ALLELE AND GENOTYPE FREQUENCIES OF THE INTERGENIC RDNA SPACER ONE REGION IN ECTOMYCORRHIZA POPULATIONS OF *XEROCOMUS PRUINATUS* ON THREE DIFFERENT HOST TREE SPECIES

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ABSTRACT

The variability of the intergenic spacer (IGS1) of the rDNA cluster in six populations of the ectomycorrhizal basidiomycete *Xerocomus pruinatus* (Fr.) Quél. is described. In *X. pruinatus* populations on European beech (*Fagus sylvatica*), sessile oak (*Quercus petraea*) and Norway spruce (*Picea abies*) the IGS1 exhibits two alleles and three genotypes.

Genetic measurements indicate that sexual reproduction is important for the genetic structure in this late stage ectomycorrhizal fungus.

In association with the two angiosperms beech and oak the homozygous genotype aa and the heterozygous genotype ab dominate, whereas in association with the gymnosperm spruce the homozygous genotype bb predominates. The corresponding allele b was statistically more frequent in the *X. pruinatus* population on Norway spruce than in populations on European beech and a population on sessile oak. Genotype preferences are discussed in the context of adaptation and coevolution.

Key words: *Xerocomus pruinatus*, *Fagus sylvatica*, *Quercus petraea*, *Picea abies*, ectomycorrhiza, population genetics, IGS1.

Introduction

Characterizing the genetic structures of populations of ectomycorrhizal fungi is a field of increasing interest. Genetic variation within species is thought to be ubiquitous in ectomycorrhizal fungi (DEBAUD *et al.* 1998) and genotypes can vary in their mycorrhizal efficiency in respect to different host tree species (BONFANTE *et al.* 1998). However, genetic variation of ectomycorrhizal populations in relation to host species has received little attention (MELTZER & ROTHE 2003).

Most studies deal with the discrimination of individuals (genets), the determination of their sizes and their persistence. From the size and persistence of genets, conclusions about the relative role of vegetative spread or spore establishment on propagation have been drawn (GHERBI *et al.* 1999, REDECKER *et al.* 2001, FIORE-DONNO & MARTIN 2001, BERGEMANN & MILLER 2002). The type of propagation differs in two opposite life strategies which are found in late stage and early stage fungi. The terms were chosen to distinguish whether fungi arise in early or late phases of forest development. One characteristic of early stage fungi is that they form many but small, short-lived genets, which establish readily from spores. In contrast, late stage fungi are more persistent with a dominant hyphal spread, which leads to the formation of large genets. Recent work showed however that populations of ectomycorrhizal fungi in mature forests can also have many small genets (GHERBI *et al.* 1999, FIORE-DONNO & MARTIN 2001, REDECKER *et al.* 2001). Therefore, we use the terms early and late stage simply to distinguish the two types of propagation modes.

Xerocomus pruinatus is a member of the Boletales. It has been observed in the north, the middle and the south of Europe (KRIEGLSTEINER 2000). Its mycorrhizae are often found in forests in central Germany (our observations, also cf. BRAND 1989). With one known exception, Xerocomaceae form ectomycorrhizae and they typically infect a broad range of host tree species which include members of the gymnosperms as well as members of the angiosperms. *X. pruinatus* was observed on Norway spruce, fir, European beech and oak. There are many examples for boletoid species that form large

genets (BONELLO *et al.* 1998, DAHLBERG & STENLID 1994, DAHLBERG *et al.* 1997). FIORE-DONNO & MARTIN (2001) reported a genet of *X. pruinatus* with a size of about 40 m^2. The intensive vegetative growth may be promoted by the presence of rhizomorphs in these species, which extend far into the substrate. So, *X. pruinatus* has been classified as a typical late stage fungus.

The intergenic spacers of the rDNA may be variable within a species of an ectomycorrhizal fungus (GUIDOT *et al.* 1999, MARTIN *et al.* 1999, SELOSSE *et al.* 1996) and are DNA regions which can be studied directly on mycorrhizae. In this study, the IGS1 was investigated to analyze the distribution of genetic variability in six *X. pruinatus* populations with a focus on the possible influence of host tree species.

