Host specificity and diversity of *Amylostereum* associated with Japanese siricids

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Abstract

The mutualism between siricid woodwasps and *Amylostereum* fungal symbionts has long been considered to be species-specific. Recent studies from North America have challenged this assumption, where native siricids and the introduced *Sirex noctilio* are clearly swapping symbionts. Whether this pattern is a consequence of invasion or an underappreciated property of siricid biology is unknown. Here we show that the native Japanese siricid, *Sirex nitobei,* carries both *Amylostereum areolatum* and *Amylostereum chailletii*, rather than only *A. areolatum* as long assumed. Furthermore, all samples from a *Urocerus* sp. unexpectedly carried, *A. chailletii* and not *Amylostereum laevigatum.* Vegetative compatibility group tests revealed extensive clonality, with one VCG present amongst three *A. areolatum* isolates and six VCGs present amongst 61 *A. chailletii* isolates. These results contribute to the understanding of insect-fungal fidelity in the siricid-*Amylostereum* association and, together with other studies, suggest that host tree influences *Amylostereum* species occurrence, perhaps more strongly than wasps.

Insect-fungal symbiosis, host fidelity, clonal population, Siricidae, Basidiomycota

Introduction

Siricid woodwasps and *Amylostereum* fungi live in mutualism that is obligatory for the wasp but not for the fungus. The numerous studies of this symbiosis have been particularly stimulated by the significant damage that a particular Eurasian species pair, *Sirex noctilio* and *Amylostereum areolatum*, have caused in areas where it is invasive and attacks commercially-grown *Pinus* trees (Slippers and Wingfield, 2012). The invasion outside the native Eurasian range was first noted around 1900 in New Zealand, followed by spread over the next decade to all major pine-growing areas of the Southern Hemisphere. In 2005, *S. noctilio* was also reported from the USA, which has brought much research interest to the field in recent years.

The siricid-*Amylostereum* mutualism is maintained across wasp generations by transmission of asexually produced propagules of the fungus by the female wasp (Slippers *et al.*, 2003; 2015). Female siricid wasps have specialized internal organs called mycangia in which they carry asexual arthrospores of *Amylostereum* which they introduce into the tree host whilst laying their eggs. This mode of reproduction and spread of asexual spores results in the widespread distribution of fungal clonal lineages. While not strictly vertically-transmitted, the offspring acquire fungal inoculum from the larval environment which was inoculated at the time of oviposition by the mother. In most cases, therefore, the maternal strain is re-acquired, during development, though the possibility of acquiring other strains during tunnelling does exist.

Prior to the widespread availability of rapid molecular tools, fungal clones were often identified and delineated using compatible growth assays to establish Vegetative Compatibility Groups (VCGs). For example, Vasiliauskas *et al*. (1998) found a VCG of *A. areolatum* across more than 600 km and from isolates made 12 years apart in Scandinavia. They also found that clonality was more prevalent in *A. areolatum* (12 VCGs from 53 isolates) than in *Amylostereum chailletii* (47 VCGs from 57 isolates). This aligns with the fact that *A. chailletii* sexual fruit bodies appear to be more common than *A. areolatum*. Slippers *et al*. (2001) used VCGs to identify a clone that has been spread with invasive *S. noctilio* across the Southern Hemisphere. In another part of the invasive range in Canada, Wooding *et al.* (2013) identified 14

VCGs from 27 isolates of *A. areolatum*. Slippers *et al*. (2015) suggest that wasps could acquire sexually produced genotypes of the fungus, which could help explain the higher VCG diversity observed in North America.

