an environment suitable for development (Talbot 1977, Madden and Coutts 1979, Slippers et al. 2003). In turn, the fungus relies on emerging adult females that collect oidia produced in the insect galleries in their mycangia for dispersal and inoculation into new trees.

A. areolatum belongs to the phylum Basidiomycota, order Agaricales, family Thelephoraceae. As the fungal symbiont of the woodwasp, *A. areolatum* is essential to the development and even the reproductive potential of its partner, *S. noctilio* (Coutts and Dolezal 1965). The larvae tunnel in the tree for one to two years, apparently feeding and tunneling in fungus-altered wood exclusively (Vasiliauskas 1999). Madden and Coutts (1979) suggest that the first, and some second, instar larvae feed exclusively on the fungus, while later larval stages feed on fungus-colonized wood. Regardless of the specific role of the fungus in larval nutrition, it also modifies environmental conditions. The wood-decaying symbiont dries the wood substrate providing a more

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suitable microenvironment for egg and larvae development. The wood degradation by the fungus facilitates tunneling of the larvae (Coutts and Dolezal 1965, Gilmour 1965, Morgan and Stewart 1966). Obviously, *S. nocitilio* derives considerable benefit from its relationship with the fungal symbiont. It seems that these entitlements awarded to the woodwasp to a great extent are linked to the wood-degrading ability of the fungal symbiont, whereby the fungus decomposes wood by degrading cellulose, hemicellulose, and lignin in host trees (Müller 1934, Francke-Grosmann 1939, Martínez et al. 2010), especially the lignin degradation by the extracellular phenoloxidase enzyme. Extracellular phenoloxidase enzyme production has been reported in a wide variety of white-rot fungi, with virtually all white-rot fungi exhibiting this type of activity (Hatakka 1994, Lankinen et al. 2005, Martínez et al. 2010). Two of these phenoloxidases, laccase and peroxidase, are the most important enzymes involved in the breakdown of lignin (Hatakka 1994, Morozova et al. 2007). Up to now, fungal laccases have been proposed to be involved in degradation of lignin (Bertrand et al. 2002). Compared with most enzymes that are highly substrate specific, fungal laccases can catalyze the oxidation of a surprisingly wide variety of substrates, including diphenols, polyphenols, diamines, and aromatic amines (Madhavi and Lele 2009, Desai and Nityanand 2011). Besides, fungal laccases have more functions, such as sporulation, pigment production, and rhizomorph formation (Coll et al. 1993). Bordeaux (2008) investigated the fungal growth characteristics of *A. areolatum* and the characterization of laccase activity, independent of the *Sirex* wasp. The author observed that *A. areolatum* produces laccase activity in both solid and liquid culture, as demonstrated by the application of the reducing agents syringaldazine and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS; Bordeaux 2008). The author also observed that the fungus is a weak pathogen that requires *S. noctilio* wasp venom secretion for the successful colonization of host trees. Earlier studies also established that the venom and the fungus injected into the tree during oviposition are responsible for the visible symptoms of *S. noctilio* attack, such as chlorosis or yellowing, especially in older needles, premature needle senescence, and loss and flagging or drooping of young needles at the fascicle sheath. Neither the venom nor the fungus alone is sufficient to kill trees, whereas the combination of the two is lethal. Thus, the *S. noctilio* venom is characterized as a "conditioning" agent that is necessary for establishing the active and overwhelming infection of the host by *A. areolatum* (Coutts 1968, 1969a,b).

To date, research has focused on the pathogenic mechanism of the *S. noctilio–A. areolatum* pine-killing pest complex on host wood. However, there is a paucity of studies on the correlation between *Sirex* venom and *A. areolatum*. Uncertainty remains about how *Sirex* venom creates the "condition" required for *A. areolatum* to infect the host. In the current study, seven isolates of *A. areolatum* were successfully isolated from *S. noctilio*, which was first detected in Daqing, Heilongjiang Province, in the northeast region in China, in 2013 (Y. L., unpublished data). Specifically, we examined the interrelationship between the insect venom and fungus by analyzing laccase activity from *A. areolatum* YQL03 in combination with several different concentrations of *Sirex* venom, as the inducer. We expect our research findings to provide a basis for subsequent studies on the interaction between *Sirex* insect venom and *A. areolatum* YQL03, focusing on the laccase gene at the transcriptional level.

Materials and Methods

Isolation of the Symbiont. The study site containing trees infested by the woodwasp *S. noctilio* was located at Xindian State Forest Farm, Daqing, Heilongjiang Province, in the northeast region of China (N 46.629777°, E 124.430925°). The infested wood were mainly distributed at three sites in the plantations of *Pinus sylvestris*, which were ~35–40 years old. At each site, one infested wood with signs of *Sirex* attack, such as resin beads and exit holes, was felled. Pieces of the stem boles were given a section number and placed upright in thick plastic tubes (diameter 40 cm, length 1 m). Each tube was sealed tightly with a plastic lid at the bottom, and fine, white netting was tied over the top (Thomsen and Harding 2011). The tubes were kept at 25°C in the standard quarantine facility of Beijing Forestry University, and were checked daily for emerged woodwasps throughout the whole emergence period (from May to October; Wermelinger and Thomsen 2012). Totally, 10 adult females and 128 adult males of *S. noctilio* were collected preflight as they emerged from infested boles. The detailed information about the collection of wasps was provided in Table 1. Immediately after emergence, 10 adult *S. noctilio* females were collected from the tubes and kept at 4°C for up to 48 h before dissection. The dissection process involved pinning a dead wasp to a Styrofoam surface with sterilized needles under a stereo microscope with 15–20 times magnification. The abdomen of the woodwasp was carefully wiped with a small brush wetted in 96% alcohol. The point of micro scissors sterilized with 96% alcohol was inserted on one side between tergum 8 and tergum 9. The scissors were used to separate tergum 8 into two parts from the middle, with the same process being used to separate the remaining tergums on the adjacent abdominal segment successively. The major internal organs of the abdominal segment, including the mycangia, venom gland, and venom reservoir, were exposed when the broken tergums were completely separated from the rest of the abdomen (Fig. 1). Then, the brush was dipped in 96% alcohol, and the surrounding tissue residue on the mycangia was very lightly brushed away. The micro 1.0-mm curette (Fine Scientific Tools, Bath, United Kingdom) sterilized with 96% alcohol was then inserted in either of the mycangia by pressing one edge of curette lightly against the wall of the sac. The sharp edge of the curette easily opened the mycangium wall, and the spore mass was caught by the curette, as it was quite viscous and stuck to the sac wall. For each female dissected, the mycangium was used to initiate a fungal culture and each

Table 1. General information on the collection of *S. noctilio* woodwasps

Section no. of stem boles	No. of the adult males	No. of the adult females	Emergence dates of the females
1–1	7	_	_
1–2	15	2	7 Aug. 2013; 25 Sept. 2013
1–3	11	1	1 Aug. 2013
1–4	2	_	_
1–5	3	1	20 June 2013
1–6	1	_	_
2–1	6	_	_
2–2	10	_	_
2–3	15	2	6 Aug. 2013; 10 Aug. 2013
2–4	12	1	10 July 2013
2–5	7	1	13 Aug. 2013
2–6	5	_	_
2–7	2	_	_
3–1	7	_	_
3–2	13	1	31 July 2013
3–3	8	1	3 Aug. 2013
3–4	2	_	_
3–5	1	_	_
3–6	1	_	_

Note: "_" means no female wasps emerged from the boles. Emergence dates of the males were not present in this table due to too many records of the males. Totally, the emergence period last ~4 mo, from the first emergence of a male wasp (3 June 2013) to the last emergence of a female wasp (25 September 2013). Sex-ratio was 12.8:1 males to females.

isolate was obtained from the mycangium of each female wasp. The fungal spore mass inside the mycangia was transferred to potato dextrose agar (PDA) containing 0.05 g/liter streptomycin sulfate as the antibiotic by scraping the curette on the surface of the agar. Growth of the subsequent cultures was on PDA without antibiotics. Culture plates were maintained at an ambient temperature (≈24°C) in darkness.