MATERIALS AND METHODS

Stand characteristics

Fine roots (d < 1 mm) which were infected at their tips with *Xerocomus pruinatus* (Fr.) Quél. were sampled in four European beech (*Fagus sylvatica* L.) stands, which are located at Bingen, Heppenheim, Rüdesheim and Taunusstein 2, one sessile oak (*Quercus petraea* Liebl.) stand located at Trippstadt and one Norway spruce (*Picea abies* (L.) Karst) stand at Taunusstein 1 in central Germany. The stands at Rüdesheim, Taunusstein 1 and Trippstadt were described recently (MELTZER & ROTHE 2003). The stands at Bingen, Heppenheim and Taunusstein 2 are described in Table 1. An overview of the location of the six stands was given recently (Fig. 1 in MELTZER & ROTHE 2003).

Sampling of mycorrhizae

Mycorrhizal root samples were collected from trees only where there was a minimum distance of 10 m between trees. The number of analyzed ectomycorrhizas ranged from 44 to 61 (Bingen: 52, Heppenheim: 62, Rüdesheim: 59, Taunusstein 1: 44, Taunusstein 2: 51, Trippstadt: 59).

Sampling and cleaning of mycorrhizas was performed as described in MELTZER & ROTHE (2000).

Species identification

The mycorrhiza were identified initially under a stereomicroscope at x25 magnification, according to morphological criteria described by BRAND (1989) and AGERER (1987 - 2002), and later by rDNA analysis (HAESE & ROTHE 2003). Species specific variabilities within the ITS region were taken to differentiate *Xerocomus pruinatus* from other mycorrhizae of similar appearance (HAESE & ROTHE 2003).

IGS1 Analysis

DNA extraction was performed as described recently (HAESE & ROTHE 2003). The primer pair CNL12 and 5SA (cf. ERLAND *et al.* 1994, HENRION *et al.* 1992) served to amplify the IGS1 region as given in HAESE & ROTHE (2003). For RFLP analysis the PCR products were cut without further purification. Three IGS1 restriction variants were identified according to their restriction patterns when applying one of the following restriction enzymes: Hae III, Hinf I, Mbo I or Rsa I (HAESE & ROTHE 2003). The enzyme Hinf I was used for routine discrimination of the three IGS1 restriction variants (i.e. genotypes) by PCR-RFLP analysis. Amplification products were separated on 0.8% agarose gels, restriction fragments on 1.7% agarose gels.

Sequencing of the IGS1 was performed on purified PCR products (GENterprise, Mainz, Germany). Sequence alignments were performed using the programs Clustal W and GeneDoc. Sequencing of the IGS1 region resulted in two different IGS1 sequences (Accession-No. AJ 305292 and AJ 305293) (HAESE & ROTHE 2003). These were termed allele α and allele β. The three observed IGS1 restriction variants were therefore interpreted as three genotypes: αα, αβ and ββ. After electrophoresis the genotypes αα and bb show one DNA band each, which are identical in size. The two homozygous genotypes could be differentiated by restriction. The genotype αβ leads to two bands, one of which consists of a heteroduplex molecule. The formation of a heteroduplex molecule in vitro was confirmed by mixing equal amounts of DNA from the two homozygous genotypes. Co-amplification resulted in an additional DNA band, the banding pattern being the very same as the pattern of the heterozygous individuals.

Exploitation of electrophoretic data

In an ectomycorrhiza the hyphae of a basidiomycete such as *X. pruinatus* are dikaryotic and genes of both nuclei are expressed. Correspondingly, genetic measures developed for diploids can be applied

Table 1. Characteristics of investigated European beech (*Fagus sylvatica*), sessile oak (*Quercus petraea*) and Norway spruce (*Picea abies*) stands in central Germany.