Since its first description in 1929 by Carthwright, the *S. noctilio-A. areolatum* symbiosis had been viewed as highly specific (Slippers *et al*., 2003). Strict co-speciation is not supported in the siricid-fungal association as the same fungus is often carried by multiple siricid species. However, it was well-entrenched in the literature that each siricid carries but a single *Amylostereum* fungus, suggesting the absence of contemporary symbiont swapping, perhaps enforced by the biology of the wasps. Recent studies from North America have called this into question, however, by showing that *A. areolatum* that presumably arrived with the invasive *S. noctilio* in 2004 is regularly carried by several native siricids. For example, *Sirex nigricornis* and *Sirex nitidus*, thought to exclusively carry *A. chailletii*, in fact regularly carry *A. areolatum*, that it putatively acquired from *S. noctilio* (Hajek *et al.*, 2013; Olatinwo *et al.*, 2013; Wooding *et al.*, 2013). Both Hajek *et al*. (2013) and Olatinwo *et al*. (2013) also found *A. areolatum* associated with *S. nigricornis* outside the range of invasive *S. noctilio,* raising the possibility that the association predates the known arrival of *S. noctilio*.

Native siricids worldwide are generally understudied, owing to their low abundance and habit of attacking only dead or highly stressed trees with little economic value. As a consequence of the global importance of *S. noctilio* – a species capable of killing healthy trees in the Southern Hemisphere – some of these native species and their fungal symbionts have received recent attention. In North America, there is a diverse native siricid fauna. However, the question of fidelity in the wasps-symbiont relationship is complicated by the invasion of *S. noctilio* since novel interactions among wasps, trees and fungi may yield different patterns that may differ in a native, co-evolved context.

In Japan, four genera of the family Siricidae, namely *Sirex*, *Urocerus*, *Xeris* and *Xoanon*, have been reported (Takeuchi, 1962; Tabata *et al.*, 2012). All species are believed to be native to the region. Two *Amylostereum* species have been confirmed to be present in Japan, namely *A. areolatum* and *Amylostereum laevigatum*. *A.*

laevigatum has been isolated from *Urocerus antennatus* and *rocerus. japonicus* and confirmed using DNA sequence data (Tabata and Abe, 1997, 1999). *Sirex nitobei* and *Xanon. matsumurae* were shown to carry *A. areolatum*, also supported by sequence data (Tabata *et al*., 2000, 2012). *A. chailletii* has been described from *U. antennatus*, but only based on culture morphology (Sano *et al.*, 1995) and was likely misidentified (Tabata, *pers. obs.*). The identification of *Amylostereum* species based on culture morphology is complicated and has led to mistaken identities in the past (Thomsen and Harding, 2011).

The aim of this study was to identify the *Amylostereum* spp. associated with two common Japanese siricid species, namely *S. nitobei* and an unknown *Urocerus* species. We used sequence data of both the nuclear internal transcribed spacer (ITS) rDNA and mitochondrial small subunit (mtSSU) rDNA to identify fungal isolates obtained from these wasps. We also considered the diversity and clonality of the *Amylostereum* isolates using VCGs. Using 64 samples across two sites and three tree species, we examined the degree of specificity between *Amylostereum* fungi in these insects in the context of the native pine ecosystem with diverse siricid fauna where no known siricid invaders occur.

Materials and Methods

Fungal sources and DNA extraction

Fifty five *Amylostereum* isolates were collected from mycangia of the *Urocerus* sp. specimens outside Komoro city in the Nagano prefecture in Japan. Nine *Amylostereum* isolates were collected from mycangia from *S. nitobei* outside Takko Town in the Aomori prefecture in Japan. Isolations were made using the techniques reported by Thomsen and Harding (2011). The nine *S. nitobei* isolates in this study were all associated with *Pinus densiflora*, while the 55 isolates from the *Urocerus* sp. were obtained from two tree hosts, *Abies homolepis* and *Larix leptolepis.* The *Urocerus* sp. in this study is a new species (unpubl. data) of woodwasp in Japan and will be described in a future study (Tabata, *pers. comm*.).

