QUANTIFICATION OF *FRANKIA* IN SOIL

by

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DEDICATION

To the loving memory of my father, Shri. Sunil P. Samant, who has been my role model for hard work, persistence and personal sacrifices.

Thank you for everything.

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ABSTRACT

The genus *Frankia* represents nitrogen fixing actinomycetes that form root nodules with more than 200 actinorhizal plant species. *Frankia* can be found in root nodules of specific host plants, a natural locale of enrichment of usually one *Frankia* population, and in soils that represent highly heterogeneous environments. Due to their low abundance in soil and difficulties in the isolation of *Frankia* from soil, molecular tools such as PCR have been evaluated for quantitative assessments of frankiae in soil. PCR-based quantification of *Frankia* in soils so far employed PCR-MPN using nested or booster PCR, but did not consider more recent technological advancements such as quantitative real-time PCR (*q*PCR) that has successfully been used for the quantification of other soil microorganisms. The work presented in this Ph.D. dissertation focuses on two basic objectives:

1) to develop detection and quantification methods that allowed us to analyze *Frankia* populations in soil, and

2) to employ these methods to address questions on the fate of indigenous and introduced frankiae in soils.

Work on the first objective resulted in the development of a SYBR Green based *q*PCR method that quantified clusters 1 and 3 of the actinomycete *Frankia* in soils by targeting *nif*H gene sequences (chapter II). Primer *nif*Hr158 was designed to be used as

reverse primer in combination with forward primer nifHf1 specifically amplifying a 191-bp fragment of the *nif*H gene of these *Frankia*. The primer combination was tested for specificity on selected pure cultures, and by comparative sequence analyses of randomly selected clones of a clone library generated with these primers from soil DNA extracts. After adjustments of DNA extraction conditions, and the determination of extraction efficiencies used for sample normalization, copy numbers of *nif*H genes representing *Frankia* of clusters 1 and 3 were quantified in different mineral soils, resulting in cell density estimates for these *Frankia* of up to 10^6 cells [g soil {dry weight}]$^{-1}$ depending on the soil. The study, however, also revealed problems in the application of *nif*H genes as targets for the quantification of frankiae. Primers developed only detected frankiae of clusters 1 and 3, but not frankiae of cluster 2 or cluster 4, and indications for *nif*H gene transfer were observed. These issues prompted us to look for other genes that could be used as target in *q*PCR applications to quantify all members of the genus *Frankia* but also to distinguish clusters or specific subgroups within the genus.

In chapter III, we report on the evaluation of 23S ribosomal RNA gene sequences as potential target for the detection of all members of the genus *Frankia* and specific subgroups within the genus. A *q*PCR with a primer combination targeting all nitrogen-fixing frankiae (clusters 1, 2 and 3) resulted in numbers similar to those obtained with a previously developed *q*PCR using *nif*H gene sequences, both with respect to introduced and indigenous *Frankia* populations. Primer combinations more specifically targeting

three subgroups of the *Alnus* host infection group (cluster 1) or members of the *Elaeagnus* host infection group (cluster 3) were specific for introduced strains of the target group, with numbers corresponding to those obtained by quantification of nitrogen-fixing frankiae with both the 23S rRNA and *nif*H genes as target. Method verification on indigenous *Frankia* populations in soils, i.e. in depth profiles from four sites at an *Alnus glutinosa* stand, revealed declining numbers in the depth profiles, with similar abundance of all nitrogen-fixing frankiae independent of 23S rRNA or *nif*H gene targets, and corresponding numbers of one group of frankiae of the *Alnus* host infection only, with no detections of frankiae representing the *Elaeagnus, Casuarina*, or a second subgroup of the *Alnus* host infection groups.

In chapter IV, we report on the application of our *q*PCR-based quantification method in the assessment of the abundance and diversity of *Frankia* in four soils with similar physicochemical characteristics, two of which were vegetated with a host plant, *Alnus glutinosa,* and two with a non-host plant, *Betula nigra*. Analyses of DAPI-stained cells at three locations, i.e. at a distance of less than 1 m (near stem), 2.5 m (middle crown) and 3-5 m (crown edge) from the stems of both tree species revealed no statistically significant differences in abundance. Frankiae generally accounted for 0.01 to 0.04% of these cells, with values between 4 and 36 x 10^5 cells (g soil)$^{-1}$. In three out of four soils, abundance of frankiae was significantly higher at locations “near stem” and/or “middle crown” compared to “crown edge”, while numbers at these locations were not

different in the fourth soil. Frankiae of the *Alnus* host infection group were dominant in all samples accounting for about 75% and more of the cells, with no obvious differences with distance to stem. In three of the soils, all of these cells were represented by strain Ag45/Mut15. In the fourth soil that was vegetated with older *A. glutinosa* trees, about half of these cells belonged to a different subgroup represented by strain ArI3. In all soils, the remaining cells belonged to the *Elaeagnus* host infection group represented by strain EAN1pec. *Casuarina*-infective frankiae were not found. Abundance and diversity of *Frankia* were similar in soils under the host plant *A. glutinosa* and the non-host plant *B. nigra*. Results did thus not reveal any specific effects of plant species on soil *Frankia* populations shown to have differing nodulation capacities for *Alnus* in past studies.

In chapter V, *q*PCR was used to follow populations dynamics of indigenous *Frankia* populations in bulk soil and the rhizosphere of *Alnus glutinosa* or *Casuarina equisetifolia* at 2 matric potentials representing “dry” (-0.005 MPa) and “wet” (-0.001 MPa) conditions. Indigenous populations of *Frankia* in bulk soil (PIATT) that was originally vegetated with *Elaeagnus umbellata* and had been stored at 4°C for about half a year, increased between 10- and 100-fold within the incubation period of 12 weeks, with usually higher numbers obtained under dry conditions. Abundance of *Frankia* in the rhizosphere and in bulk soil amended with leaf litter showed a similar pattern, though values for abundance were generally higher, with highest values obtained for *Frankia* in the rhizosphere of *C. equisetifolia*. More specific analyses revealed that all frankiae

detected at any time and treatment belonged to either subgroup I of the *Alnus* host infection group or the *Elaeagnus* host infection group. In bulk soil, growth of frankiae representing the *Elaeagnus* host infection group was usually faster than that of frankiae of *Alnus* subgroup I, resulting in higher density increases (i.e. up to 100-fold) than those of *Alnus* subgroup I (10-fold) during the incubation time. This was different in the rhizosphere of both *Alnus* and *Casuarina* plants where effects of matric potential were obtained, with more than 100-fold increases of frankiae of *Alnus* subgroup I under dry conditions compared to bulk soil at t_0, and less than 10-fold increases under wet conditions. The opposite pattern was obtained for frankiae of the *Elaeagnus* host infection group. Consequently, under dry conditions the genus *Frankia* in the rhizosphere was to a large extent (i.e. up to 95% depending on the plant species) represented by subgroup I of the *Alnus* host infection group, while under wet conditions a similar percentage of the genus in the rhizosphere of both plant species was represented by the *Elaeagnus* host infection group. Leaf litter amendment resulted in growth of frankiae of the *Elaeagnus* host infection group only, essentially matching the values obtained for genus-specific analyses.

Our results demonstrate the usefulness of the *q*PCR methodology developed in this thesis for ecological studies on frankiae in soils. However, definite conclusions about population dynamics of frankiae in general and individual groups as a function of environmental characteristics require the further reduction of variables (e.g. assessments

of population dynamics of individual strains), and the inclusion of additional resources (e.g. soil extracts) in the analyses of population dynamics. Future analyses should also include next generation sequencing techniques using either *nif*H or rRNA gene fragments as target that should provide insight on overall diversity of frankiae in terrestrial systems, and confirm coverage of our *q*PCR based analyses of all *Frankia* or specific subgroups.

