Master Thesis

Characterization of cryptogam-associated methylotrophic bacteria with focus on the genus *Methylobacterium*

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Abstract

Methylotrophic bacteria inhabit the surfaces of almost all known plants and, in particular, the genus *Methylobacterium* has been shown to have beneficial symbiotic effects. They are able to degrade toxic compounds and to produce phytohormones, such as auxines or cytokines which have a positive effect on plant growth and germination. In contrast, little is known about the occurrence of methylobacteria in bryophytes. We analyzed methylotrophic bacteria associated with two cosmopolitan peat mosses, *Sphagnum magellanicum* and *Sphagnum fallax*, and the lichen *Lobaria pulmonaria* by a multiphasic approach. Our aim was to study the effect of host species on the diversity and structure of associated methylotrophs. 16S rRNA genes of methylotrophic bacteria were amplified by nested-PCR from the metagenomic DNA by using specific primers and separated by single strand conformation polymorphism (SSCP). The results showed a clear discrimination between the three hosts. Interestingly, the fingerprints of *S. magellanicum* were more similar to those of the lichen *L. pulmonaria* than to those of *S. fallax*. Moreover, the results revealed that communities of methylotrophic bacteria are highly conserved within each investigated *Sphagnum* species and more variable in the lichen *L. pulmonaria*. Isolation of methylobacteria on selective medium yielded 186 pure cultures, differentiated from the other microorganisms due to their typical pink pigmentation. Internal transcribed spacer (ITS) analysis of the isolates led to the identification of 42 operational taxonomic units (OTUs), each including from one to 26 isolates. Sequencing of each OTU group resulted in three distinctive *Methylobacterium* species. Remarkably, only a small number of strains of *Methylobacterium* from *Sphagnum magellanicum* could be isolated, rising the hypothesis that *Methylobacterium* spp. are not part of the natural microbial flora of *S. magellanicum*. The abundance of methylotrophic bacteria in bryophytes and lichens strongly suggests that cryptogams represent an important habitat for *Methylobacterium* and other methylotrophs. The ecology of these symbiotic bacteria and their interactions with the host need to be further investigated to assess the effects and the roles played in the peat bogs and in the lichen symbioses.

Kurzfassung

Bei methylotrophen Bakterien, im Speziellen bei Spezies der Gattung *Methylobacterium,* handelt es sich um ubiquitär vorkommende, Gram-negative, aerobe Stäbchen, die eine Vielzahl von unterschiedlichen Pflanzen besiedeln. Sie zeichnen sich durch symbiontische Wechselwirkungen mit Pflanzen aus, wie zum Beispiel dem Abbau von toxischen Verbindungen oder der Produktion von unterschiedlichen Pflanzenwachstumshormonen wie Zytokinen und Auxinen. Im Fokus der Untersuchungen standen methylotrophe Bakterien der Gattung *Methylobacterium* assoziiert mit den zwei Torfmoosen *Sphagnum fallax* und *Sphagnum magellanicum* sowie mit der Flechte *Lobaria pulmonaria*. Torfmoose und Flechten stellen sich nicht zuletzt aufgrund ihrer extremen Habitate als sehr interessante Modell-Organismen dar. Ein multimethodischer Ansatz wurde gewählt, wobei kultivierungsabhängige, kultivierungsunabhängige als auch mikroskopische Arbeitstechniken zum Einsatz kamen. Das Ziel der Studie war es, die Effekte der Wirtspezies auf die Diversität und Struktur der assoziierten methylotrophen Bakterien zu untersuchen. 16S rRNA Gene der methylotrophen Bakterien wurden mithilfe spezifischer Primer aus dem metagenomischen Template amplifiziert und via Single Strand Conformation Polymorphism (SSCP) in ihre distinktiven Banden aufgetrennt. Es konnte hierbei eine klare Differenzierung zwischen den bakteriellen Populationen der unterschiedlichen Wirte gezeigt werden. Interessanterweise wurde eine höhere Ähnlichkeit der Fingerprints zwischen *L. pulmonaria* und *S. magellanicum* nachgewiesen, als zwischen den Moosen *S. fallax* und *S. magellanicum*. Des Weiteren konnte gezeigt werden, dass die methylotrophen Bakteriengemeinschaften an den Torfmoosen hoch konserviert waren. Eine höhere Variabilität wurde bei der Flechte nachgewiesen. Im Zuge der kultivierungsabhängigen Methoden wurden 186 Reinkulturen aus den drei unterschiedlichen Wirtsspezies von je vier unterschiedlichen Standorten isoliert. Die Selektion erfolgte über die typische pinke Pigmentierung der Methylobakterien. Bemerkenswert war hierbei, dass im Gegensatz zu *L. pulmonaria* und *S. fallax*, bei *S. magellanicum* kaum potentielle Kolonien gefunden wurden. Mithilfe der Ribosomal Internal Spacer Analysis (RISA) Technik wurden die Reinkulturen in einzelne Operational Taxonomic Units (OTUs) gruppiert.

Dies resultierte in 42 OTUs. Durch Sequenzierung dieser OTUs konnten drei unterschiedliche Spezies der Gattung *Methylobacterium* detektiert werden. Die Relevanz von Kryptogamen als Habitate von methylotrophen Bakterien konnte durch eine hohe Abundanz und Speziesspezifität, an Flechten und Torfmoosen gezeigt werden. Um den Einfluss auf Torfmoore oder die Flechten-Symbiose besser zu verstehen, müssen die Interaktionen zwischen den Kryptogamen und methylotrophen Bakterien weitergehend untersucht werden.

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I - INTRODUCTION

1.1 Methylotrophic bacteria and the genus *Methylobacterium*

Members of the genus *Methylobacterium* are also known as pink pigmented, facultative methylotrophic bacteria (PPFMs) due their typical pink reddish pigmentation (Fig. 1). PPFMs are rod shaped bacteria, occasionally branched and/or exhibiting polar growth. They are Gram-negative to Gram variable with a Gram-negative cell wall type (Green and Bousfield, 1982). *Methylobacterium* spp. are motile by means of a single polar, subpolar, or lateral flagellum, strict aerobic, mesophilic and chemoorganotrophic. PPFMs are highly interesting members of the order Rhizobiales and they can utilize methanol emitted by the stomata of plants, methylamine and as well C2, C3, and C4 compounds to grow (Green and Bousfield, 1983; Lidstrom and Christoserdova, 2002). In methylotrophic bacteria the enzyme methanol dehydrogenase (EC 1.1.99.8) oxidizes methanol to formaldehyde and makes it accessible as a carbon source (Anthony and Zatman, 1964; Wolf and Hanson, 1978; Machlin and Hanson, 1988; Anthony and Williams, 2003). It has been shown that strains of the genus *Methylobacterium* can form bacteriochlorophyll α under specific culture conditions, suggesting a common link to phototrophs in their ancestry (Sato, 1978). Some species like *Methylobacterium nodulans* are even able to nodulate plants and fix atmospheric nitrogen (Jourand et al., 2004). *Methylobacterium* spp. are slow growing bacteria with a high level of resistance against harsh conditions. Different strains are suggested to be part of the natural human foot flora, others have even been suggested to increase aroma compounds in strawberries or are responsible for nosocomial infections in intensive care units. (Zabetakis and Gramshaw, 1996; Zabetakis, 1997; Lidstrom and Christoserdova, 2002; Verginer et al., 2010; Lai et al., 2011). Furthermore, it was shown that some strains can activate defense response of plant antimicrobial

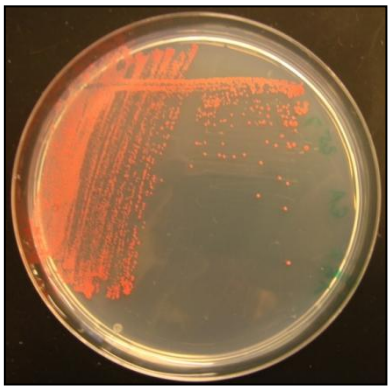

Fig. 1 Pink reddish colonies formed by *Methylobacterium* sp.

compounds (Sung et al., 2006), but in contrast, nothing is known about the occurrence and interactions of methylobacteria in the lichen symbiosis.

Previous studies on PPFMs showed that some isolates of *M. adhaesivum, M. mesophilicum* and *M. extorquens* are capable to produce *N*-Acyl homoserine lactones (AHL) (Erlacher, Cardinale and Berg, unpublished). In contrast, none of these isolates was capable to convert insoluble phosphates into soluble forms, secrete proteases or showed antifungal antagonistic activity.

1.1.1 Plant colonization by pink-pigmented facultative methylotrophic bacteria

Plant associated bacteria and, in particular, the plant epiphytes of the genus *Methylobacterium* fulfill important functions on their host. Besides the degradation of toxic compounds and the production of vitamines and phytohormones like auxines and cytokinines which have a positive effect on plant growth and germination (Ivanova et el., 2000; Delmotte et al., 2009), they are also interfere with plant metabolism. In addition, they influence fruit flavor and quality (Ivanova et al., 2000; Berg, 2009; Lugtenberg and Kamilova, 2009). Methylotrophic bacteria inhabit the surfaces of almost all known plants. Phyllosphere colonization by bacteria is an important factor for establishing plant-microbe interactions and it is suggested that *Methylobacterium* colonization of plant phyllospheres occurs via air transfer of soil particles (Whipps et al., 2008). Hornschuh et al. (2002, 2006) showed that co-cultivation of the bryophyte *Funaria hygrometrica* with methylobacteria promote the bud development as well as the total plant growth. The authors could show that this positive effect of *Methylobacterium* was due the production of auxines (Ivanova et al., 2001). The enzyme 1-Aminocyclopropane-1-carbonacid (ACC)-deaminase (EC 3.5.99.7) is produced by many PPFMs and lowers the endogenous ethylene production of plants (Idris et al., 2004, Madhaiyan et al., 2006). Some plant-associated PPFMs are also involved in the nitrogen metabolism due the production of the enzyme urease (EC 3.5.1.5) (Holland and Polacco, 1992). The methanol utilization mentioned above varies over the plant development stages. Highest methanol emission was detected on young, fast growing plants.

The methanol emission decreases with increasing ageing of the leaves. This reflects the population density of PPFMs on the phyllosphere (Hüve et al., 2007). The mentioned benefits to their host plant and the linked selective advantages, gives rise to the idea to name genus *Methylobacterium* as phytosymbiont (Hornschuh et al., 2002, Kutschera 2007).

1.2 Bryophytes of the genus *Sphagnum* and the lichen *Lobaria pulmonaria*

Lichens and bryophytes are highly interesting organisms. Bryophytes are considered as the oldest extant terrestrial plants. On the other hand, lichens are symbiotic composite organisms composed of a fungus (the mycobiont) and a photosynthetic partner (the photobiont or phycobiont), usually either a green algae or cyanobacteria or both (Rydin et al., 2006). In most cases, these symbiotic partners depend on each other and exchange specific substances. Recently Grube et al., (2009) expanded the traditional concept of the lichen symbiosis and showed that multiple bacterial partners need to be considered. So far, more than 25000 lichen species were already described (Campbell and Reece, 2006). Most lichens are able to resist extreme environmental conditions, but they are highly sensible to air pollution and they are also negatively affected by habitat loss and changes in forestry practices. In particular, the large epiphytic lichen *Lobaria pulmonaria* which consists of an ascomycete fungus, a green algal partner and a cyanobacterium living in symbiosis is described as biological indicator to air pollution (Fig. 2) (Gilbert, 1986). *L. pulmonaria* (like many others) has suffered a substantial decline in Europe during the 20th century (Wirth, 1976; Scheidegger et al., 2002).

Fig. 2 *Lobaria pulmonaria* in the "Nationalpark Gesäuse", Styria, Austria

Mosses of the genus *Sphagnum* are widely distributed and common. *Sphagnum* appears in wetlands where it accumulates high amounts of non-degraded organic matter (“peat”), the reason why they are also named peat moss. Peat bogs are wetlands where the genus *Sphagnum* is dominant. Peat bogs appear mainly on the

northern hemisphere and cover more than 3% of the land surface on earth (Fig. 5). This equals an estimated area of ca. 400 million ha what makes *Sphagnum* to one of most abundant plants of our planet (Strack, 2008). This long-existing ecosystem has been used as indicator for climate change. Peat bogs are highly sensitive to variation of a-biotic factors as a result of global climate change (Dise, 2009). Agriculture and related use of fertilizers lead to a shift in the carbon storage balance of peatlands and an elevated emission of greenhouse gases (Strack, 2008). Sphagnum exchanges protons to mineral cations for its own nutrient uptake, what results in acidification of its habitat. This leads to an inhibition of bacterial activity. Additionally, low temperatures and limited nutrients common for peat bogs contribute to inhibited bacterial growth (Campbell and Reece, 2006).

Fig. 3 *Sphagnum fallax* lawn from "Pürgschachen Moor", a peat bog in Styria, Austria

In the past, *Sphagnum* was used as diaper by Inuits and Indians and during the First World War it was used for antiseptic wound bandages. Nowadays, bog is used to increase the soil quality and as packing for plant transport. This usefulness comes, beside their antimicrobial effect, from their huge, dead, water storing cells (hyaline cells). These cells are responsible for a 20 fold uptake of water of its own weight (Campbell and Reece, 2006).

Fig. 4 *Sphagnum magellanicum* bulk from "Pürgschachen Moor", a peat bog in Styria, Austria

Besides the differences in color pigmentation and morphology of *S. fallax* and *S. magellanicum*, an interesting differentiating point accounts to their optimal pH for growth (Fig. 3 and 4). The optimal pH of *S. magellanicum* lies in very acidic areas (2.5 – 4), whereas the optimal pH ranges up to pH 6 for *S. fallax*. (Laine et al., 2009)

Fig. 5 "Pürgschachen Moor" Styria, Austria

1.3 Cultivation-dependent and -independent methods in microbial ecology

Much is known about the extraordinary sheer diversity of living creatures populating our planet. Especially vertebrates and plants are well studied up to day. Although there are millions of species of bacteria and despite arising progresses in the development of novel, high throughput techniques, the biodiversity of prokaryotes remains hidden and under-explored. More than a million species are expected within the kingdom Prokarya, but only about 10000 species are catalogued at present. The reason is that only a little fraction of natural occurring bacteria can be cultivated (Schleifer, 2004; Mora, 2011). Relief to this problem can be found using cultivation independent methods. Genetic fingerprints and *in situ* visualization techniques allows detection of non-cultivable bacteria and gives information about their structure and ecological functions. Contrariwise, enzymatic activity, experiments for optimization like protein engineering and the investigation of relevant functions can be obtained by cultivation-dependent methods.

Single strand conformation polymorphism (SSCP) was originally developed to detect gene polymorphism in human DNA (Orita et al., 1989) or genetical mutations (Hayashi, 1991). Later on, Schwieger and Tebbe (1998) found out that this fingerprinting method could also be used for the cultivation independent investigation of microbial communities in environmental samples with the advantage of detecting difficult- or non-cultivable organisms.

SSCP is a molecular method to analyze the diversity of microbial communities. DNA-fragments of similar length but with different sequence are amplified by polymerase chain reaction (PCR) (Rochelle, 2001) and digested with lambda exonuclease to obtain single stranded DNA molecules. The single strands become denaturized and develop their specific secondary structure due to intramolecular base pairing. Bands show different tracking on native polyacrylamide gel electrophoresis because of their specific conformation. Silver staining is used to visualize the different DNA-fragments (Bassam et al., 1991). With SSCP analysis, discrimination on the genus or even on species level is possible and this technique has a wide range of different specific targets. Primers developed for specific organism groups can be used to obtain specific analysis. The detection limit was proved to be 1.5 % of the total community DNA. Besides the information about the structure of bacterial communities, SSCP allows additional excision and sequencing of distinct bands for taxonomic identification and phylogenetic analysis (Tebbe et al., 2001).

Ribosomal RNA (rRNA) intergenic spacer analysis (RISA) is a fingerprinting method for discriminating genetically different bacterial isolates. RISA involves PCR amplification of the internal transcribed spacer (ITS) regions of microorganisms comprised between the small (16S) and the large (23S) subunit of the ribosomal operon. While the majority of the rRNA operon serves a structural function, portions of the 16S-23S intergenic region can encode tRNAs depending on the bacterial species. Oligonucleotide primers targeted to conserved regions within the 16S and 23S genes are used to generate ITS amplicons (Fig. 6). The taxonomic value of RISA lies in the heterogeneity in both nucleotide sequence and fragment length (Cardinale et al., 2004).

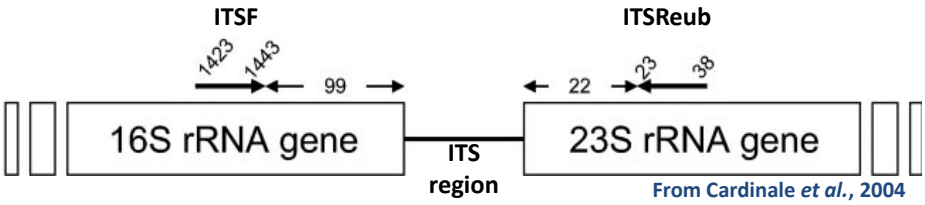

FIG. 6 Scheme showing the region between the 16S and 23S rRNA genes to be amplified for RISA analysis and sites for primer binding

1.4 Visualization of methylotrophic bacteria *in situ* using a combination of fluorescence *in situ* hybridization and confocal laser scanning microscopy

Fluorescence *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy is a powerful combined tool to detect microorganisms *in situ*. Fluorescent labeled rRNA-targeted oligonucleotide probes are used to investigate the composition and localization of microbial communities directly in their natural habitats (Amann et al., 2008). A workflow overview can be seen in Fig. 7. Originally, the FISH technique was applied to study marine environments related microbial communities. Other fields of applications are in the wastewater treatment, symbiotic organisms or the investigation of microbial communities on plants (Amann et al, 2001).

