Diversity of yeasts isolated from litter and soil of different natural forest sites in Austria

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Hefediversität in der Streuschicht und im Boden verschiedener Naturwaldstandorte

1 Introduction

About 70.000 to 120.000 fungal species have been described world-wide so far but estimations expect about 1,5 million fungal species to exist on our planet. This means that only about 5 % of all fungal species have yet been identified (HAWKSWORTH, 1991, 2001; KENDRICK, 2000). For yeasts estimations are even lower. Only about 1 % of the yeasts existing on earth have been described so far (FELL et al., 2000).

For the large majority of fungi and yeasts at least one development stage is known to take place in soil (BRIDGE and SPOONER, 2001). Soil horizons are unequally variegated with fungi and yeasts, with decreasing amounts of isolates being obtained from lower soil layers (BÅÅTH, 1981). Very often soil does not necessarily represent their natural habitat. For instance fungi normally growing on leaf surfaces get into the ground through the litter layer. For many species soil only serves as a save harbour for their resting states (BRIDGE and SPOONER, 2001). The composition of yeast flora in soil certainly depends on different factors like soil character, climate and organism communities. The amount ranges from only a few to thousands of cells per gram soil (PHAFF and STARMER, 1987). As saprotrophs soil yeasts play an important role in the decomposition and dissipitation of energy within the soil ecosystem. They have the ability to respire many carbohydrates, like e.g. the degradation products of woody material (BOTHA, 2006).

Zusammenfassung

Die Diversität der Hefen wurde in Streu- und Bodenproben aus drei österreichischen Naturwaldreservaten (Müllerboden, Saubrunn, Rotwald) mit molekularen Methoden untersucht. Es wurden 82 Hefestämme isoliert, die Sequenzierung der D1/D2 Region der 26S rDNA ergab 25 verschiedene Sequenzen. Diese konnten mit einer Ausnahme elf Gattungen zugeordnet werden. Mittels PCR-fingerprinting konnten acht Arten identifiziert werden. Die höchste Diversität wurde im Auwald des Standortes Müllerboden festgestellt. Die Basidiomycetenhefen überwogen deutlich, sieben Arten mit insgesamt 56 Isolaten konnten der Gattung *Cryptococcus* zugeordnet werden.

Schlagworte: Hefen, Diversität, Boden, Streuschicht, Naturwald.

Summary

The diversity of yeasts in soil and litter samples taken from three Austrian natural forest reserves (Müllerboden, Saubrunn, Rotwald) was investigated. In total 82 yeast strains were isolated and identified using molecular methods. Partial sequencing of the 26S rDNA gene resulted in 21 different sequences belonging to eleven genera. For one sequence it was not possible to determine the genus membership. Eight species were identified via PCR-fingerprinting. The alluvial forest at Müllerboden showed the highest yeast diversity. The vast majority of the isolated strains belong to the basidiomycete yeasts, more than the half were members of the genus *Cryptococcus* (56 isolates belonging to seven species).

Key words: Yeast, diversity, soil, litter, forest.

Several studies regarding the diversity of yeasts have been made worldwide (BABJEVA and CHERNOW, 1995; BABJEVA and RESHETOVA, 1998; MOK et al., 1984; SLÁVIKOVÁ and VADKERTIOVÁ, 2000, 2003; VISHNIAC, 1996). Species identification was done by physiological and morphological methods, but the drawback of these methods is that the results may be often misconstrued (FELL et al., 2000).

More reliable results can be achieved by the use of molecular methods. In Austria one publication dealing with yeasts isolated from soil in an alluvial forest was carried out in this way (WUCZKOWSKI and PRILLINGER, 2004).

To enhance the knowledge about soil yeasts in Austria, three different natural forest sites were investigated.

These habitats are part of a group of natural forest reserves selected for the project DIANA ("Soil Diversity in Austrian Natural Forests"), which has the aim to take stock, to monitor and to compare the biological diversity and population ecology of various groups of organisms which exist in soils of forest communities that are typically present in Austrian forests (ZECHMEISTER-BOLTENSTERN, unpublished). These locations are part of the "Natural Forest Reserves Program", which has the aim to maintain the biological diversity of forest communities not by preserving certain conditions but by allowing the forests to develop freely, without direct human impact (FRANK and MÜLLER, 2003).