CHAPTER I

GENERAL INTRODUCTION

Soil represents a complex environment that is inhabited by a large number of diverse microorganisms that interact with each other. The complexity of soil is a reason for the lack of knowledge concerning the composition of soil microbes, their diversity, their distribution at small and large spatial scales at different times, and their importance for different soil processes and functioning. The different components of soil which include inorganic material such as sand, silt and clay, as well as organic matter are responsible for the creation of highly diverse microhabitats [15]. Generally, soil is regarded as being poor in nutrients and energy resources as compared to optimal growth conditions for microorganisms *in vitro* [76]. Soil exhibits different physicochemical properties depending on location and potential interactions as experienced in bulk soil (i.e. soil not affected by plant roots and potential exudates) [69] and rhizosphere soil (i.e. soil on the root surface of plants and thus potentially affected by exudates) [33,59], or in areas with accumulated particulate organic matter [54] or animal manures [56]. These areas as well as aggregates between inorganic and organic components might provide habitat for increased biological activity. Microorganisms resident in soil are exposed to several abiotic (i.e. physicochemical and nutritional) environmental factors, such as highly diverse carbon and energy sources of different quality, temperature gradients with fast changes in time, excess or limited nutrients and organic growth factors, fast fluctuating ionic compositions and concentrations, gradients and lack of available water, highly variable air composition, pH, and oxidation–reduction potentials, as well as different surface structures, that all might be basis for spatial relationships and interaction

between microorganisms [78]. The potential of the solid phase to adsorb important biological molecules on clay minerals or entrap them by humic acids protects them (e.g. proteins and nucleic acids) against proteolysis and/or thermal and pH denaturation [45,46] so that they can maintain their activity or at least be protected from degradation. These physicochemical factors and conditions undergo constant changes in nature, thus, affecting the ecology, activity and population dynamics of microorganisms in soil.

Microbial diversity in soil ecosystems exceeds, by far, that of eukaryotic organisms [78]. A total of about 6,000 different bacterial genomes were calculated per gram of soil by taking the genome size of *Escherichia coli* as a unit for comparison in DNA reassociation analyses [79]. Modeling approaches using results from reassociation analyses resulted in diversity estimates for bacteria in soil that might be close to 10^6 species per gram of soil [14]. Soil microbial communities are often difficult to fully characterize, mainly because of their immense phenotypic and genotypic diversity, heterogeneity, and crypticity. Soils can support large microbial communities with more than 10^9 cells per gram of soil [9,89] at correspondently tremendous diversity [12,14,79,80]. Only a very small portion of bacterial cells in soil top layers at concentrations reaching 10^9 cells per gram of soil [78] could be cultured, and thus diversity and abundance of many populations could not be analyzed by traditional growth dependent methods. Thus, the fraction of the cells making up the soil microbial biomass that have been cultured and studied in any detail make up often less than 5% of the overall community [7,80]. The failure to analyze the majority of microorganisms in soils, but also other environments has led to the development or adaptation of molecular methods that allowed researchers to assess the total microbial diversity and the

abundance of specific organisms present in the soil without relying on the growth of microorganisms. These methods generally use the detection and quantification of signature sequences on DNA and relate numbers of gene sequences to numbers of the respective target organisms. Members of the genus *Frankia* that are generally described as nitrogen-fixing actinomycetes forming root nodules in symbiosis with a variety of non-leguminous woody plants [5,30,68] are a good example of organisms where the application of molecular tools has resulted in enhanced knowledge on diversity and abundance in natural environments that would not have been possible with growth-dependent methods [3,5,8,19]. In addition to root nodules, a natural locale of enrichment of usually one *Frankia* population, *Frankia* can be found in soils that represent highly heterogeneous environments. *Frankia* is thought to be present in small numbers in soils with approximately 10^4-10^5 cells per gram of soil [21,44,58]. These numbers, however, are rough estimates because quantitative analyses of *Frankia* populations in soil are methodologically extremely challenging.

It is virtually impossible to isolate *Frankia* from soil, and only one successful attempt has been reported so far [2]. The most commonly used quantification method for *Frankia* in soils is based on plant bioassays in which a quantification of the nodulation capacity on a specific host plant after inoculation with serial dilutions of soils is used to describe the infective *Frankia* population (expressed as nodulation units g^{-1} soil). Based on plant bioassays, nodulation units between 0 and 4,600 units g^{-1} soil were determined for different soils [35,43,73]. However, these bioassays are highly selective, i.e. only nodule forming populations on a specific host plant are detected, and also potentially biased because nodulation units can theoretically be represented by a single spore, a

hyphal fragment, or a colony [44]. Other drawbacks could be its inability to detect and quantify competition for infection between populations of *Frankia* [23].

Comparison of the bioassay method of quantification with molecular detection methods such as PCR in which *Frankia*-specific fragments of the 16S rRNA gene were quantified (expressed as genomic units g^{-1} soil), however, revealed that nodulation units obtained in bioassays were generally much lower than genomic units (i.e. 0.2 to 2,940 nodulation units g^{-1} soil compared to 2,000 to 92,000 genomic units g^{-1} soil) [43]. Since bioassays are highly selective, abundance of *Frankia* in soils is more likely reflected by the numbers obtained by PCR-based approaches.

In the past, many studies using molecular tools for ecological study on *Frankia* have used 16S rRNA gene sequences as target. 16S rRNA gene sequences have been found to be efficient in differentiating *Frankia* strains that belong to different host infection groups; however, it is found to be a weak target when differentiating many *Frankia* strains belonging to the same host infection group because they contained identical 16S rRNA gene sequences [22,47]. Apart from using 16S rRNA sequences as target, studies on *Frankia* involved the use of 23S rRNA due to a considerable difference and variation in its length and sequence. Also, a large insertion specific for high G+C gram-positive bacteria have been found in domain III of the 23S rRNA [65]. This large specific and highly variable insertion has been used as a target for characterizing uncultured *Frankia* populations present in root nodules by *in situ* hybridization using fluorochrome-labeled probes [90,91]. Both genes contain conserved and highly variable regions and have been proven to be useful in the analyses of *Frankia* in soil and nodules

[3,10,21,47,50,64,71,90,91]. These targets also enabled studies on the phylogeny of the cultured as well as uncultured *Frankia* (reviewed in [19]). Studies using 23S rRNA gene sequences have resulted in the assembly of a small database of about 60 sequences both from cultured and uncultured frankiae that demonstrated sufficient sequence variation to distinguish several subgroups within the genus *Frankia* [26,35].

An alternative gene target has been was used relatively recently for diversity assessments of *Frankia* and plant species effects on nodulation. These studies targeted *nif*H, the structural gene for nitrogenase reductase [28]. Comparative sequence analyses of PCR-amplified *nif*H gene fragments was used to analyze uncultured *Frankia* populations in root nodules formed in plant bioassays after inoculation of slurries of five soils from a broad geographic range, i.e., from sites in five continents (Africa, Europe, Asia, North America and South America) on six plant species, i.e., two *Morella*, three *Elaeagnus* and one *Shepherdia* species [38,39,87], but also from different *Alnus* species [60,88]. These sequences that mainly represented *Frankia* of the *Alnus*, the *Elaeagnus* and the *Casuarina* host infection groups (i.e. clusters 1 and 3) (n=453), and one of the *Dryas* host infection group (cluster 2) that had been added from the EMBL databases (i.e. of yet uncultured endophytes in nodules of *Datisca*) had sizes mostly between 522 and 606 bp [37-39,87,88].

Both rRNA and *nif*H genes have been used as targets for the detection of frankiae in soils. Quantification of *Frankia* in soils so far employed PCR-MPN using nested [43] or booster PCR [58], but did not consider more recent technological advances of PCR like quantitative real-time PCR (*q*PCR) that has successfully been used for the quantification

of other soil microorganisms [13,67]. The availability of large databases of potential target sequences (i.e. 23S rRNA and *nif*H genes) and of *q*PCR methodology applicable to quantification of soil microorganisms, was the basis for our attempts to develop molecular detection and quantification methods for the analyses of *Frankia* populations in soils. These attempts were further supported by the availability of whole genome sequences of *Frankia* (strains CcI3, ACN14a [both cluster 1], EAN1pec, EUN1f [both cluster 3], EuI1c [non-nitrogen-fixing frankiae] and the uncultured endophyte of *Datisca glomerata* [cluster 2]) and other actinomycetes (*Acidothermus*, *Geodermatophilus*, *Nakamurella*, *Streptomyces*, *Streptosporangium*, and *Kitasatospora*) in the EMBL database or specifically for rRNA the SILVA rRNA database project (www.arb-silva.de) [62]. The methods developed and evaluated will then further be used to address questions on the fate of frankiae in soils.

