Pollen associated microbiome and its relationship to pollution and allergens

Andrea Obersteiner

Helmholtz Zentrum München,

Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH),

Department für Umweltwissenschaften

Abteilung Mikroben-Pflanzen-Interaktion

Dissertation

der Fakultät für Biologie der
Ludwig-Maximilian-Universität München

zur Erlangung des akademischen Grad eines

Doktor der Naturwissenschaften
(Dr. rer. nat.)

Erstgutachter: Prof. Dr. Anton Hartmann

Zweitgutachter: Prof. Dr. Kirsten Jung

Eingereicht am: 09.08.2016

Tag der mündlichen Prüfung: 24.01.2017

LIST OF PUBLICATIONS

Obersteiner A, Gilles S, Frank U, Beck I, Häring F, Ernst D *et al*. (2016) Pollen-Associated Microbiome Correlates with Pollution Parameters and the Allergenicity of Pollen. PLoS ONE 11(2): e0149545. doi:10.1371/journal.pone.0149545

Obersteiner A*, Rauer D*, Reiger M, Gilles S, *et al*. (in preparation) The role of pollen-associated phytopathogenic bacteria in allergenic immune response.

*Co-Authors

POSTERS AND ORAL PRESENTATIONS

08/2014	Oral presentation on the ISME15 conference (International Symposium of Microbiological Ecology) in Seoul, South Korea
10/2014	Poster presentation on the DAK (Deutscher Allergie Kongress) in Wiesbaden, Germany
03/2016	Poster presentation on the DGAKI (Deutsche Gesellschaft für Allergologie und klinische Immunologie) in Mainz, Germany

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ANOSIM	Analysis of similarity
bp	Base pairs
BSA	Bovine serum albumin
Cy3	5, 5´-Disulfo-1, 1´-(γ-carbopentynyl)-3, 3, 3´, 3´-tetramethylindolocarbocyanin-N-hydroxysuccinimide ester
Cy5	Cy5.18 derivate, N-hydroxysuccinimide ester
dem	Demineralized
DNA	Deoxyribunocleic acid
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
et al.	et alteri, and others
EtOH	Absolute Ethanol
6-FAM	6-Carboxyfluorescein
Inc.	Incorporation
ISR	Induced systemic resistance
k	number of technical replicates
LSM	Laser scanning microscope
n	Biological replicates
NADPH	Nicotinamide adenine dinucleotide phosphate
p	Probability of obtaining a statistical level of significance
PALMs	Pollen-associated lipid mediators
PBS	Phosphate buffered saline solution
PCoA	Principal coordinate analyses
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGP	Plant Growth Promoting
R	Statistical similarity-parameter
r_s	Spearman´s correlation-parameter
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA
tRFs	Terminal restriction fragments

tRFLP	Terminal restriction fragment length polymorphism
Tris	Tris-(hxdroxymethyle)-aminomethane
UI	Urbanization Index
UV	Ultraviolet
x-gal	5-Brom-4-chlor-3-indoxyl-β-D-galactoside

ABSTRACT

In the past decades the prevalence of pollen-related respiratory allergies has increased rapidly. Causative agents are mostly airborne pollen grains of trees and grasses. The allergenic potential of these pollen grains depends on the amount of produced allergenic proteins (Bet v 1, Phl p 5) and non-allergenic adjuvant compounds, so called PALMs (pollen-associated lipid mediators). These compounds also play a role in the defense mechanisms of plants against biotic and abiotic stress.

The first aim of this thesis was to analyze the composition of the microbial colonization of allergenic birch and timothy grass pollen using cultivation dependent and cultivation independent molecular methods. In addition, the microbes should be localized on the pollen grains. A second aim was to uncover the relationship between urbanization-related pollution parameter (nitrogen dioxide NO_2, ozone O_3, ammonia NH_3, Urbanization Index UI), microbial α-diversity (Simpson, Shannon) colonizing the pollen and the expression of allergens and PALMs as well as stress induced NADPH-oxidase activity in the pollen.

To reach these aims, universal media were inoculated with pollen suspensions to isolate microbes and identify them by phylogenetic analyses (arb, BLAST). Furthermore microbial patterns and diversity were determined using community DNA isolated from pollen of birch and timothy grass collected in three consecutive years at different locations using terminal restriction fragment length polymorphism (tRFLP) analysis. These microbial patterns were used for calculating Spearman-correlations with pollution and allergen parameters. 16S-rDNA amplicon sequencing (Illumina MiSeq) was further used to determine abundances on bacterial family and genus levels. Localization studies were performed via Fluorescent *in situ* Hybridization (FISH) and confocal Laser-scanning microscopy (CLSM).

Most of the isolated microbes are known as environmental organisms from soil, plant and water. Several species show potential interactions with plants, but also possible pathogenicity against humans. Furthermore, a plant-species specific microbiome was

characterized on pollen of birch and timothy grass as evidenced by tRFLP- and 16S-rDNA amplicon sequencing. Accordingly, FISH- and CLSM-studies showed dominant groups of α-*Proteobacteria* on the surface of birch and γ-*Proteobacteria* on timothy grass pollen.

Correlation analyses of microbial tRFLP-pattern (bacterial diversity) to pollution parameters and the expression of allergen-related compounds showed significant results for several parameters (NO_2, O_3, UI; Bet v 1, PALMs). The results demonstrate that bacterial diversity correlates with anthropogenic factors like pollution, which in turn might impact the expression of allergens and hence, alters the immune-inflammatory potential of pollen.

ZUSAMMENFASSUNG

Das Ausmaß Pollen-induzierter Atemwegserkrankungen hat in den letzten Jahrzehnten rapide zugenommen. Der Großteil dieser Fälle wird durch luftübertragene Baum- und Graspollen ausgelöst. Das allergene Potential dieser Pollen hängt von der produzierten Menge allergener Proteine (Bet v 1, Phl p 5) und nicht-allergener Hilfssubstanzen, so genannter PALMs (Pollen-assoziierte Lipid-Mediatoren), ab. Diese Substanzen spielen auch eine Rolle in den Abwehrmechanismen von Pflanzen gegen biotischen und abiotischen Stress.

Erstes Ziel dieser Arbeit war, die Zusammensetzung der mikrobiellen Kolonisierung auf allergenen Birken- und Lieschgraspollen unter Verwendung kultivierungsabhängiger und kultivierungsunabhängiger molekularer Methoden zu analysieren. Zusätzlich sollte die Lokalisation Pollen-assoziierter Bakterien näher bestimmt werden. Das zweite Ziel war, die Beziehung zwischen urbanisierungsrelevanten Schadstoffparametern (Stickstoffdioxid NO_2, Ozon O_3, Ammoniakgas NH_3), der mikrobiellen α-Diversität (Simpson, Shannon) auf Pollen und der Expressionsrate von Allergenen und PALMs sowie stress-induzierter NADPH Oxidase-Aktivität in Pollen zu untersuchen.

Zunächst wurden Agar-Platten (Universal-Medien) mit Pollensuspensionen beimpft, um Mikroorganismen zu isolieren und diese mit Hilfe phylogenetischer Analysen (arb, BLAST) zu identifizieren. Weiterhin wurde in drei auf einander folgenden Jahren Proben von Birken- und Lieschgraspollen an verschiedenen Orten gesammelt, die mikrobielle Community-DNA extrahiert und mit Hilfe von tRFLP-Analysen (terminal restriction fragment length polymorphism) das mikrobielle Muster sowie die α-Diversität auf den Pollen bestimmt. Diese mikrobielle Diversität wurde unter Verwendung der Spearman-Korrelation mit gemessenen Konzentrationen an Luftschadstoffen und allergiebezogenen Substanzen in Beziehung gesetzt. Weiterhin wurde mit Hilfe von 16S-rDNA Amplikon Sequenzierung (Illumina MiSeq) die Häufigkeiten bakterieller Familien und Ordnungen bestimmt. Studien zur Lokalisierung

von Pollen-assoziierten Bakterien wurde unter Zuhilfenahme von Fluoreszenz *in situ* Hybridisierung (FISH) und konfokaler Laser-Scanning-Mikroskopie (CLSM) umgesetzt.

Der Großteil der von Pollen isolierten Mikroben ist bekannt als Umweltorganismen, die in Wasser, Boden und Pflanzen vorkommen. Einige der Arten zeigen Wechselwirkungen mit Pflanzen, zum Teil sogar humanpathogenes Potential. Weiterhin wurde sowohl anhand der tRFLP-Analysen als auch anhand der 16S-rDNA Amplikon Sequenzierung der Nachweis erbracht, dass es sich bei Birken- und Lieschgraspollen um ein Pflanzenarten-spezifisches Pollenmikrobiom handelt. Passend dazu konnte mittels FISH- und CLSM-Studien die Dominanz von α-*Proteobakterien* auf Birkenpollen sowie die Dominanz von γ-*Proteobakterien* auf Lieschgraspollen gezeigt werden.

Die Korrelationsstudien der tRFLP-Muster (Bakteriendiversität) zu Schadstoffkonzentrationen und der Expression allergiebezogener Substanzen zeigten einige signifikante Zusammenhänge (NO_2, O_3, UI; Bet v 1, PALMs). Die Ergebnisse deuten darauf hin, dass anthropogene Faktoren wie Luftschadstoffkonzentrationen die Pollen-assoziierte Bakteriendiversität beeinflusst, was sich wiederum auf die Expression allergiebezogener Substanzen auswirken könnte und somit das immunologisch-entzündliche Potential der Pollen verändert.

1 INTRODUCTION

In the past decades the prevalence of respiratory allergies and related diseases has been increasing all over the world (Burney *et al.* 1997; D'Amato *et al.* 2007). A prime causative agent of allergies are airborne pollen grains from anemochorous plants like trees, grasses or weeds causing e. g. allergic asthma, rhinitis or hay-fever symptoms. According to Pankwar *et al.* (2011) hundreds of millions of people worldwide suffer from rhinitis. In contrast to other diseases patients suffering from airborne-induced respiratory allergy are not able to simply avoid the exposure to the causative agent (D'Amato *et al.* 2007), because the surrounding environment is fully loaded by pollen grains during the pollination period. This fact is not only responsible for massive public health problems (D'Amato & Spieksma 1991), but consequently is leading to remarkable economic problems in society (Bauchau & Durham 2004; Gilles *et al.* 2012). Several grass species, like meadow foxtail (*Alopecurus pratensis*), orchard grass (*Dactylus glomerata*) or timothy grass (*Phleum pratense*) produce pollen grains belonging to the most abundant airborne pollen. Grass pollen mostly induce nasal and conjunctival symptoms (D'Amato *et al.* 2007), also inducing inflammatory immune responses (Djukanovic *et al.* 1996). In northern and middle Europe birch trees produce the most allergenic tree pollen in spring, followed by alder and hazel. Birch pollen allergens also induce nasal and respiratory symptoms (D'Amato *et al.* 2007). The allergenic potential of pollen depends not only on the amount of produced allergenic compounds, like the major birch allergen Bet v 1 or Phl p 5 of timothy grass, but also on low molecular weight molecules like pollen-associated lipid mediators, so called PALMs (Gilles *et al.* 2012), showing immune-modulatory and –stimulatory effects (Traidl-Hoffmann *et al.* 2009). Allergens, like for instance the major allergen of birch pollen Bet v 1, are part of the pathogenesis-related family (PR) of proteins that are involved in the defense mechanisms of the host (Swoboda *et al.* 1994). Also PALMs are suggested to play a role in the defense pathways of plants, for example when plants

are threatened by pathogens or are exposed to higher concentrations of heavy metal (Thoma *et al.* 2004; Loeffler *et al.* 2005).

The first goal of this thesis´ work was to assess the diversity of microbes (bacteria, fungi) associated with pollen from birch and timothy grass by cultivation and cultivation-independent molecular techniques. The localization of pollen-associated bacteria also was to be determined by staining techniques. Further, the hypothesis in this thesis was to investigate, if birch and timothy grass stressed by altered anthropogenic factors, also show differences in the associated microbial diversity along with an influenced expression rate of allergic and non-allergenic immune-modulatory compounds and thus changes in the inflammatory potential of pollen. Recent studies showed that enhanced ozone-concentrations in the air lead to elevated Bet v 1 content in birch pollen, as well as changes in the concentration of produced PALMs (Beck *et al.* 2013). Different factors are in focus being responsible for such a development, for example air and soil pollution or other man-made factors affecting the plant´s defense system in a long-lasting way.

1.1 The ecology and structure of pollen grains

Plants are sessile organisms, a property that makes reproduction processes complicated. For germination and fertilization, it is necessary that male microspores can contact the female reproductive organs of another plant. To overcome this distance problem plants, have evolved elements that are passively distributed and carry the male sperm over bigger distances - the pollen grain. In every flowering season the produced pollen grains are dispersed via different vectors, e. g. by insects (zoophilous), by water flow (hydrophilous) or also by wind (anemophilous), depending on morphological characteristics of the pollen (Sitte *et al.* 2002). In the sense of evolutionary development, pollen grains possess a central important role regarding reproducibility (Scott 1994). The stamen in the angiosperm flower harbor up to four

pollen sacs in which pollen grains are produced in great quantities. When a pollen grain arrives safely on other plants the fusion possibility of the pollen to the female stigma cells is highly selective. Depending on the type of incompatibility-system (sporophytic or gametophytic), either chemicals deposited on the exine by the tapetum of the pollen interacting with the stigma exudates or chemicals in the pollen tube interacting with the cell walls of the style, can inhibit a fertilization (Sitte *et al*. 2002). Hence, only pollen of appropriate species can adhere to the stigma and hydrate after signal exchange. Pollen tube germination follows, penetrating the aperture and female tissue until it reaches the female ovules. The fusion of the sperm with the egg and central cell ends in a zygote and produced endosperm (Wilhelmi & Preuss 1999; Zinkl *et al.* 1999; Figure 1).

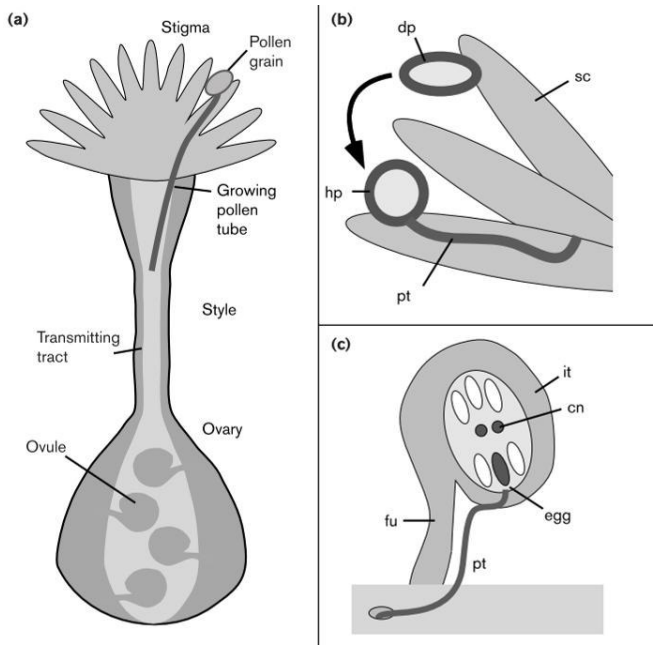

Figure 1 Anatomy of fertilization in flowering plants (Wilhelmi & Preuss 1999).
a) *Angiosperm pistil with stigma, style and ovary. Pollen tube growth and penetration of female tissue until it reaches the female ovules.*
b) *Desiccated pollen (dp) on the stigma cell surface (sc). Pollen grain hydrates (hp), when once compatible. Start of the pollen tube germination and growth (pt).*
c) *Pollen tube (pt) approaches individual ovules in the ovary. In each ovule a haploid (1n) gametophyte is arranged, containing the egg and central cell nuclei (cn). it = integument tissue, fu = funiculus.*

As already mentioned above, pollen grains are of a high evolutionary value (Scott 1994). The pollen grain acts as a biological protector of the male sperm placed in the microspore cytoplasm (Ariizumi & Toriyama 2011) inside of the pollen grain. During the period of the release from the anther to the arrival on the pistil the pollen grain is exposed to harsh conditions, ranging from desiccation, physical damage, UV-light, extreme temperatures to microbial attacks. Coping with these challenges pollen grains have developed a protective shield preventing also water loss and ensuring their viability (Scott 1994; Scott *et al.* 2004; Ariizumi & Toriyama 2011). The pollen wall consists of two main layers, the inner intine and the outer exine, also containing several layers (Figure 2).

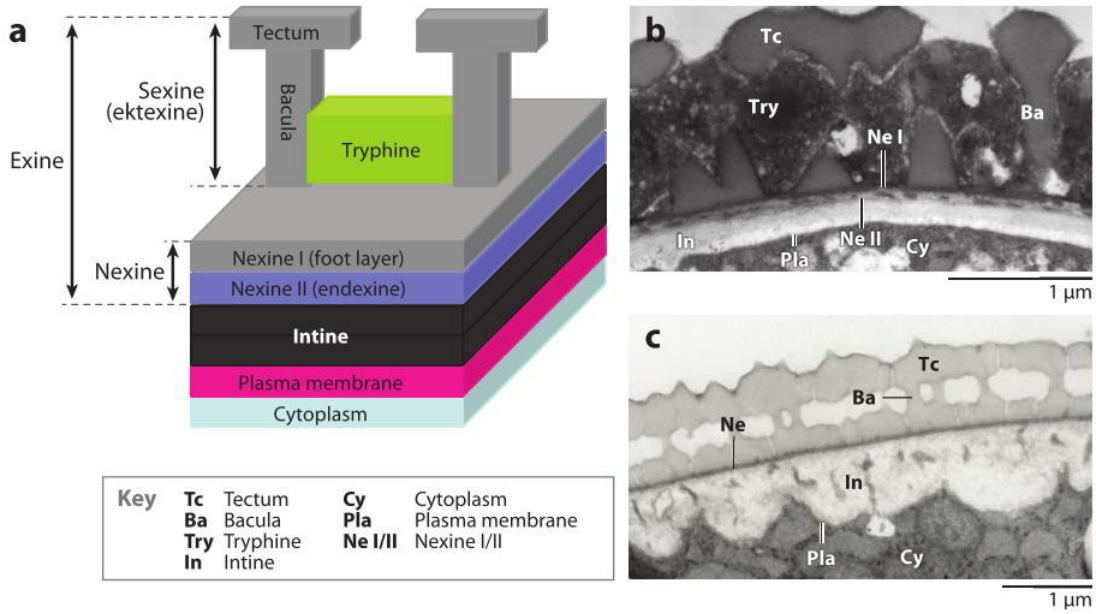

Figure 2 Typical structure of the angiosperm pollen wall (Ariizumi & Toriyama 2011).
a) *Scheme of the different appearing layers grouped mainly in exine and intine.*
b, c) *Transmission electron micrographs of cross section of exine architecture in* ***b)*** *Arabidopsis and* ***c)*** *mature rice pollen grains. This diagram represents a cross-section of a nonapertured area of pollen grains.*

The chemical composition of the intine is similar to the wall of common plant cell and made of cellulose, hemicellulose, hydrophobic proteins, hydrolytic enzymes and pectic

polymers (Wiermann & Gubatz 1992; Ariizumi & Toriyama 2011). The robust outer wall, the exine, consists of a multilayered biopolymeric material called sporopollenin appearing resistant against biological, chemical and physical degradation (Scott 1994) and thus providing strength to the exine. The wall biopolymers consists of aliphatic hydrocarbons like long chained fatty acids and phenolic compounds (Ariizumi & Toriyama 2011). The pollen wall material is related to the cell wall of fossil green algae, fern, mosses and even fungi (Scott 1994). Although the fundamental structure of the pollen walls are quite similar among taxa, a high morphological diversity, beginning by the number and arrangement of apertures (sites of pollen tube growth) to sculptural surface structures, appears. Besides the protective properties, the exine of many angiosperm species deposite compounds protecting the plant from inbreeding, making the plant self-incompatible and inadequate fertilization can be avoided (Scott 1994). The development of the exine with its special properties was an important evolutionary step for plants to adapt to and colonize the land (Ariizumi & Toriyama 2011).

1.2 Abiotic and biotic plant interactions

Plants have to deal with several environmental factors, abiotic and biotic. Among biotic factors the interaction with pathogenic and/or beneficial microbes are of great importance.

Abiotic environmental factors

Abiotic factors can lead to several changes in morphology, physiology, on both biochemical and molecular level. Different kinds of abiotic factors have damaging and harmful effects, from chemical toxicity to osmotic stress. Naturally appearing heavy metals, for example, can lead to chemical toxicity in the plant´s environment and hence cause damage to plants (John *et al.* 2009). Next to heavy metals in solid and liquid environments, air pollution is also a permanent stressor for plants.

Tiwari *et al*. (2006) studied the impact of pollutants (O_3, SO_2, NO_2) on carrot plant´s health. Plants were exposed to filtered and not-filtered air in India. Plants growing in non-filtered air showed stress symptoms like decreased photosynthetic rate, increasing lipid peroxidation or ascorbic acid content. Moreover, three further studies (Atkinson 1988; Darrall 1989; Saxe 1991) observed the negative effect of pollution on plants in terms of leaf injury, stomatal damage, premature senescence, disturbed membrane permeability and reduced growth and yield. Another extreme stress factor that can harm plants is osmotic stress, which can be induced by extreme temperatures, drought stress as well as high salinity levels. Osmotic stress causes imbalance of water-ion-relations in plant cells (Serrano *et al.* 1999). The activation and regulation of stress-related genes is started in plants in order to protect membranes, functional and structural proteins and also to get rid of free-radicals and toxic compounds (Wang *et al.* 2003).

Biotic environmental factors

Within interactions between plants and biotic environmental factors several different kinds of relationships can occur, from damaging effects of parasites or herbivores to the supporting effects of symbiotic microorganisms. In the phytosphere, meaning the interior and exterior of plants, many different organisms co-exist. A big part of these co-existing organisms is represented by microbes, although antimicrobial compounds on plants (terpenoids, benzoxacione, flavonoids, isoflavonoids) inhibit the ability of colonization for a majority of bacteria and fungi (Bais *et al.* 2006). Within this thesis we focus on the relationship between plants and microorganisms. Plants come in contact with bacteria and fungi on the surface of leaves (phyllosphere), stems and roots in the soil (rhizosphere). In addition, endophytic colonization of bacteria and fungi occurs. In the case of a phytopathogenic bacterial or fungal impact, plants are able to initially recognize invaders by certain molecular patterns (chapter 1.3), which further trigger immune responses. Also wounds inflicted by herbivores are recognized triggering wound-induced-resistance. A cascade of hormone-production (Auxin, Abscisic acid,

Gibberellin; Erb *et al.* 2012) in the plant finally induces the production of e.g. volatile defense pheromones which are effective even over greater distances (Heil & Ton 2008).

In addition to phytopathogenic microbes, being damaging and harmful for plants, also beneficial bacteria occur. These “good” bacteria can positively influence the plant growth by providing nutrients. Further, antagonistic activities against pathogenic microbes and the production of antibiotic compounds support the plant in competing to withstand the challenge of pathogen attack (Annapurna *et al.* 2013). Moreover, the production of siderophores (competition), enzymes (lysis, e. g. chitinase, ß-1,3-glutanase) and cyanid, which induces an enhanced resistance of the host plant (Kalbe *et al.* 1996), are plant-protective properties of bacteria. The bacteria, in turn, profit from the plant by an increased availability of nutrients. Last but not least, symbiotic microbes like mycorrhizal fungi or nitrogen-fixing bacteria enter specific interaction with plants, based on sophisticated recognition and interaction mechanisms (Mercado-Blanco 2015).

Endophytes

Plants are not only colonized by microbes on their outer surface but also within their various tissues. According to Mercado-Blanco (2015) every plant harbour a microbial community existing in the plant´s inside. Most endophytic microbes are soil-inhabitants and, after passing the plant´s exodermal barrier and the root pericycle, the bacteria can move inwards to even distant parts in the shoot (Compant 2005; Hardoim *et al.* 2008; Reinhold-Hurek & Hurek 2011). Only a selected number of microbes colonizing the plant´s surface are able to withstand the plant´s defense mechanism, enter and live in the interior part of plants (Hardoim *et al.* 2008). Microbes can invade via leaves, stems, roots, cotyledons, cracks or wounds. Nevertheless, this group of so called endophytes is highly divers in composition and a high variety (hundreds of taxa) of bacterial and fungal endophytes can be found in plant tissues or organs (Hallmann &

Berg 2006; Schulz 2006; Mercado-Blanco & Ludgenberg 2014). Per definition, endophytes do not cause any apparent delirious effect to the plant, but can play an important role for promoting the fitness and the development of the host plant (Mercado-Blanco 2015). The resultant, broad spectrum of possible applications in agricultural biotechnology makes the group of endophytes so interesting (Mercado-Blanco & Ludgenberg 2014). Plants provide fixed carbon and energy to plant-associated microbes, especially bacteria and fungi. So far, no information could be found about competitive interactions among endophytic microbes due to limited nutrient supply. The inside of a plant seems to provide an ecological niche with highly beneficial properties for endophytes, like protection or a constant nutrient supply. Nevertheless, the availability of specific nutrients, especially the preferably metabolized carbon sources, is assumed to represent a determining factor in forming the endophytic community (Mercado-Blanco 2015). In general, soil and plant derived conditions influuence the endophytic microbial diversity (Hardoim *et al*. 2008; van Overbeek & van Elsas 2008)

1.3 Plant defense mechanisms

Plants when being colonized by microbes and attacked by pathogens respond with several defense mechanisms similar to the innate immunity defense of lower organisms, although in plants no differentiated, mobile immune cells of adaptive immunity occur. According to Jones & Dangl (2006) the innate immune system of plants consists of two branches. Initially transmembrane pattern recognition receptors (PRR) recognize the presence of essential, conserved structures on the surface of pathogenic, non-pathogenic and also saprophytic microbes (Newman *et al.* 2013). These so called microbial-/pathogen-associated molecular patterns (MAMPs/PAMPs) contain for example bacterial lipopolysaccharide, peptidoglycans, flagellin, or fungal chitin and β-glucan, inducing specific host defense programs (Zipfel & Felix 2005; Trda *et al.* 2015). This first phase of defense is called MAMP-triggered immunity (MTI).

Subsequently the plant recognizes directly or indirectly the presence of a given effector by a group of plant resistance (R) gene products, leading to an effector triggered immunity (ETI). This interplay between plant defense and pathogenic impact is summarized in a “zigzag” model of immune response. A MAMP-induced immunity entails the production of reactive oxygen species (ROS) like nitric oxide (NO), known to trigger the synthesis of antimicrobial compounds, PALMs and also pathogenesis-related (PR) proteins (Newman *et al.* 2013; Khodai-Kalaki *et al.* 2015).

1.4 Stress-induced allergen-expression

The allergenic potential of pollen depends on the expression of allergenic proteins – the allergens – as well as on non-allergenic molecules exerting pro-inflammatory or immune-modulatory effects. Among these are NADPH oxidases, proteases, adenosine or pollen-associated lipid mediators (PALMs; Gilles *et al.* 2012). The major allergen of birch pollen, Bet v 1, belongs to the above mentioned PR-family of proteins (chapter 1.3) and is therefore involved in the defense system of the plant (Swoboda *et al.* 1994). Previous data imply that birch trees exposed to elevated ozone levels produce pollen grains with elevated levels of Bet v 1 and an altered PALMs composition, leading to higher pro-inflammatory potential (Beck *et al.* 2013). In other studies, PALMs specifically were assumed to play a role in the plant´s stress response to pathogens or heavy metal exposure (Thoma *et al.* 2004; Loeffler *et al.* 2005). Moreover, the influence of abiotic stress may have a modifying effect altering the susceptibility of plants to microbial pathogens (Bostock *et al.* 2014). Therefore, plants in a defense mode that was induced by microbes might show changes in the expression of allergens and adjuvant substances like PALMs.

1.5 Pollen impact on human health

Roughly six month per year pollen are produced by plants in huge quantities and widely dispersed in order to bring in the plant´s gene pool into the next generation. During this pollination period many people suffer from allergic diseases like pollinosis, rhinitis, asthma and others induced by airborne pollen. Higher organisms developed a highly evolved defense mechanism against undesirable invaders, the immune system. Besides the predetermined, innate immunity an additional and even more effective defense mechanism was developed by vertebrates, the so called specific, adaptive immunity. This enables vertebrates to deal with abnormal or degenerated cells produced by their own body, but also with foreign, potentially threatening organisms like parasites, viruses or microbes (Müller & Frings 2007).

The adaptive immune system of the human body consists of several different cells like T-lymphocytes, B-lymphocytes (B-cells), Plasma- and Memory cells. By an invasion of foreign or abnormal substances (= antigen), specific antigen fragments are recognized. After a cascade of complex biochemical reactions the immune response is taking place by presenting an antigen-complex on the surface of dendritic cells. Molecular interactions with T- and B-cells induce the attack of infected cells (= cell-mediated immunity) or the production of antibodies (=immune globulie, Ig) by Plasma cells (=humoral immunity). In terms of allergenic reactions an immune response is mediated by produced IgE-antibodies (Müller & Frings 2007).

Certain compounds produced by pollen also lead to an IgE-mediated immune response. Allergenic compounds, like the major allergen of birch pollen Bet v 1, and non-allergenic, immune-modulatory and -stimulatory compounds like pollen-associated lipid mediators (PALMs), can trigger the progress of an allergenic immune response and further allergic symptoms (Traidl-Hoffmann *et al.* 2009). Beside the well-known respiratory symptoms rhino-conjunctivitis and asthma, also organs like skin (atopic eczema), gastrointestinal tract (pollen associated food allergy) can be affected

by an allergenic reaction. Even most dangerous health crisis like anaphylactic shock can be triggered by pollen (reviewed in Gilles *et al.* 2012).

With regard to the increasing sensitization rates to pollen allergens, which are estimated to range from 20% to 30% in Germany (Boehme *et al.* 2013), in Europe even up to 40% (D'Amato *et al.* 2007), and the fact that environmental factors may lead to alteration in the inflammatory potential of pollen grains, this thesis would shed light on certain abiotic (pollution) and biotic (microbial load) factors due to their inter-relationship to the expression of allergen-related compounds in major allergenic pollen grains of timothy grass and birch tree.

1.6 Modern methods of molecular microbiology

With the advancement of cultivation-independent molecular genetic methods (tRFLP, 454-Pyrrosequencing, 16S-rDNA amplicon sequencing) in the last 20 years, the determination of bacterial compositions has come into more focus. No matter what kind of habitat - from the human skin, over the entomological gut to plant tissue - the colonizing bacterial community can be determined on a broad spectrum. Next to the frequently studied microbial community associated to the phyllosphere and rhizosphere, the bacterial colonization on plant pollen has also received more attention. So far, however, the studies about pollen microbiome were mostly in association to insects, like honey bees, carpenter bees or other hymenopteran species in terms of food storage and brood provision (Keller *et al.* 2013; Lozo *et al.* 2015; McFrederick & Rehan 2016). The bacterial community associated to allergenic pollen directly collected from trees or spikes were only rarely analyzed so far. Cultivation dependent analyses were performed regarding the immunological impact of pollen-associated *Bacillus sp*. to dendritic cells (Heydenreich *et al.* 2012).

In relation to the actual analyses used in this study to determine the composition of pollen-associated microbial community, these methods in molecular microbial ecology are explained in short:

Traditional cultivation methods are able to provide only a biased view of the members of microbial communities in environmental habitats, such as soils or plants. Usually only a small percentage of bacterial and fungal species can be isolated in standard laboratory media and culture conditions. This so called “plate count anomaly” is caused by conditions that are not suitable for several microorganisms (Amann *et al*. 1995; Staley & Konopka 1985), e. g. too much oxygen, low humidity, surrounding microbial community (competing/symbiotic), or many microbes that entered a non-cultivable state (Madigan & Martinko 2009). To get a comprehensive overview of the bacterial community by cultivation-dependent methods approaches including a high number of different selective media would be necessary. But, nevertheless, all the so far unknown species that have never been cultivated before would still be missing (Amann *et al*. 1995). But, still this is the only way to get a chance to study certain microbial species later in detail. Restriction fragment length polymorphism (RFLP), a molecular method for creating DNA-fragments with specific length and combined with comparative gel electrophoresis, provides a reliable tool to distinguish even morphologically very similar strains.

For the determination of bacterial (16S-rRNA coding genes) and fungal diversity (Internal Transcribed Spacer Region ITS1-2) and to compare community structure of microbes colonizing environmental habitats in a rather unbiased manner, several DNA-based molecular methods are in common use. Besides using a denaturation gradient gel electrophoresis (DGGE) or clone libraries, also a terminal restriction length polymorphism (tRFLP) and 16S-rDNA based amplicon-sequencing (Illumina, 454-Pyrosequencing) arose. As described as highly sensitive (reviewed in Dickie & FitzJohn 2007), the tRFLP-analysis was the method of choice for determining bacterial and fungal pattern composition in this thesis. Thereby three DNA based methods are

combined: 1) Amplification of labeled DNA fragments by polymerase chain reaction (PCR). 2) Amplicon-digestion with restriction endonucleases creates terminal restricted fragments. 3) A separation of DNA fragments according to their size by performing a nucleic acid electrophoresis. Insofar as no identification of the detected DNA-fragments happened in terms of tRFLP-analyses, another modern technique was used to identify the bacterial community composition: In the last years the interest in microbial community composition increased, leading to a rapid development in molecular analysis, which so far climaxed in the relative cost-efficient, high-throughput competent amplicon sequencing of the Illumina HiSeq/MiSeq platforms. Although, technical issues are mentioned in literature like PCR primer bias or a different efficiencies in DNA-extraction (Caporaso *et al.* 2012), this method can provide a preferably good overview of the pollen-associated microbiome.

Furthermore, to complete the bacterial community analyses and show actual colonization, images of pollen colonized by specific bacteria were provided to round up this thesis. A well-established staining method, the Fluorescent *in situ* Hybridization (Amann *et al*. 1990) that is able to distinguish between several bacterial levels (from class to species), was used. The technique of Fluorescent *in situ* Hybridization basically uses short DNA sequences (probes), targeting and binding on the rRNA of bacterial cells. Various and highly conserved sequence domains make the differentiation of different bacteria in a community possible (Amann & Fuchs 2008). The method is easy to handle, cost- and time-efficient and provided a further possibilityto confirm the results of other community analyses in a relative attractive way.

1.7 An overview of the thesis

Beginning with cultivation-dependent approaches, the colonization of allergenic pollen was demonstrated by identification of bacterial and fungal species isolated from birch and timothy grass pollen. Next, 16S-rDNA amplicon sequencing (Illumina MiSeq) provided a deeper insight in abundances of identified bacteria on family and genus levels. The colonization of birch and timothy grass pollen was illustrated by microscope images after the application of a Fluorescent *in situ* Hybridization method and confocal Laser-scanning microscopy (CLSM). Furthermore, bacterial and fungal colonization patterns of different pollen species were analyzed by cultivation-independent methods like terminal restricted fragment length polymorphism (tRFLP), resulting in the calculation of α-diversity indices and the illustration via ordination and dendrogram plots. The potential influence of environmental pollution parameters to the diversity of bacterial and fungal communities were studied based on correlation patterns and scatterplots. Also different contents of produced allergen concentrations in the pollen were correlated to the extent of microbial diversity.

In this doctoral thesis, data about plant parameters were provided from the cooperation partner Dr. Ulrike Frank (Institut für Biochemische Pflanzenpathologie, Helmholtz Zentrum München). Pollen-collections, allergenicity-related and environmental parameters were provided by the group of the cooperation partner Dr. Stefanie Gilles (Institut für Umweltmedizin, UNIKA-T, Augsburg).

2 MATERIAL AND METHODS

2.1 Sample collection and preparation

Catkins from birch trees (*Betula pendula*) were collected in the flowering seasons April 2013 (n = 40), 2014 (n = 55) and 2015 (n = 57) at rural and urban locations in and around Augsburg by the working group of Dr. Stefanie Gilles (UNIKA-T, TUM).

Timothy grass (*Phleum pratense*) spikes were collected from meadows in urban and suburban areas of Munich as well as from rural meadows in Bavaria during the flowering season in June of 2013 (n = 13), 2014 (n = 20) and 2015 (n = 11), in collaboration with Dr. Ulrike Frank (BIOP, Helmholtz Zentrum München) and Dr. Stefanie Gilles (UNIKA-T, Augsburg).

Freshly collected inflorescences were dried overnight in order to extract pollen preferably quantitative using sieves (Retsch®, Haan, Germany) with pore-sizes of 100 µm and 71 µm. For culturing approaches pollen grains were placed in 25%-glycerol and stored at -80 °C Freezer (Thermo Electron LED GmbH, Langenseibold, Germany) and for culture-independent methods pollen grains were prepared as described in the following chapter 2.2 and stored at -20 °C Freezer (Liebherr, Darmstadt, Germany).

2.1.1 Preparation of culture stocks

Liquid cultures of bacterial and fungal isolates were grown over night in respective media, transferred in 2 ml reaction tubes (Eppendorf) and pelleted with a centrifugation (7000 rpm for 5 min; 5417R, Eppendorf). Furthermore the pellets were washed twice in 1xPBS and re-suspended in 25%-glycerol. The cell suspension was transferred to 1.8 ml cryotubes (Karl Roth GmbH, Karlsruhe, Germany) and stored at -80 °C.

2.1.2 Preparation for community analysis

80-85 mg of freshly sieved pollen were re-suspended in 330 µl of 1xPBS and stored at -20 °C. After vortexing 100 µl of the homogenous suspension was used for DNA-isolation and further community analysis.

The remaining volume served as backup for eventually required replicates.

10x PBS	NaH_2PO_4 (100 mM)	12 g
	Na_2HPO_4 (100 mM)	14.2 g
	NaCl (1.3 M)	76 g
	ad H_2O_{dem}	1000 ml
	pH	7.2-7.4
1x PBS	10x PBS	100 ml
	H_2O_{dem}	900 ml

2.2 Cultivation of pollen-associated microbes

2.2.1 Selection of pollen samples for cultivation

First of all it was important to proof, if cultivation of pollen-associated microbes is feasible at all. Previously cultivation based approaches were performed with timothy grass pollen sampled from five different urban and rural locations in 2013 (explained in the next chapters). As this was done before the beginning of this dissertation work, unfortunately no images could have been taken showing the morphology of the single isolates. Nevertheless, the colonies isolated from timothy grass were identified as a part of this work and therefore they are also listed in the result part. Inoculation-conditions like temperature and media were similar to those described for the inoculation of birch pollen sample (chapter 2.2.2 and 2.2.3).

For further cultivation approaches performed with birch pollen a limited number (n = 5) of samples were selected from the whole pollen set collected in 2014 according

to extreme nitrogen dioxide (NO_2)-pollution values in the air, extreme content of the allergen Bet v 1 in pollen as well as high and low levels of terminal restriction fragments reflecting the bacterial contamination (Table 1).

Table 1 *Overview of the selected birch pollen samples for microbial isolations according to their content of the allergen Bet v 1, the number of different bacterial fragments (n(tRFs)) and the NO_2 pollution values at the sample sites. The values were compared to the mean value of the whole birch pollen data set 2014.*

sample site	**n(tRFs)**	**NO_2-pollution [µg/m^3]**	**Bet v 1 content [ng/10mg]**
#16	62	37	7127.41
#34	93	11	4025.97
#52	130	22.6	19870.59
#55	148	not reported	7310.69
#61	140	23.1	24382.76
mean value over all 55 pollen samples	96.8	24.2	10010.7

Color scale: low middle high

2.2.2 Preparation of media for cultivation

Media were prepared with deionized water (H_2O_{dem}) and 1.5 % agar was added (**Table 2**). For media sterilization autoclaving systems (Systec D65 and Systec V65, Systec GmbH Labor-Systemtechnik, Wettenberg, Germany) were used (121 °C, 1.013 x 10^5 Pa, 20 min).

Table 2 *Ingredients of media used for cultivation and isolation of bacteria and fungi colonizing pollen from birch and timothy grass.*

Medium	Ingredients	Amount
LB (Luria-Bertani)-medium	Peptone (casein)	10 g
Bertani 1951 (mod.)	yeast-extract	5 g
	NaCl	10 g
	H_2O_{dem}	1000 ml
	pH 7.0	
PDA (Potato-Dextrose)-Agar	Potato-extract	4 g
(Fluka, Buch, Schweiz)	Dextrose	20 g
	H_2O_{dem}	1000 ml
	pH 7.0	
BHI-Agar	Brain-heart-infusion	37 g
(Fluca, Sigma-Aldrich,	H_2O_{dem}	1000 ml
Germany)	pH 7.0	
R2A-Agar	Yeast-extract	0.5 g
(Merck, Germany)	Proteose peptone	0.5 g
	Casamino acids	0.5 g
	Glucose	0.5 g
	Soluble starch	0.5 g
	Na-pyruvate	0.3 g
	K_2HPO_4	0.3 g
	$MgSO_4 \times 7H_2O$	0.05 g
	H_2O_{dem}	1000 ml
	pH 7.0	
SDA	Glucose	32 g
	Peptone (casein)	8 g
	H_2O_{dem}	1000 ml
	pH 7.0	

2.2.3 Conditions of cultivation of microbes

Pollen samples (n = 5) suspended in 1xPBS have been used for inoculation of five different media (BHI, PDA, LB, R2A, SDA) which were incubated at three different temperatures (RT, 30°C, 37°C) for 2-3 weeks. Morphologically different colonies were transferred on new and sterile agar plates. Subsequently these isolated cultures were used for preparing long term stocks and DNA-extraction via liquid overnight culture. Therefore test tubes were filled with 4-5 ml appropriate liquid media and inoculated with a single colony. Test tubes were incubated aerobically at 175 rpm and the appropriate temperature in incubators (Innova 4200, New Brunnswick Scientific, Edison, NJ, USA; Memmert, Schwabach, Germany).

