CHARACTERIZATION AND FREQUENCIES OF THE IGS1 ALLELES OF THE RIBOSOMAL DNA OF *XEROCOMUS PRUINATUS* MYCORRHIZAE

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ABSTRACT

Ectomycorrhizae of the basidiomycete *Xerocomus pruinatus* in association with fine roots of European beech (*Fagus sylvatica*) were investigated for inter- and intra-species genetic variability using the spacer regions of their rDNA cluster. PCR-RFLP analysis of the internal transcribed spacer (ITS) region of the nuclear rDNA allowed separation of *X. pruinatus* from related mycorrhizae with similar morphological appearance such as *X. chrysenteron, X. badius* or *X. subtomentosus*. PCR-RFLP analysis of the intergenic spacer (IGS1) region revealed intra-species variability. The investigated population showed in total three different groups of individuals with respect to IGS1 amplification products and their RFLPs. Sequence analysis of the IGS1 region revealed two allelic variants. Thus the three groups are interpreted as three genotypes ($\alpha\alpha$, $\beta\beta$, $\alpha\beta$) of which the populational distribution was $\alpha\alpha = 42$ %, $\beta\beta = 16$ % and $\alpha\beta = 42$ %, following Hardy-Weinberg's theorem. The sequence of the IGS1 region is compared to that of other species and its substructure is discussed.

Key words: alleles, ectomycorrhiza, *Fagus sylvatica*, IGS1-RFLP, ITS-RFLP, *Xerocomus pruinatus*

INTRODUCTION

Most tree species in European forests form a mutualistic association with soil fungi. In this relationship, referred to as ectomycorrhiza, the fungi improve the water and nutrient uptake, while the hosts support the fungi with carbohydrates (HARLEY & SMITH 1983, SMITH & READ 1997).

While the genetic structure of many forest tree species such as European beech have been studied intensively (*e.g.* SANDER *et al.* 2001), studies on the genetic structure of their fungal symbionts are still rare and are in most cases limited to conifers (GRYTA *et al.* 1997; GUIDOT *et al.* 1999) and/or fruiting bodies (basidiocarps) (GRYTA *et al.* 1997, GHERBI *et al.* 1999, GUIDOT *et al.* 1999).

The fungal root symbiont which we studied here is *Xerocomus pruinatus* (Fr.) Quél. Its fruiting bodies may be found in mixed forests (PHILLIPS 1998). We specifically located its forms on both broad leaved and coniferous trees, such as European beech (*Fagus sylvatica* L.), sessile oak (*Quercus petraea* (Matt.) Liebl.) and Norway spruce (*Picea abies* (L.) Karst.). On root tips, *X. pruinatus* forms a silvery-white to light yellow hyphal mantle, encapsulating the tips. It possesses clearly recognizable rhizomorphs which arise from the base of the ectomycorrhizal structure (BRAND 1989). The mycorrhiza of *X. pruinatus* is morphologically similar to those of *X. badius* (Fr.: Fr.) Kühn.: Gilbert, *X. chrysenteron* (Bull.) Quél., *X. subtomentosus* (L.: Fr.) Quél. and *Boletus edulis* Bull.: Fr. (AGERER 1987 – 1998).

We investigated the rDNA region of *X. pruinatus* infecting a population of European beech growing in Central Germany. First, the corresponding ITS region was studied to see whether it may differ in morphologically similar mycorrhizae so that they can be clearly differentiated. Second, the IGS1 region was studied to see whether it might possess enough variability to be used in population genetic studies.

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MATERIALS AND METHODS

Sampling of mycorrhizae

Thirty-eight mycorrhizae of *X. pruinatus/Fagus sylvatica* were sampled in a beech forest in Central Germany near the town Bingen on the Rhine. Mycorrhizal root samples were collected from trees only with a minimum distance of 10 m between trees. For detailed sampling and stand characteristics see MELTZER & ROTHE (2000).