DNA Extraction. Fungal mycelia were collected by scraping the mycelium from the growing fungal cultures ($N = 10$) and were immediately frozen in liquid nitrogen. The frozen mycelia were ground to powder with a mortar and pestle. Total genomic fungal DNA was extracted using the EZgene Fungal gDNA Kit (Biomiga, San Diego, CA) following manufacturer's protocol. The DNA was eluted with 50 µl elution buffer and stored at −20°C until use.

PCR Amplification Conditions. The internally transcribed spacer regions (ITS) were amplified using the general primer ITS1 (5′TCCGTAGGTGAACCT GCGG′3) and ITS4 (5′TCCTCCGCTTATTGATA TGC′3). Amplification by PCR was performed in 25 µl mixture, which contained 2 µl fungal DNA template, 2.5 µl 10× PCR buffer (Qiagen Inc., Valencia, CA), 0.2 µM of each primer, 200 µM of each dNTP (Qiagen), and 2.5 U Taq polymerase (Qiagen). The thermocycling procedure included an initial denaturation step of 2 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. A final extension step at 72°C for 10 min ended the program.

Clone and DNA Sequencing. PCR products were subjected to electrophoresis on 1.5% (w/v) ethidium bromide-stained gel and were visualized under UV illumination. The products were purified from the gel using the Prep-A-Gene kit (Bio-Rad, Hercules, CA) and ligated into the vector pMD18-T vector (Takara, Dalian, China). Ligation mixtures were transformed into competent *Escherichia coli* (DH5a) cells (Novagen, Darmstadt, Germany). Transformants were screened on Luria Bertani (LB) ampicillin agar plates using the blue and white phenotype. Plasmid DNA was isolated using the Wizard *Plus* SV Minipreps DNA Purification System protocol (Promega, Madison, WI). Screening for positive colonies containing the insert was carried out by PCR using the M13U and M13R primers. BLAST searches of the sequences were performed at the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 15 April 2015) to identify individual isolates.

Phylogenetic Analyses. For the phylogenetic analyses, the 7 ITS sequences identified in this study and 19 ITS sequences from five *Amylostereum* species available from the GenBank were included in the analyses. Two *Echinodontium* species were selected as the outgroup. The GenBank accession numbers and published sources for the sequences analyzed are provided in Table 2. The sequences were aligned using ClustalX 1.83 (Chenna et al. 2003). Alignments were optimized manually in BioEdit (Hall 1999). Maximum parsimony (MP) and Bayesian analysis were conducted for the ITS dataset. All characters were equally weighted and gaps were treated as missing data. Maximum parsimony analyses were performed using PAUP* (Swofford 2002). Gaps in the alignments were treated as missing data. Trees were generated using 1000 replicates of random stepwise addition and a tree-bisection reconnection (TBR) branch-swapping algorithm, with all characteristics being given equal weighting. Branch support for all parsimony analyses was estimated by performing 1,000 bootstrap replicates (Felsenstein 1985), with a heuristic search of 10 random-addition replicates for each bootstrap replicate. Tree length (TL), consistency indices (CI), retention indices (RI), rescaled consistency indices (RC), and the homoplasy index (HI) were calculated for each generated tree. Trees were created in TreeView (Page 1996). Bayesian analysis with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) implementing the Markov Chain Monte Carlo technique and parameters predetermined with MrMODELTEST2.3 (Posada and Crandall 1998, Nylander 2004) was performed, and the parameters in MrBayes were set as follows: lset nst = 6, and rates = gamma. Four simultaneous Markov chains were run with two million generations, starting from random trees, and keeping one tree every 1,000th generation.

Somatic Compatibility Tests. Seven isolates of *A. areolatum* newly generated in this study were made for tests of somatic compatibility. All isolates were maintained on PDA. Inoculations were made with 7-mm plugs cut from the edge of 14-d-old growing cultures. Following the methods of Thomsen and Koch (1999), tests were made on PDA in 6-cm Petri dishes with two or four isolates per dish and all tests were performed at