2.2.4 Differentiation of isolated pure cultures by Restriction Fragment Length Polymorphism (RFLP)

According to obtain pure cultures bacterial and fungal isolates were separated. Although colonies with different morphologies were selected, the possibility existed that some isolates were identical. Different colors of the used media led to variable appearance of the colonies. For a distinct differentiation of isolates the extracted DNA was firstly amplified performing a polymerase chain reaction (PCR). Therefore the universal primer pairs binding on eubacterial 16S rRNA coding genes (27f/1492r) and the internal transcript spacer region of fungal DNA (ITS1/ITS4; Table 3) were used. The PCR was performed using the TopTaq Polymerase Master Mix (Qiagen). A total amount of 50 µl of this master mix contained 10 ng of extracted microbial DNA. The further amplification procedure of bacterial 16S-rDNA was performed with a thermocycler (PeqLab) and performed as followed: initial denaturation at 94°C for 5 min, 30 cycles of denaturation (94°C, 45 sec), annealing (59°C, 45 sec) and elongation (72°C, 45 sec) and final elongation with an extension at 72°C for 5 min.

Table 3 *Primer pairs used for amplification of bacterial 16S-rRNA coding genes or fungal ITS1-ITS2 region. Binding positions of 16S-rDNA primers 27f/1492r refer to binding position in E.coli (Brosius et al. 1978).*

primer	5´-3´-sequence	Binding position	Ref.
ITS1	TCC GTA GGT GAA CCT GCG G	ITS1 - SSU	(White *et al.* 1990)
ITS4	TCC TCC GCT TAT TGA TAT G	ITS2 - LSU	(Gardes & Bruns 1993)
27f	AGA GTT TGA TCM TGG CTC AG	8-27	(Edwards *et al.* 1989)
1492r	GGT TAC CTT GTT ACG ACT T	1492-1513	(Turner *et al.* 1999)

For the amplification of the fungal ITS region also 10 ng of extracted DNA was used and the PCR was processed as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation (94°C, 45 sec), annealing (57°C, 45 sec) and elongation (72°C, 45 sec) and closing the procedure with a final extension at 72°C for 5 min.

In further steps amplified bacterial DNA was digested using restriction endonucleases MspI (HpaII; 5´-C^CGG-3´, incubation in Tango buffer at 37 °C for 2h; Thermofisher Scientific) and RsaI (5´-GT^AC-3´, incubation in Buffer R at 65 °C for 3h; Thermofisher Scientific) for bacterial isolates (Buddrus-Schiemann 2008) and TaiI (MaeII; 5´-ACGT-3´, incubation in buffer R at 65°C for 3h; Thermofisher Scientific) for fungal isolates (Weikl *et al.* 2016). The restriction procedure followed the manufacturer´s instruction.

R**estriction-Mastermix:**	nuclease free water	7 µl
	recommended buffer	2 µl
	restriction enzyme	1 µl
	PCR-product (10 ng)	10 µl

Thereafter, fragments of specific length were created and analyzed by horizontal agarose gel electrophoresis (chapter 2.2.5). Modified settings (75V, 120 min) led to a distinct separation of band patterns on the gel which allowed a differentiation of morphologically similar colonies (Figure 23 and Figure 24, pp. 137).

2.2.5 Horizontal agarose gel electrophoresis

DNA fragments can be separated in an electric field. Depending on the molecular weight the DNA fragments move in different speed from the cathode (+) to the anode (-). With a standard ladder, containing fragments with different length, it is easy to determine the sequence length of DNA-fragments.

To verify the correct length of amplified 16S-/ITS-DNA-fragments a horizontal 1%-agarose gel-electrophoresis (120 V, 45 min) was performed using ethidium bromide staining for visualizing the DNA-bands under UV-light. The ethidium bromide (3µg/100 ml) was included in the gel.

The gel was placed in 1x TAE-buffer. After loading the wells with the DNA samples mixed with a 6x Loading Dye (Fermentas) a separation was induced by applying 80-120 V, 100W.

2.2.6 Identification of microbial isolates from pollen

Amplified purified 16S-rRNA coding genes and ITS-regions were sequenced by SequiServe GmbH (Vaterstetten, Germany) using Sanger technology. At first the sequences were edited using the freeware BioEdit Sequence Alignment Editor version 7.2.3 (Hall 1999) and then for a rapid classification the NCBI (National Center for Biotechnology Information) online database BLAST was used. Herein the sequences were compared to other already known sequences for homologous similarities (Altschul *et al.* 1997).

For further calculation of phylogenetic trees, the sequences were aligned using the SINA alignment tool on the homepage of silva, a high quality rRNA database (http://www.arb-silva.de/aligner/; Pruesse *et al.* 2012). The calculation of phylogenetic trees of the isolated sequences together with the nearest relatives was performed using the Linux-based software package Arb (Strunk & Ludwig 1997). After proof-reading of the sequences by comparing uncertain bases to the respective electropherogram, phylogenetic trees were created using Neighbor-joining method based on a pairwise distance matrix (Saitou & Nei 1987).

2.3 Molecular-biological standard techniques

2.3.1 Extraction of chromosomal DNA from isolated pure cultures

Isolated pure cultures were grown overnight by inoculation of liquid media and incubating in a rotator at 37 °C. Then liquid cultures were centrifuged (5 min, 7000 rpm) and the pellet was transferred to a Matrix lysing tube of the FastDNA Spin Kit (MP Biomedicals). Chromosomal DNA was extracted according to the manufacturer's instruction. For cell homogenization a FastPrep 24 (40 sec, 6 m/s; MP Biomedicals) was used.

2.3.2 Extraction of microbial community-DNA

Previously several methods for bacterial/fungal DNA-extraction (phase- or spin-column techniques) including different kinds of cell-lysis-steps (mechanical or enzymatic) were compared. Based on the results of these pre-experiments the DNA-extraction with the FastDNA Spin kit for soil (MP Biomedicals) was selected to be the best in terms of reproducibility, which was tested by technical replicates (k = 3). An enhanced effectivity was achieved by including an additional enzymatic cell-lysis step to the already recommended mechanic cell-lysis in the manufacturer's instruction. Therefore

the pollen-PBS-suspension was incubated with lysozyme (1µg/µl) at 37°C for 1h. Apart from that the manufacturer's instructions were followed.

2.3.3 Purification of isolated DNA

DNA extracted from pure cultures as well as from pollen-associated microbial community were released from components of enzymatic reactions (e. g. amplification, restriction digestions) using the NucleoSpin Gel and PCR Clean up kit (Macherey & Nagel), according to the manufacturer's instruction. After purification the concentration of the amplified DNA was determined with the Nanodrop 1000 spectrophotometer (chapter 2.3.4).

2.3.4 Determination of DNA-concentrations

Nucleic acids were measured photo metrically at wavelength λ = 260 nm using a spectrophotometer (Nanodrop 1000, Thermo Scientific). To ensure the purity of DNA the purity coefficients have been calculated. The ratio of sample absorbance at λ = 260/280 nm should be approximately 1.8 for pure DNA; lower values would indicate the presence of phenol and proteins. The coefficients of absorbance at λ = 260/230 nm should be in the range of 1.8-2.2 to ensure the purity of nucleic acids. Lower values of this coefficient indicate the co-purification of other contaminants (Nanodrop 1000 v3.7 user manual).

2.4 Bacterial community determined by amplicon sequencing

Using the Illumina MiSeq platform (Illumina Inc., USA) the composition of a bacterial community can be sequenced and hence identified. The preparation of the amplicon library was basically performed by following the protocol "16S Metagenomic Sequencing Library Preparation" (Illumina Inc., USA). In the case of pollen-associated

bacterial community a relatively high abundance of plastid DNA (mitochondria, chloroplasts) was co-amplified. In order to avoid a misinterpretation of the results of bacterial community based on high amounts of plastid sequences, it was necessary to exclude them.

2.4.1 Sample selection

Initial experiments of Illumina Sequencing with 799f/1492r primers led to positive results, so a bigger sample set was analyzed. Based on statistical results of other experiments in this doctoral thesis birch pollen samples with different allergen contents (Bet v 1, Table 4), birch pollen samples collected in areas with different NO_2 concentrations (Table 5) and further grass pollen containing different amount of $PALM_{PGE2}$ (Table 6) were chosen. It is also important to mention, that pollen samples which were used for cultivation approaches in chapter 2.2 were also included in the Illumina approach.

Table 4 Selection of birch pollen samples with increasing amount of Bet v 1 allergen. *All birch pollen samples were collected in and around Augsburg, Germany.*

ID	c (Bet v 1) [ng/10 mg pollen]
01	3136
02	5071
03	5911
04	7310
05	9701
06	13833
07	16969
08	21443
09	24382
10	28147

Table 5 Selection of birch pollen sample-sites with increasing air concentration of NO_2. *All birch pollen samples were collected in and around Augsburg, Germany.*

ID	c (NO_2) [µg/m^3]
11	10.8
06	13.7
12	16.2
13	19.2
14	22.6
15	24.7
16	30.4
01	34.7
17	37.3
18	41.7

Table 6 Selection of timothy grass pollen samples with increasing amount of $PALM_{PGE2}$. *All the samples of timothy grass pollen were collected in and around Munich, Germany.*

ID	c ($PALM_{PGE2}$) [pg/ml]
19	2.96
20	3.84
21	5.31
22	6.49
23	7.39
24	8.19
25	12.55
26	16.83

2.4.2 Separation of plastid DNA performing 16S-PCR with 799f/1492r

Illumina sequencing of the bacterial 16S-community were separated from mitochondrial and chloroplast DNA by amplifying 16S-rDNA using the 799f forward primer in combination with the 1492r reverse primer (Chelius & Triplett 2001). For amplification 12.5 µl of the NEBNext High Fidelity 2x PCR Mastermix (New England Biolabs, Inc.) was used in combination with the forward and reverse primers (each 5 pmol, Table 7), 1% BSA and 100 ng of DNA in a complete approach of 25 µl per sample. The conditions of thermal cycling were as followed: 95°C for 3 min, followed by 28 cycles of 94°C for 20 sec (denaturation), 53°C for 40 sec (annealing) and 72°C for 40 sec (elongation). The final extension step was at 72°C for 7 min.

Table 7 Primer sequences. *Forward and reverse primers used for plastid exclusion doing Illumina sequencing. Binding positions of 16S primers refer to E.coli sequence (Brosius et al. 1978).*

primer	5´-3´-sequence	Binding positior	Ref.
799f	AAC MGG ATT AGA TACC CKG	781-799	(Chelius & Triplett 2001)
1492r	GGT TAC CTT GTT ACG ACT T	1492-1513	(Turner *et al.* 1999; Chelius & Triplett 2001)

Different binding positions in 16S-DNA and mitochondrial DNA led to different band lengths which were visible in a 2% agarose gel electrophoresis (chapter 2.2.5). After electrophoretic separation the 735 bp band of the amplified bacterial 16S-rDNA was eluted from the gel (Figure 3) and purified with NucleoSpin Gel and PCR Clean up kit (Macherey &Nagel; chapter 2.3.3). In order to keep the amount of randomly amplified artifacts on low level, the PCR products were produced in triplicates and pooled after purification (as described in chapter 2.3.3). Moreover, the quality of the pooled PCR-triplicates was additionally checked using the Bioanalyzer (Chips 7500 DNA, Agilent Technologies).

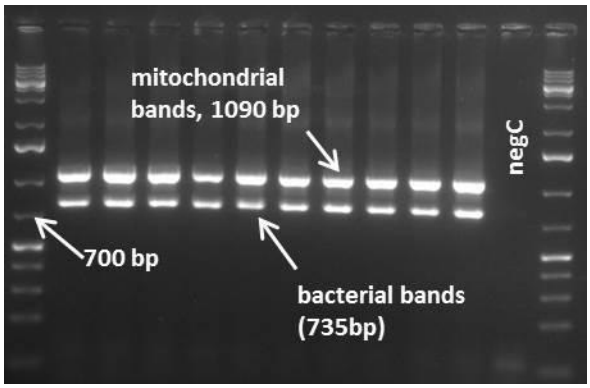

Figure 3 Agarose gel electrophoresis of amplified 16S-rRNA coding genes of **Bacteria** ***and mitochondria****. Used primer 799f binds on different positions in mitochondrial and bacterial DNA, therefore bands of different length appear in the gel and can easily be separated. negC = negative control. Standard = 1kb plus ladder (Fermetnas).*

2.4.3 Nested-PCR

Since the PCR-products obtained by the above amplification step were too long for Illumina Sequencing, a Nested-PCR with the internal primer pair 1115f and 1492r including Illumina specific adaptors (Table 8) had to be performed. NEBNext 2x PCR Mastermix and thermal cycler conditions were set up analogous to the first 16S-PCR approach for plastid separation (chapter 2.4.2). In order to keep the bias co-produced during the PCR process on a low level, the number of cycles in the Nested-PCR was 25 instead of 28 (Suzuki & Giovanonni 1996).

Table 8 Primer sequences. *Primer pairs including Illumina specific adaptors used for Nested-PCR. Binding positions of 16S primers refer to E.coli sequence (Brosius et al. 1978).*

primer	5´-adaptor-primer-3	Binding position	Ref.
illum1115f	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CAA CGA GCG CAA CCC T	1100-1115	(Redford *et al.* 2010) mod.
illum1492r	GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G GGT TAC CTT GTT ACG ACT T	1492-1513	(Turner *et al.* 1999)

Although the 16S-DNA bands were eluted the amplified products were again be tested for good quality using the Bioanalyzer (Chips 7500 DNA, Agilent Technologies).

2.4.4 Quantification of DNA with PicoGreen

To prepare a plate for quantitative measurement of the DNA concentration on the photometer the protocol of the QuantIT Kit (life technologies, Oregon, USA) was used. PicoGreen solution was added to the DNA samples and a standard dilution series was prepared with the following concentrations: 25 ng/µl, 12.5 ng/µl, 6.25 ng/µl, 3.13 ng/µl, 1.56 ng/µl, 0.78 ng/µl and 0.39 ng/µl. The dilution series was used to create a straight calibration line for an exact calculation of DNA-concentration. The coefficient

of determination R^2 of the regression line had to fit most perfectly with the standard series, at least 99.9%.

2.4.5 Indexing PCR

Firstly the DNA was diluted to 10 ng/µl, hence 1 µl was dispensed in Mastermix of the Indexing-PCR. This PCR approach is similar to the first 16S-DNA amplification at the beginning of Illumina procedure. The Mastermix with a total amount of 25 µl per sample contained 12.5 µl of NEBNext High Fidelity 2x PCR master mix (New England Biolabs, Inc.), 2.5 µl of specific indices (Table 9 and Table 10) and 10 ng of amplified 16S-rDNA.

Table 9 *The matrix shows the index-primer pairs combination (set C) for every sample for Illumina indexing-PCR. E. g. sample with ID01 was performed with primer pair N703 (reverse) and S521 (forward).*

Primer	N703	N704	N705	N706
S513		3	11	19
S515		4	12	20
S516		5	13	21
S517		6	14	22
S518		7	15	23
S520		8	16	24
S521	1	9	17	25
S522	2	10	18	26

Table 10 *Sequences of the used primers for Illumina sequencing.*
Source: http://seq.liai.org/204-2/

ID	5´-index-3
S513 forward	TCGACTAG
S515 forward	TTCTAGCT
S516 forward	CCTAGAGT
S517 forward	CGCTAAGA
S518 forward	CTATTAAG
S520 forward	AAGGCTAT
S521 forward	GAGCCTTA
S522 forward	TTATGCGA
N703 forward	AGGCAGAA
N704 forward	TCCTGAGC
N705 forward	GGACTCCT
N706 forward	TAGGCATG

The thermal cycling procedure had the following set up: heating up to 98°C for 30 sec, followed by 8 cycles containing an denaturation step at 98°C for 10 sec, a primer annealing at 55°C for 30 sec and the elongation step at 72°C for 30 sec. The final elongation was about 5 min at 72°C. Indexed PCR-products were again purified with the NucleoSpin Gel and PCR Clean up Kit (Macherey & Nagel, chapter 2.3.3).

2.4.6 Equimolar pooling

At first the DNA-concentration of the index-PCR products was quantified with a photometer using the QuantIT kit from Roche, as already explained above. Then, for pooling the index-PCR products in equimolar concentration, the DNA-concentration was calculated in nM, based on the molar mass of the four nucleic bases and the size of DNA amplicons as it was determined by the Bioanalyzer (Agilent Technologies).

$$\text{DNA-concentration in nM} = \frac{\text{DNA concentration [ng/µl]} * 10^6}{660\ \text{g/mol} * \text{average amplicon size}}$$

The DNA was diluted with recommended resuspension buffer and 4 nM of each index-PCR product was pooled and aliquots of 5 µl were prepared for Illumina sequencing. The amplicon library was sequenced with the Illumina MiSeq System (Illumina Inc., USA) using the MiSeq Reagent kit v3 (600 cycles) (Illumina Inc., USA).

2.4.7 Illumina BaseSpace Data Analysis

The final analysis of the bacterial community data performed with Illumina MiSeq was done by Dr. Matthias Reiger (UNIKA-T, Augsburg) using the Illumina BaseSpace, a platform included for Illumina-data analysis and recommended by the manufacturer (Roche, Munich).

2.5 Fluorescence *in situ* Hybridization (FISH)

For detection of bacteria on pollen from timothy grass (*Phleum pratense*) the culture-independent method Fluorescent *in situ* Hybridization (FISH) was performed. Therein fluorescently labeled probes bind complementarily to specific regions of the rRNA of bacteria, so that the occurrence of bacteria on pollen can be shown with epifluorescence (Axioplan2, Zeiss, Germany) or confocal microscopy (LSM-510-Meta, Zeiss, Germany).

2.5.1 Fixation in paraformaldehyde (PFA)

PFA induces a cross-linking of the murein layer of bacteria leading to a higher density of cell-wall. Thus stabilized, the bacteria keep their shape even over longer storage period. Freshly sieved birch and grass pollen was transferred to a 1.5 ml reaction tube, covered with 500 µl of 4 % PFA (PFA/PBS = 3:1) and stored at least 1 h at 4 °C. After

treating with PFA the pollen-bacteria suspension was washed twice with 1x PBS (centrifugation steps: 2 min ant 7500 rpm; Eppendorf Centrifuge 5417R) to get rid of remaining PFA. Fixatives were stored in Ethanol/1xPBS-solution (3:2) at -20 °C (Amann *et al.* 1990).

2.5.2 Hybridization

For hybridization, 1-3 µl of pollen suspension was placed on a microscope slide containing 8 sample wells (Roth, Karlsruhe, Germany). After evaporation of the EtOH/PBS content the pollen samples were dehydrated with Ethanol (50%, 80%, 100%) for each 3 min to enhance the osmotic pressure of the bacterial cells (Amann *et al.* 1990; Hoshino *et al.* 2008). After air drying a hybridization buffer was added including 35 % of formamide, which simulate the necessary hybridization temperature for probe binding complementarily on the rRNA sequence. The higher the amount of included formamide, the higher is the stringency during hybridization (Alqueres *et al.* 2013).

Hybridization buffer:		
	NaCl (5M)	360 µl
	Tris/HCL (1 M, pH 8)	40 µl
	Formamide	700 µl
	H_2O_{dest}	900 µl
	SDS (10% w/v)	2 µl

Previous experiments showed that the pollen grain itself, no matter if obtained from birch or timothy grass, show a rather high auto-fluorescence. This fact had to be considered when choosing the staining dye of the probes, because it turned out that probes including Fluorescein (Fluos) were not suitable for use. With a lower fluorescence the structure of the pollen was to be fine, but bacteria could not be detected; a brighter fluorescence, in contrast, led to nicely illustrated bacteria in the surrounding area of the pollen, but the pollen grain itself was much too bright as to see any structures.

The staining-dyes Cy3 and Cy5 visible in red and blue channel worked better, therefore a mixtures of the following 16S rRNA targeted oligonucleotide probes were used: EUB-338-I, II, III Cy5 binding on most bacteria and used for both plant pollen species, ALF-1b Cy3 specific for most α-*Proteobacteria* and used for birch pollen samples, and GAM-42a Cy3 specific for γ-*Proteobacteria* and used for timothy grass pollen (Table 11).

Table 11 *Probes including staining-dye (Cy3 = red; Cy5 = blue) used for FISH. Probes: EUB-338 universal for most Bacteria, ALF1b specific for most α-Proteobacteria, GAM-42a specific for γ-Proteobacteria. Binding positions of 16S probes refer to E.coli 16S sequence (Brosius et al. 1978).*

probe	sequence 5´-3´	Binding position	Reference
EUB-338	GCTGCCTCCCGTA GGAGT	338-355	(Amann *et al.* 1990)
EUB-338-II	GCAGCCACCCGTA GGTGT	338-355	(Daims *et al.* 1999)
EUB-338-III	GCTGCCACCCGTA GGTGT	338-355	(Daims *et al.* 1999)
ALF-1b	CGTTCG(C/T)TCT GAGCCAG	19-35	(Manz *et al.* 1992; Manz *et al.* 1994)
GAM-42a	GCCTTCCCACATC GTTT	1027-1043	(Manz *et al.* 1992; Manz *et al.* 1994)

1 µl of a probe working solution (30µg/ml) together with 8 µl hybridization buffer was pipetted on to the pollens sample and the slide was placed in a humid chamber. Therein the samples were incubated for at least 1.5 hours at 46 °C. Furthermore the hybridized samples were washed with pre-warmed (48 °C) washing buffer and incubated for 20 min at 48 °C (Stoffels *et al.* 2001; Hoshino *et al.* 2008) to get rid of unspecific bounded probes. Subsequently, the slide were rinsed with H_2O_{milliQ} and dried by air in the dark.

Washing buffer:	Tris/HCL (1 M, pH 8)	1 ml
	EDTA (0.5 M, pH 8)	500 µl
	NaCl (5 M)	700 µl
	H_2O_{dest}	50 ml
	SDS (10 % w/v)	50 µl

2.5.3 Confocal Laser Scanning- and Epifluorescence Microscopy

For microscopic analysis samples on the slide were embedded in Citifluor-AF1 (CITIFLUOR Ltd, London, Great Britain) and covered with cover slip (Stoffels *et al.* 2001). The bacteria colonizing pollen were visualized using a confocal laser scanning microscope LSM-510-Meta (Zeiss, Oberkochen, Germany) was used. The excitation of the fluorophores Cy3 (543 nm; red) and Cy5 (633 nm; blue) was realized with two helium neon lasers, for Fluos (488 nm; green) the microscope was equipped with an argon laser. For taking and editing images the LSM Image Browser software version 2.80 (Zeiss) was used.

2.6 Environmental and allergenicity related parameter

Pollution parameters have been measured on every sample site where birch pollen was collected in 2014 (n = 55). The ambient air-pollution concentrations of nitrogen dioxide (NO_2), ozone (O_3) and ammonia (NH_3) were measured on-site the trees by the working group of Dr. Stefanie Gilles (UNIKA-T, TUM), as well as the calculation of the Urbanization Index (UI) was performed. Moreover the content of allergens (Bet v 1; Phl p 5) and PALMs ($PALM_{PGE2}$, $PALM_{LTB4}$) were determined by the group of Dr. Stefanie Gilles via ELISA as described in Beck *et al.* (2013). All the raw data related to environmental allergen parameters were shared under an agreed cooperation for further statistical correlation analysis.

The raw data of the stress induced NADPH-oxidase activity in birch pollen was measured and provided by the collaboration partner Dr. Ulrike Frank (Institute of Biochemical Plantpathology BIOP, Helmholtz Zentrum München).

2.7 Terminal restriction fragment length polymorphism (tRFLP)

2.7.1 Amplification of bacterial 16S-rDNA fragments and fungal internal transcribed spacer region (ITS 1-2)

For tRFLP analysis an amplification of the 16S-rDNA of the bacterial community was performed. When set up the PCR 20 ng of DNA template was mixed with the master mix of TopTaq DNA polymerase kit (Qiagen). The 6-FAM-labeled forward primer 27f (Edwards *et al.* 1989) was used with the reverse primer 907r (Lane *et al.* 1985), both binding to the 16S-rRNA coding gene of most bacteria (Table 12). The further amplification procedure was performed with a thermocycler (PeqLab) and performed as followed: initial denaturation at 94°C for 5 min, 30 cycles of denaturation (94°C, 45 sec), annealing (59°C, 45 sec) and elongation (72°C, 45 sec) and final elongation with an extension at 72°C for 5 min.

Table 12 *FAM-labeled PCR-primer pair used for the amplification of 16S-rRNA. Binding positions of 16S primers refer to E.coli sequence (Brosius et al. 1978).*

primer	5´-3´-sequence	Binding position	Ref.
27f	[6-FAM] - AGA GTT TGA TCM TGG CTC AG	8-27	(Edwards *et al.* 1989)
907r	CCG TCA ATT CMT TTR AGT TT	907-926	(Lane *et al.* 1985)

To analyze fungal diversity patterns on pollen the non-coding rDNA internal spacer region (ITS) was amplified, consisting of highly variable sequence and taxonomic resolution (Lord *et al.* 2002; Anderson *et al.* 2003b). For amplification of fungal templates in mixed DNA communities the fluorescently labeled primer ITS1F (Gardes & Bruns 1993) in combination with the reverse primer ITS4 (White *et al.* 1990) was used (Table 13), as repeatedly recommended (Chen & Cairney 2002; Dickie *et al.* 2002; Lord *et al.* 2002; Anderson *et al.* 2003a; Anderson *et al.* 2003b). For amplification procedure the same conditions as described above were used, except the annealing temperature of 56 °C.

Table 13 *FAM-labeled PCR-primer pair used for the amplification of fungal ITS region.*

primer	5´-3´-sequence	Binding position	Ref.
ITS1F	[6-FAM]-CTT GGT CAT TTA GAG GAA GTA A	ITS1-SSU	(Gardes & Bruns 1993)
ITS4	TCC TCC GCT TAT TGA TAT G	ITS2-LSU	(White *et al.* 1990)

To verify the correct length of amplified 16S-/ITS-rDNA-fragments a horizontal 1%-agarose gel-electrophoresis (120 V, 45 min) was performed (chapter 2.2.5).

2.7.2 Enzymatic restriction digest

For restriction digestion approximately 400 ng of bacterial amplicon-DNA were incubated at 37°C for 2h with 5U of MspI (HpaII) (Chen *et al.* 2013) with a 4-bp recognition site (C^CGG) in recommended Tango buffer (Thermo Sientific). Fungal amplicon-DNA (approximately 100 ng) was digested at 65 °C for 3h with 5U of TaiI (MaeII; HpyCH4IV; ACGT^) in recommended buffer R (Thermo Sientific) (Weikl *et al*. 2016). After purifying the samples again with NucleoSpin Gel and PCR Clean up kit (Macherey & Nagel; chapter 2.3.3) 5 ng of the fragments were transferred to 13 µl of

HiDi™ Formamid (Applied Biosystems). This induces the DNA molecules to remain single-stranded after denaturation of fragments (5 min, 95°C). Subsequently the DNA fragments were separated by electrophoresis based on capillary system on an automated DNA sequencer (ABI 3730, Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany).

The former inclusion of the internal standard MapMarker® ROX1000 (1:400; Bioventures) ensures the determination of fragment length together with the validation of data quality. This was done by using the online software package Peak Scanner™ Software v1.0 (Life technologies).

2.7.3 tRFLP data-analysis

The tRFLP-data were transferred to online software tool t-REX (http://trex.biohpc.org/) for improving data quality: Background noise was filtered (peak area, standard deviation multiplier “1”), tRFs were aligned (clustering threshold “1bp”) and true peaks were determined, characterized by a size bigger 50bp and more than 50 fluorescent units (Culman *et al.* 2008; Chowdhury *et al.* 2013). Data Matrix of bacterial pattern was exported and statistically analyzed in PAST ver. 2.17c (Hammer *et al.* 2001). In the following analytical processes of microbial pattern fragments (tRF) with the same length were assumed to be dissipated from the same group of microorganism throughout the whole sample set.

2.7.4 Statistical analysis of tRFLP-data

The pattern of colonizing bacteria and fungi on pollen of birch (n = 55) and timothy grass (n = 20) was compared to find significant differences in composition by creating Principal Coordinates Analysis (PCoA; Index: Bray-Curtis) as well as a Cluster analysis (Index: Bray-Curtis). Furthermore the R- and p-value of a one-way ANOSIM (Analysis of Similarity) were calculated (Clarke 1993). The ANOSIM R-value describes the similarity

in community structure of groups (R = 0) or if the groups are separated and different (R = 1) from each other. An R-value up to 0.25 characterize the case of well-overlapping and similar groups, an R-value up to 0.75 means the compared groups are overlapping but still slightly separated and an R-value ranged from 0.75 to 1 describes well-separated, different groups (Clarke & Gorley 2001). The creation of the ordination diagram, the cluster dendrogram and the ANOSIM calculation was done in PAST ver. 2.17c (Hammer *et al.* 2001). Beside the absolute number of different fragments (tRFs) of a pollen sample of birch (n = 55; 2014) and timothy grass (n = 20; 2014) also two diversity indices were calculated in R (Rstudio Inc. version 0.98.953). To assign the bacterial and fungal α-diversity weighing high abundant fragments the inverse Simpson-Index (1-D), weighing less abundant fragments Shannon-Index H was calculated (Köhler *et al.* 2007). To compare bacterial and fungal diversity on pollen from the two species the significance level p was calculated by using Man U Whitney-test for not-Gaussian distributed data. For Gaussian distributed data the F-test was used to calculate the homogeneity of data. Further t-test was performed for equal/unequal data to obtain the p-value.

In order to correlate pollution and allergenicity parameters to diversity-indices of bacterial/fungal contamination of the pollen, a Spearman´s permutation p-value was calculated for not-Gaussian distributed samples. To test Gaussian distribution of data p-value of Shapiro-Wilk test was calculated (Köhler *et al.* 2007). For univariate statistics of mean/median comparison as well as for correlation analysis the statistical program PAST ver. 2.17c was used (Hammer *et al.* 2001).

2.7.5 Exclusion of plastid DNA from tRFLP-data

2.7.5.1 Determining the length of plastid terminal restricted fragment

During the amplification of the bacterial 16S-rDNA for tRFLP-analysis also sequences of plastid DNA from plant cells were co-amplified. To analyze only the composition of bacterial community on the pollen, DNA-fragments of plant originated chloroplasts and mitochondria had to be identified and excluded. Therefore the length of plastid fragments was determined by doing tRFLP-procedure using the plastid-specific reverse primer CYA781r paired with a fluorescently labeled forward primer 27f for amplification (Table 14). Further steps of amplification and restriction processes were performed analogically to the 16S-tRFLP-analysis described in chapter 2.7.1 and chapter 2.7.2.

Table 14 *Primer combination specific for the determination of plastid DNA fragments. Binding positions of 16S primers refer to E.coli sequence (Brosius et al. 1978).*

primer	5´-3´-sequence	Binding position	Ref.
27f	[6-FAM] AGA GTT TGA TCM TGG CTC AG	8-27	(Edwards *et al.* 1989)
CYA781r	GAC TAC TGG GGT ATC TAA TCC CAT T	781-805	(Nubel *et al.* 1997)

2.7.5.2 Sequencing of plastid fragment after cloning

To identify the obtained fragments as mitochondrial or chloroplast-DNA, respectively, the extracted bacterial community DNA was amplified with the same primer combination used for tRFLP-analysis, 27f/907r (chapter 2.7.1). Subsequently a cloning process was followed by using the StrataClone cloning kit (Agilent technologies). After heat shock transformation of fragments in *E.coli* plasmid pSC-A-amp/Kan and incubation over night at 37°C picked colonies were amplified by colony-PCR. For that the forward primer T3 was used in combination with the reverse primer T7 (Table 15),

both recommended in the manual of StrataClone Cloning kit because of the binding regions in plasmid sequence. Amplified fragments were purified with the NucleoSpin plasmid clean up kit (Macherey & Nagel) and sequenced by SequiServe GmbH (Vaterstetten) according to the Sanger technology.

Table 15 *Strata Clone primer pair binding in E.coli plasmid pSC-A-Amp/Kan for cloning. The binding positions of the primer refer the sequence of the plasmid pSC-A-amp/Kan.*

primer	5´-3´-sequence	Binding position	Ref.
T3 f	TTA ATT GGG AGT GAT TTC CCT	80-100	StrataClone cloning kit
T7 r	CAT TTT GCT GCC GGT C	80-100	StrataClone cloning kit

3 RESULTS

3.1 Identification of cultivable pollen-associated microbes

In the following chapter the results of identified bacterial and fungal pure cultures isolated from birch (*Betula pendula,* n = 5) and timothy grass pollen (*Phleum pratense,* n = 5) are described.

3.1.1 Fungal strains isolated from timothy grass pollen

Isolated fungi belonged to different classes, the most fungi could be cultivated from pollen samples collected on the sampling site Echinger See (ES), only one strain could be isolated from pollen samples collected in Scheyern2 (S2), Garching-Hochbrück (GH) and Eching (E). No fungi grew on plates inoculated with pollen samples from Scheyern1 (S1).

Identified fungal species belonged to *Fusarium sporotrichoides* and *Aspergillus* sp. (related to *A. fumigatus, A. niger, A. tubingensis*). Moreover two strains of *Rhodotorula glutinis* and *Rhizopus microsporus* could be cultivated, also one strain related to *Aureobasisium pullulans* (Table 16).

Table 16 *Isolated fungal species obtained from timothy grass pollen (Phleum pratense) 2013 sampled in the surrounding are of Munich, Germany. Listed is the taxonomic position, the declaration of the isolate, the nearest relative (accession number) and also the relative similarity. Sample sites (=Loc): S2 = Scheyern 2, GH = Garching-Hochbrück,E = Eching, ES = Echinger See.*

Loc	Taxonomic position	Isolate	Nearest relative (BLAST)	% similarity
S2	Sordariomycetes	PP13-58	*Fusarium sporotrichioides* ITS1-2 complete (AY188917)	100
GH	Eurotiomycetes	PP13-56	*Aspergillus niger* WHAK1 (JQ929761)	99
E	Microbotryomycetes	PP13-60	*Rhodotorula glutinis* R63 (HG532073)	100

→	Tab. 16			
Loc	**Taxonomic position**	**Isolate**	**Nearest relative (BLAST)**	**% similarity**
ES	Mucoromycotina	PP13-51	*Rhizopus microsporus* ITS1-2 complete (HQ285720)	99
	Dothideomycetes	PP13-53	*Aureobasidium pullulan*s R124 (HG532077)	99
	Eurotiomycetes	PP13-54	*Aspergillus tubingensis* USMG08 (KF669469)	100
	Eurotiomycetes	PP13-62	*Aspergillus fumigatus* FC2-2 (KF611906)	100

In the attachment images of the fungal colonies isolated from timothy grass pollen are demonstrated (Figure 21, p. 135).

3.1.2 Fungal strains isolated from birch pollen

Most of the fungal strains isolated from birch pollen samples were affiliated to the classes *Eurotiomycetes* and *Dothideomycetes*, only one strain belonging to *Ascomycetes* and one strain to *Tremellomycetes* were cultivated.

Fungi were isolated from every inoculated birch pollen sample. Strains which were mostly cultivated belong to the genera *Penicillium* (related to *P. pancosmium, P. australiense, P. vancouverense*) and *Aurobasidium* (*A. pullulans*). Further strains belonged to *Aspergillus fumigatus*, C*ladosporium australiense*, *Kwoniella betulae*, *Alternaia alternata*, *Dothideales* sp., *Cryptosphaeria eunomia* and *Davidiella macrospora* (Table 17).

Table 17 *Isolated fungi from birch pollen (Betula pendula) sampled in 2014 sampled in the surrounding area of Munich. Listed is the taxonomic position, the declaration of the isolates, the nearest relative (accession number) and also the relative similarity. Sample sites (= Loc) = #16,#34, #52, #55, #61.*

Loc	Taxonomic position	Isolate	Nearest relative (BLAST)	% similarity
#16	*Eurotiomycetes*	BP14-31	*Aspergillus fumigatus* WL002 (KR732648)	100
	Eurotiomycetes	BP14-32	*Penicilium* sp.0109CI39M4 (FN598944)	100
	Eurotiomycetes	BP14-33	*Penicilium* pancosmium DTO:268-G7 (KP329841)	100
	Dothideomycetes	BP14-34	*Cladosporium australiense* DTO 255-F3 (KP701978)	99
	Eurotiomycetes	BP14-35	*Penicillium vancouverense* CBS 126323 (NR_121512)	100
#34	*Eurotiomycetes*	BP14-36	*Penicilium* sp. 0109CI39M4 (FN598944)	100
	Tremellomycetes	BP14-37	*Kwoniella betulae* yHKS286 (KM384103)	99
	Dothideomycetes	BP14-38	*Aureobasidium pullulans* NRRL 58012 (JX462673)	100
	Dothideomycetes	BP14-34	*Cladosporium australiense* DTO 255-F3 (KP701978)	100
#52		BP14-39	Fungal endophyte sp. CZ9 (FJ176471)	98
	Dothideomycetes	BP14-40	*Aureobasidium pullulans* NRRL 58012 (JX462673)	100
	Dothideomycetes	BP14-41	*Alternaria alternata* BS14 (KP985749)	100
	Dothideomycetes	BP14-40	*Aureobasidium pullulans* NRRL 58012 (JX462673)	100
#55	*Eurotiomycetes*	BP14-32	*Penicilium* sp. 0109CI39M4 (FN598944)	100
	Dothideomycetes	BP14-42	*Dothideales* sp. P44C001 (JX188157)	97
	Dothideomycetes	BP14-40	*Aureobasidium pullulans* NRRL 58012 (JX462673)	100
#61	*Eurotiomycetes*	BP14-33	*Penicillium pancosmium* DTO:268-G7 (KP329841)	100

→ **Tab. 17**

Loc.	Taxonomic position	Isolate	Nearest relative (BLAST)	% similarity
#61	*Ascomycetes*	BP14-43	*Cryptosphaeria eunomia* var. *fraxini* CBS223.87 (KT425231)	96
	Dothideomycetes	BP14-44	*Davidiella macrospora* P134_D2_23 (JF311953)	100

In the attachment images of the fungal colonies isolated from birch pollen are shown (Figure 22, p. 136).

3.1.3 Bacterial strains isolated from timothy grass pollen

Cultivated bacterial strains that could be isolated from timothy grass pollen samples and identified according to their 16S-rDNA sequences mostly belong to Gram-positive bacteria with low DNA G+C content (*Firmicutes*) and Gram-negative γ-*Proteobateria* (Table 18). Phylogenetic tree (Figure 25-29, pp. 139) and calculated distance matrices (Figure 33-35, pp. 147) are attached..

The class *Firmicutes* was represented mainly by *Bacillus* strains (related to *B. pumilus, B. subtilis, B. amyloliquefaciens, B. savensis, B. thuringiensis, B. clausii*), which were found on all samples of the five locations studied. *Exiguobacterium* strains (related to *E. undae, E. sibiricum*), also belonging to the class of Bacilli in the phylum *Firmicutes*, was isolated from samples of three locations (Scheyern S2, Garching-Hochbrück GH, Echinger See ES). Strains which could be isolated only once and belong also to *Firmicutes* were related to *Carnobacterium maltaromanicum (*Scheyern S2) and *Enterococcus rottae* (Scheyern S2).

The class of γ-*Proteobacteria* was mainly represented by *Pseudomonas* sp. (related to *P. fluorescens, P. putida, P. orientalis*). Other isolates from this class belonged to *Erwinia persicina*, *Stenotrophomonas rhizophila* and *Pantoea anantis*.