2 Materials and Methods

2.1 Sampling

To record data from undisturbed natural habitats, three different natural forest sites were selected: Müllerboden, Burgenland (48°00‘ latitude N, 16°42‘ longitude E, sea level: 160m, flood plain forest, soil type: calcaric fluvisol, pH: 6,9, dominant trees: *Alnus glutinosa*, *Fraxinus excelsior*), Saubrunn, Lower Austria (48°32‘ latitude N, 15°33‘ longitude E, sea level: 550m, acidophilous-beech grove, soil type: dystric planosol, pH: 3,2, dominant tree: *Fagus sylvatica*) and Rotwald, Lower Austria (47°46‘ latitude N, 15°07‘ longitude E, sea level: 1035 m, spruce-fir-beech grove, soil type: chromic cambisol, pH: 5,3, dominant tree: *Fagus sylvatica*), see Fig. 1. For detailed soil and vegetation characteristics see HACKL et al. (2000, 2004). Sampling has been carried out between November 2001 and June 2002. At the first two sites, samples were taken from four different zones: litter, soil at 0–5 cm, 10–15 cm and 30–35 cm depth. Due to digging restrictions within the habitat Rotwald, the soil zones at 10–15 cm and 30–35 cm depth could not be sampled. This would anyway have been a hard task to be performed at the selected transect points, due to the craggy soil properties present at this location. The material was taken from six different sampling points within each horizon and habitat and mixed well.

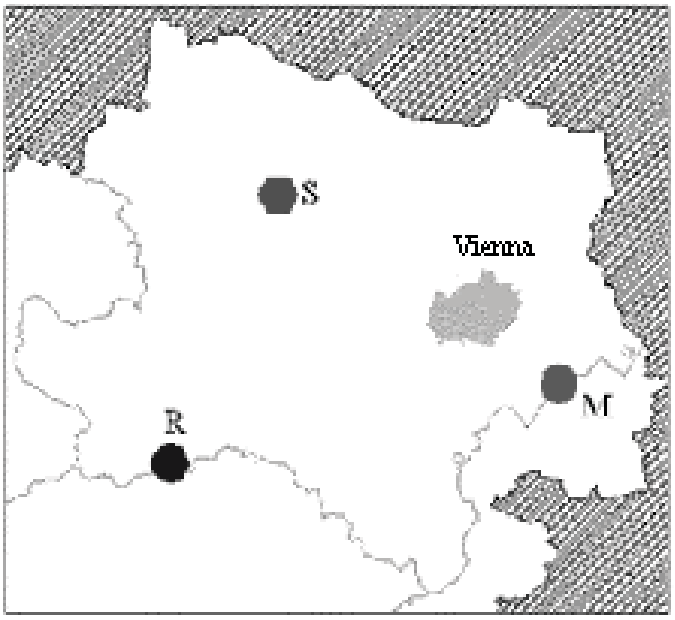

Figure 1: Map showing eastern Austria where the sampling sites are located. M … Müllerboden, S … Saubrunn, R … Rotwald (Map from DIANA homepage http://bfw.ac.at/300/2200.html, with modifications)

Abbildung 1: Lage der untersuchten Naturwaldstandorte in Ostösterreich. M … Müllerboden, S … Saubrunn, R … Rotwald. (Plan siehe DIANA homepage http://bfw.ac.at/300/2200.html, mit Modifikationen)

2.2 Isolation of yeasts

The litter samples were shaken with 1/4 strength Ringer solution (Merck, Darmstadt, Germany) and plated out. Soil was processed according to the soil washing technique developed by Gams (GAMS and DOMSCH, 1967). For detailed description see WUCZKOWSKI et al. (2003).

2.3 Identification and conservation

For rapid distinction between asco- and basidiomycetous yeasts, the diazonium blue B (DBB) test was performed as described in WUCZKOWSKI et al. (2004). The strains were then conserved in the ACBR culture collection.

For DNA extraction, two loopfuls of cells were suspended in 500µl lysing buffer (50 mmol l^{-1} Tris, 250 mmol l^{-1} NaCl,

50 mmol l^{-1} EDTA, 0.3 % w/v SDS, pH 8) and the equivalent to a volume of 200 µl of 425–600 mm glass beads was added. The tubes were then vortexed for 3 min, incubated for 1 h at 65 °C and again vortexed for 3 min. After centrifugation for 10 min at full speed, the supernatant was transferred to a new tube. A dilution of 1:150 was used in the PCR reaction (GADANHO et al., 2003, with modifications).

The D1/D2 region of the 26S rDNA was sequenced, the procedure is described in WUCZKOWSKI et al. (2003).

