New Phytol. (1998), **139**, 751–758

Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from northern Europe

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(*Received 9 October 1997; accepted 7 May 1998*)

SUMMARY

Genetic variation within and between vegetative compatibility groups (VCGs) of *Amylostereum areolatum* (Fr.) Boid. and *Amylostereum chailletii* (Pers.: Fr.) Boid. isolates was investigated. DNA fingerprints were made using the M13 core sequence as a primer. A total of 53 isolates of *A. areolatum* and 57 isolates of *A. chailletii* from Lithuania, Sweden, Denmark and Great Britain were studied. In all cases isolates belonging to the same VCG showed identical DNA banding patterns, suggesting that VCGs correspond to clones. In *A. areolatum* the vast majority of the isolates (spread by *Sirex juvencus* L.) were assigned to dispersive clones, that have wide geographical distribution (i.e. the same genotypes were detected in Lithuania, Sweden and Denmark), with low genetic variation between the different clones. By contrast, *A. chailletii* population structure was consistent with the spread of airborne basidiospores produced by outcrossing. Only a small fraction of *A. chailletii* isolates studied, could be assigned to dispersal clones with a local distribution, spread by *Urocerus gigas* L. Overall, M13 fingerprinting detected low genetic differentiation in both species in the samples we studied.

Key words: *Amylostereum* spp., arbitrary primed DNA, genetic variation, clones, populations.

INTRODUCTION

Amylostereum areolatum and *A. chailletii* are saprotrophic decay fungi occurring on fallen trunks and stumps of conifers (Eriksson & Ryvarden, 1973; Eriksson, Hjortstam & Ryvarden, 1978; Breitenbach & Kränzlin, 1986). In central and northern Europe these fungi are usually also found in open wounds on living stems of *Picea abies* (Pechmann & Aufsess, 1971; Schönhar, 1975; Vasiliauskas, Stenlid & Johansson, 1996). In time the fungi cause extensive rot within damaged stems, reaching a length of approx. 2·8 m in 10 yr (Vasiliauskas, unpublished).

Both *A. areolatum* and *A. chailletii* are associated with woodwasps of the genera *Sirex* and *Urocerus* that introduce the fungi into living trees (Stillwell, 1966; Coutts & Dolezal, 1969). During the 1940s and 1950s, the combined effect of *Sirex noctilio* and *A. areolatum* devastated hundreds of thousands of ha of *Pinus radiata* plantations in New Zealand, Tasmania and Australia (Talbot, 1977), demonstrating the high efficiency of insects in disseminating this fungus over huge areas. Since the females of *Sirex* carry and introduce the fungus in the form of vegetative mycelium fragmented into oidia or arthrospores (Francke-Grossmann, 1939), single genotypes of *A. areolatum* can become widespread, thus forming dispersive fungal clones (Anderson & Kohn, 1995).

Recent studies based on vegetative (or somatic) incompatibility revealed several VCGs of both *A. areolatum* and *A. chailletii* in the Baltic sea region, indicating the importance of insect vectors in the spread of the fungi and formation of dispersive clones or VCGs (Thomsen, 1996; Vasiliauskas & Stenlid, unpublished). Representatives of the same VCGs were repeatedly isolated from woodwasp females and from decayed wood (Thomsen, 1996).

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However, the extent of clonality, as expressed by assignment of individual isolates from spatially separated resource units to VCGs, was different between these two species. In *A. areolatum*, the vast majority of isolates was assigned to VCGs with wide geographical distribution and was detected in localities separated by hundreds of km and the Baltic sea, in Denmark, Sweden and Lithuania (Thomsen, 1996; Vasiliauskas & Stenlid, unpublished). Occurrence of compatible pairings between isolates of *A. areolatum* from Sweden and Lithuania was 33·3 % within sample plots, and 29·2 % between samples from the two countries showing uniform distribution of VCGs in all spatial scales of investigation (Vasiliauskas & Stenlid, unpublished). By contrast, the proportion of compatible isolates of *A. chailletii* was significantly lower, only 4·3 % within the sample plots, and 0 % between samples drawn from a larger geographical area (Vasiliauskas & Stenlid, unpublished). Also in Denmark, VCGs of *A. chailletii* had only a local distribution and compatible isolates were never separated by > 75 km (Thomsen, 1996).