The class of *Actinobacteria* is represented only by two isolates, namely by *Brachybacterium alimentarium* and *Streptomyces rutgersensis.*

Table 18 *Bacterial isolates obtained from timothy grass pollen (Phleum pratense) 2013 sampled in the surrounding area of Munich, Germany. Listed is the taxonomic position, the declaration of the isolate, the nearest relative (accession number) and also the relative similarity. Sample sites (=Loc): S2 = Scheyern 2, GH = Garching-Hochbrück,E = Eching, ES = Echinger See.*

Loc.	Taxonomic position (Gram)	Isolate	Nearest relative (BLAST)	% similarity
S2	γ–*Proteobacteria* (-)	PP13-27	*Erwinia persicina* LMG2691 (AJ001190)	98
	γ-Proteobacteria (-)	PP13-21=22	*Pseudomonas poae* DSM 14936T (AJ692829)	99
	γ-Proteobacteria (-)	PP13-26	*Pseudomonas trivialis* DSM 14937T (AJ692831)	99
	Actinobacteria (-)	PP13-34	*Brachybacterium alimentarium* CNRZ 925 (X91031)	99
	Actinobacteria (-)	PP13-25	*Curtobacterium flaccumfaciens* DSM20129 (AM410688)	98
	Firmicutes (+)	PP13-30	*Bacillus pumilus* MTCCB6033 (CP007436)	96
	Firmicutes (+)	PP13-19	*Bacillus amyloliquefaciens* DSM 7 (FN597644)	99
	Firmicutes (+)	PP13-29	*Exiguobacterium undae* GLPB13 (FN870072)	96
	Firmicutes (+)	PP13-24	*Exiguobacterium sibiricum* 255-15 (NR_075006)	99
	Firmicutes (+)	PP13-31/32	*Carnobacterium maltaromaticum* LMA28 (HE999757.2)	99
	Firmicutes (+)	PP13-33	*Enterococcus rottae* CCM4629 (AJ276352)	99
GH	γ–*Proteobacteria* (-)	PP13-40	*Pseudomonas fluorescens* SBW25 (AM181176.4)	99
	Firmicutes (+)	PP13-35	*Bacillus subtilis* 16S gene (FJ686816)	99
	Firmicutes (+)	PP13-36	*Exiguobacterium undae* 16S gene (F870072)	99
	Firmicutes (+)	PP13-37	*Bacillus amyloliquefaciens* DSM 7 (FN597644)	99

→ **Tab. 18**

Loc.	Taxonomic position (Gram)	Isolate	Nearest relative (BLAST)	% similarity
GH	Firmicutes (+)	PP13-38	*Bacillus pumilus* S9 (AY548955)	99
	Firmicutes (+)	PP13-39	*Bacillus safensis* PG1 (KF804070)	99
E	γ -*Proteobacteria* (-)	PP13-42	*Pseudomonas fluorescens* SBW25 (AM181176.4)	99
	γ -*Proteobacteria* (-)	PP13-43	*Pseudomonas orientalis* CFML96-170 (NR_024909)	99
	γ -*Proteobacteria* (-)	PP13-44	*Stenotrophomonas rhizophila* Asd M1-7 (FM955853)	99
	γ -*Proteobacteria* (-)	PP13-46	*Pseudomonas putida* PC36 (DQ178233)	99
	Firmicutes (+)	PP13-57	*Bacillus subtilis* VCRC B546 (AB598736)	97
	Firmicutes (+)	PP13-47=48	*Bacillus amyloliquefaciens subsp. DSM 7* (FN597644)	99
ES	γ -*Proteobacteria* (-)	PP13-03	*Stenotrophomonas rhizophila* S8 (HG421016)	97
	γ -*Proteobacteria* (-)	PP13-04	*Pseudomonas orientalis* 16S gene (AF064457)	99
	γ -*Proteobacteria* (-)	PP13-07	*Pantoea agglomerans* KB38 (JF327464)	99
	γ -*Proteobacteria* (-)	PP13-08	*Pseudomonas fluorescens* LMG 5329 (JQ974027)	99
	Firmicutes (+)	PP13-02	*Bacillus stratosphericus* 41KF2a (NR_118441)	100
	Firmicutes (+)	PP13-05	*Exiguobacterium sibiricum* 255-15 (NR_075006)	99
S1	Actinobacteria (-)	PP13-10	*Streptomyces rutgersensis* DSM 40077 (NR_119349)	99
	γ-Proteobacteria (-)	PP13-17	*Pseudomonas* sp. HC2-31 (JF312670)	93
	Firmicutes (+)	PP13-09	*Bacillus amyloliquefaciens* DSM 7 (FN597644)	99
	Firmicutes (+)	PP13-11	*Bacillus pumilus* CTSP16 (EU855197)	98
	Firmicutes (+)	PP13-18	*Bacillus amyloliquefaciens* DSM 7 (FN597644)	99

3.1.4 Bacterial strains isolated from birch pollen

The dominating group among isolated bacteria from birch pollen samples identified according to their 16S-rDNA sequence similarities was also the class *Firmicutes*, similar to the isolates from timothy grass pollen. Further a few γ-*Proteobacteria* and *Actinobacteria* were cultivable, as well as one strain related to α-*Proteobacteria* (Table 19).

Most of the *Firmicutes* related isolates were affiliated to the genus *Bacillus* (related to *B. thuringiensis, B. thuringiensis serovar israelensis, B. subtilis, B. pumilus, B. licheniformis, B. oleronius, B. simplex)* and to *Laceyella sacchari*.

Each of the four isolated strains clustered within the phylum γ-*Proteobacteria* and was affiliated to the genera *Serratia* (*S. liquefaciens*), *Erwinia* (*E. billiningiae*), *Pseudomonas* (*P. graminis*) and *Pantoea* (*P. agglomaerans*).

Nearest relatives of isolated *Actinobacteria* were *Micrococcus luteus*, *Streptomyces mutabilis* and *Curtobacterium flaccumfaciens*.

The isolated strain of *Paracraurococcus sp.* belonging to the family of *Acetobacteriaceae* is the only one belonging to α-*Proteobacteria*.

Table 19 *Bacterial isolates obtained from birch pollen (Betula pendula) 2014 sampled in the surrounding area of Augsburg, Germany. Listed is the taxonomic position, the declaration of the isolates, the nearest relative (accession number) and also the relative similarity. Sample sites (= Loc) = #16,#34, #52, #55, #61. Status: 20.06.2016*

Loc.	Taxonomic position (gram)	Isolate	Nearest relative (BLAST)	% similarity
#16	γ-*Proteoabcteria* (-)	BP14-01	*Serratia liquefaciens* ATCC 27592 (NR_122057)	100
	γ-*Proteoabcteria* (-)	BP14-02	*Erwinia billingiae* EB661 (NR_102820)	100
	γ-*Proteoabcteria* (-)	BP14-04	*Pseudomonas graminis* DSM 11363 (NR_026395)	99

→ **Table 19**

Loc.	Taxonomic position (gram)	Isolate	Nearest relative (BLAST)	% similarity
#16	Firmicutes (+)	BP14-03	*Bacillus thuringiensis serovar israelensis* AM65-52 (CP013275)	99
	Firmicutes (+)	BP14-013	*Bacillus subtilis* BY-3 (KC961634)	99
	Firmicutes (+)	BP14-17	*Bacillus pumilus* 38 (KF923448)	99
	Firmicutes (+)	BP14-18	*Laceyella sacchari* VTT E-062990 (EU430566)	99
#34	α-*Proteobacteria* (-)	BP14-20	*Paracraurococcus sp. 1N-11 16S (EU379242)*	99
	Firmicutes (+)	BP14-05	*Bacillus licheniformis* W61 (KC441866)	99
	Firmicutes (+)	BP14-19	*Bacillus oleronius* W22 (KC441834)	99
#52	γ-*Proteoabcteria* (-)	BP14-07	*Pantoea agglomerans* 16S (EU598802)	99
	Actinobacteria (-)	BP14-09	*Miccrococcus luteus* JGTA-S5 (KT805418)	99
	Actinobacteria (-)	BP14-24	*Streptomyces mutabilis* C.B.239 (KF991631)	99
	Actinobacteria (-)	BP14-25	*Curtobacterium flaccumfaciens* SAP758.3 (JX067681)	99
	Firmicutes (+)	BP14-13	*Bacillus subtilis* BY-3 (KC961634)	99
	Firmicutes (+)	BP14-21	*Bacillus thuringiensis HS18-1 (CP012099)*	99
	Firmicutes (+)	BP14-22	*Bacillus simplex* N25 (GU086427)	99
	Firmicutes (+)	BP14-23	*Bacillus licheniformis* MS5-14 (EU718490)	99
#61	Firmicutes (+)	BP14-03	*Bacillus thuringiensis serovar israelensis* strain AM65-52 (CP013275)	99
	Firmicutes (+)	BP14-30	*Bacillus pumilus HBP8 (DQ275671.1)*	100
	Firmicutes (+)	BP14-03	*Bacillus thuringiensis serovar israelensis* strain AM65-52 (CP013275)	99
	Firmicutes (+)	BP14-13	*Bacillus subtilis* BY-3 (KC961634)	100
	Firmicutes (+)	BP14-15	*Bacillus pumilus HBP8 (DQ275671.1)*	99
	Firmicutes (+)	BP14-27	*Bacillus pumilus* BFB30 (KF512664)	99
	Firmicutes (+)	BP14-28	*Bacillus simplex* TP-snow-C5 (HQ327114)	99
#55	γ-*Proteobacteria* (-)	BP14-28	*Pseudomonas graminis* DSM 11363 (NR_026395)	98

→ **Table 19**

Loc.	Taxonomic position (gram)	Isolate	Nearest relative (BLAST)	% similarity
#55	Firmicutes (+)	BP14-11	*Bacillus subtilis* H-3 (KT273284)	99
	Firmicutes (+)	BP14-13	*Bacillus subtilis* BY-3 (KC961634)	100
	Firmicutes (+)	BP14-15	*Bacillus pumilus* HBP8 (DQ275671)	99
	Firmicutes (+)	BP14- 21	*Bacillus thuringiensis HS18-1 (CP012099)*	99

The morphology of the bacterial colonies isolated from birch pollen is demonstrated in the attachment section (Figure 20, p. 134). Phylogenetic trees (**Figure 30**-32, pp. 144) and distance matrices (Figure 36-38, pp. 149) are attached.

3.2 Diversity analysis of microbial community

In the following chapter the results of bacterial and fungal community analyses obtained from birch (*Betula pendula*) and timothy grass pollen (*Phleum pratense*) collected in three consecutive years, analyzed by tRFLP-pattern, α-diversity and community composition are described.

3.2.1 Fungal tRFLP-pattern analysis

The comparison of the fungal composition on birch- and timothy grass-pollen collected in 2013, 2014 and 2015 repeatedly resulted in significant differences. By performing a PCoA and Cluster analysis (Figure 4) it was shown that for all three years 2013 (Figure 4A/B), 2014 (Figure 4C/D) and 2015 (Figure 4E/F) the 95 %-confidence interval of PCoA slightly overlaps and the branches of the cluster analysis formed clustered groups. According to an ANOSIM calculation the differences of pollen from birch and timothy grass sampled in 2013 (R_{2013} = 0.34, p_{2013} = 0.0001), 2014 (R_{2014} = 0.51,

p_{2014} = 0.0001) and 2015 (R_{2015} = 0.35, p_{2015} = 0.0004) are significantly divers regarding the fungal composition.

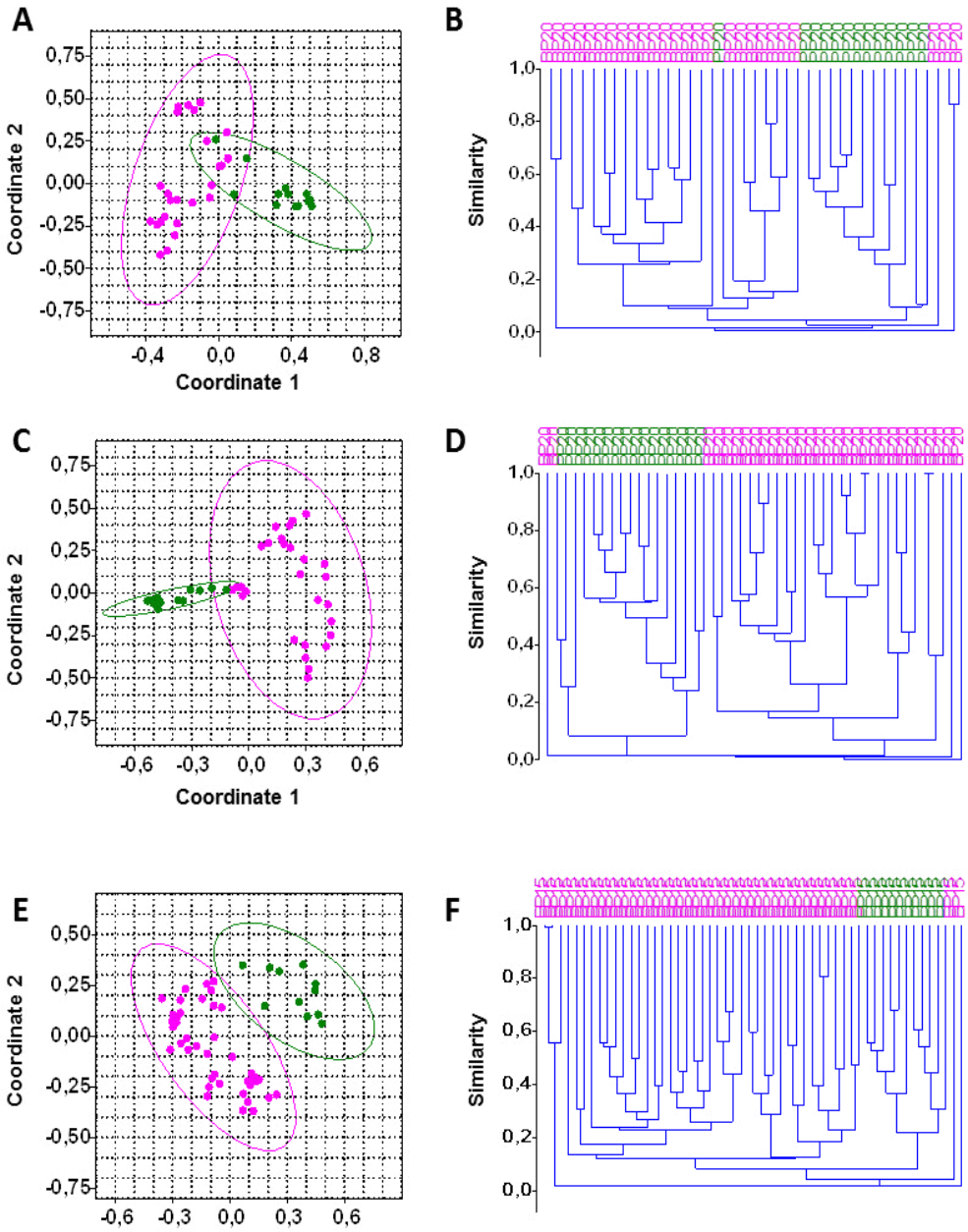

Figure 4 Comparison of fungal pattern on pollen from birch and timothy grass.
A, B) <u>Pollen collected in 2013:</u> *PCoA (Bray-Curtis) performed to compare fungal pattern on birch- (**Betula pendula**; n = 27; **magenta**) and timothy grass-pollen (**Phleum pratense**; n = 13; **green**). Hardly overlapping 95%-confidence intervals show distinct differences, the significance*

could be confirmed by ANOSIM (R = 0.34, p = 0.0001). In the cluster dendrogram (Bray-Curtis) pollen from both species are forming separated groups which are not so distinct separated, but significant.
C, D) ***Pollen collected in 2014:*** *PCoA (Bray-Curtis) performed to compare fungal pattern on birch- (**Betula pendula**; n = 31; **magenta**) and timothy grass-pollen (**Phleum pratense**; n = 15; **green**). Hardly overlapping 95%-confidence intervals show a distinct difference, the significance could be confirmed by ANOSIM (R = 0.51, p = 0.0001). The cluster dendrogram (Bray-Curtis) also demonstrates that the separation of the two pollen species due to bacterial pattern (Obersteiner et al. 2016).*
E, F) ***Pollen collected in 2015:*** *PCoA (Bray-Curtis) performed to compare fungal pattern on birch- (**Betula pendula**; n = 43; **magenta**) and timothy grass-pollen (**Phleum pratense**; n = 11; **green**). Completely separated 95%-confidence intervals show distinct differences, the significance could be confirmed by ANOSIM (R = 0.35, p = 0.0004). In the cluster dendrogram (Bray-Curtis) pollen from both plant species are forming separated groups also attesting the significant difference.*

3.2.2 Fungal α-diversity based on tRFLP patterns

In season 2013 and 2014 the fungal diversity differed significantly comparing birch and timothy grass pollen, whereas the diversity on timothy grass pollen was higher for both years (2013: $P_{shan} < 0.001$, $p_{simp} < 0.001$, Figure 5A/B; 2014: $p_{shan} = 0.005$, $p_{simp} = 0.006$, Figure 5C/D). In the year 2015 the fungal diversity on birch- and timothy grass-pollen showed no significant distinctions ($p_{shan} = 0.771$, $p_{simp} = 0.673$, Figure 5E/F).

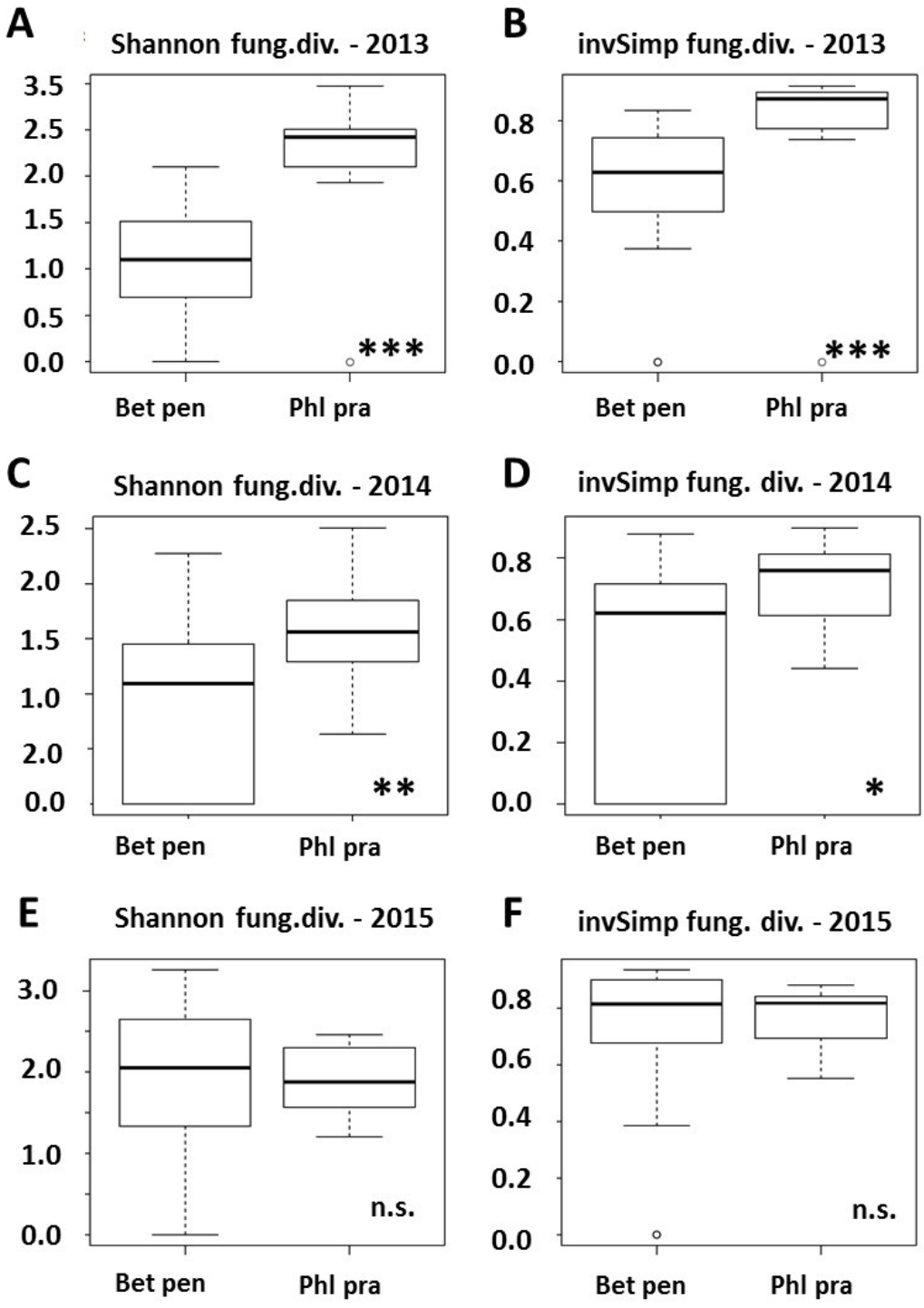

Figure 5 Comparison of fungal diversity on pollen from birch and timothy grass.
A, B) <u>Pollen collected in 2013:</u> *Boxplot of Shannon diversity Index H and invers Simpson diversity Index of fungal community on birch- (Betula pendula; n = 27; Bet pen) and timothy grass-pollen (Phleum pratense, n = 13; Phl pra) collected in 2013. In comparison the two groups*

differs significantly due to Shannon diversity (U-test, p < 0.001) and invSimpson diversity 1-D (U-Test, p < 0.001) of fungal composition.
***C, D) <u>Pollen collected in 2014:</u>** Boxplot of Shannon diversity Index H and invers Simpson diversity Index of fungal community on birch- (Betula pendula; n = 30; Bet pen) and timothy grass-pollen (Phleum pratense, n = 16; Phl pra) collected in 2014. In comparison the two groups differs significantly due to Shannon diversity (U-test, p = 0.005) and invSimpson diversity 1-D (U-Test, p = 0.006) of fungal composition (Obersteiner et al. 2016).*
***E, F) <u>Pollen collected in 2015:</u>** Boxplot of Shannon diversity Index H and invers Simpson diversity Index of fungal community on birch- (Betula pendula; n = 43; Bet pen) and timothy grass-pollen (Phleum pratense, n = 11; Phl pra) collected in 2015. In comparison the two groups differs not significantly due to Shannon diversity (U-test, p = 0.771) nor due to invSimpson diversity 1-D (U-Test, p = 0.673) of fungal composition.*

3.2.3 Bacterial tRFLP-pattern analysis

The bacterial pattern on pollen from timothy grass differed significantly to the bacterial pattern on pollen from birch tree. Comparing the calculated ANOSIM-values of microbial patterns of birch and timothy grass pollen significant differences in all three pollen-collection seasons 2013 (R = 0.99; p = 0.001), 2014 (R = 0.81, p = 0.0001) and 2015 (R = 0.66, p = 0.0001) appear. The 95%-confidence intervals of performed PCoA are not or hardly overlapping (Figure 6A/C/E). In the cluster dendrogram the separation of pollen-associated bacterial pattern between the two plant species is distinct for samples collected in 2013, 2014 as well as in 2015 (Figure 6B/D/F).

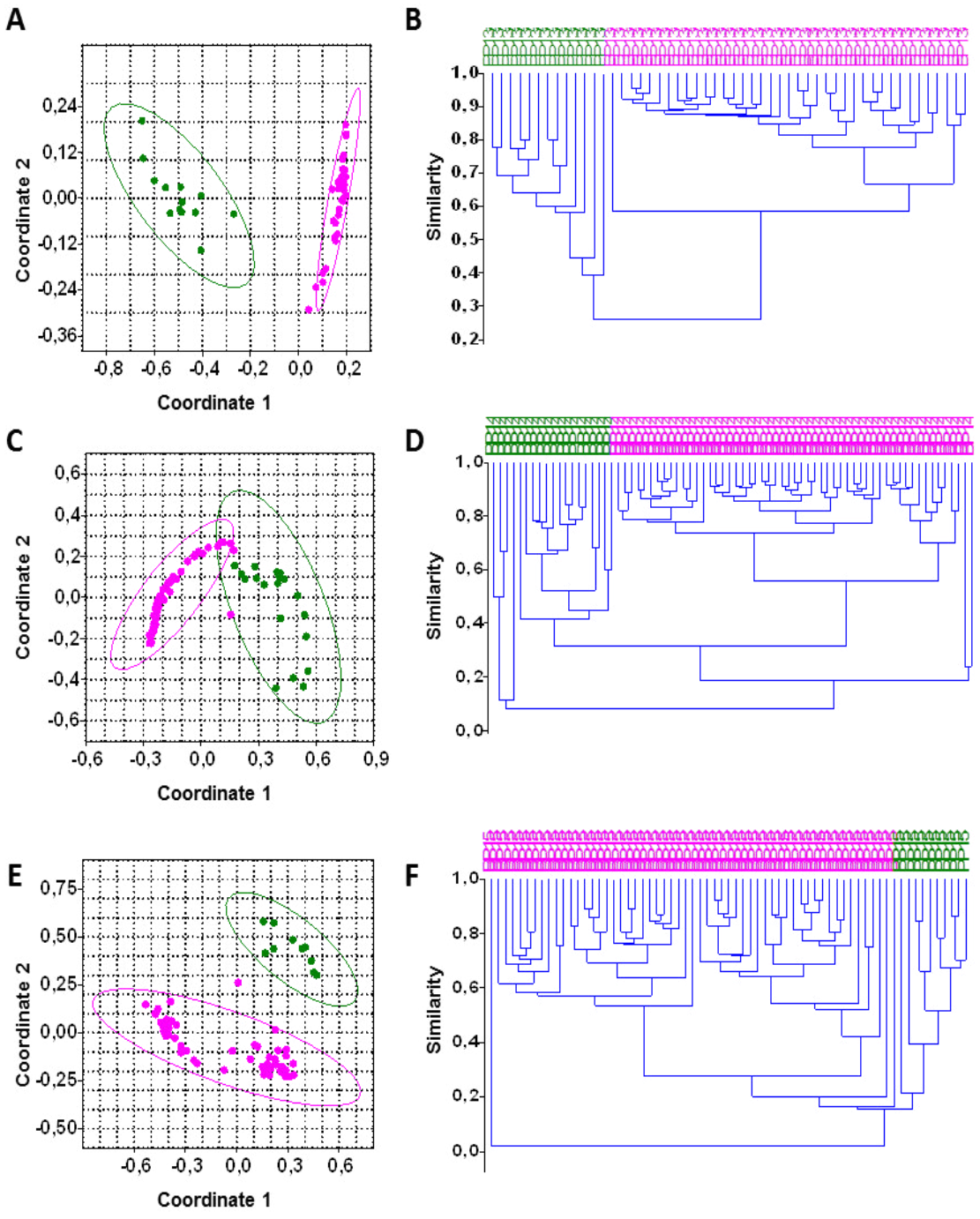

Figure 6 Comparison of bacterial pattern on pollen from birch and timothy grass.
A, B) ***<u>Pollen collected in 2013:</u>*** *PCoA (Bray-Curtis) performed to compare bacterial pattern on birch- (**Betula pendula**; n = 40; **magenta**) and timothy grass-pollen (**Phleum pratense**; n = 13; **green**). Completely separated 95%-confidence intervals show distinct differences, the significance could be confirmed by ANOSIM (R = 0.99, p = 0.001). In the cluster dendrogram (Bray-Curtis) pollen from both plant species collected in 2013 are forming separated groups*

also attesting the significant difference.
C, D) ***Pollen collected in 2014:*** *PCoA (Bray-Curtis) performed to compare bacterial pattern on birch- (Betula pendula; n = 55;* ***magenta****) and timothy grass-pollen (****Phleum pratense****; n = 20;* ***green****). Hardly overlapping 95%-confidence intervals show a distinct difference, the significance could be confirmed by ANOSIM (R = 0.81, p = 0.0001). The cluster dendrogram (Bray-Curtis) also demonstrates that the separation of the two pollen species collected in 2014 due to bacterial pattern (Obersteiner et al. 2016).*
E, F) ***Pollen collected in 2015:*** *PCoA (Bray-Curtis) performed to compare bacterial pattern on birch- (****Betula pendula****; n = 57;* ***magenta****) and timothy grass-pollen (****Phleum pratense****; n = 11;* ***green****). Completely separated 95%-confidence intervals show distinct differences, the significance could be confirmed by ANOSIM (R = 0.66, p = 0.0001). In the cluster dendrogram (Bray-Curtis) pollen from both plant species collected in 2015 are forming separated groups also attesting the significant difference.*

3.2.4 Bacterial α-diversity based on tRFLP-patterns

Timothy grass and birch pollen collected in 2013, 2014 and 2015 differ significantly according to the diversity of bacterial colonization (Figure 7). When indexing the bacterial diversity for all three seasons, birch pollen showed a significant higher diversity due to rare species (Shannon; $p_{2013} = 0.006$, Figure 7A; $p_{2014} = 0.009$, Figure 7C; $p_{2015} < 0.001$, Figure 7E). Also for dominant species (Simpson inv. index) on birch pollen a significant higher diversity occurred in year 2013 ($p < 0.001$, Figure 7B) and 2015 ($p = 0.013$, Figure 7F), but not so in 2014 ($p = 0.78$, Figure 7D). The bacterial diversity on pollen from birch as well as from timothy grass in 2015 generally seemed to be lower than in the former two year

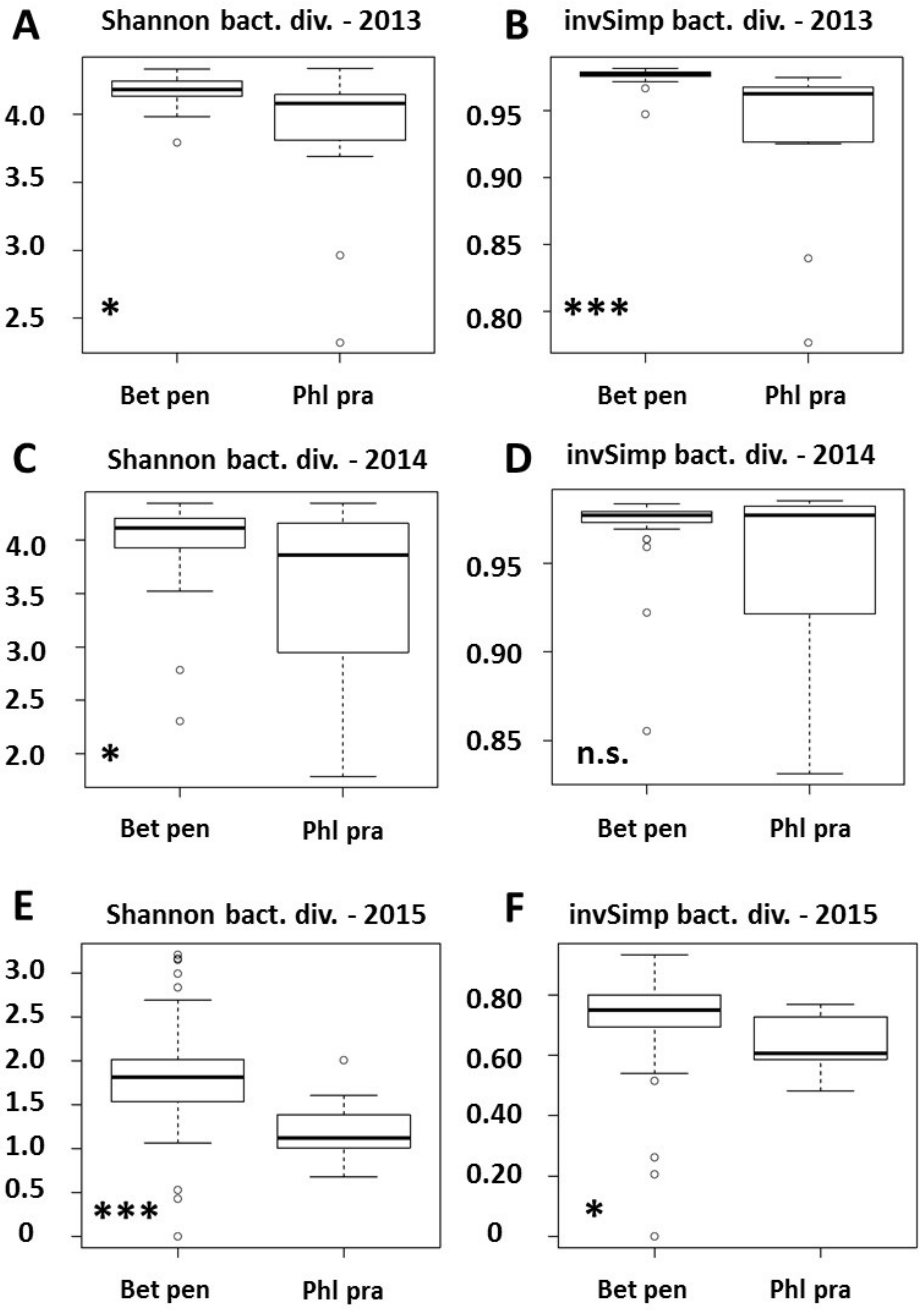

Figure 7 Comparison of bacterial diversity on pollen from birch and timothy grass.
A, B) <u>Pollen collected in 2013:</u> *Boxplots of Shannon diversity Index H and invers Simpson diversity Index (1-D) of bacterial community on birch (Betula pendula, n = 40; Bet pen) and timothy grass pollen (Phleum pratense, n = 13; Phl pra). In comparison the two groups differs significantly due to Shannon diversity (U-test, p = 0.006), and also significantly due to invSimpson diversity 1-D of bacterial composition (U-test, p < 0.001).*
C, D) <u>Pollen collected in 2014:</u> *Boxplots of Shannon diversity Index H and invers Simpson*

diversity Index (1-D) of bacterial community on birch (Betula pendula, n = 55; Bet pen) and timothy grass-pollen (Phleum pratense, n = 20; Phl pra). In comparison the two groups differs significantly due to Shannon diversity (U-test, p = 0.009), but not significantly due to invSimpson diversity 1-D of bacterial composition (U-test, p = 0.78) (Obersteiner et al. 2016). ***E, F) Pollen collected in 2015:*** *Boxplots of Shannon diversity Index H and invers Simpson diversity Index (1-D) of bacterial community on birch- (Betula pendula, n = 57; Bet pen) and timothy grass-pollen (Phleum pratense, n = 11; Phl pra). In comparison the two groups differs significantly due to Shannon diversity (U-test, p < 0.001), and also significantly due to invSimpson diversity 1-D of bacterial composition (U-test, p = 0.013).*

3.2.5 Bacterial community identification by 16S-rDNA amplicon sequencing

3.2.5.1 Bacterial composition on timothy grass pollen

On class-level the bacterial community composition associated to pollen from timothy grass did not show a consistent level in bacterial abundances over the eight analyzed samples, although γ-*Proteobacteria* were dominant in most cases (Figure 8a).

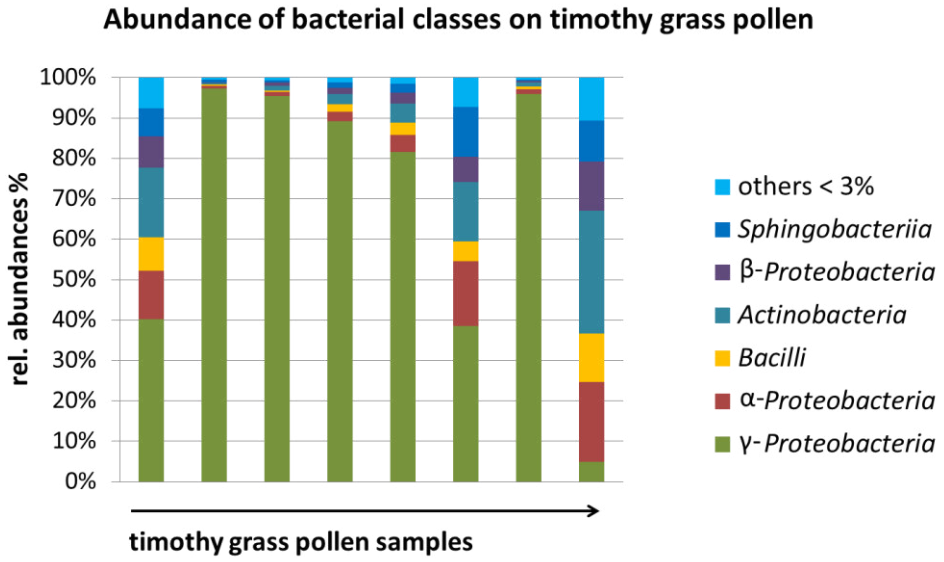

Figure 8a Timothy grass pollen associated bacterial community on class level. *Composition of bacterial community on class-level isolated from timothy grass pollen (Phleum pratense, n = 8) and determined by 16S-rDNA amplicon sequencing. Given are the relative abundances.*

When examining the mean-values of bacterial abundances a distinct dominant class arises. The bacterial composition on timothy grass pollen contained predominantly species of γ-*Proteobacteria* (mean 68% ± 35). Several classes appear with an abundance lower 10%, like *Actinobacteria* (mean 9% ±9), α-*Proteobacteria* (mean 7%±7), *Bacilli* (mean 4% ±4), β-*Proteobacteria* (mean 4%±4) and *Sphingobacteriia* (mean 4%±4).The classes occurring less often than 3% are cumulatively named others (mean 4%±4) (Figure 8b).

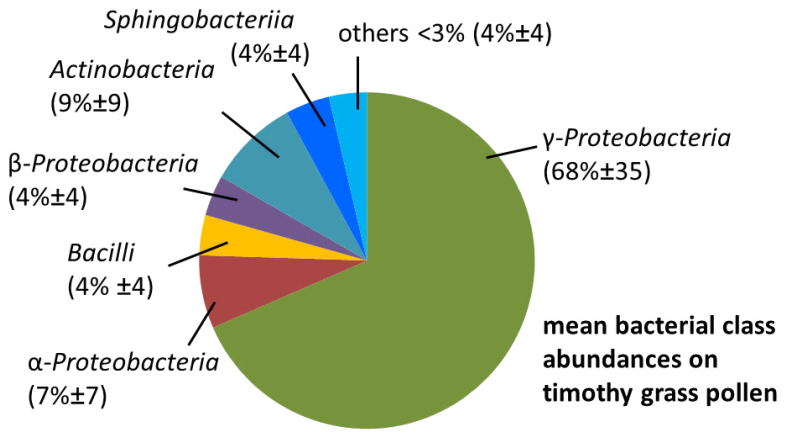

Figure 8b Timothy grass pollen associated bacterial community on class level. *Composition of bacterial community on class-level isolated from timothy grass pollen (Phleum pratense, n = 8) and determined by 16S-rDNA amplicon sequencing. Given are the mean values of the relative abundances and the standard deviation.*

On genus-level the amplicon sequencing of bacterial community colonizing timothy grass pollen resulted in a similar inconsistent abundance distribution due to different samples.The genera *Dickeya*, *Erwinia* (both γ-*Proteobacteria*) and *Pseudomonas* (*Bacilli*) belong to already detected dominant classes. *Hymenobacter* is a genus belonging to the class of *Bacterioidetes*, cumulatively shown in the group of others (Figure 9a).

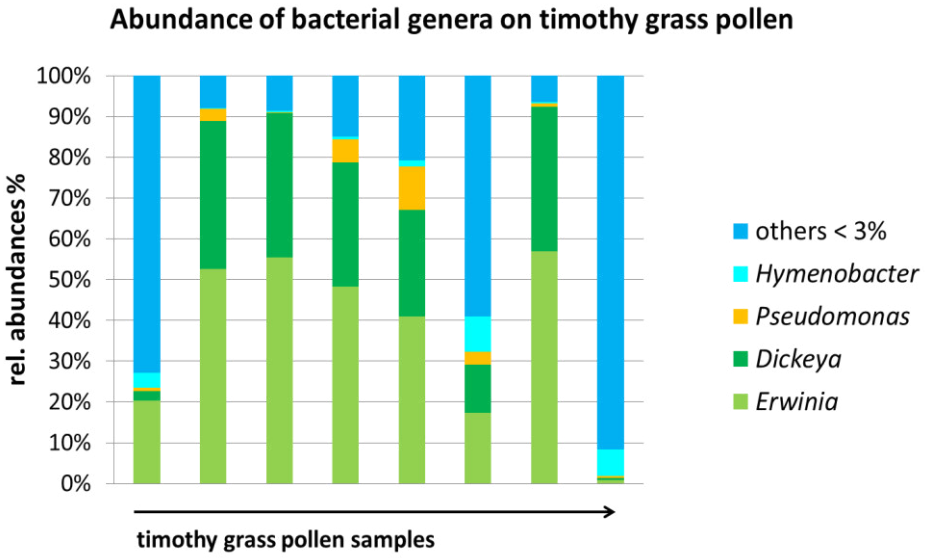

Figure 9a Timothy grass pollen associated bacterial community on genus level. *Composition of bacterial community on* genus-level isolated from timothy grass pollen (Phleum pratense, n = 8) *and determined by 16S-rDNA amplicon sequencing*. Given are the relative abundances.