DNA Extraction

Total DNA was extracted from mycorrhizae according to the following protocol: A single mycorrhizal system of approximately 15 mg fresh weight was put into a sterile 1.7 ml Eppendorf Safe-Lock tube, frozen in liquid nitrogen and then homogenized with a plastic minipestle. The powdered material was suspensed in 300 µl of extraction buffer (100 mM Tris-HCl (pH 8.0), 1.4 mM NaCl, 20 mM Na_2-EDTA, 2 % (w/v) CTAB (DOYLE 1991)), incubated for 60–75 min in a waterbath at 65 °C and was vortexed four to six times during incubation. The resulting slurry was centrifuged for 10 min at 10000× g and the supernatant transferred into a sterile 1.7 ml Eppendorf Safe-Lock tube. Then 390 µl of isopropanol (99.7 %) and 20 µl of sodiumacetate solution (3 M) were added, and the extracts were gently mixed and incubated at –20 °C over night. The next day the nucleic acids were precipitated by centrifugation (10 min, 6700× g, 4 °C). The precipitate was washed twice with 600 µl of 70 % ethanol and dried at room temperature. Then 100 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM Na_2-EDTA) was added to the pellet which was then incubated at 65 °C for about 15 minutes. The resulting DNA solution was diluted to 1:50 and 1:500 with autoclaved water and stored at –20 °C until used for PCR. DNA was also extracted from fruiting bodies by the same procedure with the exception that material was homogenized without liquid nitrogen.

PCR-RFLP Analysis

PCR was performed using the primer pair ITS1 (WHITE *et al.* 1990) and ITS4B (GARDES & BRUNS 1993) to amplify the ITS region. The primer pair CNL12 and 5SA (cf. ERLAND *et al.* 1994) served to amplifiy the IGS1 region. The components for a 20 µl PCR reaction mixture were: dNTP's (200 µM of each), primers (1 pM per primer), BioTherm polymerase (1 Unit, GeneCraft, Münster, Germany), the appropriate buffer (according to the manufacturer) and 10 µl DNA solution. Null controls contained autoclaved water instead of DNA extract.

Amplifications were performed, using the following protocol: (a) denaturation (3 min, 94 °C), (b) 35 cycles for amplification (consisting of 45 sec at 94 °C, 45 sec at 58 °C and 90 sec at 72 °C) and (c) final extension (72 °C for 10 min). The PCR products were cut without further purification. A total of seven restriction enzymes were used to study species specific variabilities within the ITS region: *Alu* I, *Cla* I, *Eco* RI, *Hind* III, *Hinf* I, *Mbo* I and *Tai* I. A total of six restriction enzymes were used to study intra-specific variability of the IGS1 region: *Alu* I, *Hae* III, *Hinf* I, *Mbo* I, *Rsa* I and *Tai* I.

Species identification

Samples of fruiting bodies obtained from HAHN (http://www.botanik.biologie.uni-muenchen.de/botsyst/hahn.htm) were used for confirmation of species identication.

The basidiocarps of *Xerocomus badius* (leg./det. Ch. Hahn, 13.10.1998, CH 388/98), *Xerocomus pruinatus* (leg./det. Ch. Hahn, 27.10.99, CH 305/99) and *Xerocomus subtomentosus* s. str. (leg./det. Ch. Hahn, 22.8. 1998, CH 217/98) were collected in Bavaria, Germany. The basidiocarp of *Xerocomus chrysenteron* s. str. (leg./det. Ch. Hahn, 4.9.1997, CH 186/97) was from Poland.

Sequencing

Sequencing of PCR products was done by GENterprise (Mainz, Germany). Two new primers (Prigs 3 and Prigs 10) were designed to determine the complete sequence of the amplified IGS1 product, including the binding sites for the primers CNL12 and 5SA. The sequences of the new primers are:
Prigs 3: 5'- CAGTACCCCGTTCCCATTCA -3',
Prigs 10: 5'-GCCTAGACTTTGGTGCGC-3'.

Exploitation of electrophoretic data

The allele frequencies and the Hardy-Weinberg distribution of the three assumed genotypes were calculated according to ROTHE (1994).