It is well known that growth of frankiae in soil is influenced by the availability of carbon resources. Several studies have shown that *Frankia* can survive and retain infectivity in the rhizosphere soils of host plants [1,27,31,73,74,84,86] and non-host plants, which could be attributed to root exudates that are known to be rich in carbon sources. In addition, studies under axenic conditions have shown that the presence of organic material in soil affects the nodulation capacity of *Frankia* strains Ag45/Mut15 and ArI3 (subgroups within the *Alnus* host infection group) [48], however, except for microcosm studies [37,40] growth of *Frankia* has not been directly proven under these conditions. Thus, very little is known about the survival and possible proliferation of indigenous *Frankia* in the rhizosphere of host or non-host plants or the surrounding soil. Effects of plant species and root exudates have been indicated and were studied as part of

our method application assessments.

The goal of this PhD study was to develop detection and quantification methods that allowed us to analyze *Frankia* populations in soil. The experimental setup of this study covered four basic objectives:

1) Method development and evaluation

a) development of a SYBR Green *q*PCR method for the quantification of *Frankia* in soils. This research was based on the availability of a large database of *nif*H gene sequences (about 500 sequences obtained from pure cultures and uncultured populations from nodules and soils) that was used to develop primers aimed at detecting all nitrogen-fixing members of the genus. This study also addressed questions concerned with soil DNA extraction efficiencies and *q*PCR methodology that are affecting quantification of target organisms (chapter II).

b) evaluation of the usefulness of 23S rRNA gene sequences as an alternative to *nif*H gene sequences for the analyses of nitrogen-fixing members of the genus, as well as for subgroups within the genus (chapter III).

2) Method application

a) application of the developed methodology to assess the effects of plant species (*Alnus*, *Betula*) and sampling location (rhizosphere, crown cover, crown edge) on the abundance and diversity of indigenous *Frankia* strains (chapter IV)

b) elucidation of the effects of specific environmental conditions (i.e. matric potential, plant species, leaf litter amendment) on population dynamics of indigenous *Frankia* strains in soil microcosms (chapter V).

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CHAPTER II

QUANTIFICATION OF *FRANKIA* IN SOILS USING SYBR GREEN BASED *q*PCR

Abstract

A SYBR Green based *q*PCR method was developed for the quantification of clusters 1 and 3 of the actinomycete *Frankia* in soils. Primer *nif*Hr158 was designed to be used as reverse primer in combination with forward primer *nif*Hf1 specifically amplifying a 191-bp fragment of the *nif*H gene of these *Frankia*. The primer combination was tested for specificity on selected pure cultures, and by comparative sequence analyses of randomly selected clones of a clone library generated with these primers from soil DNA extracts. After adjustments of DNA extraction conditions, and the determination of extraction efficiencies used for sample normalization, copy numbers of *nif*H genes representing *Frankia* of clusters 1 and 3 were quantified in different mineral soils, resulting in cell density estimates for these *Frankia* of up to 10^6 cells [g soil {dry weight}]$^{-1}$ depending on the soil. Despite indications that the *nif*H gene is not a perfect target for the quantification of *Frankia*, the *q*PCR method described here provides a new tool for the quantification and thus a more complete examination of the ecology of *Frankia* in soils.

Keywords: Clone libraries, *Frankia*, *nif*H, Nitrogenase, Quantitative PCR, Root nodules

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Introduction

Members of the genus *Frankia* are nitrogen-fixing actinomycetes that form root nodules in symbiosis with a variety of non-leguminous woody plants [2,17,41]. *Frankia* can be found in root nodules, a natural locale of enrichment of usually one *Frankia* population, and in soils that represent highly heterogeneous environments. Soils can support large microbial communities with more than 10^9 cells g^{-1} soil [4,54] at a tremendous diversity [6,8,46,47]. *Frankia* are thought to be present in small numbers in soils with approximately 10^4–10^5 cells g^{-1} soil [11,28,36]. These numbers, however, are rough estimates because quantitative analyses of *Frankia* populations in soil are methodologically extremely challenging.

It is virtually impossible to isolate *Frankia* from soil, and only one successful attempt has been reported so far [1]. The most commonly used quantification method for *Frankia* in soils is based on plant bioassays in which a quantification of the nodulation capacity on a specific host plant after inoculation with serial dilutions of soils is used to describe the infective *Frankia* population (expressed as nodulation units g^{-1} soil). Based on plant bioassays, nodulation units between 0 and 4600 units g^{-1} soil were determined for different soils [20,29,43]. Comparison of this method of quantification with molecular detection methods such as PCR in which *Frankia*-specific fragments of the 16S rRNA gene were quantified (expressed as genomic units g^{-1} soil), however, revealed that nodulation units obtained in bioassays were generally much lower than genomic units (i.e. 0.2–2940 nodulation units g^{-1} soil compared to 2000–92,000 genomic units g^{-1} soil) [29]. Since bioassays are highly selective, i.e. only nodule forming populations on a specific host plant are detected, and potentially biased because nodulation units can

theoretically be represented by a single spore, a hyphal fragment, or a colony [28], abundance of *Frankia* in soils is more likely reflected by the numbers obtained by PCR-based approaches.

PCR-based quantification of *Frankia* in soils so far employed PCR-MPN using nested [29] or booster PCR [36], but did not consider more recent technological advances of PCR like quantitative real-time PCR (*q*PCR) that has successfully been used for the quantification of other soil microorganisms [7,40]. The aim of this study was therefore to develop a *q*PCR method for the quantification of *Frankia* in soils. The method development took advantage of the availability of a large database of *nif*H gene sequences for *Frankia* in our laboratory, with about 500 sequences of pure cultures and uncultured *Frankia* from nodules and from soils.

Materials and Methods

Primer design and specificity evaluation

Sequences that mainly represented *Frankia* of the *Alnus*, the *Elaeagnus* and the *Casuarina* host infection groups (i.e. clusters 1 and 3) (n = 453), and one of the *Dryas* host infection group (cluster 2) that had been added from the EMBL databases (i.e. of yet uncultured endophytes in nodules of *Datisca*) with sizes mostly between 522 and 606 bp [23-25,52,53] were aligned in Geneious 4.8.3 (Biomatters Ltd., Auckland, New Zealand), and screened for conserved regions. While several conserved regions were obtained, none of these covered the sequence of the uncultured endophyte of the *Dryas* host infection group (cluster 2). Therefore, primer *nif*Hr158 (5′ GAC GCA CTT GAT GCC CCA) was designed to be used as reverse primer in combination with forward primer *nif*Hf1 [23] for

the detection of frankiae of clusters 1 and 3 only.

The specificity of both primers for *Frankia* of clusters 1 and 3 was initially checked in GenBank/EMBL databases using the BLAST algorithm [34], and anneal temperatures for primer combination *nif*Hf1/*nif*H158 tested in end-point PCRs with DNA of representative *Frankia* strains of the *Alnus*, *Elaeagnus* and *Casuarina* host infection groups (n = 54) [12], with 64 °C resulting in specific amplification on all strains tested (data not shown). The specificity of amplification with 64 °C anneal temperature was further tested by analysis of a gene clone library generated with DNA extracted from soil (site I, Fort McCoy, Wisconsin, USA, 43°58′49″N, 90°43′36″W) as described in detail in a previous study (Mirza *et al.*, 2009c). Sequences of clones selected at random that were generated at the DNA Sequencing Facility of the Institute for Cellular and Molecular Biology at the University of Texas at Austin, TX, were compared to GenBank/EMBL databases using BLAST (Pearson and Lipman, 1988). Sequences of clones representing *nif*H gene fragments from *Frankia* (n = 34), were trimmed to 154 bp (i.e. primer sequences were removed from the amplicon of 191 bp), aligned with trimmed sequences of *nif*H fragments from pure cultures of *Frankia* (n = 34) (Welsh *et al.*, 2009a), and the alignment analyzed using maximum parsimony (MP), neighbor joining (NJ), Bayesian and maximum likelihood (ML) methods as outlined in detail in Welsh et al. (Welsh *et al.*, 2009a).