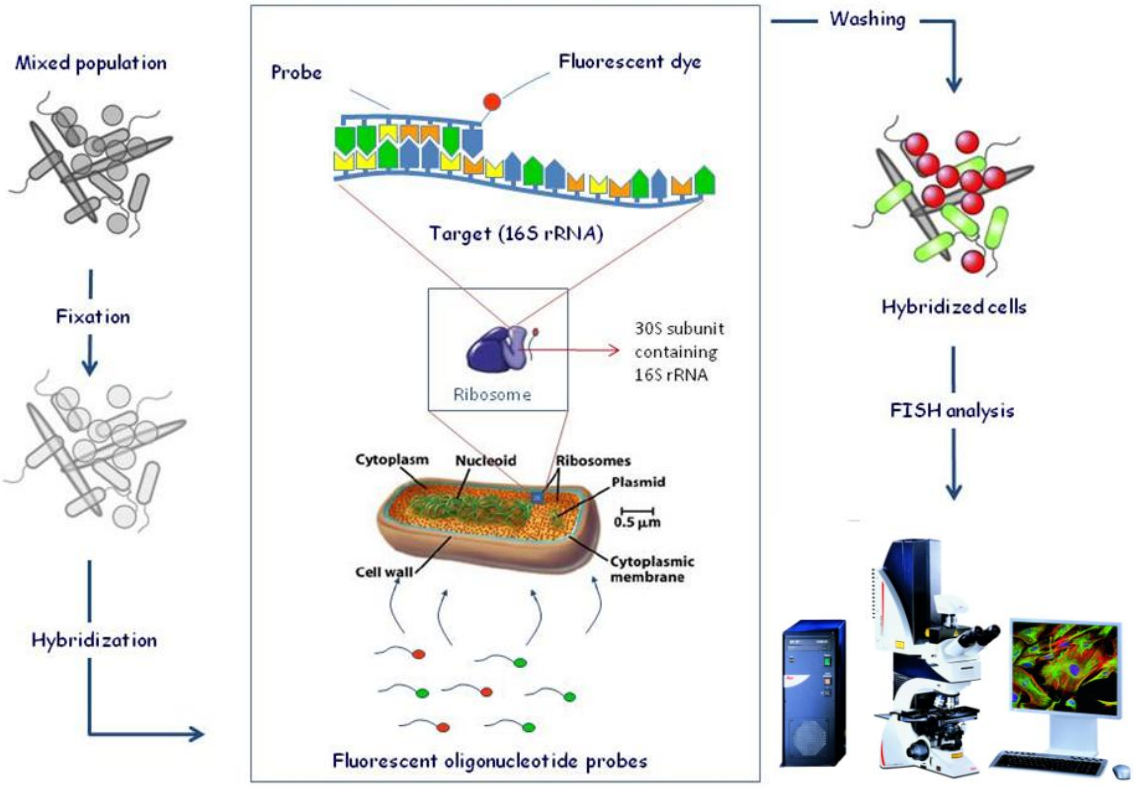

Fig. 7 Process of FISH using rRNA-targeted probes (adapted from http://www.biovisible.com/FISHex.jpg (20.01.2012); http://www.leica-microsystems.com/typo3temp/pics/TCS_SPE_1_54c882c596.jpg (15.12.2011))

1.5 Objectives of the study

A lot of research is done on *Methylobacterium* on higher plants but in contrast little is known about their role on cryptogams like bryophytes and lichens. Therefore, methylotrophic bacteria associated with two cosmopolitan peat mosses, *Sphagnum magellanicum* and *Sphagnum fallax*, and the lichen *Lobaria pulmonaria* were analyzed by a multiphasic approach. Aim was to study the effect of host species on the diversity, structure and colonization pattern of associated methylotrophs and the investigation of further possible biotechnological potentials of isolated *Methylobacterium* strains.

II - MATERIALS AND METHODS

2.1 Sampling

Samples of the peat mosses *Sphagnum magellanicum* and *Sphagnum fallax* were collected in the Pürgschachen peat bog (Ardning, Styria, Austria, GPS N47.592215,E14.364796). *Lobaria pulmonaria* samples were taken from the woods in Nationalpark Gesäuse near Admont (Gstatterboden, Styria, Austria, GPS N47.579131,E14.613533). Both samplings took place on November 16th 2011. For each species, four independent replicates consisting of approximately 30 gametophytes plantlets or five to 10 lichen thalli, respectively, were collected and stored in sterile plastic-bags on ice. The samples were processed and treated according Opelt & Berg, 2004 (see below).

2.2 Cultivation-dependent investigation

2.2.1 Isolation of moss-associated *Metyhlobacterium* spp. on selective medium

Cultivation of *Methylobacterium* sp. was achieved with imprinting sample-tissues on selective agarose methanol inorganic salt medium (MIS). This agar impression method as described by Corpe (1985) was used in order to reveal any presence of PPFMs. For each sample we repeated the process on four MIS-plates using different growth parameters: 2x MIS, 30°C; 1x MIS, 20 µg/mL cycloheximide added (Green and Bousfield, 1983), 30°C; 1x MIS, RT. These 48 inoculated plates were incubated over 9 days. From every plate with distinctive colony growth, five single colonies were transferred on fresh plates to obtain pure cultures. The pink-pigmented colonies were repeatedly transferred with sterile toothpicks onto fresh selective MIS-plates until only one type of colony remained.

2.2.2 Cryo-conservation of the isolates for long-time storage

For each of the 186 isolates we prepared two cryo-stocks in 20% glycerol. 700 µL MIS media in 1.5 mL Eppendorf tubes were inoculated with a single colony from every obtained pure culture and incubated at 30 °C, 200 rpm, for 7 days. After incubation, additional 700 µL of 40% sterile glycerol were added. These 20% glycerol stock suspensions were transferred into liquid nitrogen and placed at -70 °C for long-time storage.

2.2.3 Ribosomal internal spacer analysis (RISA) to group isolates into operational taxonomic units (OTUs)

DNA for internal transcribed spacer (ITS) PCR was gathered by breaking the cell walls following the Quick & Dirty method described by Nishio et al., 1997. *Metyhlobacterium* colonies were transferred into 150 µL $H_2O_{ultra\ pure}$ with an inoculating loop. After incubating at 96 °C for 10 minutes and centrifugation ($13x10^3$ rpm, 4 °C, 10 min) the supernatant was used directly for the ITS-PCR.

Table 1 Primer used for ITS analysis (Sigma-Aldrich, Vienna, Austria; Biomers.net; Wiener Neudorf, Austria)

Primer	Sequence	Target	Reference
ITS F	5´-GTCGTAACAAGGTAGCCGTA-3´	Bacteria	Cardinale et al., 2004
ITS R eub	5´-GCCAAGGCATCCACC-3´	Bacteria	Cardinale et al., 2004

Table 2 PCR components - RISA PCR

Taq-&GO^{TM} (QBiogen) [5x]	6 µL
Primer ITS F [10 µM]	1.2 µL
Primer ITS R eub [10 µM]	1.2 µL
$H_2O_{Ultra\ pure}$	19.1 µL
Template	2.5 µL
Total volume	30 µL

Table 3 Thermal cycling parameters -RISA PCR

	[T]	[t]	
Initial denaturation	94°C	5 min	
Denaturation	94°C	45 s	32 cycles
Annealing	55°C	1 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	7 min	
Pause	4°C	∞	

ITS fragments were separated on 1.5% agarose gel electrophoresis. To prepare a gel, the needed amount of agarose (Agarose NEED ultra Qualität, Rotigarose für DNA/RNA Elektrophorese), 1.5% (w/v) was added to the electrophoresis buffer (0.5x TBE). 0.5x TBE buffer was also used as running buffer. 1 kb DNA Ladder (Fermentas) was used as a reference to estimate the size of DNA molecules. After electrophoresis the gel was stained with ethidium bromide to visualize the DNA bands under ultraviolet light. The duration of each run was set to four hours.

GelComparII software (version 4.1; Applied Maths, Kortrijk, Belgium) was used for grouping the ITS profiles into OTU based on fingerprint similarity.

2.2.4 DNA isolation from Methylobacteria and preparation of the samples for 16S rRNA gene sequencing.

DNA Isolation was carried out using 0.40 mm - 0.60 mm glass pearls. Therefore, single colonies from the pure cultures and glass pearls were given into 2 mL screw tubes together with 200 µl $H_2O_{ultra\ pure}$. The bacterial cells were broken down using mechanical shear rates in a Ribolyzer (4 m/s, 20 s) and in following heatshock at 96°C for 10 min. After that, the cells were set immediately on ice for 5 minutes followed by a centrifugation step (13 x 10^3 rpm, 4 °C, 15 min). One µL of the supernatant was used for amplification of the 16S rDNA gene (Tab. 4 to 6). The PCR products were prepared for sequencing according the requirements. Sequencing was performed by ZMF - CENTER FOR MEDICAL RESEARCH, Graz, Austria.

Table 4 Primers suitable to generate 16S rRNA fragments for identification (Sigma-Aldrich, Vienna, Austria; Biomers.net; Wiener Neudorf, Austria)

Primer	Sequence	Target	Reference
27f	5´-AGAGTTTGATCCTGGCTCAG-3´	Bacteria	Lane et al., 1991
1492r	5´-TACGGYTACCTTGTTACGACTT-3´	Bacteria	Blackwood et al., 2005

Table 5 PCR components – Bacteria

Taq-&GO™ (QBiogen) [5x]	6 µL
Primer 27f [10 µM]	0.8 µL
Primer 1492r [10 µM]	0.8 µL
$H_2O_{Ultra\ pure}$	21.8 µL
Template	1 µL
Total volume	30 µL

Table 6 Thermal cycling parameters – Bacteria

	[T]	[t]	
Initial denaturation	95°C	5 min	
Denaturation	95°C	30 s	30 cycles
Annealing	55°C	30 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	5 min	
Pause	4°C	∞	

2.2.5 Screening for plant growth promotion (PGP) activity of *Methylobacterium adhaesivum* and *M. mesophilicum* strains on lettuce seedlings

Plant growth promotion (PGP) assay was done, according to Faltin *et al*. (2004) with modifications (Zachow et al., 2010). Surface-sterilised (1% NaOCl, 5 min) lettuce seeds (*Lactuca sativa*) cv. 'Neusiedler Gelber Winter' (Austrosaat, Vienna, Austria) were pre-germinated for two days in Petri dishes containing tab water solidified with 7 g/l plant agar (Duchefa Biochemie, Haarlem, The Netherlands) in moist chambers at 22°C for 2 days. Sterility of seeds was proved by imprinting onto nutrient agar. Standard 24-well microplates (Roth, Karlsruhe, Germany) were filled with 2 mL of plant agar. One pre-germinated seed was placed in each well followed by the addition of 10 µl of bacterial suspension (10^5 cells mL^{-1}). Cell count of grown bacterial suspensions was evaluated in a Thoma cell count chamber. Bacterial cells were obtained by harvesting cells of a culture grown in MIS broth (9 days, 30°C, 120 rpm) by centrifugation and re-suspension in physiological sodium chloride solution (0.85%). As control, lettuce seedlings were treated with 10 µl of a sterile 0.85% NaCl solution. Each strain was tested in 24 replicates. As negative control 72 replicates were utilized. After two weeks of incubation in a phytochamber (22°C, 16/8 h day/night, and artificial light) the assay were evaluated. For a comprehensive evaluation of the experiment, all the growth parameters were measured, including weight and length of the plantlets, roots and leaves. The data was statistically significantly enhanced using confidence interval at $p \leq 0.05$. The dry weights were determined after drying the plantlets at 110°C for 24 hours.

2.3 Cultivation independent approach

2.3.1 Cell-extraction of the moss- and lichen-associated bacterial fraction

For the cell-extraction procedure, 10 mL 0,85% NaCl were added to 5 g plant material of each sample replicate and well disrupted and homogenized using mortar and pestle. After centrifugation (20 min; 13000 rpm; 4 °C) the supernatants were discarded and the pellets were transferred into 2 mL Eppendorf tubes and stored at -70°C for further processing (Opelt & Berg 2004).

2.3.2 Total DNA extraction

Total DNA from the samples was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol and using a FastPrep Instrument (BIO101 Systems; Qbiogene, Carlsbad, CA, USA) where samples were homogenized twice for 30 seconds at 5.0 m/s (Opelt and Berg, 2004).

2.3.3 PCR amplification using methylotrophic bacteria-specific primers

The methylotrophic bacterial division was amplified from the metagenomic templates via polymerase chain reaction (Mullis et al., 1986; Saiki et al., 1988). Therefore, three different primer sets were evaluated, to achieve highest possible differentiation of the target species from the total bacterial fraction. Amplification conditions and reagents are displayed in Tables 7 and 8. The primers 2f and 3r were then further used in this study. (Nishio et al., 1997).

Table 7 Primers (Sigma-Aldrich, Vienna, Austria; Biomers.net; Wiener Neudorf, Austria) tested in this study

Primer	Sequence	Position [bp]	Tm	Reference
2f	5´-GATCGGCCCGCGTCTGATTAG-3´	Pos= 226-246	60.4°C	Nishio et al, 1997
3r	5´-GGCTTATCACCGGCAGTCTCC-3´	Pos= 1153-1173	57.4°C	Nishio et al, 1997
1319f	5´-ACTCGRGTGCATGAAGGCGG-3´	-	-	Knief et al., 2008
444lof	5´-CGGGACGATAATGACGGTACCGGDDGAA-3´	-	-	Knief et al., 2008
1003f	5´-GCGGCACCAACTGGGGCTGGT-3´		67°C	McDonald et al., 1997
1561r	5´-GGGCAGCATGAAGGGCTCCC-3´		62°C	McDonald et al., 1997

Table 8 PCR components using Primer 2f and 3r

Taq-&GO™ (QBiogen) [5x]	6 µL
Primer 2f [10 µM]	1.2 µL
Primer 3r [10 µM]	1.2 µL
$H_2O_{Ultra\ pure}$	19.1 µL
Total DNA (1:10)	2.5 µL
Total volume	30 µL

Table 9 Thermal cycling parameters of Primer 2f/3r

	[T]	[t]	
Initial denaturation	95°C	5 min	
Denaturation	95°C	20 s	32 cycles
Annealing	55°C	30 s	
Extension	72°C	60 s	
Final extension	72°C	10 min	
Pause	4°C	∞	

2.3.4 Separation of DNA fragments by agarose gel electrophoresis

To prepare a gel, the needed amount of agarose (Agarose NEED ultra Qualität, Rotigarose für DNA/RNA Elektrophorese), typically 0.8-1%, was added to the electrophoresis buffer (1 x TAE). 1 x TAE buffer was used as running buffer. 1 kb or 100 bp DNA Ladder (Fermentas, St. Leon-Rot, Germany) were used as a reference to estimate the size of DNA molecules. After electrophoresis the gel was stained with ethidium bromide to visualize the separation under ultraviolet light.

2.3.5 Microbial fingerprinting using single strand conformation polymorphism (SSCP)

SSCP analysis was used to analyze the amplicons of methylotrophic bacteria with regard to their diversity. Fingerprinting of the bacterial community was carried out as described by Schwieger and Tebbe (1998). Unibac II 515f and Unibac II 927r^{p} primers were used to produce 412 bp fragments with one phosphorylated strand (Tab. 10-12).

To purify the PCR products, Wizard SV Gel & PCR Clean – Up System (Promega) was used according to the manufacturer´s protocol. To each of the purified samples 2.4 µl lambda exonuclease (Lambda Exonuklease, 5 U/mL, Biolabs, New England) and 3.6 µl 10 x reaction buffer (Lambda Exonuklease Buffer 10 x, New England, Biolabs) were added. For denaturation and folding of the single strands, the loading buffer was prepared by mixing 950 µl formamide (95%), 4 µl 2.5 M NaOH (10 mM), 5 µl 5% bromophenol blue-solution (0.025%) and 41 µl deionized water. Thirty µl of loading buffer was added to the samples, heated for 2-5 min at 98°C and incubated on ice. Samples were loaded onto the gel or stored at -20°C.

Methylotrophic bacteria-specific PCR amplicons were separated on an 8% (w/v) acryl amide gel for 26 hours (Tab. 13). The TGGE Maxi system (Biometra, Göttingen, Germany) was used at 400 V and 26 °C. Silver-staining was performed according to Bassam et al. (1991) with modifications (Opelt and Berg, 2004; Opelt et al., 2007b).

GelComparII software (version 4.1; Applied Maths, Kortrijk, Belgium) was used for evaluation of bacterial community profiles obtained by SSCP as shown in Opelt et al. (2007b).

Table 10 Primers for SSCP-analysis (Sigma-Aldrich, Vienna, Austria; Biomers.net; Wiener Neudorf, Austria)

Primer	Sequence	Target	Reference
Unibac-II-515f	5´-GTGCCAGCAGCCGC-3´	Bacteria	Lieber et al, 2002
Unibac-II-927r^{P}	5´-CCCGTCAATTYMTTTGAG-3´	Bacteria	Lieber et al, 2002

Table 11 PCR components – UnibacII

Taq-&GO^{TM} (QBiogen) [5x]	12 µL
Primer Unibac-II-515f [5 µM]	2.4 µL
Primer Unibac-II-927r^{P} [5 µM]	2.4 µL
$H_2O_{Ultra\ pure}$	38.2 µL
2f/3r PCR product [diluted 1:100]	5 µL
Total volume	60 µL

Table 12 Thermal cycling parameters – UnibacII

	[T]	[t]	
Initial denaturation	95°C	5 min	
Denaturation	95°C	20 s	32 cycles
Annealing	55°C	15 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	
Pause	4°C	∞	

Table 13 Polyacrylamid gel components for SSCP analysis

Polyacrylamide gel ingredients	8%
Deionized H2O	23.1 mL
TBE puffer [5x]	10.4 mL
MDE solution [2x]	17.1 mL
TEMED	23 µL
APS [10%]	230 µL

2.3.6 Identification of the SSCP excised bands

Besides the determination of the total SSCP profile, obtained SSCP bands were excised and prepared for further sequencing using the Crush & Soak protocol (Sambrook et al., 1989) . Therefore, a sterile razorblade was used to excise the bands and the gel slabs were placed in tubes filled with 150 µL Crush & Soak buffer (Tab. 14) and stored/incubated at 4°C for 5 days. 428.6 µL EtOH 70% was added to each supernatant after centrifugation at $13x10^3$ rpm, 4°C, 10 min and precipitated over night at -20°C. After centrifugation ($13x10^3$ rpm, 4°C, 20 min) supernatants were discarded and the precipitated ssDNA fragments were resuspended in 50 µL 10 mM Tris-HCl (pH 8) buffer. After an additional UnibacII-PCR, samples were set for Sanger sequencing.

Table 14 Crush & Soak buffer recipe

Sodium acetate	300 mM
EDTA (pH 8.0)	1 mM
SDS (optional)	0.1%

2.4 Fluorescence *in situ* hybridization combined with confocal laser scanning microscopy (FISH-CLSM)

2.4.1 Sample Fixation for FISH

The following procedure (Cardinale et al. 2008) was performed to fix the samples. Within 3 h after collection, samples were washed with 1% phosphate-buffered saline (PBS) and incubated in 3:1 4% paraformaldehyde/PBS for 6 h at -4°C. After four washing steps in ice-cold PBS the samples were covered with 1:1 PBS/96% ethanol and stored at -20°C.

2.4.2 FISH probes and fluorochromes

Detailed information about the probes used for FISH and their optimal stringency conditions are provided in Table 19. Cy3- and Cy5-labelled probes were applied sequentially according their different formamide concentrations. Multiple probes were simultaneously observed, taking advantage of the non-overlapping emission wavelengths of the fluorochromes (max. excitation/emission: Cy3 548/562 nm and Cy5 650/670 nm).