All isolates were maintained on potato dextrose agar (PDA; Merck (Pty) Ltd South Africa) and are deposited in the Culture Collection of the Tree Protection

Cooperative Programme (CMW) (Supplementary Table 1). The mycelia were collected and phenol-chloroform extraction performed. DNA was precipitated overnight using 0.1 volume of NaAc and 1 volume of 100% $EtOH_2$. After centrifugation (Eppendorf 5417C, Hamburg, Germany) and clean-up with 70% $EtOH_2$ the samples were vacuum dried (Concentrator 5301, Eppendorf, Hamburg, Germany) at 45°C for 5 min. The dried DNA was resuspended in 100µl sterile distilled water.

Primer amplification and DNA sequencing

To identify the *Amylostereum* isolates the mtSSU rDNA primer pair MS1 and MS2 (White *et al.*, 1990) were used to amplify a portion of the mitochondrial small subunit, as well as the ITS1 and ITS4 primers to amplify the internal transcribed spacer (ITS rDNA) region (White *et al.*, 1990). PCR volume for MS1 and MS2 was 25 µl, 5 µl of MyTaq™ Reaction Buffer (Bioline USA, Taunton, Massachusetts), 0.1 µM of both MS1 and MS2, 0.5 µl of MyTaq™ DNA polymerase (Bioline USA, Taunton, Massachusetts) and around 100 ng of the template DNA was added. PCR conditions were as follows: preincubation of 95°C 3 min, 35 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 1 min, and a final extension of 72°C for 10 min. For amplification of the ITS rDNA gene the PCR protocol and cycling conditions from Wooding *et al.* (2013) were followed. The PCR amplicons were sequenced using the ABI Prism™ 3500xL automated DNA sequencer (Applied Biosystems USA, Foster City, California). All sequence data was submitted to GenBank (ITS: **KU870238 - KU870275**; mtSSU: **KU870276 - KU870311**).

Phylogenetic analyses

Sequence data representing authentic isolates of described *Amylostereum* spp. were obtained from GenBank and alignment of the entire dataset constructed online by means of MAFFT v7 (Katoh and Standley, 2013). This was verified with ClustalW in MEGA v6 (Tamura *et al.*, 2011). While ITS and mtSSU data were present for all species, both could not always be found for the same isolates. The best fit model was selected for the resulting datasets using jModelTest v2.1.3 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012) and used to construct the maximum likelihood tree (ML) using PhyML v3.1 (Guindon and Gascuel, 2003). Maximum parsimony

(MP) phylogenetic analyses were also done using PAUP v4.0b10 (Swofford, 2003). Measures such as tree length (Lombardero *et al.*), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) were recorded. Statistical support for branching for both analyses were determined through 1000 bootstrap replicates.

Vegetative compatibility analysis

VCG analysis was used to investigate clonality among *A. areolatum* and *A. chailletii* isolates as described by Vasiliauskas *et al*. (1998) and Slippers *et al*. (2001). All the isolates from each of the two prefectures were crossed in all possible combinations on PDA. Hyphal plugs of 0.5 cm^2 were cut from the edge of actively growing cultures and placed around 2 cm apart on a 9 cm Petri dish with PDA, and incubated at 23°C. After 3 - 4 weeks compatibility was determined by scoring the presence or absence of a brown demarcation zone. The presence of the demarcation zone was considered as an incompatible reaction whereas the mergence of the two cultures showed compatibility between the cultures.

Results

DNA sequencing and phylogenetic analyses

Based on sequence data the ITS amplicon was 604 bp for *A. chailletii* and 607 bp for *A. areolatum*. For the mitochondrial small subunit region the amplicon was calculated to be 519 bp for *A. areolatum* and 516 bp for *A. chailletii.* Analyses on the two gene regions were performed separately. The dataset for ITS after alignment consisted of 439 characters (30 parsimony-uninformative, 24 parsimony-informative, 385 constant characters). One thousand most parsimonious trees were found (TL = 38, CI = 0.763, RI = 0.906, and RC = 0.692). The mtSSU dataset consisted of 493 characters after alignment (23 parsimony-uninformative, 28 parsimony-informative, 442 constant characters). Forty-two most parsimonious trees were found (TL = 40, CI = 0.900, RI = 0.970, and RC = 0.873).