_q_PCR design and evaluation

*q*PCR quantification of *Frankia* was performed for each sample in triplicate in a total volume of 10 µl containing 5 µl of Quanta Mix (BioSciences Inc., PA), 0.2 µl of

each primer (20 ng each) and 1 μl of template in an Eco Real-time PCR system (Illumina, San Diego, CA) using an initial denaturation at 95 °C for 5 min, and 40 cycles of denaturation at 95 °C, annealing at 64 °C, and extension at 72 °C, each for 30 s. The amplification was followed by a melting curve analysis. Quantification was based on standard curves generated from cloned *nif*H gene fragments of strain ArI3 (606 bp in pGEM-Teasy; Promega, Madison, WI). Values for *nif*H gene copy numbers were only accepted when all 3 controls remained negative for the entire 40 cycles.

Initial attempts to quantify *Frankia* used 10-fold dilutions of cells of *Frankia* strain ArI3 [27] in pure culture with estimated cell densities of 10^5–0 cells μl^{-1}. Strain ArI3 was grown in a 500 ml bottle containing 100 ml Defined Propionate Medium [21] at 30 °C for 3 weeks. Filamentous cell aggregates were harvested with a pipette, and disrupted in 5 ml-subsamples in a tapered 40 ml glass tissue homogenizer (Krackeler Scientific, Albany, NY). Filaments in combined homogenates (20 ml) were further disrupted with a Sonifier® S-250A ultrasonic processor (Branson Ultrasonics Corp., Danbury, CT) with a tapered microtip (5 mm) at 20% output for 10 s. Cells were harvested by centrifugation at 4000 x g for 15 min, and washed with autoclaved tap water twice. Cell numbers were determined after in situ hybridization of fixed subsamples and subsequent epifluorescence microscopy [13]. The analysis revealed cell densities of $1.7 \pm 0.6 \times 10^8$ cells ml^{-1}, with cells generally present in filaments ($2.4 \pm 0.7 \times 10^7$ filaments ml^{-1}) of quite variable sizes (7.5 ± 2.8 cells $filament^{-1}$). Quantification by in situ hybridization and *q*PCR allowed us to relate *Frankia* cell numbers to *nif*H gene copy numbers.

Quantification of frankiae in soils

Initially, three commercially available soil DNA extraction kits were tested on soil samples (site I, Fort McCoy, Wisconsin, USA) using the manufacturer's protocols, though modified by using bead-beating for 2 min in a Mini-Bead-Beater-8 (Table 1). Based on performance (i.e. highest DNA yields, best purity and most reproducible quantification of *Frankia*), the SurePrep^{TM} Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) was used for further modifications aiming to increase DNA yields by optimizing soil/bead/buffer ratios during the lysis procedure. These modifications included doubling recommended values for soil or beads in combination with the use of beads of a smaller size (Ø0.1 mm), and/or a 50% reduction in extraction buffer during lysis, the harvest of the supernatant and the subsequent re-extraction of soil pellets with the remaining buffer (Table 1). In a separate attempt, ethanol precipitation after re-extraction and removal of proteins and debris was used instead of column purification to reduce potential losses during the purification steps (Table 1). Extractions of all combinations were done in triplicate, and assessed for yield in the final extract (ng μl^{-1}, in a final volume of 100 µl water), yield per gram of soil (µg [g{dry weight}]$^{-1}$), purity (absorbance ratios at 260/280 and 260/230), and the abundance of *Frankia* using 1 µl of the final extract or a 10-fold dilution as template (Table 1.1). Abundance of *Frankia* (cells [g soil {dry weight}]$^{-1}$) was estimated using the *nif*H gene copy numbers detected as being equivalent to cell numbers.

An optimized protocol that included modifications in the lysis procedure (i.e. a 50% reduction of the lysis buffer, the addition of 0.5 g of zirconium beads [Ø0.1 mm] instead of those provided, and bead-beating for 2 min in a Mini-Bead-Beater-8 [BioSpec

Products, Inc., Bartlesville, OK]), and included the re-extraction of the soil pellets with the remaining 50% of buffer after harvest of the supernatant, was then used to quantify cells of *Frankia* strain ArI3 inoculated into 250 mg of a sandy soil (Bastrop, TX) with low content of organic matter (<0.1%). This soil was chosen because it was always negative in *nif*H gene detection attempts. Inoculations were done with estimated cell densities of 10^5–0 cells μl^{-1}, using the same suspensions as described above for pure cultures in order to retrieve information on detection limits and extraction efficiencies.

The usefulness of the extraction and *q*PCR procedure was further assessed on DNA extracts obtained from different soils using the optimized extraction protocol (Table 2). Extraction efficiencies on these soils were determined as the ratio of inoculated *Salmonella enterica* cells detected before and after extraction by *q*PCR-based quantification of a 268-bp *inv*A gene fragment [38], using the same conditions as described above for *nif*H gene quantification. Extraction efficiencies were used to normalize copy numbers of *nif*H genes detected in 1 μl of the final extract or a 10-fold dilution as template. Copy numbers were used as being equivalent to cell numbers.

Results and Discussion

Primer design and specificity evaluation

Primer *nif*Hr158 had 6 mismatches to the *nif*H gene sequence of the uncultured *Frankia* strain in root nodules of *Datisca cannabina* (X76398), but was 100% complementary to all other *Frankia* sequences in our database, except for 16 sequences with 8 sequences representing pure cultures and another 8 sequences uncultured *Frankia* that had a single nucleotide difference at the last base at the 3′ end (C–G). Due to the

location of this mismatch at the first base at the 5/ end of the primer, this mismatch was not considered to negatively influence the amplification. This assumption was supported by both end-point and *q*PCR applications with DNA of four of these pure cultures (strains AgB32, AgKG'84/4, AiPa1, and AiPs4) as template (data not shown).

Database searches for target sequences of primers *nif*Hf1 and *nif*Hr158 using the BLAST algorithm detected mainly *Frankia nif*H gene sequences, however, none of the primers was entirely specific for *Frankia*. The combination of both primers, however, was specific for the detection of *nif*H genes representing *Frankia*, with one exception. Both primers detected the *nif*H gene of *Micromonospora lupini*, strain Lupac 08 (DSM 44870) that was isolated from root nodules of *Lupinus* [48]. The *nif*H gene fragment (435 bp) of this strain (FN395238) showed up to 99.3% sequence similarity to *nif*H gene fragments of confirmed members of the genus *Frankia* (i.e. strains ACN14a, AgP1P1, AgP1R2, AgP1R3, and AvcI1). Sequence similarities to *nif*H gene fragments of other *Micromonospora* isolates were between 99.3 and 99.7%, while intrageneric variation in *nif*H sequence similarities of confirmed *Frankia* strains has been found with lowest values just above 93% [23,25,52,53]. Horizontal gene transfer of nif genes has been discussed for several organisms [14,19] including *Frankia* [15,31,32] and can be speculated about in this case as well, with obvious consequences for quantitative analyses based upon *nif*H gene detection. The extent of these consequences for quantification of *Frankia*, however, remains highly speculative since information on the ecology of nitrogen-fixing *Micromonospora sp.*, including those isolated from root surfaces or nodules of actinorhizal plants (Trujillo *et al.*, 2007; Valdés *et al.*, 2005) is scarce.

The analysis of 40 clones from a gene clone library generated with DNA extracted from soil revealed six clones that contained vector only, while the remaining 34 clones represented *nif*H gene fragments with high similarity to *Frankia* (GenBank accession numbers FN824287–FN824320), demonstrating a high specificity of the primer combination *nif*Hf1/*nif*Hr158 for the *nif*H gene of the genus *Frankia*. One of the 34 clones, clone 32 (FN824308) carried a *nif*H gene fragment with identical sequence to that of *Frankia* strain ACN14a. This sequence was also identical to *nif*H gene fragments of 6 *Micromonospora* strains, further emphasizing the potential impact on quantitative analyses of *Frankia* based upon *nif*H gene detection. Meta-analysis consistently assigned sequences of all clones to *Frankia* of the *Elaeagnus* and the *Alnus* host infection groups, regardless of the phylogenetic method used (data not shown). The overall topology of these analyses was consistent with previous analyses that used sequences of 522–606 bp fragments of the *nif*H gene of both cultured and uncultured *Frankia* [23,52,53], although the much smaller size of the fragments used in this analyses (154 bp) resulted in hardly any support by bootstrap and posterior probability methods (Fig. 1). This result was similar to one of our previous studies with small fragments (252 bp) [25], that was used to relate *nif*H gene sequences retrieved from soil organisms to those of confirmed *Frankia* strains. Since the presence of *Frankia* of the *Casuarina* host infection group was suggested to be dependent on co-introduction with this exotic plant species [5,55], they were not expected to be present in this soil, and thus our results suggest non-preferential amplification or absence of primer biases toward a particular group of targeted *Frankia* strains (Fig. 1).