Owing to a limited number of loci and alleles coding for the vegetative incompatibility (Hansen, Stenlid & Johansson, 1993; Rizzo, Rentmeester & Burdsall, 1995), fungal VCGs do not necessarily represent clones, and in this case DNA fingerprinting can confirm whether VCGs correspond to clonal lineages (Anderson & Kohn, 1995). Although in wood destroying basidiomycetes that tend to form territorial clones, such as *Phellinus weirii* and *Armillaria* spp., DNA markers are not always consistent with the assignment of isolates to VCGs, the discrepancies are regarded as minor, since DNA patterns exhibited by the majority of compatible isolates are identical or nearly so (Bae, Hansen & Strauss, 1994; Guillaumin *et al.*, 1996). On the contrary, DNA fingerprinting revealed substantial genetic variation among vegetatively compatible individuals of the ectomycorrhizal fungus *Suillus granulatus* (Jacobson, Miller & Turner, 1993), and within dispersive VCGs of the sclerotia-propagated snow-mould basidiomycete *Typhula ishikariensis* (Matsumoto, Uchiyama & Tsushima, 1996). Our recent study has shown the incidence of non-identical banding patterns within dispersive VCGs of the non-outcrossing wood decayer *Stereum sanguinolentum* that is spread in nature by airborne basidiospores. Moreover, genetic differentiation within VCGs was more pronounced among isolates collected on a larger geographical scale (Stenlid & Vasiliauskas, 1998). Vegetative incompatibility studies and DNA markers can measure genetic variation independently, complementing each other. Thus, in *Armillaria ostoyae*, nuclear DNA markers delineated three genets within one VCG, whereas incompatibility pairings differentiated several genets with identical nuclear markers (Rizzo, Blanchette & May, 1995).

The aim of the present study was to check whether the VCGs of *A. areolatum* and *A. chailletii* detected in our investigations are invariable clonal lines or whether DNA variation could be found within these VCGs. The second aim was to investigate relatedness and genetic differentiation among isolates within and between populations sampled over wide geographical distances.

MATERIALS AND METHODS

Fungal isolates

Isolates of *A. areolatum* (Fr.) Boid. and *A. chailletii* (Pers.: Fr.) Boid. from Denmark, Sweden and Lithuania were collected as described in our earlier studies. In Denmark, the fungal cultures were obtained from wood or fruit bodies growing on logs and stumps of *Picea* spp., *Abies* spp. and *Pseudotsuga* spp., and from fungi isolated from the glands of woodwasps *Sirex juvencus* L. (in the case of *A. areolatum*) and *Urocerus gigas* L. (in the case of *A. chailletii*) (Thomsen, 1996). All strains of both species from Sweden and Lithuania were isolated from wood of living wounded stems of *P. abies* (Vasiliauskas *et al.*, 1996). Pure cultures of *A. chailletii* from Great Britain were provided by Dr D. Redfern. They were obtained from fruit bodies and decayed wood of *Picea* spp. and *Larix* spp., and from mycangia of *U. gigas*.

All isolates were tested in pairs in pure culture and assigned to different VCGs as previously described by Thomsen (1996) and Vasiliauskas and Stenlid (1998*a*). The number of *A. areolatum* and *A. chailletii* isolates included in the present study, together with their geographic origin and assignment to VCGs is presented in Table 1 and Figure 1.

DNA extraction and PCR conditions

Before DNA extraction, the isolates were cultured in liquid Hagem media (Stenlid, 1985) for 2 wk, harvested by vacuum filtration and lyophilized. DNA extraction and PCR conditions were identical to those described in previous studies (Karlsson, 1994; Stenlid, Karlsson & Högberg, 1994; Högberg, Stenlid, & Karlsson, 1995; Stenlid & Vasiliauskas, 1998; Vasiliauskas & Stenlid, 1998*b*).

Amplification of fungal DNA was carried out using the core sequence of M13 minisatellite DNA - GAGGGTGGCGGTTCT as a primer (Karlsson, 1994; Stenlid *et al.*, 1994; Högberg *et al.*, 1995; Stenlid & Vasiliauskas, 1998; Vasiliauskas & Stenlid, 1998*b*). The amplified products were subjected to electrophoresis on 1·4 % agarose gels, stained with ethidium bromide, and the banding patterns were visualized using u.v. light.