Examining the mean-value of bacterial genus-abundances the community was dominated by *Erwinia* sp. (mean 36%±21) and *Dickeya* sp. (mean 22%±15), both belonging to the class of γ-*Proteobacteria*. The genera of *Pseudomonas sp.* (mean 3%±3; class: *Bacilli*), and *Hymenobacter* sp. (mean 3.5%±3; class: *Bacterioidetes*) occur with more than 3% abundance, whereas a high relation up to 29% ±26 of the bacterial genera in the community appeared with an abundance < 3% (Figure 9b).

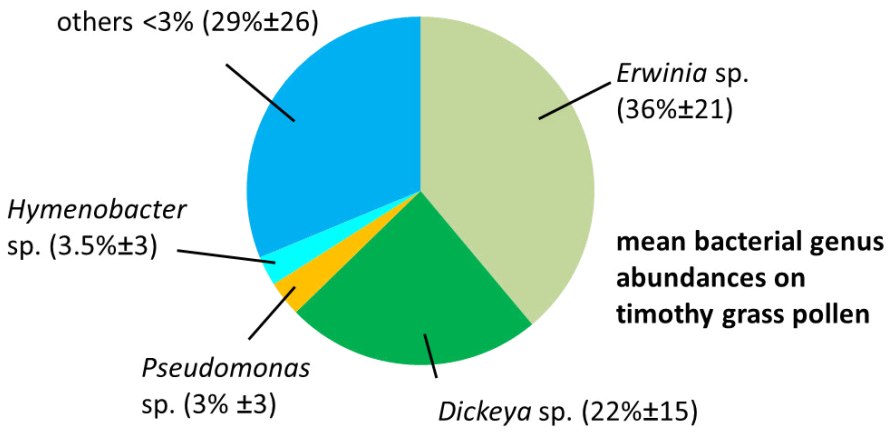

Figure 9bTimothy grass pollen associated bacterial community on genus level. *Composition of bacterial community on* genus-level isolated from timothy grass pollen (Phleum pratense, n = 8) *and determined by 16S-rDNA amplicon sequencing*. Given are the mean-values of the relative abundance and the standard deviation.

3.2.5.2 Bacterial composition on birch pollen

On class-level the bacterial community composition associated to pollen from birch showed a higher consistency level in bacterial abundances over the 10 analyzed samples, than the bacterial community on timothy grass pollen. Predominantly the classes of α-*Proteobacteria* and *Bacilli* were dominant in all cases (Figure 10a).

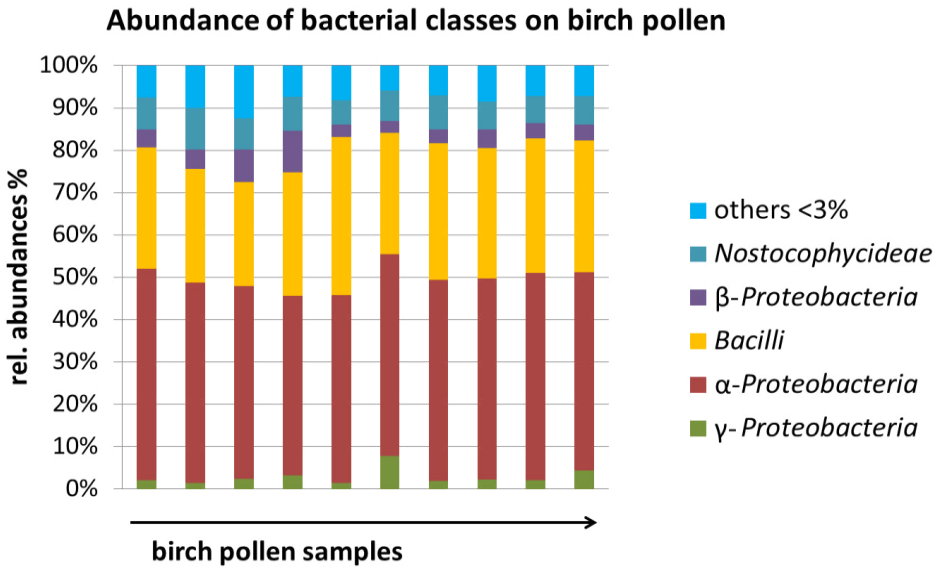

Figure 10a Birch pollen associated bacterial community on class level. *Composition of bacterial community on* class-level isolated from birch pollen (Betula pendula, n = 10) *and determined by 16S-rDNA amplicon sequencing*. Given are the relative abundances.

Examining the mean-values of bacterial class abundances community on birch pollen was dominated by α-*Proteobacteria* (mean 43%±6) and Gram-positive *Bacilli* (mean 27%±4). The classes of *Nostocophycideae* (mean 7%±2), β-*Proetobacteria* (mean 4%±2) and γ-*Proteobacteria* (mean 3%±0.6) occur with more than 3% abundance, whereas 5% (±0.5) of the bacterial classes in the community appeared with an abundance < 3% (Figure 10b).

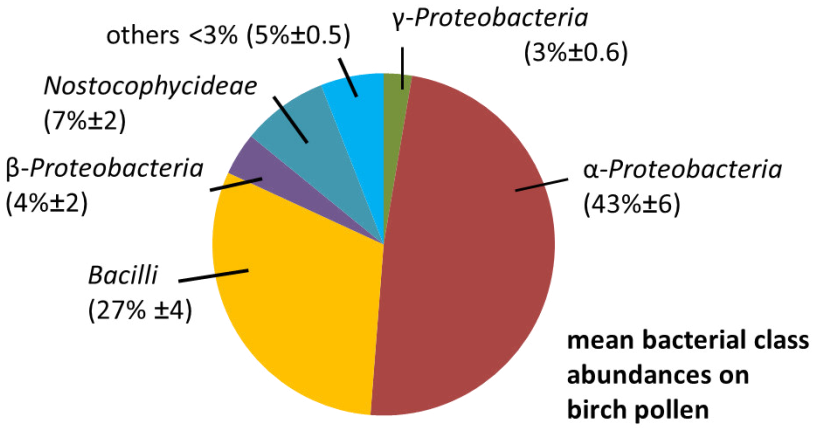

Figure 10b Birch pollen associated bacterial community on class-level. *Composition of bacterial community on class-level isolated from birch pollen (Betula pendula, n = 10) and determined by 16S-rDNA amplicon sequencing. Given are the mean values of the relative abundance and the standard deviation.*

On genus-level amplicon sequencing of bacterial community colonizing birch pollen resulted in a similar consistency due to abundance distribution in different samples (Figure 11a).The genera *Devosia* (γ-*Proteobacteria*) and *Alkalibacillus* (*Bacilli*) belong to already detected dominant classes. *Thermodesulfovibrio* is a genus belonging to the class of *Nitrospira,* whereas *Burkholderia* spp. are sorted in to the class of β-*Proteobacteria*.

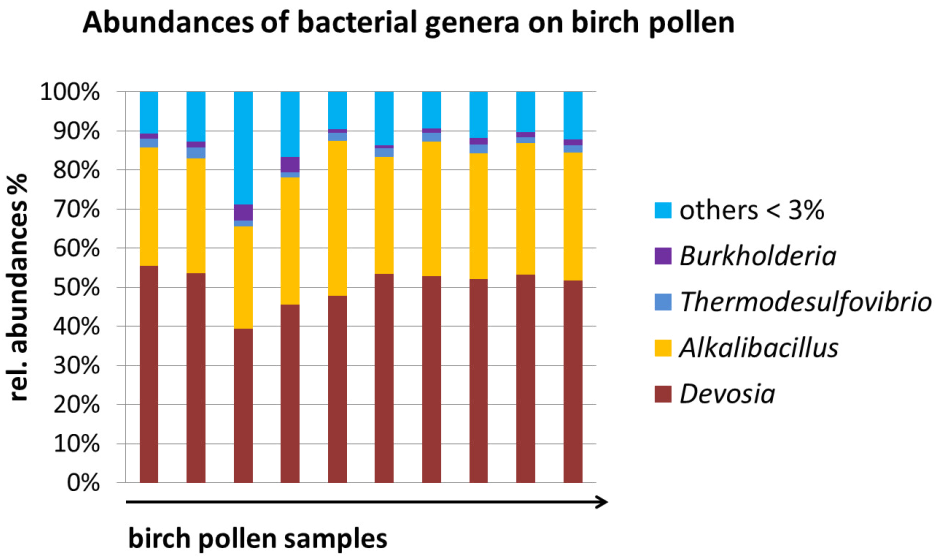

Figure 11a Birch pollen associated bacterial community on class-level. *Composition of bacterial community on class-level isolated from birch pollen (Betula pendula, n = 10) and determined by 16S-rDNA amplicon sequencing. Given are the mean values of the relative abundance and the standard deviation.*

Examining the mean-values of bacterial genus-abundances the community composition associated to pollen from birch contains predominantly species of *Devosia* (mean 51%±4; class: α-*Proteobacteria*), followed by the genus *Alkalibacillus* sp. (mean 31%±3; class: α-*Proteobacteria*). Other genera appear with abundance lower 3%, like *Thermodesulfovibrio* sp. (mean 3%±0.7; phylum: *Nitrospira*) and *Burkholderia* sp. (mean 3.5%±0.6; class: β-*Proteobacteria*). The genera occurring less often than 3% are cumulatively named others (mean 5%±0.5) (Figure 11b).

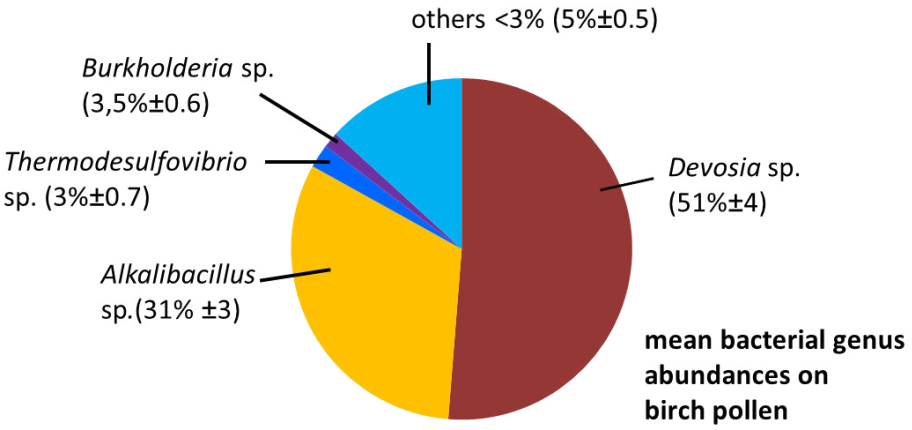

Figure 11b Birch pollen associated bacterial community on genus-level. *Composition of bacterial community on genus-level isolated from birch pollen (Betula pendula, n = 18) and determined by 16S-rDNA amplicon sequencing. Given are the mean values of relative abundance and the standard deviation.*

Hence, the significant differences in bacterial community composition associated to pollen from timothy grass and birch as determined by the tRFLP-pattern-analysis (chapter 3.2.3), are confirmed by the 16S-rDNA amplicon sequencing analysis.

3.3 Fluorescence *in situ* Hybridization (FISH)

In the following chapter stained class-specific bacterial colonization of birch (*Betula pendula*) and timothy grass pollen (*Phleum pratense*) is demonstrated visually via microscope imaging.

3.3.1 γ-*Proteobacteria* on timothy grass pollen

The colonization of timothy grass pollen was also stained with probes including blue and red staining dyes and firstly viewed through an epifluorescence microscope. Bacteria treated with the EUB-338-I, II, III Cy5 probe could be seen in the blue channel (Figure 12B) and γ-*Proteobacteria* treated with ALF-1b Cy3 probe were visualized in the red channel (Figure 12C). Figure 14A shows the overlap of the blue and red channel, so

that the two taxonomic levels can be distinguished. In Figure 12D the autofluorescence of timothy grass pollen are visible since no dye staining was used (chapter 2.5.2).

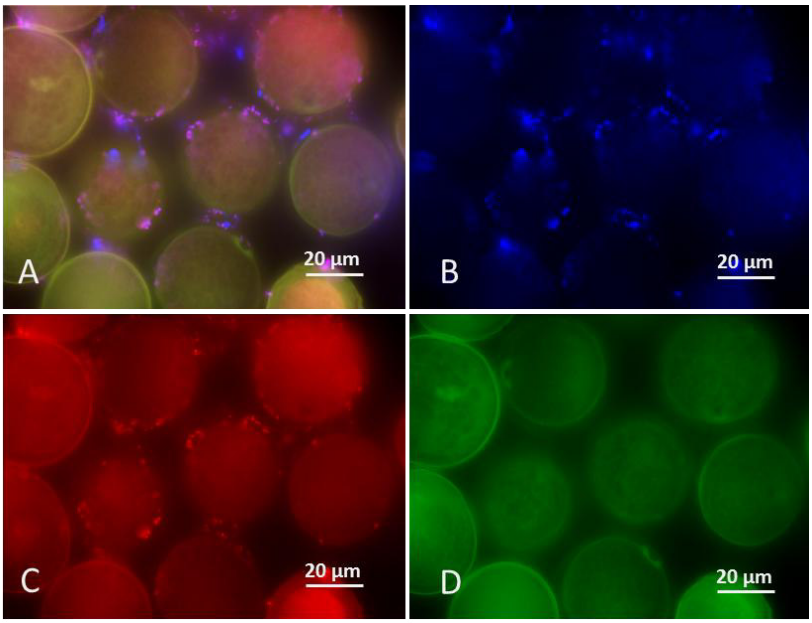

Figure 12 Bacteria colonizing timothy grass pollen*. Shown is the view through an epifluorescence microscope. Probes: universal Bacteria EUB-338-I, II, III Cy5 (blue); GAM-42a Cy3 (red) specific for γ-Proteobacteria.*
A) *Overlay of the channels red, blue and green.*
B) *Timothy grass pollen grains colonized by* ***blue stained Bacteria*** *in the blue channel.*
C) *Timothy grass pollen grains colonized by* ***magenta stained γ-Proteobacteria*** *in the red channel.*
D) *Autofluorescence of timothy grass pollen grains in the green channel.*

In the CLSM-images of FISH-stained bacteria on timothy grass pollen the surface attachment of the bacteria could be demonstrated well illustrated. The blue group of Bacteria is shown in Figure 13A, together with magenta colored γ-*Proteobacteria*. In Figure 13B-D only single channel images show the bacteria localization treated with the appropriate staining dye. In Figure 13D the autofluorescence of pollen grains are shown in the green channel.

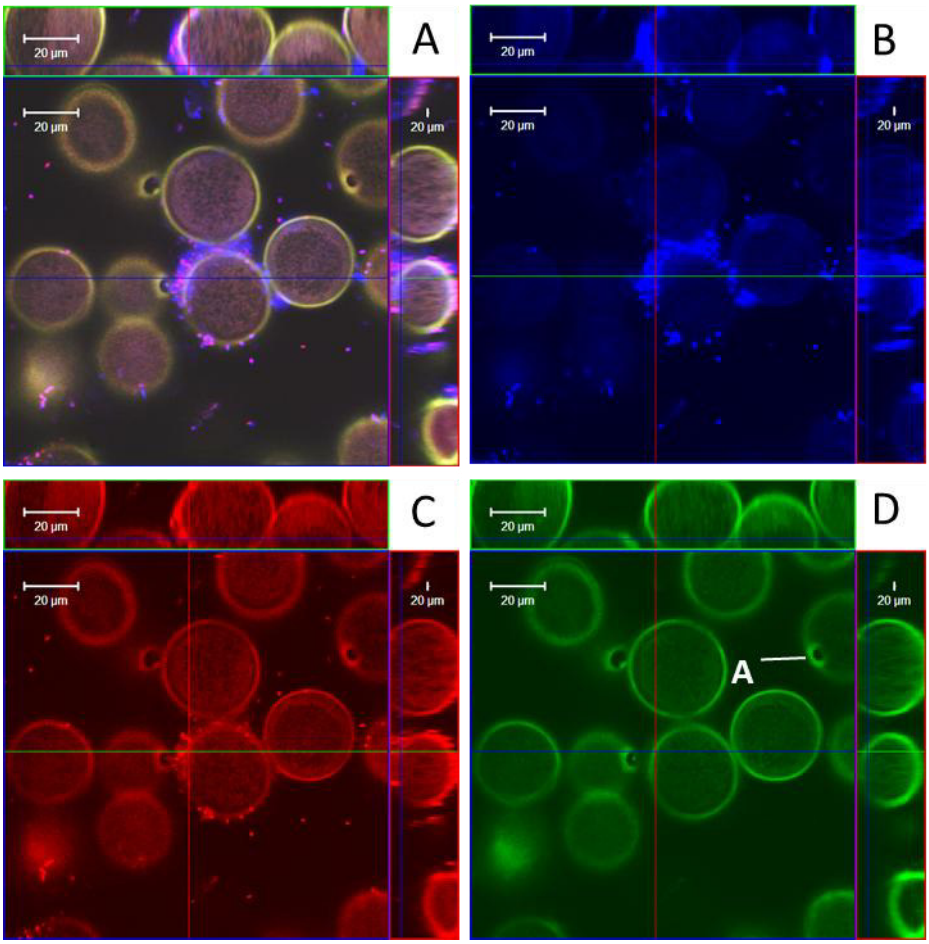

Figure 13 Confocal laser-scanning microscopy (CLSM) *of FISH-stained γ-Proteobacteria (probe: GAM-42a Cy3) and universal Bacteria (EUB-338-I, II, III Cy5).*
A) *Overlay of the channels red, blue and green.*
B) *Timothy grass pollen grains colonized by* ***blue stained Bacteria*** *in the blue channel.*
C) *Timothy grass pollen grains colonized by* ***magenta stained γ-Proteobacteria*** *in the red channel.*
D) *Autofluorescence of timothy grass pollen grains in the green channel. A = aperture.*

3.3.2 α-*Proteobacteria* on birch pollen

In Figure 14 the colonization of birch pollen grains is illustrated through an epifluorescence microscope, wherein Fluorescent probes containing two different staining dyes (Cy3 in red, Cy5 in blue) were used in parallel. Figure 14B with the adjusted blue channel illustrate all Eubacteria are identified with universal EUB-338-I, II, III Cy5 probe. In Figure 14C bacteria belonging to the class α-*Proteobacteria* that

bound the ALF-1b Cy3 probe were visible in the red channel. In Figure 14A the overlap of the blue, red and green channel is shown by visible blue Eubacteria, magenta α-*Proteobacteria* and white/green birch pollen. In Figure 14D the pollen autofluorescence is shown, because no fluorescence labeling was performed (chapter 2.5.2).

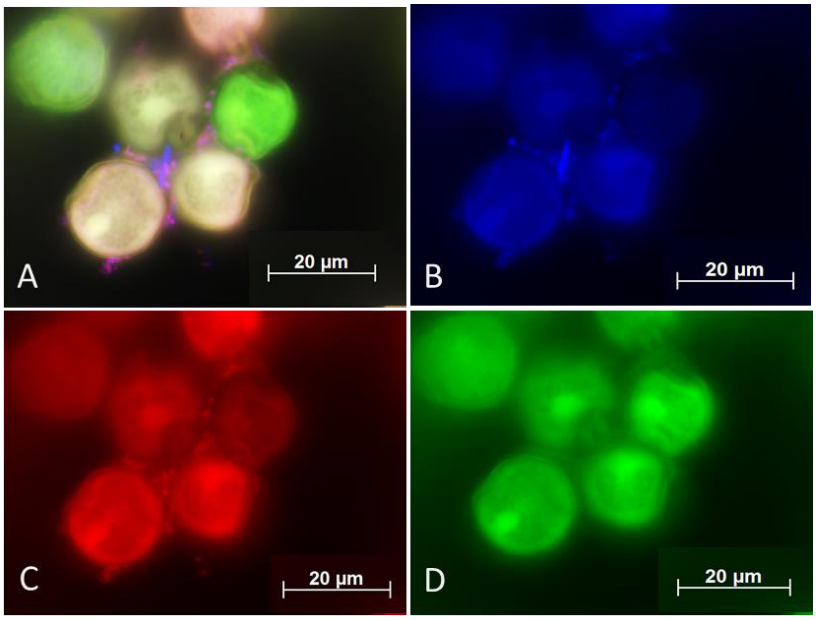

Figure 14 Bacteria colonizing birch pollen. *Shown is the view through a epifluorescence microscope. Probes: universal Bacteria EUB-338-I, II, III Cy5 (blue); ALF-1b Cy3 (red) specifically for α-Proteobacteria.*
A) Overlay of the channels red, blue and green.
B) Birch pollen grains colonized by ***blue stained Bacteria*** *in the blue channel.*
C) Birch pollen grains colonized by ***magenta stained α-Proteobacteria*** *in the red channel.*
D) Autofluorescence of birch pollen grains in the green channel.

The orthogonal view of a stack-image taken via confocal Laser-scanning microscope (CLSM) enables one to see in detail, where the colonization takes place – on the surface of the pollen or even in the inside. In case of birch pollen it can distinctly be detected that the colonization is an extrinsic one. Magenta colored α-*Proteobacteria*

were illustrated. Moreover the three apertures of birch pollen and also the granules of the starch core is visible (Figure 15).

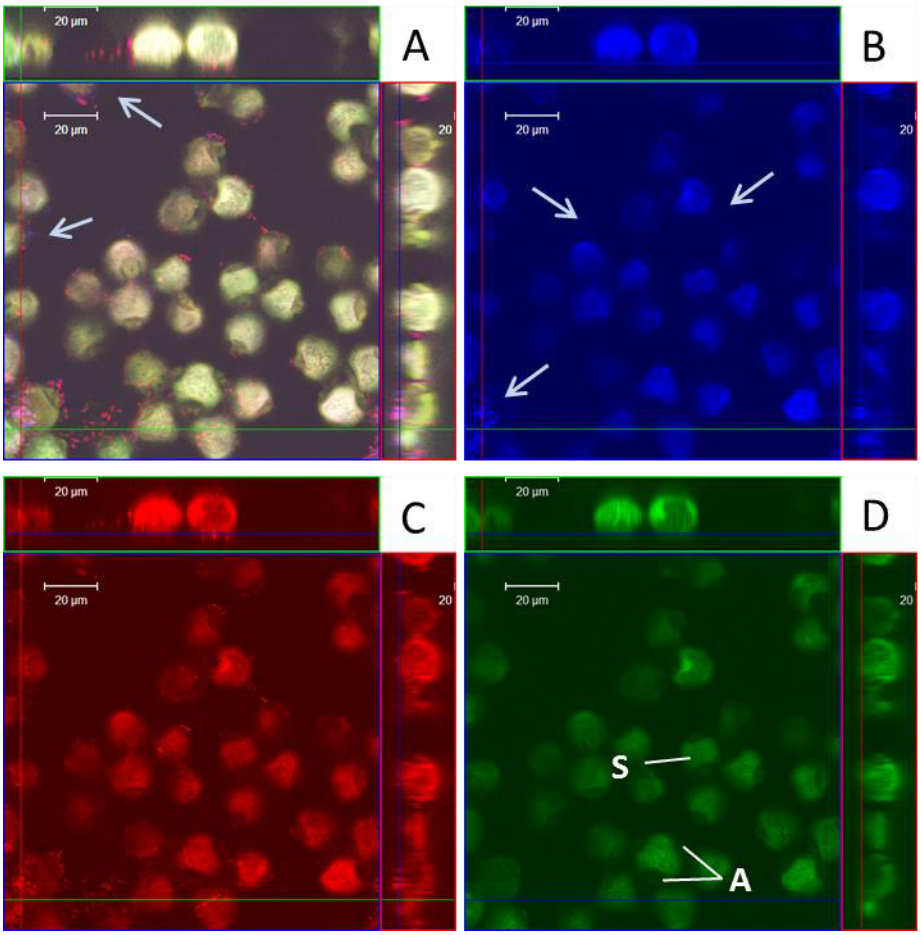

Figure 15 Confocal laser-scanning microscopy *of FISH-stained α-Proteobacteria (probe: ALF-1b Cy3) and universal Bacteria (EUB-338-I, II, III Cy5). Bacteria visible in blue are especially pointed.*
A) *Overlay of the channels red, blue and green.*
B) *Birch pollen grains colonized by* ***blue stained Bacteria*** *in the blue channel.*
C) *Birch pollen grains colonized by* ***magenta stained α-Proteobacteria*** *in the red channel.*
D) *Autofluorescence of birch pollen grains in the green channel. S = starch core, A = apertures.*

3.4 Air pollution and pollen-associated microbial composition

In the following chapter the influence of urbanization on microbial composition of pollen samples from birch (*Betula pendula*) and timothy grass (*Phleum pratense*) is demonstrated by the comparison of urban/rural tRFLP-pattern and correlation-analyses of pollution values to α-diversity of birch pollen-associated microbe

3.4.1 Comparison of microbial patterns on pollen from rural and urban locations

The microbial colonization on birch pollen differed partly significantly, when sorting the pollen samples according to urban and rural sample sites. The calculation of an ANOSIM indeed resulted in no significant differences in fungal ($R = 0.04$, $p = 0.62$; Figure 16C), but significantly in bacterial composition ($R = 0.23$, $p = 0.003$; Figure 16A), considering rural or urban sample sites. In case of timothy grass pollen no significant differences in bacterial ($R = 0.02$; $p = 0.36$; Figure 16B) or fungal colonization patterns ($R_{grass} = 0.09$, $p_{grass} = 0.69$; Figure 16D) were detected between urban and rural sample sites.

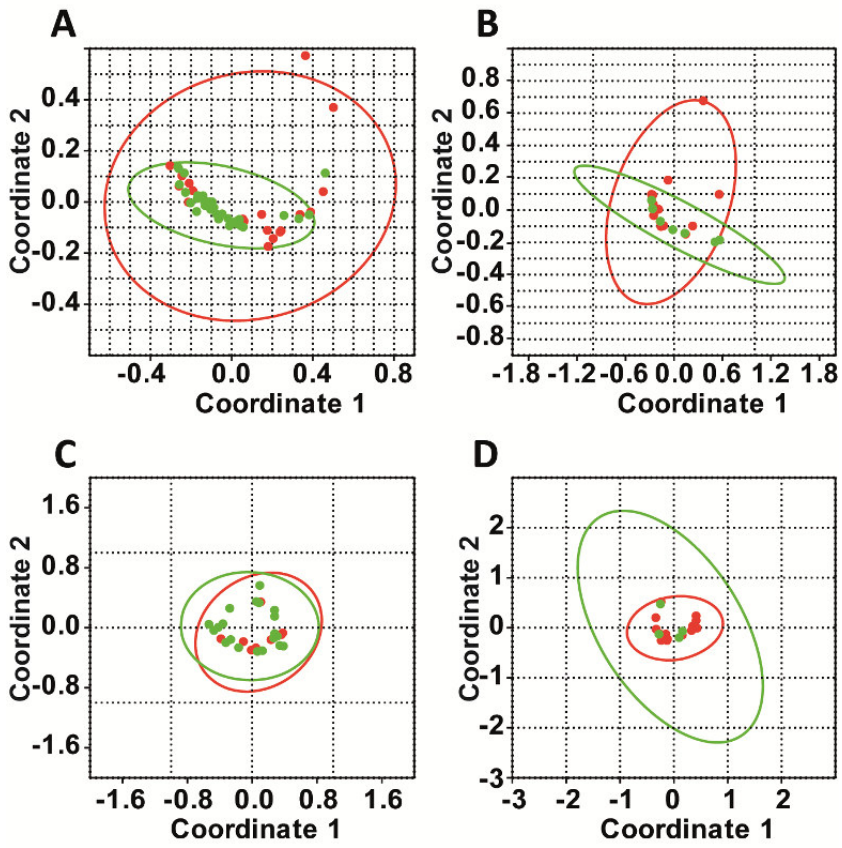

Figure 16 Comparison of microbial pattern colonizing urban and rural pollen via PCoA (Obersteiner et al. 2016).
A) *Bacterial composition on* ***urban*** *and* ***rural*** *birch pollen (Betula pendula, n_{urban} = 20, n_{rural} = 35). R = 0.23, p = 0.003.*
B) *Bacterial composition on* ***urban*** *and* ***rural*** *timothy grass pollen (Phleum pratense, n_{urban} = 13, n_{rural} = 4). R = 0.02; p = 0.36.*
C) *Fungal composition on* ***urban*** *and* ***rural*** *birch pollen (Betula pendula, n_{urban} = 9, n_{rural} = 22). R = 0.04, p = 0.62.*
D) *Fungal composition on* ***urban*** *and* ***rural*** *timothy grass pollen (Phleum pratense, n_{urban} = 14, n_{rural} = 4). R_{grass} = 0.09, p_{grass} = 0.69.*

3.4.2 Pollution parameters correlated to bacterial diversity on birch pollen

Diversity indices based on bacterial tRFLP patterns on birch pollen collected in 2014 partly correlate significantly with pollution parameters of the respective pollen sampling sides. The number of different fragments n(TRFs) significantly decreases with increasing concentration of nitrogen dioxide NO_2 (r_s = -0.31, p = 0.027; Figure 17B) in

the air, but there was no correlation with ozone O_3 (r_s = 0.21, p = 0.13; Figure 17A) or ammonia NH_3 (r_s =0.14, p = 0.34; Figure 17C). Simpson diversity Index 1-D weighing the high abundant fragments correlates significantly positive with O_3 (r_s = 0.39, p = 0.0039; Figure 17D) and significantly negative with NO_2 (r_s = -0.54, p < 0.0001; Figure 17E), but not with NH_3 (r_s = 0.01, p = 0.94; Figure 17F). Shannon diversity Index H weighing the less abundant fragments occurs in no significant adaption to one of the measured pollution parameters ($r_s(O_3)$ = 0.19, $p(O_3)$ = 0.16, Figure 17G; $r_s(NO_2)$ = 0.14, $p(NO_2)$ = 0.31, Figure 17H; $r_s(NH_3)$ = 0.01, $p(NH_3)$ = 0.89, Figure 17I).

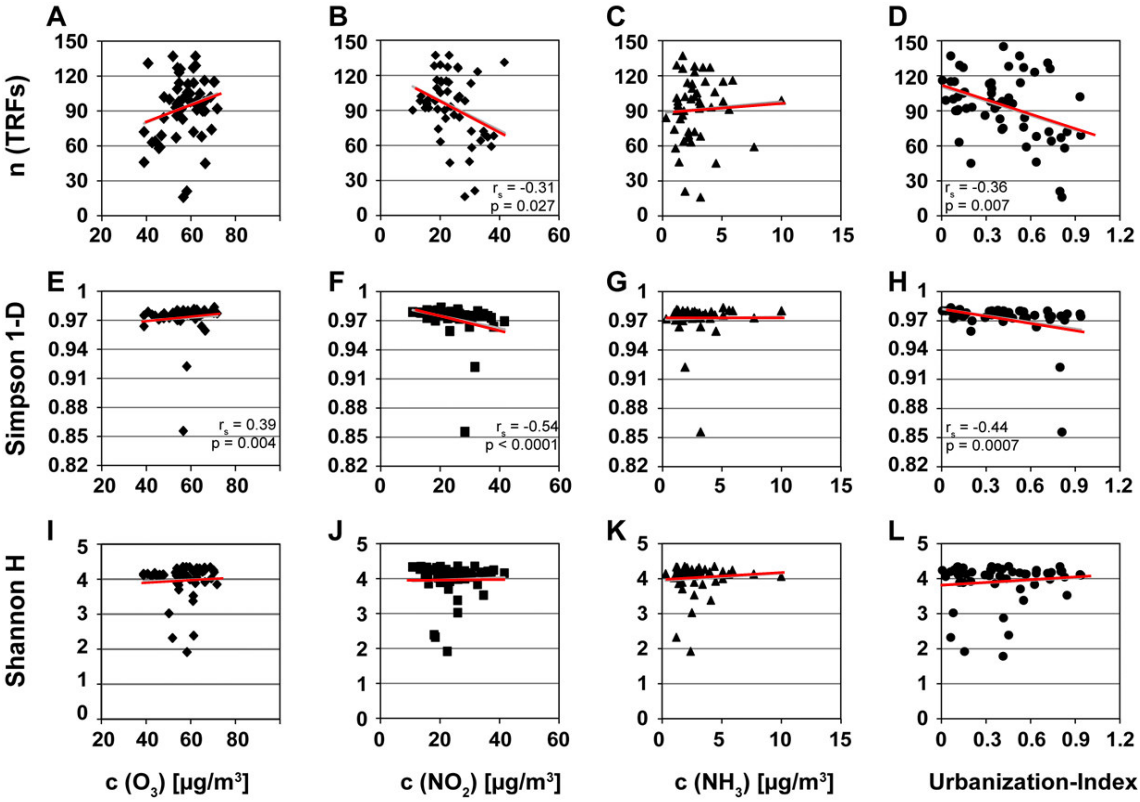

Figure 17 Correlation of bacterial diversity on birch pollen to pollution parameter (Obersteiner et al. 2016). *Scatterplot including linear trend line and spearman correlation values r_s and significance level p of diversity indices (Shannon/Simpson) and parameter (number of fragments n(tRFs)) of bacterial pattern on birch pollen (Betula pendula 2014, n = 55) with pollution measurement*

A-C) *Correlation of n(tRFs) and pollution parameters ozone O_3 (r_s = 0.21, p = 0.13), nitrogen dioxide NO_2 (r_s = -0.31, p = 0.027) and ammonia NH_3 (r_s = 0.14, p = 0.34).*

D-F) *Correlation of Simpson diversity Index 1-D and pollution parameters O_3 (r_s = 0.39,*

p = 0.004), NO_2 (r_s = -0.54, p < 0.0001) and NH_3 (r_s = 0.01, p = 0.94).
***G-I)** Correlation of Shannon diversity Index H and pollution parameters O_3 (r_s = 0.19, p = 0.17), NO_2 (r_s = -0.14, p = 0.305) and NH_3 (r_s = 0.01, p = 0.89).*

3.4.3 Pollution parameters correlated to fungal diversity on birch pollen

In contrast to the bacterial composition the fungal diversity on birch pollen grains were not significantly correlated to any pollution-related parameter (NO_2, O_3, NH_3; Table 20).

***Table 20 Correlation of fungal diversity on birch pollen to pollution parameter**. Spearman correlation of fungal diversity-indices (Simpson, Shannon) and the absolute number of different fragments (n(tRFs)) analyzed from birch pollen (Betula pendula, n = 2014) to air pollution concentration (NO_2, O_3, NH_3; n = 31) and the calculated Urbanization Index (UI, n = 18). p = significance level.*

Div. Index	NO_2 [µg/m³]	O_3 [µg/m³]	NH_3 [µg/m³]	UI
Simpson 1-D	p = 0.53	p = 0.86	p = 0.95	p = 0.29
Shannon H	p = 0.54	p = 0.65	p = 0.95	p = 0.33
N(tRFs)	p = 0.79	p = 0.39	p = 0.98	p = 0.77

3.4.4 Urbanization Index correlated to bacterial and fungal diversity on timothy grass pollen

Correlation analysis of the Urbanization Index UI to bacterial diversity indices partly led to significant results (p_{simp} = 0.04, p_{shan} = 0.14, p_{tRFs} = 0.44), whereas the fungal diversity did not significantly correlate (p_{simp} = 0.20, p_{shan} = 0.36, p_{tRFs} = 0.64) to the UI.

3.5 Allergen-related compounds and pollen-associated microbial composition

In the following chapter the influence of microbial community on the allergenicity of pollen samples from birch (*Betula pendula*) and timothy grass (*Phleum pratense*) was demonstrated by the correlation-analyses of microbial α-diversity to allergens, allergen-related compounds (PALMs) and stress-induced enzyme activity (NADPH oxidase).

3.5.1 Allergen/stress-related compounds correlated to bacterial diversity on birch pollen

Plotting diversity indices (Shannon, Simpson, n(tRFs)) calculated for the bacterial community on birch pollen against parameters defining the pollen´s allergenic potential (content of Bet v 1, PALMs, NADPH oxidase), significant correlations were observed. Specifically, the number of different fragments (n(tRFs)) is positively correlated to the concentration of Bet v 1 (r_s = 0.28, p = 0.038; Figure 18A), but not to the concentration of $PALM_{LTB4}$ (r_s = 0,14, p = 0.33; Figure 18B), $PALM_{PGE2}$ (r_s = 0.17, p = 0.22; Figure 18C) or NADPH oxidase (r_s = -0.04, p = 0.79; Figure 18D). Simpson diversity Index shows no significant correlation with any of the measured parameters of allergenic potential (r_s(Bet v 1) = 0.25, p(Bet v 1) = 0.068, Figure 18E; $r_s(PALM_{LTB4})$ = 0.08, $p(PALM_{LTB4})$ = 0.53, Figure 18F; $r_s(PALM_{PGE2})$ = 0.12, $p(PALM_{PGE2})$ = 0.366, Figure 18G; r_s(NADPHox.) = 0.05, p(NADPHox.) = 0.75, Figure 18H). Shannon diversity Index, however, is negatively correlated to the concentration of $PALM_{LTB4}$ (r_s = -0.36, p = 0.006; Figure 18J) and $PALM_{PGE2}$ (r_s = -0.04, p = 0.001; Figure 18K), but not with the concentration of Bet v 1 (r_s = 0.14, p = 0.317; Figure 18I) or NADPH oxidase (r_s = 0.2, p = 0.19; Figure 18L).

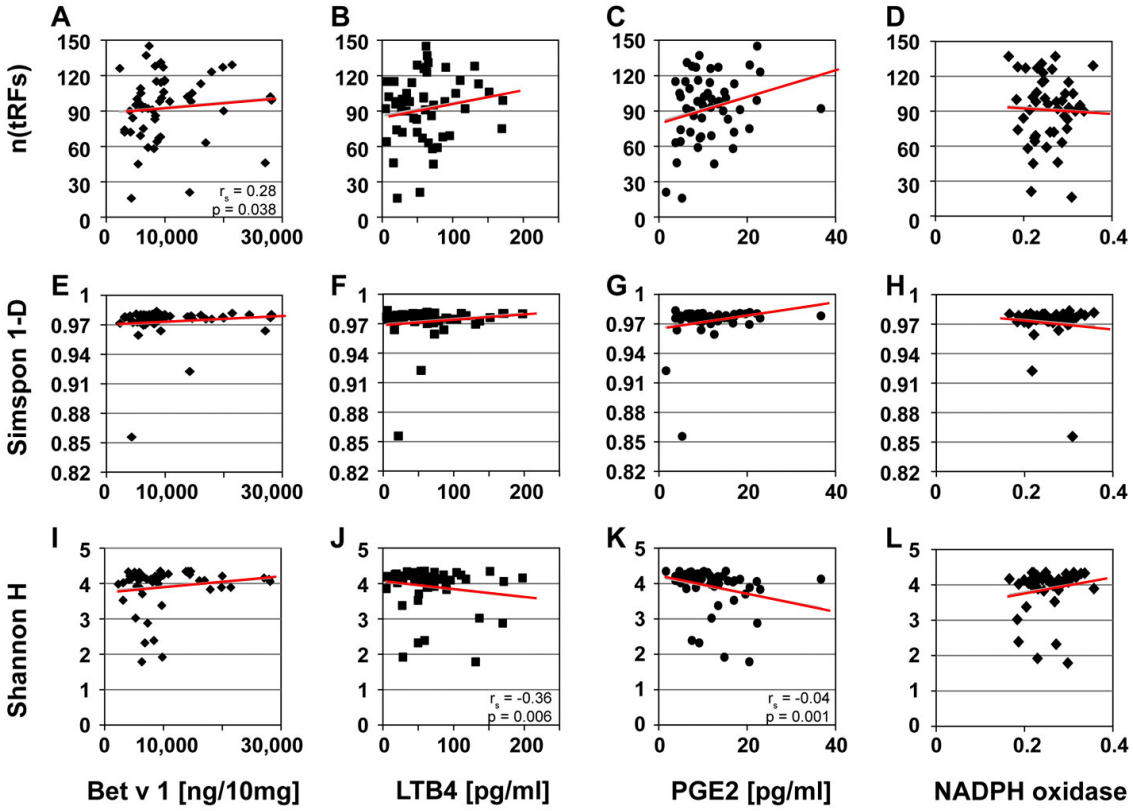

Figure 18 Correlation of bacterial diversity on birch pollen to the content of allergens/PALMs and the activity of NADPH oxidase (Obersteiner et al. 2016). *Scatterplot including linear trend line and spearman correlation values r_s and significance level p of diversity indices (Shannon H/Simpson 1-D) and parameter (number of fragments n(tRFs)) of bacterial pattern on birch pollen (Betula pendula 2014, n = 55) with allergen concentration in pollen.*
A-C) *Correlation of n(tRFs) and allergenicity parameters Bet v 1 (r_s = 0.28, p = 0.038), $PALM_{LTB4}$ (r_s = 0.14, p = 0.33) and $PALM_{PGE2}$ (r_s = 0.17, p = 0.22).*
D-F) *Correlation of Simpson diversity Index 1-D and allergenicity parameters Bet v 1 (r_s = 0.25, p = 0.068), $PALM_{LTB4}$ (r_s = 0.08, p = 0.53) and $PALM_{PGE2}$ (r_s = 0.12, p = 0.366).*
G-I) *Correlation of Shannon diversity Index H and allergenicity parameters Bet v 1 (r_s = 0.14, p = 0.317), $PALM_{LTB4}$ (r_s = -0.36, p = 0.006) and $PALM_{PGE2}$ (r_s = -0.04, p = 0.0015).*

3.5.2 Allergen/stress-related compounds correlated to fungal diversity on birch pollen

In contrast to the bacterial patterns the composition of fungi on birch pollen grains was not significantly correlated to any allergenicity parameters (Bet v 1, $PALM_{PGE2}$, $PALM_{LTB4}$, NADPHoxidase) tested (Table 21).