2.4.3 FISH procedure

FISH of the *Sphagnum* samples was performed according the FISH protocol for lichen-associated bacteria (Cardinale et al., 2008). RHIZ1244 and 3R-FISH probes covalently bound at the 5´ end to a Cy5 dye were utilized in two separate experiments in order to detect methylotrophic bacteria. A Cy3-labeled EUBmix probe was used to stain all bacteria. NonEUB probes labeled to fluorochromes analogous to the positive probes were used as negative controls.

The fixed *Sphagnum* samples were separated into small sections using a sterile razorblade and placed in 1.5 mL microcentrifuge tubes. After rinsing the sections with PBS, 200 µL of 1 mg/mL Lysozyme (Sigma-Aldrich) was added and incubated at RT for 10 minutes in order to increase the permeability of the bacterial cell wall. Sections were rinsed twice previous to an ethanol series (50%-70%-96%; 3 min each) applied in order to destabilize the bacterial membranes. The samples were rinsed again and subsequently washed for 3 minutes with ice cold PBS. In the next step, the hybridization buffer (Rhizo1244/3r-FISH) in adequate composition for each probe was added and the samples were incubated for 120 minutes at 43 °C in the dark. After eliminating the hybridization buffer, the samples were rinsed twice with 500 µL of pre warmed (44 °C) washing buffer, and then incubated with 1 mL washing buffer for 15 minutes. After eliminating the buffer, a second hybridization with the EUBmix probe (which requires lower stringency conditions) was performed. After eliminating the hybridization buffer again and applying a second washing step, the samples were rinsed with ice cold ddH_2O in order to eliminate residual salts.

FISH sections were now placed on microscope glass slides, quickly dried with soft compressed air and immediately mounted with ProLong Gold or SlowFade Gold antifadent (Molecular Probes). Cover slips were placed onto the samples and sealed with nail polish.

Table 15 Composition of hybridization buffers for each probe according to the required formamide concentration. Volumes are expressed in microliter

	EUBmix (300 mg/mL) 10%	NonEUB-Cy3 (300 mg/mL) 10%	NonEUB-Cy5 (300 mg/mL) 10%	RHIZ1244 (500 mg/mL) 50%	3r-FISH (500 mg/mL) 35%
5 M NaCl	36	36	36	36	36
1 M Tris/HCl	4	4	4	4	4
2% SDS	1	1	1	1	1
Formamide	20	20	20	100	70
$H_2O_{Ultra\ pure}$	138	138	138	58	88
FISH probe	1	1	1	1	1

Table 16 Composition of washing buffers for each probe according to required formamide concentration in the hybridization buffer. Volumes are expressed in microliter

	EUBmix (300 mg/mL) 10%	NonEUB Cy3 (300 mg/mL) 10%	NonEUB-Cy5 (300 mg/mL) 10%	RHIZ1244 (500 mg/mL) 50%	3r-FISH (500 mg/mL) 35%
5 M NaCl	450	450	450	18	70
1 M Tris/HCl	100	100	100	100	100
0.5 M EDTA	0	0	0	50	50
$H_2O_{Ultra\ pure}$	4450	4450	4450	4820	4780

Table 17 Probes (Biomers.net, Wiener Neudorf, Austria) with attributes utilized for FISH in this study (Cardinale et al., 2008)

Name	Sequence (5'- -3')	Fluorescent	target	% FA	Reference
EUB338*	GCTGCCTCC CGTAGGAGT	Cy3	**Most bacteria**	10	Amann et al., (1990)
EUB338II*	GCAGCCACC CGTAGGTGT	Cy3	**Planctomycetales**	10	Daims et al., (1999)
EUB338III*	GCTGCCACC CGTAGGTGT	Cy3	**Verrucomicrobiales**	10	Daims et al., (1999)
NONEUB-Cy5	ACTCCTACGGGA GGCAGC	Cy5	**/**	**	Wallner et al., (1993)
NONEUB-Cy3	ACTCCTACGGGA GGCAGC	Cy3	**/**	**	Wallner et al., (1993)
RHIZ1244	TCGCTGCCCAGT CACC	Cy5	**Rhizobiales**	50	Thayanuku et al., (2010)
3R-FISH	GGCTTATCACGT CTCC	Cy5	**Metylotrophs/ Bradyrhizobium**	35	Nishio et al., (1997)

*** probes were used in equimolar concentration. ** NONEUB was applied as negative control. Formamide concentrations were analog to the positive FISH probes**

2.4.4 Confocal laser-scanning microscopy

Stained samples of *S. fallax* and *S. magellanicum* were observed with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with solid state and UV lasers. Up to three confocal light channels were observed sequentially plus a further bright-field channel. The filter settings were adjusted to achieve best signals, while avoiding noise. Maximum projections of an appropriate number of optical slices were applied to visualize the *Sphagnum* tissue (confocal stacks); the Z-step was 0.15−0.5 µm, depending on the magnification and the objective used. The software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for both the volume rendering of confocal stacks and the creation of 3D isosurface-spot models.

2.5 Growth media and solutions

If not specifically mentioned otherwise, all chemicals, culture media, ready-for-use kits and hardware were obtained from the following companies: Epicentre (Madison, USA), Eppendorf (Hamburg, Germany), Greiner Bio-One (Kremsmünster, Austria), Fluka (Buchs, Switzerland), Invitrogen (Lofer, Austria), Lactan (Graz, Austria), Leica Microsystems (Wetzlar, Germany), Merck (Darmstadt, Germany), MP Biomedicals (Eschwege, Germany), Sarstedt (Nümbrecht ,Germany), Sifin (Berlin, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, USA), Sorvall (Buckinghamshire, England).

2.5.1 Growth media

Table 18 MIS-medium components

$(NH_4)_2SO_4$	1.8 g/L	
$MgSO_4$	0.2 g/L	
NaH_2PO_4	1.4 g/L	
K_2HPO_4	1.9 g/L	
MeOH	5 mL/L	Added after autoclavation
Trace elements	1 mL/L	
Agarose	2% (w/v)	

Table 19 Components for MIS-medium trace elements stock solution

EDTA	500 mg/L
$FeSO_4 \times 7H_2O$	200 mgl/L
$ZnSO_4 \times 7H_2O$	10 mg/L
$MnCl_2 \times 4H_2O$	3 mg/L
H_3BO_3	20 mg/L
$CoCl_2 \times 6H_2O$	20 mg/L
$CuCl_2 \times 6H_2O$	1 mg/L
$NiCl_2 \times 6H_2O$	2 mg/L
$Na_2MoO_4 \times 2H_2O$	3 mg/L

Plant Growth Agar (Duchefa Biochemie, Haarlem, The Netherlands)

2.5.2 Liquids and solutions

Crush and Soak buffer: (50 mL) (Sambrook / Fritsch / Manatio, 1989)

105.75 mg magnesium acetate
1.93 g ammonium acetate
18.5 mg EDTA (pH 8.0)
250 µl 20% SDS
Up to 50 mL deionised water

0.5 M EDTA-Stock- solution (pH 8.0, 180 mL)

37.3 g EDTA
12 g 10 M NaOH
8 g 1 M NaOH
Filled up with 18 mL deionised water

Loading- buffer 6x (Sambrook / Fritsch / Manatio, 1989)

30% glycerol in deionised water
0.25% bromphenol blue
0.25% xylencyanol

50x TAE buffer

242 g Tris (99.9%)
57 mL glacial acetic acid
100 mL 0.5 M EDTA
Filled up with 1 L deionised water

5x TBE buffer

54 g Tris HCl
27.5 g boric acid
20 mL 0.5 M EDTA
Filled up with 1 L deionised water

III - RESULTS

3.1 Cultivation-dependent investigation

3.1.1 Isolation of moss-associated *Metyhlobacterium* spp. on selective medium

Methylobacterium growth, characterized by the typical pink pigmentation, was visible after nine days on plates from *Sphagnum fallax* and *Lobaria pulmonaria* isolation (Fig. 8). No colony growth could be observed on *Sphagnum magellanicum* plates. From the remaining 32 plates (*L. pulmonaria* and *S. fallax*) single colonies were chosen based on their morphological diversity and minimal contamination, picked with sterile toothpicks and transferred on clean selective MIS plates to purity.

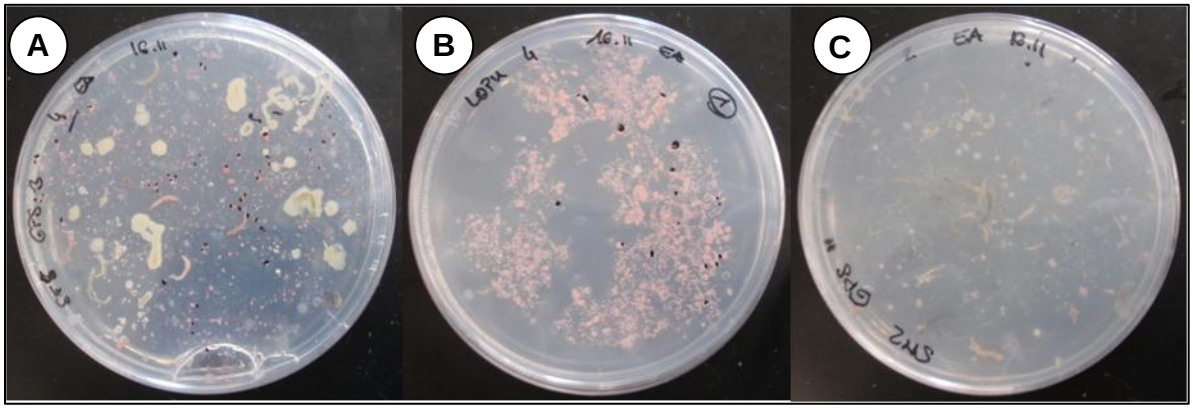

Fig. 8 Examples of MIS-plates imprinted with tissue from mosses and lichens after nine days of incubation. A) *Sphagnum fallax*; B) *Lobaria pulmonaria*; C) *Sphagnum magellanicum*. Black dots on the Petri dish bottoms mark PPFMs colonies

Streak out of pure cultures resulted in 186 single isolates (Fig. 9, 10). The isolates were named concerning a given consecutive number, abbreviated name of the host organism, number of the sample site and recapitulation of the imprinting method. All the isolates can be found in the Appendix attached to this work.

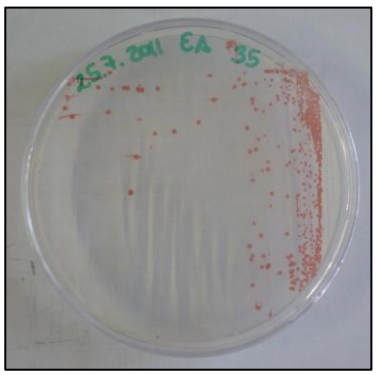

Fig. 9 Pure culture of Isolate 35LOPU23

3.1.2 Ribosomal intergenic spacer analysis (RISA) to group isolates into operational taxonomic units

The pure cultures of *Methylobacterium* strains (186 total) isolated from *S. fallax* (60), *S. magellanicum* (6), *L. pulmonaria* (116) and, as additional control, *Brassica napus* (4) (isolated from seedlings grown in the plant growth chamber of the Institute of Environmental Biotechnology, Graz University of Technology) were subjected to RISA analysis. This resulted in ITS profiles of the isolates as shown in Fig 11. Similar banding patterns were grouped with GelComparII and, among 149 total isolates, 42 groups were formed (Fig. 12-14). Every group represents an individual operational taxonomic unit (OTU). As settings UPGMA with dice coefficient (*Unweighted Pair Group Method with Arithmetic mean*) algorithm, 1% optimization and 1% tolerance was used to compare the set of variables. Out of each OTU group, representative strains were chosen to partially sequence the 16S rRNA gene to perform the taxonomical identification.

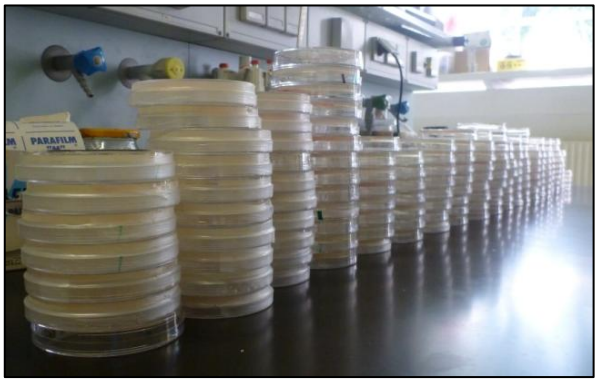

Fig. 10 Petri dishes with *Methylobacterium* isolates sorted regarding their sampling site origin and imprint recapitulation

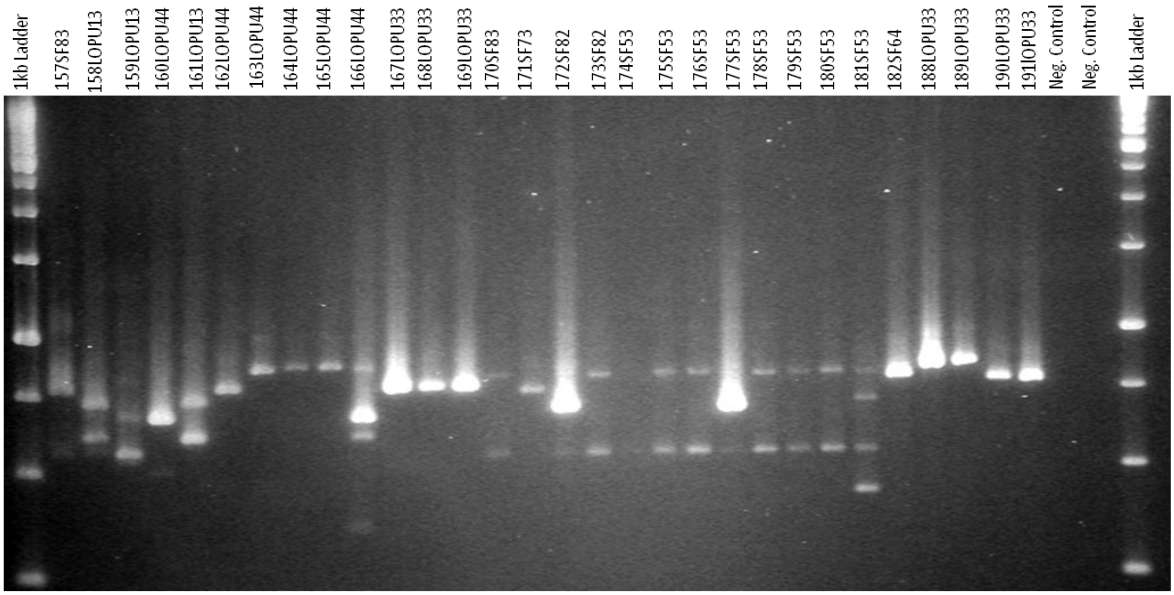

Fig. 11 Examples for ITS profiles of *Methylobacterium* isolates

For taxonomical identification and phylogenetic analysis, sequences were aligned using NCBI (National Center for Biotechnology Information) database with blastn algorithm and resulted in the identification of three different species within the genus *Methylobacterium*: *Methylobacterium adhaesivum*, *Methylobacterium mesophilicum* and *Methylobacterium rhodesianum.* RISA analysis revealed 42 distinctive OTUs within 149 selected isolates. The most abundant OTUs harbor isolates from all species but with different frequencies (Fig.15). Mentionable was the OTU group 20 (Fig.16), which gathered 11 isolates from *L. pulmonaria,* but only one additional isolate from the poorly colonized *S. magellanicum.* Species-specificity was found among minor OTU groups. Two isolates from *Brassica napus* were additionally analyzed and both isolates were linked into an own OTU group with unique band patterns in the RISA analysis (Fig.13). The total distribution of all OTUs shows high strain diversity; as shown in Fig. 17, 33 OTUs out of 42 undergo 4% of relative abundance.

For identification and phylogenetic analysis, out of this 42 OTU groups, 1465 bp long fragments of the 16S region from representative isolates were partially sequenced. One isolate from minor groups and from major groups at least two isolates were selected. Three distinct species groups could be detected within the genus *Methylobacterium*. Four isolates belonged to *Methylobacterium rhodesanium*, 12 belonged to *Methylobacterium mesophilicum* and the highest number of identified amplicons accounted to *Methylobacterium adhaesivum*. However, it was interesting that *M. adhaesivum* and *M. mesophilicum* could be identified from both *S. fallax* and *L.* pulmonaria. Contrariwise*, M. rhodesanium* could only be detected from *S. fallax*. The two sequenced amplicons from *S. magellanicum* and *B. napus* were both identified as *M. adhaesivum*.