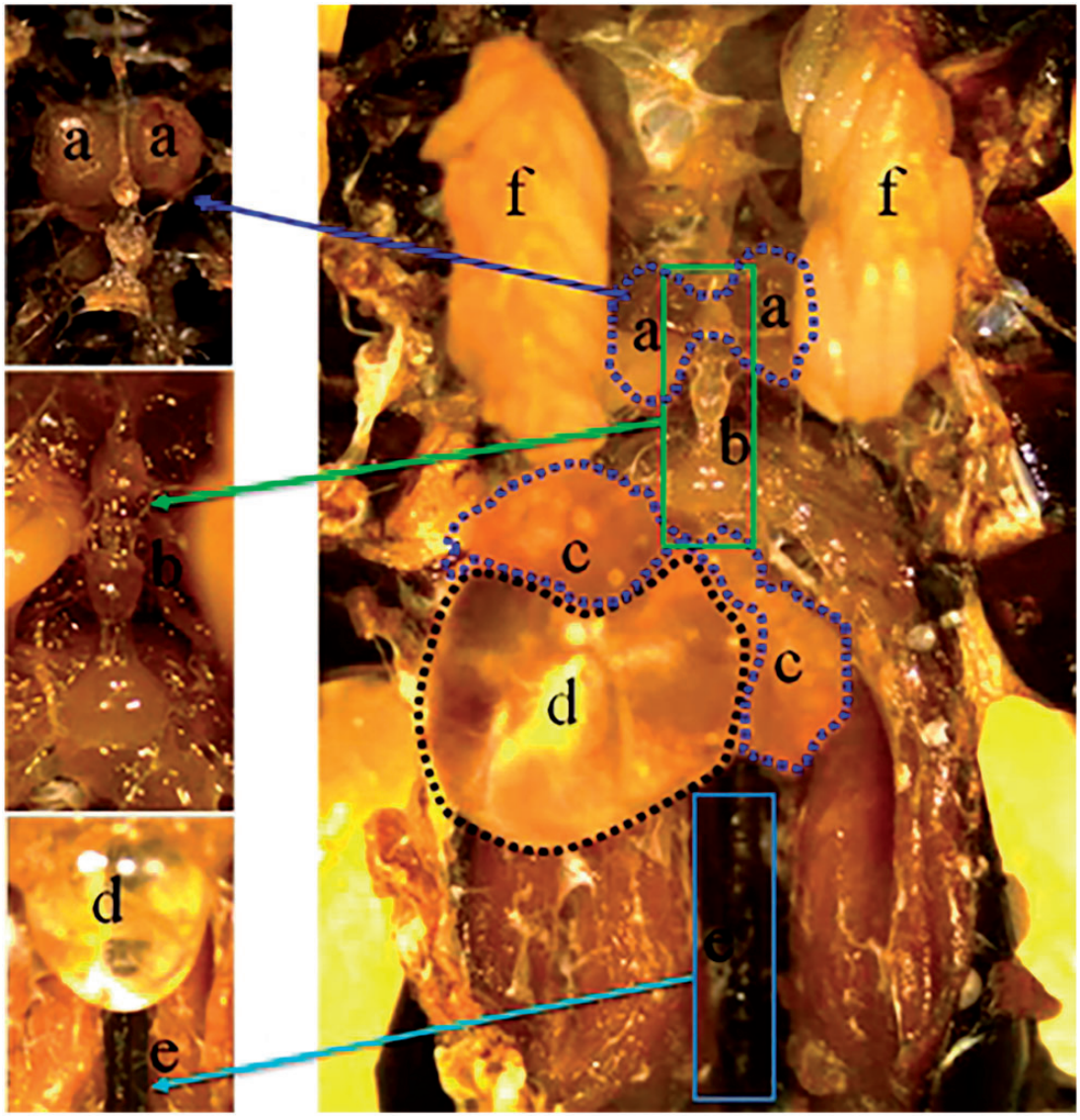

Fig. 1. Major organs of the 2–8 abdominal segments of an adult female *S. noctilio* woodwasp (dorsal view). a: mycangia; b: abdominal ganglion; c: venom gland; d: venom reservoir; e: ovipositor; f: ovaries.

three replicates. In tests with two isolates the plugs were placed at the opposite edges of the dish. In tests with four isolates the plugs were placed in the four "corners" of a square (2.5 by 2.5 cm) in the center of the dish. The inoculated plugs were placed 1.5–2 cm apart from the edge of the dish, in order that the reaction could be seen over the whole width of the dish. The dishes were incubated at an ambient temperature (≈24°C) in darkness for 15–25 d. Fungal isolates were considered to be vegetatively compatible when their interacting hyphae were able to merge. In contrast, incompatible reactions were generally characterized by the appearance of brown demarcation lines and sparse mycelia growth in confrontation zones.

Preparation of the Venom Solutions. The venom gland and reservoir were removed whole from the female wasps kept at 4°C for up to 48 h at the same time that the symbiotic fungus was isolated. Using two different processing methods, two types of venom solution were obtained: crude supernatant and autoclaved supernatant. The treatment involved the venom gland and reservoir being crushed together in reagent grade water using a Dounce homogenizer. The resulting suspension was diluted to a final concentration of 20 mg/ml, based on whole tissue weight. The suspension solution was centrifuged at 17000 g for 8 min, and the resulting supernatant was retained (crude supernatant). The autoclaved supernatant was prepared in the same way as the crude supernatant, except that it was autoclaved. Two different types of venom solution were dispensed into 1-ml aliquots and were stored at −20°C for use (Bordeaux et al. 2014).

Table 2. List of species, voucher specimen and isolate information, and accession numbers of ITS sequences used in the phylogenetic analysis

Species	Specimen/isolate	Host insect or tree	Locality	ITS accession no.
Amylostereum areolatum	YQL01	*Sirex noctilio*	Heilongjiang, China	KP313569[a]
	YQL02	*Sirex noctilio*	Heilongjiang, China	KP313570[a]
	YQL03	*Sirex noctilio*	Heilongjiang, China	KP313571[a]
	YQL04	*Sirex noctilio*	Heilongjiang, China	KP313572[a]
	YQL05	*Sirex noctilio*	Heilongjiang, China	KP313573[a]
	YQL06	*Sirex noctilio*	Heilongjiang, China	KP313574[a]
	YQL07	*Sirex noctilio*	Heilongjiang, China	KP313575[a]
	B1350	*Sirex nitobei*	Japan	AF218389
	CBS334.66	*Unknown*	France	AF454428
	S227	*Picea abies*	Sweden	AY781245
	SIR9-1-2	*Pinus sylvestris*	Canada	EU249344
	N36	*Picea abies*	Latvia	FJ903375
	AH1-01	*Sirex noctilio*	US	GQ422464
	Ecogrow	*S. juvencus*	Hungary	GQ422466
	CBS655.93	—	Denmark	HM461074
	Yuan 2059	*Abies sp.*	Yunnan, China	JX049993
	CMW8900	—	South Africa	KC865582
	CMW3300	—	New Zealand	KC865584
A. chailletii	B1354	*Urocerus gigas*	United Kingdom	AF218391
	RLG8273	—	US	HM461077
A. ferreum	CBS637.84	*Podocarpus lambertii*	Brazil	HM461082
	CBS634.84	*Podocarpus lambertii*	Brazil	AF218390
A. laevigatum	Kotiranta 20108	*Juniperus sp.*	Finland	JX049990
	CBS624.84	*Juniperus nana*	France	AF218396
A. orientale	He 468	*Cunninghamia lanceolata*	China	JX049989
	He 480	*Cunninghamia lanceolata*	China	JX049987
Echinodontium tsugicola	B 1377	*Tsuga diversifolia*	Japan	AF218398
E. tinctorium	B 1122	*Tsuga sp.*	US	AF218397
	DAOM 16666	—	—	AY854088

[a] Sequences obtained from the current study.

Medium and Culture Conditions. Because all seven isolates shared high sequence identity (≥99%) and belonged to one clone, a random sample of *A. areolatum* YQL03 was chosen for this analysis (details are provided in the results section). The fungus was maintained on PDA medium. Potato dextrose broth (PDB, sterilized for 20 min at 121°C) medium was used for the cultures. Five agar plugs obtained from the outer circumference of a 14-d-old *A. areolatum* YQL03 culture colony growing on PDA plates were inoculated in 100-ml volumes of PDB in 250-ml Erlenmeyer flasks. The fungus was grown at 24°C with agitation at 150 rpm for 18 d. For the insect venom induction studies, 25, 50, and 100 µl of the two types of venom solution (crude and autoclaved supernatant) were added to actively growing 5-day-old cultures of *A. areolatum* YQL03. After adding the venom solutions, the fungal cultures were then grown at 24°C with continuous agitation at 150 rpm. Samples from each flask were collected every 2 d, centrifuged, and the clear supernatant was used to measure enzyme activity. A reference culture without any venom solution was used as the control. All experiments were performed in triplicate. Previous studies have shown that natural venom contains amylase, esterase, laccase, and proteolytic enzyme activity, with it being suggested that these enzymes may be partly responsible for the "natural" disaggregation process (Wong and Crowden 1976). To clarify enzyme activity in the venom, a bioassay on the laccase activity of venom solution was conducted at four dilution levels (1, 1/10, 1/100, and 1/500).

Enzyme Activity Assay. Laccase activity was quantitatively determined by measuring the maximum absorption of oxidation of ABTS at A_{420} ($\varepsilon = 36{,}000 M^{-1}cm^{-1}$) after incubation for 3 min at 30°C. Assay mixtures (2 ml) contained 100 µl of 10 mmol/liter ABTS, 1.8 ml of 50 mmol/liter sodium acetate buffer solution (pH = 4.8), and 100 µl enzyme sample or 100 µl venom solution (bioassay on the laccase activity of venom). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min (Collins and Dobson 1997).

