11994

To Prof. Michael Wingfield 1999. 11. 11
with many thanks for the review, and
with the best wishes. Romvil

MYCOTAXON

Volume LXXI, pp. 155-161 April-June 1999

MOLECULAR RELATIONSHIPS WITHIN THE GENUS AMYLOSTEREUM AS DETERMINED BY INTERNAL TRANSCRIBED SPACER SEQUENCES OF THE RIBOSOMAL DNA

R. VASILIAUSKAS[1], H. JOHANNESSON[2] AND J. STENLID[2]

[1]Lithuanian Forest Research Institute, LT-4312 Kaunas - Girionys, Lithuania.
Fax + 370 7 547446 (corresponding author)
[2]Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, S-750 07 Uppsala, Sweden

Abstract

Intraspecific and interspecific variation in ITS sequences of ribosomal DNA was studied among different genets (clonal lines) of *Amylostereum areolatum*, *A. chailletii* and *A. laevigatum*. No intraspecific variation was found within *A. chailletii* and *A. laevigatum* isolates. The sequences of *A. areolatum* isolates from Denmark and Sweden were also identical, but differed from Lithuanian strain by a single base. High sequence homology (> 97 %) was revealed among the three species from the genus *Amylostereum*. Results of the study indicated a closer relationship between *A. chailletii* and *A. laevigatum*, than between the latter two species and *A. areolatum*.

Introduction

In Europe, the basidiomycete genus *Amylostereum* Boid. (family *Stereaceae* Pilat) consists of three species, *A. areolatum* (Fr.) Boid., *A. chailletii* (Pers.: Fr.) Boid., and *A. laevigatum* (Fr.) Boid., which inhabit wood of various conifer trees (Eriksson & Ryvarden, 1973; Eriksson *et al.*, 1978; Jahn, 1979; Breitenbach & Kränzlin, 1986). All three species are completely intersterile and produce basidiospores that possess tetrapolar outcrossing mating systems (Boidin & Lanquetin, 1984). Besides spread by airborne basidiospores, *A. areolatum* and *A. chailletii* are additionally distributed by siricid woodwasps as oidia or arthrospores (vegetative mycelium) (Stillwell, 1966; Coutts & Dolezal, 1969; Kobayashi *et al.*, 1978; Thomsen & Koch, 1993, 1999; Thomsen, 1996).

In nature, fungal spread by vegetative propagules may result in formation of vegetative compatibility groups (VCGs) that are genetically isolated from each other and consist of spatially separated isolates (Anderson & Kohn, 1995). In fact, VCGs of *A. areolatum* (carried by *Sirex juvencus* L.) and *A. chailletii* (carried by *Urocerus gigas* L.) have already been detected in northern Europe (Thomsen, 1996; Thomsen

& Koch, 1999; Vasiliauskas & Stenlid, 1999). Furthermore, DNA fingerprinting has shown that those VCGs correspond to clonal lineages, which may remain stable over space and time (Vasiliauskas *et al.*, 1998).

The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is reported to exhibit a high degree of polymorphism between species, but to be highly conserved within species, thus providing information on the molecular relationships between fungal taxonomic units (Bruns *et al.*, 1991; Gardes *et al.*, 1991). A number of morphological differences between *A. areolatum* and *A. chailletii* have already been reported (Thomsen, 1998). The aim of the present study was, by means of ITS sequence analyses, to study relatedness between the three *Amylostereum* species and to check for possible within species variation among different genets (clonal lines) of *A. areolatum*, *A. chailletii* and *A. laevigatum*.

Materials and methods

The isolates used in this study are listed in Table 1. Each of them was already found to be genetically distinct from each other, and the isolates of *A. areolatum* and *A. chailletii* were the representatives from different clonal lineages (Thomsen, 1996; Vasiliauskas *et al.*, 1998; Thomsen & Koch, 1999; Vasiliauskas & Stenlid, 1999, unpublished data). Subcultured mycelia were maintained in pure culture on Hagem agar medium at 4 °C. Mycelia for DNA extraction were grown for two weeks on liquid Hagem medium in static cultures at room temperature.

Table 1. List of *Amylostereum* spp. isolates used in this study.