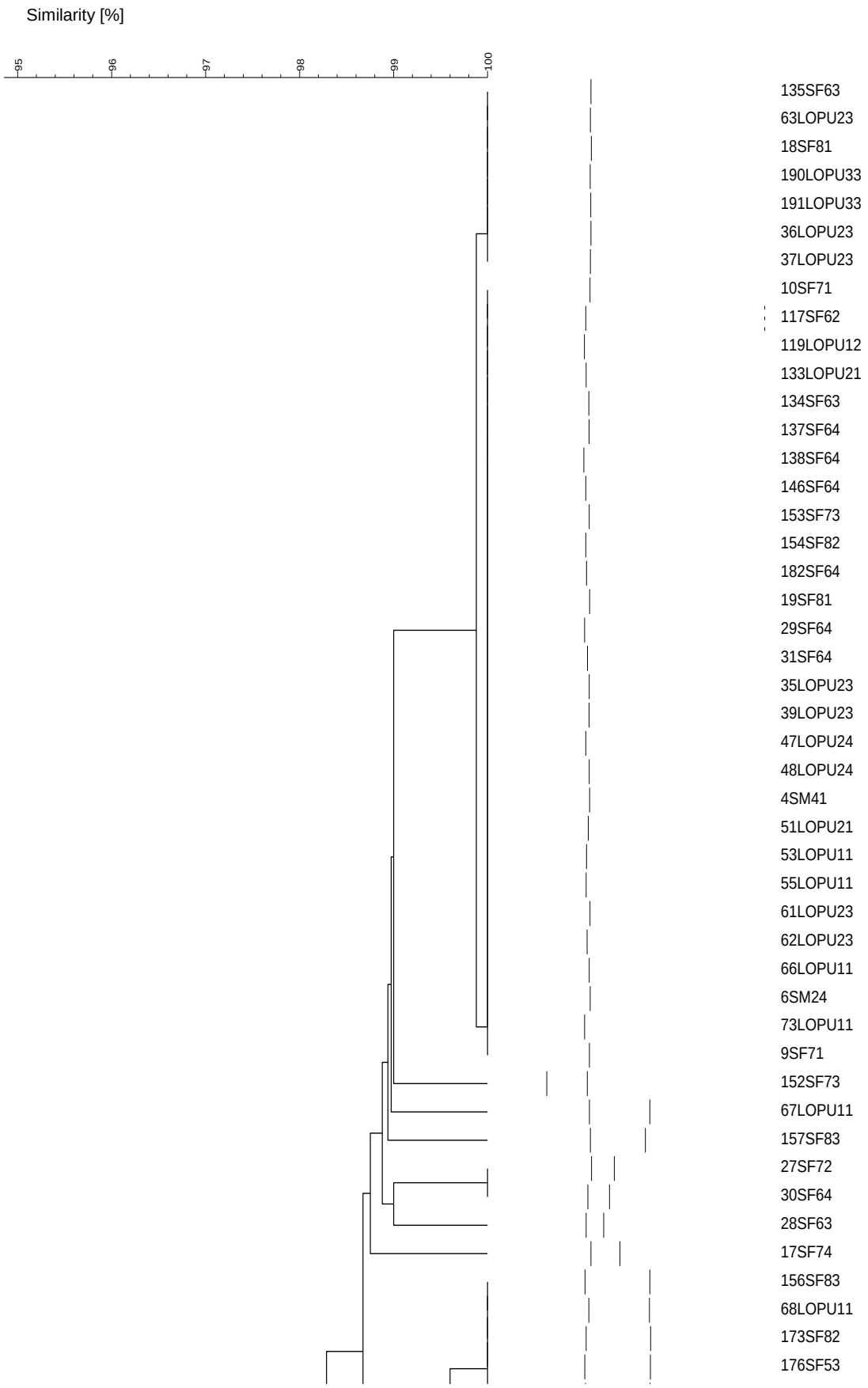

Fig. 12 Dendrogram of selected *Methylobacterium* sp. grouped by their RISA fingerprints. The dendrogram was constructed using the software GelComparII and grouped with UPGMA. Part 1 / 3

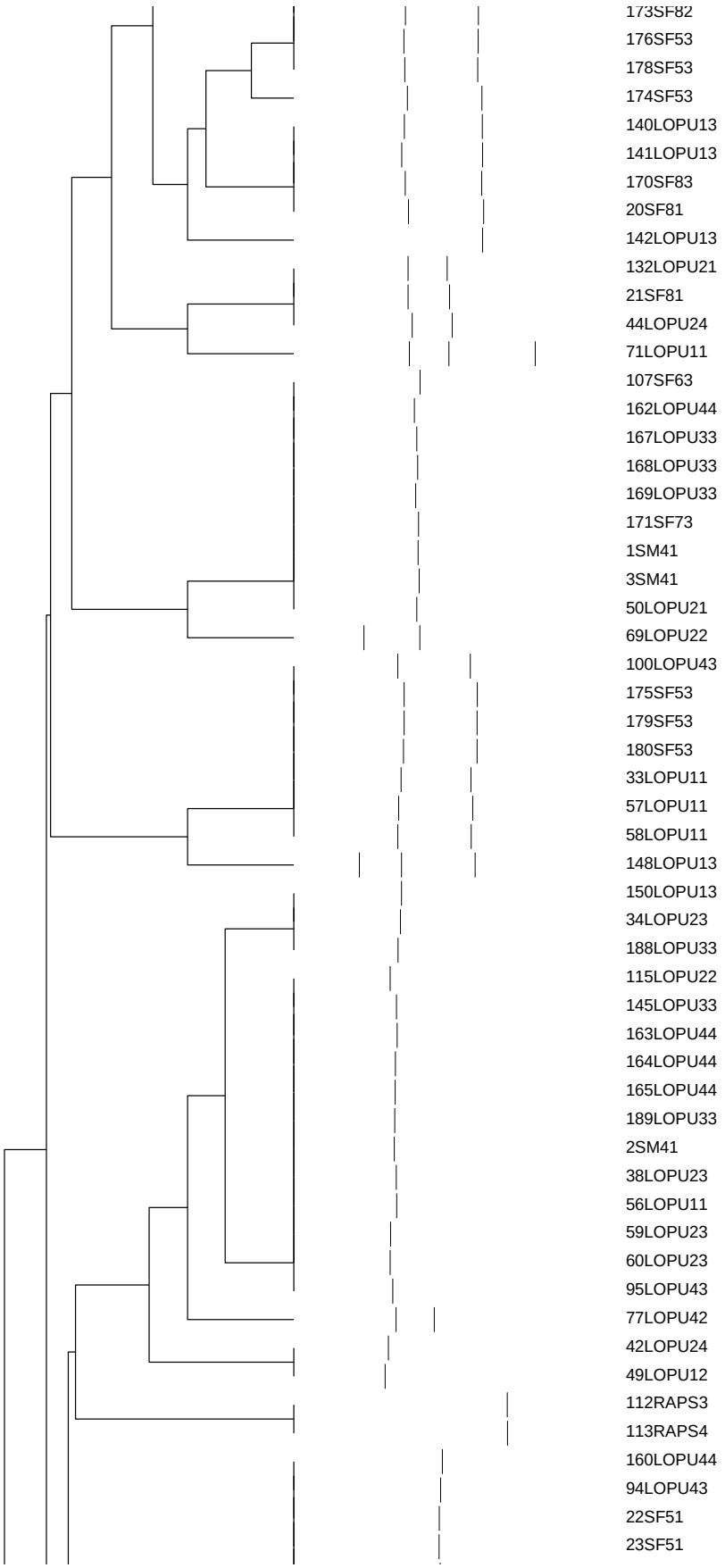

Fig. 13 Dendrogram of selected *Methylobacterium* sp. grouped by their OTUs. The dendrogram was constructed using the software GelCompar II and grouped with UPGMA. Part 2 / 3

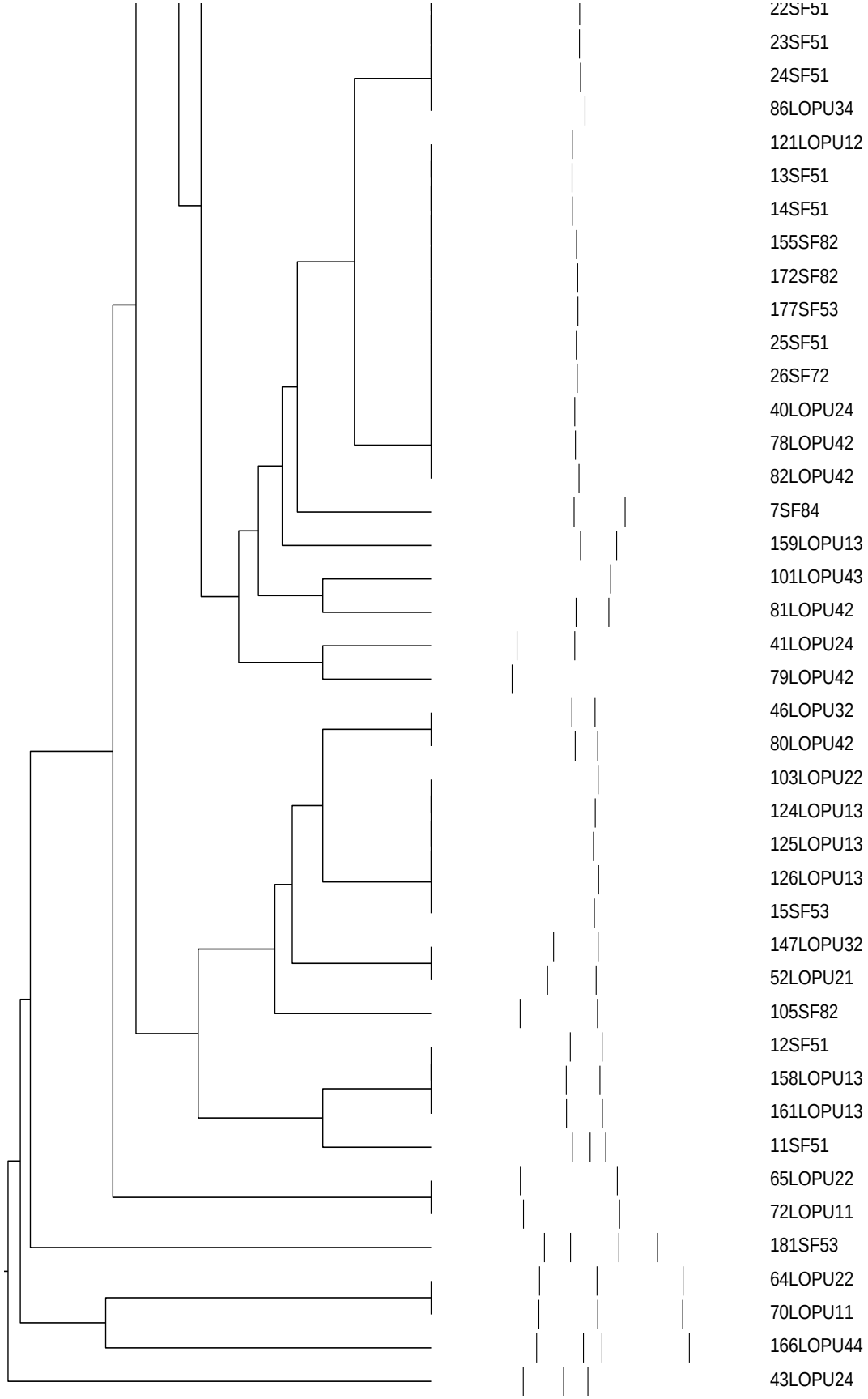

Fig. 14 Dendrogram of selected *Methylobacterium* sp. grouped by their OTUs. The dendrogram was constructed using the software GelCompar II and grouped with UPGMA. Part 3 / 3

The number of isolates among the host *S. magellanicum* was negligible small. Only 6 isolates could be obtained. Therefore only the proportion of isolates per OTU could be investigated for *S. fallax* and *L. pulmonaria* shown in Fig. 1.

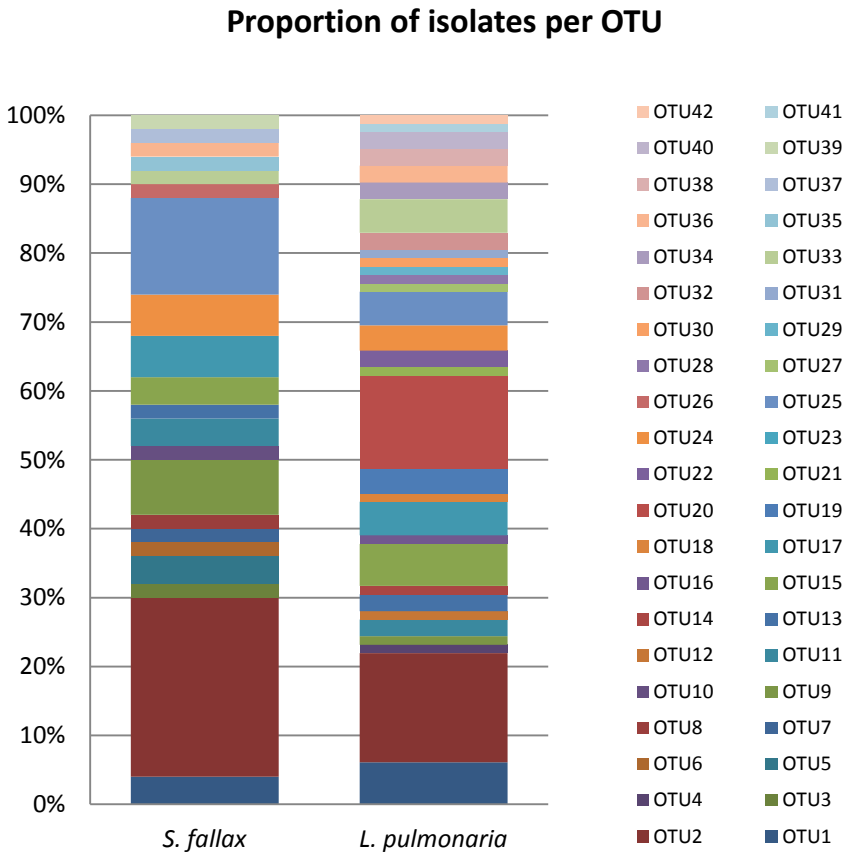

Fig. 15 Relative distribution of *Methylobacterium* OTUs as resulted from ribosomal intergenic spacer analysis (RISA) of 149 isolates from *S. fallax* and *L. pulmonaria*

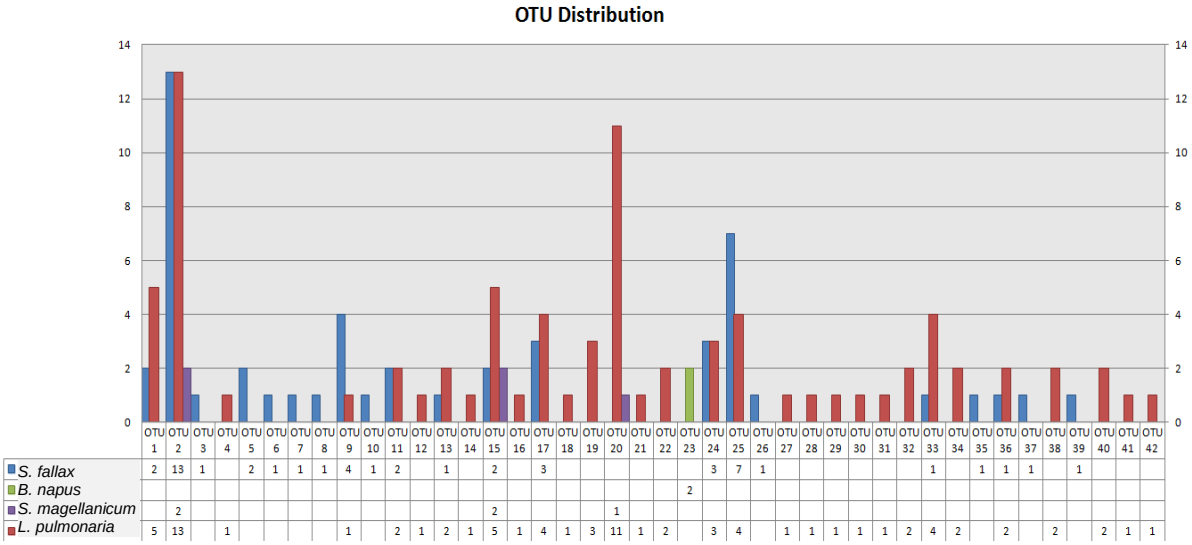

Fig. 16 Distribution and number of isolates within the generated operational taxonomic units

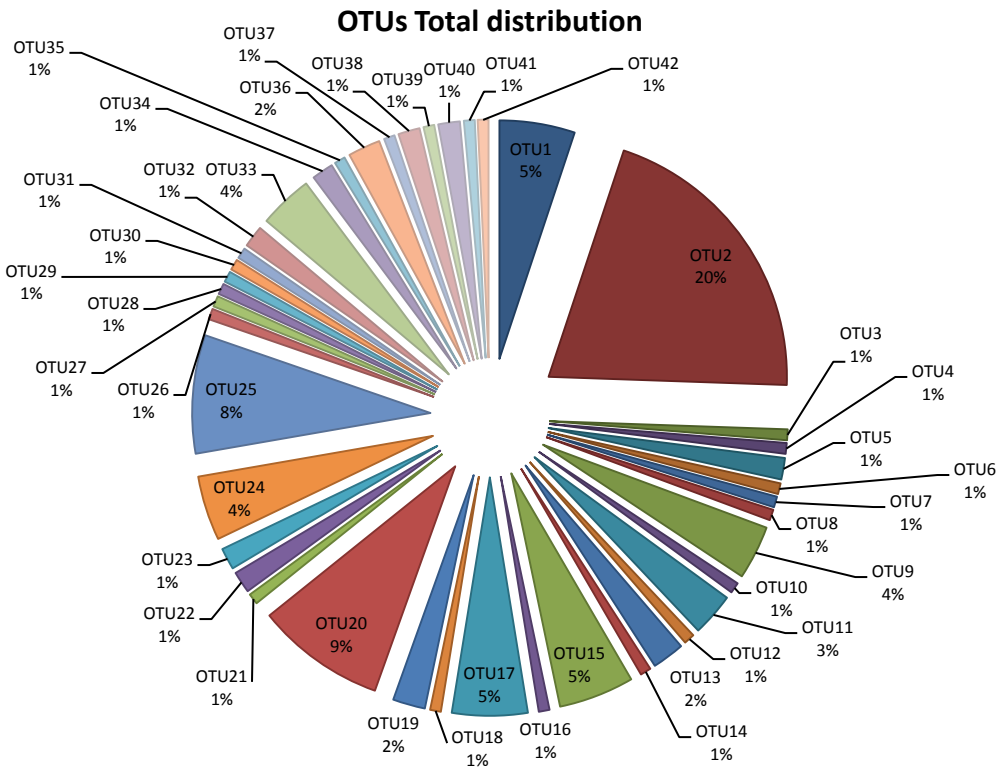

Fig. 17 Diagram visualizing the total identified OTU groups of all hosts

The phylogenetic tree in Fig. 18 shows the relationships between 16S rRNA gene sequences of methylotrophs isolated in this study and reference sequences. Type strains and additional strains derived from the NCBI-database were compared with different isolates representative of the operational taxonomic units (OTUs). The tree was constructed with the maximum-likelihood algorithm using PHYLIP. Bootstrap values (calculated with maximum likelihood/neighbor joining method) are based on 100 data resamplings. Interestingly, two specific clusters, within the *Methylobacterium adhaesivum and Methylobacterium mesophilicum* group were detected.

Fig. 18 Phylogenetic tree showing the relationships between 16S rRNA gene sequences of methylotrophs isolated in this study and reference sequences. 1) Outgroup containing two sequences of the genus *Microvirga*. 2) Isolates within the *M. adhaesivum* cluster. 3) Isolates within the *M. mesophilicum* cluster. 4) Isolates 11SF51, 17SF74, 12SF51 and 23SF51 identified as *M. rhodesianum.* 5) Other *Methylobacterium* type strains

3.1.3 Lettuce growth assay

In an *in vitro* bioassay, the PGP effect of the *Methylobacterium* strains on lettuce was evaluated. The assay was performed in 24-well plates plates according to Faltin et al. *(*2004) and Zachow et al. (2010). Eight distinct strains (53LOPU11 *M. adhaesivum,* 70LOPU11 *M. adhaesivum,* 137SF64 *M. adhaesivum,* 153SF73 *M. adhaesivum,* 166LOPU44 *M. adhaesivum,* 105SF82 *M. mesophilicum,* 148LOPU13 *M. mesophilicum,* 175SF53 *M. mesophilicum*, Fig. 15) of *M. adhaesivum* and *M. mesophilicum,* isolated from *S. fallax* and *L. pulmonaria* were chosen on the basis of their phylogenetic distance to each other, as deduced from the phylogenetic tree (Fig. 18). The strains were grown in liquid MIS-medium and showed different growth parameters in terms to flocculation, color and turbidity of the cell suspension (Tab. 20). Lettuce seedlings treated with bacteria, or untreated, were cultivated in 24-well plates under constant phytochamber conditions. The PGP effect of *Methylobacterium* produced plant growth hormones to the seedlings, was monitored by measuring the fresh weight, the area of leaves and the length of leaves and roots. The effects on plant growth are shown in Fig. 19-24.