Strains showing high similarity to known species (99 or 100 %) were compared with the appropriate type strain via PCR-fingerprinting. Reactions were performed as described in MESSNER et al. (1996). The following primers were used: M13 (5'-GAGGGTGGCGGTTCT-3'), GTG5 (5'-GTGGTGGTGGTGG-3') and V5 (5'-TGCCGAGCTG-3'), see PRILLINGER et al. (1999), SAMPAIO et al. (2001).

3 Results and discussion

In total 82 yeast strains were isolated, 40 from the alluvial forest at Müllerboden, 32 from the acidophilous-beech grove at Saubrunn and ten from the spruce-fir-beech grove at Rotwald (Table 1). At Müllerboden the largest number of isolates was obtained from litter (27), followed by soil at 0–5 cm (7) and soil at 10–15 respectively 30–35 cm depth (3 each). At Saubrunn the majority of the yeasts was isolated from soil at 0–5 cm depth (21), followed by litter (8), soil at 10–15 cm (2) and soil at 30–35 cm depth (1). At Rotwald yeast strains were only isolated from soil at 0–5 cm depth.

Partial sequence analysis of the D1/D2 region of the 26S rDNA section of the strains led to 25 different sequences, they could be allocated to eleven genera and one sequence

Table 1: Species distribution on the different sampling points, L … litter, 0 … 0–5 cm depth, 1 … 10–15 cm depth, 3 … 30–35 cm depth
Tabelle 1: Verteilung der Arten auf die verschiedenen Probenahmestellen, L … Streuschicht, 0 … 0–5 cm, 1 … 10–15 cm, 3 … 30–35 cm Tiefe

	Müllerboden				Saubrunn				Rotwald	
	L	0	1	3	L	0	1	3	L	0
Aureobasidium pullulans	1									
Cryptococcus carnescens	2									
gastricus					4	1				
laurentii	1									
magnus		2								
podzolicus						1	1	1		
terricola						15	1			10
victoriae	10	4		2						
Cystofilobasidium capitatum	4			1						
infirmo-miniatum		2								
Dioszegia crocea		1								
hungarica	1									
Leucosporidium fellii	2									
Rhodotorula bacarum	1									
sp.	1									
Sporobolomyces roseus	1									
salicinus	1									
Taphrina wiesneri	1									
Tremella moriformis	1									
Trichosporon porosum					4	2				
Williopsis saturnus			1							
unidentified						2				

Table 2: List of yeast species showing the results of sequencing (sequence identity), the results of PCR-fingerprinting, ACBR- and Nucleotide Sequence Database accession numbers

Tabelle 2: Liste der isolierten Hefearten mit den Resultaten der Sequenzierung, des PCR-fingerprinting, den ACBR- und Genbanknummern

Results of sequence comparison		Sequence homology	confirmed by PCR fingerprinting	Fig. no.	Strain	EMBL No.
Genus	species					
Aureobasidium	*pullulans*	100	+	2	HB1133	AM040215
Cryptococcus	*carnescens*	100			HB1134	AM039432
	gastricus	99	+	3	HB1099	AM039433
	laurentii	100	–		HB1122	AM039434
	magnus	100	–		HB1163	AM039666
	podzolicus	99			HB1104	AM039667
	terricola	100	+	4	HB1105	AM039670
	victoriae	99			HB1144	AM039668
	victoriae	100			HB1155	AM039669
	victoriae	100			HB1147	AM039671
	victoriae	100			HB1160	AM039672
Cystofilobasidium	*capitatum*	100	+	5	HB1123	AM039673
	infirmo-miniatum	99			HB1156	AM039674
Dioszegia	*crocea*	99	–		HB1157	AM039675
	hungarica	99	+	6	HB1137	AM039676
Leucosporidium	*fellii*	99	–		HB1121	AM039677
Rhodotorula	*bacarum*	100	–		HB1141	AM039678
	sp.	96			HB1139	AM039679
Sporobolomyces	*roseus*	100	–		HB1153	AM039680
	salicinus	99	+	7	HB1140	AM039681
Taphrina	*wiesneri*	99	–		HA1613	AM039682
Tremella	*moriformis*	99			HB1142	AM039683
Trichosporon	*porosum*	100			HB1168	AM039684
Williopsis	*saturnus*	99	–		HA1617	AM039685
unidentified					HA1600	AM039686

for which it was not possible to determine the genus membership (Table 2). By comparison with their respective type strains via PCR-fingerprinting 39 strains belonging to eight species could be identified, which based on the total number of strains are approximately 49 %.