_q_PCR design and evaluation

_q_PCR conditions with 64 °C annealing temperature resulted in the production of a single fragment melting at T_m = 87.9 °C with both *Frankia* cells or standards (i.e. cloned *nif*H fragments) as template. Quantification of cells in pure cultures of *Frankia* strain ArI3 using 1 μl of 10-fold dilutions of cell suspensions without further treatments resulted in the determination of *nif*H gene copy numbers of $2.3 \pm 0.0 \times 10^5$ in the least diluted samples which corresponded to cell densities determined by in situ hybridization after correction for dilution (i.e. cell densities of $1.7 \pm 0.6 \times 10^8$ cells ml^{-1}). This result indicated adequate lysis of *Frankia* filaments and amplification of *nif*H gene fragments during the _q_PCR reaction, and suggested that a direct conversion of copy to cell numbers was permitted.

Quantification of frankiae in soils

DNA extractions from soil using 3 commercially available soil DNA kits resulted in similar yields, however, large differences in purity and *nif*H gene copy numbers of *Frankia* detected, with the SurePrep™ Soil DNA Isolation Kit providing the highest purity of DNA and the most reproducible quantification of frankiae at two template dilutions, i.e. 1 μl of the final extract or a 10-fold dilution (Table 1). Dilutions are commonly recommended to circumvent potential effects of inhibitory substances co-extracted with DNA [10,37]. While dilutions are an option for the quantification of target organisms present in large numbers, and for the detection of our target sequences by individual kits, the large variability in the quantification of *nif*H copy numbers for *Frankia* in the dilutions suggested target concentrations close to the detection limit, and thus negative effects of the 10-fold dilution on the reliability of the quantification.

Attempts to address low target concentrations focused on increasing DNA yields in the extraction while maintaining DNA purity. Maintaining purity required column purification, because substitution of this step by ethanol precipitation resulted in lower 260/280 and higher 260/230 absorbance values and no amplification without dilution (Table 1). All modifications in the lysis and extraction procedure of the SurePrep™ Soil DNA Isolation Kit resulted in higher DNA yields compared to the original protocol (i.e. 2–7 times more DNA) (Table 1). Values between 6.1 $\pm$ 0.6 and 35.3 $\pm$ 1.0 μg (g soil)$^{-1}$ reflect concentrations of DNA often obtained from [35,39]. Purity generally remained high, however, quantification of *Frankia* at both template concentrations was still quite variable, with higher values for *nif*H copy numbers not necessarily related to higher DNA yield (Table 1). Copy numbers were all in the same range, with 0.4 $\pm$ 0.2 to 2.7 $\pm$ 0.2 x 10^5 copies (g soil)$^{-1}$ in 1 μl of the final extract, and 0.6 $\pm$ 0.3 to 4.6 + 2.0 x 10^5 copies (g soil)$^{-1}$ in 10-fold dilutions (Table 1). Since none of the protocols produced any outstanding values for the detection of copy numbers, the protocol producing the highest yield of DNA was chosen for further analyses (Table 1, protocol B.2.b).

Quantification of cells of *Frankia* strain ArI3 in extracts retrieved by using this DNA extraction protocol resulted in the detection of 1.4 $\pm$ 0.9 x 10^5 copies at the highest inoculated cell concentration which corresponded to a recovery of about 60%. *Nif*H genes were detected in all dilutions of pure cultures and inoculated soils, including the estimated 0 dilution, while non-inoculated soil remained negative at all times. Values, however, became highly variable with increasing dilution (i.e. non-linear in dilutions, and recoveries between 13 and 65%) which is most likely a consequence of increasing inaccuracies during the sequential transfer of small filaments to lower concentrations.

Consequently, reliable assessments of detection limits with introduced *Frankia* strains in soil, as well as the determination of recoveries using comparative analyses of pure cultures and inoculated cells in soil at lower dilutions were not achieved.

Because non inoculated soils remained negative for the entire 40 cycles, the low values in soils inoculated with the most diluted cell suspension (about 10 copies at C_T around 35, and single fragment melting at T_m = 87.9 °C like those generated from cells or standards) suggested a detection limit of *Frankia* cells in soils in numbers that corresponded to about 4 x 10^3 cells (g soil)$^{-1}$, and thus 2 orders of magnitude below numbers obtained for indigenous populations in our soil from Wisconsin (Table 1). Because the recovery of DNA from soils and other environments is often far below the 60% obtained with our sandy soil (i.e. about 10% and less) [3,26], abundances of up to 10^6 cells (g soil)$^{-1}$ would probably depict a more accurate picture of abundances of indigenous *Frankia* populations in the Wisconsin soil.

Extraction efficiencies can be determined by the quantification of internal standards added to the soil samples [33,51]. In our study, the addition and quantification of *Salmonella* cells in soil samples resulted in highly variable extraction efficiencies (Table 2). The use of template dilutions always resulted in much higher extraction efficiencies than the use of non-diluted extracts suggesting inhibition of the amplification, most likely due to contaminating humic acids [10,45], though effects of large amounts of non-target DNA [18] cannot be excluded (Table 2).

Extraction efficiencies were also affected by the soil with high variation between replicate samples, demonstrating the need for the determination of extraction efficiencies

for each sample to normalize for variation in DNA recoveries. Quantification of *nif*H gene copies and subsequent normalization indicated abundances of more than 10^6 cells (g soil)$^{-1}$ in 3 out of 6 soils, with similar values obtained with diluted and non-diluted template (Table 2). *Nif*H genes of frankiae were not detected in the soil originally used for our inoculation studies (i.e. Bastrop), both with diluted and non-diluted template, even though *q*PCR quantification of the *inv*A gene of Salmonella was achieved with high recoveries (Table 2). For the remaining 2 soils, only the use of diluted template resulted in the detection of amplification products for both *inv*A and *nif*H genes suggesting inhibition by the non-diluted extract. Thus, our extraction procedure needs adjustments for soils rich in organic material, potentially by using pretreatments [39], or by using alternative purification procedures [42,44].

Despite high yields of DNA extracted from these two soils, copy numbers of *nif*H genes were much lower than in the other soils. These differences in copy numbers of *nif*H genes or abundance of *Frankia* between, but also within soils might reflect effects of environmental conditions that include microheterogeneities within a soil as demonstrated for other bacteria and environments [9,16,30]. Although it has been shown that *Frankia* strains can grow saprophytically in filamentous form in the rhizosphere of host and non-host plants, and some even with leaf litter as a nutrient resource [22], it is currently unknown whether *Frankia* occur in soils in single cells, in hyphal fragments or even in colonies, and how its fate and occurrence in soil is affected by environmental conditions. Despite indications that the *nif*H gene is not a perfect target for the quantification of *Frankia*, the *q*PCR method described here should allow for a more complete examination of the ecology of indigenous and introduced *Frankia* in soils.