RESULTS

ITS region

The silvery-white to light yellow mycorrhiza of *X. pruinatus* may be confounded with related mycorrhizal

species of similar appearances, especially with *X. chrysenteron*, which can also occur on European beech, sessile oak and Norway spruce. The silvery-white to light yellow ectomycorrhiza was initially identified as *X. chrysenteron* according to the morphological descriptions recorded by BRAND (1989). PCR-RFLP analysis of the ITS region of the rDNA gene cluster showed that all of the individuals in this study belonged to the same species. During the course of the research however, it was discovered that the ectomycorrhizal species described by BRAND (1989) was possibly not *Xerocomus chrysenteron* but rather *Xerocomus pruinatus* (AGERER 2002, personal communication). Subsequently, the species identification using PCR-RFLP analysis was re-examined. The analysis showed beyond a doubt that the ectomycorrhizae used in our research generated the same PCR-RFLP banding patterns as *Xerocomus pruinatus* but a different pattern compared to *Xerocomus chrysenteron* (referring to the basidiocarps received from HAHN).

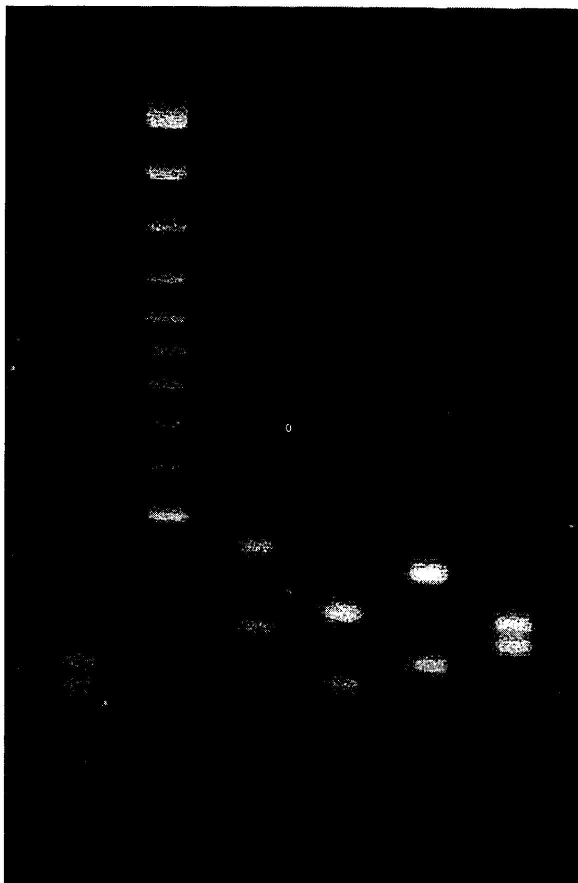

Figure 1. Discrimination between five ectomycorrhizal species of the Boletaceae by restriction of the ITS region with *Hinf*I. From left to right: *Boletus edulis*, standard, *Xerocomus chrysenteron, X. pruinatus, X. subtomentosus* and *X. badius*. Standard: 100 bp ladder (3000, 2500, 2000, 1500, 1031, 900, 800, 700, 600, **500**, 400, 300, 200, (100 bp)).

The sequence of the ITS region, which comprised the ITS1 spacer, the 5.8S gene and the ITS2 spacer, turned out to be invariant within the investigated *X. pruinatus* population. The amplification product was about 900 bp long and no polymorphisms were observed, when the seven restriction enzymes were applied. Different restriction patterns, however, were observed, for the ectomycorrhizal fungi *X. badius*, *X. chrysenteron*, *X. subtomentosus* and *Boletus edulis* so that these species can be clearly distinguished (Fig. 1).

IGS1

The intergenic spacer region IGS1 was polymorphic within the investigated *X. pruinatus* population on European beech (Fig. 2).

By PCR-RFLP analysis each of the 38 individuals could be classified as belonging to one of three groups: Mycorrhizae of one group (group A) differed from the rest by the presence of two amplification products (about 850 and 750 bp). The remainder of individuals belonging to the other two groups (groups B and C) had only one amplification product of identical size (about 750 bp). Members of the groups B and C could be separated from each other by restriction with one of the enzymes *Hae* III, *Hinf* I, *Mbo* I or *Rsa* I. As an example the restriction pattern of *Mbo* I is shown in Fig. 2.