Table 20 Cell suspensions of chosen isolates for PGP bioassay, showing differences in their growth properties. "+++/++/+" indicate the intensity of the related attribute, whereas "-" accounts to the absence of the property. Accession number and max ident values were evaluated using NCBI database and the blastn tool.

Isolate	Species	Accessionnumber	Max ident	Color intensity	Turbid	Flocculating
53LOPU11	*Methylobacterium adhaesivum*	EU867318	99%	+	-	+
70LOPU11	*Methylobacterium adhaesivum*	NR_042409	98%	-	-	+
137SF64	*Methylobacterium adhaesivum*	GU983169	100%	+	++	-
153SF73	*Methylobacterium adhaesivum*	GU992358	98%	-	-	+++
166LOPU44	*Methylobacterium adhaesivum*	GU992364	99%	-	-	+
105SF82	*Methylobacterium mesophilicum*	GU992365	96%	++	++	-
148LOPU13	*Methylobacterium mesophilicum*	GU992365	99%	-	-	2-3 big aggregates
175SF53	*Methylobacterium mesophilicum*	GU992365	99%	++	++	-

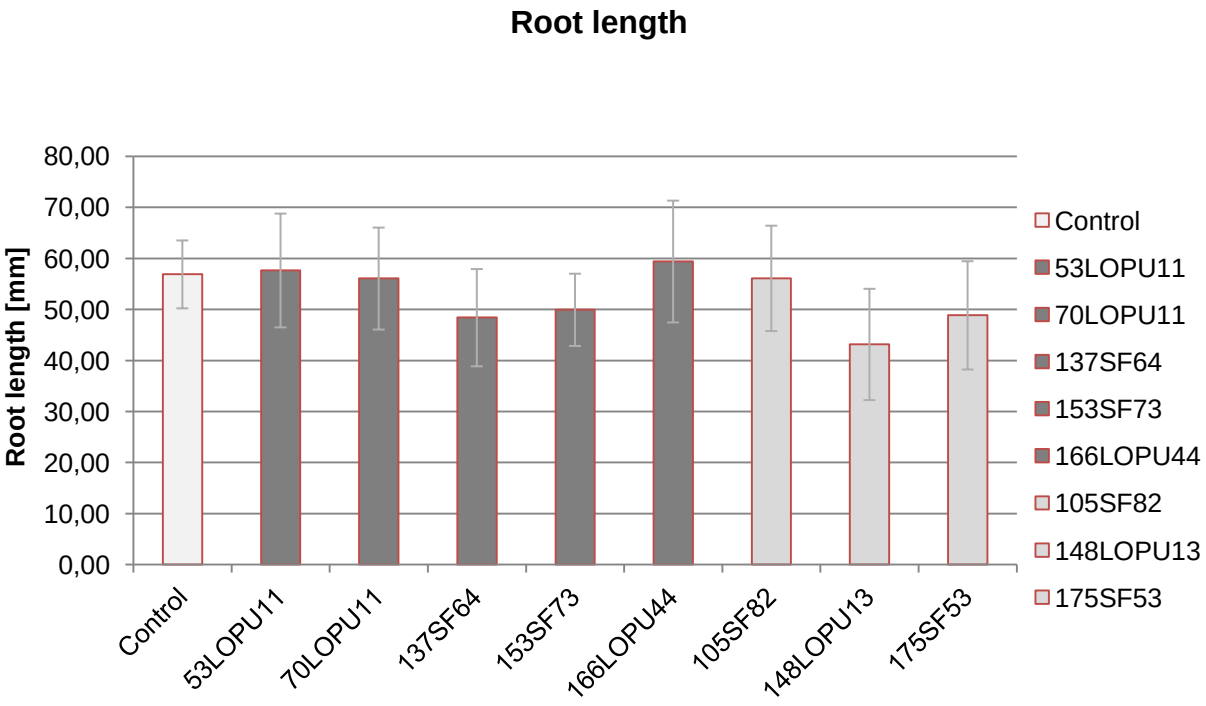

Fig. 19 PGP effect of elected *Methylobacterium* strains on seedling root length. Error bars indicate the confidence interval at $p \leq 0.05$

Fig. 20 Lettuce plantlets collected in paper cups for further determination of the dry weight.

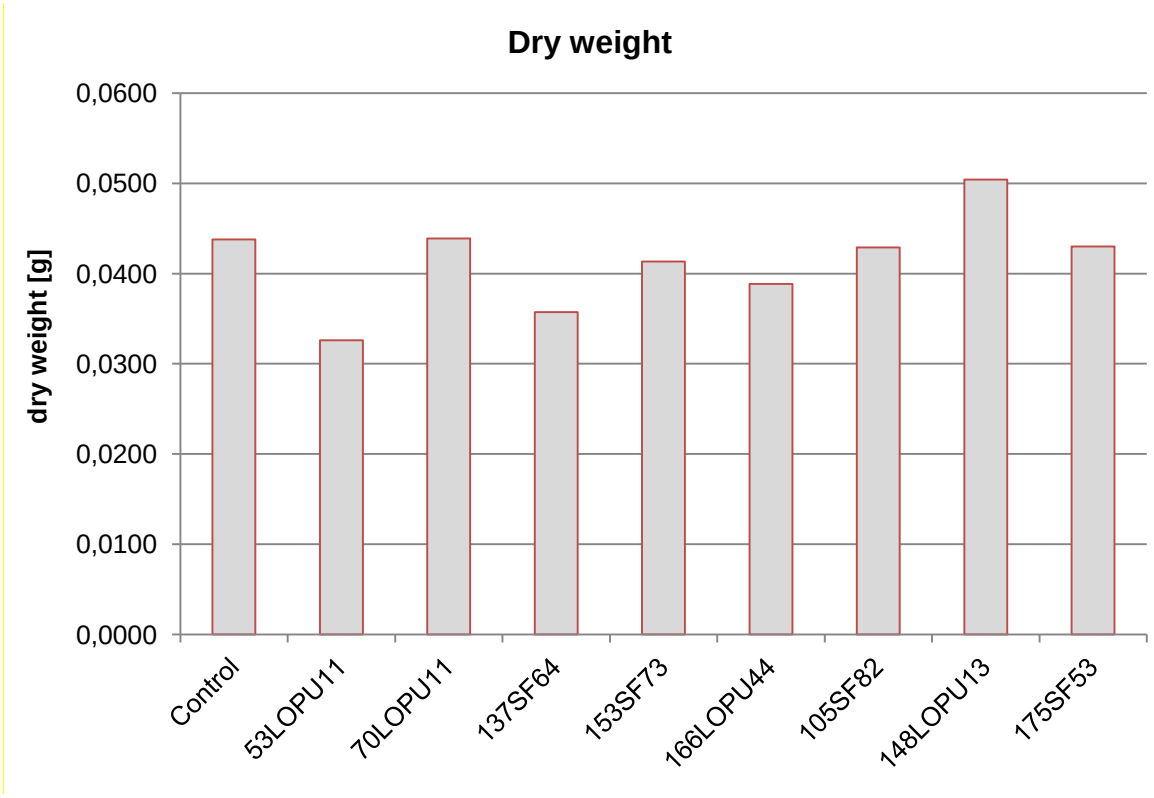

Fig. 21 PGP effect of elected *Methylobacterium* strains on seedling dry weight

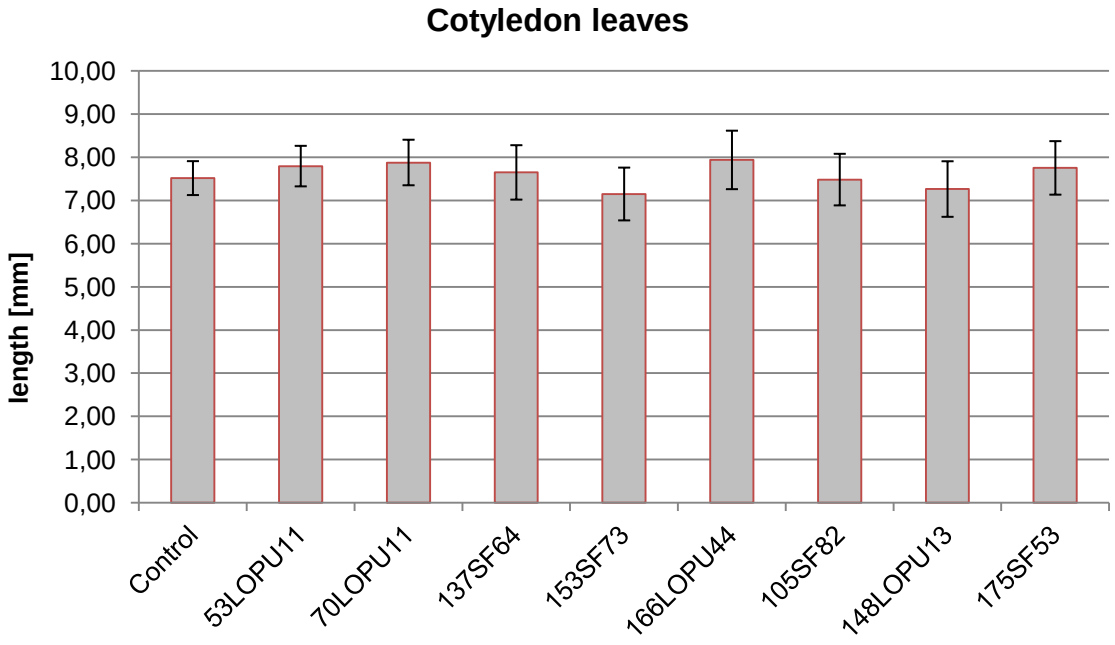

Fig. 22 PGP effect of elected *Methylobacterium* strains on seedling cotyledon leaves. Error bars indicate the confidence interval at $p \leq 0.05$

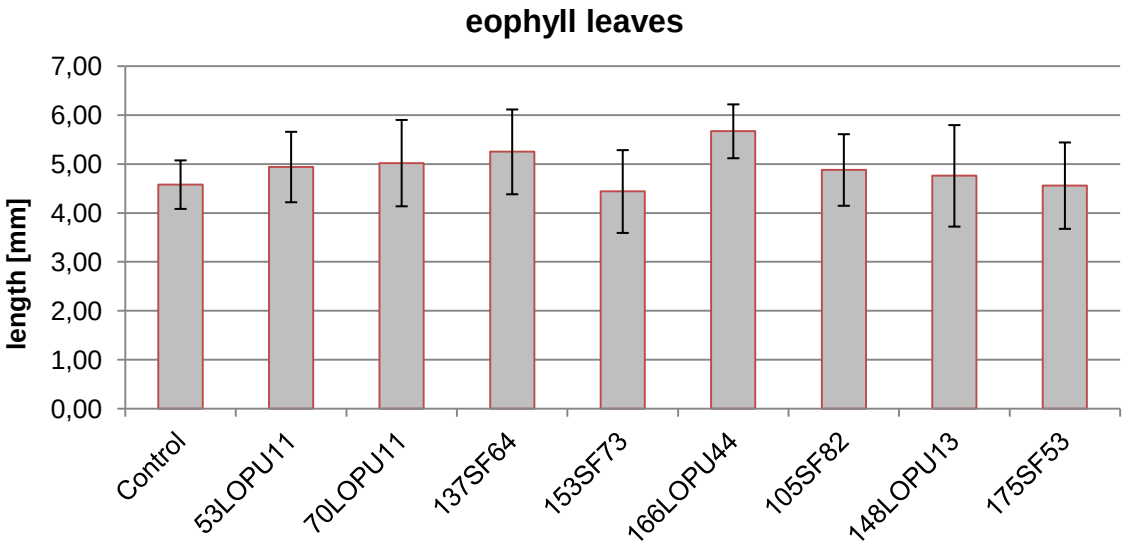

Fig. 23 PGP effect of elected *Methylobacterium* strains on seedling eophyll leaves. Error bars indicate the confidence interval at $p \leq 0.05$

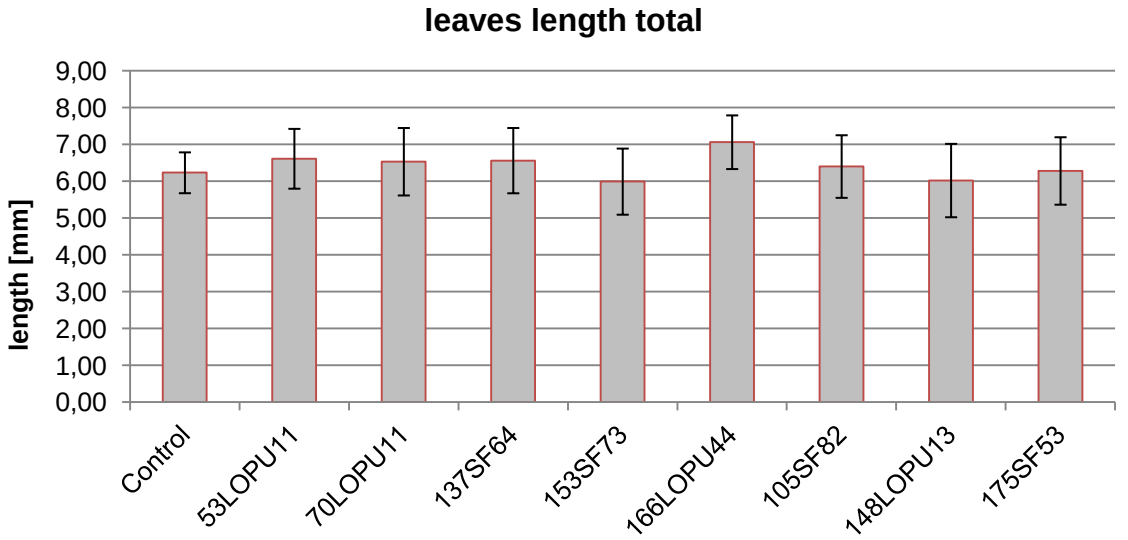

Fig. 24 PGP effect of elected *Methylobacterium* strains on seedling leaves total length. Error bars indicate the confidence interval at $p \leq 0.05$

In the bioassay minor, but not statistically significant positive effects on both the length and the dry weight of the roots were shown. Also the length of the cotyledon and eophyll leaves gave no significant rise to a positive plant-growth promotion. The most promising strain was 166LOPU44 (*Methylobacterium adhaesivum*) which showed a PGP tendency among the length of measured leaves.

3.2 Cultivation-independent approach

3.2.1 Total DNA extraction

Metagenomic DNA was successfully isolated from all samples, by using Fast Spin Kit for Soil (MP Biolabs) (Fig. 25; data for *Sphagnum fallax* not shown).

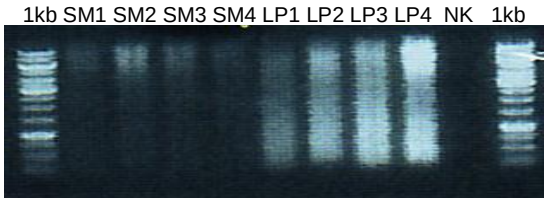

FIG. 25 Electrophoresis gel of total DNA isolated from *S. magellanicum* (SM) and *L. pulmonaria* (LP) by using a DNA extraction kit. NK = negative control; 1kb Ladder (Fermentas)

3.2.1 Microbial fingerprinting using single strand conformation polymorphism (SSCP)

The fingerprinting method SSCP was chosen for the comparison of the methylotrophic bacterial fraction composition associated with *S. magellanicum*, *S. fallax* and the lichen *L. pulmonaria*. Selective primer targeting methylotrophic bacteria were used to amplify from the total metagenomic DNA. In the total DNA only fragments within the detection range of the primer pair 2f/3r were amplified. As control, random isolates from pure cultures were additionally amplified (Fig. 26 and 27).

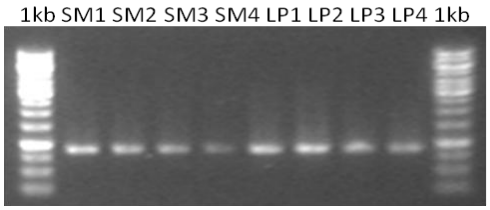

Fig. 26 Electrophoresis gel of obtained PCR products using the primer pair 2f/3r. SM = *Sphagnum magellanicum*; LP = *Lobaria pulmonaria*; 1kb ladder (Fermentas)

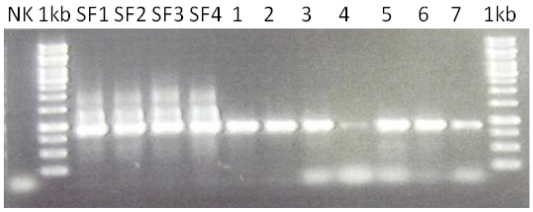

Fig. 27 Electrophoresis gel of obtained PCR products using the primer pair 2f/3r. 1-7 amplified DNA extracted from *Methylobacterium* pure cultures. SF = *Sphagnum fallax*; NK = negative control; 1kb ladder (Fermentas)

A nested PCR was performed in succession using primer which generating amplicons suitable for SSCP. Results were controlled on an electrophoresis gel as shown in Fig. 28 and 29. The phosphorylated complimentary strand of the generated dsDNA was digested by Lambda exonucleases and the residual strand became denatured. The renatured single strand amplicons were used for the SSCP approach. SSCP profiles were evaluated using both, visual observation and UPGMA (band-based with dice) grouped dendrograms of the SSCP-gel profiles. The SSCP fingerprints of amplified communities associated with the cryptogams are shown in Fig. 30. SSCP sequence analysis is shown in Fig. 31 and Tab. 21.