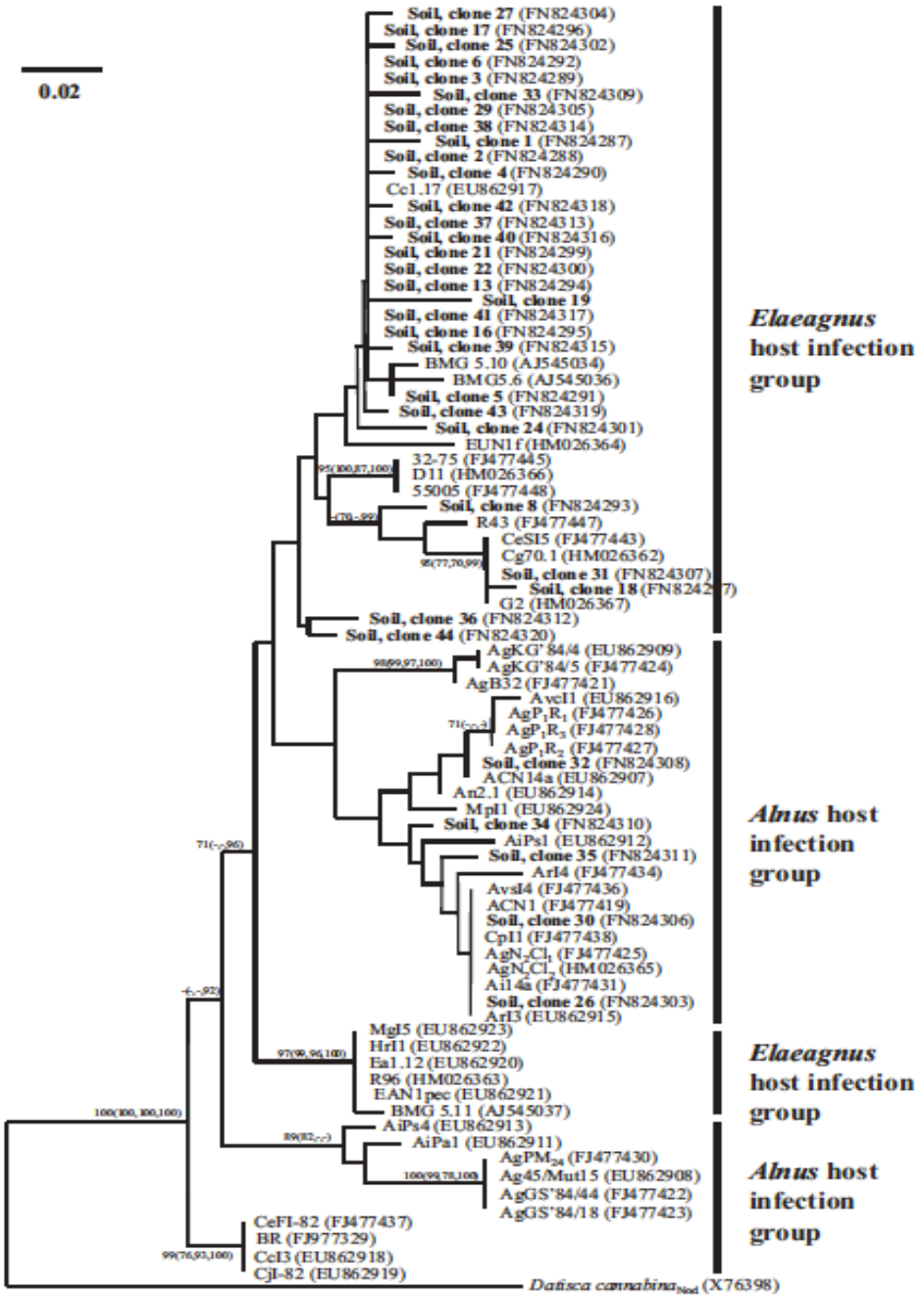

Fig. 1. Maximum likelihood-based tree generated using 154 bp of the *nif*H gene of 34 clones obtained from soil in this study, and trimmed sequences of *nif*H fragments obtained in previous studies from pure cultures of *Frankia* (n = 34) [52]. Numbers at nodes reflect maximum likelihood BS support measures and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, Bayesian, and maximum parsimony analyses, respectively, obtained as outlined in detail in Welsh et al. [52]. The outgroup was a sequence from an uncultured *Frankia* population from nodules of *Datisca cannabina*

Table 1. *q*PCR-based enumeration of frankiae in a sandy loam (native prairie, Fort McCoy, WI) after DNA extraction using the SurePrep™ Soil DNA Isolation Kit

Treatment (n=3)[a]	DNA Yield ($ng\ \mu l^{-1}$)[b]	DNA Yield ($\mu g\ [g\ soil]^{-1}$)	Absorbance		Number of *Frankia* cells ($x\ 10^5\ [g\ soil]^{-1}$)[d]	
			260/280	260/230	1 µl DNA	0.1 µl DNA
A. Selection of soil DNA extraction kit using the manufacturer's protocol						
1. UltraClean™ Soil DNA Kit (MoBio)	24.3 (2.6)	9.7 (1.0)	1.1 (0.0)	nd[c]	0	3.4 (5.9)
2. PowerSoil™ DNA Kit (MoBio)	19.4 (1.3)	7.8 (0.5)	0.7 (0.1)	nd	4.8 (3.6)	3.9 (3.4)
3. SurePrep™ Soil DNA Isolation Kit (Fisher)	29.7 (1.3)	11.9 (0.5)	1.9 (0.1)	1.0 (0.0)	1.4 (0.3)	3.7 (1.1)
B. Modifications to the SurePrep™ Soil DNA Isolation Kit (Fisher)						
1. Changes to soil/bead ratio or in bead size						
a. 0.25 g soil, 0.5 g beads	42.0 (2.7)	16.8 (1.1)	1.8 (0.0)	1.0 (0.0)	2.5 (0.2)	4.1 (1.6)
b. 0.25 g soil, 1 g original beads	59.0 (1.0)	23.6 (0.4)	1.8 (0.0)	1.0 (0.0)	1.8 (0.1)	1.7 (1.2)
c. 0.25 g soil, 1 g beads (Ø: 0.1 mm)	39.5 (2.0)	15.8 (0.8)	1.8 (0.1)	1.3 (0.0)	2.7 (0.2)	4.6 (2.0)
d. 0.5 g soil, 1 g original beads	30.4 (3.1)	6.1 (0.6)	1.8 (0.1)	0.9 (0.1)	0.6 (0.2)	0.6 (0.3)
e. 0.5 g soil, 1 g beads (Ø: 0.1 mm)	48.5 (3.4)	9.7 (0.7)	1.9 (0.0)	1.1 (0.1)	1.0 (0.5)	1.0 (0.4)
2. 50% reduction of extraction buffer, harvest of supernatant and subsequent re-extraction of soil with the remaining buffer						
a. 0.25 g soil, 0.5 g original beads	55.3 (4.8)	22.1 (1.9)	1.9	1.0	2.1 (1.1)	1.1 (0.3)

				(0.0)	(0.0)		
b.	0.25 g soil, 0.5 g beads (Ø: 0.1 mm)	88.1 (2.3)	35.3 (1.0)	1.9 (0.0)	1.3 (0.0)	1.8 (1.1)	1.5 (0.6)
c.	0.25 g soil, 1 g original beads	26.6 (2.1)	10.6 (0.8)	1.7 (0.1)	0.9 (0.1)	0.7 (0.3)	0.7 (0.0)
d.	0.25 g soil, 1 g beads (Ø: 0.1 mm)	75.9 (3.6)	30.4 (1.4)	1.8 (0.0)	1.3 (0.1)	0.8 (0.2)	1.6 (1.0)
e.	0.5 g soil, 1 g original beads	39.5 (3.5)	7.9 (0.7)	1.8 (0.0)	0.9 (0.0)	0.4 (0.2)	1.0 (0.8)
f.	0.5 g soil, 1 g beads (Ø: 0.1 mm)	65.4 (9.9)	13.1 (2.0)	1.9 (0.0)	1.0 (0.2)	0	0.6 (0.3)
3.	Ethanol precipitation after re-extraction and removal of proteins and debris (i.e. omission of column purification steps)						
a.	0.25 g soil, 0.5 g beads (Ø: 0.1 mm)	116.4 (15.3)	46.6 (1.5)	1.7 (0.0)	1.3 (0.0)	0	2.0 (1.1)
b.	0.5 g soil, 1 g beads (Ø: 0.1 mm)	140.7 (7.4)	28.1 (1.5)	1.7 (0.0)	1.1 (0.0)	0	0.2 (0.0)

[a]calculations are based on 3 independent extractions and 3 replicate measurements for each
[b]DNA yield (ng μl^{-1}) in a final volume of 100 µl
[c]not determined
[d]the quantification is based on a direct conversion of *nif*H gene copy numbers to *Frankia* cell numbers

Table 2. *q*PCR-based enumeration of frankiae in different soils after DNA extraction with the SurePrep™ Soil DNA Isolation Kit, using the manufacturer's purification protocol with modifications in the lysis and extraction procedure