The alluvial forest at Müllerboden was the sampling site showing the highest diversity. The isolated strains could be assigned to 17 different species of ten genera. In the acidophilous-beech grove at Saubrunn members belonging to four species of two genera were found. At the spruce-fir-beech grove Rotwald only one species was isolated. As above mentioned, at the latter location samples were taken only from the upper soil core (0–5 cm). In comparison with the other sites it is surprising that at Saubrunn (pH 3,2) the highest number of species was isolated from this core, whereas in the Rotwald (pH 5,3) forest only one species was found.

The majority (55) of the isolated strains was assigned to seven species of the genus *Cryptococcus*. Within this genus, *C. terricola* was the most frequently isolated species (26) and the

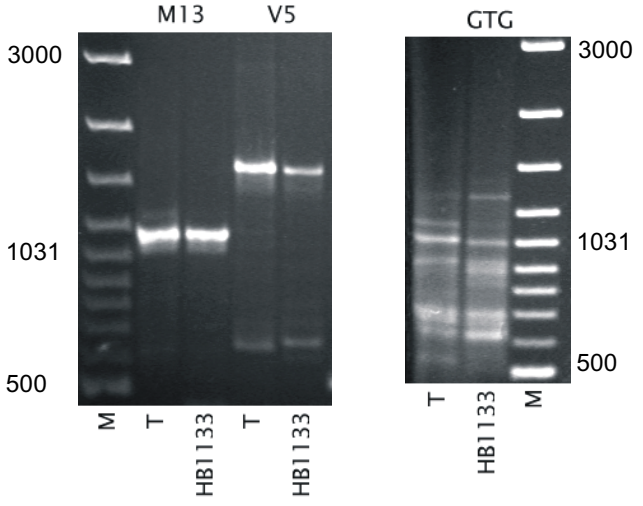

Figure 2: PCR-fingerprint of *Aureobasidium pullulans*. T … type strain *(A. pullulans var. pullulans)*, M … length marker

Abbildung 2: PCR-fingerprint von *Aureobasidium pullulans*. T … Typstamm *(A. pullulans var. pullulans)*, M … Längenstandard

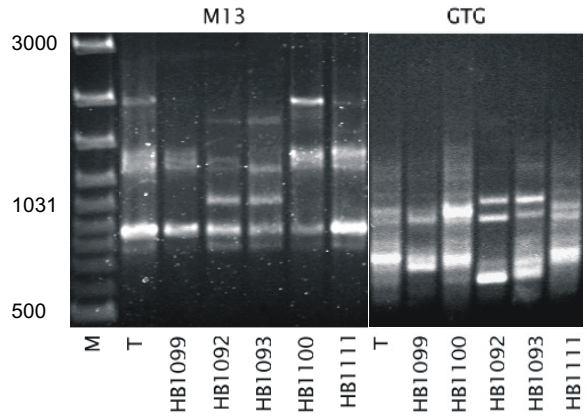

Figure 3: PCR-fingerprint of *Cryptococcus gastricus,* T … type strain, M … length marker.
Abbildung 3: PCR-fingerprint von *Cryptococcus gastricus,* T … Typstamm, M … Längenstandard.

only species that was found at two sampling sites (Saubrunn and Rotwald). Furthermore it was the only yeast species that was isolated at the spruce-fir-beech grove at all. Sequence analyses of strains of both sampling sites led to identical partial sequences. Also PCR-fingerprinting showed a high homology of all isolates with their type strain (see Figure 4).