Statistical Analysis. All statistical analyses were conducted using SPSS (SPSS.18). When appropriate, the data were checked for normality and homoscedasticity before performing ANOVA analysis. The least significance differences test (LSD, a = 0.05) was used to compare the laccase activities of venom solution at different dilution levels, the laccase activities of *A.areolatum* YQL03 under different culture conditions on the same day and that under the same culture conditions on different days, respectively.

Results

Culture Morphology. The fungus was successfully obtained after being isolated from the mycangia and transferred to new dishes twice. The morphological characteristics of the colony and hypha are shown in Figs. 2A and 3, respectively. When grown on PDA, the fungus produced branched, thin-walled, hyaline

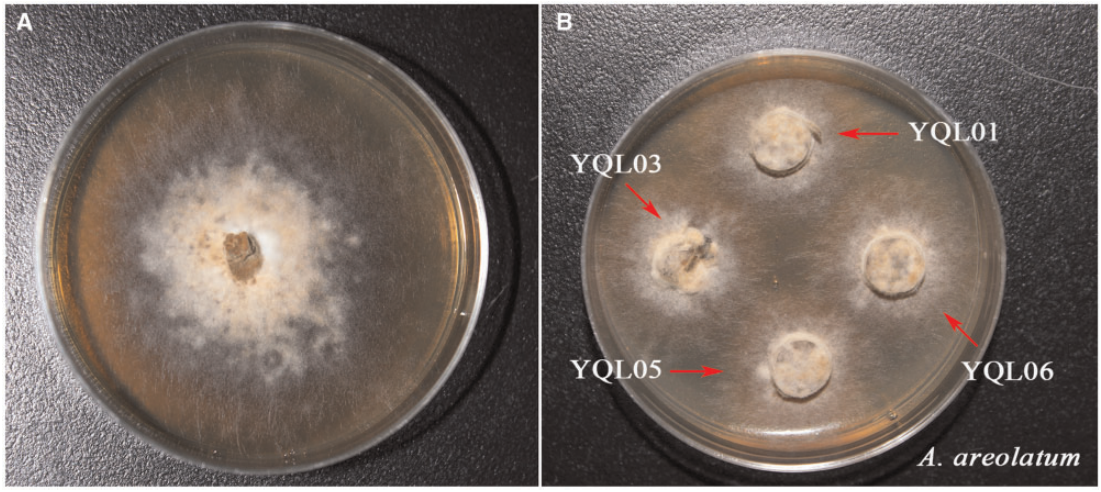

Fig. 2. (A) Fungal culture on PDA 4 wk after the spore mass was isolated from the mycangia of female *S. noctilio* woodwasps reared from infested wood. (B) Example of somatic compatibility between different fungal isolates of *A. areolatum*. Note the free intermingling of mycelia with no antagonistic reaction between the four isolates (*A. areolatum* YQL03, YQL05, YQL06, and YQL03) growing in the same petri dish. Picture was taken on day 20 after the inoculation.

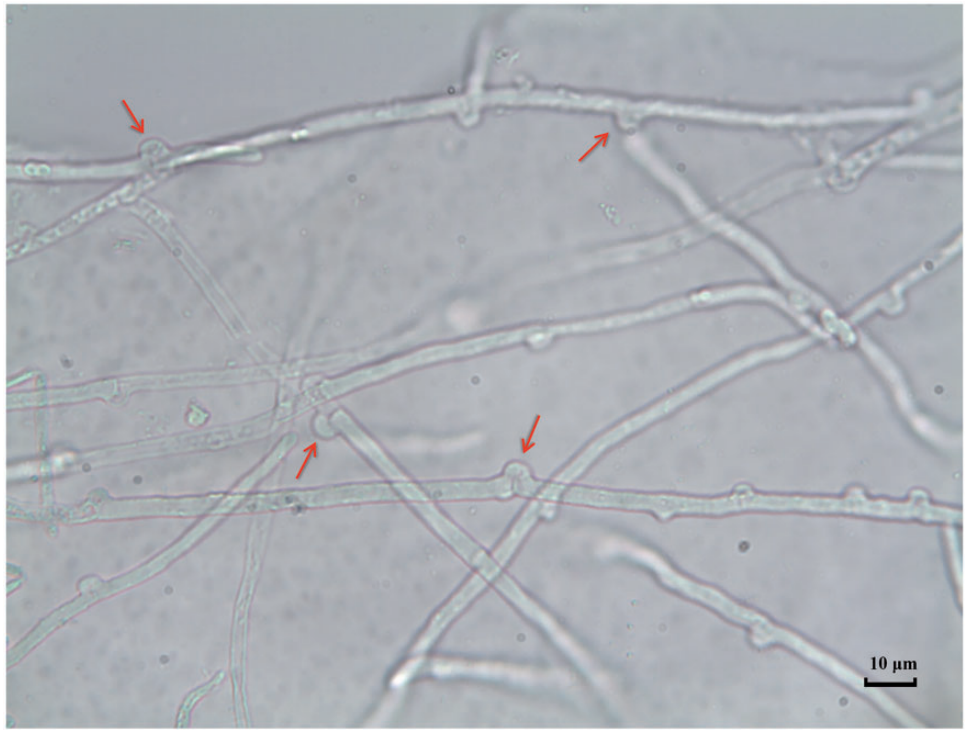

Fig. 3. Hyphal fragments of the fungus from an isolate cultured on PDA. Note the numerous clamp connections (indicated by red arrows) on the hyphae, which are structures unique to the phylum Basidiomycota (Talbot 1964, Gilmour 1965, Thomsen and Harding 2011).

generative hyphae, with abundant simple clamp connections (Fig. 3). After 3–4 wk, the older parts of the mycelium developed cystidia, which were hyphal ends that were pointed and encrusted. The numerous clamp connections (indicated by red arrows) on the hyphae represent structures that are unique to the phylum Basidiomycota. Besides, the fungus could produce oidia in culture (Fig. 4), which was the most important feature of *A. areolatum* for identification (Nobles 1965). It was the presence of oidia together with clamp