Table 21 Correlations of fungal diversity on birch pollen to allergen and PALMs-content. *Spearman-Correlation of fungal diversity-indices (Simpson, Shannon) and the absolute number of different fragments (n(tRFs)) analyzed from birch pollen (Betula pendula 2014, n = 18) to the produced amount of allergens and PALMs (Bet v 1, $PALM_{PGE2}$, $PALM_{LTB4}$) and the activity of NADPH oxidase. p = significance level.*

Div.-Index	**allergenicity**			**physiology**
	Bet v 1 [ng/10mg]	**$PALM_{PGE2}$ [pg/ml]**	**$PALM_{LTB4}$ [pg/ml]**	**NADPH oxidase**
Simpson 1-D	p = 0.69	p = 0.61	p = 0.21	p = 0.96
Shannon H	p = 0.64	p = 0.58	p = 0.15	p = 0.82
n(tRFs)	p = 0.69	p = 0.36	p = 0.13	p = 0.55

3.5.3 Allergen-related compounds correlated to bacterial diversity on timothy grass pollen

The statistical analysis of bacterial pattern obtained from timothy grass pollen (*Phleum pratense*, n = 20; 2014) resulted in a significantly negative correlation between the diversity-index Simpson 1-D and the concentration of the allergen $PALM_{PGE2}$ (r_s = -0.51, p = 0.039). In all other cases no significant relationship between bacterial diversity-indices and allergen concentrations could be identified (Table 22).

Table 22 Correlation of bacterial diversity on timothy grass pollen to allergen and PALMs. *p-value of spearman-correlation of bacterial diversity-indices (Simpson, Shannon) and the absolute number of different fragments (N(tRFs)) analyzed from timothy grass pollen (Phleum pratense 2014, n = 20) to the produced amount of allergens (Phl p 5, $PALM_{PGE2}$, $PALM_{LTB4}$).*

Div.-Index	Phl p 5 [pg/ml]	$PALM_{PGE2}$ [pg/ml]	$PALM_{LTB4}$ [pg/ml]
Simpson 1-D	p = 0.70	p = 0.039	p = 0.06
Shannon H	p = 0.46	p = 0.57	p = 0.67
n(tRFs)	p = 0.25	p = 0.26	p = 0.10

3.5.4 Allergen-related compounds correlated to fungal diversity on timothy grass pollen

The statistical analysis of fungal pattern obtained from timothy grass pollen (n = 20; 2014) resulted in no significant correlation between the diversity-indices (Simpson 1-D; Shannon H; n(tRFs)) and the concentration of the allergens (Phl p5, $PALM_{PGE2}$, $PALM_{LTB4}$; Table 23).

Table 23 Correlation of fungal diversity on timothy grass pollen to allergen and PALMs-content. *p-value of spearman-correlation of fungal diversity-indices (Simpson, Shannon) and the absolute number of different fragments (n(tRFs)) analyzed from timothy grass pollen (Phleum pratense 2014, n = 20) to the produced amount of allergens (Phl p 5, $PALM_{PGE2}$, $PALM_{LTB4}$).*

Div.-Index	Phl p 5 [pg/ml]	$PALM_{PGE2}$ [pg/ml]	$PALM_{LTB4}$ [pg/ml]
Simpson 1-D	p = 0.64	p = 0.81	p = 0.09
Shannon H	p = 0.64	p = 0.92	p = 0.21
n(tRFs)	p = 0.35	p = 0.85	p = 0.84

4 DISCUSSION

In the first part of the discussion, the plant- and human relevant properties of cultured microbial isolates (chapter 3.1, pp. 51) as well as the characteristics of bacterial genera determined by 16S amplicon sequencing, occurring in the pollen-associated communities of birch (*Betula pendula*) and timothy grass pollen (*Phleum pratense*) (chapter 3.2.5, pp. 67) are discussed. In the second part, the localization of bacteria on pollen surface, as revealed by FISH-CLSM technique (chapter 3.3, pp. 74), are discussed and questions about the kind of colonization (surface or endophytic colonization) are elucidated. Furthermore, the influence of environmental factors and sampling conditions (pollution) on microbial communities of birch and timothy grass pollen (chapter 3.4, pp. 79), which potentially may lead to significant differences in plant-specific fungal and bacterial diversity patterns on pollen grains, is addressed in the third part. Additionally a further impact of changes in birch pollen bacterial α-diversity to the expression of allergenicity-related substances (chapter 3.5, pp. 83) is discussed in the last part.

4.1 Identification of pollen-associated microbiome

The first aim of this thesis was to isolate and identify microbes living on pollen grains of birch (*Betula pendula*) or timothy grass (*Phleum pratense*) using cultivation dependent isolation approaches on different laboratory media and different temperatures. Considering the so called “plate count anomaly” (chapter 1.6, p. 21) it is to be expected, that only a small percentage of the whole microbial diversity is cultivable under standard laboratory conditions. Microbes belonging to the ecological group of r-strategists are predicted to dominate under such nutrient rich conditions that are offered by the used universal media. R-strategists are characterized by high nutritional requirements, a weak substrate affinity and a fast growth. In contrast, microbes showing stronger substrate affinity and a lower growth rate, are cumulatively named

k-strategists, would have advantage in habitats with limiting conditions (selective media; Chen *et al*. 2016). It should be considered that in case of pollen inhalation the conditions in the human nose and mucosa would also present a quite selective and specific habitat for bacteria and fungi to colonize and establish within the epithelial community.

A more representative insight into the composition of microbial colonization of pollen was determined by cultivation-independent methods (chapter 3.2, p. 59) using tRFLP fingerprinting (bacterial 16S-rDNA and fungal ITS 1-2 region), followed by 16S-rDNA amplicon sequencing.

4.1.1 Cultivation dependent analysis of Fungi

4.1.1.1 Frequently isolated fungi

Pollen samples from both birch and timothy grass generally showed fungal colonization (Table 16 and Table 17, pp. 51) by *Aspergillus fumigatus* and *Aureobasidium pullulans. A. fumigatus* was described firstly by Fresenius (1863) isolated from bronchi and alveoli obtained from bustard. The fungus is able to invade the lung leading to aspergillosis in immune-suppressed individuals, high numbers of spores are necessary for infection of healthy people. *A. pullulans* is known as the black yeast producing the polysaccharide pullulan, which is used in several areas, e. g. medicine, pharmacy and food industry (Cheng *et al.* 2011). Moreover it produces compounds interacting with the human IgG antibodies and is suggested to influence the immune-system (Muramatsu *et al.* 2012; Gostinčar *et al.* 2014).

On birch pollen several fungi could be isolated and identified. Among them we found species of the genus *Penicillium* (affiliated to *P. pancosmium, P. vancouverense*), a ubiquitous, saprophytic and also airborne fungal genus producing spores inducing respiratory allergy problem in humans. Especially in immunosuppressed people this fungus can cause infections in different areas of the body like the external ear, the

urinary and respiratory tract or the lung, including pneumonia. Furthermore *Penicillium* sp. is known for their production of mycotoxins (Pitt 1994). *P. vancouverense* belongs to the section *Citrina*. Species of this section mostly appear in soil (Houbraken *et al.* 2011). For *P. vancouverense* or *P. pancosmium* no hints were found in the literature regarding the production of mycotoxins or any other human or plant threatening (pathogenic) property.

4.1.1.2 Occasionally isolated fungi

Another fungal strain colonizing birch pollen is affiliated to *Cladosporium australiense*. Species of the genus *Cladosporium* are found cosmopolitan in plant, soil, food, paint, textiles or other organic material. They are known as common endophytes and also as causative agents of leaf spots and lesions (Bensch *et al.* 2012).

Kwoniella betulae, isolated from birch pollen, is a novel yeast species described recently in 2015 isolated from birch trees in the USA (Sylvester *et al.* 2015). So far not much information is found about pathogenic of beneficial properties of this fungus.

Alternaria alternata, a mold isolated from birch pollen, is known as a major causative agent for fungal allergies and occur ubiquitously all over the world (Achatz *et al.* 1995). Moreover this filamentous fungus contains seven different variants producing host-specific phytopathogenic toxins leading to necrosis in different plants (Nishimura & Kohmoto 1983; Hatta *et al.* 2002).

Isolated strains were also affiliated to *Cryptosphaeria eunomia var. fraxini* and *Davidiella macrospora*. Rather poor information about these species were found. *D. machrospora* was found to be highly adapted to colonize wheat roots, stem base and stem, neither beneficial nor pathogenic properties are mentioned (Grudzinska-Sterno *et al.* 2016).

Other fungal strains that were occasionally isolated from timothy grass pollen were affiliated to the mycotoxin producing species *Aspergillus tubingensis* and *A. niger,* two closely related species. Recent studies showed that the fungus *A. tubingensis* is able to produce an enzyme (glucose oxidase) inhibiting the growth and spore production of the plant pathogen *Fusarium solani* (Kriaa *et al.* 2015). *A. tubingensis* as well as *A. niger* belong to the group of fungus which were shown to produce the Ochratoxin A (OTA), a toxin exhibiting several problems in human health and agricultural products, including grapes, wine (Perrone *et al.* 2006) and roasted Chilean coffee (Galarce-Bustos *et al.* 2014). Summed up in Medina *et al.* (2005) OTA is considered as teratogenic, carcinogenic, cytotoxic in hepatic cell lines and leads to nephrotoxicity, immunosuppression and intestinal fragility.

Another phytopathogenic fungus is *Fusarium sporotrichioides* (Marasas *et al.* 1984), to which one strain isolated from timothy grass pollen was affiliated to. The genus *Fusarium* sp. is frequently studied and a well-known for its pathogenic properties, for example on wheat (Grudzinska-Sterno *et al.* 2016). Several species, among them also the soil-borne *F. sporotrichioides,* were found to induce head blight in cereal crops worldwide. It was also reported that overwintered cereals that were contaminated with. *F. sporotrichioides* led to the death of more than 1000 people during World War II (Asano *et al.* 2012).

A strain isolated from timothy grass pollen was also affiliated to *Rhizopus microsporus.* This fungus contains an endosymbiotic bacterium producing the phytotoxin rhizoxin (Partida-Martinez *et al.* 2007), a peptide also highly toxic to mammals leading to serious hepatic lesions (Wilson *et al.* 1984). Some subspecies of *Rhizopus* sp. are used in fermentation processes in soybeans, which could cause a potential risk for human health (Scherlach *et al.* 2013).

Rhodotorula glutinis, yeast isolated from timothy grass pollen, is considered as an ubiquitous saprophytic yeast, isolated from different environments. It is known for its

ability as biocontrol agent and its antagonistic activities against the postharvest pathogens (Chen *et al.* 2015), for example *Penicillium expansum* which was shown to be the causative agent for blue rot in harvested apples (Calvente *et al.* 1999). Moreover single cases of human infections were reported associated to *R. glutinis*, like liver cirrhosis, systemic lupus erythematous or nasopharyngeal carcinoma (Wirth & Goldani 2012).

4.1.2 Cultivation dependent analysis of Bacteria

Only selected bacterial species respond well to the *in vitro* cultivation conditions (Table **18** and Table 19, pp. 55; phylogenetic trees Figure 25-32, pp. 139). In the following section, the properties of cultivated bacterial isolates are presented in more detail.

Bacterial cultures isolated from timothy grass pollen as well as from birch pollen comprise three main phyla: *Proteobacteria*, *Firmicutes* and *Actinobacteria*. According to several studies (e. g. Bulgarelli *et al.* 2012; Vorholt 2012; Bai *et al.* 2015) these three phyla belong to the main co-occurring phyla which colonize the plant´s phyllosphere and rhizosphere.

4.1.2.1 Frequently isolated *Bacillus* spp.

Species belonging to the genus *Bacillus* were isolated from every birch and timothy grass pollen samples that were collected from five different locations and inoculated on universal media. Species of *Bacillus sp.* (Skerman *et al.* 1980) are rod shaped, Gram-positive, aerobic to facultative aerobic and known to build endospores colonizing wide spread environmental habitats like soil, water sources or plants (Madigan & Martinko 2009). Species affiliated to *B. thuringiensis* were isolated even from insect carcasses (Schneider *et al.* 2015). Several studies showed that certain species of Bacilli show antagonistic activities by limiting the number of phytopathogens and also are able to influence the growth and development of the host plant positively by plant

growth promoting effects (Shternshis *et al.* 2015). Furthermore, *Bacillus* spp. are known for their ability to induce systemic resistance (ISR) (Kloepper *et al.* 2004).

B. thuringiensis (Skerman *et al.* 1980), isolated from birch and timothy grass pollen, belongs to the *B. cereus* group and the production of crystalline, proteinaceous δ-endotoxins enables this bacterial species as highly successful for biological pest-control. Recent studies showed that *B. subtilis* (Zhang *et al.* 2010) and also *B. licheniformis* (Probanza *et al.* 2001) strains are able to suppress the growth of the phytopathogenic fungus *Botrytis cinerea* on strawberry plants (Shternshis *et al.* 2015). Another beneficial characteristic of *Bacillus spp*. is the frequently studied and well known ability of plant-growth-promotion in the plant rhizosphere (PGPR). Next to *B. pumilus* (Probanza *et al.* 2001), *B. subtilis* (Zhang *et al.* 2010), *B. thuringiensis* (Bai *et al.* 2003) also *B. licheniformis* (Probanza *et al.* 2001) show this special property (Gutierrez-Manero *et al.* 2001; Schwartz *et al.* 2013). Moreover *B. licheniformis* and also *B. simplex* (Priest *et al.* 1988) recently were shown to produce a heat-stable toxin, which is not studied in more detail so far, but with similar physical characteristics to the *Bacillus cereus* emetic toxin “cereulide” (Taylor *et al.* 2005). *B. subtilis* and its close relatives are known to play an important role in industry in terms of enzymes like amylases and proteases (Kunst *et al.* 1997). *Bacillus oleronius* (Kuhnigk *et al.* 1995; Heyndrickx *et al.* 2012) is known as a symbiont for insects. In 1995, *B. oleronius* was firstly isolated from the midgut of the termite *Reticulitermes santonensis* (Kuhnigk *et al.* 1995). Moreover it was found in the *Demodex* mite, obtained from a patient suffering from the Rosaceae skin disease (Lacey *et al.* 2007; Li *et al.* 2010). The bacterium is suggested to produce proteins supporting the course of Rosaceae disease (Jarmuda *et al.* 2012) by activating neutrophils which produce inflammatory cytokines (Jarmuda *et al.* 2012; O'Reilly *et al.* 2012). Strains of *Bacillus amyloliquefaciens* (Priest *et al.* 1988) are frequently reported in literature due to plant growth promoting effects, e. g. in lettuce (Chowdhury *et al.* 2013; Gamez *et al.* 2015) and the production of volatile compounds possessing antifungal activities against *Fusarium* sp. mold (Yuan

et al. 2012). The isolated strains from timothy grass, which was closely related to *B. safensis* (Satomi *et al.* 2006), is reported to be tolerant to saline conditions (Kothari *et al.* 2013) and osmotic stress, and further show plant growth promoting effects in wheat plants (Chakraborty *et al.* 2013). *B. clausii* (Nielsen 1995), also isolated from timothy grass pollen, was recently studied and a compound was found that show bactericidal activity against *Agrobacterium tumefaciens* and fungistatic mode against *Candida tropicalis* (Mouloud *et al.* 2013).

The high number of isolated species affiliated to *Bacillus* may be because of their ability to produce spores which are dormant and heat-/desiccation-tolerant (Schwartz *et al.* 2013). This provides an advantage for survival in severe stress situation compared to Gram-negative bacteria lacking spore formation. Therefore, after sampling and sieving the pollen, *Bacillus sp.* may have survived in fitter conditions which led to their abundance on media in competing community.

4.1.2.2 Frequently isolated *Pseudomonas* spp.

Also bacterial species affiliated to the genus of *Pseudomonas*, originally defined by Migula (1894), could be isolated both from birch (affiliated to: 2x *P. graminis*) and timothy grass pollen (affiliated to: 1x *P. graminis*, 4x *P. fluorescens*, 1x *P. poae*, 1x *P. trivialis*, 2x *P. orientalis*, 1x *P. putida*, 1x *P. rhodesiae*). The genus *Pseudomonas* belongs to the subphylum of γ-*Proteobacteria*, are Gram-negative, aerobic, rod-shaped with polar flagella growing in different environmental habitats like, water, soil or air (Schreiber *et al.* 2005). They are known for their broad functional spectrum, from the decomposition of organic material, to plant growth promoting or pathogenic effects (Anwar *et al.* 2016). Together with other bacterial genera, like *Pantoea* sp. (Gavini *et al.* 1989) which were also isolated from birch pollen (affiliated to: 1x *P. agglomerans*), bacteria of the genus *Pseudomonas* form yellow-pigmented colonies and are commonly known to occur in the rhizosphere as well as in the phyllosphere of plants (Behrendt *et al.* 1999). Pseudomonads even represent one of the dominant bacterial

genera colonizing leaf surfaces (Lindow & Brandl 2003). The isolated strain was affiliated to *P. graminis* (Behrendt *et al.* 1999) which firstly was described in 1999 after isolation from the grass phyllosphere. It is a non-spore-forming, straight rod and motile bacterium (Behrendt *et al.* 1999). Strains of *P. graminis* recently showed antagonistic affects to foodborne pathogens of apples and peaches (Alegre *et al.* 2013). Studies performed in 2005 showed an enhancing effect of *P. graminis* strain due to the water permeability in the leaves cuticle (Schreiber *et al.* 2005). Isolated strains were also related to *P. poae* and *P. trivialis* both belonging to fluorescent pseudomodas, occurring typically in the phyllosphere and first described in 2003 by Behrendt *et al*. (2003). For *P. trivialis,* an antagonistic effect to the phytopathogenic fungus *Rhizoctonia solani* could be shown, as well as for *P. fluorescens* (Skerman *et al.* 1980; Kai *et al.* 2007). *P. fluorescens* is also known for their ability to promote plant growth, as it is shown for some graminaceous plants. Another strain isolated from timothy grass pollen was affiliated to *P. orientalis* which was first isolated in Lebanese spring water (Dabboussi *et al.* 1999). No special properties regarding supporting or stressing plants were found in literature. One isolate from timothy grass pollen was affiliated to *P. putida* (Chun *et al.* 2001) which is known as a plant-associated antagonistic species against e. g. the phytopathogenic fungus *Verticillium dahlia* by the production of hydrolytic enzymes and secondary metabolites (Berg *et al.* 2005). Furthermore, *P. putida* is well studied in association to plant growth promoting properties, even in combination with an induced systemic resistance of the host plant (Annapurna *et al.* 2013). *P. rhodesiae*, the last pseudomonad isolated from timothy grass pollen, was firstly described in 1996 (Coroler *et al.* 1996). This species was found to support crops like tomato (*Solanum lycopersicum*), chilli (*Capsicum annuum*) or brinjal (*Solanum melongena*) highly effective in enhancing their total biomass (Kalita *et al.* 2015).

4.1.2.3 Occasionally isolated Gram-negative bacterial species

Several bacterial isolates were cultivated once from one birch or timothy grass pollen samples, respectively; they belong to the following bacterial species:

Serratia sp. (affiliated to S. *liquefaciens*, Skerman *et al.* 1980) are reported as antagonistic bacteria and isolated from birch pollen. Gram-negative *Serratia* spp. frequently occur in environmental and plant associated habitats showing antibiotic and sometimes antifungal activity. *Serratia liquefaciens* strain C63 for example showed good protection abilities in carnation rhizosphere against root cutting (Kalbe *et al.* 1996).

Isolates belonging to the Gram-negative genus *Erwinia* (originally described 1920 by Winslow *et al*.) were cultivated from pollen of both plant species. The genus *Erwinia* is known to contain pathogenic as well as non-pathogen bacterial species. *E. billingiae* (Mergaert *et al.* 1999), also isolated from birch pollen, shows a tendency to colonize saprophytically necrotic tissue of plants. Moreover, the ability to act as an antagonist to the fire blight pathogen has been shown for this epiphytic bacterium (Jakovljevic *et al.* 2008). From timothy grass pollen, a strain isolated was closely related to *E. persicina*, a phytopathogenic bacterium according to Zhan *et al.* 2014. In addition to wilted and necrotic plants, this bacterium had been isolated also from healthy plants like tomato, banana, apple and others. It was found in the urinary track of humans. *E. persicina* is highly adaptive, which probably explains the wide range of environmental habitats, even under arid, saline and alkaline conditions (Zhang & Nan 2014).

Another bacterium isolated from birch pollen belonged to the closely related genus *Pantoea* (affiliated to *P. agglomerans*, former known as *Erwinia herbicola*, (Gavini *et al.* 1989). *Pantoea* species are found to colonize feculent material, plants and soil (Andersson *et al.* 1999; Cruz *et al.* 2007). The bacterium *Pantoea agglomerans* was

commonly isolated from human samples, leading to soft tissue and bone infections (Kratz *et al.* 2003; Cruz *et al.* 2007).

Another strain isolated from birch pollen was affiliated to the genus *Paracraurococcus* sp. (Saitoh *et al.* 1998), Gram-negative coccoid bacterium. *Paracraurococcus* spp. are non-motile and form irregular, red colonies. *Paracraurococcus* sp. is not known to have any pathogenic or beneficial effect to plants or humans.

Two isolated strains from timothy grass were closely related to the genus *Stenotrophomonas* sp. and specifically to *S. rhizophila* (Wolf 2002). Antagonistic activities against the pathogenic fungus *Rhizoctonia solani* by the production of volatile compounds were demonstrated (Kai *et al.* 2007). Furthermore, plant growth promoting effects of *S. rhizophila* were shown by affecting deleterious fungal communities in the rhizosphere of tomato and sweet pepper plants. Therefore, this bacterium is of biotechnological interest, especially in the case of disease control in cotton plants (Schmidt *et al.* 2012).

4.1.2.4 Occasionally isolated Gram-positive bacterial species

An isolate obtained from birch pollen was related to the genus *Laceyella* sp. (Lacey 1971).The affiliated species *L. sacchari* is also known as *Thermoactinomyces sacchari* (Yoon *et al.* 2005). The endospore producing cells are gram positive, aerobic and form kind of mycelia (Yoon 2005). *T. sacchari* was found to be responsible for respiratory problems by inducing an allergic alveolitis, also known as hypersensitivity pneumonitis. Therein are several diseases which can be induced by an infection with *T. sacchari*, for example the “Mushroom workers lung”, Bagassosis or the “Ventilation lung” (Selman *et al.* 2009).

Another strain isolated from birch pollen was affiliated to *Micrococcus luteus* (Skerman *et al.* 1980) forming Gram-positive cells. It is often reported in clinical article causing

intracranial abscesses (Selladurai *et al.* 2009), pneumonia (Souhami *et al.* 1979), and septic arthritis in patients with a suppressed immune-system (Altuntas *et al.* 2004). Furthermore it was found in the catheter of a haemodialysis patient, inducing bacteraemia and was not responding to several antibiotic therapies (Peces *et al.* 1997). A case of meningitis due to *M. luteus* (Fosse *et al.* 1985) was also reported.

Other isolates were affiliated to *Streptomyces* spp. (Skerman *et al.* 1980) which are filamentous, spore-forming bacteria belonging to the phylum of *Actinobacteria*. They can be found in soil and as endophytes in plants. Beneficial effects due to the host plant regarding growth promoting activities. *Streptomyces* sp. are also well known for their production of antibiotic compounds which further protects the plant against pathogens. A minority is also known as phytopathogens themselves (Rasimus-Sahari *et al.* 2016). S. *mutabilis*, closely related to a strain isolated from birch pollen, was described originally by Preobrazhenskaya and Ryabova (1957) is not only suitable for biocontrol of pathogens, but also show plant growth promoting effects in wheat plants (Toumatia *et al.* 2016). Another strain, isolated from timothy grass pollen, was closely related to *Streptomyces rutgersensis* (Skerman *et al.* 1980), originally described by Waksman and Curtis (1916). Since then only rare information is available in the literature.

Isolates affiliated to *Curtobacterium flaccumfaciens* (Collins & Jones 1983) were cultivated both from birch and timothy grass pollen. *Curtobacterium* spp. was originally described as *Corynebacterium flaccumfaciens* by Hedges (1922). It was shown to be responsible for bacterial wilt in dry beans worldwide. Firstly appearing in 1926 in the USA, the pathogen was recently isolated in Germany (Osdaghi *et al.* 2015).

From timothy grass pollen, bacterial isolates belonging to the genus *Exiguobacterium* were isolated. Bacteria of this genus are rod-shaped, facultative anaerobic, non-spore-forming, motile cells with peritrichous flagella, positioned in the Gram-positive phylum of *Firmicutes* (Fruhling 2002). *E. undae* (Fruhling 2002), isolated on two sample sites,

was firstly isolated from surface water in Wolfenbüttel in 2001. No information about pathogen or beneficial relation to plants or human could be found. Another strain affiliated to *Exiguobacterium* is closely related to *E. sibiricum* (Rodrigues *et al.* 2006). *E. sibiricum* was firstly isolated from Siberian permafrost in 2005, a challenging habitat for any organism.

A strain affiliated to *Brachybacterium alimentarium* was isolated from one location of sampled timothy grass pollen. Originally this coryneform bacterium was isolated from Beaufort cheeses (Schubert *et al*. 1996). *Brachybacterium* spp. (Collins *et al.* 1988) are gram-positive, non-motile cells varying in shape from coccoid to rods in their exponential phase. *Brachybacterium* spp. (*B. saurashtrense*) was reported as halotolerant species and to be able to promote the growth of the halophyte *Salicornia brachiate* (Iha *et al*. 2012).

Another isolate belong to the genus *Carnobacterium*, specifically affiliated to *C. maltaromaticum* (Mora *et al.* 2003). This lactic acid bacterium was former known as *C. piscicola*. The cells are non-motile and occur partly in chains (Mora *et al.* 2003). The facultative anaerobic *C. maltaromanicum* is frequently isolated from natural environments and foods and often reported as inhibitor for the growth of pathogenic and spoilage microorganisms, like *Listeria* sp. in fish and meat samples (Leisner *et al.* 2007).

4.1.3 Bacterial community analyses based on cultivation-independent 16S-rDNA amplicon sequencing

To get insights into the bacterial colonization of pollen independent from cultivation bias, DNA-based molecular community analyses were performed which also provided bacterial phylogenetic characterization (chapter 3.2.5, pp. 67). It is not surprising that a completely different community picture arose from these studies.

4.1.3.1 Exclusion of bias

Universal primers were used for PCR procedures, and attention was paid on weak gel-bands. PCR-runs were performed in triplicates to avoid random over-amplification, however, the risk biased amplification could not be excluded. Due to a very high ratio of plastid DNA, two PCR runs were necessary, which increase the risk of bias in case of available preferred binding sites by the used primers. However, in this study the same primers and procedures were performed for grass and birch pollen, so that direct comparison could be made. On both pollen (plant) species the dominant genus *Dickeya* sp. (birch pollen < 3%, grass pollen 50 %) and *Devosia* sp. (birch pollen 54 %, grass pollen 0.8%) appear, but in a contrary abundance. If the difference would be caused by any artificial bias, the distribution of the dominant genera would be more random, not so distinctly assigned to plant species.

The problem of plastid contamination was solved by separation of the amplified bands belonging to the plastid and 16S-rDNA respectively (chapter 2.4.2, pp.36), before starting amplicon sequencing. Eluted bacterial 16S-DNA-band were used for further amplification and sequencing processes.

4.1.3.2 Bacterial community associated with timothy grass pollen

Timothy grass pollen was dominated by γ-*Proteobacteria*, specifically by the enterobacterial genera *Dickeya* (former called *Erwinia chrysanthemi*) and *Erwinia* (Figure 8ab and 9ab, pp. 67). Species of *Dickeya* are phytopathogenic members of the pectolytic *Enterobacteriaceae,* occurring worldwide causing soft rot diseases on several crop and ornamental plants (Toth *et al.* 2011). Showing a wide host range in the angiosperm clade *Dickeya* sp. was also found in several species of *Poaceae* (*Oryza sativa* L., *Saccharum officinarum* L., *Zea mays* L.; Ma *et al.* 2007; Toth *et al.* 2011). Six pathovars arose from the reclassification of *Erwinia chrysanthemi* basically named after host specificity (Samson *et al.* 2005). Three species were detected to appear on timothy grass pollen: *D. paradisiaca* with highest abundance, *D. dianthicola* and

D. dadantii in traces. Besides phytopathogenic characteristics, single species of *Dickeya* (*D. dadantii*) also appear to be highly infectious for insects like the pea aphid *Acyrthosiphon pisum* (Grenier *et al.* 2006). Although, closely related *Erwinia* spp. could be isolated, no *Dickeya* culture could be isolated from timothy grass pollen.

The aggressiveness amongst *Dickeya* species is highly variable, wherein *D. dianthicola* currently belong to the most effective one. The bacteria are able to survive in soil and are transmitted to plants by irrigation water, insects and trading in seed tubers – and as we know now - also by anemochorous pollen. This is an important fact in ecological and economical terms, as far as 25% of potato blackleg incidences in the last 40 years in Netherlands, Belgium and France were attributed to *Dickeya* spp. (Toth *et al.* 2011).

The second dominating genus on timothy grass pollen is the group of the γ-*Proteobacterium Erwinia sp*., a genus containing plant-associated bacteria. On timothy grass pollen the group is represented by the species *E. tasmaniensis* (5±4.9 %), *E. billingiae* (< 3%) and partially in traces *E. soli* and *E. dispersa* (< 3%). *E. tasmaniensis* as well as *E. billingieae* (Mergaert *et al.* 1999) are non-pathogenic, epiphytic species isolated from the surface of apple and pear trees showing antagonistic activities to fire blight disease by competition the pathogens (Geider *et al.* 2006; Jakovljevic *et al.* 2008; Kube *et al.* 2010). Several isolates of *Erwinia* could be cultivated from both birch and timothy grass pollen.

4.1.3.3 Bacterial community associated to birch pollen

Birch pollen was colonized by a completely different bacterial community consisting of genera of Gram-negative *Devosia* (α-*Proteobacteria*) and Gram-positive *Alkalibacillus* (*Firmicutes*) (Figure 10ab and Figure 11ab, pp. 73).

Species of *Alkalibacillus* belong to the Gram-positive *Bacillaceae*, a family of robust spore-forming, rod-shaped bacteria, which were found to play important roles in soil ecology, plant health and plant growth promotion. Bacilli are widespread in natural

environments. *Alkalibacillus* spp. are strictly aerobic and moderately halotolerant. The dominating species on birch pollen, *A. salilacus*, was firstly isolated from salt lakes in China (Jeon *et al.* 2005), but also from brine, camel dung, loam, mud (Mandic-Mulec *et al.* 2015) and various saline environments (Wen *et al.* 2009). No reports about phytopathogenic characteristics or even plant colonizing activities of *Alkalibacillus* spp. could be found in literature. Therefore, this high abundance of *Alkalibacillus* spp. in bacterial composition on European birch pollen is the more interesting as isolates were obtained from such different and distant habitats so far. Because of the spore forming characteristics species of *Bacillaceae* are able to survive also adverse conditions for even longer periods. This empowers them also to travel greater distances, even over continents by air (Smith *et al.* 2013; Mandic-Mulec *et al.* 2015). However, no cultivated strain belonging to *Alkalibacillus* was obtained from pollen; although other Bacilli were isolated frequently.

The second dominating genus on birch pollen is the group of α-*Proteobacterium Devosia* sp., predominantly by *D. hwasunensis* (53.6±3.3 %), and also in small numbers *D. geojeni. D. hwasunensis* appears in aerobic granular sludge important for industrial waste water treatment, but information about plant colonization is rare. *Devosia* spp. generally are known for their biodegradability in contaminated soil and waste water (Li *et al.* 2012).

4.1.4 Conclusion: microbial colonization on allergenic pollen

Cultivated bacteria isolated from birch and timothy grass pollen do not represent the actually occurring abundances of bacterial populations. It is well known that in bacterial populations, a richer biodiversity can be determined when analyzed by culture-independent, DNA-based methods (Rastogi *et al.* 2012). Indeed, only a selected number of bacterial strains dominated under the isolation conditions using inoculated agar media. This is an important fact to consider, because similar condensed conditions might appear in the nose or mucosa of allergic patients after inhaling

pollen. Bacteria and fungi, which are able to grow under such conditions, may have the potential to influence the human immune-system, after contaminated pollen come in contact to the epithelia. Most of the isolated bacteria and fungi in this study are inhabitants of natural environments like plant, soil and water, interacting with plants in beneficial and also pathogenic way. However, some of the isolates also occur commonly on human or animal material threatening their health. Taken together, the isolated strains represent the potential to interact with the plant or the human defense mechanisms.

Predominant genera of the microbes colonizing pollen may have a substantial impact on the pollen and plant metabolome, interacting with the plant defense mechanisms. The results due to this hypothesis (chapter 3.4 and chapter 3.5, pp. 79) are discussed in one of the following chapters (chapter 4.4). For further studies it would be interesting, whether bacterial communities isolated from human nose and mucosa have some resemblance with the pollen-associated, isolated microbes. Such a comparison might give a hint, if beside allergenic compounds; certain microbes colonizing allergenic pollen also contribute directly to the allergic reaction of patients.

4.2 Fluorescence *in situ* Hybridization (FISH)

The staining approach by doing FISH is the third method that shows the significant difference in colonization of pollen obtained from birch and timothy grass pollen (chapter 3.3, pp. 74). On timothy grass pollen a distinct predominance of γ-*Proteobacteria* were visible, whereas α-*Proteobacteria* dominated on birch pollen.

FISH-CLSM-images of pollen-associated bacteria show an effective colonization of the surface of the pollen wall (including the so called aperture area) by γ-*Proteobacteria* (Figure 12 and Figure 13, pp. 76), to which *Dickeya* spp. and *Erwinia* spp. belong. γ-*Proteobacteria* are found located to the so called aperture area of pollen grains, where the pectin polymers of the intine partly appear (Li *et al.* 1994; Taylor &

Hepler 1997). On these sites the pectolytic effects of *Dickeya* sp. (Collmer & Keen 1986; Toth *et al.* 2011) may impact the pollen and hence may influence defense-induced mechanism in the plants.

Although pollen and bacteria were dehydrated during FISH procedure the possibility has to be mentioned that the probes eventually could not reach the inside of the pollen to stain eventually occurring endophytic bacteria. The outer layer (exine) is covered by a robust coat made of sporopollenin creating a border for many physical and chemical influences (Ariizumi & Toriyama 2011). The images also show the apertures of timothy grass and birch pollen possessing only very small opening, thus the probes possibly were not able to enter the inside of the pollen.

4.3 Possible influences on the pollen-associated microbiome

Bacterial and fungal pattern composition as well as the α-diversity differs significantly between microbiome of birch and timothy grass pollen as determined by tRFLP fingerprinting and 16S-rDNA community analyses (chapter 3.2, pp.59). The extent of bacterial diversity on birch pollen is significantly higher regarding highly abundant species than on timothy grass pollen; whereas the fungal diversity on birch pollen is significantly lower than on timothy grass pollen.

The distinct differences in bacterial colonization on birch and timothy grass pollen can be explained by their different ecology, pollen morphology and physiology. Timothy grass and birch trees are exposed to different environmental conditions caused by their height, root depth, exudates and blooming period, exhibiting various biotic (other plants, insects) and abiotic factors (wind, temperature, humidity) influencing the microbial composition on natural samples (Finkel *et al.* 2012; Rastogi *et al.* 2012).

Pollen from birch catkins contains three apertures, whereas timothy grass pollen only has one. Pollen from different plant species develop a different surface structure. The

inner wall (intine) consists of compounds similar to the wall of common plant cells (cellulose, hemicellulose, pectic polymers, enzymes, proteins), the outer layer (exine), however, consists of sporopollenin. This is a robust and very tough material resistant against biological, chemical and physical degradation (Scott 1994) consisting of biopolymers derived mainly from long-chained fatty acids or long aliphatic chains (Scott 1994; Bubert *et al.* 2002). Despite the morphological variations the fundamental structure of the pollen walls are quite similar among taxa and therefore further studies are needed for detecting the influences that may affect the bacterial composition on pollen. Nevertheless, we found specific dominant bacteria with different potential pathogenic characteristics colonizing birch and timothy grass pollen. This pathogenic potential of the dominant bacteria could considerably affect the plant-defense-system and an enhancement of the allergen expression may appear. This could especially be the case, since some allergens (like Bet v 1) belong to the pathogenesis-related (PR) family of proteins, which are involved in the plant defense system (Swoboda *et al.* 1994).

4.3.1 Influence of sampling locations

Birch pollen were sampled in Augsburg, roughly 70 km away from the Munich area, where timothy grass pollen were collected. Therefore, the microbiome-related differences may not really be species-specific, but also could be traced back to probably different conditions of the sampling sites located apart. To further investigate these phenomena, samples of timothy grass pollen were collected in Augsburg and Munich and a microbiome-related pattern Analysis of Similarity (ANOSIM) was performed. The result showed a quite similar composition of tRFLP fingerprint on the timothy grass pollen collected in Augsburg and Munich ($p = 0.2359$, $R = 0.1058$). Hence, it can be excluded, that different sampling site of birch and timothy grass can explain the plant-species specific differences.

4.3.2 Influence of environmental factors

The environment itself could influence the composition of bacterial and fungal community on the surface of pollen in the three major ways: Firstly, the environment can act as a source of inoculum. At different locations, different microbial communities may be in the air, in the soil or near plants, which represents different sink-source-dynamics. Secondly, at different locations foreign inoculation can happen by rain-splash, overhead irrigation, wind or insects. Third, the environment can act as a driver of microbial activity by altering temperature and humidity parameter (Finkel *et al.* 2012; Rastogi *et al.* 2012).

Pollen of timothy grass (*Phleum pratense*) develops in June/July under different environmental and climatic conditions compared to pollen of birch (*Betula pendula*), which flowers in April. Caused by a growth near the ground and flowering period in July grass pollen would probably be exposed to higher temperature, which further leads to higher evaporation rate and thus lower humidity. Catkins from birch are flowering in April when it is colder and often rainy. Catkins dispersed in the wind may also have more contact to other plant organs (leaves, stems) whereas the inflorescence of timothy grass is rather rigid.

4.3.3 Influence of seasonal climatic conditions

Another fact that may explain the significantly different microbial pattern detected on birch and timothy grass pollen is the different climatic condition of the pollination periods. As mentioned earlier, the pollination and sampling period of birch catkins took place from March to April, whereas the pollination and sampling period of timothy grass spikes took place in June. With the support of the Deutsche Wetterdienst, providing the access to annual climate data (www.dwd.de/cdc), a statistical comparison of the temperature and humidity conditions during pollination periods in all three sampling years 2013-2015 was possible.

As expected, the **air temperatures** in April differed significantly to the temperatures in June in all three sampling seasons in 2013 to 2015 ($p_{2013} < 0.001$, $p_{2014} < 0.001$, $p_{2015} < 0.001$). However, comparing the temperature of birch pollination periods 2013, 2014 and 2015 with each other, also significant differences appear ($p_{13/14} < 0.001$, $p_{14/15} = 0.008$, $p_{13/15} = 0.005$), whereas the pollination periods of timothy grass 2013, 2014 and 2015 did not differ significantly ($p_{13/14} = 0.08$, $p_{14/15} = 0.8$, $p_{13/15} = 0.1$). In summary, not only April and June differ significantly due to the temperature, but also the April temperatures among the three sampling years. While the differences in microbiome patterns on birch and timothy grass pollen were consistent in all three sampling years, it indicates that temperature differences might not be the responsible factor for microbial colonization of pollen.

Concerning the **relative humidity** in the air, which could also have an important influence on the growth of bacteria or fungi, April and June differed significantly in 2013 ($p = 0.02$) and 2014 ($p < 0.001$), but not so in 2015 ($p = 0.07$). Nevertheless, in 2015 the microbiome patterns on birch and timothy grass pollen differed also significantly which indicates that relative humidity is also probably not a responsible factor governing the microbial colonization of pollen.