Based on phylogenetic analyses, 61 of 64 isolates in our study were grouped strongly with known *A. chailletii* sequences (ML/MP; Fig. 1). The remaining three samples were confirmed as *A. areolatum* (ML/MP; Fig. 1). The *A. chailletii* isolates

were isolated from both species of woodwasps, *S. nitobei* and the *Urocerus* sp. *A. chailletii* was collected from both the Nagano and Aomori prefecture (Fig 2). The three *A. areolatum* isolates all originated from *S. nitobei* wasps attacking *P. densiflora* collected from the Aomori prefecture.

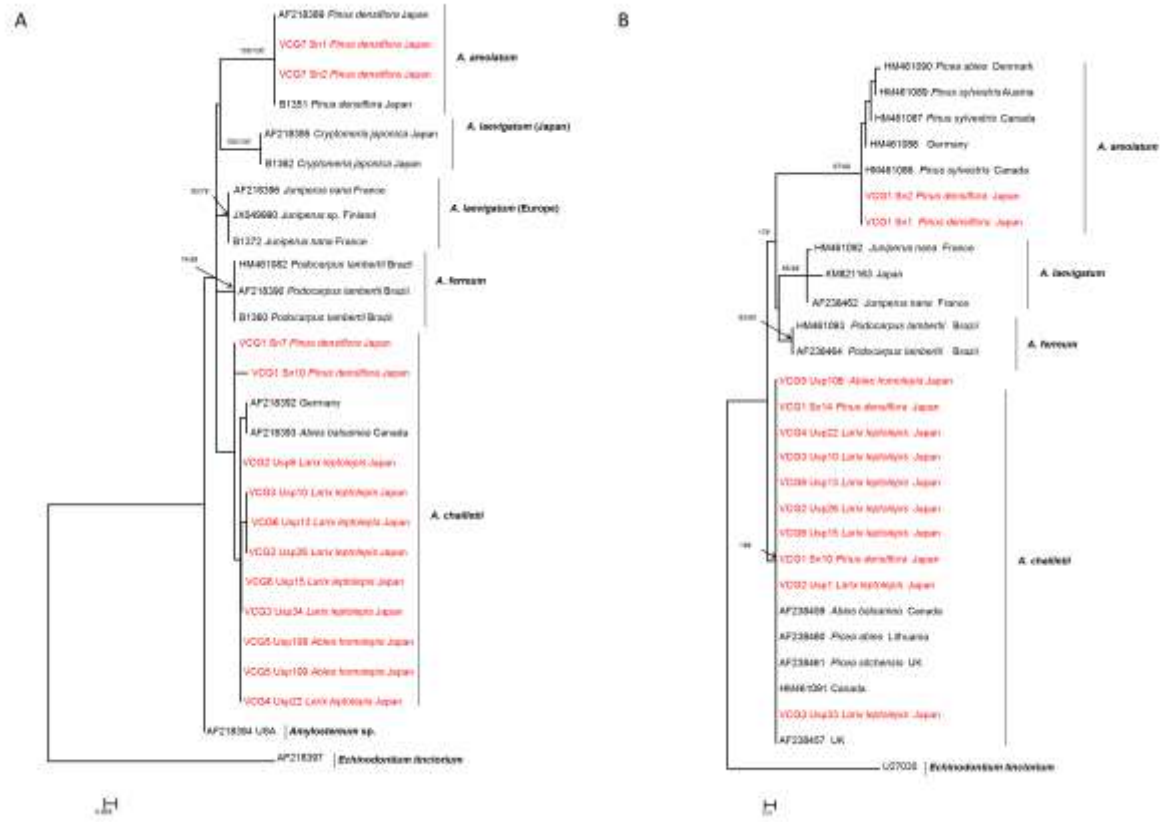

Fig. 1. Identification of *Amylostereum*. Phylogenetic analysis using both Maximum Likelihood (ML) and Maximum Parsimony (MP). (A) A Maximum Likelihood phylogenetic tree of a subset of sequences of the internal transcribed spacer region (ITS-rDNA), based on 439 bp, and (B) the mitochondrial small subunit (mtSSU rDNA), based on 493 bp. Both trees are rooted to *Echinodontium tinctorium*. Samples obtained from *Urocerus* sp. are abbreviated with Usp, whereas the samples from *Sirex nitobei* with Sn. Statistical support was determined by 1000 bootstrap replicates and all the bootstrap values above 70% are indicated for ML (roman) and Maximum Parsimony (italics) at the nodes. * indicates that the bootstrap values were below the threshold. Isolates in red are the isolates from this study.

Vegetative compatibility analysis

Seven VCGs were identified from the 64 *Amylostereum* isolates (Supplementary Table 1), Six out of the seven VCGs came from the *A. chailletii* isolates. There was a geographic structure in the distribution of VCGs in *A. chailletii,* with VCG1 being found only in the Aomori prefecture and the remaining five in the Nagano prefecture

(Fig. 2). The most frequent VCG in *A. chailletii* contained 36 isolates, all from the Nagano prefecture. One *A. chailletii* VCG (VCG5) was collected from both *L. leptolepis* and *A. homolepis*. In *A. areolatum,* one VCG was identified representing three isolates from the Aomori prefecture. The VCGs did not overlap between samples from *S. nitobei* and the *Urocerus* sp.

Fig. 2. Map of Japan highlighting the sampling areas from the various studies. The various shapes represent the three different *Amylostereum* species present in Japan: the square (■) represents *A. laevigatum*, the triangle (▲) *A. chailletii* and the circle (●) *A. areolatum*. The two encircled locations identify the areas sampled for the current study. Names of the prefectures of sampling sites are indicated.