All interpretations were made from photographic prints. Presence or absence of amplified fragments

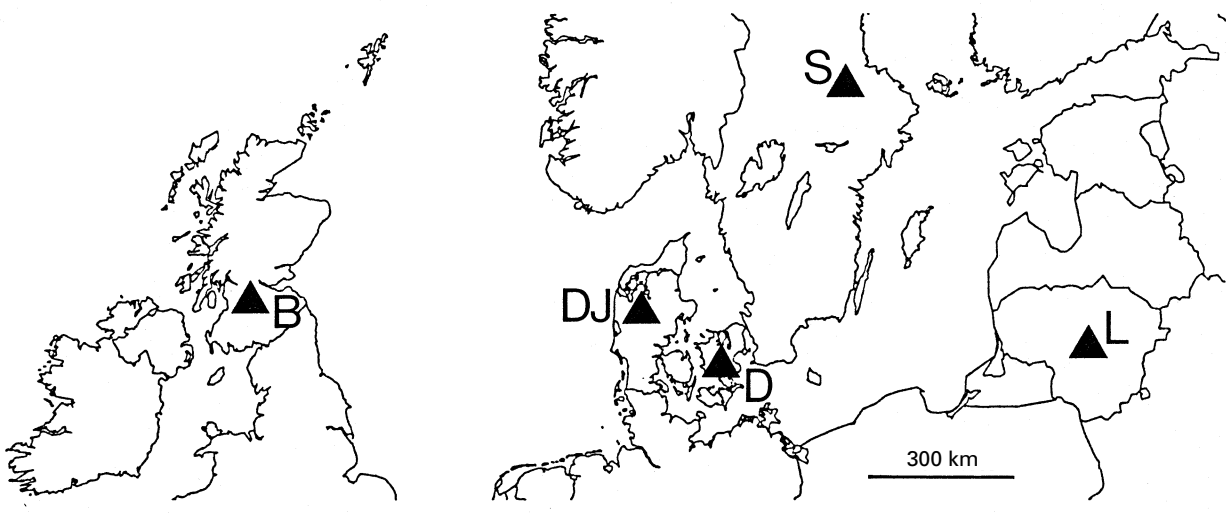

Figure 1. Map showing sampling localities of *Amylostereum areolatum* and *A. chailletii* in Lithuania (L), in Sweden (S), in Great Britain (B), and in Denmark, in Zealand island (D) and Jutland peninsula (DJ). Isolates from each locality were collected within a radius not exceeding 100 km.

Table 1. *Number of studied* Amylostereum areolatum *and* Amylostereum chailletii *isolates listed according to their sampling locations and vegetative compatibility groups*

	Number of isolates from:				
VCG	Lithuania	Sweden	Denmark	G. Britain	Total no.
		Amylostereum areolatum			
A1	2	1	17	—	20
A2	6	8	—	—	14
B	—	—	4	—	4
C	—	—	3	—	3
A3	3	—	—	—	3
A4	2	—	—	—	2
A5	2	—	—	—	2
Incompatible isolates	4	—	1	—	5
All isolates	19	9	25	—	53
		Amylostereum chailletii			
1	—	—	3	—	3
2	—	—	2	—	2
3	—	—	5	—	5
C1	2	—	—	—	2
C2	2	—	—	—	2
C3	2	—	—	—	2
Incompatible isolates	2	1	32	6	41
All isolates	8	1	42	6	57

was scored in relation to molecular weight markers. Only clear and distinct bands were included in the analysis.

Statistical analyses

For every pair of *A. areolatum* and every pair of *A. chailletii* isolates, a similarity index was calculated as the number of shared bands (S_{XY}) divided by the number of bands in strain X (S_X) and strain Y (S_Y) from the formula $2S_{XY}/(S_X+S_Y)$ (Lynch, 1990). The corresponding similarity matrix was constructed using the program SIMQUAL from the numerical taxonomy package NTSYS-pc, version 1.70 (Rohlf, 1992). Similarity between the isolates was expressed in percentage terms, as a band sharing index (BSI), calculated as 100 × the value from similarity matrix. The average band sharing index (ABSI) was calculated for all strain comparisons within and between any two populations (Stenlid *et al.*, 1994). Two populations in which all bands are identical for all individual strains have an ABSI of 100, and two populations having no identical bands in common have an ABSI of 0.

Principal component analysis (Sirius for Windows, 1993) was carried out directly on the presence/absence matrix for *A. chailletii* to determine how the various isolates and populations are related to each other.