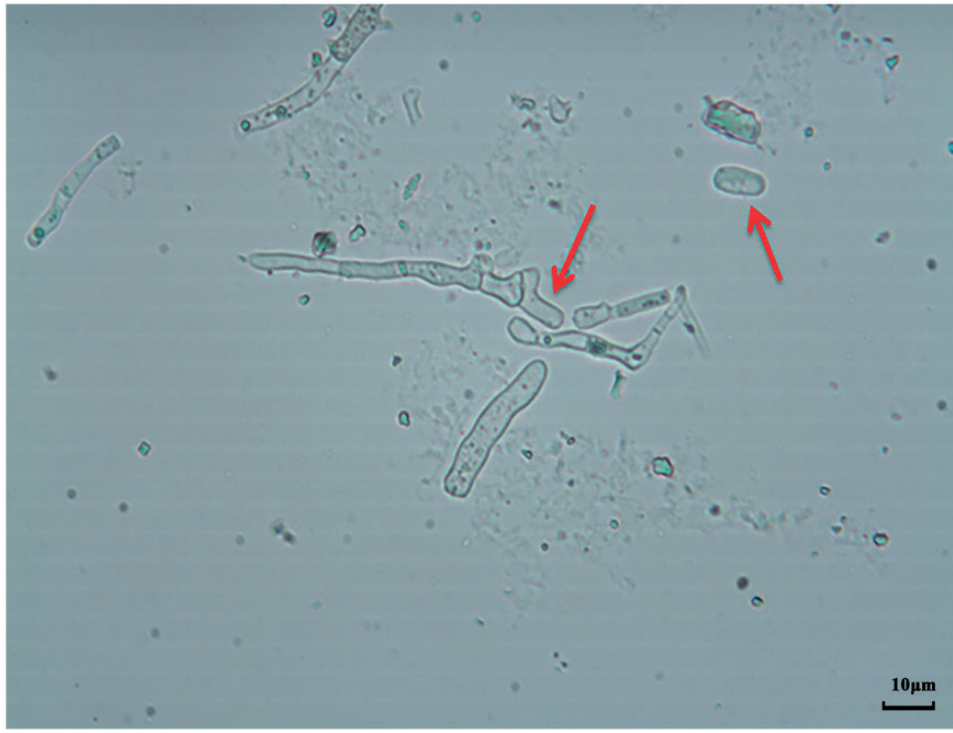

Fig. 4. Hyphal fragments (arthrospores), often called oidia of *A. areolatum* from an isolate cultured on PDA.

connections and cystidia which could give the precise ID of *A. areolatum* (Talbot 1964, Gilmour 1965, Nobles 1965, Thomsen and Harding 2011).

Identification of Isolates. The nuc-ITS-rDNA region targeted with primers ITS4 and ITS1 was successfully amplified for the 10 fungal isolates from the mycangia. By sequence alignment on NCBI, 7 out of the 10 fungal isolates were identified as *A. areolatum*. The remaining three isolates were identified as *Trichoderma viride*, *Verticillium dahlia*, and *Geosmithia pallida*, which were probably contaminants that were introduced during mycangia-spore extraction process.

Phylogenetic Analyses. The seven ITS sequences from the seven isolates of *A. areolatum* were newly generated in this study (GenBank KP313569–KP313575, Table 1). The alignment of 29 ITS sequences from the seven species resulted in 536 base pairs with 67 parsimony informative characters. Three equally parsimonious trees were obtained from the analysis with TL = 261, CI = 0.878, RI = 0.942, RC = 0.827, and HI = 0.122 (Fig. 5). The 50% majority consensus tree generated by the Bayesian analysis showed a similar topology with the strict consensus MP tree, and only the topology from MP analysis is presented, while both BT values and BPPs are shown at the nodes (Fig. 5). The strict consensus tree showed that the *Amylostereum* species was well separated from the outgroup. Phylogenetic analyses of the ITS locus confirmed that the seven isolates (YQL01, YQL02, YQL03, YQL04, YQL05, YQL06, and YQL07) from this study were present within the *A. areolatum* population. The seven isolates shared ≥99% sequence identity to each other.

Somatic Compatibility Tests. Within all the seven isolates of *A. areolatum*, there was a high degree of somatic compatibility between isolates. The free intermingling of mycelia with no antagonistic reaction was observed in every treatment. All the seven isolates belonged to one clone or vegetative compatibility group. More details are shown in Table 3 and Figure 2B.

Bioassay on the Laccase Activity of Venom. As shown in Fig. 6, a maximum activity of 392 U/liter was detected in the crude supernatant when diluted 100 times, which was significantly higher than the laccase activity recorded at other dilution levels (96 U/liter for the initial concentration, 183 U/liter at 10 times the dilution level, and 223 U/liter at 500 times the dilution level). However, laccase activity was not detected in the autoclaved supernatant at any dilution level, which was probably due to the thermal denaturation of laccase protein during the preparation of venom solutions.

Effect of Venom on the Production of Laccase by *A. areolatum* YQL03. The effect of *Sirex* venom on extracellular laccase production by *A. areolatum* YQL03 was determined from 5-day-old cultures treated with different levels of the two types of venom solution (autoclaved and crude supernatant), with laccase activity produced by the fungus being measured at subsequent time intervals. As shown in Fig. 7A and B, extracellular laccase activity remained relatively low throughout incubation in the absence of either

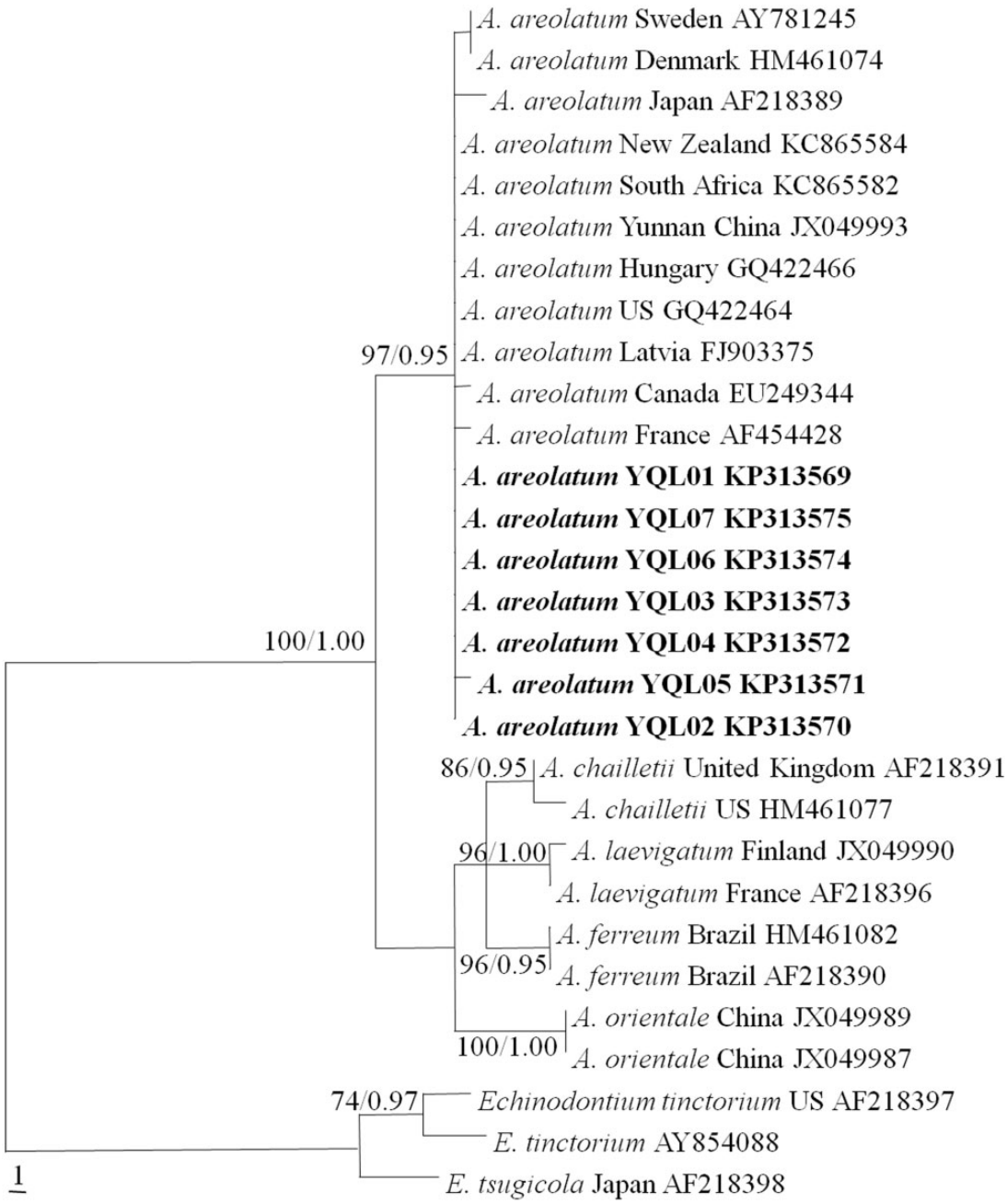

Fig. 5. Strict consensus tree obtained from the Maximum Parsimony analysis of the ITS sequences of *Amylostereum*. *Echinodontium* was used as the outgroup. Branchers are labeled with parsimony bootstrap proportions (before the slash markers) higher than 70% and Bayesian posterior probabilities (after the slash markers) more than 0.95.