Discussion

The *S. nitobei* wasps sampled for this study from *P. densiflora* trees in the Aomori prefecture in Japan were associated with both *A. chailletii* and *A. areolatum*. This result confirms recent North America findings that the paradigm of obligate fidelity to a single fungus per wasp species should be discarded, even in a native context devoid of non-coevolved invaders. The native range of *S. nitobei* is eastern Asia. Terashita (1970) first identified *A. areolatum* as a symbiont of *S. nitobei* in Japan using morphological data. This was later confirmed by Tabata *et al*. (2000) using DNA sequence data that grouped three *Amylostereum* isolates from *S. nitobei* (Kochi prefecture) with European isolates of *A. areolatum*. These results illustrate a lack of host-symbiont fidelity in *S. nitobei* that is similar to that observed in *S. nigricornis* and other North American species in the past 2 yrs. It is not clear, however, how widespread or prevalent either of these species are over the wider distribution of *S. nitobei*.

Urocerus species are common throughout Japan. In this study the unknown *Urocerus* species yielded only isolates of *A. chailletii.* Kanamitsu (1978) and Sano *et al*. (1995) described *A. chailletii* from mycangia of *U. antennatus* from *Abies sachalinensis* and *Picea jezoensis* in Hokkaido and *Cryptomeria japonica* in the Mie prefecture using culture morphology. *Amylostereum* spp. are difficult to separate in culture, so it is hard to confirm this identification of *A. chailletii*. In subsequent studies from the Ibaraki, Kochi and Nagasaki prefectures, the fungal species associated with this woodwasp species was recognised as *A. laevigatum* (Tabata and Abe, 1997, 1999) using both morphological and DNA sequence data (Tabata *et al*. 2000). These sampling areas for the studies prior to 2000 are located in the central and southern part of Japan, stretching over an area of around 1000 km. One of the new sampling sites of this study now incorporates the northern part of Japan, whereas the second site adds to the existing data from the central area of Japan. The data from this study and that of Tabata *et al*. (2000) confirm that *Urocerus* species in Japan are associated with both *A. laevigatum* and *A. chailletii*.