Species	Isolate	Geographic origin	Host	Collector, year
A. areolatum	DK-540	Denmark	*Sirex juvencus*	I.M. Thomsen, 1994
A. areolatum	S3-25	Sweden	*Picea abies*	J. Stenlid, 1993
A. areolatum	LI-9	Lithuania	*Picea abies*	R. Vasiliauskas, 1994
A. chailletii	BC-623	Scotland	*Picea sitchensis*	D. Redfern, 1981
A. chailletii	S1-22	Sweden	*Picea abies*	J. Stenlid, 1993
A. chailletii	LI-116	Lithuania	*Picea abies*	R. Vasiliauskas, 1994
A. laevigatum	BL-84	England	*Thuja plicata*	B. Greig, 1982
A. laevigatum	BL-174	England	*Thuja plicata*	B. Greig, 1980
A. laevigatum	BL-278	England	*Thuja occidentalis*	B. Greig, 1979

DNA from the isolates was obtained during earlier studies (Vasiliauskas *et al.*, 1998). PCR amplifications and DNA sequencing procedures followed the study of Kåren *et al.* (1997). PCR amplifications were performed with a Perkin-Elmer Cetus DNA thermal cycler (model GenAmp 2400). The primer pair ITS1/ITS4 was used to amplify the ITS region (White *et al.*, 1990). Reaction components for the PCR were: 0.01-1.0 ng/µl of total DNA, 0.1µM of each primer, 0.025 U/µl of Taq-polymerase, 200 µM dNTP, 10 mM Tris-HCl, 1.5-3.0 mM $MgCl_2$ and 5 µM KCl. Cycling parameters were an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 2 min, annealing at 53 °C for 22 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Negative controls, without DNA

template, were prepared in every series of amplification in order to exclude the possibility of contamination. Electrophoresis of PCR products was carried out on a 1.0 % agarose gel (LE-agarose, FMC BioProducts, Rockland, USA). Each PCR product was cleaned using the QIAquick PCR purification kit (QIAGEN Inc., Chatsworth, USA).

Sequences were determined with an Applied Biosystems (Foster City, CA, USA) 310 sequencer with the *Taq* DyeDeoxi Terminator™ cycle system (Perkin Elmer), using each of the primers ITS1, ITS2 and ITS3 (White *et al.*, 1990). The alignment of the sequences was performed manually. The whole procedure of ITS amplification and sequencing was performed twice for all isolates with identical results.

Results

A single product of uniform size resulted from all PCR amplifications, corresponding to approx. 600 bp in size (data not shown). There was no intraspecific variation in the ITS region of studied *A. chailletii* and *A. laevigatum* isolates. ITS sequences of *A. areolatum* isolates from Denmark and Sweden were also identical, but differed from Lithuanian strain by a single base (A → G) at 279 bp position.

Aligned sequences of the ITS region of all three species are presented in Fig. 1. In general, high sequence homology (> 97 %) was revealed among the three *Amylostereum* species. Thus, similarity in the ITS region between *A. chailletii* and *A. laevigatum* was 98.8 % (7 variable sites), between *A. chailletii* and *A. areolatum* 97.2 % (16 variable sites), and between *A. laevigatum* and *A. areolatum* 97.4 % (15 variable sites). However, when proportion of similar sites between *A. chailletii* and *A. laevigatum* was compared by χ^2-test with that between *A. chailletii* and *A. areolatum*, the difference was statistically insignificant ($\chi^2 = 3.80$, $p > 0.05$). Therefore the area sequenced was too similar to resolve clearly the question of the relationships between the three species. Despite that it indicated that *A. chailletii* and *A. laevigatum* might be more closely related than *A. chailletii* and *A. areolatum*.

Discussion

In the present study, identical intraspecific ITS sequences of rDNA are reported for *A. chailletii* and *A. laevigatum*. Nearly identical intraspecific sequences were found in *A. areolatum* (within species variation was at one nucleotide site). Except for *A. laevigatum*, isolates of the species studied were collected within rather large geographical scale (Table 1) and were separated in nature by ca. 800 km and open seas. In addition, examined strains of *A. areolatum* and *A. chailletii* represented clonal lineages that are genetically isolated within each species (Vasiliauskas *et al.*, 1998).

In other studies, no intraspecific variation was found in the ITS region among isolates from ectomycorrhizal basidiomycetes *Cortinarius armillatus* (collected ca. 350 km apart) and *C. traganus* (collected ca. 650 km apart) (Kåren *et al.*, 1997). In *Suillus luteus*, only one base difference was detected even between isolates originating from Europe and North America (Kretzer *et al.*, 1996).