4.3.4 Influence of endophytic microbial colonization

It is possible that due to the mechanical and enzymatic lysis steps used during DNA-extraction, the DNA from the endophytic microbes within pollen could have been liberated. Some of the bacterial species which were identified in 16S-rDNA analysis have also been described previously as endophytes of plants. For example, a strain of *Enterobacter cloacae* has been characterized as obligate endophyte in surface sterilized pollen of several *Pine* species, and also in their roots and shoots (Madmony *et al.* 2005).

It is known that several plant-specific parameters can influence the diversity of the bacterial community in the rhizosphere, for example root exudates attracting specific microbes, altering the pH- milieu or selecting via antimicrobial compounds (Hartmann *et al*. 2009). Thus, plants harbor a specific microbiome recruited from the soil microbiome. This also determines the endophytic composition and hence also different bacteria and fungi may occur in pollen of different plant species. Plant endophytes may vary with changing environmental conditions, like pollution or climate.

4.3.5 Influence of the geno-/phenotype of plants

The genotype of a plant can influence the bacterial community composition (Vorholt 2012). The so called “common garden” experiments (different plant species were grown under similar environmental conditions) showed that mutants from *Arabidopsis thaliana,* with an altered cuticular wax biosynthesis, presented a significant different microbial composition on leaves than the wildtype. The plant specific differences on pollen already occur in the rough structure as well as the number of apertures on the outer layer (exine) (Blackmore *et al.* 2007), by which microbes may enter the inside of pollen.

Although the fundamental chemical structure of the outer pollen walls is quite similar among taxa, the morphology is highly variable. To overcome the immobility plants have evolved to distinguish between native and foreign pollen grains to avoid inappropriate fertilization. The pollen-stigma adhesion is communicated by specific chemical and physical impulses delivered by the pollen exine (Zinkl *et al.* 1999). These specific signals of the exine may have an influence on the pollen-associated microbiome. However, further studies are needed to investigate this hypothesis.

4.3.6 Conclusion: Pollen microbiome depends on plant species

In natural environments a combination of several biotic and abiotic factors are responsible for influencing and characterizing ecosystems. Therefore, it is difficult to find one special factor which has the highest impact on the nature of the ecosystem. The fact that a constant difference in pollen-associated microbiome on the two different plant species studied here was observed indicates a core pollen-microbiome for a plant species. Further studies are required to exclude the potential impact of different factors separately under controlled conditions to determine which one has the highest influence on the composition of pollen-microbiome structure.

4.4 Relationship of bacterial diversity determined by tRFLP-analysis to pollution and allergenicity

4.4.1 Impact of urban pollution on bacterial diversity

As it was shown from the microbiome community analysis of pollen samples from different locations (Figure 17, pp. 81), an influence of environmental conditions could be found. With a higher urbanization rate, a higher air concentration of NO_2 and a lower concentration of O_3 occur (Figure 19).

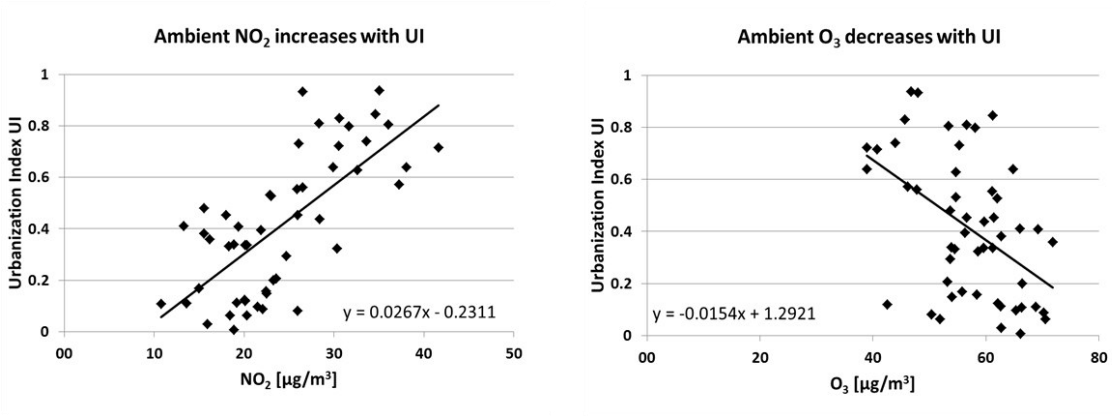

Figure 19 *Spearman correlation of ambient NO_2- and O_3-concentraion to an increasing Urbanization Index (UI) on locations of birch pollen sampling in 2014 (each n = 55).*

Although the Spearman r_s-value was partly quite low indicating a high variance among the correlation data, the pollution parameters correlated significantly to the bacterial diversity on birch pollen, which was calculated on the basis of tRFLP pattern analysis. Ambient NO_2 concentrations as well as the Urbanization Index correlate significantly negative with the bacterial diversity (n(tRFs), Simpson Index). In the case of O_3 the bacterial diversity (Simpson Index) increased significantly with higher pollutant concentration in the air. Considering together the correlation results of the urbanization-parameter UI, NO_2 and O_3, a lower bacterial diversity on pollen was observed in more urbanized areas. Apparently, the high number of different abiotic stress factors occurring in a city, also influence plants and their associated microbes. Beside higher air pollution values also soil pollution, nutrient shortage, heat and drought can occur in high intensity in urbanized areas and may affect and stress the organism living there (Bostock *et al.* 2014). Due to the fact that no other urbanization-related abiotic stress factor beside air-pollution was analyzed, a direct impact of pollution to the reduced bacterial diversity on the pollen cannot be certainly concluded. Nevertheless, it is possible that one of the abiotic factors mentioned above may have an impact on the microbial diversity associated to pollen. Another study analyzing microbial communities in dust showed distinct differences in bacterial

diversity when comparing samples from urban and rural areas. Similar to our observations, the dust-associated microbial community showed a lower diversity in urban area as compared to rural areas (Barberán *et al.* 2015).

4.4.2 Impact of bacterial diversity on allergenicity

In addition to environmental parameters, some allergenicity parameters showed significant correlations to the bacterial diversity on pollen (Figure 18, pp. 84). The major birch allergen Bet v 1 increased with higher bacterial diversity (nTRFs), whereas the content of $PALM_{LTB4}$ and $PALM_{PGE2}$ decreased (Shannon H). The analysis of timothy grass pollen showed a significantly negative correlation of the bacterial diversity (Simpson 1-D) and the content of $PALM_{PGE2}$ (Table 22, pp. 86). Plants are able to recognize microbes on their surface via pattern recognition receptors (PRR), which induces a broad spectrum of defense mechanisms (Zipfel 2014). Both allergens (like Bet v 1) and non-allergenic, immune-modulatory compounds (like PALMs) are involved in this defense mechanisms of plants. Cell culture experiments with birch pollen showed that in the presence of bacteria the gene expression encoding Bet v 1 isoforms was activated (Swoboda *et al.* 1994). This effect was not shown by other abiotic stress factor like heavy metal exposure. In the same study of Swoboda *et al.* (1994) a potential antibiotic effect of Bet v 1 was tested negative. Concerning the immune-modulating PALMs only little information is available about their physiological functions. An involvement of certain PALMs, like B_1-Phytoprostanes, on the defense response of plants against chemical and oxidative stress has been shown (Thoma *et al.* 2004; Loeffler *et al.* 2005).

5 CONCLUSION

Caused by the production of allergenic proteins and immune-modulating compounds special pollen grains lead to health problems in many humans. Initial studies have shown that factors influencing plants may lead to an altered allergenic potential of their pollen grains. This work has tried to uncover potential interrelationships between the microbial load on allergenic pollen, the influence of extrinsic environmental pollution and the impact on the allergenic potential of pollen.

In terms of a systematic characterization of bacteria and fungi colonizing pollen grains from birch tree and timothy grass it was shown, that the pollen-associated microbial load contains species both potentially beneficial and deleterious for plants. In further studies regarding pollen-allergenicity and pollen-associated microbes, dominant species/genera gained from both cultivation and cultivation-independent approaches should be considered. Further results showed that microbial compositions on pollen differ significantly between the two studied plant species. This fact was confirmed by three different molecular techniques (tRFLP, Illumina MiSeq, FISH) over three consecutive years. Correlation analyses of tRFLP fingerprinting showed a decreasing bacterial diversity in more urbanized areas. A further, significant correlation between high bacterial diversity, high allergen (Bet v 1) and lower PALMs-content was detected. Therefore, the hypothesis that alterations of anthropogenic factors might induce changes in the allergenicity of pollen by microbial interactions was supported in this study. According to the obtained results, changing pollution may impact pollen-associated bacterial diversity, which in turn might lead to altered gene expressions of allergens and allergen-related compound

In regard to the increasing number of incidences in allergenic diseases all over the world, subsequent studies about anthropogenic influences enhancing the allergenic potential of pollen, and their interaction with pollen-associated microbes are of high

importance. Also the possibility of an impact of the whole plant microbiome on the allergenicity-level of their pollen should be considered in future studies.

6 REFERENCES

Achatz G, Oberkofler H, Lechenbauer E, Simon B, Unger A*, et al.* (1995) Molecular clonig of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Mol Immunol* 32: 213-227.

Alegre I, Viñas I, Usall J, Anguera M, Altisent R & Abadias M (2013) Antagonistic effect of Pseudomonas graminis CPA-7 against foodborne pathogens in fresh-cut apples under simulated commercial conditions. *Food Microbiol* 33: 139-148.

Alqueres S, Meneses C, Rouws L, Rothballer M, Baldani I, Schmid M & Hartmann A (2013) The bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by Gluconacetobacter diazotrophicus PAL5. *Mol Plant Microbe Interact* 26: 937-945. doi: 10.1094/MPMI-12-12-0286-R.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.

Altuntas F, Yildiz O, Eser B, Gundogan K, Sumerkan B & Cetin M (2004) Catheter-related bacteremia due to Kocuria rosea in a patient undergoing peripheral blood stem cell transplantation. *BMC Infect Dis* 4: 62. doi: 10.1186/1471-2334-4-62.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. *Appl Environ Microbiol* 56: 1919-1925.

Amann RI & Fuchs BM (2008) Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nat Rev Microbiol* 6: 339-348. doi: 10.1038/nrmicro1888.

Amann RI, Ludwig W, Schleifer K-H (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* 59(1):143-169.

Anderson IC, Campbell CD & Prosser JI (2003a) Diversity of fungi in organic soils under a moorland - Scots pine (Pinus sylvestris L.) gradient. *Environ Microbiol* 5: 1121-1132. doi: 10.1046/j.1462-2920.2003.00522.x.

Anderson IC, Campbell CD & Prosser JI (2003b) Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ Microbiol* 5: 36-47.

Andersson AM, Weiss N, Rainey F & Salkinoja-Salonen M (1999) Dust-borne bacteria in animal sheds, school and children´s day care centres. *J Appl Microbiol* 86: 622-634.

Annapurna K, Kumar A, Kumar LV, Govindasamy V, Bose P & Ramadoss D (2013) PGPR-Induced Systemic Resistance (ISR) in Plant Disease Management. *Bacteria in Agrobiology: Disease Management.* (ed. Maheshwari DK), pp. 405-425. Springer-Verlag, Berlin Heidelberg.

Anwar N, Abaydulla G, Zayadan B, Abdurahman M, Hamood B*, et al.* (2016) *Pseudomonas populi* sp. nov., an endophytic bacterium isolated from *Populus euphratica*. *Int J Syst Evol Microbiol* 66: 1419-1425. doi: 10.1099/ijsem.0.000896.

Ariizumi T & Toriyama K (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. *Annu Rev Plant Biol* 62: 437-460. doi: 10.1146/annurev-arplant-042809-112312.

Asano T, Kimura M & Nishiuchi T (2012) The defense response in *Arabidopsis thalliana* against *Fusarium sporotrichioides. Proteome Sci* 10:61.

Atkinson CJ, Robe, V. and Winner, W. E. (1988) The relationship between changes in photosynthesis and growth for radish plants fumigated with SO_2 and NO_2. *New Phytol* 110: 173-184.

Bai Y, Zhou X & Smith DL (2003) Enhanced Soybean Plant Growth Resulting from Coinoculation of Bacillus Strains with *Bradyrhizobium japonicum*. *Crop Sci* 43: 1774-1781. doi: 10.2135/cropsci2003.1774.

Bai Y, Muller DB, Srinivas G, Garrido-Oter R, Potthoff E*, et al.* (2015) Functional overlap of the Arabidopsis leaf and root microbiota. *Nature* 528: 364-369. doi: 10.1038/nature16192.

Bais HP, Weir TL, Perry LG, Gilroy S & Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* 57: 233-266. doi: 10.1146/.

Barberán A, Ladau J, Leff JW, Pollard KS, Menninger HL, Dunn RR & Fierer N (2015) Continental-scale distributions of dust-associated bacteria and fungi. *PNAS* 112: 5756-5761.

Bauchau V & Durham SR (2004) Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 24: 758-764. doi: 10.1183/09031936.04.00013904.

Beck I, Jochner S, Gilles S, McIntyre M, Buters JT*, et al.* (2013) High environmental ozone levels lead to enhanced allergenicity of birch pollen. *PLoS One* 8. doi: 10.1371/journal.pone.0080147.

Behrendt U, Ulrich A & Schumann P (2003) Fluorescent pseudomonads associated with the phyllosphere of grasses; *Pseudomonas trivialis* sp. nov., *Pseudomonas poae*

sp. nov. and *Pseudomonas congelans* sp. nov.. *Int J Syst Evol Microbiol* 53: 1461-1469. doi: 10.1099/ijs.0.02567-0.

Behrendt U, Ulrich A, Schumann P, Erler W, Burghardt J & Seyfarth W (1999) A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov.. *Int J Syst Evol Microbiol* 49: 297-308.

Bensch K, Braun U, Groenewald JZ & Crous PW (2012) The genus Cladosporium. *Stud Mycol* 72: 1-401. doi: 10.3114/sim0003.

Berg G, Krechel A, Ditz M, Sikora RA, Ulrich A & Hallmann J (2005) Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiol Ecol* 51: 215-229. doi: 10.1016/j.femsec.2004.08.006.

Blackmore S, Wortley AH, Skvarla JJ & Rowley JR (2007) Pollen wall development in flowering plants. *New Phytol* 174: 483-498. doi: 10.1111/j.1469-8137.2007.02060.x.

Boehme MW, Kompauer I, Weidner U, Piechotowski I, Gabrio T & Behrendt H (2013) Respiratory symptoms and sensitization to airborne pollen of ragweed and mugwort of adults in Southwest Germany. *Deutsche Medizinische Wochenschrift (1946)* 138: 1651-1658.

Bostock RM, Pye MF & Roubtsova TV (2014) Predisposition in plant disease: exploiting the nexus in abiotic and biotic stress perception and response. *Annu Rev Phytopathol* 52: 517-549. doi: 10.1146/annurev-phyto-081211-172902.

Brosius J, Palmer ML, Kennedy PJ & Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci* 75: 4801-4805.

Bubert H, Lambert J, Steuernagel S, Ahlers F & Wiermann R (2002) Continuous decomposition of sporopollenin from pollen of *Typha angustifolia* L. by acidic methanolysis. *Z Naturforsch* Teil C 57: 1035-1041.

Buddrus-Schiemann KEM (2008) Wirkung des biologischen Pflanzenstärkungsmittels Proradix® (*Pseudomonas fluorescens*) auf das Wachstum von Gerste (*Hordeum vulgare* L. cv. Barke) und auf die bakterielle Gemeinschaft in der Rhizosphäre. Thesis, Ludwig-Maximilia-Universität (LMU), München.

Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N*, et al.* (2012) Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488: 91-95. doi: 10.1038/nature11336.

Burney P, Malmberg E, Chinn S, Jarvis D, Luczynska C & Lai E (1997) The distribution of total and specific serum IgE in the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 99: 314-322.

Calvente V, Benuzzi D & de Tosetti MIS (1999) Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. *Int Biodeterior Biodegradation* 43: 167-172.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J*, et al.* (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 6: 1621-1624. doi: 10.1038/ismej.2012.8.

Chakraborty U, Chakraborty BN, Chakraborty AP & Dey PL (2013) Water stress amelioration and plant growth promotion in wheat plants by osmotic stress tolerant bacteria. *World J Microbiol Biotechnol* 29: 789-803. doi: 10.1007/s11274-012-1234-8.

Chelius MK & Triplett EW (2001) The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L.. *Microb Ecol* 41: 252-263. doi: 10.1007/s002480000087.

Chen DM & Cairney JWG (2002) Investigation of the influence of prescribed burning on ITS profiles of ectomycorrhizal and other soil fungi at three Australian sclerophyll forest sites. *Mycol Res* 106: 532-540.

Chen J, Li B, Qin G & Tian S (2015) Mechanism of H_2O_2-induced oxidative stress regulating viability and biocontrol ability of *Rhodotorula glutinis. Int J Food Microbiol* 193: 152-158. doi: 10.1016/j.ijfoodmicro.2014.10.025.

Chen J, Liu X, Zheng J, Zhang B, Lu H*, et al.* (2013) Biochar soil amendment increased bacterial but decreased fungal gene abundance with shifts in community structure in a slightly acid rice paddy from Southwest China. *Appl Soil Ecol* 71: 33-44. doi: 10.1016/j.apsoil.2013.05.003.

Chen Y, Chen G, Robinson D, Yang Z, Guo J *et al.* (2016) Large amounts of easily decomposable carbon stored in subtropical forest subsoil are associated with r-strategy-dominated soil microbes. *Soil Biol Biochem* 95:233-242.

Cheng KC, Demirci A & Catchmark JM (2011) Pullulan: biosynthesis, production, and applications. *Appl Microbiol Biotechnol* 92: 29-44. doi: 10.1007/s00253-011-3477-y.

Chowdhury SP, Dietel K, Rändler M, Schmid M, Junge H*, et al.* (2013) Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. *PLoS ONE* 8: e68818. doi: 10.1371/journal.pone.0068818.

Chun J, Rhee M-S, Han J-I & Bae KS (2001) *Arthrobacter siderocapsulatus* Dubinina and Zhdanov 1975 is a later subjective synonym of *Pseudomonas putida* (Trevisan 1889) Migula 1895. *Int J Syst Evol Microbiol* 51:169-170.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. *Austral Ecol* 18: 117-143.

Clarke KR & Gorley RN (2001) Primer v5: User manual/tutorial, Primer-E. *Plymouth UK* 91.

Coca AF (1929) The Skin as a shock tissue. *B New York Acad Med* V: 223-231.

Collins MD & Jones D (1983) Reclassification of *Corynebacterium flaccumfaciens*, *Corynebacterium betae*, *Corynebacterium oortii* and *Corynebacterium poinsettiae* in the genus Curtobacterium, as *Curtobacteiium flaccumfaciens* comb. nov.. *J Gen Microbiol* 129: 3545-3548.

Collins MD, Brown J & Jones D (1988) *Brachybacterium faecium* gen. nov., sp. nov., a Coryneform Bacterium from poultry deep litter. *Int J Syst Evol Microbiol* 38: 45-48.

Collmer A & Keen NT (1986) The role of pectic enzymes in plant pathogenesi*Annu Rev Phytopathol* 24: 383-409.

Compant S, Duffy, B., Nowak, J., Clement, C., Barka, E. A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* 71: 4951-4959. doi: 10.1128/AEM.71.9.4951-4959.2005.

Coroler L, Elomari M, Hoste B, Gillis M, Izard D & Leclerc H (1996) *Pseudomonas rhodesiae* sp. nov., a new species isolated from natural mineral water. *Syst Appl Microbiol* 19: 600-607. doi: 10.1016/s0723-2020(96)80032-4.

Cruz AT, Cazacu AC & Allen CH (2007) *Pantoea agglomerans*, a plant pathogen causing human disease. *J Clin Microbiol* 45: 1989-1992. doi: 10.1128/JCM.00632-07.

Culman SW, Gauch HG, Blackwood CB & Thies JE (2008) Analysis of tRFLP data using analysis of variance and ordination methods: A comparative study. *J Microbiol Methods* 75: 55-63.

D'Amato G & Spieksma FTM (1991) Allergenic pollen in europe. *Grana* 30: 67-70. doi: 10.1080/00173139109427772.

D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I*, et al.* (2007) Allergenic pollen and pollen allergy in Europe. *Allergy* 62: 976-990. doi: 10.1111/j.1398-9995.2007.01393.x.

Dabboussi F, Hamze M, Elomari M, Verhille S, Baida N, Izard D & Leclerc H (1999) Taxonomic study of bacteria isolated from Lebanese spring waters: proposal for *Pseudomonas cedrella* sp. nov. and *P. orientalis* sp. nov.. *Res Microbiol.* 150: 303-316.

Daims H, Brühl A, Amann R, Schleifer K-H & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* 22: 434-444. doi: 10.1016/s0723-2020(99)80053-8.

Darrall NM (1989) The effect of air pollutants on physiological processes in plants. *Plant Cell Environ* 12: 1-30.

Dickie IA & FitzJohn RG (2007) Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: a methods review. *Mycorrhiza* 17: 259-270. doi: 10.1007/s00572-007-0129-2.

Dickie IA, Xu B & Koide RT (2002) Vertical distribution of ectomycorrhizal hyphae in soil as shown by T-RFLP analysi*New Phytol* 527-535.

Djukanovic R, Feather I, Gratziou C, Walls A, Peroni D*, et al.* (1996) Effect of natural allergen exposure during the grass pollen season on airways inflammatory cells and asthma symptom*Thorax* 51: 575-581.

Edwards U, Rogall T, Blocker H, Emde M & Bottger EC (1989) Isolation and direct complete nucleotide determination of entire geneCharacterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 17: 7843-7853.

Erb M, Meldau S & Howe GA (2012) Role of phytohormones in insect-specific plant reaction*Trends Plant Sci* 17: 250-259. doi: 10.1016/j.tplants.2012.01.003.

Finkel OM, Burch AY, Elad T, Huse SM, Lindow SE, Post AF & Belkin S (2012) Distance-decay relationships partially determine diversity patterns of phyllosphere bacteria on *Tamarix* trees across the Sonoran Desert. *Appl Environ Microbiol* 78: 6187-6193. doi: 10.1128/AEM.00888-12.

Fosse T, Peloux Y, Granthil C, Toga B, Bertrando J & Sethian M (1985) Meningitis Due to *Micrococcus luteuInfection* 13: 280-281.

Fruhling A (2002) *Exiguobacterium undae* sp. nov. and *Exiguobacterium antarcticum* sp. nov.. *Int J Syst Evol Microbiol* 52: 1171-1176. doi: 10.1099/ijs.0.02185-0.

Galarce-Bustos O, Alvarado M, Vega M & Aranda M (2014) Occurrence of ochratoxin A in roasted and instant coffees in Chilean market. *Food Control* 46: 102-107. doi: 10.1016/j.foodcont.2014.05.014.

Gamez RM, Rodríguez F, Bernal JF, Agarwala R, Landsman D & Mariño-Ramírezc L (2015) Genome sequence of the banana plant growth-promoting rhizobacterium *Bacillus amyloliquefaciens* BS006. *Genome Announc* 3. doi: 01391-15. doi: 10.1128/genomeA.01391-15.

Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rust. *Mol Ecol* 2: 113-118. doi: 10.1111/j.1365-294X.1993.tb00005.x.

Gavini F, Mergaert J, Beji A, Mielcarek C, Izard D, Kersters K & De Ley J (1989) Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov.. *Int J Syst Evol Microbiol* 39: 337-345.

Geider K, Auling G, Du Z, Jakovljevic V, Jock S & Volksch B (2006) *Erwinia tasmaniensis* sp. nov., a non-phytopathogenic bacterium from apple and pear trees. *Int J Syst Evol Microbiol* 56: 2937-2943. doi: 10.1099/ijs.0.64032-0.

Gilles S, Behrendt H, Ring J & Traidl-Hoffmann C (2012) The pollen enigma: modulation of the allergic immune response by non-allergenic, pollen-derived compounds. *Curr Pharm Des* 18: 2314-2319.

Gostinčar C, Ohm RA, Kogej T, Sonjak S, Turk M*, et al.* (2014) Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. *BMC Genomics* 15: 1-28.

Grenier AM, Duport G, Pages S, Condemine G & Rahbe Y (2006) The phytopathogen *Dickeya dadantii* (*Erwinia chrysanthemi* 3937) is a pathogen of the pea aphid. *Appl Environ Microbiol* 72: 1956-1965. doi: 10.1128/AEM.72.3.1956-1965.2006.

Grudzinska-Sterno M, Yuen J, Stenlid J & Djurle A (2016) Fungal communities in organically grown winter wheat affected by plant organ and development stage. *Eur J Plant Pathol*. doi: DOI 10.1007/s10658-016-0927-5.

Gutierrez-Manero FJ, Ramos-Solano B, Probanza A, Mehouachi J, Tadeo FR & Talon M (2001) The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellin. *Physiol plant* 111: 206-211.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. AcidSymp.* 41: 95-98.

Hallmann J & Berg G (2006) Spectrum and population dynamics of bacterial root enodphyte*Microbial Root EndophyteSoil biology,* Vol. 9 (edSchulz BJE, Boyle CJC & Sieber TN), pp. 15-31. Springer Berlin Heidelberg.

Hammer O, Harper DAT & Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontol Electronica* 4(1). URL: http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Hardoim PR, Van Overbeek LS & Van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 16: 463-471.

Hatta R, Ito K, Hosaki Y, Tanaka T, Tanaka A*, et al.* (2002) A Conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata. Genetics* 161: 59-70.

Heil M & Ton J (2008) Long-distance signalling in plant defence. *Trends Plant Sci* 13: 264-272. doi: 10.1016/j.tplants.2008.03.005.

Heydenreich B, Bellinghausen I, Konig B, Becker WM, Grabbe S, Petersen A & Saloga J (2012) Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy* 42: 76-84. doi: 10.1111/j.1365-2222.2011.03888.x.

Heyndrickx M, Coorevits A, Scheldeman P, Lebbe L, Schumann P*, et al.* (2012) Emended descriptions of *Bacillus sporothermodurans* and *Bacillus oleronius* with the inclusion of dairy farm isolates of both species. Int J Syst Evol Microbiol 62: 307-314. doi: 10.1099/ijs.0.026740-0.

Hoshino T, Yilmaz LS, Noguera DR, Daims H & Wagner M (2008) Quantification of target molecules needed to detect microorganisms by fluorescence *in situ* hybridization (FISH) and catalyzed reporter deposition-FISH. *Appl Environ Microbiol* 74: 5068-5077. doi: 10.1128/AEM.00208-08.

Jha B, Gontia I & Hartmann A (2012) The roots of the halophyte *Salicornia brachiata* are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential. *Plant Soil* 356(1):265-277.

Houbraken J, Frisvad JC & Samson RA (2011) Taxonomy of *Penicillium* section Citrina. *Stud Mycol* 70: 53-138. doi: 10.3114/sim.2011.70.02.

Jakovljevic V, Jock S, Du Z & Geider K (2008) Hypersensitive response and acyl-homoserine lactone production of the fire blight antagonists *Erwinia tasmaniensis* and *Erwinia billingiae*. *Microb Biotechnol* 1: 416-424. doi: 10.1111/j.1751-7915.2008.00043.x.

Jarmuda S, O'Reilly N, Zaba R, Jakubowicz O, Szkaradkiewicz A & Kavanagh K (2012) Potential role of Demodex mites and bacteria in the induction of rosacea. *J med microbiol* 61: 1504-1510. doi: 10.1099/jmm.0.048090-0.

Jeon CO, Lim JM, Lee JM, Xu LH, Jiang CL & Kim CJ (2005) Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* 55: 1891-1896. doi: 10.1099/ijs.0.63456-0.

John R, Ahmad P, Gadgil K & Sharma S (2009) Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation in *Brassica juncea* L. *Int J plant pro* 3: 65-76.

Jones JD & Dangl JL (2006) The plant immune system. *Nature* 444: 323-329. doi: 10.1038/nature05286.

Kai M, Effmert U, Berg G & Piechulla B (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch Microbiol* 187: 351-360. doi: 10.1007/s00203-006-0199-0.

Kalbe C, Marten P & Berg G (1996) Strains of the genus Serratia as beneficial rhizobacteria of oilseed rape with antifungal properties. *Microbiol Res* 151: 433-439. doi: 10.1016/s0944-5013(96)80014-0.

Kalita M, Bharadwaz M, Dey T, Gogoi K, Dowarah P*, et al.* (2015) Developing novel bacterial based bioformulation having PGPR properties for enhanced production of agricultural crops. *Indian J Exp Biol* 53: 56-60.

Keller A, Grimmer G & Steffan-Dewenter I (2013) Diverse microbiota identified in whole intact nest chambers of the red mason bee *Osmia bicornis* (Linnaeus 1758). *PLoS One* 8: e78296. doi: 10.1371/journal.pone.0078296.

Khodai-Kalaki M, Andrade A, Fathy Mohamed Y & Valvano MA (2015) *Burkholderia cenocepacia* lipopolysaccharide modification and flagellin glycosylation affect virulence but not innate immune recognition in plants. *MBio* 6: e00679-00615. doi: 10.1128/mBio.00679-15.

Kloepper JW, Ryu C-M & Zhang S (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp.. *Phytopathology* 94: 1259-1266.

Köhler W, Schachtel G & Voleske P (eds., 2007) *Biostatistik. Einführung für Biologen und Agrarwissenschaftler.* Springer-Verlag Berlin Heidelberg.

Kothari VV, Kothari RK, Kothari CR, Bhatt VD, Nathani NM*, et al.* (2013) Genome Sequence of Salt-Tolerant *Bacillus safensis* Strain VK, isolated from saline desert area of Gujarat, India. *Genome Announc* 1. doi: i:10.1128/genomeA.00671-13.

Kratz A, Greenberg D, Barki Y, Cohen E & Lifshitz M (2003) *Pantoea agglomerans* as a cause of septic arthritis after palm tree thorn injury; case report and literature review. *Arch Dis Child* 88: 542-544.

Kriaa M, Hammami I, Sahnoun M, Azebou MC, Triki MA & Kammoun R (2015) Biocontrol of tomato plant diseases caused by *Fusarium solani* using a new isolated *Aspergillus tubingensis* CTM 507 glucose oxidase. *Comptes Rendus Biologies* 338: 666-677.

Kube M, Migdoll AM, Gehring I, Heitmann K, Mayer Y*, et al.* (2010) Genome comparison of the epiphytic bacteria *Erwinia billingiae* and *E. tasmaniensis* with the pear pathogen *E. pyrifoliae*. *BMC Genomics* 11.

Kuhnigk T, Borst E-M, Breunig A, König H, Collins MD, Hutson RA & Kämpfer P (1995) *Bacillus oleronius* sp.nov., a member of the hindgut flora of the termite *Reticulitermes santonensis* (Feytaud). *Can J Microbiol* 41: 699-706. doi: 10.1139/m95-096.

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G*, et al.* (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtiliNature* 390: 249-256.

Lacey N, Delaney S, Kavanagh K & Powell FC (2007) Mite-related bacterial antigens stimulate inflammatory cells in rosacea. *Br J Dermatol* 157: 474-481. doi: 10.1111/j.1365-2133.2007.08028.x.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML & Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *PNAS* 82: 6955-6959.

Leisner JJ, Laursen BG, Prevost H, Drider D & Dalgaard P (2007) Carnobacterium: positive and negative effects in the environment and in foods. *FEMS Microbiol Rev* 31: 592-613. doi: 10.1111/j.1574-6976.2007.00080.x.

Li J, O'Reilly N, Sheha H, Katz R, Raju VK, Kavanagh K & Scheffer Tseng CG (2010) Correlation between ocular Demodex infestation and serum immunoreactivity to Bacillus proteins in patients with facial Rosacea. *Ophthalmol* 117: 870-877.

Li L, Zhao C, Liu Q & Zhang YQ (2012) Isolation and genetic identification of dibenzothiophene degrading bacteria from contaminated soil. *Advanced material research* 610-613: 292-295.

Li YQ, Chen F, Linskens HF & Cresti M (1994) Distribution of unesterified and esterified pectins in cell walls of pollen tubes of flowering plants. *Sex Plant Reprod* 7:145-152.

Lindow SE & Brandl MT (2003) Microbiology of the phyllosphere. *Appl Environ Microbiol* 69: 1875-1883. doi: 10.1128/aem.69.4.1875-1883.2003.

Loeffler C, Berger S, Guy A, Durand T, Bringmann G*, et al.* (2005) B1-phytoprostanes trigger plant defense and detoxification response*J Plant physiol* 137: 328-340. doi: 10.1104/pp.104.051714.

Lord NS, Kaplan CW, Shank P, Kitts CL & Elrod SL (2002) Asessement of fungal diversity. *FEMS Microbiol Ecol* 42: 327-337.

Lozo J, Beric T, Terzic-Vidojevic A, Stankovic S, Fira D & Stanisavljevic L (2015) Microbiota associated with pollen, bee bread, larvae and adults of solitary bee *Osmia cornuta* (Hymenoptera: *Megachilidae*). *Bull Entomol Res* 105: 470-476. doi: 10.1017/S0007485315000292.

Ma B, Hibbing ME, Kim HS, Reedy RM, Yedidia I*, et al.* (2007) Host range and molecular phylogenies of the soft rot enterobacterial genera pectobacterium and *Dickeya*. *Phytopathology* 97: 1150-1163. doi: 10.1094/PHYTO-97-9-1150.

Madigan MT & Martinko JM (eds., 2009) *Brock: Mikrobiologie.* Pearson Studium, München.

Madmony A, Chernin L, Pleban S, Peleg E & Riov J (2005) *Enterobacter cloacae*, an obligatory endophyte of pollen grains of mediterranean pines. *Folia Microbiol* 50: 209-216.

Mandic-Mulec I, Stefanic P & Van Elsas JD (2015) Ecology of *Bacillaceae*. *Microbiol Spectr* 3. doi: 10.1128/microbiolspec.TBS-0017-2013.

Manz W, Wagner M, Amann R & Schleifer K-K (1994) *In situ* characterization of the microbial consortia active in two wastewater treatment plants. *Water Res* 28: 1715-1712.

Manz W, Amann R, Ludwig W, Wagner W & Schleifer K-H (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* 15: 593-600.

Marasas WFO, Nelson PE & Toussoun TA (1984) *Toxigenic Fusarium species. Identity and mycotoxicology.* Pennsylvania State University, University Park, USA.

Mattina MI, Lannucci-Berger W, Musante C & White JC (2003) Concurrent plant uptake of heavy metals and persistent organic pollutants from soil. *Environ Pollut* 124: 375-378. doi: 10.1016/s0269-7491(03)00060-5.

McFrederick QS & Rehan SM (2016) Characterization of pollen and bacterial community composition in brood provisions of a small carpenter bee. *Mol Ecol* 25: 2302-2311. doi: 10.1111/mec.13608.

Medina A, Mateo R, Lopez-Ocana L, Valle-Algarra FM & Jimenez M (2005) Study of spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section n*igri*. *Appl Environ Microbiol* 71: 4696-4702. doi: 10.1128/AEM.71.8.4696-4702.2005.

Mercado-Blanco J (2015) Life of microbes inside the plant. *Principles of Plant-Microbe-Interactions: Microbes for sustainable Agriculture.* (ed. Lugtenberg B), pp. 25-32. Springer International Publishing Switzerland, Cham Heidelberg.

Mercado-Blanco J & Lugtenberg BJJ (2014) Biotechnological applications of bacterial endophytes. *Curr Biotechnol* 3: 60-75(16).

Mergaert J, Hauben L, Cnockaert MC & Swings J (1999) Reclassification of non-pigmented *Erwinia herbicola* strains from trees as *Erwinia billingiae* sp. nov.. *Int J Syst Bacteriol* 49: 377-383.

Mora D, Scarpellini M, Franzetti L, Colombo S & Galli A (2003) Reclassification of *Lactobacillus maltaromicus* (Miller et al. 1974) DSM 20342(T) and DSM 20344 and *Carnobacterium piscicola* (Collins et al. 1987) DSM 20730(T) and DSM 20722 as *Carnobacterium maltaromaticum* comb. nov.. *Int J Syst Evol Microbiol* 53: 675-678. doi: 10.1099/ijs.0.02405-0.

Mouloud G, Daoud H, Bassem J, Laribi Atef I & Hani B (2013) New bacteriocin from *Bacillus clausii* strainGM17: purification, characterization, and biological activity. *Appl Biochem Biotechnol* 171: 2186-2200. doi: 10.1007/s12010-013-0489-3.

Müller W & Frings S (eds., 2007) *Tier- und Humanphysiologie*. Springer-Verlag, Berlin-Heidelberg.

Muramatsu D, Iwai A, Aoki S, Uchiyama H, Kawata K*, et al.* (2012) Beta-glucan derived from *Aureobasidium pullulans* is effective for the prevention of influenza in mice. *PLoS One* 7: e41399. doi: 10.1371/journal.pone.0041399.

Newman MA, Sundelin T, Nielsen JT & Erbs G (2013) MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front Plant Sci* 4: 1-14. doi: 10.3389/fpls.2013.00139.

Nielsen P, Fritze D, Priest FG (1995) Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new specie*Microbiology* 141: 1745-1761.

Nishimura S & Kohmoto K (1983) Host-specific toxins and chemical structures from *Alternaria* species. *Annu Rev Phytopathol* 21: 87-116.

Nübel U, Garcia-Pichel F & Muyzer G (1997) PCR primers to amplify 16S rRNA genes from *Cyanobacteria*. *Appl Environ Microbiol* 63: 3327-3332.

O'Reilly N, Bergin D, Reeves EP, McElvaney NG & Kavanagh K (2012) *Demodex*-associated bacterial proteins induce neutrophil activation. *Br J Dermatol* 166: 753-760. doi: 10.1111/j.1365-2133.2011.10746.x.

Obersteiner A, Gilles S, Frank U, Beck I, Haring F*, et al.* (2016) Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. *PLoS One* 11: e0149545. doi: 10.1371/journal.pone.0149545.

Osdaghi E, Taghavi SM, Fazliarab A, Elahifard E & Lamichhane JR (2015) Characterization, geographic distribution and host range of *Curtobacterium flaccumfaciens*: An emerging bacterial pathogen in Iran. *Crop Prot* 78: 185-192. doi: 10.1016/j.cropro.2015.09.015.

Partida-Martinez LP, de Looss CF, Ishida K, Ishida M, Roth M, Buder K & Hertweck C (2007) Rhizonin, the first mycotoxin isolated from the *Zygomycota*, is not a fungal metabolite but is produced by bacterial endosymbionts. *Appl Environ Microbiol* 73: 793-797. doi: 10.1128/AEM.01784-06.

Pawankar R, Canonica GW, Holgate ST & Lockey RFe (2011) *WAO White Book on Allergy*. World Allergy Organization, Milwaukee, USA.

Peces R, Gago E, Tejada F, Laures AS & Alvarez-Grande J (1997) Relapsing bacteraemia due to *Micrococcus luteus* in a haemodialysis patient with a Perm-Cath catheter. *Nephrol Dial Transplant* 12: 2428-2429.

Peix A, Ramírez-Bahena M-H & Velázquez E (2009) Historical evolution and current status of the taxonomy of genus *Pseudomonas. Infect Genet Evol* 9: 1132-1147. doi: 10.1016/j.meegid.2009.08.001.

Perrone G, Mule G, Susca A, Battilani P, Pietri A & Logrieco A (2006) Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Appl Environ Microbiol* 72: 680-685. doi: 10.1128/AEM.72.1.680-685.2006.

Pitt JI (1994) The current role of *Aspergillus* and *Penicillium* in human and animal health. *J Med Vet Mycol* 32: 17-32.

Priest FG, Goodfellow M & Todd C (1988) A numerical classification of the genus *Bacillus. J Gen Microbiol* 134: 1847-1882.

Probanza A, Mateos JL, Lucas Garcia JA, Ramos B, De Felipe MR & Gutierrez Manero FJ (2001) Effects of Inoculation with PGPR *Bacillus* and *Pisolithus tinctorius* on *Pinus pinea* L. growth, bacterial rhizosphere colonization, and mycorrhizal infection. *Microb Ecol* 41: 140-148. doi: 10.1007/s002480000081.

Pruesse E, Peplies J & Glockner FO (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28: 1823-1829. doi: 10.1093/bioinformatics/bts252.

Rasimus-Sahari S, Mikkola R, Andersson MA, Jestoi M & Salkinoja-Salonen M (2016) *Streptomyces* strains producing mitochondriotoxic antimycin A found in cereal grains. *Int J Food Microbiol* 218: 78-85. doi: 10.1016/j.ijfoodmicro.2015.11.007.

Rastogi G, Sbodio A, Tech JJ, Suslow TV, Coaker GL & Leveau JH (2012) Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J* 6: 1812-1822. doi: 10.1038/ismej.2012.32.

Redford AJ, Bowers RM, Knight R, Linhart Y & Fierer N (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environ Microbiol* 12: 2885-2893. doi: 10.1111/j.1462-2920.2010.02258.x.