RESULTS

Amplification of *A. areolatum* DNA using the M13 primer produced 14 different fragments ranging from 300 to 2000 base pairs (bp) in size. Seven markers (50 %) were common to all 53 isolates and no country specific markers were observed, indicating low genetic variation and differentiation of the fungus within the population sample studied. This finding was further supported by analysis of distribution of amplified fragments in the isolates within and among VCGs. All isolates, within each VCG, exhibited identical banding patterns, indicating that VCGs correspond to clones (Fig. 2). Variation among vegetative incompatible isolates (clones) was generally low (BSIs ranging from 74 to 96 %), but there was one case when two different VCGs shared the same DNA fingerprint (Table 2). Clonal spread has resulted in high genetic similarity indices in populations of *A. areolatum* at all geographical scales, covering distance ranges up to 1000 km across Denmark, Sweden, Lithuania and the Baltic sea. ABSI for populations within countries, estimated only between different VCGs (clones) was 86·6 %. However, when all the sampled isolates, together with the clonal representatives, were included in the analysis, similarity increased to 94·3 %: this difference being statistically significant (Table 3). The same tendency was noted also on a larger geographical scale in all the populations of *A. areolatum* we studied. Thus the ABSI value increased from 86·9 % when only different VCGs were considered, to 90·4 % when all isolates, together with the clonal ones, were included in the analysis (Table 3). Geographical scale on its own had a profound impact on regional differentiation of the fungus only when all isolates, including clonal duplicates, were taken into account, indicating that individuals of particular clones were characteristic of particular countries (Table 3). However, when only single representatives from different VCGs were analysed, no significant difference in similarity indices within and among countries was observed, suggesting the occurrence of the same genotypes in Denmark, Sweden and Lithuania (Table 3).

Amplification of *A. chailletii* DNA using the M13 primer produced 24 different fragments of 300–4000 bp in size. Among those, six markers (25 %) were common to all 57 isolates. One marker that was totally absent in strains from Great Britain was present in all Lithuanian strains and occurred in 86 % of Danish strains. No other country-specific marker was observed. Also, in *A. chailletii*, all compatible isolates within each VCG exhibited identical banding patterns, indicating that VCGs correspond to clones. For isolates from different VCGs, typically there was a distinct pattern of bands amplified from each individual strain (Fig. 3). However, in two cases, incompatible *A. chailletii* isolates (different VCGs) showed identical banding patterns. This was noted between VCG C3 from Lithuania and one Danish isolate, and between VCG C1 and another isolate from the same forest stand in Lithuania.

The first two axes in the principal component analysis (PCA) explained only 33·3 % of the total