Stand characteristics	Bingen	Heppenheim	Taunusstein 2
Forest district	Bingen	Heppenheim	Chausseehaus
Forest site	Jägerhaus	Kirschhausen Stadtwald Heppenheim	Altenstein, Staatswald Chausseehaus
Stand number	56 + 54b2	101	164a1
Stand area	9.6 ha	701	5.5 ha
Partition of tree species	mostly beech, some spruce and oak	beech: 85 %, oak: 5%, pine: 5 %, larch: 5 %	beech: 91% oak: 9%
Host species	beech	beech	beech
Altitude (m above sea level)	465–515	305–350	470–490
Mean temperature (°C) over the year (in the vegetation period)	7.5–8 (14)	7.5 (13)	7.5 (13)
Mean precipitation (mm) per year (per vegetation period)	675–750 (350)	750 (370)	800 (200)
Parent rock	quartzite, with loamy sand	granite with loess loam	quartzite with loess loam
Liming (year)	yes (1989)	no	yes (1987)
Application rate ($t \cdot ha^{-1}$)	3		3
Age of trees in years	98–103	108	158
Number of mycorrhizae sampled	52	62	51
Date of sampling of the mycorrhizas	autumn 1998	spring 1998	spring 2000

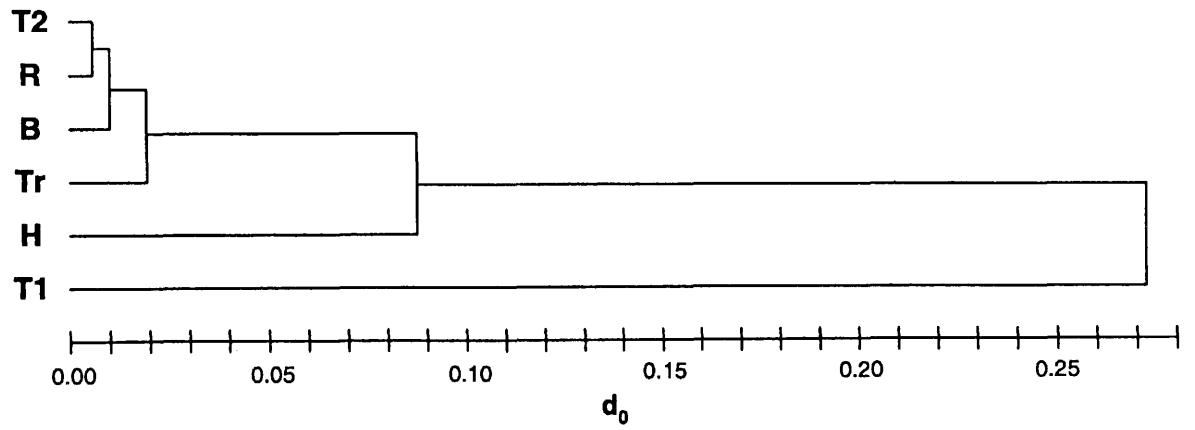

Figure 1. Dendrogram based on the genetic distance (d_0) values for the six *X. pruinatus* populations investigated. **B**: Bingen, **H**: Heppenheim, **R**: Rüdesheim, **T1**: Taunusstein 1, **T2**: Taunusstein 2, **Tr**: Trippstadt.

(MELTZER & ROTHE 2000).

Analysis of allelic structures:

- Allele frequencies: Frequencies (p) of the alleles α and β were calculated by use of the formula p_α = (2 [αα] + [αβ]) / 2N where [αα] represents the number of αα homozygotes and [αβ] the number heterozygotes αβ. The frequency of allele β resulted from $p_\beta = 1 - p_\alpha$.
- Genetic identity (I): The amount of joined alleles in two populations was calculated by use of Nei's formula (NEI 1972):

$$I_{XY} = \left(\sum X_i Y_i\right) / \left(\sum X_i^2 \sum Y_i^2\right)^{1/2}$$

where X_i = frequency of the i-th allele in population X and Y_i = frequency of the i-th allele in population Y.

- Genetic distance (d_0): genetic distance was also calculated according to GREGORIUS (1974), using the formula

$$d_0 = \frac{1}{2}\sum_{i=1}^{n} X_i - Y_i$$

where X_i = frequency of the i-th allele at the IGS1 locus in population X and Y_i = frequency of the i-th allele at the IGS1 locus in population Y.