Fig. 28 Electrophoresis gel of the nested approach. NK = negative control; LP = *Lobaria pulmonaria*; 1kb ladder (Fermentas)

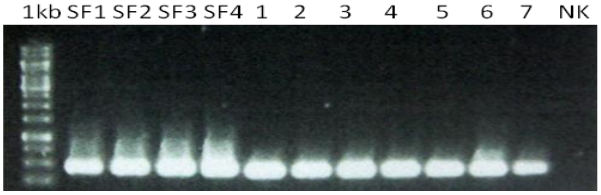

Fig. 29 Electrophoresis gel of the nested approach. NK = negative control; SF = *Sphagnum fallax*; 1kb ladder (Fermentas)

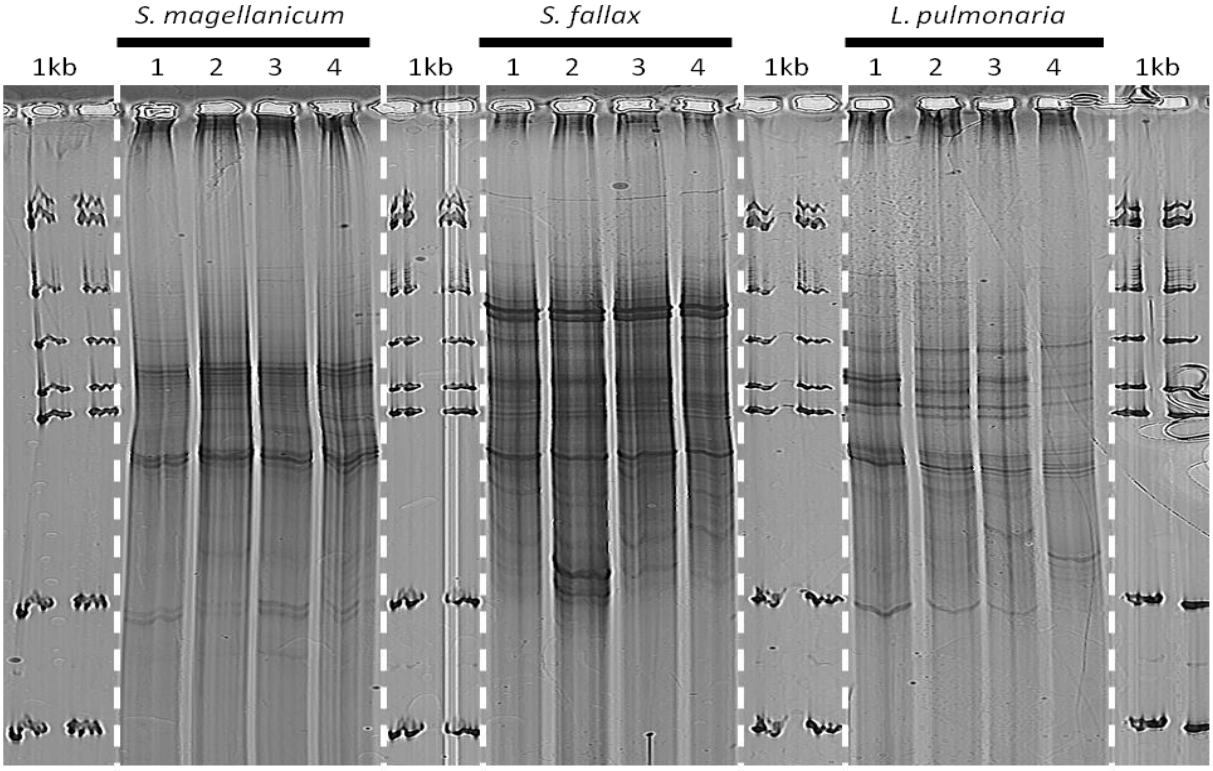

Fig. 30 SSCP profiles of methylotrophic bacteria communities associated with *S. magellanicum*, *S. fallax* and *L. pulmonaria*. The fingerprints were generated due the specific secondary structure of the selectively amplified 16S rRNA templates. The numbers 1, 2, 3 and 4 represent the replicate number for each habitat. 1kb ladder was used as reference.

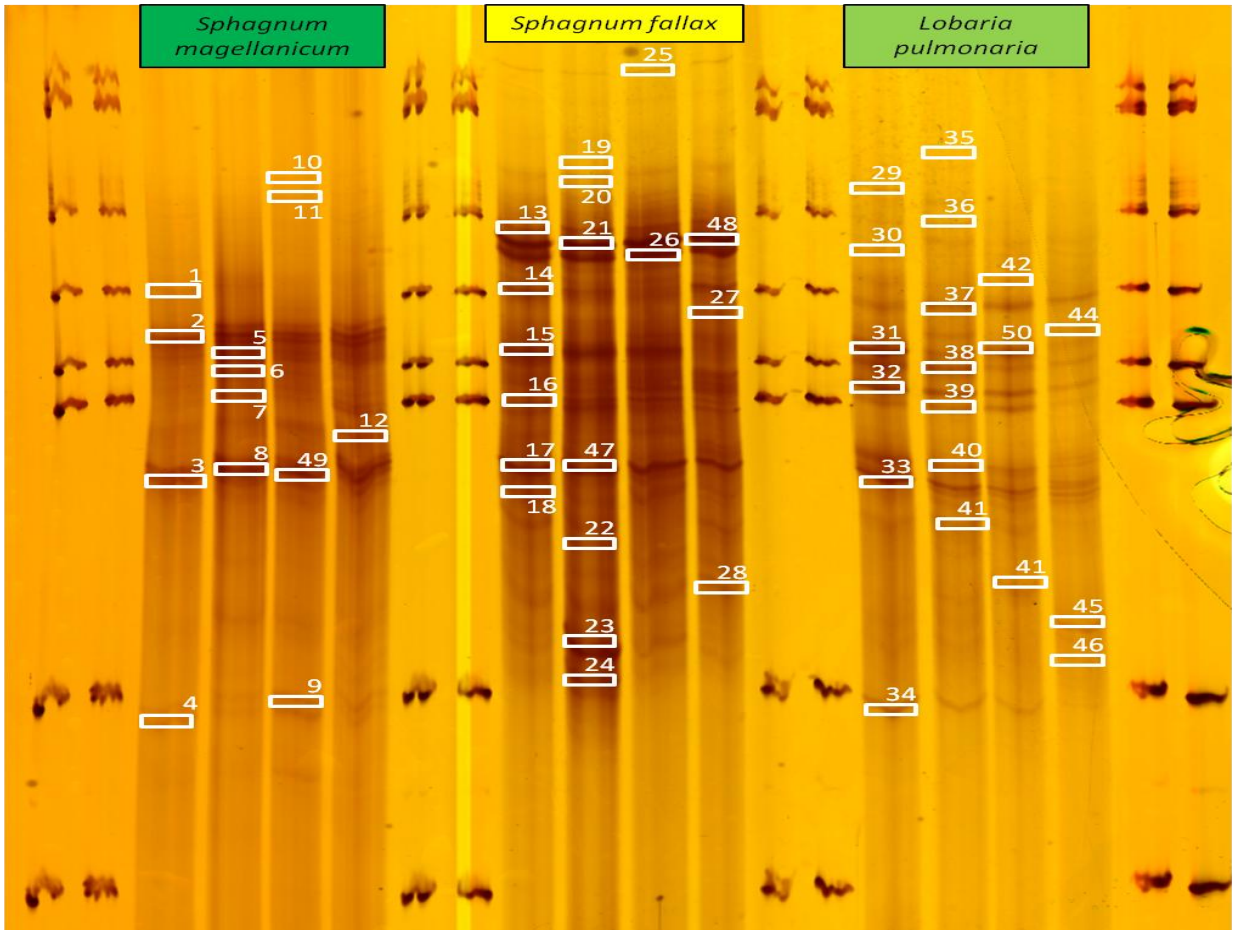

Fig. 31 SSCP analysis of specifically amplified 16S rRNA genes of methylotrophic bacteria. For each habitat (*S. fallax*, *S. magellanicum*, *L. pulmonaria*) four independent replicates were collected. Taxonomic identification of 46 excised bands (white boxes) is shown. 1kb ladder was used as reference

Tab. 21 Results of the taxonomic identification as shown in Fig. 31. Sequenced data was compared against NCBI 16S ribosomal RNA sequence database using the blastn algorithm. Accession numbers are provided within the brackets. The Identity value is based on NCBI-blast max ident results.

Excised band	Identified species (Accession number)	Identity
1	*Bradyrhizobium japonicum* (NR_036865) / *Rhizobium lupini* (NR_044869)	96%
2	*Rhodoplanes piscinae* (NR_029125)	97%
3	*Bradyrhizobium japonicum* (NR_036865) / *Rhizobium lupini* (NR_044869)	99%
4	*Afipia massiliensis* (NR_025646)	98%
5	*Rhodoplanes elegans* (NR_029125)	97%

6	*Methylosinus trichosporium* (NR_044947)	97%
7	*Beijerinckia* sp. (NR_042182) / *Methylosinus trichosporium* (NR_044947)	97%
8	*Bradyrhizobium japonicum* (NR_036865) / *Nitrobacter* sp. (NR_042449) / *Rhizobium lupini* (NR_044869)	99%
9	*Afipia massiliensis* (NR_025646)	96%
10	*Methylobacterium* sp. (NR_042409)	96%
11	*Bradyrhizobium yuanmingense* (NR_028768) / *Afipia massiliensis* (NR_025646)	95%
12	*Bradyrhizobium yuanmingense* (NR_028768)	96%
13	*Methylobacterium* sp. (NR_042409)	93%
14	*Methlyosinus trichosporium* (NR_044947)	98%
15	*Beijerinckia mobilis* (NR_042180)	97%
16	*Beijerinckia* sp. (NR_042182) / *Methylosinus trichosporium* (NR_044947)	97%
17	*Bradyrhizobium japonicum* (NR_036865) / *Nitrobacter* sp. (NR_024920) / *Rhizobium lupini* (NR_044869)	99%
18	*Bradyrhizobium japonicum* (NR_036865) / *Nitrobacter* sp. (NR_024920) / *Rhizobium lupini* (NR_044869)	99%
19	*Rhodopseudomonas rhenobacensis* (NR_028641)	96%
20	*Methylobacterium adhaesivum* (GU992348)	86%
21	Cyanobacterium	-
22	*Bradyrhizobium japonicum* (NR_036865) / *Afipia massiliensis* (NR_025646) / *Nitrobacter alkalicus* (NR_024920) / *Rhizobium lupini* (NR_044869)	97%
23	*Rhodopseudomonas rhenobacensis* (NR_028641) / *Rhodopseudomonas palustris* (NR_036771)	99%
24	*Rhodopseudomonas rhenobacensis* (NR_028641)	98%
25	*Methylobacterium adhaesivum* (NR_042409)	96%
26	Cyanobacterium	-
27	*Methylosinus trichosporium* (NR_044947)	94%
28	*Methylocapsa acidiphila* (NR_028923)	97%
29	*Methylobacterium* sp. (NR_041441)	89%
30	*Methylocystis* sp. (NR_025544)	94%
31	*Methylobacterium mesophilicum* (NR_041026) / *Methylobacterium brachiatum* (NR_041032)	97%

32	*Methylosinus sporium* (NR_026512)	96%
33	*Methylobacterium organophilum* (NR_041027)	97%
34	*Chelatococcus daeguensi* (NR_044297)	92%
35	*Methylobacterium* sp. (NR_043878)	87%
36	*Methylobacterium jeotgali* (NR_043878) / *Methylobacterium adhaesivum* (NR_042409)	93%
37	*Methylosinus sporium* (NR_026512)	96%
38	*Beijerinckia mobilis* (NR_042180)	96%
39	*Methylocystis rosea* (NR_042108) / *Beijerinckia mobilis* (NR_042180)	95%
40	*Microvirga guangxiensis* (NR_044563)	98%
41	*Methylobacterium organophilum* (NR_041027)	95%
42	*Methylosinus sporium* (NR_026512)	96%
43	*Methylosinus trichosporium* (NR_026512)	94%
44	*Methylocystis echinoides* (NR_025544)	96%
45	*Rhodopseudomonas* sp. (NR_028641)	96%
46	*Rhodopseudomonas palustris* (NR_028641)	97%
47	*Bradyrhizobium japonicum* (NR_036865) / *Rhizobium lupini* (NR_044869)	99%
48	Cyanobacterium	-
49	*Bradyrhizobium japonicum* (NR_036865) / *Nitrobacter vulgaris* (NR_042449) / *Rhizobium lupini* (NR_044869)	99%
50	*Beijerinckia mobilis* (NR_042180)	92%

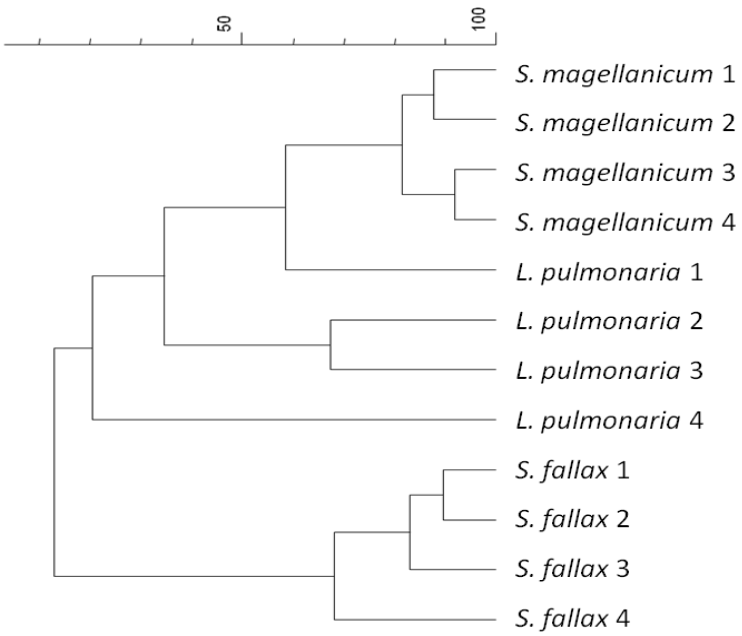

Fig. 32 Dendrogram based on SSCP fingerprint of amplified 16S rRNA gene fragments of methylotropic bacterial communities associated with *S. fallax*, *S. magellanicum* and *L. pulmonaria*. Patterns grouped by UPGMA

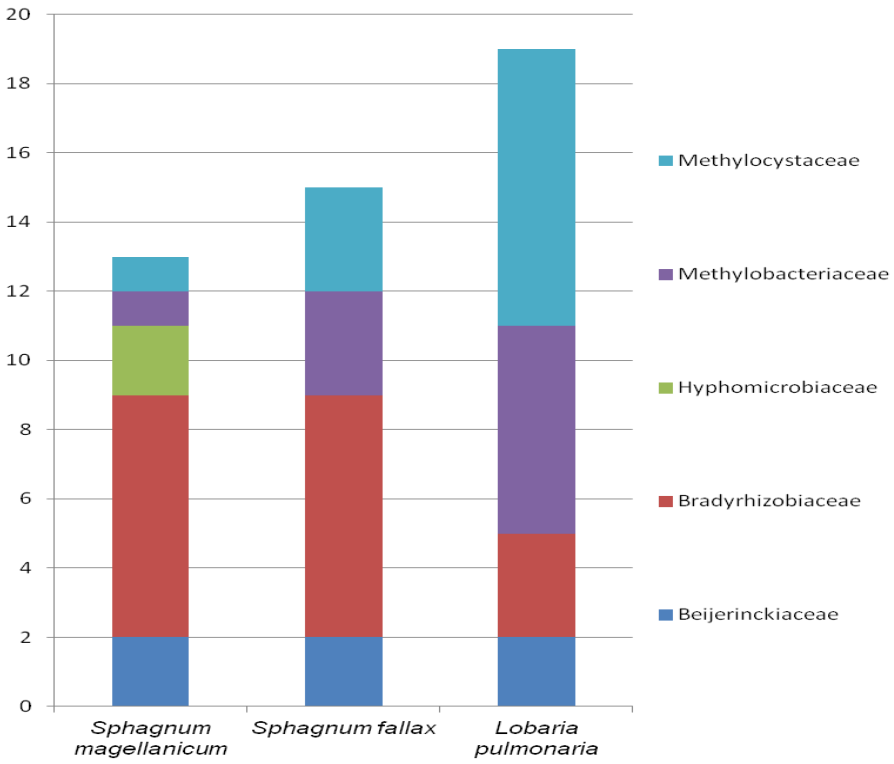

Fig. 33 Composition of bacterial families detect by SSCP, by using selective primers. Colors indicate different bacterial families

By SSCP fingerprinting it could be demonstrated that methylotrophic communities were more variable between than within host individuals (Fig. 32). The variability was higher within *L. pulmonaria*, in comparison to the *Sphagnum* species. Comparing the two mosses, the variability was higher in *S. fallax*. An overview of the detected bacterial families is given in Fig. 33.

3.3 Fluorescence *in situ* hybridization combined with confocal laser scanning microscopy

The application of FISH combined with CLSM allowed the investigation of the differences in abundance and spatial organization of bacterial communities associated with *Sphagnum fallax* and *Sphagnum magellanicum*. Two specific oligonucleotide probes for *Rhizobiales* were utilized in this work. CLSM observation on *Sphagnum* tissue revealed highest bacterial colonization inside the hyaline cells (Fig. 34-39). These big dead cells are used by the mosses as water reservoirs, and are responsible for the enormous water uptake ability of *Sphagnum*. The first utilized oligonucleotide probe RHIZ1244 was applied onto the fixed samples using two different stringency conditions (50% and 40% formamide at 43°C) in two separate sequential approaches. However, no specific hybridization could be detected. The second oligonucleotide probe was constructed based on the sequence of the primer 3f used in this work for selective amplification of methylotrophic bacteria (see above). This DNA probe was applied using 35% formamide. The group-specific amplification had shown the presence of bacteria belonging to the order Rhizobiales. No bacteria were detected using a nonsense FISH probe as negative controls.