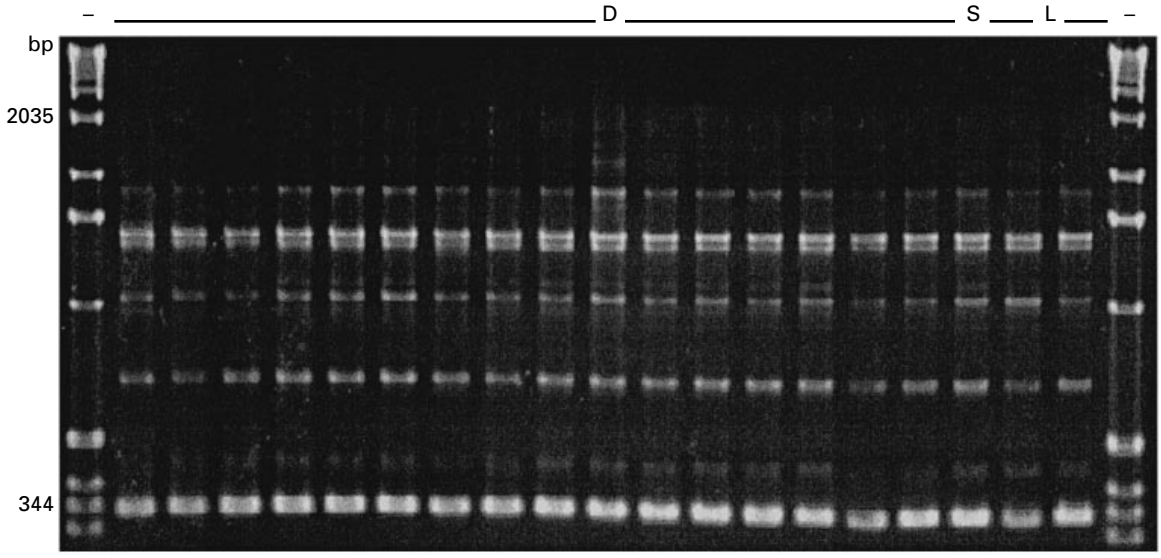

Figure 2. Gel of amplified DNA of 19 *Amylostereum areolatum* isolates from vegetative compatibility group A1. Lanes —, molecular weight marker (number of basepairs indicated on the left). Lanes indicated by the letter D and the bar show banding patterns of 16 isolates from Denmark (first 10 from Zealand island and the following six from Jutland peninsula). Lane S shows the banding pattern of one isolate from Sweden. Lanes indicated by the letter L and the bar are banding patterns of two isolates from Lithuania.

Table 2. *Matrix of average band sharing indices* (*ABSIs*, %) *among different vegetative compatibility groups* (*VCGs*) *and incompatible isolates of* Amylostereum areolatum

VCG	A1	A2	B	C	A3	A4	A5	L160	L180	L157	L002	D566
A1	100·0											
A2	84·2	100·0										
B	94·1	90·0	100·0									
C	100·0	84·2	94·1	100·0								
A3	84·2	90·0	90·0	84·2	100·0							
A4	88·9	85·7	84·2	88·9	85·7	100·0						
A5	88·9	95·2	94·7	88·9	85·7	80·0	100·0					
L160	80·0	87·0	85·7	80·0	95·7	90·9	81·8	100·0				
L180	88·9	85·7	94·7	88·9	85·7	80·0	90·0	81·8	100·0			
L157	88·9	95·2	94·7	88·9	95·2	90·0	90·0	90·9	90·0	100·0		
L002	87·5	73·7	82·4	87·5	73·7	88·9	77·8	80·0	77·8	77·8	100·0	
D566	84·2	81·8	90·0	84·2	81·8	85·7	85·7	87·0	95·2	85·7	84·2	100·0

Incompatible isolates from Lithuania indicated by L, incompatible isolate from Denmark indicated by D, and VCGs as in Table 1.

Table 3. *Average band sharing indices* (*ABSIs*) *in Lithuanian, Swedish and Danish populations of* Amylostereum areolatum *and* Amylostereum chailletii, *and similarity comparisons of populations at different spatial scales of distribution*

For isolates from populations	ABSI, %(no. of comparisons) for *A. areolatum*	ABSI, %(no. of comparisons) for *A. chailletii*	Difference significant at
Within countries			
Only from different VCGs (clonal duplicates excluded)	86·6 (43)	84·8 (610)	$P < 0{\cdot}05$
All sampled individuals (clonal duplicates included)	94·3 (518)	84·7 (889)	$P < 0{\cdot}001$
Difference significant at	$P < 0{\cdot}001$	n.s.	
Among countries			
Only from different VCGs (clonal duplicates excluded)	87·4 (59)	86·0 (215)	n.s.
All sampled individuals (clonal duplicates included)	88·1 (871)	86·7 (385)	$P < 0{\cdot}001$
Difference significant at	n.s.	n.s.	
Within vs. among country comparisons of			
Different VCGs (clonal duplicates excluded)	n.s.	$P < 0{\cdot}01$	
All sampled individuals (clonal duplicates included)	$P < 0{\cdot}001$	$P < 0{\cdot}001$	
All study area			
Only from different VCGs (clonal duplicates excluded)	86·9 (66)	85·1 (825)	$P < 0{\cdot}05$
All sampled individuals (clonal duplicates included)	90·4 (1389)	85·3 (1274)	$P < 0{\cdot}001$
Difference significant at	$P < 0{\cdot}001$	n.s.	
Within country vs. all study area comparisons of			
Different VCGs (clonal duplicates excluded)	n.s.	n.s.	
All sampled individuals (clonal duplicates included)	$P < 0{\cdot}001$	$P < 0{\cdot}05$	

variation in the material. Furthermore, isolates of *A. chailletii* from different geographical populations did not fall into discrete clusters on the PCA (Fig. 4), suggesting high diversity and low level of genetic differentiation between the samples from different geographical areas. This was further supported by analysis of the figures for ABSIs; the similarity among isolates from different countries increased as compared with the similarity of isolates within countries (Table 3). ABSI values for British *A. chailletii*, as well as PCA analysis, showed no regional differentiation among populations of the fungus: genetic similarity among isolates from Britain was 79·6 %. Between isolates from Britain and Denmark separated by *c.* 1000 km and the North sea, the ABSI was 77·5 % (according to a *t*-test, this difference was not statistically significant), and between isolates from Britain and Lithuania (situated an additional 1000 km further eastwards across the Baltic sea) the genetic similarity was 79·6 %.