Reinhold-Hurek B & Hurek T (2011) Living inside plants: bacterial endophytes. *Curr Opin Plant Biol* 14: 435-443. doi: 10.1016/j.pbi.2011.04.004.

Rodrigues DF, Goris J, Vishnivetskaya T, Gilichinsky D, Thomashow MF & Tiedje JM (2006) Characterization of *Exiguobacterium* isolates from the siberian permafrost. Description of *Exiguobacterium sibiricum* sp. nov.. *Extremophiles* 10: 285-294. doi: 10.1007/s00792-005-0497-5.

Saitoh S, Suzuki T & Nishimura Y (1998) Proposal of *Craurococcus roseus* gen. nov., sp. nov. and *Paracraurococcus ruber* gen. nov., sp. nov., novel aerobic bacteriochlorophyll a-containing bacteria from soil. *Int J Syst Evol Microbiol* 48: 1043-1047.

Saitou N & Nei M (1987) The Neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol Evol* 4: 406-425.

Samson R, Legendre J, Christen R, Fischer-Le Saux M, Achouak W & Gardan L (2005) Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., Dic*keya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov.. *Int J Syst Evol Microbiol* 55: 1415-1427.

Satomi M, La Duc MT & Venkateswaran K (2006) *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *Int J Syst Evol Microbiol* 56: 1735-1740. doi: 10.1099/ijs.0.64189-0.

Saxe H (1991) Photosynthesis and stomatal responses to polluted air, and the use of physiological and biochemical responses for early detection and diagnostic tools. *Advances in Botanical Research* (ed. Callow JA). Academic Press, London, UK.

Scherlach K, Graupner K & Hertweck C (2013) Molecular bacteria-fungi interactions: effects on environment, food, and medicine. *Annu Rev Microbiol* 67: 375-397. doi: 10.1146/annurev-micro-092412-155702.

Schmidt CS, Alavi M, Cardinale M, Müller H & Berg G (2012) *Stenotrophomonas rhizophila* DSM14405T promotes plant growth probably by altering fungal communities in the rhizosphere. *Biology and Fertility of Soils* 48: 947-960. doi: 10.1007/s00374-012-0688-z.

Schneider S, Hendriksen NB, Melin P, Lundstrom JO & Sundh I (2015) Chromosome-directed PCR-based detection and quantification of *Bacillus cereus* group members with focus on *B. thuringiensis Serovar israelensis* active against nematoceran larvae. *Appl Environ Microbiol* 81: 4894-4903. doi: 10.1128/AEM.00671-15.

Schreiber L, Krimm U, Knoll D, Sayed M, Auling G & Kroppenstedt RM (2005) Plant-microbe interactions: identification of epiphytic bacteria and their ability to alter leaf surface permeability. *New Phytol* 166: 589-594. doi: 10.1111/j.1469-8137.2005.01343.x.

Schubert K, Ludwig W, Springer N, Kroppenstedt RM, Accol J-P & Fiedler F (1996) Two coryneform bacteria isolated from the surface of french Gruykre and Beaufort Cheeses are new species of the genus *Brachybacterium*: *Brachybacterium alimentarium* sp. nav. and *Brachybacterium tyrofernentans* sp. nov.. *Int. J. Syst. Bacterio* 46(1):81-87.

Schulz B (2006) Mutualistic interactions with fungal root endophytes. *Microbial root endophyteSoil Biology,* Vol. 9 (edSchulz BJE, Boyle CJC & Sieber TN), pp. 261-279. Springer Berlin Heidelberg.

Schwartz A, Ortiz I, Maymon M, Herbold C, Fujishige N*, et al.* (2013) *Bacillus simplex* - a little known PGPB with anti-fungal activity - alters pea legume root architecture and nodule morphology when coinoculated with *Rhizobium leguminosarum* bv. *viciae*. *Agronomy* 3: 595-620. doi: 10.3390/agronomy3040595.

Scott R (1994) Pollen exine: the sporopollenin enigma and the physics of pattern. *Molecular and cellular aspects of plant reproduction.* (eds. Scott R & Stead M), pp. 49-81. Cambridge univ. press, Cambridge, NY.

Scott RJ, Spielman M & Dickinson HG (2004) Stamen structure and function. *The Plant cell* 16 Suppl: S46-60. doi: 10.1105/tpc.017012.

Selladurai BM, Sivakumaran S, Aiyar S & Mohamad AR (2009) Intracranial suppuration caused by *Micrococcus luteus. Br J Neurosurg* 7: 205-207. doi: 10.3109/02688699309103481.

Selman M, Carrillo G, Navarro C & Gaxiola M (2009) Hypersensitivity Pneumoniti*Pulmonary Arterial Hypertension and Interstitial Lung Disease*(eds. Baughman RP, Carbone RG & Bottino G), pp. 139-157. Humana Presdoi: 10.1007/978-1-60327-074-8_7.

Serrano R, Mulet JM, Rios G, Marquez JA, de Larrinoa IF*, et al.* (1999) A glimpse of the mechanisms of ion homeostasis during salt stres*J Exp Bot* 50: 1023-1036.

Shternshis MV, Belyaev AA, Shpatova TV & Lelyak AA (2015) Influence of *Bacillus* spp. on strawberry gray-mold causing agent and host plant resistance to disease. *Contemp Probl Ecol* 8: 390-396. doi: 10.1134/s1995425515030130.

Sitte P, Weiler EW, Kadereit JW, Bresinsiky A & Körner C (2002) *Straßburger: Lehrbuch der Botanik*. Spektrum Akademischer Verlag, Heidelberg, Berlin.

Skerman VBD, McGowan V & Sneath PHA (1980) Approved Lists of Bacterial Names. *Int. J. Syst. Bacterio* 30: 225-420.

Smith DJ, Timonen HJ, Jaffe DA, Griffin DW, Birmele MN*, et al.* (2013) Intercontinental dispersal of bacteria and archaea by transpacific winds. *Appl Environ Microbiol* 79: 1134-1139.

Souhami L, Feld R, Tuffnell PG & Feller T (1979) *Micrococcus luteus* pneumonia: A case report and review. *Med Pediatr Oncol* 7: 309-314.

Staley JT & Konopka A (1985) Measurement of *in situ* activities of non-photosynthetic microorganisms in aquatic and terrestrial habitats. *Ann Rev Microbiol* 39:321-346.

Stoffels M, Castellanos T & Hartmann A (2001) Design and application of new 16S rRNA-targeted oligonucleotide probes for the *Azospirillum-Skermanella-Rhodocista*-cluster. *Syst Appl Microbiol* 24: 83-97. doi: 10.1078/0723-2020-00011.

Strunk O & Ludwig W (1997) ARB software program package. URL: http://www.biol.chemie.tu-muenchen.de/pub/ARB/

Suzuki MT & Giovanonni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* 62: 625-630.

Swoboda I, Scheiner O, Kraft D, Breitenbach M, Heberle-Bors E & Vicente O (1994) A birch gene family encoding pollen allergens and pathogenesis-related proteins. *Biochim Biophys Acta* 1219: 457-464**.**

Sylvester K, Wang QM, James B, Mendez R, Hulfachor AB & Hittinger CT (2015) Temperature and host preferences drive the diversification of *Saccharomyces* and other yeasts: a survey and the discovery of eight new yeast species. *FEMS yeast res* 15. doi: 10.1093/femsyr/fov002.

Taylor JM, Sutherland AD, Aidoo KE & Logan NA (2005) Heat-stable toxin production by strains of B*acillus cereus, Bacillus firmus, Bacillus megaterium, Bacillus simplex* and *Bacillus licheniformis. FEMS Microbiol Lett* 242: 313-317. doi: 10.1016/j.femsle.2004.11.022.

Taylor LP & Hepler PK (1997) Pollen germination and tube growth. *Annu Rev Plant Physiol Plant Mol Biol* 48: 461-491.

Thoma I, Krischke M, Loeffler C & Mueller MJ (2004) The isoprostanoid pathway in plants. *Chem Phys Lipids* 128: 135-148. doi: 10.1016/j.chemphyslip.2003.10.002.

Tiwari S, Agrawal M & Marshall FM (2006) Evaluation of ambient air pollution impact on carrot plants at a suburban site using open top chambers. *Environ Monit Assess* 119: 15-30. doi: 10.1007/s10661-005-9001-z.

Toth IK, van der Wolf JM, Saddler G, Lojkowska E, Hélias V*, et al.* (2011) *Dickeya* species: an emerging problem for potato production in Europe. *Plant Pathology* 60: 385-399.

Toumatia O, Compant S, Yekkour A, Goudjal Y, Sabaou N*, et al.* (2016) Biocontrol and plant growth promoting properties of *Streptomyces mutabilis* strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization. *S Afr J Bot* 105: 234-239.

Traidl-Hoffmann C, Jakob T & Behrendt H (2009) Determinants of allergenicity. *J Allergy Clin Immunol* 123: 558-566. doi: 10.1016/j.jaci.2008.12.003.

Trda L, Boutrot F, Claverie J, Brule D, Dorey S & Poinssot B (2015) Perception of pathogenic or beneficial bacteria and their evasion of host immunity: pattern recognition receptors in the frontline. *Front Plant Sci* 6. doi: 10.3389/fpls.2015.00219.

Turner S, Pryer KM, Mia VPW & Palmer JD (1999) Investigating deep phylogenetic relationships among *Cyanobacteria* and plastids by small subunit rRNA sequence analysis. *J Eukaryot Microbiol* 46: 327-338.

van Overbeek L & van Elsas JD (2008) Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.). *FEMS Microbiol Ecol* 64: 283-296. doi: 10.1111/j.1574-6941.2008.00469.x.

Vorholt JA (2012) Microbial life in the phyllosphere. *Nat Rev Microbiol* 10: 828-840. doi: 10.1038/nrmicro2910.

Wang W, Vinocur B & Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218: 1-14. doi: 10.1007/s00425-003-1105-5.

Weikl F, Tischer C, Probst AJ, Heinrich J, Markevych I, Jochner S & Pritsch K (2016) Fungal and bacterial communities in indoor dust follow different environmental determinants. *PLoS One* 11: e0154131. doi: 10.1371/journal.pone.0154131.

Wen H, Yang L, Shen L, Hu B, Li Z & Jin Q (2009) Isolation and characterization of culturable halophilic microorganisms of salt ponds in Lianyungang, China. *World J Microbiol Biotechnol* 25: 1727-1732. doi: 10.1007/s11274-009-0068-5.

White TJ, Bruns T, Lee S & Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic*PCR Protocols: A Guide to Methods and Application*(eds. Innis MA, Gelfand DH, Sninsky JJ & T.J. W), pp. 315-322. Academic Press, Inc., New York.

Wiermann R & Gubatz S (1992) Pollen wall and sporopollenin. *Sexual reproduction in flowering plant* Vol. 140 (eds. Russel SD & Dumas C), pp. 35-67. International review of cytology. Academic Press, Inc., San Diego, California.

Wilhelmi LK & Preuss D (1999) The mating game: pollination and fertilization in flowering plants. *Curr Opin Plant Biol* 2: 18-22.

Wilson T, Rabie CJ, Fincham JE, Steyn PS & Schipper MAA (1984) Toxicity of Rhizonin A, isolated from *Rhizopus microsporus*, in laboratory animals. *Fd chem toxic* 22: 275-281.

Wirth F & Goldani LZ (2012) Epidemiology of *Rhodotorula*: an emerging pathogen. *Interdiscip Perspect Infect Dis* 2012: 465717. doi: 10.1155/2012/465717.

Wolf A (2002) *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. *Int J Syst Evol Microbiol* 52: 1937-1944. doi: 10.1099/ijs.0.02135-0.

Yoon JH, Kim IG, Shin YK & Park YH (2005) Proposal of the genus *Thermoactinomyces* sensu stricto and three new genera, *Laceyella*, *Thermoflavimicrobium* and *Seinonella*,

on the basis of phenotypic, phylogenetic and chemotaxonomic analyses. *Int J Syst Evol Microbiol* 55: 395-400. doi: 10.1099/ijs.0.63203-0.

Yuan J, Raza W, Shen Q & Huang Q (2012) Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. *Appl Environ Microbiol* 78: 5942-5944. doi: 10.1128/AEM.01357-12.

Zhang H, Murzello C, Sun Y, Kim M-S, Xie X*, et al.* (2010) Choline and osmotic-stress tolerance induced in *Arabidopsis* by the soil microbe *Bacillus subtilis* (GB03). *Mol Plant Microbe Interact* 23: 1097-1014. doi: 10.1094/mpmi.

Zhang Y, Bielory L, Mi Z, Cai T, Robock A & Georgopoulos P (2014) Allergenic pollen season variations in the past two decades under changing climate in the United States. *Glob Chang Biol*. doi: 10.1111/gcb.12755.

Zhang Z & Nan Z (2014) *Erwinia persicina*, a possible new necrosis and wilt threat to forage or grain legumes production. *Eur J Plant Pathol* 139: 349-358. doi: 10.1007/s10658-014-0390-0.

Zinkl GM, Zwiebel BI, Grier DG & Preuss D (1999) Pollen-stigma adhesion in *Arabidopsis:* a species-specific interaction mediated by lipophilic molecules in the pollen exine. *Development* 126: 5431-5440.

Zipfel C (2014) Plant pattern-recognition receptors.*Trends Immunol* 35: 345-351. doi: 10.1016/j.it.2014.05.004.

Zipfel C & Felix G (2005) Plants and animals: a different taste for microbes? *Curr Opin Plant Biol* 8: 353-360. doi: 10.1016/j.pbi.2005.05.004.

ATTACHMENT

Figure 20 Isolated bacterial colonies obtained from birch pollen *(n = 5) which were sampled in and around Augsburg, Germany in season 2014. The universal media LB, PDA, R2A, SDA and BHI was used. The declaration of the single species is the same as in the respective Table 17.*

Figure 21 Isolated fungal colonies obtained from timothy grass pollen *(n = 5) which were sampled in and around Munich, Germany in season 2013. Fungal isolates preferably grew on PDA-medium. The declaration of the single species is the same as in the respective Table 18.*

Figure 22 Isolated fungal colonies obtained from birch pollen *(n = 5) which were sampled in and around Augsburg, Germany in season 2014. Fungal isolates preferably grew on PDA-medium. The declaration of the single species is the same as in the respective Table 19.*

Figure 23 MspI-Restricted fragment polymorphism in bacteria*. Differentiation of bacterial colonies with similar morphology isolated from birch pollen. After restriction digest with MspI, different species show different band patterns in agarose gelelectrophoresi#16-#61 = sample sites selected for cultivation approacheNumbered isolates only show Lab-IDs which are not analog to the Isolate-IDs in the running text. Standard: GeneRuler™ 100bp Plus DNA Ladder (Fermentas).*

Figure 24 RsaI-Restricted fragment polymorphism in bacteria*. Differentiation of bacterial colonies with similar morphology isolated from birch pollen. After restriction digest with RsaI different species show different band patterns in agarose gel electrophoresi#16-#61 = sample sites selected for cultivation approacheNumbered isolates only show Lab-IDs which are not analog to the Isolate-IDs in the running text. Standard: GeneRuler™ 100bp Plus DNA Ladder (Fermentas.)*

Figure 25 *Extracted 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of γ-Proteobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

Figure 26 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of γ-Proteobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

Figure 27 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Firmicutes in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

Figure 28 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Firmicutes in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

Figure 29 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA*

sequences of Actinobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.

Figure 30 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from birch pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Proteobacteria in the arb gene bank. Isolates are highlighted.Scale of sequencing differences: 10%*

Figure 31 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from birch pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Actinobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

Figure 32 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from birch pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Firmicutes in the arb gene bank. Isolates are highlighted.Scale of sequencing differences: 10%.*

Figure 33-35 *Distance Matrices calculated in arb for isolated bacterial strains obtained from timothy grass pollen (n = 5). Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*
Fig. 33) Actinobacteria
Fig. 34) Proteobacteria
Fig. 35) Firmicutes (next page)

Figure 36 *Distance Matrices calculated in arb for isolated bacterial strains obtained from birch pollen (n = 5) and affiliated to Actinobacteria. Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*

Figure 37 *Distance Matrices calculated in arb for isolated bacterial strains obtained from birch pollen (n = 5) and affiliated to Proteobacteria. Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*

Figure 38 *Distance Matrices calculated in arb for isolated bacterial strains obtained from birch pollen (n = 5) and affiliated to Firmicutes. Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*

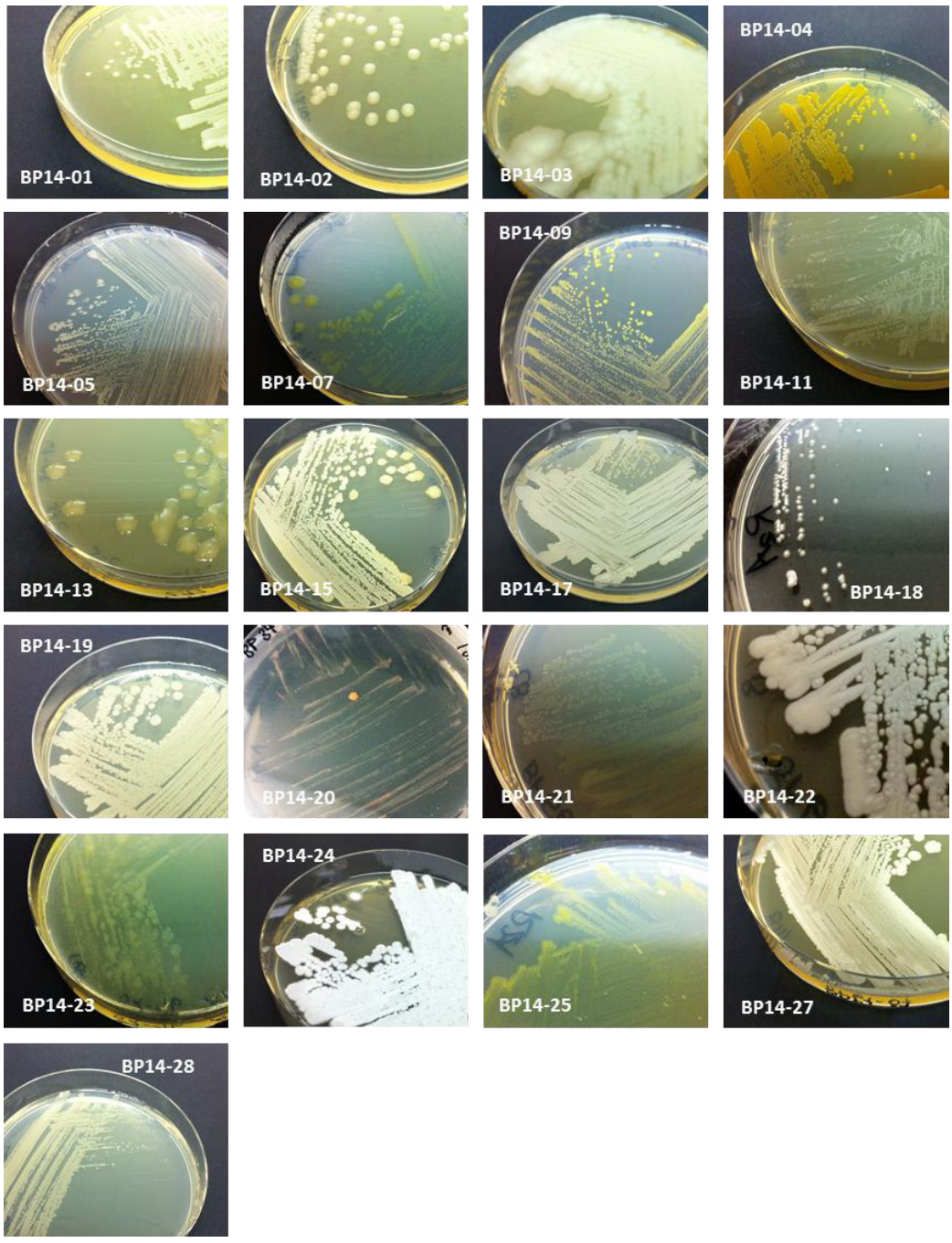

Figure 20 Isolated bacterial colonies obtained from birch pollen *(n = 5) which were sampled in and around Augsburg, Germany in season 2014. The universal media LB, PDA, R2A, SDA and BHI was used. The declaration of the single species is the same as in the respective Table 17.*

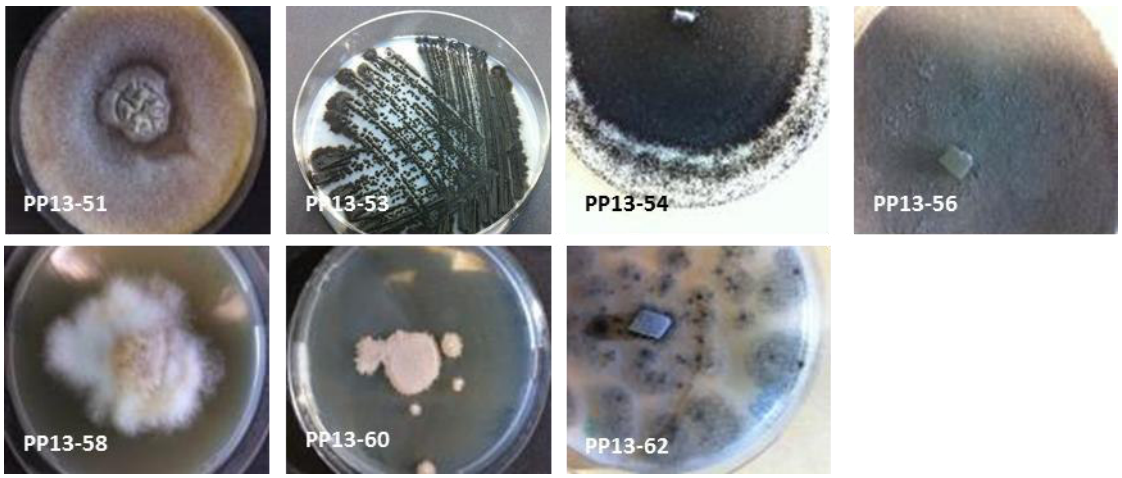

Figure 21 Isolated fungal colonies obtained from timothy grass pollen *(n = 5) which were sampled in and around Munich, Germany in season 2013. Fungal isolates preferably grew on PDA-medium. The declaration of the single species is the same as in the respective Table 18.*

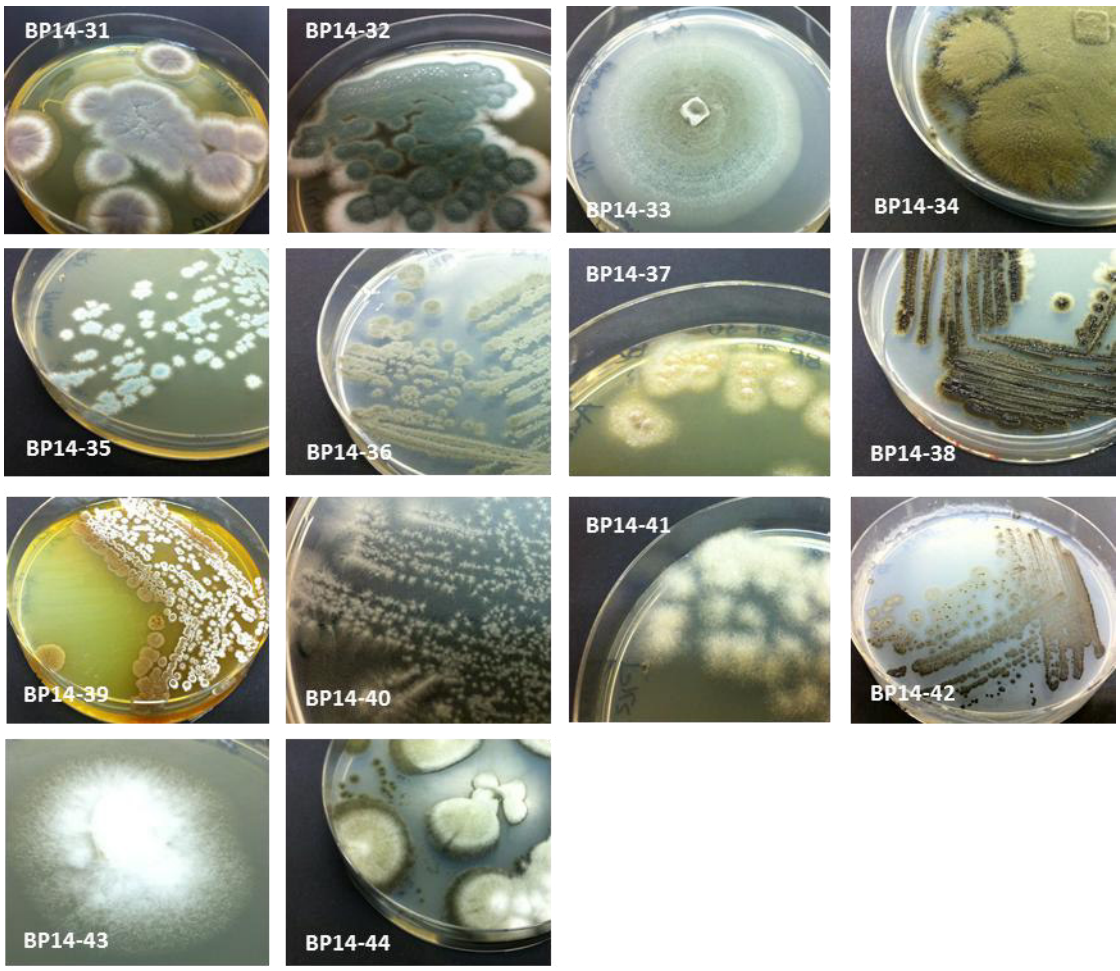

Figure 22 Isolated fungal colonies obtained from birch pollen *(n = 5) which were sampled in and around Augsburg, Germany in season 2014. Fungal isolates preferably grew on PDA-medium. The declaration of the single species is the same as in the respective Table 19.*

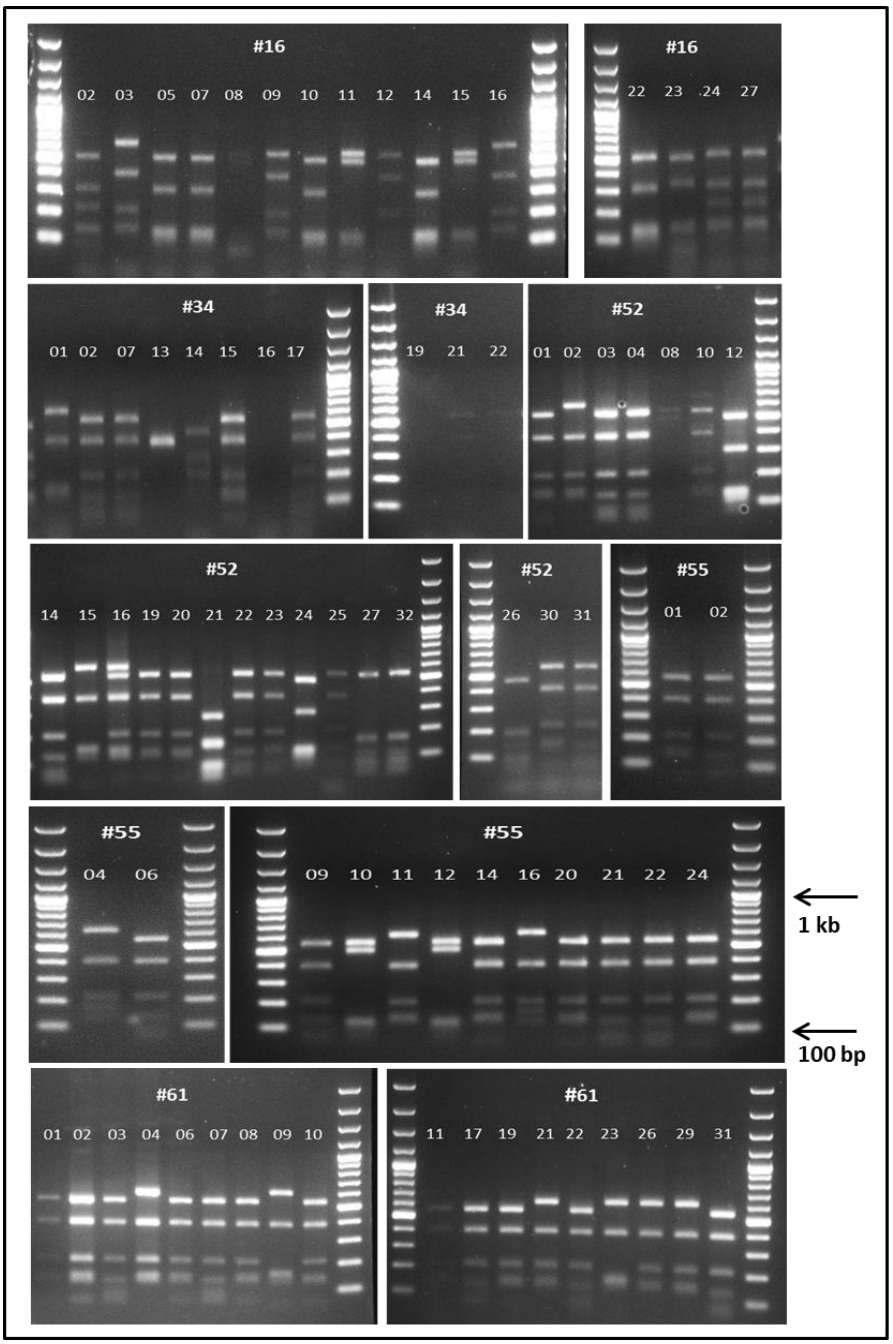

Figure 23 MspI-Restricted fragment polymorphism in bacteria*. Differentiation of bacterial colonies with similar morphology isolated from birch pollen. After restriction digest with MspI, different species show different band patterns in agarose gelelectrophoresi#16-#61 = sample sites selected for cultivation approacheNumbered isolates only show Lab-IDs which are not analog to the Isolate-IDs in the running text. Standard: GeneRuler™ 100bp Plus DNA Ladder (Fermentas).*

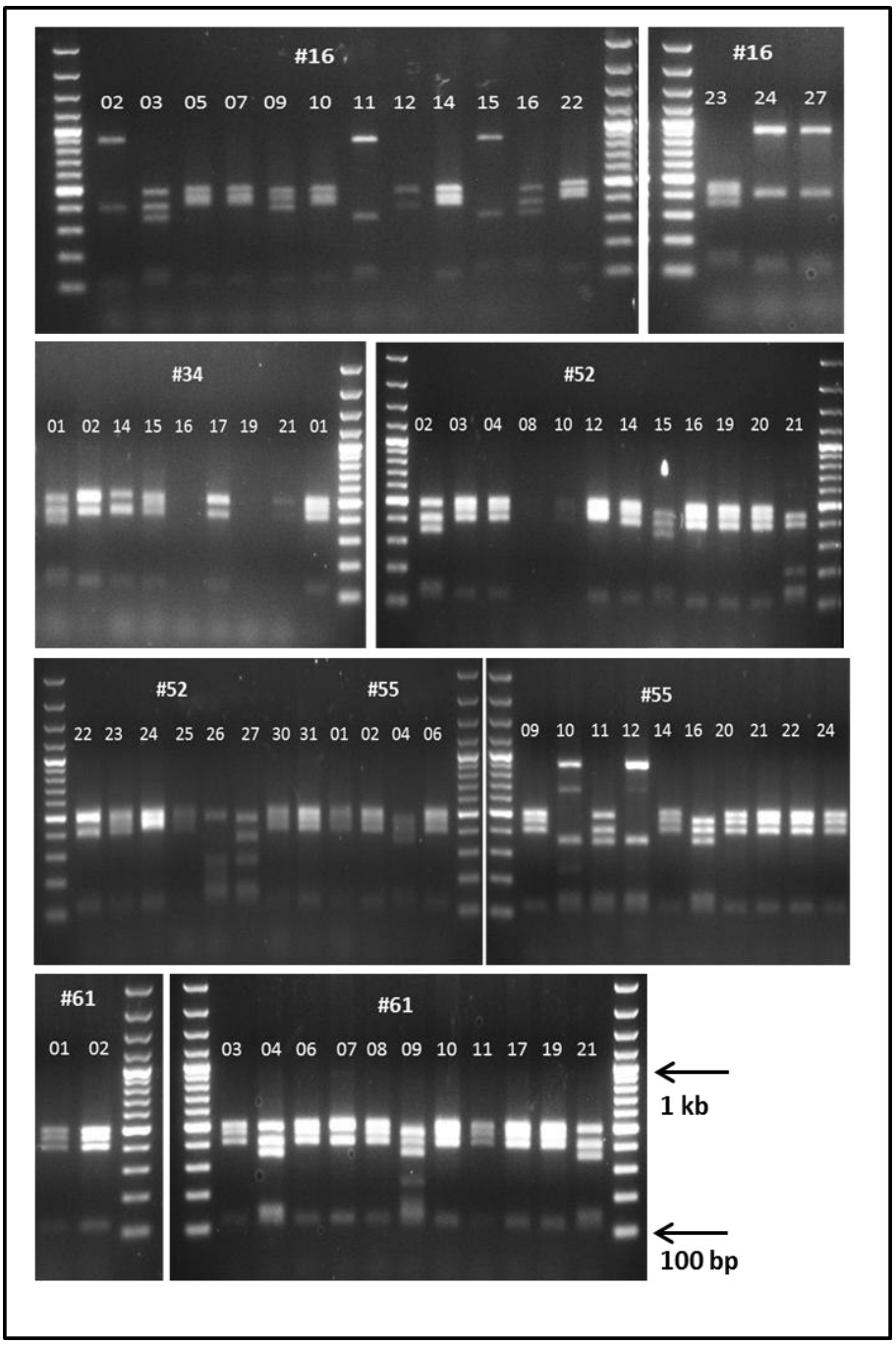

Figure 24 RsaI-Restricted fragment polymorphism in bacteria*. Differentiation of bacterial colonies with similar morphology isolated from birch pollen. After restriction digest with RsaI different species show different band patterns in agarose gel electrophoresi#16-#61 = sample sites selected for cultivation approaches. Numbered isolates only show Lab-IDs which are not analog to the Isolate-IDs in the running text. Standard: GeneRuler™ 100bp Plus DNA Ladder (Fermentas.)*

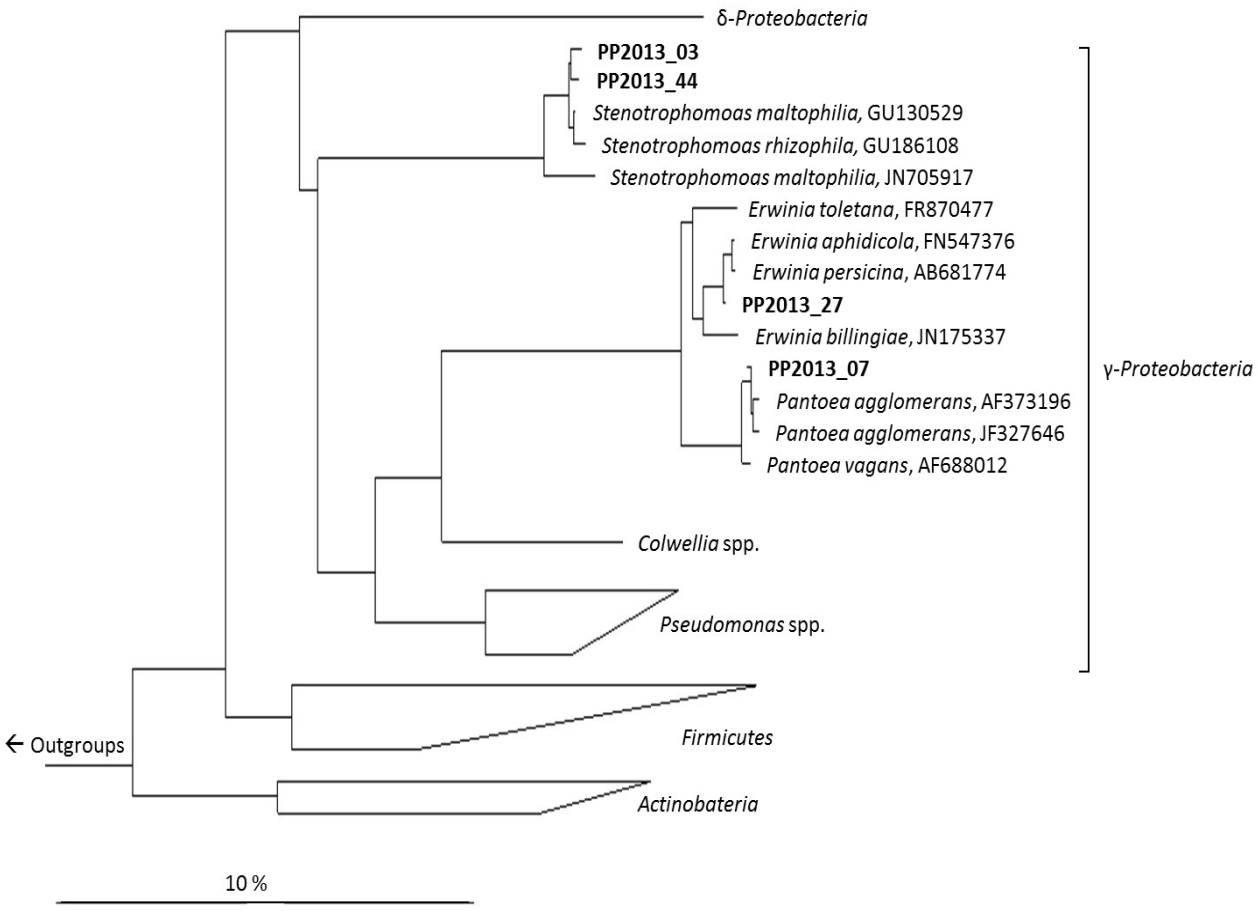

Figure 25 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of γ-Proteobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

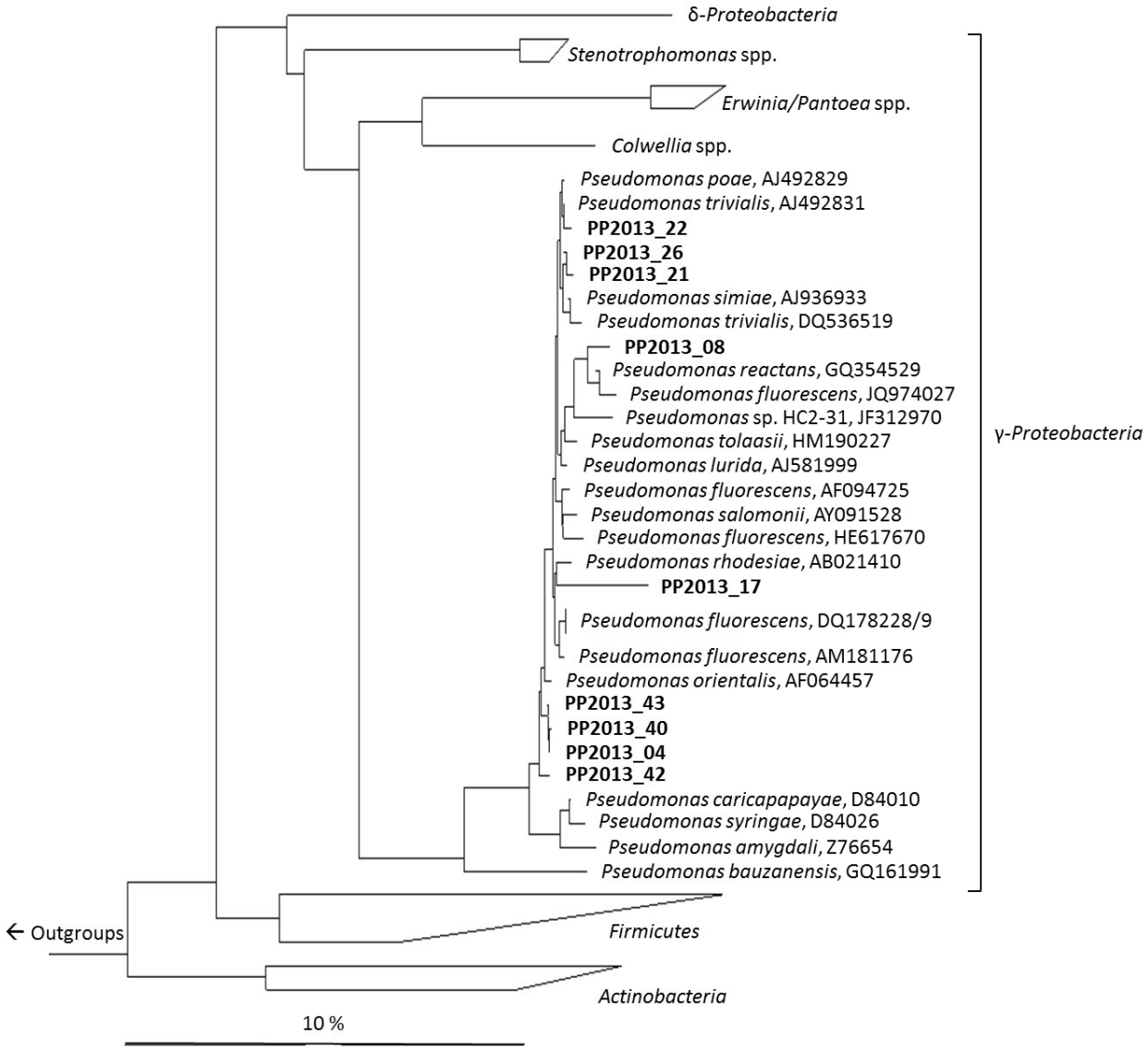

Figure 26 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of γ-Proteobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