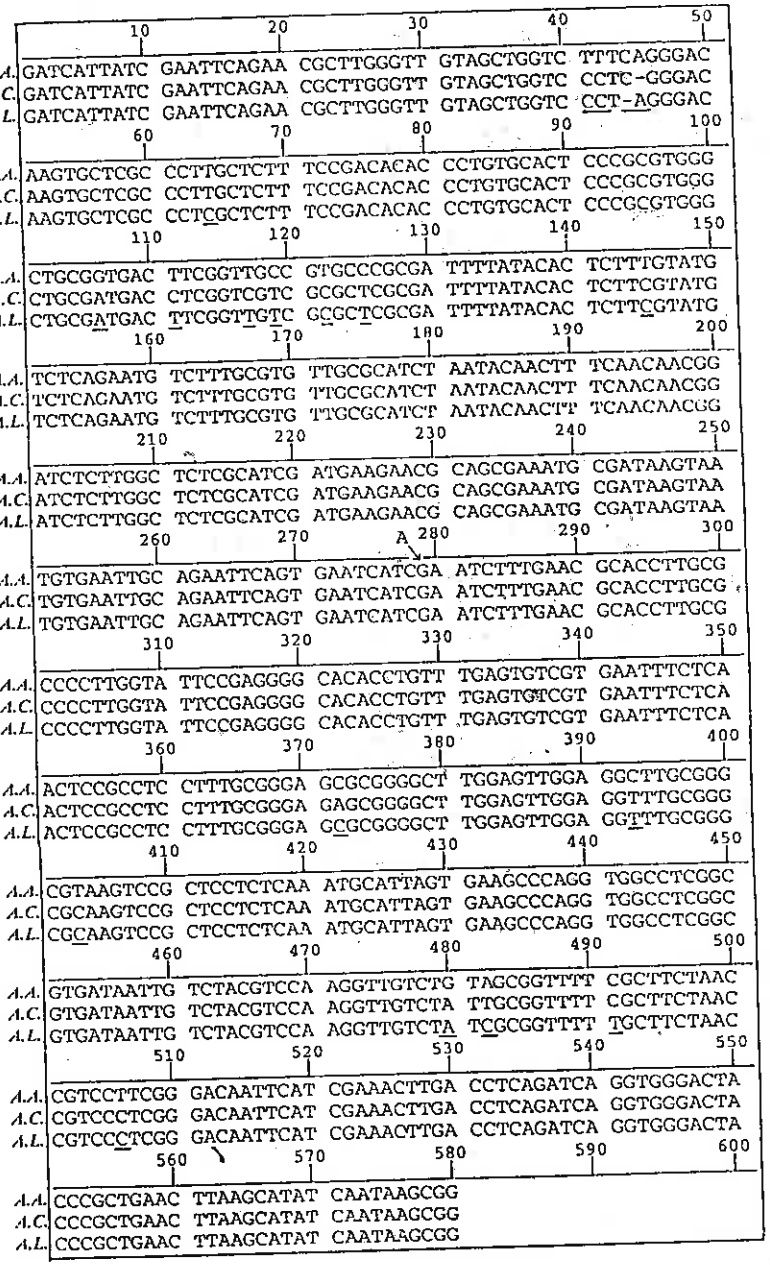

Fig. 1. Aligned sequences of the ITS region of *Amylostereum areolatum* (*A.A.*), *A. chailletii* (*A.C.*), and *A. laevigatum* (*A.L.*). Site differences within the genus *Amylostereum* are underlined. Site change within the species *A. areolatum* (279 bp) indicated by arrow.

Despite a wide geographical range of collected strains, interspecific variation within the genus *Amylostereum* (among the three species studied) was very low, especially if compared with ITS sequence variation observed in other basidiomycete macrofungi. Thus, it approximated to intraspecific variation reported within certain species of *Lentinus* (Hibbet & Vilgalys, 1993), *Cantharellus* and *Craterellus* (Feibelman *et al.*, 1997), *Pisolithus* (Anderson *et al.*, 1998), and European *Armillaria* (Chillali *et al.*, 1998). Rather similar level of ITS sequence variation was noted also among intersterile groups (biological species) within complexes of *Heterobasidion* (Kasuga *et al.*, 1993; Kasuga & Mitchelson, 1993), and that of North American *Armillaria* (Anderson & Stasovski, 1992).