venom solution. Maximum activity was just 133 U/liter on day 13. However, extracellular laccase production might be significantly stimulated by the two types of venom solution. After 13 days of incubation in the presence of the venom solution, extracellular laccase activity increased to 2,899 U/liter (100 µl autoclaved supernatant, Fig. 7A) and 2,799 U/liter (100 µl crude supernatant, Fig. 7B). Compared to the control, laccase production increased by 20.8-fold and 20-fold in the presence of the two types of venom, respectively. We also observed that extracellular laccase activity increased as the dosage of venom solution increased (Fig. 7A and B).

Based on these results, comparison of the effects of the two types of venom solution on extracellular laccase production by *A. areolatum* YQL03 was carried out.

Table 3. Somatic compatibility tests of the seven isolates of *A. areolatum*

Isolate	Host insect	Locality	YQL01	YQL02	YQL03	YQL04	YQL05	YQL06	YQL07
YQL01	*S. noctilio*	HLJ	+	+	+	+	+	+	+
YQL02	*S. noctilio*	HLJ	+	+	+	+	+	+	+
YQL03	*S. noctilio*	HLJ	+	+	+	+	+	+	+
YQL04	*S. noctilio*	HLJ	+	+	+	+	+	+	+
YQL05	*S. noctilio*	HLJ	+	+	+	+	+	+	+
YQL06	*S. noctilio*	HLJ	+	+	+	+	+	+	+
YQL07	*S. noctilio*	HLJ	+	+	+	+	+	+	+

Note: "+" means the reaction between the isolates was fully compatible. HLJ means Heilongjiang Province, China.

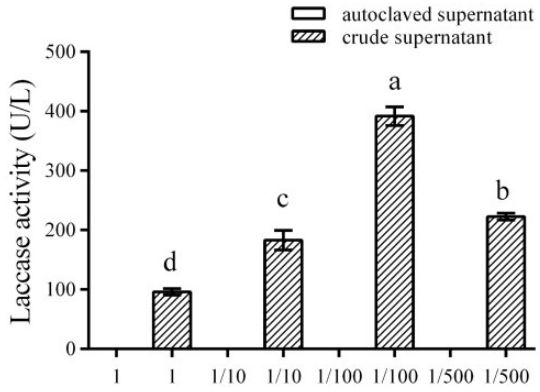

Fig. 6. Laccase activities of two types of venom solution (autoclaved supernatant and crude supernatant) at four dilution levels (1, 1/10, 1/100, and 1/500). Error bars represent the standard deviation (SD). Different lowercase letters indicate a significant difference among laccase activities at different dilution levels ($P < 0.05$).

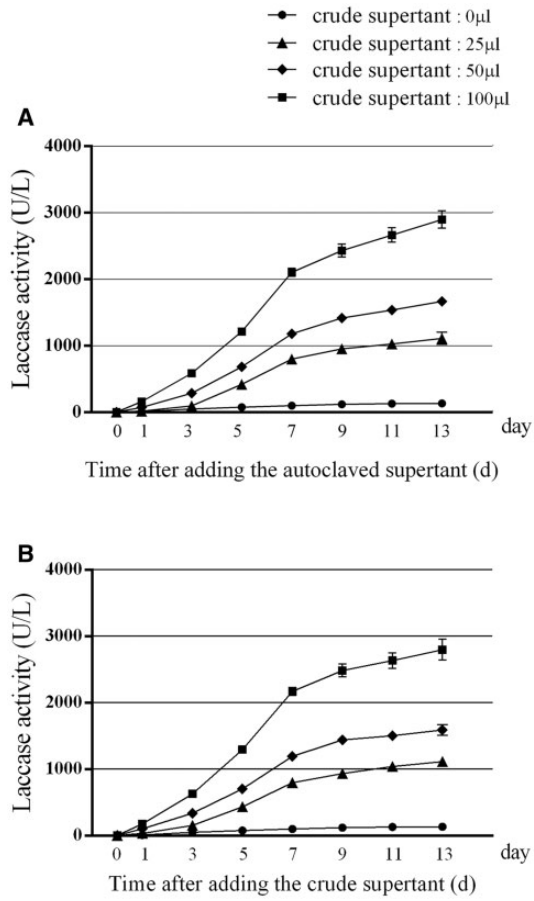

Fig. 7. Effect of two types of venom solution (autoclaved supernatant and crude supernatant) on extracellular laccase production by *A. areolatum* YQL03. Error bars represent the standard deviation (SD). (A) Time course of laccase activity in extracellular fluid of *A. areolatum* YQL03 culture after the addition of different dosages of autoclaved supernatant. (B) Time course of laccase activity in the extracellular fluid of *A. areolatum* YQL03 culture after the addition of different dosages of crude supernatant.

As shown in Fig. 8A, B, and C, extracellular laccase production was noticeably stimulated by both types of venom solution, regardless of dosage, with laccase production being significantly higher than the control throughout the whole incubation period after adding venom solution. There was no significant difference between the two types of venom during most of the cultivation period. However, laccase activity was more stimulated by crude supernatant than the autoclaved supernatant during the early incubation period. For example, laccase activity increased to 34 U/liter on day 1 and 155 U/liter on day 3 after adding 25 µl crude supernatant following 5 days cultivation (Fig. 8A). In comparison, laccase activity was 19 U/liter and 99 U/liter on day 1 and 3, respectively, after adding 25 µl autoclaved supernatant (Fig. 8A). Similar cases were documented in the other two concentration levels (Fig. 8B and C).

Discussion

In our study, seven isolates of *A. areolatum* were successfully obtained from the mycangia of adult *S. noctilio* females reared in a standard quarantine facility. Phylogenetic analyses of the ITS sequences from the seven isolates obtained in our study and other isolates available from the GenBank confirmed the identity of the fungal symbiont of *S. noctilio* detected in China (Y. L., unpublished data).