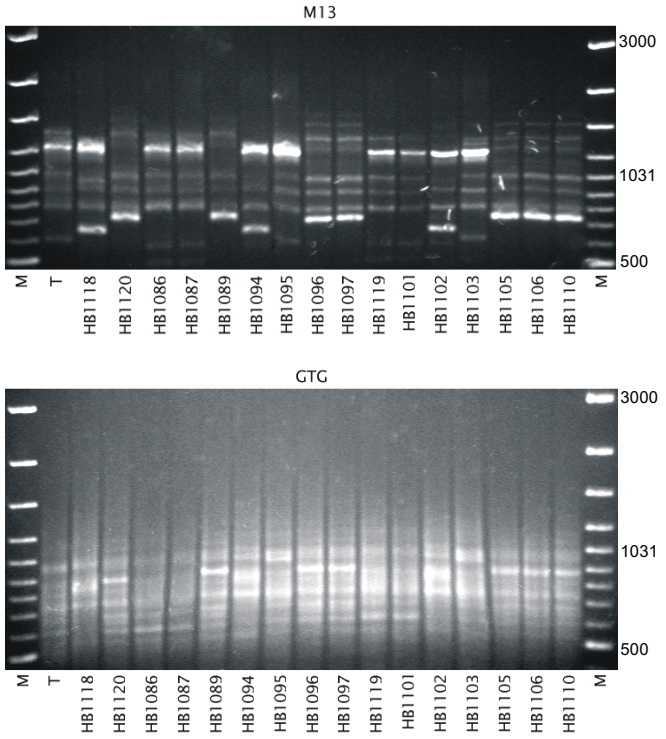

Figure 4: PCR-fingerprint of *Cryptococcus terricola* (Isolates from Saubrunn), T … type strain, M … length marker.
Abbildung 4: PCR-fingerprint von *Cryptococcus terricola* (Isolate aus Saubrunn), T … Typstamm, M … Längenstandard.

Sixteen strains belong to the *Cryptococcus victoriae* group, including four different sequence types. Seven of these strains (HB1135, HB1138, HB1143, HB1144, HB1146, HB1162, HA1616) showed 99 % partial-sequence homology with an isolate (HB1052) obtained from an alluvial forest at Mannswörth, Lower Austria. Five isolates (HB1151, HB1155, HB1159, HB1165, HA1618) showed 100 % partial sequence homology with an isolate (HB1042) obtained from an alluvial forest at Gr. Enzersdorf, Lower Austria (for both isolates, see WUCZKOWSKI M., 2004).

C. gastricus (5 strains) and *C. podzolicus* (3 strains) were exclusively isolated from the acidophilous-beech forest, whereas *C. victoriae* (16 strains), *C. magnus* (2 strains), *C. carnescens* (2 strains) and *C. laurentii* (1 isolate) were only found in the alluvial forest at Müllerboden (Table 1).

By PCR-fingerprinting three of five *C. gastricus* strains (HB1099, HB1100, HB1111; Figure 3) were acknowledged whereas *C. laurentii*, *C. magnus* and *C. victoriae* could not be confirmed.

Five strains of *Cystofilobasidium capitatum* and two of *Cystofilobasidium infirmo-miniatum* were isolated at the alluvial forest. The PCR-fingerprints of all *C. capitatum* strains showed a high similarity with their type strain (Figure 5).

Six isolates were identified as *Trichosporon porosum* by partial sequence analysis but not confirmed by PCR-fingerprinting. They were isolated from litter and soil at 0–5 cm depth at Saubrunn.

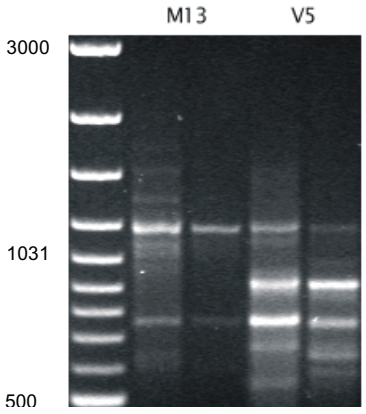

Figure 5: PCR-fingerprint of *Cystofilobasidium capitatum,* T … type strain, M … length marker.
Abbildung 5: PCR-fingerprint von *Cystofilobasidium capitatum,* T … Typstamm, M … Längenstandard.

Two strains, isolated from litter samples taken at the alluvial forest, were identified as *Leucosporidium fellii*. The comparison with their respective type strain could not confirm the results of the partial sequence analysis.

Two isolates which are identical in their D1/D2 partial sequences did not show significant homology to known species, it was not even possible to identify the genus.

One isolate showed 96 % homology with members of the genus *Rhodotorula*, it was therefore classified as *Rhodotorula sp*.

All other strains were isolated only once (Table 2). The results of the partial sequence comparison could be confirmed by PCR-fingerprinting for *Aureobasidium pullulans* (Figure 2), *Dioszegia hungarica* (Figure 6) and *Sporobolomyces salicinus* (Figure 7). For *Dioszegia crocea, Rhodotorula bacarum*, *Sporobolomyces roseus, Taphrina wiesneri* and *Williopsis saturnus* the identities obtained by partial sequence analyses could not be confirmed by PCR-fingerprinting.