Analysis of genotypic structures:

- Genotype frequencies: Relative genotype frequencies (g_r) were calculated by use of the formula $g_r = (g_a \quad 100)/N$ with g_a representing the observed frequency in the number N of investigated individuals. The variance υ of g_r results from $= \pm (g_a (100 - g_a)/N)^{1/2}$.

At a significance level of α = 0.05, υ' = 1.96 υ. If, from the higher relative frequency g_r in one population the corresponding υ' value is subtracted while from the lower g_r value in another population the respective υ' value is added and the resulting values do not overlap, then the two g_r values are different at a significance level of α = 5 %. If they overlap they are not significantly different at that level (e. g. population A: g_a = 75 %, N = 54 and υ = 33.2, i.e. = 41.8; population B: g_a = 38 %, N = 54 and υ' = 6.6 i.e. υ'_B = 31.4. Since υ'_A and υ'_B do not overlap the genotype frequencies at population A and B differ at a significance level of α = 5 % (B. Thiebout, personal communication).

- Fixation index (F): This index links the amount of observed and expected heterozygosities in two populations; its values range from -1 to 1 (WRIGHT 1978, NEI 1987). F was calculated by use of the formula: $F = 1 - (h_o/h_e)$ with h_o = observed heterozygosity and h_e = expected heterozygosity.
- Genetic diversity within populations (H_S) and between populations (D_{ST}): The total genetic diversity (H_T) according to NEI (1973) results from $H_T = 1 - \sum X_k^2$ with $X_k = \sum (p_{ik}^2/n)$ where p_{ik} respresents the frequency of the k-th allele at a locus within the i-th population and n represents the number of populations. The total genetic diversity is composed of the diversity between populations (D_{ST}) and the diversity within populations (H_S): $H_T = H_S + D_{ST}$.
- Coefficient of genetic differentiation (G_{ST}): the genetic diversity between populations may also be characterized by the quotient of diversity between populations (D_{ST}) and the total genetic diversity (H_T) (NEI 1973): $G_{ST} = D_{ST}/H_T$.
- Dendrogram: Dendrogram construction was based on the d_0 values using the unweighted pair-group method with arithmetic mean (UPGMA) (cf. ROTHE 1994).

All calculations were performed with the computer program Excel (Microsoft). For further details see for example ROTHE (1994).

RESULTS

Genotype frequencies

The three IGS1 genotypes aa, ab and bb were observed in all of the six *Xerocomus populations* investigated in central Germany (Table 2). In four populations on European beech (Bingen, Heppenheim, Rüdesheim and Taunusstein 2) the homozygous genotype aa and the heterozygous genotype ab appeared in similar frequencies which were lower than 50 % (aa: 34–46 %, ab: 41–48 %). In the population on sessile oak (Trippstadt) the frequencies of both genotypes were comparable (aa: 51 %, ab: 34 %), while they were much lower in the population on Norway spruce (aa: 25 %, ab: 21 %). Accordingly, the frequency of the genotype bb was low in the populations on beech and oak (bb: 12 to 18 %) as compared to the population on Norway spruce (bb: 55 %).The later frequency differences are significant at a level of α = 5 % (Table 3). The investigated populations were at Hardy-Weinberg equilibrium (α = 5 %), except the one infecting Norway spruce.

Fixation index

The population of *X. pruinatus* on sessile oak (Trippstadt) and especially the one on Norway

Table 2. IGS1 genotype frequencies in the *X. pruinatus* populations infecting European beech, sessile oak or Norway spruce.