In contrast to *A. areolatum*, evidence suggests that clones in *A. chailletii* were uncommon. No significant differences in ABSIs were detected for (*a*) all the *A. chailletii* isolates taken together, including clonal duplicates with identical banding patterns, and (*b*) ABSIs calculated for representatives from different VCGs, both within country and between countries (Table 3).

Comparison of genetic variation between *A. areolatum* and *A. chailletii*, detected by the M13

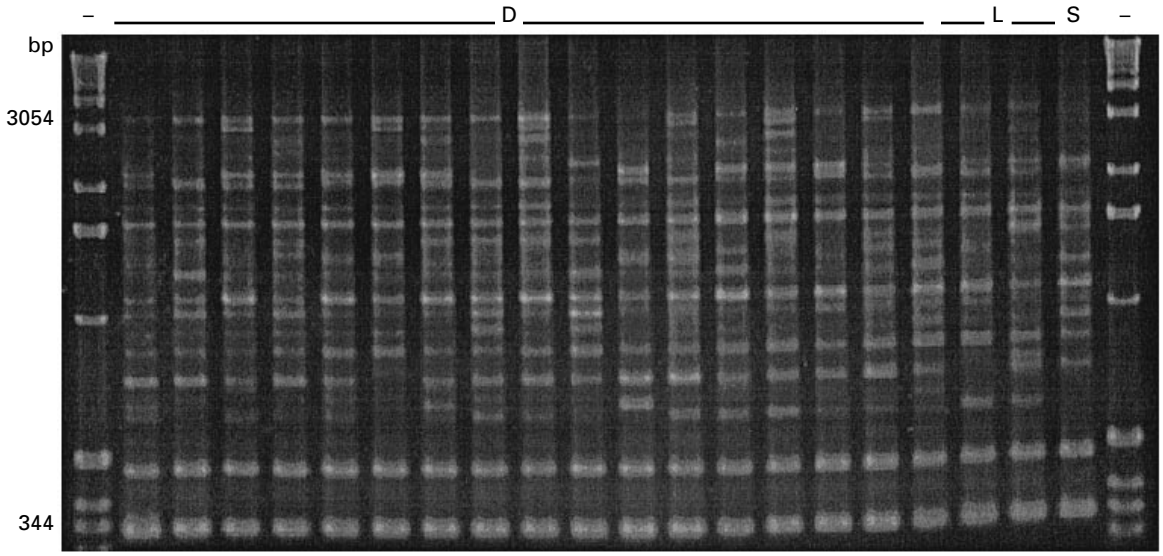

Figure 3. Gel of amplified *Amylostereum chailletii* DNA. Lanes marked —, molecular weight markers (number of basepairs indicated on the left). Lanes indicated by the letter D and the bar show banding patterns of 17 different isolates from Denmark. Lanes indicated by the letter L and the bar show banding patterns of two different isolates from Lithuania. Lane S shows the banding pattern of one isolate from Sweden.

primer, revealed significantly higher variation in *A. chailletii* (Table 3). However, when different VCGs of both species were considered, clonal duplicates having been excluded, the differences were less pronounced (Table 3).