The test of the *Sirex* venom in a laccase assay showed that the native venom contained laccase, which confirmed previous assumptions about the bioactivity

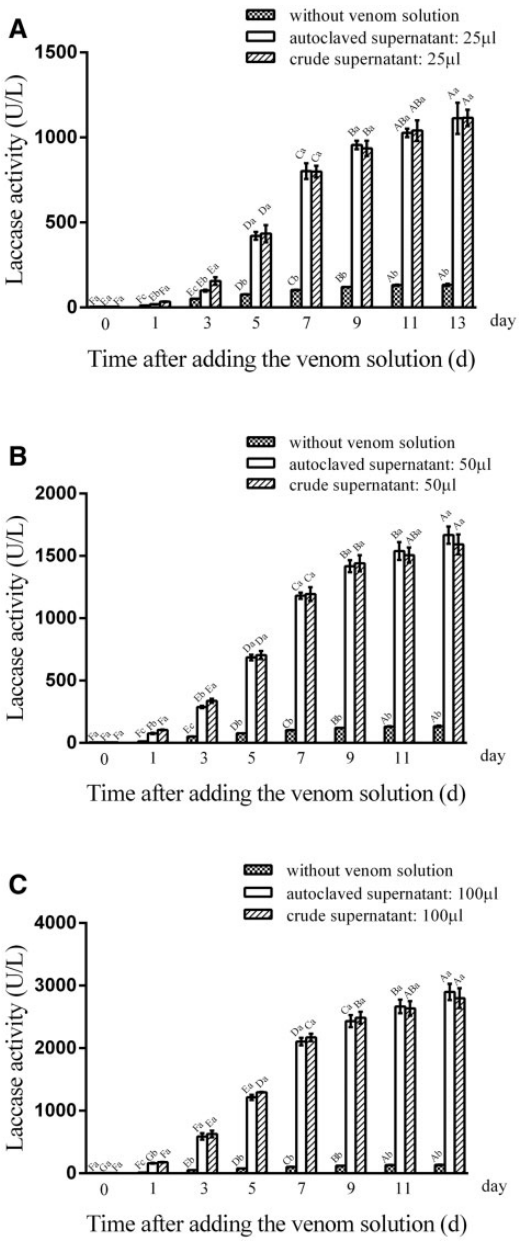

Fig. 8. Comparison of the effect of two types of venom solution (autoclaved supernatant and crude supernatant) on extracellular laccase production by *A. areolatum* YQL03. (A) 25 μl. (B) 50 μl. (C) 100 μl. Different lowercase letters indicate a significant difference between laccase activity under different culture conditions on the same day ($P < 0.05$); different uppercase letters indicate a significant difference between laccase activity under the same culture conditions on different days ($P < 0.05$). Error bars represent the standard deviation (SD).

of *Sirex* venom (Wong and Crowden 1976). The absence of laccase activity in the autoclaved supernatant proved that active laccase protein is only present in crude supernatant that is not subjected to autoclaving. Thus, venom solution may contain one or several heat-stable factors that are crucial for noticeably increasing laccase production by *A. areolatum* YQL03, as both types of venom solutions are able to significantly stimulate extracellular laccase production by the fungus, with no significant difference between the two during most of the cultivation period. Several studies have investigated the properties of *Sirex* venom. For instance, Bordeaux et al. (2014) used size fractionation and reversed-phase high-performance liquid chromatography together with activity assays based on defense gene induction of the host to determine the bioactive factor that is responsible for attacking the host in *S. noctilio* venom. The authors revealed that Noctilisin, which is a venom glycopeptide of *S. noctilio*, causes needle wilt and induces defense gene expression in attacked conifers. These results are expected to guide our future studies on the purification and characterization of the heat-stable factors in *Sirex* venom that are expected to significantly stimulate laccase production by *A.areolatum* YQL03.

In this work, it was found that the laccase production by *A. areolatum* YQL03 could be prominently stimulated by insect venom. Naturally, it was interesting to make clear the mechanism underlying this phenomenon, in particular, how these putative heat-stable factors in venom affected the fungus. To our knowledge, there have been few reports on the effect of *Sirex* venom on the laccase production in its fungal symbiont. However, previous researchers have made brilliant achievement from the study on the laccase of other white-rot fungi. For instance, Yang et al. (2013) have found that laccase production and laccase gene transcription in *Trametes velutina* 5930 can be stimulated by a range of factors including different metal ions and aromatic compounds. Some putative cis-acting elements present in the promoter region of *lac5930-1* gene (metal-responsive elements, xenobiotic-responsive elements, and aromatic-responsive elements) may be closely related to the induction of laccase gene transcription by different factors. Furthermore, addition of some aromatic compounds can decrease the extracellular proteolytic activity, thus increasing the laccase stability. Based on the results and analysis above, they speculate that the higher laccase activity induced by some aromatic compounds may be due to not only the increase of laccase gene transcription, but also the improvement of the enzyme stability. Because *S. noctilio* venom was described by early researchers as an acid mucopolysaccharide–protein complex (Boros 1968, Wong and Crowden 1976), some kind of substance in the venom, especially the putative heat-stable factors, may share the similar characteristics and functions with the metal ions and aromatic compounds mentioned above.

In conclusion, this study confirms the presence of *A. areolatum* in *S. noctilio* specimens from China. We confirm that laccase activity in the fungus is prominently enhanced by insect venom. This result supports the "condition" hypothesis, whereby the insect venom is assumed to facilitate the establishment of the fungus in pine trees, which, in turn, helps generate the ideal environment for the development of insect larvae. We believe that further analysis of the putative heat-stable factors identified in *S. noctilio* venom may

contribute novel information about the pathogenic mechanism of the *S. noctilio–A. areolatum* pine-killing pest complex on host trees. Such information could be used to combat the severe damage caused to introduced North American pine plantations, leading to major economic losses, in countries of the Southern Hemisphere.

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References Cited

Bertrand, T., C. Jolivalt, P. Briozzo, E. Caminade, N. Joly, C. Madzak, and C. Mougin. 2002. Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry 41: 7325–7333.

Borchert, D., G. Fowler, and L. Jackson. 2007. Organism pest risk analysis: Risks to the conterminous United States associated with the woodwasp, *Sirex noctilio* Fabricius, and the symbiotic fungus, *Amylostereum areolatum*, (Fries: Fries) Boidin. USDA-APHIS-PPQ-CPHST-PERAL. Rev. 1. (www.aphis.usda.gov/plant_health/plant_pest_info/sirex/downloads/sirex-pra.pdf) (accessed 15 April 2015).

Bordeaux, J. M. 2008. Characterization of growth conditions for production of a laccase-like phenoloxidase by *Amylostereum areolatum*, a fungal pathogen of pines and other conifers. M.S. thesis, University of Georgia, Athens, GA.

Bordeaux, J. M., W. W. Lorenz, D. Johnson, M. J. Badgett, J. Glushka, R. Orlando, and J. F. Dean. 2014. Noctilisin, a venom glycopeptide of *Sirex noctilio* (Hymenoptera: Siricidae), causes needle wilt and defense gene responses in pines. J. Econ. Entomol. 107: 1931–1945.

Boros, C. B. 1968. The relationship between the woodwasp *Sirex Noctilio* F. and the wood-rot fungus *Amylostereum* sp. Master of Agricultural Science M. S., University of Adelaide, Adelaide, Australia.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31: 3497–3500.

Ciesla, W. M. 2003. European woodwasp: a potential threat to North America's conifer forests. J. For. 101: 18–23.

Collins, P. J., and A. Dobson. 1997. Regulation of laccase gene transcription in *Trametes versicolor*. Appl. Environ. Microb. 63: 3444–3450.