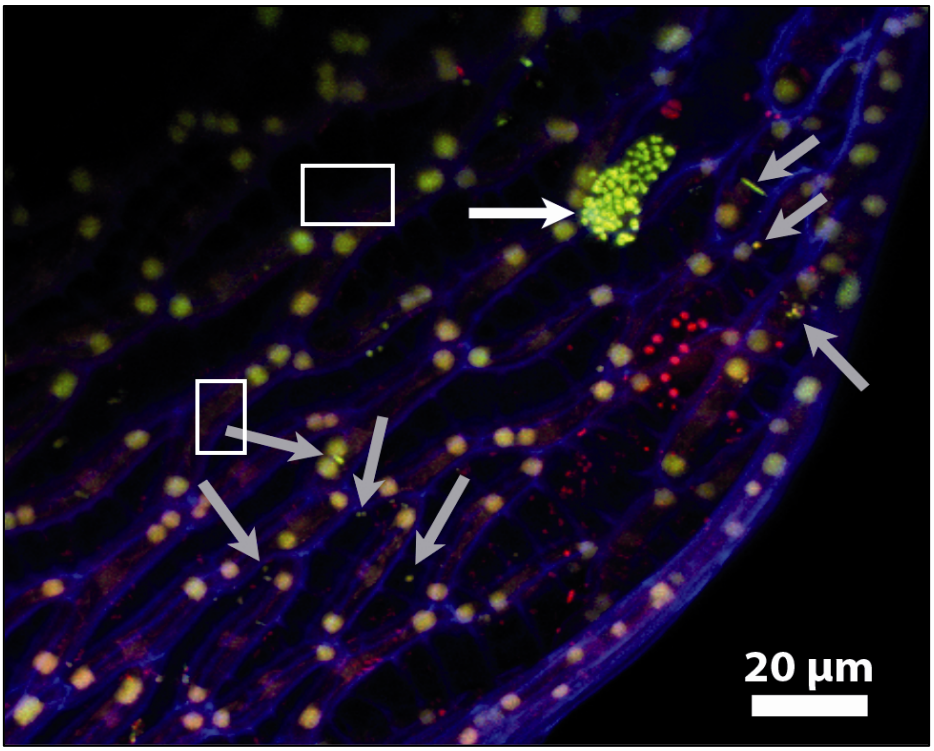

Fig. 34 Confocal laser scanning microscopy picture of *Sphagnum fallax.* Maximum projection showing Bacteria (red) and Rhizobiales (yellow/green, small dots/bacilli) colonizing *S. fallax* tissue. Green/yellow, larger circular objects are chloroplasts (squares); blue/pink: *Sphagnum* cell walls. A large Rhizobiales colony (white arrow), as well as single cells (grey arrows), were detected

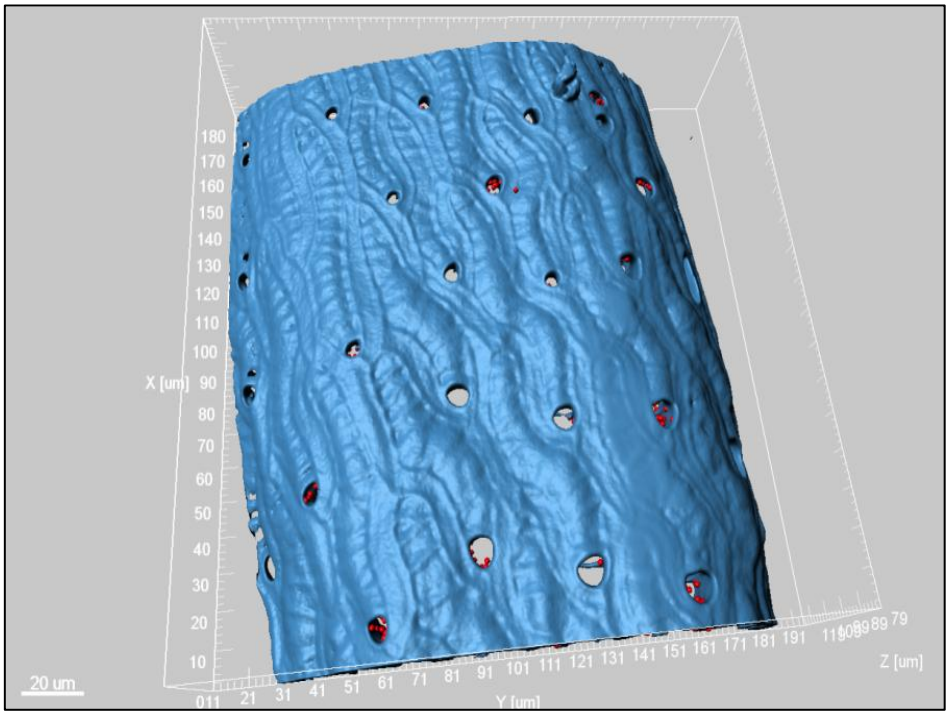

Fig. 35 Three-dimensional model showing the outer surface of *Sphagnum fallax* stem-leaves with characteristic holes for water accumulation. Bacteria (red) colonizing the inner tissues are visible through the holes

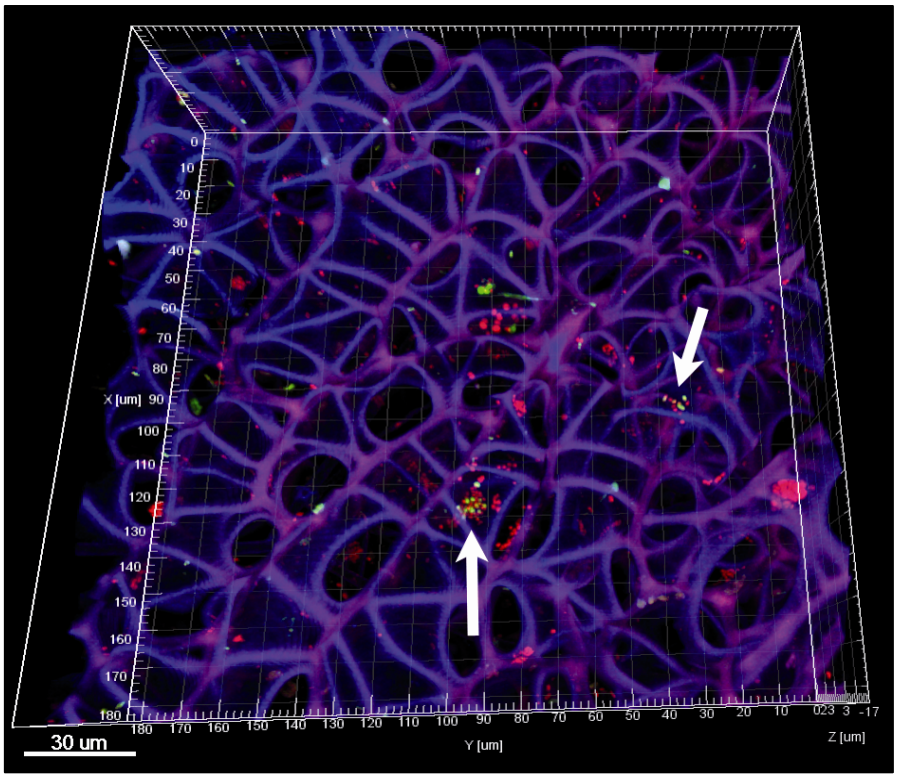

Fig. 36 Volume rendering showing *Sphagnum magellanicum* tissue colonized by Bacteria (red) and Rhizobiales (yellow). The arrows point out mixed colonies, where Rhizobiales and other Bacteria are intermingled

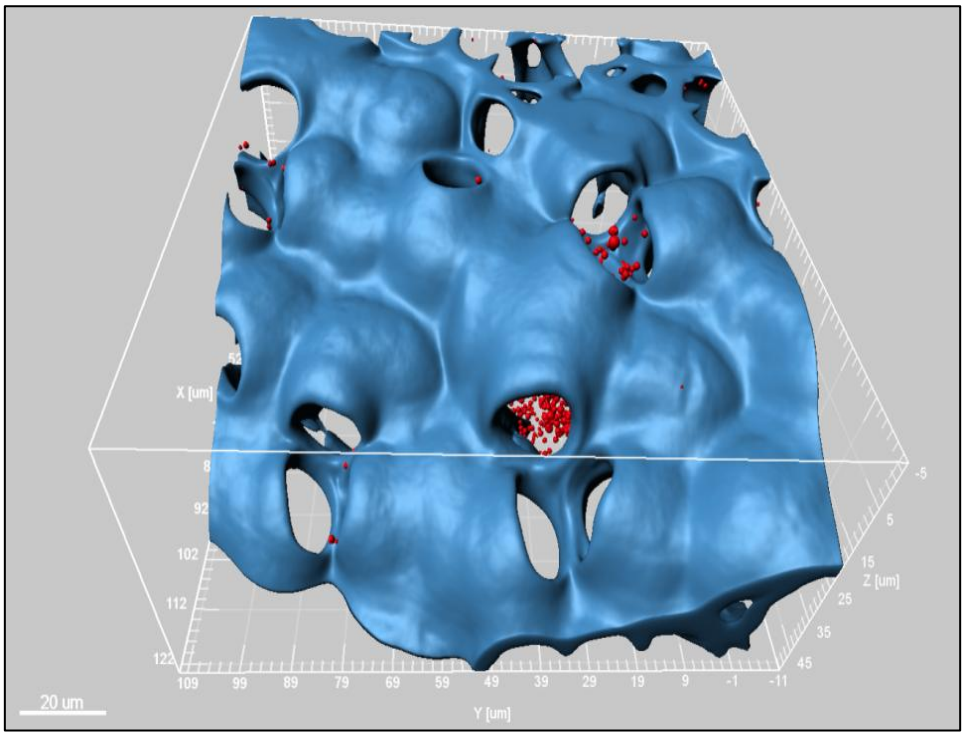

Fig. 37 Three-dimensional model showing the outer surface of *Sphagnum magellanicum*. A dense bacterial colonization (red) is visible in the the inner side of the halocytes. There is a remarkable morphological difference between *S. fallax* and *S. magellanicum*

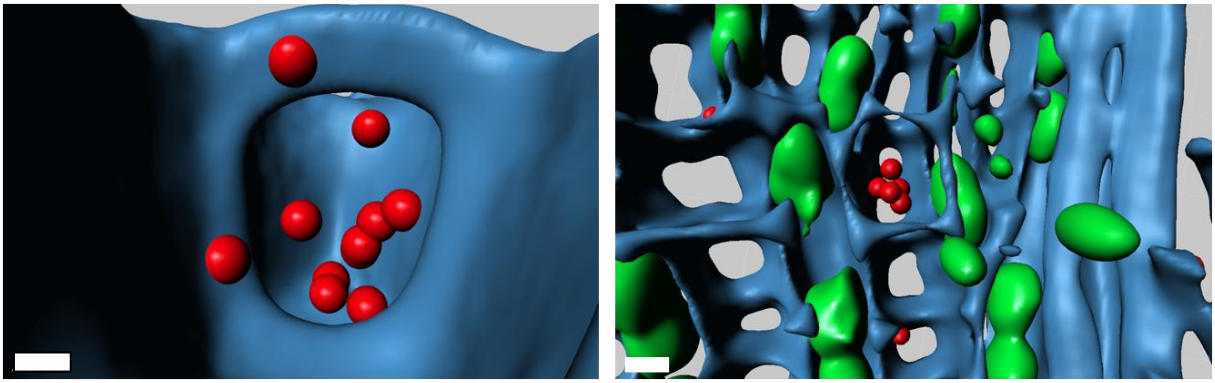

Fig. 38 Bacteria cells (red) in branch leaves inside hyaline cells of *S. fallax*. Photosynthetic cells (green) between the hyaline cells are visible. Scale bar = 4 µm (left), 3 µm (right)

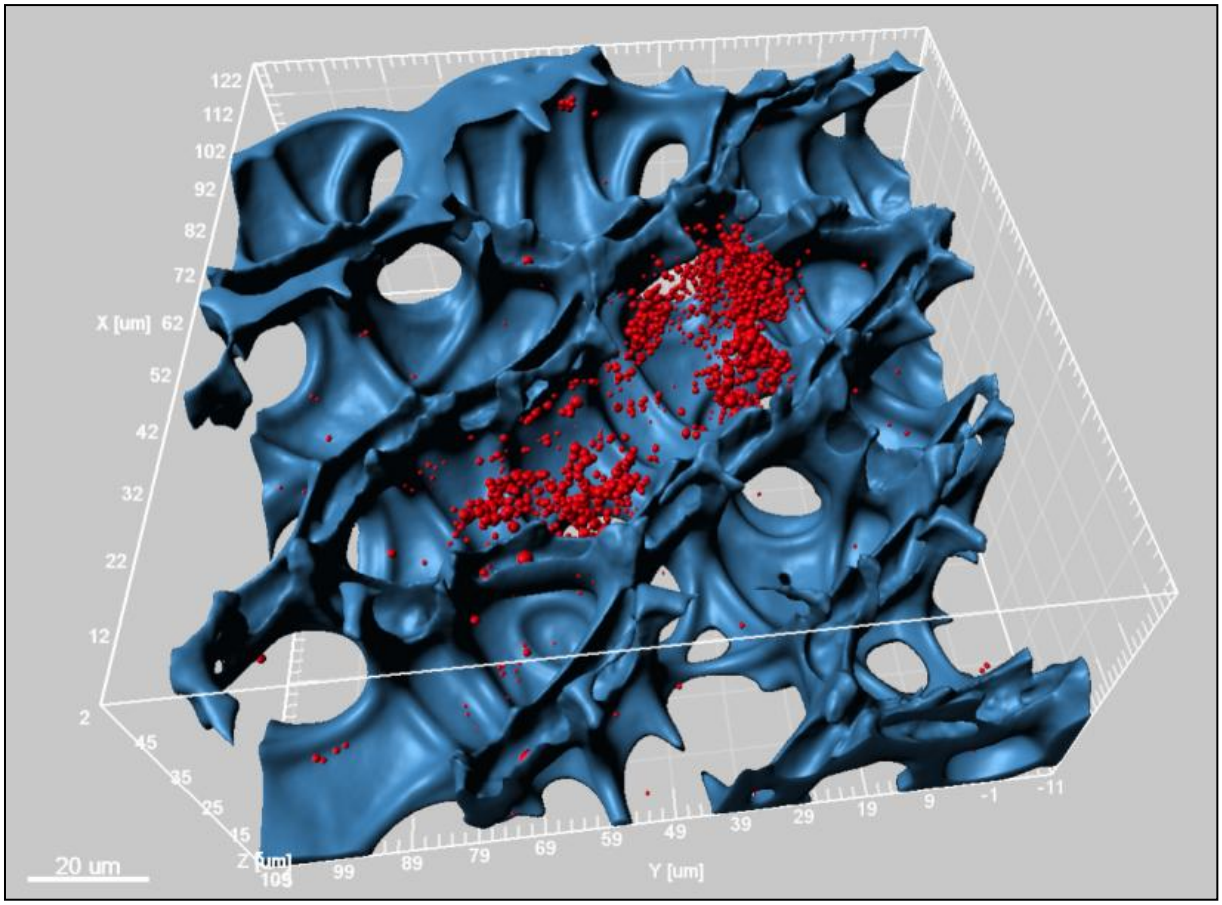

Fig. 39 Branch leaves of *Sphagnum magellanicum*. A huge bacterial colony was found in hyaline cells

IV - DISCUSSION

The aim of this study was to detect and investigate the genus *Methylobacterium* and closely related methylotrophic bacteria of the Rhizobiales family on cryptogams, with respect to their diversity and spatial structure on the outer plant tissue. It was attempted to clarify which species are dominant and how do they contribute to composition of host-associated bacterial communities. However, remarkable differences could be found between *S. fallax*, *S. magellanicum* and *L. pulomaria*. Molecular- and microbiological techniques allowed deeper insight into the methylotrophic bacterial communities associated with our investigated organisms. Cultivation-dependent methods focus on the investigation of the small proportion of aerobic cultivable organisms, while the analysis of the total DNA and FISH provides information about a broader spectrum of the microbial community. Combined approaches yield comprehensive results and reduce the biases of utilizing only monophasic approaches (Berg & Smalla, 2009). Confocal laser scanning microscopy combined with FISH led to stunning results, showing the bacterial colonization *in situ*. A bioassay was performed to evaluate possible PGP effects of selected PPFM strains. Using both, cultivation independent and cultivation dependent approaches permitted the investigation in terms of bacterial structure, diversity, function and activity.

In the cultivation-dependent analysis, 186 isolates from three distinct habitats with the impression technique were achieved (Corpe, 1985). The morphology of pink colonies was highly diverse on the isolation plates regarding different habitats. Highest cell counts were observed on *L. pulmonaria.* We gained 60 pure cultures from *Sphagnum fallax* and 116 from *Lobaria pulmonaria*, whereas only 6 pure cultures could be achieved from *S. magellanicum*. Mentionable is that PPFMs could only be isolated from *S. magellanicum* when plant debris of additional plants was visible as contamination. A reason might be the remarkable difference in the soil reaction value and nutrient value; Bragina et al. (2011) showed that the habitat of *S. magellanicum* is extremely acidic and poor in nutrients, and these two abiotic parameters may represent a growth limit for possible plant colonization by *Methylobacterium* sp.

Forty-two distinctive OTUs could be defined within 149 isolates by ribosomal intergenic spacer analysis. The most abundant OTUs harbor isolates from all species

but with different frequencies. In the OTU 20, 11 isolates from *L. pulmonaria* and one isolate from *S. magellanicum* were gathered. Among minor OTU groups species-specificity was found. Two isolates from *Brassica napus* showed unique band patterns. The variation and composition of the bacterial communities is influenced by the site (van Overbeek and van Elsas, 2008) and host species (Opelt et al., 2007c). Knief et al., (2010) could show that different plant species are colonized by a set of species or strains of the genus *Methylobacterium* which are present at the respective site where the plants grow. The close geographical distance between sampling sites and the presence of strains isolated from different species within the same OTU groups support to the mentioned thesis. On the other hand, minor OTUs suggest species specificity. Additionally the high strain diversity is mentionable. Within 42 OTUs, 33 undergo a relative abundance of 4%. Sequencing of this 42 OTU groups led to the identification of *Methylobacterium rhodesanium, Methylobacterium mesophilicum and Methylobacterium adhaesivum.* The highest number of identified amplicons accunted to the Species M. adhaesivum. However, it was interesting that *M. adhaesivum* and *M. mesophilicum* could be identified from both *S. fallax* and *L. pulmonaria*. Contrariwise, *M. rhodesanium* could only be detected from *S. fallax*. *M. mesophilicum* former classified as *Pseudomonas mesophilica* (Green & Bousfield, 1983) was first described by Austin & Goodfellow (1979). Interestingly, Kaye et al., (1992) has publicized this species as rare cause of opportunistic infections in immunocomprimised patients. The source of infection in cases as described by Sanders et al. (2000) are attributed ranging from tap water, flowers delivered to hospitalized patients or the ingestion from raw vegetables. Altogether the occurrence of *Methylobacterium* is often described as ubiquitous but with limits, as shown on *S. magellanicum.*

The relationship between identified OTUs in comparison with a wide spectrum of described species and strains within the genus *Methylobacterium* was shown in a phylogenetic tree based upon their 16s rRNA gene sequences. An interesting cluster was formed within the *M. adhaesivum* group including the isolates 69LOPU22, 70LOPU22, 112RAPS3, 153SF73 and 3SM41. This cluster was spectacular, because it is monophyletic and it is composed of five isolates from four different hosts. Further, the clustering of identified *Methylobacterium rhodesianum* strains was between type strains of *M. chorlomethanicum, M. populi, M. zatmanii* and *M.*

cyanatum, showing a close phylogenetic relation between *Methylobacterium* species using partial 16s rRNA gene sequences.