Stand (host species)	N^{1}	Genotype	Genotype frequencies		Variance (%)[2]
			absolute	relative (%)	
Bingen	52	αα	22	42.31	±13.43
(*Fagus sylvatica*)		αβ	24	46.15	±13.55
		ββ	6	11.64	±8.68
Heppenheim	62	αα	21	33.87	±11.78
(*Fagus sylvatica*)		αβ	30	48.39	±12.44
		ββ	11	17.74	±9.51
Rüdesheim	59	αα	27	45.76	±12.71
(*Fagus sylvatica*)		αβ	24	40.68	±12.53
		ββ	8	13.56	±8.74
Taunusstein 2	51	αα	23	45.10	±13.66
(*Fagus sylvatica*)		αβ	22	43.14	±13.59
		ββ	6	11.76	±8.84
Trippstadt	59	αα	30	50.85	±12.76
(*Quercus petraea*)		αβ	20	33.90	±12.08
		ββ	9	15.25	±9.17
Taunusstein 1	44	αα	11	25.00	±12.79
(*Picea abies*)		αβ	9	20.45	±11.92
		ββ	24	54.55	±14.71

[1] number of investigated mycorrhizae per stand [2] calculation of variances cf. MELTZER & ROTHE 2000

Table 3. Significant differences in IGS1 genotype frequencies between six *X. pruinatus* populations, four on European beech, one on sessile oak and one on Norway spruce.

Stand	geno-type	Bin (*F.s.*)			Hep (*F. s.*)			Rüd (*F. s.*)			Tau 2 (*F. s.*)			Trip (*Q. p.*)		
		αα	ββ	αβ	αα	ββ	αβ	αα	ββ	αβ	αα	ββ	αβ	αα	ββ	αβ
Tau 1	αα								*			*			*	*
(*P. a.*)	ββ		*			*										
	αβ						*									

The asterix (*) indicate significant differences (α = 5%) in frequencies between populations. *F. s.*: *Fagus sylvatica*, *P. a.*: *Picea abies*, *Q. p.*: *Quercus petraea*. Stands: Bin: Bingen, Hep: Heppenheim, Rüd: Rüdesheim, Tau: Taunusstein, Trip: Trippstadt.

spruce (Taunusstein 1) exhibited an excess in homozygous genotypes ($F = 0.224$ and $F = 0.552$, respectively), while the fungal populations on European beech revealed inbreeding coefficients near zero (Bingen: $F = -0.020$, Heppenheim: $F = 0.006$, Rüdesheim: 0.092, and Taunusstein 2: $F = 0.029$).

Allele frequencies

The IGS1 allele a was most abundant with frequencies ranging from 58 to 68 % within the *X. pruinatus* populations on the angiosperm species *Fagus sylvatica* and *Quercus petraea*. In the population on the gymnosperm species *Picea abies* the allele b was dominant with a frequency of 65 % (Table 4). Allele frequencies between the population on Norway

spruce on the one hand, and those on European beech and sessile oak on the other, were significant at a level of a = 5 %.

Genetic identity (I), distance (d_0) and diversity (H_T)

The genetic identity between the six populations of *X. pruinatus* was found to be high (0.80 < I < 1.00). Correspondingly, the genetic distances between populations at the IGS1 region were low. The low distance (d_0 = 0.006) was found between the *X. pruinatus* populations on beech at Rüdesheim and the population on beech at Taunusstein 2. The relatively large distance, related to the IGS1 region, was calculated for the population on oak (Trippstadt) and the one on spruce (Taunusstein 1), represented by d_0 = 0.326. Figure 1 shows the dendrogramm based on the d_0 values.

The total amount of genetic diversity H_T was found to be H_T = 0.46 for the four populations on beech and H_T = 0.48 for all six populations. The percentage of genetic diversity between populations (G_{ST}) related to the total amount of genetic diversity (H_T) was 0.5 % for the populations on beech and 5.5 % overall for the populations studied.

DISCUSSION

Several investigations on ectomycorrhizal fungi proved the existence of alleles at the IGS1 region of the rDNA gene cluster. In the *Laccaria bicolor* S238N isolate two IGS1 alleles were described (SELOSSE *et al.* 1996, MARTIN *et al.* 1999), in isolates of *Paxillus involutus* the existence of three alleles was inferred (HÖNIG *et al.* 2000) and in *Laccaria amethystina* seven alleles were observed (GHERBI *et al.* 1999). So, in *Xerocomus pruinatus* the presence of two alleles at the IGS1 is relatively low, but within the range of other ectomycorrhizal fungi. Isozyme data showed that individuals with the same IGS1 genotypes were not clones (cf. HAESE & ROTHE 2003).