DISCUSSION

These results provide strong support for our earlier work based on vegetative compatibility tests (Thomsen, 1996; Vasiliauskas & Stenlid, unpublished), showing low genetic differentiation among geographically separated populations of both *A. areolatum* and *A. chailletii*. Despite the fact that both species have tetrapolar heterothallic mating systems (Boidin & Lanquetin, 1984), the reasons for low genetic differentiation in their population structures are different. In *A. areolatum*, it is a consequence of widespread clonal spread (the vast majority of the isolates were assigned to dispersive clones spread by *S. juvencus*), wide geographical distribution of clones (same genotypes detected in Lithuania, Sweden and Denmark) and low genetic variation between clones. In contrast, low differentiation in *A. chailletii* results from the wide dispersal of airborne outcrossing basidiospores. The population structure of this fungus therefore closely resembles that of natural populations of other airborne wood decaying basidiomycetes studied using DNA markers. In earlier studies high population variation and minor regional differentiation, both within large and small geographical areas, was revealed in *Heterobasidion annosum* (Karlsson, 1994; Stenlid *et al.*, 1994; La Porta *et al.*, 1997), *Fomitopsis pinicola* (Högberg *et al.*, 1995), *F. rosea*. (Högberg & Stenlid, 1994), *Chondrostereum purpureum* (Gosselin, Jobidon & Bernier, 1996; Ramsfield *et al.*, 1996), *Cylindrobasidium evolvens* (Vasiliauskas & Stenlid, 1998*b*), *Phlebiopsis gigantea* (Vainio, Korhonen & Hantula, 1998), and *Stereum sanguinolentum* (Stenlid & Vasiliauskas, 1998).

Only a small fraction of *A. chailletii* isolates could be assigned to dispersal clones spread by *U. gigas*, such clones consisting of a few individuals with a local distribution. In contrast to *A. areolatum*, clones of *A. chailletii* did not have any significant impact on overall genetic structure of populations of the fungus (Table 3).

Similar results from vegetative compatibility reactions and DNA fingerprints suggest that dispersive clones of both *Amylostereum* species are stable over space and time. In addition to the previously reported wide geographical distribution, representatives of some VCGs (or clones) have been repeatedly isolated over long time-periods. For example, representatives from the *A. areolatum* VCG (Fig. 2) were collected during the period 1983–1995, and one VCG of *A. chailletii*, between 1985 and 1993 (Thomsen, 1996).

One possible explanation for wide geographical distribution of *A. areolatum* clones is that they were gradually spread by insects across Europe from one forest stand to another and therefore represent old clonal lineages in a well adapted *Sirex* – *A. areolatum* association. The crucial importance of an insect vector for the spread of *A. areolatum* to living trees was demonstrated in a Japanese study, where experiments of direct inoculation of mycelium to pines were unsuccessful, and successful infections were only obtained when female adults of *Sirex* introduced the fungus to the stems (Kobayashi, Sasaki & Enda, 1978). Regarding establishment on wood surfaces, woodwasps probably are of less importance to the fungus, because basidiospores of