The length of restriction fragments for the two alleles based on the nukleotid sequences are presented in Table 1.

Sequence of IGS1

Sequencing was successful for individuals of groups B

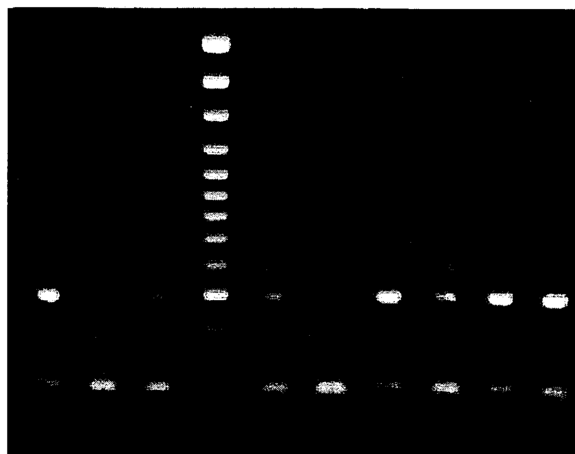

Figure 2. RFLP patterns of nine different individual ectomycorrhizae of *X. pruinatus* after digestion of the IGS1 region with *Mbo* I. Individual mycorrhizae could be classified as belonging to one of three groups: A (genotype AB), B (genotype BB) and C (genotype AA). From left to right: C, B, A, standard, A, B, C, A, C, C. Standard: 100 bp ladder.

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Table 2. Abundance of genotypes and allele frequencies in the investigated ectomycorrhizal population of *Xerocomus pruinatus* on European beech.

Relative abundance (%) of restriction groups / genotypes			Allele frequency (h_i)		χ^2	p
A	B	C				
αβ	ββ	αα	α	β		
42.1	15.8	42.1	0.632	0.368	0.192	0.662

p: probability value corresponding to the χ^2 value. The p value of 0.662 indicates that the three genotypes are distributed according to Hardy-Weinberg's theorem.

and C only. The end of the 28S gene and the beginning of the 5S gene within the amplificate of the IGS1 region were determined by comparison with a known basidiomycetal sequence (EMBL database of the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/embl/index.html), Accession Nr.: L 25898). If the sequences of the two alleles α and β of the IGS1 region of *X. pruinatus* were compared, one can see conserved and variable parts (Fig. 3). Following the 28S rDNA gene the IGS1 is comprised of a conserved region (Fig. 4) of about 100 bp which is fol

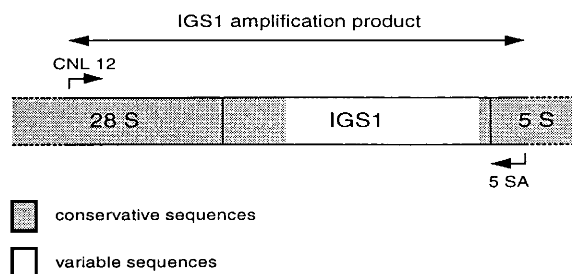

Figure 3. Location of conservative and variable parts and genes in the amplification product obtained with the primers CNL12 and 5SA.

lowed by a variable region of about 340 bp. The sequence ends with a short conserved region of 15 bp before the 5S rDNA starts. The variable part is characterized by some point mutations and two larger indels (insertions/deletions) (Fig. 5).

The comparison of individuals within the same group showed homogeneosity. This led to the conclusion that two types of IGS1 exist in the investigated *Xerocomus pruinatus* population.