The present study showed that more similarity in ITS nucleotide sequence exists between *A. chailletii* and *A. laevigatum*, than between *A. chailletii* and *A. areolatum*. This was unexpected for several reasons. First, both *A. chailletii* and *A. areolatum* in Europe commonly occur on wood of the same host, e.g. spruce (*Picea*) (Eriksson & Ryvarden, 1973; Eriksson *et al.*, 1978; Breitenbach & Kränzlin, 1986). In contrast, *A. laevigatum* has a very distinctive host range and grows only on wood of *Juniperus*, *Taxus* and *Thuja* (Eriksson & Ryvarden, 1973; Jahn, 1979; Breitenbach & Kränzlin, 1986). Second, decay patterns in spruce of both *A. chailletii* and *A. areolatum* are very similar (Vasiliauskas, 1999). Third, both *A. chailletii* and *A. areolatum* are symbiotic with siricid woodwasps, whereas *A. laevigatum* is not (Talbot, 1977). In insect-associated ascomycetes, for example, related taxonomic lineages were thought to diverge depending on association with different beetle species (Blackwell & Jones, 1997).

Some evidence is available to suggest that *A. chailletii* and *A. laevigatum* are more closely related to each other than to *A. areolatum*. Thus Boidin & Lanquetin (1984) proposed to expand the genus *Amylostereum* and to include the tropical species, *A. ferreum* Berk. et Curt. Mating tests had shown that *A. ferreum* was partially fertile both with *A. chailletii* and with *A. laevigatum*, but was completely intersterile with *A. areolatum* (Boidin & Lanquetin, 1984). In the recent study, mitochondrial small subunit rDNA and the intergenic spacer region of the nuclear rDNA of *Amylostereum* species were sequenced, and it was found that *A. leavigatum* and *A. ferreum* are phylogenetically more closely related to *A. chailletii* than to *A. areolatum* (Slippers *et al.*, 1998).

A. laevigatum in Europe was suspected to contain two different taxa - one occurring on *Juniperus* and possessing shorter basidiospores, and another occurring on *Taxus* and *Thuja* with the longer basidiospores (Eriksson & Ryvarden, 1973; Hallenberg & Hallingbäck, 1974). Despite the fact that both forms appeared to be completely

intersterile (Hallenberg & Hallingbäck, 1974), it might be of interest in future studies to consider genetic variation among both types of *A. laevigatum* (in this work only isolates from *Thuja* were included), and also to include strains of *A. ferreum*.

Acknowledgements

We wish to thank Dr. Iben M. Thomsen for useful comments on the manuscript. The Swedish Institute supported the study with a scolarship for R. Vasiliauskas.

Literature

Anderson, I.C., Chambers, S.M. & Cairney, J.W.G. (1998) Molecular determination of genetic variation in *Pisolithus* isolates from a defined region in New South Wales, Australia. New Phytologist, 138: 151-162.

Anderson, J.B. & Kohn, L.M. (1995) Clonality in soilborne, plant-pathogenic fungi. Annual Review of Phytopathology, 33: 369-391.

Anderson, J.B. & Stasovski, E. (1992) Molecular phylogeny of northern hemisphere species of *Armillaria*. Mycologia, 84: 505-516.

Blackwell, M. & Jones, K. (1997) Taxonomic diversity and interactions of insect-associated ascomycetes. Biodiversity and Conservation, 6: 689-699.

Boidin, J. & Lanquetin, P. (1984) Le genre *Amylostereum* (*Basidiomycetes*) intercompatibilités partielles entre espèces allopatriques. Bulletin de la Société Mycologique de France, 100: 211-236.

Breitenbach, J. & Kränzlin, F. (1986) Fungi of Switzerland. Volume 2. Heterobasidiomycetes, Aphyllophorales, Gasteromycetes. Verlag Mykologia, Luzern. 412 pp.

Bruns, T.D., White, T.J. & Taylor, J.W. (1991) Fungal molecular systematic. Annual Review of Ecology and Systematic, 22: 525-564.

Chillali, M., Wipf, D., Guillaumin, J.J., Mohammed, C. & Botton, B. (1998) Delineation of the European *Armillaria* species based on the sequences of the internal transcribed spacer (ITS) of ribosomal DNA. New Phytologist, 138: 553-561.

Coutts, M.P. & Dolezal, J.E. (1969) Emplacement of fungal spores by the woodwasp, *Sirex noctilio*, during oviposition. Forest Science, 15: 412-416.

Eriksson, J., Hjortstam, K. & Ryvarden, L. (1978) The Corticiaceae of North Europe. Volume 5, pp. 890-893. Fungiflora, Oslo.

Eriksson, J. & Ryvarden, L. (1973) The Corticiaceae of North Europe. Volume 2, pp. 91-95. Fungiflora, Oslo.