VCG assays revealed a high degree of clonality in the 64 *Amylostereum* isolates studied. All three *A. areolatum* isolates were of the same VCG, while all 61 *A.*

chailletii isolates belonged to 5 VCGs. Previous studies have revealed much higher levels of diversity of *A. chailletii* in native regions such as Europe (Vasiliauskas *et al*. 1998). In Europe *A. chailletii* frequently produces sexual structures and apparently spreads through basidiospores as well as in association with the woodwasp. In Japan the fruit bodies of neither *A. areolatum* nor *A. chailletii* have been recorded. Together with the clonality observed here, it seems most likely that these species are spread predominantly in association with woodwasps rather than via sexual spore production and wind, at least in the areas that we studied here. There was no VCG overlap in the *A. chailletii* isolates carried by *S. nitobei* and the *Urocerus* sp., but this could be driven by the fact that they were not sampled in the same site. Further isolates are required to understand clonal distribution and the degree to which clones are specific to wasp or tree species.

The collection data from this and other studies in Japan and Europe (Tabata and Abe, 1997; Vasiliauskas *et al.*, 1998; Tabata and Abe, 1999; Vasiliauskas, 1999; Vasiliauskas and Stenlid, 1999; Tabata *et al.*, 2000) appears to suggest that the tree host may be a non-trivial driver of the fungal and wasp species association. For example, *A. chailletii* always appears to be associated with tree hosts in the Pinaceae, but outside the genus *Pinus* (i.e. *Picea* spp., *Larix* spp., *Abies* spp. and rarely *Pinus* spp.), irrespective of the wasp species. On the other hand, *A. areolatum* is most commonly found in association with *Pinus*. The Cupressaceae (*Cupressus* spp., *C. japonica* and *Chamaecyparis* spp.) appears to primarily support *A. laevigatum*, which may drive the association of this fungus with siricids of the genus *Urocerus* that attack trees within this family. While *Urocerus* carries *A. laevigatum* on Cupressaceae (Tabata and Abe, 1997, 1999), it carried *A. chailletii* on Pinaceae (Tabata *et al*., 2000 and results from this study). While interesting, the extent of the role of tree species in structuring siricid-*Amylostereum* interactions awaits further study.

Apart from the unexpected prominence of *A. chailletii* and the absence of *A. laevigatum*, the study reinforced the recent discovery that woodwasps do not always carry the same fungal species. How dynamic the dominance of a particular species as fungal symbiont is over time, is an intriguing question. The mechanism through which such switching happens has not been directly observed, but is likely through

wasps ovipositing in trees that have already been attacked by other woodwasps or infected by *Amylostereum* by basidiospores (Wooding *et al*. 2013, Slippers *et al*. 2015). The frequency, mechanisms and conditions of symbiont switching, as well as its ecological consequences deserves closer study. The Japanese forest resource appears to offer ideal opportunities to do so. As pointed out by Wooding *et al*. (2013), this could be significant in terms of virulence of associated fungi, or changes in interactions with parasites such as *Deladenus* spp. or *Ibalia* spp. that are dependent on the fungus for food or signals to find its host.

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Supplementary Table1. *Amylostereum* isolates used for phylogenetic analysis in this study.