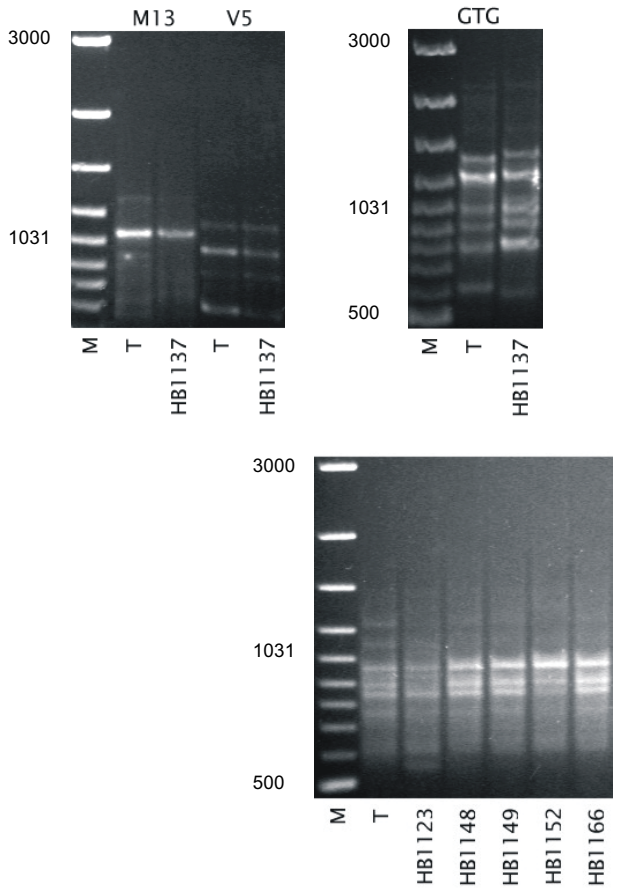

Figure 6: PCR-fingerprint of *Dioszegia hungarica*, T … type strain, M … length marker

Abbildung 6: PCR-fingerprint von *Dioszegia hungarica,* T … Typstamm, M … Längenstandard

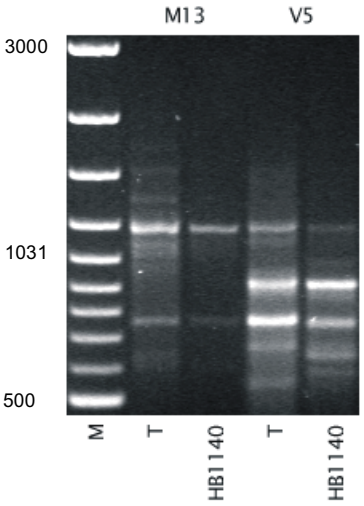

Figure 7: PCR-fingerprint of *Sporobolomyces salicinus*, T … type strain, M … length marker

Abbildung 7: PCR-fingerprint von *Sporobolomyces salicinus*, T … Typstamm, M … Längenstandard

The majority of the yeast strains isolated from litter and soil were Basidiomycetes which confirms results of former studies (Slávikovᡠand Vadkertiovᡠ2000, Wuczkowski and Prillinger, 2004). Thereof, members of the genus *Cryptococcus* are commonly found in soil because, as they belong to the group of encapsulated yeasts, they are well prepared to survive under unfavourable conditions (Slávikovᡠand Vadkertiovᡠ2000; Spencer and Spencer, 1997). The highest biodiversity was by far observed in the alluvial forest at Müllerboden where also the largest number of yeast isolates was obtained. The acidophilous-beech grove at Saubrunn and the spruce-fir-beech grove at Rotwald followed second and third in this respect.

It is difficult to compare the results of this study with earlier studies dealing with yeast biodiversity in nature due to the fact that most of them were carried out by using classical methods like physiology which do not provide reliable identification. In comparison with a publication appeared recently and based also on molecular identification (Wuczkowski and Prillinger, 2004), some interesting findings emerged, assisting the results regarding the yeast flora within the different forest types. Within this publication, yeasts from soils of an alluvial forest in Austria were investigated, which is a similar habitat to the Müllerboden site. Nearly half of the yeasts which were isolated at Müllerboden occurred also in the aforementioned alluvial forest, but almost all coinciding species did not occur in the other forest types of this study (Saubrunn and Rotwald). *Cryptococcus terricola* was isolated only at Saubrunn and Rotwald,

this is also in accordance with the results of the above mentioned work, where this species was found only once. *Cryptococcus victoriae*, a heterogeneous species (HERZBERG et al., 2002; SCORZETTI et al., 2002) was very frequently isolated and seems to be common in forest soil, especially in alluvial forests.

The results of this study also show that a lot of yeasts isolated from soil are still unknown and need to be described.

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