Heteroduplex analysis and interpretation

Co-amplification of DNA extracted from one individual of group B and one individual of group C resulted in

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            *        20         *        40         *        60
B  TCTGAACCGCCTCTAAGTCAGAATCCGTGCTGGAAGTGACGATGTTTTGGTCCCGCACATGTAAATGG
C  --------------------------------------------------------------------

    *        80         *       100         *       120         *
B  AGTTGAGATAGAGCTTTGCTCGTGAACCAAATCAGGTGGACTAGGCTCGTCGGACGGAAACGTCTGGC
C  ---------------------------------------G----------------------------

    140        *       160         *       180         *       200
B  GGGTTCGTCTACGAATTGCAATCATGATATGCGCGGGGGTGAATCCTTTGCAGACGACTTGAATGGGA
C  --------------------------------------------------------------------

        *       220         *       240         *       260         *
B  ACGGGGTACTGTAAGCGGTAGAGTAGCCTTGTTGCTACGATCCGCTGAGGTTAACCCCTTGTTCTATA
C  ------------------------------------------------------G-------------

        280         *       300         *       320         *       340
B  GATTTGTTCAGCCTTTTCAAAGGGTTGGACTTTCTTCTTTTCTTTGTCACGTCCTCTACTTCAGGGCC
C  --------------------------------------------------------------------

            *       360         *
B  GGTTCACTAGAACTTGGCTTTGTGGTGGACATGTCGATG      379
C  ---------------------------------------      379
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Figure 4. Nucleotide sequences of the conserved parts of the IGS1 amplification products from group B and C ranging from position 1 to 379. Dashes represent nucleotides identical to those of the top sequence.

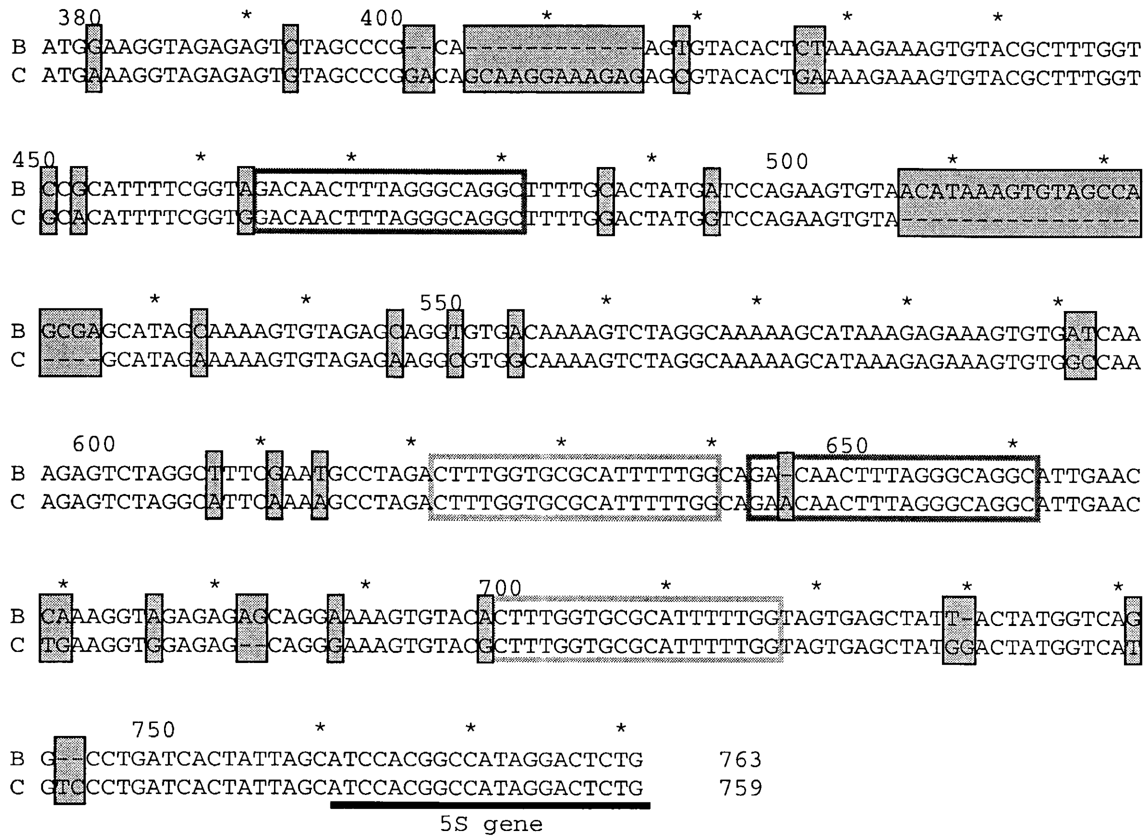

Figure 5. Nucleotide sequence of the second halfs of the IGS1 region of the groups B and C (including 5' part of the 5S gene; black bar). Grey boxes indicate mutations. Grey frames indicate repetition elements.