Feibelman, T.P., Doudrick, R.L., Cibula, W.G. & Bennett, J.W. (1997) Phylogenetic relationships within the Cantharellaceae inferred from sequence analysis of the nuclear large subunit rDNA. Mycological Research, 101: 1423-1430.

Gardes, M., White, T.J., Fortin, J.A., Bruns, T.D. & Taylor, J.W. (1991) Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Canadian Journal of Botany, 69: 180-190.

Hallenberg, N. & Hallingbäck, T. (1974) Interfertility and polarity in *Amylostereum laevigatum* (Fr.) Boid. and *Peniophora lycii* (Pers.) Höhn. et Litsch. Göteborgs Svampklubs Årsskrift: 18-21.

Hibbett, D.S. & Vilgalys, R. (1993) Phylogenetic relationships of *Lentinus* (Basidiomycotina) inferred from molecular and morphological characters. Systematical Botany, 18: 409-433.

Jahn, H. (1979) Pilze die an Holz wachsen. Bussesche Verlagshandlung, Herford. 268 pp.

Kåren, O., Högberg, N., Dahlberg, A., Jonsson, L. & Nylund, J.E. (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytologist, 136: 313-325.

Kasuga, T. & Mitchelson, K. (1993) Determination of the DNA sequence of the 5.8S ribosomal gene of *Heterobasidion annosum* and *Heterobasidion araucariae*. Nucleic Acids Research, 21: 1320.

Kasuga, T., Woods, C., Woodward, S. & Mitchelson, K. (1993) *Heterobasidion annosum* 5.8s ribosomal DNA and internal transcribed spacer sequence: rapid identification of European intersterility groups by ribosomal DNA restriction polymorphism. Current Genetics, 24: 433-436.

Kobayashi, T., Sasaki, K. & Enda, N. (1978) Correlation between *Sirex nitobei* and *Amylostereum areolatum*, associated with the death of Japanese pine trees during winter season. Journal of the Japanese Forestry Society, 60: 405-411.

Kretzer, A., Li, Y., Szaro, T.M. & Bruns, T.D. (1996) Internal transcribed spacer sequences from 38 recognized species of *Suillus* sensu lato: phylogenetic and taxonomic implications. Mycologia, 88: 776-785.

Slippers, B., Wingfield, M.J., Coutinho, T.A & Wingfield, B.D. (1998) The identity and possible origin of the *Amylostereum* symbiont of *Sirex noctilio* in South Africa. In: Abstracts of the 7th International Congress of Plant Pathology, Edinburgh, Scotland 9-16 August 1998. Volume 3 (3.7.55).

Stillwell, M.A. (1966) Woodwasps (Siricidae) in conifers and the associated fungus, *Stereum chailletii*, in eastern Canada. Forest Science, 12: 121-128.

Talbot, P.H.B. (1977) The *Sirex-Amylostereum-Pinus* association. Annual Review of Phytopathology, 15: 41-54.

Thomsen, I.M. (1996) *Amylostereum areolatum* and *Amylostereum chailletii*, symbiotic fungi of woodwasps (*Sirex* sp. and *Urocerus* sp.). Ph. D. thesis, Danish Forest and Landscape Research Institute, Hørsholm, Denmark. 130 pp.

Thomsen, I.M. (1998) Characters of fruitbodies, basidiospores and cultures useful for recognizing *Amylostereum areolatum* and *A. chailletii*. Mycotaxon, 69: 419-428.

Thomsen, I.M. & Koch, J. (1993) *Amylostereum areolatum* og *A. chailletii* - to ejendommelige rådsvampe på nåletræ i Danmark. Svampe, 28: 23-25.

Thomsen, I.M. & Koch, J. (1999) Somatic compatibility in *Amylostereum areolatum* and *A. chailletii*, symbionts of siricid woodwasps. Mycological Research, 103 (in press).

Vasiliauskas, R. (1999) Spread of *Amylostereum areolatum* and *A. chailletii* decay in living stems of *Picea abies*. Forestry, 72 (in press).

Vasiliauskas, R. & Stenlid, J. (1999) Vegetative compatibility groups of *Amylostereum areolatum* and *A. chailletii* from Sweden and Lithuania. Mycological Research, 103 (in press).

Vasiliauskas, R., Stenlid, J. & Thomsen, I.M. (1998) Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from northern Europe. New Phytologist, 139: 751-758.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications. Edited by M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White. Academic Press, San Diego. pp. 315-322.