Coll, P. M., J. Fernandez-Abalos, J. Villanueva, R. Santamaria, and P. Perez. 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl. Environ. Microb. 59: 2607–2613.

Coutts, M. 1968. Rapid physiological change in *Pinus radiata* following attack by *Sirex noctilio* and its associated fungus, *Amylostereum* sp. Aust. J. Sci. 30: 275–277.

Coutts, M. 1969a. The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* II. Effects of *S. noctilio* mucus. Aust. J. Biol. Sci. 22: 1153–1162.

Coutts, M. 1969b. The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata* I. Effects of the symbiotic fungus *Amylostereum* sp. (Thelophoraceae). Aust. J. Biol. Sci. 22: 915–924.

Coutts, M. 1969c. The formation of polyphenols in small blocks of *Pinus radiata* sapwood with and without the fungal symbiont of *Sirex*. Aust. For. Res. 4: 29–34.

Coutts, M., and J. Dolezal. 1965. *Sirex noctilio*, its associated fungus, and some aspects of wood moisture content. Aust. For. Res. 1: 3–13.

Coutts, M., and J. Dolezal. 1966. Some effects of bark cincturing on the physiology of *Pinus radiata*, and on *Sirex* attack. Aust. For. Res. 2: 17–28.

Desai, S., and C. Nityanand. 2011. Microbial laccases and their applications: a review. Asian J. Biotechnol. 3: 98–124.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

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

Gilmour, J. 1965. The life cycle of the fungal symbiont of *Sirex noctilio*. N. Z. J. For. 10: 80–89.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, pp. 95–98. *In* T. A. Hall, and K. Ursula (eds.), Nucleic Acids Symposium Series. Oxford University Press, New York, NY.

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. FEMS Microbiol. Rev. 13: 125–135.

Haugen, D. A. 2007. *Sirex* woodwasp: biology, ecology and management, p. 48. *In* Proceedings, 17th US Department of Agriculture Interagency Research Forum on Gypsy Moth and Other Invasive Species, 10–13 January 2006, Annapolis, MD. USDA Forest Service, Delaware, OH.

Hurley, B. P., B. Slippers, and M. J. Wingfield. 2007. A comparison of control results for the alien invasive woodwasp, *Sirex noctilio*, in the southern hemisphere. Agric. For. Entomol. 9: 159–171.

Iede, E. T., S.R.C. Penteado, and E. G. Schaitza. 1988. *Sirex noctilio* problem in Brazil-detection, evaluation and control, pp. 45–52. *In* E. Iede, E. Shaitza, S. Penteado, R. Reardon, and T. Murphy (eds.), Proceedings of a Conference: Training in the Control of *Sirex noctilio* by use of Natural Enemies. U.S. Department of Agriculture, Forest Service, Morgantown, WV (FHTET 98-13).

Lankinen, P., K. Hildén, N. Aro, M. Salkinoja-Salonen, and A. Hatakka. 2005. Manganese peroxidase of Agaricus bisporus: grain bran-promoted production and gene characterization. Appl. Microbiol. Biot. 66: 401–407.

Müller, W. 1934. Untersuchungen über die Symbiose von Tieren mit Pilzen und Bakterien. Arch. Microbiol. 5: 84–147.

Madden, J. L. 1988. *Sirex* in Australasia, pp. 407–429. *In* A. A. Berryman (eds.), Dynamics of forest insect populations. Plenum Publishing Corporation, New York, NY.

Madden, J. L., and M. Coutts. 1979. The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). Insect–Fungus Symbiosis: 165–174.

Madhavi, V., and S. Lele. 2009. Laccase: properties and applications. BioResources 4: 1694–1717.

Martínez, Á. T., M. Speranza, F. J. Ruiz-Dueñas, P. Ferreira, S. Camarero, F. Guillén, M. J. Martínez, A. Gutiérrez, and J. C. del Río. 2010. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. 8: 195–204.

Morgan, F. D., and N. C. Stewart. 1966. The biology and behaviour of the wood-wasp *Sirex noctilio* F. in New Zealand. Trans. R. Soc. N. Z 7: 195–204.

Morozova, O., G. Shumakovich, M. Gorbacheva, S. Shleev, and A. Yaropolov. 2007. "Blue" laccases. Biochemistry (Moscow) 72: 1136–1150.

Nobles, M. K. 1965. Identification of cultures of wood-inhabiting Hymenomycetes. Can. J. Bot. 43: 1097–1139.

Nylander, J. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University 2, Sweden.

Page, R.M.D. 1996. Treeview: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12: 357–358.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Rawlings, G. 1948. Recent observations on the *Sirex noctilio* population in *Pinus radiata* forest in New Zealand. N. Z. J. For. 5: 411–421.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Slippers, B., T. A. Coutinho, B. D. Wingfield, and M. J. Wingfield. 2003. A review of the genus *Amylostereum* and its association with woodwasps. S. Afr. J. Sci. 99: 70–74.

Spradbery, J. P., and A. Kirk. 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. Bull. Entomol. Res. 68: 341–359.

Swofford, D. L. 2002. Phylogenetic analysis using parsimony and other program (PAUP*), ver. 4.0 b10. Sinauer Associates, Sunderland, MA.

Talbot, P.H.B. 1964. Taxonomy of the fungus associated with *Sirex noctilio*. Aust. J. Bot. 12: 46–52.

Talbot, P.H.B. 1977. The *Sirex-Amylostereum-Pinus* association. Annu. Rev. Phytopathol. 15: 41–54.

Taylor, K. 1981. The *Sirex* woodwasp: ecology and control of an introduced forest insect. pp. 231–248. *In* R. L., Kitching and R. E., Jones (eds.), The Ecology of Pests. (CSIRO) Commonwealth Scientific and Industrial Research Orginazation, Melbourne, Australia.

Thomsen, I. M., and J. Koch. 1999. Somatic compatibility in *Amylostereum areolatum* and *A. chailletii* as a consequence of symbiosis with siricid woodwasps. Mycol. Res. 103: 817–823.

Thomsen, I. M., and S. Harding. 2011. Fungal symbionts of siricid woodwasps: Isolation techniques and identification. For. Pathol. 41: 325–333.

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

Vasiliauskas, R. 1999. Spread of *Amylostereum areolatum* and *A. chailletii* decay in living stems of *Picea abies*. Forestry 72: 95–102.

Wermelinger, B., and I. M. Thomsen. 2012. The woodwasp *Sirex noctilio* and its associated fungus *Amylostereum areolatum* in Europe, pp. 65–80. *In* B. Slippers, P., de Groot and M. J. Wingfield (eds.), The *Sirex* Woodwasp and its Fungal Symbiont. Springer, Dordrecht, Netherlands.

Wong, L. K., and R. K. Crowden. 1976. Preliminary studies on the mucus secretion of the wood wasp, *Sirex noctilio* F. I Physicochemical and biochemical properties. Aust. J. Biol. Sci. 29: 21–32.

Yang, Y., F. Wei, R. Zhuo, F. Fan, H. Liu, C. Zhang, L. Ma, M. Jiang, and X. Zhang. 2013. Enhancing the laccase production and laccase gene expression in the white-rot fungus *Trametes velutina* 5930 with great potential for biotechnological applications by different metal ions and aromatic compounds. PloS ONE 8: e79307.

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