two amplification products of a length of 850 bp and 750 bp as found in individuals of group A. Group B is interpreted as being homokaryotic for a two allelic system of the IGS1 region (ββ) while group C is thought to be homokaryotic with respect to a different allele (α). Individuals being heterokaryotic with respect to their IGS1 region would then possess both alleles (αβ) and heteroduplex formation (PCR artifact) would result in two bands. The "larger" band (850 bp) consisting of the α and β heteroduplex molecule is retarded because of loop formation between non-homologeous regions (insertions/deletions, cf. Fig. 5) and therefore appears to have a size of 850 bp. The smaller band (750 bp) consists of homoduplexes of αα and ββ, having the same length but differing in their sequences.

Intra-population variability

Within the investigated population, the two alleles of the IGS1 region were distributed as shown in Table 2. The genotypes aa and ab were more frequently (42 %, respectively) found among the 38 individuals screened than genotype bb (16 %) (Table 2). The distribution of the observed genotypes was found to follow Hardy-Weinberg's theorem (Table 2). Thus the two allelic system of the IGS1 region of *X. pruinatus* can be used in combination with other genetic markers to study its population structure. Combination of PCR-RFLP and isozyme data (MELTZER & ROTHE 2000) showed individual multilocus genotypes for 35 of 38 mycorrhiza. So these ectomycorrhizae possessing identical genotypes at the IGS1 locus do not belong to the same genet.

DISCUSSION

In the literature there is scarely any reference at all of *Xerocomus pruinatus* which is described as a rare occuring mushroom (PHILLIPS 1998). This stands in contradiction to our field observations on mycorrhizae. In the investigated stand in Central Germany, the ectomycorrhizae of this species were relative abundant.

The appearance of fruiting bodies is sporadic (GARDES & BRUNS 1996; DAHLBERG *et al.* 1997), occurring from the end of summer to mid autumn. It is

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not quite clear, if the above-ground ectomycorrhiza population also represents the below-ground ectomycorrhiza population. It has been shown that species which are rarely seen as fruiting bodies can occur in large quantities as mycorrhizae and *vice versa* (GARDES & BRUNS 1996). So, since the mycorrhizae are more universally available, they were used to study the diversity of the IGS1 region of a *X. pruinatus* population.

The ITS is a DNA cut out, which can exhibit variability at the inter- or intra-species level (ERLAND *et al.* 1994), but for the most part it varies between species. Therefore it is widely used for species identification of fungi (KÅREN *et al.* 1997). By comparison of the ITS structure of mycorrhizae and fruiting bodies which we collected in the beech forest with material from a herbarium (HAHN, http://www.botanik.biologie.uni-muenchen.de/botsyst/hahn.htm) we were able to identify our mycorrhizal population as belonging to the basidiomycetal species *Xerocomus pruinatus*. We demonstrated, that between the species *X. pruinatus*, *X. badius*, *X. chrysenteron*, *X. subtomentosus* and *Boletus edulis* the ITS region is variable enough to differentiate the morphologically similar mycorrhizae (cf. Fig. 1) while within the species *X. pruinatus* it is invariant.

Analysis of the rDNA was supposed to be an appropriate tool for population analysis of ECM fungi (EGGER 1995). Especially the IGS region of ectomycorrhizal fungi may exhibit intra-populational variability as shown for the fungus *Hebeloma cylindrosporum* on *Pinus* where more than 20 alleles were observed (GUIDOT *et al.* 1999).