Alleles of the IGS are codominantly inherited as found out for the basidiomycetes *Laccaria bicolor* S238N (SELOSSE *et al.* 1996, MARTIN *et al.* 1999) and *Phellinus nigrolimitatus* (KAUSERUD & SCHUHMACHER 2001) and therefore they can be used for populational studies.

Based on the analysis of the IGS1 the genetic identity of the six *X. pruinatus* populations studied was high (0.80<I<1.00). Similar values were observed in isozyme studies on the very same populations (MELTZER & ROTHE 2000, MELTZER & ROTHE 2003). Correspondingly, the genetic diversity between the population was low, with G_{ST}-values of 5.5 % for all six populations. Isozyme analysis of these populations resulted in G_{ST}-values of 10.9 % (MELTZER & ROTHE 2003).

A low genetic diversity is found between fungal populations with a moderate gene flow, for example the G_{ST}-value was 4 % in the canker pathogen *Crumenulopsis sororia* (ENNOS & SWALES 1991), 3.9 % in the foliar pathogen *Mycosphaerella graminicola* or 3 % in *Phaeosphaeria nodorum* (HARVEY *et al.* 2001 and references therein). In contrast to this situation relatively high genetic diversities are found between vegetatively propagating fungi such as in the host specialized varieties of *Leptographium wageneri* (G_{ST}: 86 %, ZAMBINO & HARRINGTON 1989).

Between populations of the ectomycorrhizal fungus *Cenococcum geophilum* a genetic variability of 42 % was observed (JANY *et al.* 2002). Here, the asexual spores are released in the ground. If, on the other hand, asexual spores are spread by the wind, the genetic variability between fungal populations is low, as observed in the phytopathogen *Phytophtora infestans* where G_{ST}-values of 6 % and 12–14 % were determined (FRY *et al.* 1992, GOODWIN *et al.* 1992). So, the way of spore dispersal strongly influences the genetic structures in ectomycorrhizal fungi. *X. pruinatus* forms above ground fruiting bodies which release haploid spores which may be spread by the wind. Even if the distances for spore dispersal are generally assumed to be not very far, it seems to be sufficient to allow gene flow between populations, which explains the low genetic diversities observed in this study and in isozyme studies (MELTZER & ROTHE 2000, MELTZER & ROTHE 2003). As a result, sexual reproduction can also influence the populational structure in late stage ectomycorrhizal fungi as *X. pruinatus*.

The IGS1 genotypes and the corresponding alleles showed significantly different frequencies in populations of *X. pruinatus* on European beech and sessile oak on the one hand and in the one on Norway spruce on the other. Soil and climatic reasons can be excluded for this unequal distribution. Rather, the reasons must be given in the different host species.

Clearly the IGS1 genotype bb has a preference for association with Norway spruce (or *vice versa*). The deviation from Hardy-Weinberg equilibrium could point to selection processes acting on the population on Norway spruce.

A pair wise coevolution between myco- and phytobionts is possible, even when both partners are generalists and able to associate with different species (TAYLOR 2000). During the last glaciations

different genotype combinations (host races) of *X. pruinatus* might have evolved in separated areas. DOVER (1982, 1986) postulates that different alleles of DNA loci, which evolve in a concerted manner as the rDNA does, can only develop in reproductively separated populations. These races may have kept their ability to infect hosts from the Fagaceae family as well as hosts from the Pinaceae family such as spruce but they differ in the success of their interaction.

The role of the IGS1 region concerning the adaptation is not known. It is likely that it serves as a marker for other unknown DNA loci, which are involved in this process. Whether the IGS1 region could serve as a marker for host adaptation of ectomycorrhizal fungi in general has to be ruled out in further studies.

Adaptation of *X. pruinatus* to its hosts can also be seen in the different expression patterns of diaphorase genes as shown by MELTZER & ROTHE (2003).

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