A bioassay was performed in order to detect possible plant growth promotion of eight strains from the two dominant PPFMs species investigated in the cultivation dependent approach. Remarkable differences in their growth behavior in liquid MIS media was found. The isolates were classified according to their growth properties relating the color intensity of the whole culture, the turbidity of the suspension and the way how they flocculate in liquid culture. Abanda-Nkpwatt et al., (2006) could show a plant growth-promoting effect of *Methylobacterium extorquens* ME4 on seedlings of *Lycopersicum esculentum*, *Fragaria. vesca*, *Nicotiana tabacum* and *Salix alba*. But this PGP effect did not generally apply for additional tested seeds, like *Triticum aestivum, Zea mays* or *Pisum sativum*. In the bioassay minor, but not statistically significant positive effects on both the length and the dry weight of the roots were shown. Also the length of the cotyledon and eophyll leaves gave no significant rise to a positive plant-growth promotion. The most promising strain was 166LOPU44 (*Methylobacterium adhaesivum*) which showed a PGP tendency among the length of measured leaves. A reason for the non-significant plant growth promotion may in part be explained by the slow generation time of *Methylobacterium* spp. resulting in a short time given to PPFMS for colonizing the plants. Another explanation might be found in the selection of the host plant. The plant-specificity is a key factor, and a bioassay using *Sphagnum* species could show more promising results. However, the data provided by the bioassay is too fragmentary and insignificant to draw any general conclusion about a PGP effect of *Methylobacterium adhaesivum* and *Methylobacterium mesophilicum.* Trends can be found within the data but with lack of significance.

Many plants and in particular *Sphagnum* species have an extensive influence on the structure and function of their associated microbial populations (Berg & Smalla, 2009; Bragina et. al, 2012). SSCP fingerprinting could demonstrate that methylotrophic communities were more variable between than within host individuals. The variability was higher within *L. pulmonaria* samples, in comparison to the *Sphagnum* species. Comparing the two mosses, the variability was higher in *S. fallax*. One explanation might be the differences in distinct environmental conditions and bioactive secondary metabolites. Typically, the habitats for *S. magellanicum* are very acidic and

oligotrophic, whereas *S. fallax* grows in mesotrophic and weaker acidic conditions (Opelt et al., 2007a). However, in both Sphagnum species a high similarity of SSCP patterns of associated methylotrophic microbial communities was shown. Opelt et al., (2007b) demonstrated that *Sphagnum fallax* and *Sphagnum magellanicum* associated bacteria are highly specific for the moss species. This is partially consistent to present results and it was observed that also the group of methylotrophic bacteria implies to this thesis.

In a dendrogram based on amplified 16s rRNA genes of methylotrophic bacterial communities using UPGMA algorithm, it was shown that *L. pulmonaria* is closer to *S. magellanicum*, than the *Sphagnum* species to each other. In all investigated cryptogams members of the families Methylocystaceae, Methylobacteriaceae, Bradyrhizobiaceae and Beijerinckiaceae were identified. In addition, the family Hyphomicrobiaceae could only be detected in *Sphagnum magellanicum.* With the primer used for the nested PCR approach distinct members of the family Rhizobiales were detected. In the cultivation dependent-approach we could detect all those PPFMs, which were able to develop colony growth on selective MIS Agar. Using the cultivation-independent methods, the detection of PPFMs was determined by the specificity of the oligonucleotide primer used in the polyphasic approach. The primer set 2f/3r could not detect Methylobacteriaceae exclusively. Bacterial members of the families Hyphomicrobiaceae, Methylocystaceae, Beijerinckiaceae, Bradyrhizobiaceae were also amplified and detected. Alternatively, a set of several primers was utilized as described by Nishio (1997) or *mxaF* gene homologous primer (McDonald, 1997) to improve the exclusive detection of the genus *Methylobacterium*.

Our FISH-CLSM experiments revealed an abundant bacterial colonization in the investigated peat mosses, which range from single cells attached to dense biofilm-like aggregations. Furthermore, the CLSM analysis of *Sphagnum magellanicum* and *S. fallax* showed that bacteria mainly colonize hyaline cells. Older tissues, indicated by deformed and partially degraded chloroplasts, showed higher bacterial colonization. Two group-specific probes where applied, whereas only the 3r probe showed results. No distinct colonies or cells could be detected with the Rhiz1244 probe utilizing different stringency conditions. It was mainly focused on the moss branch leaves in order to detect Rhizobiales, because of the lower colonization of bacteria on the stem leaves. (Bragina et al., 2011). The detection limit of the 3r probe

is yet not exactly known, but trough the nested approach in the cultivation-independent investigation and probe blast, a good coverage for the family Rhizobiales is assumed.

V - CONCLUSIONS

In conclusion, *Sphagnum* peat mosses and lichens are interesting habitats for studying methylotrophic bacteria. *Methylobacterium mesophilicum* and *M. adhaesivum* were the most abundant species of the genus *Methylobacterium* in both habitats, as shown by cultivation-independent and depended-methods. The ease with which PPFMs could be recovered from *S. fallax* and *L. pulomaria* and purified into pure cultures suggests they are a ubiquitous part of their microbiome. On the other hand, problems encountered isolating PPFMs from *S. magellanicum* and this very low abundance gives rise to the hypothesis that *Methylobacterium* spp. might not colonize *S magellanicum*. Altogether, our multiphasic approach led to insight into the methylotrophic bacterial communities, with focus on the genus *Methylobacterium*, on cryptogams. But not to forget, among this interesting moss species and the lichen *Lobaria pulmonaria,* there could be still a hidden fraction of less abundant methylotrophic bacteria, not detectable by the methods used in this study. The chosen habitats as models to study plant-associated methylotrophic bacteria harbor an enormous potential. Reasons are numerous: besides the fact that bryophytes were the first plants colonizing our planet, they have an interesting ombrotrophic lifestyle and thereby they are not influenced by soil. They are exceedingly important for the climate on our earth. But also lichens are highly attractive as study model, in particular because of their fascinating symbiosis of different kingdoms and with their manner how they cope with extreme environmental conditions.

VI - REFERENCES

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VII - LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
Bp	base pair
CLSM	confocal laser scanning microscopy
°C	degree centigrade
DNA	deoxyribonucleic acid
dNTP	desoxyribonukleosid-5‟ -triphosphate
Et al.	et alteri
Fig	figure
FISH	fluorescence *in situ* hybridisation
h	hour
H_2O	water
kb	kilo base
L	liter
µ	micro
MIS	Minimal-inorganic-salts-Medium
m	milli
M	molar
min	minute
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ONC	over night culture
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
%	percentage
PGP	plant growth promotion
pH	negative decimal logarithm of the hydrogen ion activity in a solution
r	ribosomal
RNA	ribonucleic acid

rpm	rounds per minute
SDS	sodiumdodecylsulfate
sec	second
SSCP	single-strand conformation polymorphism
Tab	table
TAE	Tris-Acetat-EDTA
TBE	Tris-Borat-EDTA
TEMED	N,N,N,N-Tetramethylethylendiamin
Tris	Tris-Hydroxymethyl-Amminomethan
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
ZMF	Zentrum für medizinische Grundlagenforschung

VIII - LIST OF TABLES

IX - LIST OF FIGURES

X - APPENDIX

Isolate number	Origin	Sample site	Sample number	Growth temperature	Date of cryo conservation	Date of isolation
1	SM	4	1	RT*	09.Dez	16.Nov
2	SM	4	1	RT*	09.Dez	16.Nov
3	SM	4	1	RT*	09.Dez	16.Nov
4	SM	4	1	RT*	09.Dez	16.Nov
5	SM	1	2	30°C	09.Dez	16.Nov
6	SM	2	4	30°C	09.Dez	16.Nov
7	SF	8	4	30°C	09.Dez	16.Nov
8	SF	7	1	RT*	09.Dez	16.Nov
9	SF	7	1	RT*	09.Dez	16.Nov
10	SF	7	1	RT*	09.Dez	16.Nov
11	SF	5	1	RT*	09.Dez	16.Nov
12	SF	5	1	RT*	09.Dez	16.Nov
13	SF	5	1	RT*	09.Dez	16.Nov
14	SF	5	1	RT*	09.Dez	16.Nov
15	SF	5	3	30°C	09.Dez	16.Nov
16	SF	5	3	30°C	09.Dez	16.Nov
17	SF	7	4	30°C	09.Dez	16.Nov
18	SF	8	1	RT*	09.Dez	16.Nov
19	SF	8	1	RT*	09.Dez	16.Nov
20	SF	8	1	RT*	09.Dez	16.Nov
21	SF	8	1	RT*	09.Dez	16.Nov
22	SF	5	1	RT*	09.Dez	16.Nov
23	SF	5	1	RT*	09.Dez	16.Nov
24	SF	5	1	RT*	09.Dez	16.Nov
25	SF	5	1	RT*	09.Dez	16.Nov
26	SF	7	2	30°C	10.Dez	16.Nov
27	SF	7	2	30°C	10.Dez	16.Nov
28	SF	6	3	30°C	10.Dez	16.Nov
29	SF	6	4	30°C	10.Dez	16.Nov
30	SF	6	4	30°C	10.Dez	16.Nov
31	SF	6	4	30°C	10.Dez	16.Nov
32	LOPU	1	1	RT*	09.Dez	16.Nov
33	LOPU	1	1	RT*	09.Dez	16.Nov
34	LOPU	2	3	30°C	10.Dez	16.Nov
35	LOPU	2	3	30°C	10.Dez	16.Nov
36	LOPU	2	3	30°C	10.Dez	16.Nov
37	LOPU	2	3	30°C	10.Dez	16.Nov
38	LOPU	2	3	30°C	10.Dez	16.Nov
39	LOPU	2	3	30°C	10.Dez	16.Nov
40	LOPU	2	4	30°C	10.Dez	16.Nov

41	LOPU	2	4	30°C	10.Dez	16.Nov
42	LOPU	2	4	30°C	10.Dez	16.Nov
43	LOPU	2	4	30°C	10.Dez	16.Nov
44	LOPU	2	4	30°C	10.Dez	16.Nov
45	LOPU	2	4	30°C	10.Dez	16.Nov
46	LOPU	3	2	30°C	09.Dez	16.Nov
47	LOPU	2	4	30°C	09.Dez	16.Nov
48	LOPU	2	4	30°C	09.Dez	16.Nov
49	LOPU	1	2	30°C	09.Dez	16.Nov
50	LOPU	2	1	RT*	09.Dez	16.Nov
51	LOPU	2	1	RT*	09.Dez	16.Nov
52	LOPU	2	1	RT*	09.Dez	16.Nov
53	LOPU	1	1	RT*	09.Dez	16.Nov
54	LOPU	1	1	RT*	09.Dez	16.Nov
55	LOPU	1	1	RT*	09.Dez	16.Nov
56	LOPU	1	1	RT*	09.Dez	16.Nov
57	LOPU	1	1	RT*	09.Dez	16.Nov
58	LOPU	1	1	RT*	09.Dez	16.Nov
59	LOPU	2	3	30°C	10.Dez	16.Nov
60	LOPU	2	3	30°C	10.Dez	16.Nov
61	LOPU	2	3	30°C	10.Dez	16.Nov
62	LOPU	2	3	30°C	10.Dez	16.Nov
63	LOPU	2	3	30°C	10.Dez	16.Nov
64	LOPU	2	2	30°C	10.Dez	16.Nov
65	LOPU	2	2	30°C	10.Dez	16.Nov
66	LOPU	1	1	RT*	10.Dez	16.Nov
67	LOPU	1	1	RT*	10.Dez	16.Nov
68	LOPU	1	1	RT*	10.Dez	16.Nov
69	LOPU	2	2	30°C	10.Dez	16.Nov
70	LOPU	2	2	30°C	10.Dez	16.Nov
71	LOPU	2	2	30°C	10.Dez	16.Nov
72	LOPU	2	2	30°C	10.Dez	16.Nov
73	LOPU	2	2	30°C	10.Dez	16.Nov
74	LOPU	3	4	30°C	10.Dez	16.Nov
75	LOPU	3	4	30°C	10.Dez	16.Nov
76	LOPU	3	4	30°C	10.Dez	16.Nov
77	LOPU	4	2	30°C	10.Dez	16.Nov
78	LOPU	4	2	30°C	10.Dez	16.Nov
79	LOPU	4	2	30°C	10.Dez	16.Nov
80	LOPU	4	2	30°C	10.Dez	16.Nov
81	LOPU	4	2	30°C	10.Dez	16.Nov
82	LOPU	4	2	30°C	10.Dez	16.Nov
83	LOPU	4	2	30°C	10.Dez	16.Nov
84	LOPU	3	4	30°C	10.Dez	16.Nov
85	LOPU	3	4	30°C	10.Dez	16.Nov

86	LOPU	3	4	30°C	10.Dez	16.Nov
87	LOPU	3	4	30°C	10.Dez	16.Nov
88	LOPU	3	4	30°C	10.Dez	16.Nov
89	LOPU	3	4	30°C	10.Dez	16.Nov
90	LOPU	3	4	30°C	10.Dez	16.Nov
91	LOPU	3	4	30°C	10.Dez	16.Nov
92	LOPU	3	4	30°C	10.Dez	16.Nov
93	LOPU	4	3	30°C	10.Dez	16.Nov
94	LOPU	4	3	30°C	10.Dez	16.Nov
95	LOPU	4	3	30°C	10.Dez	16.Nov
96	LOPU	4	3	30°C	10.Dez	16.Nov
97	LOPU	4	3	30°C	10.Dez	16.Nov
98	LOPU	4	3	30°C	10.Dez	16.Nov
99	LOPU	4	3	30°C	10.Dez	16.Nov
100	LOPU	4	3	30°C	10.Dez	16.Nov
101	LOPU	4	3	30°C	10.Dez	16.Nov
102	LOPU	2	1	30°C	09.Dez	16.Nov
103	LOPU	2	2	30°C	09.Dez	16.Nov
104	SF	7	3	30°C	14.Dez	16.Nov
105	SF	8	2	30°C	14.Dez	16.Nov
106	SF	6	3	30°C	14.Dez	16.Nov
107	SF	6	3	30°C	14.Dez	16.Nov
108	LOPU	4	1	30°C	14.Dez	16.Nov
109	SF	7	1	30°C	14.Jän	16.Nov
110	RAPS	1		30°C	14.Dez	23.Nov
111	RAPS	2		30°C	14.Dez	23.Nov
112	RAPS	3		30°C	14.Dez	23.Nov
113	RAPS	4		30°C	14.Dez	23.Nov
114	LOPU	4	2	30°C	14.Dez	16.Nov
115	LOPU	2	2	30°C	14.Dez	16.Nov
116	LOPU	3	1	30°C	14.Dez	16.Nov
117	SF	6	2	30°C	14.Dez	16.Nov
118	LOPU	1	2	30°C	14.Dez	16.Nov
119	LOPU	1	2	30°C	14.Dez	16.Nov
120	LOPU	1	2	30°C	14.Dez	16.Nov
121	LOPU	1	2	30°C	14.Dez	16.Nov
122	LOPU	1	2	30°C	14.Dez	16.Nov
123	LOPU	1	2	30°C	14.Dez	16.Nov
124	LOPU	1	3	30°C	14.Dez	16.Nov
125	LOPU	1	3	30°C	14.Dez	16.Nov
126	LOPU	1	3	30°C	14.Dez	16.Nov
127	LOPU	1	3	30°C	14.Dez	16.Nov
128	SF	6	1	30°C	14.Dez	16.Nov
129	SF	6	1	30°C	14.Dez	16.Nov
130	LOPU	2	1	30°C	14.Dez	16.Nov

131	SF	8	2	30°C	14.Dez	16.Nov
132	LOPU	2	1	30°C	14.Dez	16.Nov
133	LOPU	2	1	30°C	14.Dez	16.Nov
134	SF	6	3	30°C	14.Dez	16.Nov
135	SF	6	3	30°C	14.Dez	16.Nov
136	SF	5	4	30°C	14.Dez	16.Nov
137	SF	6	4	30°C	14.Dez	16.Nov
138	SF	6	4	30°C	14.Dez	16.Nov
139	SF	7	4	30°C	14.Dez	16.Nov
140	LOPU	1	3	30°C	14.Dez	16.Nov
141	LOPU	1	3	30°C	14.Dez	16.Nov
142	LOPU	1	3	30°C	14.Dez	16.Nov
143	LOPU	1	3	30°C	14.Dez	16.Nov
144	LOPU	3	3	30°C	14.Dez	16.Nov
145	LOPU	3	3	30°C	14.Dez	16.Nov
146	SF	6	4	30°C	14.Dez	16.Nov
147	LOPU	3	2	30°C	14.Dez	16.Nov
148	LOPU	1	3	30°C	14.Dez	16.Nov
149	LOPU	1	3	30°C	14.Dez	16.Nov
150	LOPU	1	3	30°C	14.Dez	16.Nov
151	LOPU	1	3	30°C	14.Dez	16.Nov
152	SF	7	3	30°C	14.Dez	16.Nov
153	SF	7	3	30°C	14.Dez	16.Nov
154	SF	7	3	30°C	14.Dez	16.Nov
155	SF	8	2	30°C	14.Dez	16.Nov
156	SF	8	3	30°C	14.Dez	16.Nov
157	SF	8	3	30°C	14.Dez	16.Nov
158	LOPU	1	3	30°C	14.Dez	16.Nov
159	LOPU	1	3	30°C	14.Dez	16.Nov
160	LOPU	4	4	30°C	14.Dez	16.Nov
161	LOPU	1	3	30°C	14.Dez	16.Nov
162	LOPU	4	4	30°C	14.Dez	16.Nov
163	LOPU	4	4	30°C	14.Dez	16.Nov
164	LOPU	4	4	30°C	14.Dez	16.Nov
165	LOPU	4	4	30°C	14.Dez	16.Nov
166	LOPU	4	4	30°C	14.Dez	16.Nov
167	LOPU	3	3	30°C	14.Dez	16.Nov
168	LOPU	3	3	30°C	14.Dez	16.Nov
169	LOPU	3	3	30°C	14.Dez	16.Nov
170	SF	8	3	30°C	14.Dez	16.Nov
171	SF	7	3	30°C	14.Dez	16.Nov
172	SF	8	2	30°C	14.Dez	16.Nov
173	SF	8	2	30°C	14.Dez	16.Nov
174	SF	5	3	30°C	14.Dez	16.Nov
175	SF	5	3	30°C	14.Dez	16.Nov

176	SF	5	3	30°C	14.Dez	16.Nov
177	SF	5	3	30°C	14.Dez	16.Nov
178	SF	5	3	30°C	14.Dez	16.Nov
179	SF	5	3	30°C	14.Dez	16.Nov
180	SF	5	3	30°C	14.Dez	16.Nov
181	SF	5	3	30°C	14.Dez	16.Nov
182	SF	6	4	30°C	14.Dez	16.Nov
188	LOPU	3	3	30°C	14.Dez	16.Nov
189	LOPU	3	3	30°C	14.Dez	16.Nov
190	LOPU	3	3	30°C	14.Dez	16.Nov
191	LOPU	3	3	30°C	14.Dez	16.Nov

*RT = Room temperature