Allelic variation at the IGS locus in ectomycorrhizal fungi can be based on length variation (different number of subrepeats or indels) or pointmutations. In the case of *H. cylindrosporum* allelic differences were mainly caused by a varying number of a short repeated motif occuring in the IGS2 (GUIDOT *et al.* 1999). In *Laccaria bicolor* also two alleles were observed which varied with respect to the repeat number of a short motif (SELOSSE *et al.* 1996, MARTIN *et al.* 1999). In contrast the differences between the IGS1 alleles of *Xerocomus pruinatus* were due to point mutations and indels. A comparable situation was found for *Laccaria amethystina* where two sequenced alleles of IGS1 also differed because of an indel and a few point mutations (GHERBI *et al.* 1999).

Within the *X. pruinatus* populations on European beech we found the two alleles at the IGS1-DNA region with the corresponding three genotypes distributed according to Hardy-Weinberg´s theorem. From our results it is concluded that the IGS1 region of *Xerocomus pruinatus* does show measurable allelic variation and therefore could be used as an additional marker for genetic analysis of *X. pruinatus* populations.

Further studies will investigate the population structure of *X. pruinatus* species associated with different host trees combining isozyme and PCR-RFLP analysis.

The sequence analysis of the IGS1 of *X. pruinatus* demonstrated that beside the variable part the spacer contains conservative areas, too.
The preserved sequence of the first part of the IGS1 of *X. pruinatus* indicates, that functional areas may be located here. Special functional sequences (also called SalI boxes), which are involved in the termination process, are described by GRUMMT *et al.* (1986). No terminal sequence, similar to the ones described, could be recognized in the IGS1 of *X. pruinatus*. However, a functional structure, that also appears in organisms of different taxa, is the T-run (LANG & REEDER 1995). The nucleotide sequences of these elements vary among different organisms, but all have a high proportion of thymidines (T) in common. Such T-aggregations can be involved in termination events for RNA polymerases. For example, in *Saccharomyces cerevisiae*, a T-run is involved in termination of polymerase I (LANG & REEDER 1995, REEDER *et al.* 1999). Within the conservative IGS1 region of *X. pruinatus*, the part ranging from nucleotide 302 to nucleotide 316 contains four blocks with 2 to 4 Ts, separated by C. In total, there are 12 Ts. These thymidines may play a role in termination.

In the IGS1 of *X. pruinatus* two motifs occur which's sequences are similar to a TATA box sequence. They are located in an area just before the beginning of the 5S rDNA. The sequence of the first box deviates in the both alleles slightly from each other, while in the second box the sequences are identical. But in both alleles, the position of the boxes with respect to the beginning of the gene is relatively consistent. The preservation of the sequence and its location indicates a possible functional relationship to the 5S gene.

The 5S rRNA genes as well as the tRNA-genes generally possess internal promotors. But there also exist genes, transcribed by RNA polymerase III (*e.g.* the signal recognition particle RNA-gene family in the tomato (RIEDEL *et al.* 1996)) where TATA box motifs occur. These are necessary for the transcription of corresponding genes. Likewise in some plant species, similar TATA box sequences were found shortly before transcription start codons of 5S rRNA genes (VENTKATESWARLU *et al.* 1991). Among different plant species such as lupine, wheat, pea and tomato the sequence of this motif is not very strongly preserved but its position is. In these cases, all TATA boxes are found in an area of –30/–22 before the transcription starting point. In *X. pruinatus*, the position of the first TATA box lies between –35/–27, the second one is

located directly before the beginning of the 5S rRNA gene. Conserved sequences including a TATA box were found in the upstream regions (-29 to -27) of the dispersed 5S RNA genes in *Neurospora crassa*, an ascomycete (SELKER *et al.* 1986). In *Laccaria amethystina* a TATA box motif also occurs in a distance of -27 to -24 before the beginning of the 5S rDNA (cf. EMBL database (http://www.ebi.ac.uk/ embl/index.html), Accession No.: AF 124340 and AF 124341).

In addition to the TATA box, some of the polymerase III transcripted genes, further own conserved, external regulatory sequences, PSE (proximal sequence element) and DSE (distal sequence element). These are involved in the transcription of the corresponding genes. So, two preserved sequence blocks observed in the IGS1 of *X. pruinatus*, which each occur twice within the variable part (cf. Fig. 5), may have a corresponding function.

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