Soil origin	Major vegetation	Soil texture/organic matter content (%)[a]	DNA Yield (µg [g soil]$^{-1}$)[a]	Extraction efficiency (%)[b/c]		Number of *Frankia* cells (x 10^5 [g soil]$^{-1}$)[b/d]	
				1 µl DNA	0.1 µl DNA	1 µl DNA	0.1 µl DNA
Fort McCoy, WI site I	native prairie, perennial grasses, pines	Sandy loam/3.4 (1.1)	25.1 (1.6)	10.6 (3.5)	38.3 (11.4)	13.3 (6.2)	16.9 (5.5)
site II	native prairie, perennial grasses, pines	Sandy loam/3.9 (0.2)	25.1 (11.8)	10.4 (4.1)	42.0 (21.7)	9.4 (1.2)	12.1 (3.5)
site III	native prairie, perennial grasses, pines	Sandy loam/5.5 (0.4)	24.2 (11.4)	0	49.8 (27.7)	0	3.1 (1.1)
Poudre Canyon, CO	Ponderosa pine forest, grasses, forbs, shrubs	Sandy loam/9.4 (0.4)	32.3 (3.2)	0	89.0 (21.0)	0	4.5 (2.1)
Bastrop, TX	Loblolly pine forest, with Eastern juniper	Sand/0.1 (0.1)	8.3 (1.0)	39.9 (9.5)	99.8 (39.5)	0	0
Morton Arboretum, IL	planted alder species	Silty loam/10.2 (0.1)	7.6 (2.5)	1.0 (0.8)	8.2 (4.0)	13.0 (0.9)	12.0 (0.7)

[a]all analyses were performed in triplicate and presented as mean (standard deviation)
[b]calculations are based on 3 independent extractions and 3 replicate measurements for each and presented as mean (standard deviation)
[c]extraction efficiencies were determined as the ratio of inoculated *Salmonella* cells detected before and after purification using 268-bp *inv*A gene fragments for detection by *q*PCR. The first column used 1 µl of the DNA dissolved a final volume of 100 µl, while the second column represents a 10-fold dilution (0.1 µl)
[d]number of *Frankia* cells were corrected for DNA loss as indicated by extraction efficiency value

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CHAPTER III

EVALUATION OF THE 23S rRNA GENE AS TARGET FOR *q*PCR BASED QUANTIFICATION OF *FRANKIA* IN SOILS

Abstract

The 23S rRNA gene was evaluated as target for the development of SYBR Green-based quantitative PCR (*q*PCR) for the analysis of nitrogen-fixing members of the genus *Frankia* or subgroups of these in soil. A *q*PCR with a primer combination targeting all nitrogen-fixing frankiae (clusters 1, 2 and 3) resulted in numbers similar to those obtained with a previously developed *q*PCR using *nif*H gene sequences, both with respect to introduced and indigenous *Frankia* populations. Primer combinations more specifically targeting three subgroups of the *Alnus* host infection group (cluster 1) or members of the *Elaeagnus* host infection group (cluster 3) were specific for introduced strains of the target group, with numbers corresponding to those obtained by quantification of nitrogen-fixing frankiae with both the 23S rRNA and *nif*H genes as target. Method verification on indigenous *Frankia* populations in soils, i.e. in depth profiles from four sites at an *Alnus glutinosa* stand, revealed declining numbers in the depth profiles, with similar abundance of all nitrogen-fixing frankiae independent of 23S rRNA or *nif*H gene targets, and corresponding numbers of one group of frankiae of the *Alnus* host infection only, with no detections of frankiae representing the *Elaeagnus, Casuarina*, or a second subgroup of the *Alnus* host infection groups.

Keywords: *Frankia*, *nif*H, Nitrogenase, 23S rRNA, Quantitative PCR, Root nodules, *Alnus*

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Introduction

The genus *Frankia* represents nitrogen- and non-nitrogen-fixing actinomycetes that form root nodules on some non-leguminous woody plants [4,10]. Root nodule formation is host plant-specific, with clusters 1, 2, and 3 representing nitrogen-fixing frankiae of the *Alnus*, *Dryas* and *Elaeagnus* host infection groups [2], and cluster 4 representing non nitrogen-fixing strains [39]. Root nodules resemble highly homogeneous environments occupied by enrichments of generally one major *Frankia* population only which allowed for extensive studies on nodule-forming *Frankia* populations by both growth-dependent and -independent methods (e.g. [4,10,14]). Frankiae inhabit a second ecological niche, i.e. soils, with vastly contrasting conditions compared to root nodules. Soils are highly heterogeneous environments that can support a large microbial community with more than 10^9 cells g^{-1} soil [7,40] at a tremendous diversity [8,9,33,34], and frankiae generally present in small numbers only (approx. 10^4–10^6 cells g^{-1} soil) [11,22,26,31]. The complexity of soils and the low abundance of frankiae negatively affects population analyses by both growth-dependent and -independent methods, and consequently information on populations of *Frankia* in soil is much more limited than that on *Frankia* in root nodules (see [6]) for review).

We recently reported on the development of a SYBR Green based *q*PCR method for the quantification of the genus *Frankia* in soils using *nif*H gene sequences as target [31]. This *q*PCR method allowed us to quantify frankiae in different mineral soils, with cell density estimates for frankiae of up to 10^6 cells [g soil {dry wt.}]$^{-1}$ depending on the soil [31]. The study, however, also revealed some problems using *nif*H genes as target for the quantification of frankiae. First, primers developed only detected frankiae of the

Alnus and *Elaeagnus* host infection groups (clusters 1 and 3, respectively), but not frankiae of the *Dryas* host infection group (cluster 2) or non-nitrogen-fixing strains (cluster 4). Second, indications for *nif*H gene transfer were observed with up to 99.3% sequence similarity of nifH gene fragments of confirmed members of the genus *Frankia* to those of *Micromonospora lupini* that was isolated from root nodules of *Lupinus angustifolius* [35]. These issues prompted us to look for other genes that could be used as target in *q*PCR applications to quantify all members of the genus *Frankia* but also to distinguish clusters or specific subgroups within the genus. A promising target was found in an actinomycetes-specific insertion in Domain III of the 23S rRNA and its gene [28] that had been used as target for oligonucleotide probing before [29]. A small database of about 60 sequences of this insertion from different *Frankia* strains was available from previous taxonomic studies that demonstrated sufficient sequence variation to distinguish several subgroups within the genus *Frankia* [13,16]. This database was amended with published and unpublished sequences obtained from whole genome sequencing projects for *Frankia* [25].

The aim of this work was to evaluate the usefulness of this 23S rRNA insertion as target for *q*PCR applications aimed at the specific detection and quantification of *Frankia* in soils. The evaluation was set up as a comparative study with our previously developed SYBR Green based *q*PCR method using *nif*H gene sequences as target [31]. For this purpose, the 23S rRNA insertion was evaluated as target for the detection of all nitrogen-fixing frankiae only, excluding the non-nitrogen-fixing members of the genus, and within the nitrogen-fixing frankiae on the subgroup level using host plant group assignments of frankiae (i.e. frankiae of the *Elaeagnus* and *Alnus* host infection groups, with *Casuarina*-

infective strains separated from the latter).

Materials and Methods

Primer design and evaluation

Partial sequences of about 160 bp that represented nitrogen fixing frankiae of the *Alnus* host infection group including *Casuarina*-infective strains (cluster 1, n = 35 and 5, respectively), the *Elaeagnus* host infection group (cluster 3, n = 12), and the *Dryas* host infection group (cluster 2, n = 1) and non-nitrogen fixing strains (cluster 4, n = 12) [13,16] were aligned in Geneious 5.5.7 (Biomatters Ltd, Auckland, New Zealand). This alignment was further amended with sequences of *Frankia* strains CcI3, ACN14a [both cluster 1], EAN1pec, EUN1f [both cluster 3], EuI1c [non-nitrogen fixing frankiae] and the uncultured endophyte of *Datisca glomerata* [cluster 2], and other actinomycetes (*Acidothermus*, *Geodermatophilus*, *Nakamurella*, *Streptomyces*, *Streptosporangium,* and *Kitasatospora*) retrieved from the SILVA rRNA database project (www.arb-silva.de, accessed 08/18/2011) [27]. The SILVA rRNA database also provided information on copy numbers of the 23S rRNA gene per genome, with two copies in strains CcI3, ACN14a and the endophyte in *D. glomerata*, and three copies in strains EAN1pec, EUN1f and EuI1c.