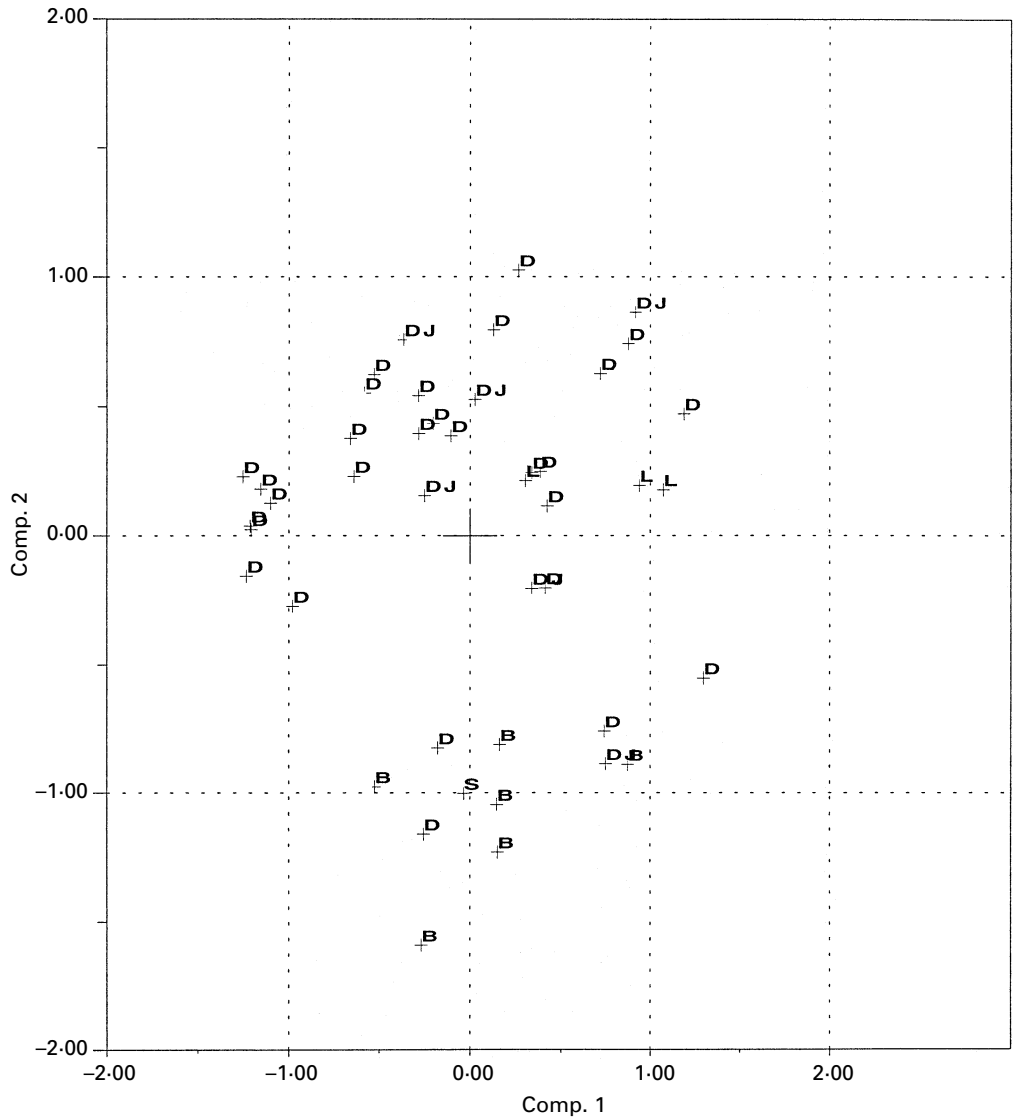

Figure 4. Principal component analysis of *Amylostereum chailletii* isolates. Each + symbol indicates a single isolate. Letters indicate the sampling localities of isolates as in Figure 1: D, isolates from Zealand island in Denmark; DJ, isolates from Jutland peninsula in Denmark; L, isolates from Lithuania; B, isolates from Great Britain; S, an isolate from Sweden.

A. areolatum are able to colonize freshly cut wood discs of spruce (Pechmann *et al.*, 1967).

Despite being well adapted to spread by *S. juvencus*, *A. areolatum* genotypes might exhibit different ecological strategies, since the genotype A1 (Fig. 2) was isolated from dead logs, stumps and living trees of *Picea abies* (Thomsen, 1996; Vasiliauskas & Stenlid, unpublished). In the earlier studies, wider ecological amplitude was observed within VCGs of *S. sanguinolentum*, where isolates belonging to the same VCG have been obtained from living trees and dead wood of different conifer genera (Rayner & Boddy, 1988; Vasiliauskas & Stenlid, 1998*a*).

Besides natural spread, the fungi might have been present in exports of wood from one country to another. Since the life cycle of *Sirex* woodwasps usually lasts 3–4 yr (Schwerdtfeger, 1957), it is possible for the larvae to be transported within timber along with mycelium of *A. areolatum*. Emerging insect females can enter new areas in this manner and establish local clonal populations of the fungus, as happened in New Zealand and Australia (Talbot, 1977). However, export of wood from Lithuania to Sweden has occurred only recently, in 1992 (V. Vaiciúnas, Ministry of Agriculture and Forestry of Lithuania, pers. comm.), and therefore it is unlikely that timber transport could have strongly influenced the fungal population structure we investigated.

In the present study, no DNA variation was observed within VCGs of *A. areolatum* and *A. chailletii*. It is noteworthy, however, that vegetative incompatibility tests differentiated several genets with identical DNA markers (one case in *A. areolatum*, and two cases in *A. chailletii*). The higher resolving power of vegetative compatibility tests, as compared with DNA fingerprinting, has already

been reported in earlier studies on *Armillaria ostoyae* (Rizzo *et al.*, 1995) and the plant pathogenic ascomycete *Phomopsis subordinaria* (Meijer, Megnegneau & Linders, 1994).

ACKNOWLEDGEMENTS

We wish to thank Dr Derek Redfern for providing isolates of *Amylostereum chailletii* from Great Britain. The study was supported by a scholarship to R. Vasiliauskas from funds of the Bilateral Research Cooperation with Eastern Europe at the Swedish University of Agricultural Sciences.

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