Species	Isolate No.*	Other isolate No.	Host	Insect	Location	Vegetative compatibility groups	GenBank Accession Nos. (ITS)	GenBank Accession Nos. (mt-SSU-rDNA)
A. areolatum		B1350	*Pinus densiflora*	*S. nitobei*	Japan		AF218389	
A. areolatum		B1351	*Pinus densiflora*	*S. nitobei*	Japan			
A. areolatum	Sn1	CMW42090	*Pinus densiflora*	*S. nitobei*	Japan	VCG7	KU870238	KU870299
A. areolatum	Sn2	CMW42091	*Pinus densiflora*	*S. nitobei*	Japan	VCG7	KU870239	KU870307
A. areolatum		DAOM:239281	*Pinus sylvestris*		Canada			HM461086
A. areolatum		DAOM Francke Sirex 1			Germany			HM461088
A. areolatum		DAOM 239284	*Pinus sylvestris*		Canada			HM461087
A. areolatum		CBS:655.93	*Picea abies*		Denmark			HM461090
A. areolatum		AtII28			Austria			HM461089
A. chailletii		B1387		*U. gigas*	Germany		AF218392	
A. chailletii		B1355	*Abies balsamea*		Canada		AF218393	
A. chailletii		DOAM X-589			Canada			HM461091
A. chailletii		54-95	*Tusga* sp.		Canada			AF238458
A. chailletii		DOAM 21327	*Abies balsamea*		Canada			AF238459
A. chailletii		L234	*Picea abies*		Lithuania			AF238460
A. chailletii		SC62.8	*Picea sitchensis*		United Kingdom			AF238461
A. chailletii		CBS 483.83		*U. gigas*	United Kingdom			AF238457
A. chailletii	Sn7	CMW40400	*Pinus densiflora*	*S. nitobei*	Japan	VCG1	KU870241	
A. chailletii	Sn10	CMW40401	*Pinus densiflora*	*S. nitobei*	Japan	VCG1	KU870240	KU870305
A. chailletii	Sn14		*Pinus densiflora*	*S. nitobei*	Japan	VCG1		KU870306
A. chailletii	Usp1	CMW42092	*Larix densiflora*	*Urocerus sp.*	Japan	VCG2		KU870306
A. chailletii	Usp6		*Larix densiflora*	*Urocerus sp.*	Japan	VCG2	KU870268	KU870303
A. chailletii	Usp26	CMW40402	*Larix densiflora*	*Urocerus sp.*	Japan	VCG2	KU870245	KU870280
A. chailletii	Usp10	CMW40403	*Larix densiflora*	*Urocerus sp.*	Japan	VCG3	KU870249	KU870276
A. chailletii	Usp33	CMW42096	*Larix densiflora*	*Urocerus sp.*	Japan	VCG3		KU870281
A. chailletii	Usp34		*Larix densiflora*	*Urocerus sp.*	Japan	VCG3	KU870257	

A. chailletii	Usp22	CMW40404	*Larix densiflora*	*Urocerus sp.*	Japan	VCG4	KU870247	KU870279
A. chailletii	Usp108	CMW40406	*Abies homolepis*	*Urocerus sp.*	Japan	VCG5	KU870248	KU870277
A. chailletii	Usp109	CMW40407	*Abies homolepis*	*Urocerus sp.*	Japan	VCG5	KU870244	
A. chailletii	Usp13		*Larix densiflora*	*Urocerus sp.*	Japan	VCG6	KU870271	KU870311
A. chailletii	Usp15	CMW40409	*Larix densiflora*	*Urocerus sp.*	Japan	VCG6	KU870258	KU870278
A. laevigatum		B1361	*Cryptomeria japonica*	*U. japonicas*	Japan		AF218395	
A. laevigatum		B1362	*Cryptomeria japonica*	*U. japonicas*	Japan		AF218395	
A. laevigatum		B1371	*Juniperus nana*		France		AF218396	
A. laevigatum		B1372	*Juniperus nana*		France		AF218396	
A. laevigatum		CBS 626.84	*Juniperus nana*		France			HM461092
A. laevigatum		CBS 624.84	*Juniperus nana*		France			AF238462
A. laevigatum		Voucher F2	*Juniperus* sp.		Finland		JX049990	
A. ferreum		B1359	*Podocarpus lambertii*		Brazil		AF218390	
A. ferreum		B1360	*Podocarpus lambertii*		Brazil		AF218390	
A. ferreum		CBS 637.84	*Podocarpus lambertii*		Brazil		HM461082	HM461093
A. ferreum		CBA 633.84	*Podocarpus lambertii*		Brazil			AF238464
A. sp.		B1393			USA		AF218391	
E.tinctorium		B1122					AF218397	
E.tinctorium		DAOM16666						U27035