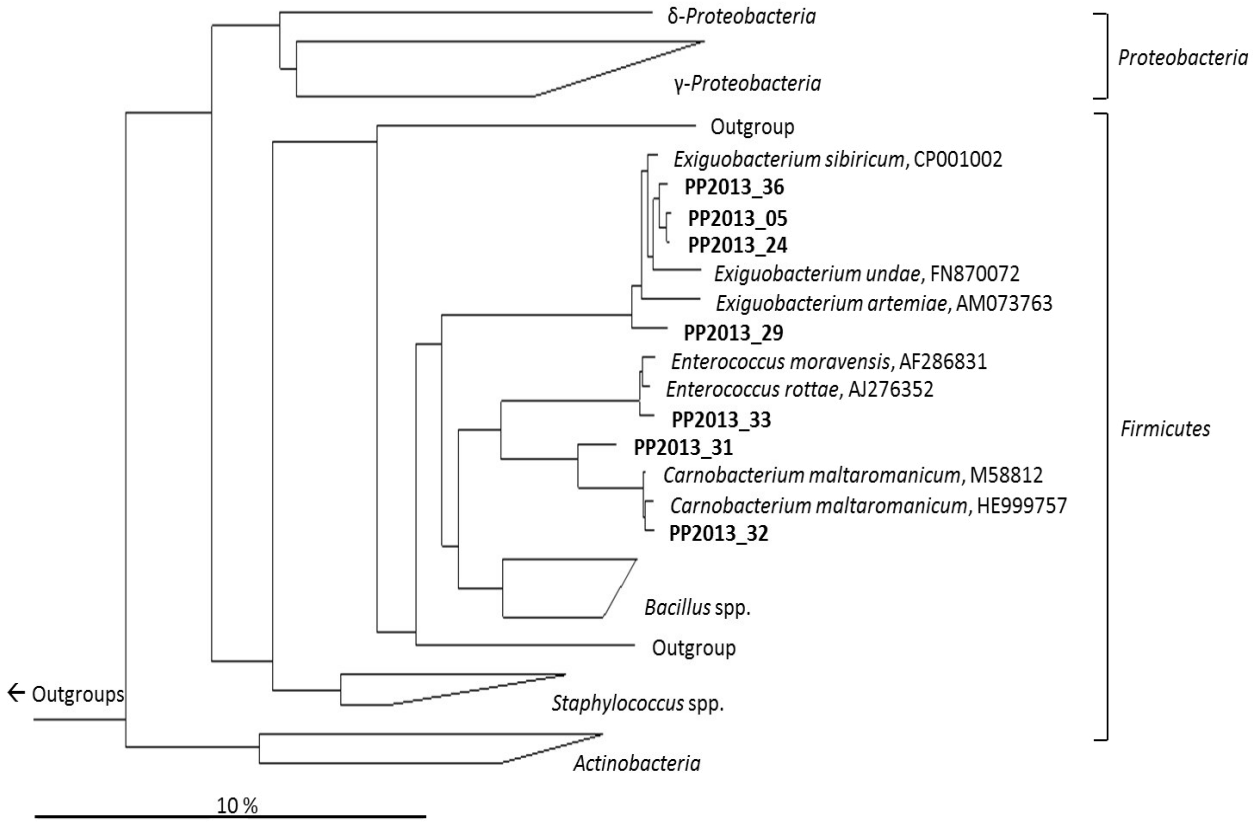

Figure 27 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Firmicutes in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

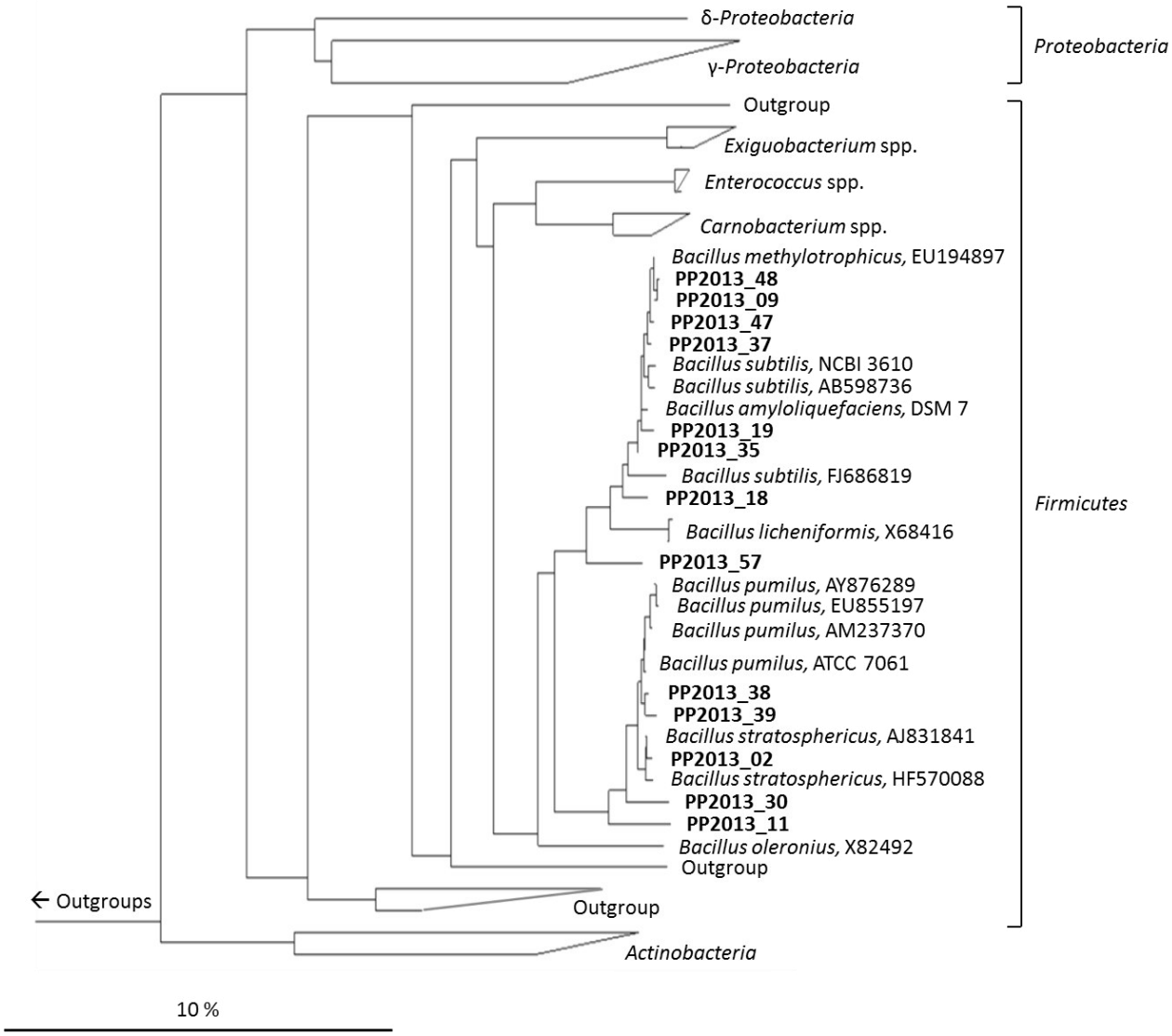

Figure 28 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Firmicutes in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

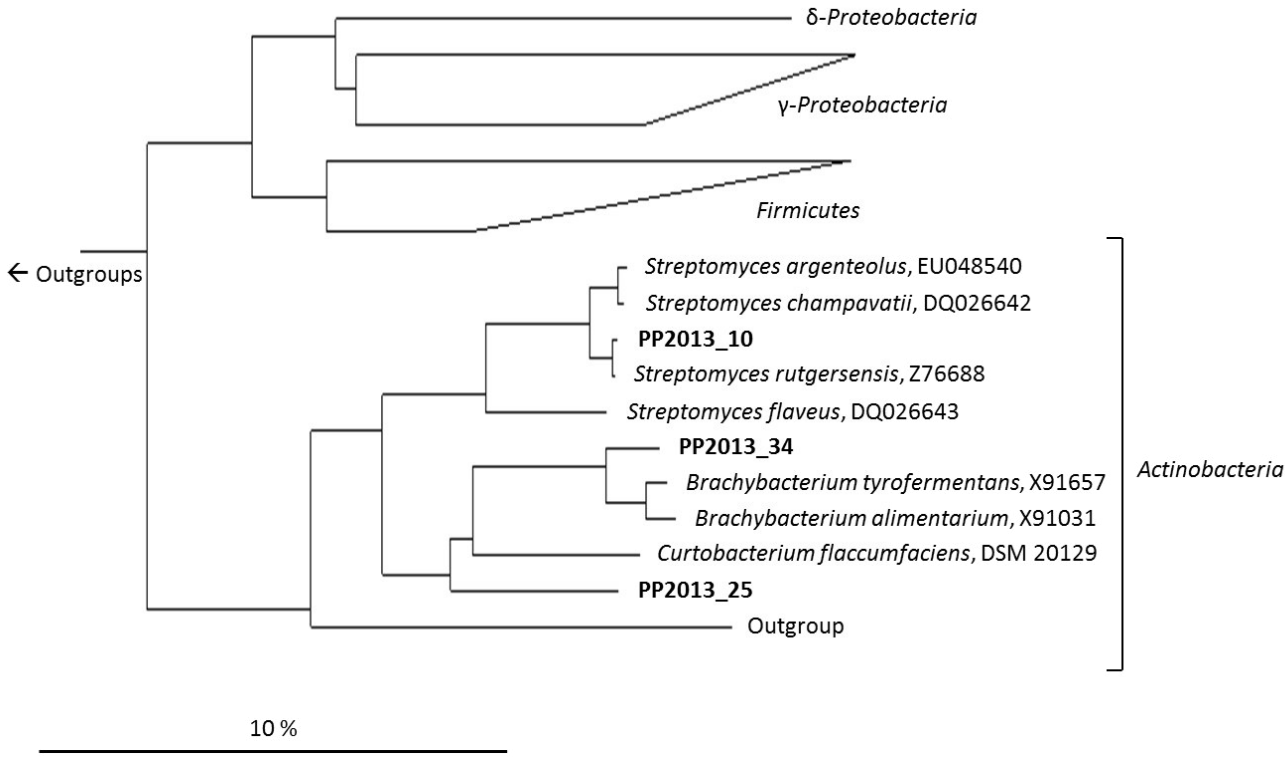

Figure 29 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from timothy grass pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Actinobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

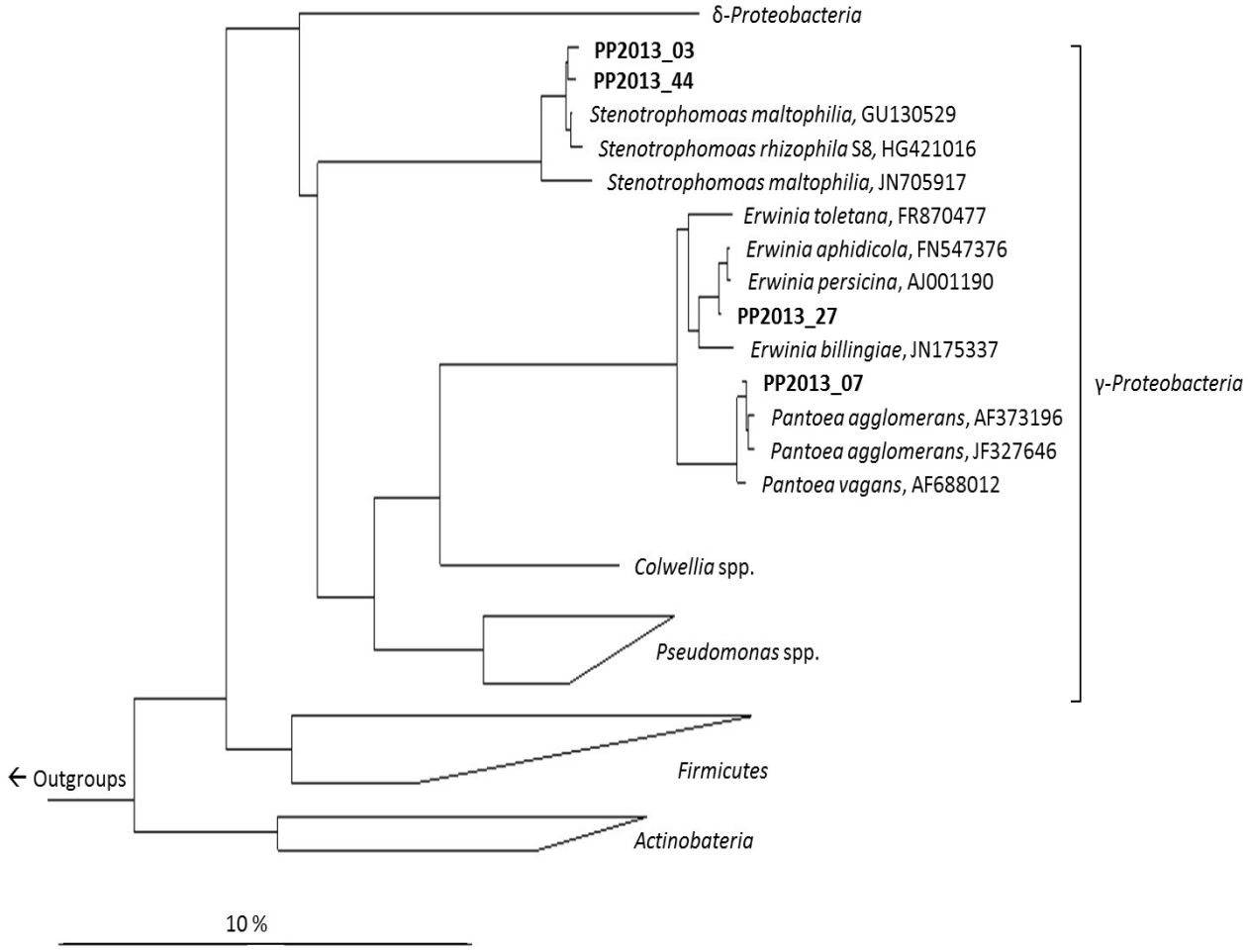

Figure 30 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from birch pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Proteobacteria in the arb gene bank. Isolates are highlighted.Scale of sequencing differences: 10%*

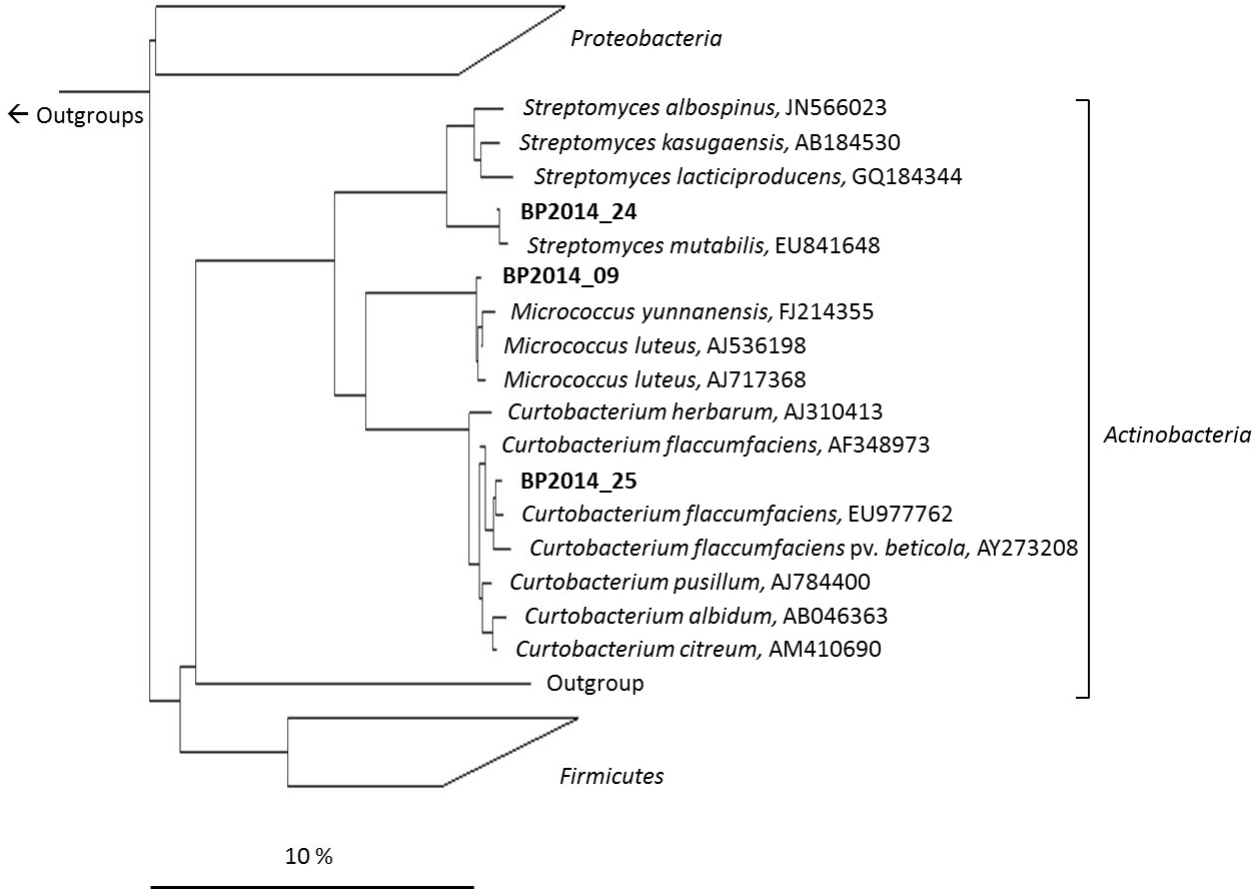

Figure 31 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from birch pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Actinobacteria in the arb gene bank. Isolates are highlighted. Scale of sequencing differences: 10%.*

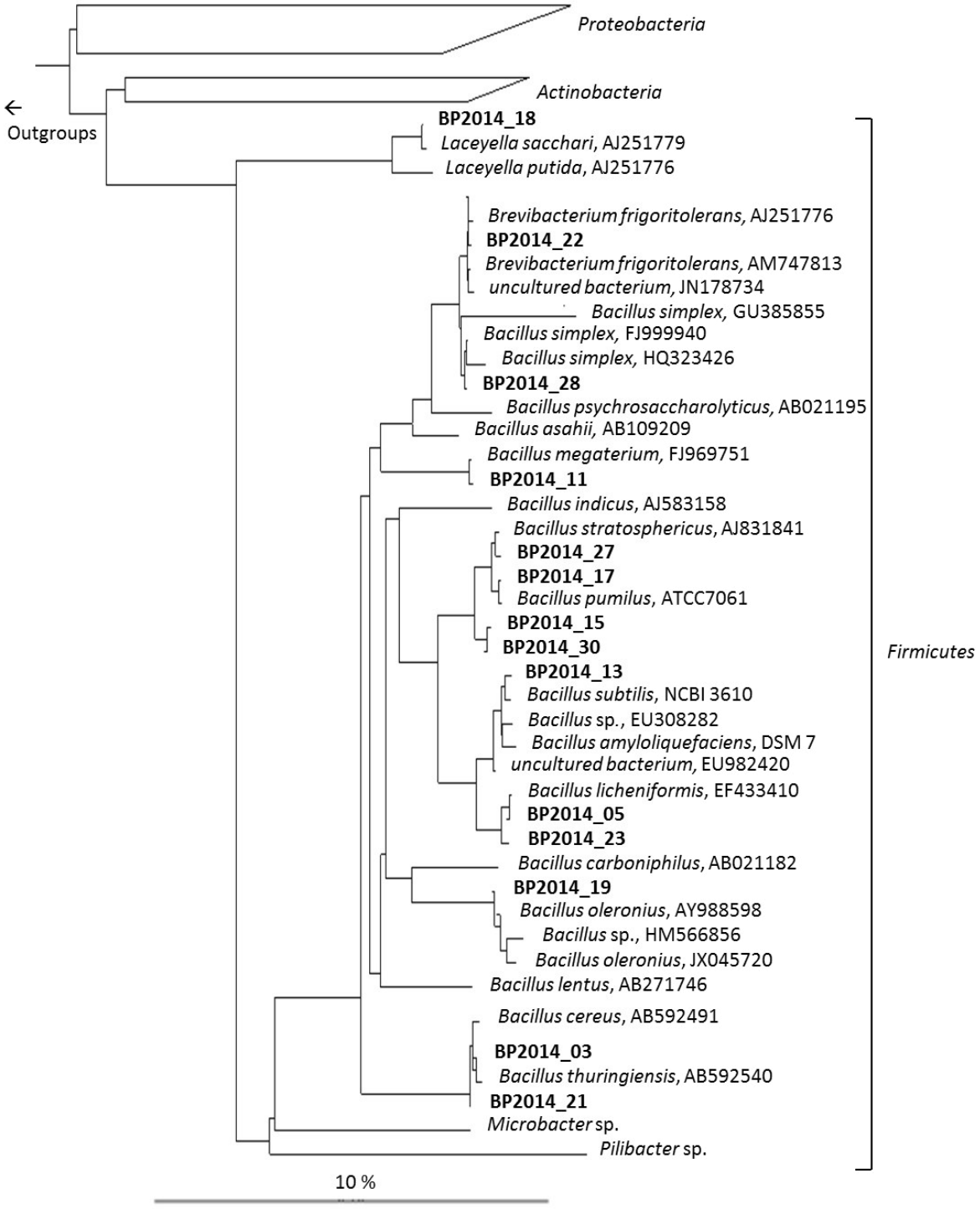

Figure 32 *Extracted and amplified 16S-rDNA from bacterial cultures isolated from birch pollen samples (n = 5). The sequences were compared to known cultured 16S-rDNA sequences of Firmicutes in the arb gene bank. Isolates are highlighted.Scale of sequencing differences: 10%.*

Figure 33-35 *Distance Matrices calculated in arb for isolated bacterial strains obtained from timothy grass pollen (n = 5). Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*
Fig. 33) Actinobacteria
Fig. 34) Proteobacteria
Fig. 35) Firmicutes (next page)

Figure 33: Actinobacteria isolated frim timothy grass pollen (PP2013)

	#10	#34	#25
EU048540, *Streptomyces argenteolus*	98.6	89.4	91.2
DQ026642, *Streptomyces champavatii*	98.8	89.8	91.2
Z76688, *Streptomyces rutgersensis*	**99.9**	90.3	91.4
DQ026643, *Streptomyces flaveus*	95.9	89.3	89.4
X91657, *Brachybacterium tyrofermentans*	89.6	97.6	92
X91031, *Brachybacterium alimentarium*	89.9	**98**	92
FJ969847, *Bogoriellaceae bacterium* YIM 93306	91.2	92.8	**93.6**

Figure 34: Proteobacteria isolated from timothy grass pollen (PP2013)

	#03	#44	#27	#07	#22	#26	#21	#08	#17	#43	#40	#04	#42
GU130529, *Stenotrophomonas maltophilia*	99.3	**99.6**	83.5	82.6	87.4	86.5	87.5	87.1	85.5	87.8	87.4	87.5	87.5
HG421016, *Stenotrophomonas rhizophila*	**99.6**	99.3	84.1	82.8	87.1	86.6	86.9	86.6	85.3	88	87.6	87.1	87.7
FN547376, *Erwinia aphidicola*	83.8	83.7	**99.8**	96.6	87.1	87.2	86.9	86.8	84.9	87	86.7	86.6	86.7
JF327464, *Pantoea agglomerans*	83	82.7	96.4	**100**	85.5	85.7	85.5	85.3	83.4	85.5	85.3	85.2	85.3
AJ492831, *Pseudomonas trivialis*	87.2	87.2	87.4	85.4	**99.8**	**100**	99.5	98.5	97.4	99.2	99.1	99.2	99.1
AJ936933, *Pseudomonas simiae*	87.1	87.3	86.5	85	99.5	99.9	**99.6**	98.4	97.1	99.2	99.1	99.1	99.1
GQ354529, *Pseudomonas reactans*	87	87.2	87.1	85.4	98.9	98.5	98.8	**99.6**	96.8	98.4	98.3	98.4	98.3
AB021410, *Pseudomonas rhodesiae*	87.2	87.2	87.4	85.4	99.7	99.8	99.5	98.6	**97.5**	99.1	99	99.1	99
AF064457, *Pseudomonas orientalis*	87.4	87.6	87.2	85.4	99.4	99.3	99.1	98.2	97.2	**99.7**	**99.6**	**99.7**	**99.6**

Figure 35: Firmicutes isolated from timothy grass pollen (PP2013)

	#36	#05	#24	#29	#33	#31	#32	#48	#09	#47	#37	#19	#35	#18	#57	#38	#39	#02	#30	#11
CP001022, *Exiguobacterium sibiricum* 255-15	99.4	99.8	99	98.5	89.7	90.6	90.4	89.3	89.6	89.9	90	90.1	90.6	90.3	91.1	91.2	90.8	90.8	91.8	93.4
FN870072, *Exiguobacterium undae*	98.1	98.9	98.1	98.1	88.1	89.1	88.9	88.7	88.7	88.2	88.3	88.3	90	89.5	90.5	89.3	90	89.7	90.4	91.9
AM072763, *Exiguobacterium artemiae*	97.9	98	97.9	96.8	88	88.6	88.5	87.9	87.9	88.2	88.2	88.2	89	88.5	89.8	89.3	89.2	89.1	90.3	92.1
AF286831, *Enterococcus moraviensis*	89.7	89.7	89.5	89.1	99.3	93	94.1	90.3	90.3	90.4	90.5	90.5	91.2	90.6	92.1	91.5	91.6	91.8	91.2	90.6
AJ276352, *Enterococcus rottae*	89.9	89.8	90.1	89.4	99.5	93.1	94.4	90.3	90.7	90.8	90.8	90.8	91.2	90.9	92.1	91.8	91.5	91.5	91.3	90.7
M58812, *Carnobacterium maltaromaticum*	90.2	90	90.2	89.9	94.2	97.2	99.5	90.9	90.9	91	91.1	91	91.4	91.1	92.6	91.4	91.2	91.1	91.6	91.1
HE999757, *Carnobacterium maltaromaticum*	89.8	89.7	89.6	89.6	94.1	97.1	99.7	90.6	90.8	90.9	91	91	91	90.9	92.3	91.4	90.9	90.8	91	90.7
EU194897, *Bacillus methylotrophicus*	89.7	89.6	89.5	89.8	90.2	92.9	90.7	99.9	99.9	99.7	99.8	99.5	99.7	99.1	97.2	97.4	97.1	97.6	96.3	94.7
ABQL01000004, *Bacillus subtilis* NCIB 3610	89.7	89.6	89.8	89.9	90.6	93.3	91.2	99.4	99.4	99.3	99.3	99.2	99.2	98.7	96.7	97	96.6	97.1	96	94.3
AB598736, *Bacillus subtilis*	89.9	89.7	89.9	90.1	90.6	93.4	91.2	99.7	99.7	99.7	99.7	99.5	99.5	99.1	97	97.3	96.9	97.4	96.3	94.6
FN597644, *Bacillus amyloliquefaciens* DSM 7	89.6	89.5	89.4	89.8	90.8	93.2	91.2	100	99.9	99.8	99.9	99.6	99.8	99.2	97.3	97.5	97	97.6	96	94.7
FJ686819, *Bacillus subtilis*	89.4	89.5	89.4	89.7	89.6	92.1	90	98.8	98.8	98.8	98.8	98.6	98.8	98.1	96.8	96.3	95.8	96.5	95.3	93.6
X68416, *Bacillus licheniformis*	89.8	89.7	89.3	90	90.8	93	91.2	98.4	97.7	98.1	97.7	97.5	98.6	97.1	96.5	95.8	96.1	96.7	94.6	93.8
EF433410, *Bacillus licheniformis*	89.8	89.7	89.8	90	91.3	93.6	91.6	98.4	98.4	98.4	98.5	98.1	98.6	97.9	96.5	96.5	96.1	96.7	95.5	93.8
AY876289, *Bacillus pumilus*	90.8	90.9	90.9	90.9	91.2	93.8	90.9	97.4	97.4	97.4	97.4	97.4	97.4	96.8	96.1	99.9	99.4	99.6	98.3	96.8
EU855197, *Bacillus pumilus*	90.9	90.9	90.6	91	91	93.5	90.7	97.4	97	96.9	97	97	97.4	96.4	96.1	99.3	99.4	99.6	97.9	96.8
AM237370, *Bacillus pumilus*	90.9	90.7	90.9	90.9	91.4	93.9	91.2	97.6	97.6	97.6	97.6	97.6	97.5	96.9	96.3	99.7	99.3	99.5	98.2	96.8
ABRX01000007, *Bacillus pumilus* ATCC 7061	90.9	90.9	91.2	91	91.5	94	91.3	97.4	97.4	97.4	97.4	97.5	97.4	96.8	96	99.7	99.3	99.6	98.2	96.7
AJ831841, *Bacillus stratosphericus*	90.7	90.5	90.8	90.8	91.6	93.9	91.2	97.7	97.8	97.7	97.8	97.8	97.7	97.2	96.1	99.5	99.1	100	98.5	96.4
HF570088, *Bacillus stratosphericus*	90.5	90.3	90.7	90.6	91.4	93.7	91	97.5	97.6	97.5	97.6	97.6	97.5	97	95.8	99.3	98.9	99.7	98.4	96.2
X82492, *Bacillus oleronius*	89.8	89.8	90.3	89.8	91.4	91.8	91.7	94.6	94.8	94.8	94.9	94.8	94.9	94.7	95.4	94.5	94.2	94.5	94.6	93.1

Figure 36 *Distance Matrices calculated in arb for isolated bacterial strains obtained from birch pollen (n = 5) and affiliated to Actinobacteria. Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*

Figure 36: Actinobacteria isolated from birch pollen (BP2014)

	#24	#09	#25
JN566023, *Streptomyces albospinus*	97.5	90.8	90.2
AB184530, *Streptomyces kasugaensis*	96.4	90.6	91.2
GQ184344, *Streptomyces lacticiproducens*	96.8	90.1	90.9
EU841648, *Streptomyces mutabilis*	99.9	91.1	90.4
FJ214355, *Micrococcus yunnanensis*	90.8	99.6	93.1
AJ536198, *Micrococcus luteus*	91.2	99.5	93.8
AJ717368, *Micrococcus luteus*	91.1	99.7	93.4
AJ310413, *Curtobacterium herbarum*	90.2	92.7	98.7
AF348973, *Curtobacterium flaccumfaciens*	91	93.3	99.7
EU977762, *Curtobacterium flaccumfaciens*	90.5	93.4	99.6
AY273208, *Curtobacterium flaccumfaciens pv. beticola*	90.3	93.2	99.5
AJ784400, *Curtobacterium pusillum*	90.5	93.3	99.3
AB046363, *Curtobacterium albidum*	90.4	92.9	99.0
AM410690, *Curtobacterium citreum*	90.6	93.4	99.5

Figure 37 *Distance Matrices calculated in arb for isolated bacterial strains obtained from birch pollen (n = 5) and affiliated to Proteobacteria. Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*

Figure 37: Proteobacteria isolated from birch pollen (BP 2014)

	#07	#02	#01	#04	#20
AM184091, *Pantoea agglomerans*	99.1	97.3	94.7	85.2	78.9
U80202, *Pantoea agglomerans*	98.8	96.8	94.6	84.9	78.3
EF688012, *Pantoea vagans*	98.9	97	95.3	84.9	78.9
Z96083, *Pantoea agglomerans*	99.3	97.3	95	85.2	78.8
FP236843, *Erwinia billingiae*	97	100	96.1	86.2	79
JQ929658, *Erwinia billingiae*	97.1	100	96.2	85.9	78.6
AM055716, *Erwinia tasmaniensis*	97	98.6	95.5	85.8	78.9
AF130910, *Erwinia toletana*	98.5	99	96.7	87.2	81
AJ306725, *Serratia liquefaciens*	95.5	96.4	99.6	85.2	77.2
AJ233430, *Serratia grimesii*	95.7	96.7	99.6	85.7	77.7
AJ233434, *Serratia proteamaculans*	95.8	96.6	99.6	85.9	78
AJ233435, *Serratia proteamaculans*	95.6	96.4	99.3	86.1	77.9
AF286872, *Serratia plymuthica*	94.8	95.8	98.2	85.2	77.2
AM411059, *Pseudomonas putida*	85.2	85.8	85.7	98.7	80.8
Y11150, *Pseudomonas graminis*	85.2	85.8	86	99.5	80.7
AJ011504, *Pseudomonas abietaniphila*	84.8	85.7	85.4	98.6	80.2
Z76652, *Pseudomonas agarici*	85.2	86	85.8	98	80.4
JQ390519, *Acetobacteraceae bacterium* GIMN 1.017	79	78.7	77.8	80.6	99.4
EU379242, *Paracraurococcus* sp. 1N-11	78.4	78	77.1	80.4	99
HQ882802, *Humitalea rosea*	79	79.4	79	81.3	93.4

Figure 38 *Distance Matrices calculated in arb for isolated bacterial strains obtained from birch pollen (n = 5) and affiliated to Firmicutes. Shown are the rel. similarities (%) of the 16S-rDNA to the next related known sequences in arb gene bank.*

Figure 38: Firmicutes isolated from birch pollen (BP 2014)

	#18	#22	#28	#11	#27	#17	#15	#30	#13	#05	#23	#19	#03	#21
AJ251779, *Laceyella sacchari*	**99.9**	89.4	89.4	89.7	89.8	89.7	89.7	89.9	89.7	90	89.8	89.7	90.1	90.1
AJ251776, *Laceyella putida*	98.6	89.4	89.4	89.7	90.1	90.1	89.9	90	89.9	90.3	90.1	89.8	89.9	90
GU252128, *[Brevibacterium] frigoritolerans*	89.5	**100**	99.6	95.5	94.8	94.8	95	95.1	94.3	94.7	94.6	94.8	95	95
AM747813, *[Brevibacterium] frigoritolerans*	89.5	**99.9**	99.5	95.4	94.7	94.8	94.9	95.1	94.2	94.7	94.5	94.7	94.9	94.9
JN178734, uncultured bacterium	89.4	99.7	99.4	95.5	94.6	94.6	94.8	95	94.1	94.4	94.4	94.7	94.9	94.9
AB109209, *Bacillus asahii*	90	97.4	97.8	95.8	95.1	95.2	95.4	95.6	94.9	95.2	95	95.7	95	94.9
FJ969751, *Bacillus megaterium*	89.4	95.6	96	**99.9**	95.2	94.9	95.1	95.3	94.5	94.4	94.6	94.6	94.9	94.9
AJ583158, *Bacillus indicus*	90	95	95	95.7	96.1	96.2	96.1	96.3	95.3	95.4	95.1	94.9	94.2	94.2
AJ831841, *Bacillus stratosphericus*	90.1	94.8	94.8	94.9	**99.8**	99.5	**99.2**	**99.3**	97.5	96.9	96.9	94.9	94.7	94.9
ABRX01000007, *Bacillus pumilus* ATCC 7061	89.7	94.7	94.7	94.7	99.5	**99.8**	99.1	99.2	97.2	96.6	96.6	94.7	94.6	94.7
ABQL01000004, *Bacillus subtilis* NCIB 3610	89.9	94.2	94.3	94.2	97.4	97.4	97.4	97.8	**99.7**	98.5	98.7	95.4	94.4	94.4
EU308282, *Bacillus* sp. FIA02_2	89.7	94.2	94.3	94.2	97.3	97.2	97.4	97.8	99.6	98.4	98.6	95.1	94.1	94.2
FN597644, *Bacillus amyloliquefaciens* DSM 7	90	94	94.1	94	97.6	97.4	97.2	97.6	99.5	98.6	98.7	95.2	94.4	94.4
EU982420, uncultured bacterium	90.4	94.6	94.6	94.7	97.6	97.5	97.8	98.2	**99.7**	98.8	98.8	95.2	94.4	94.6
EF433410, *Bacillus licheniformis*	90	94.6	94.5	94.1	96.7	96.5	96.8	97.1	98.6	**100**	**99.7**	95.6	94.4	94.4
AB021182, *Bacillus carboniphilus*	89.3	94.5	94.8	94.1	94.5	94.5	94.5	94.7	95.8	96.5	96.3	96.4	94.1	94.1
AY988598, *Bacillus oleronius*	90	94.7	95.1	94.3	94.8	94.7	94.9	95	95.3	95.8	95.6	**100**	94.2	94.1
HM566856, *Bacillus* sp. DB142(2010)	88.7	94	94.4	93.9	94.1	94	94.3	94.4	94.5	94.9	94.7	99.7	93.4	93.4
JX045720, *Bacillus oleronius*	89.6	94	94.3	94.3	94.3	94.2	94.4	94.5	94.6	95	94.9	99.5	93.8	93.8
AB271746, *Bacillus lentus*	90.2	95.3	95.4	95.7	94.9	94.9	95.1	95.2	94.5	94.5	94.6	95.9	94.7	94.8
AB592491, *Bacillus cereus*	89.9	94.8	95.2	94.9	94.6	94.5	94.3	94.5	94.2	94.3	94.4	94	99.7	99.6
AB598737, *Bacillus cereus*	90.1	94.9	95.3	95.1	94.7	94.6	94.3	94.5	94.3	94.4	94.5	94.1	99.7	99.7
AB592540, *Bacillus thuringiensis*	89.6	94.8	95.1	94.8	94.7	94.7	94.4	94.6	94.2	94.2	94.4	93.9	**100**	**100**

DANKSAGUNG

An dieser Stelle möchte ich mich bei folgenden Personen ganz herzlich bedanken:

Herrn Prof Dr. Anton Hartmann gilt mein besonderer Dank für die Möglichkeit, meine Doktorarbeit am Helmholtz Zentrum anfertigen zu dürfen, für die Möglichkeit, das zur Verfügung gestellte Projekt weitestgehend selbstständig und frei bearbeiten zu dürfen.

Frau Prof. Dr. Jung für die Erstellung des Zweitgutachtens sowie den weiteren Mitgliedern der Prüfungskommission.

In stillem Gedenken an Herrn Dr. Michael Schmid, der im letzten Juni leider von uns ging. Ihm gilt mein Dank für seine Hilfe und Unterstützung in sämtlichen administrativen Angelegenheiten.

Herrn Dr. Michael Rothballer für die Möglichkeit, jederzeit mit Problemen und Fragen im wissenschaftlichen Bereich zu ihm kommen zu können.

Angelo Weiss, der am allermeisten für mich da war. Für die Motivation und Unterstützung im Labor, für die Erarbeitung der Vorversuche ohne die dieses Projekt vermutlich nicht in der Form zustande gekommen wär. Für unsere so herrlichen „Kaffeepausen“ auch spät abends. Dafür, dass du mir in jeder nur erdenklichen Situation zur Seite gestanden, mir zugehört und immer wieder Aufwind zur rechten Zeit gegeben hast.

Dr. Soumitra Paul-Chowdhury für die mentale Unterstützung, die lustigen Kaffeepausen und das Korrigieren der englischen Sprache in Paper und Dissertation.

Fabian Weikl für die guten Tipps zum Enzymverdau von Pilz-DNA!

Simone Rankl, Andres Sauvetre, Rudi Harpaintner und allen anderen aktuellen und ehemaligen AMP-Kollegen für die tolle Zeit und super Atmosphäre.

Dr. Ulrike Frank und Dr. Stefanie Gilles-Stein für die nette und ergiebige Zusammenarbeit im Rahmen des Pollen-Mikrobiom-Projektes.

Dieses Projektes wurde durch das Environmental Health Portfolio finanziert, wofür ich mich beim Helmholtz Zentrum München recht bedanken will.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, Andrea Obersteiner, an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur unter Zuhilfenahme der ausgewiesenen Hilfsmittel angefertigt habe. Sämtliche Stellen der Arbeit, die im Wortlaut oder dem Sinn nach auf Publikationen oder Vorträgen anderer Autoren beruhen, habe ich durch genaue Quellenangaben kenntlich gemacht.

Diese Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt.

München, den 09.08.2016 _____

(Andrea Obersteiner)