The alignment was screened for conserved as well as for group-specific sequences within the genus *Frankia*. Selected primers were checked for low potential of self- and hetero-dimer formation using OligoAnalyzer 3.1 (www.idtdna.com/analyzer/ Applications/OligoAnalyzer) and for target specificity using Test-Prime 1.0 [15] from the SILVA rRNA database project (accessed 12/06/2012).

_q_PCR design and evaluation

Annealing temperatures for all primer combinations were tested in *q*PCRs with DNA of representative *Frankia* strains of the *Elaeagnus* and *Alnus* host infection groups and of *Casuarina* infective strains (n = 54) [12]. Non-nitrogen-fixing *Frankia* strain AgB1.9 (cluster 4) and several streptomycetes (*S. albireticuli* NRRLB5493, *S. flavogriseus* NRRL-B1671, *S. erythrogriseus* NRRL-B3808 and *S. griseus* NRRL-B2682) retrieved from database searches with small mismatches in the forward and reverse primer, respectively, served as non-target organisms. *q*PCR quantification of frankiae was performed for each sample in triplicate in a total volume of 10 µl containing 5 µl of SsoADV SybrGreen Mix (BioRad, Hercules, CA), 0.2 µl of each primer (100 nM each) and 1 µl of template in an Eco Real-time PCR system (Illumina, San Diego, CA) using an initial denaturation at 95 °C for 5 min, and 40 cycles of denaturation at 95 °C, annealing at 64 or 66 °C depending on the primer combination (Table 3), and extension at 72 °C, each for 30 s, as in our previous study [31]. The amplification was followed by a melting curve analysis. Quantification was based on standard curves generated from purified PCR products of strains EAN1pec, ArI3, CcI3 or Ag45/Mut15 depending on the primer combination. Amplicons were generated using the genus-specific primers, and concentrations measured with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Copy numbers were calculated from concentrations (http://www.uri.edu/research/gsc/resources/cndna.html) and normalized after *q*PCR quantification with the primer combination targeting all nitrogen-fixing frankiae. Copy numbers were divided by copy numbers of the 23S rRNA gene per genome (2 or 3 for pure cultures, 2.5 for mixtures or unknown populations) to relate copy numbers to

Frankia cell numbers.

Initial attempts to quantify all nitrogen-fixing frankiae and subgroups used cells of *Frankia* strains Ag45/Mut15, ArI3, EAN1pec, and CcI3 as representatives of the specific target groups and a mixture of these strains inoculated into 250 mg of a sandy soil (Bastrop, TX) with low content of organic matter (<0.1%). This soil was chosen because it was always negative in previous quantification attempts that targeted the *nif*H gene of frankiae in SybrGreen-based *q*PCR [31]. Cells of all strains were grown in 500 ml bottles containing 100 ml Defined Propionate Medium [17] at 30 °C for three weeks. Cells were harvested with a pipette, filamentous cell aggregates disrupted in 5 ml-subsamples in a tapered 40 ml glass tissue homogenizer (Krackeler Scientific, Albany, NY), and filaments in combined homogenates (20 ml) further disrupted with a Sonifier® S-250A ultrasonic processor (Branson Ultrasonics Corp., Danbury, CT) with a tapered microtip (5 mm) at 20% output for 10 s [31]. Cells were harvested by centrifugation at 4000 x g for 15 min, washed with autoclaved tap water twice and resuspended in 20 ml autoclaved tap water. Subsamples of 100 µl containing an estimated cell density of 10^6 cells were inoculated into the soil, with negative controls being soil that remained un-inoculated.

DNA was extracted from soils using the SurePrepTM Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) with small modifications as described before [31]. Extractions of all combinations were done in triplicate, and assessed for the abundance of frankiae by *q*PCR using 1 µl of the final extract (100 µl) as template (Table 4). Abundance of frankiae as number of cells [g soil {dry wt.}]$^{-1}$ was estimated after

correction for gene copy numbers per genome, and compared to numbers obtained by *q*PCR using the *nif*H gene as target for all frankiae [31].

*q*PCR verification on indigenous *Frankia* populations in soil

The usefulness of the *q*PCR procedures was further assessed on DNA extracts obtained from soils harboring indigenous frankiae (Table 4). The sampling site (53.9963 N, 10.0257 E) was located in the sandy barren flats of the "geest", close to the village Boostedt in the center of Schleswig-Holstein, the northernmost of the states of Germany, at a steep slope at the shore of a pond. This pond was previously an obstacle of equestrian cross country events but was abandoned in the early 1980s. Since that time, natural re-vegetation of the slope resulted in the establishment of an *Alnus glutinosa* stand (Fig. 2. A). Using a Purkhauer auger system (2 cm diameter), soil samples were taken from 4 locations (A-D) from the upper 20 cm in 5 cm intervals (1–4) on June 21, 2010. All samples consisted of fine sand with generally declining concentrations of organic material in depth (Fig. 2. A). All samples from location D were water-saturated, while those from locations A-C were not. DNA was extracted from triplicate samples as described above, with 10-fold dilutions used as template in *q*PCR analyses. Results of these analyses were corrected after assessments of extraction efficiencies determined as the ratio of inoculated *Salmonella enterica* cells detected by *q*PCR-based quantification of a 268-bp *inv*A gene fragment before and after extraction as described previously [31].

Quantitative analyses were also performed on a small number of nodules (n = 3 per site) that were only found in the upper 5 cm of the soils (A1 to D1). The epidermis from one lobe from each of the nodules preserved in 70% isopropanol was removed and

the remaining material crushed with a mortar and pistil. Crushed nodule lobes were washed with 1 ml of PBS twice, and finally resuspended in 50 μl of distilled water [20,37]. One μl of this suspension was then used without further treatments as template in *q*PCR-based quantification using primer combinations for all nitrogen-fixing frankiae and the subgroups.

Results and Discussion

Primer design and evaluation

Primer combination 23Fra1655f/23Fra1769r was selected to detect all nitrogen-fixing *Frankia* strains (clusters 1, 2 and 3) (Table 3), but not non-nitrogen-fixing strains (cluster 4). Primer 23Fra1769r was also used as reverse primer for the detection of more specific groups of frankiae, with forward primers 23Ar/Cas1579f, 23Mut1555f and Cas1610f targeting subgroups I and II as well as *Casuarina*-infective strains within the *Alnus* host infection group (cluster 1), respectively, and primer 23EAN1579f targeting frankiae of the *Elaeagnus* host infection group (cluster 3) (Table 3). Primers targeting members of clusters 2 and 4 were not designed.

TestPrime 1.0 analyses revealed that all primers and primer combination retrieved their respective target sequences available in the SILVA databases with no mismatches. Forward primers detecting specific groups were highly specific, with 4–6 mismatches to non-target sequences. This specificity was not impacted in combination with the reverse primer 23Fra1769r, even though this primer was not only identical to sequences of frankiae, but also to those of *Streptomyces* and *Kitasatospora*. Forward primer 23Fra1655f used in combination with this primer to detect all nitrogen-fixing frankiae

was also not specific for frankiae, but was identical to sequences from *Geodermatophilus*, *Acidothermus*, *Blastococcus* and *Gordonia*. Their combination, however, was specific for nitrogen-fixing frankiae. Specificity, however, was not strong, with sequences of the genus *Acidothermus* displaying only 1 mismatch to the reverse primer, sequences of a few *Streptomyces* strains showing 2 mismatches to the forward primer, and those of non-nitrogen-fixing *Frankia* strains having 2 and 1 mismatch in the forward and reverse primers, respectively. These results indicate a high potential of the 23S rRNA gene as target for the specific quantification of subgroups of frankiae due to the large number of mismatches to non-target sequences, but demonstrate limitations for specific quantification of all nitrogen-fixing frankiae consequent to the small number of mismatches to some non-target sequences, with none of the mismatches located at or near the 3′-terminal position meant to result in the largest reduction of non-target amplification [1,5,32].

_q_PCR design and evaluation

As indicated by the large mismatches in forward primers to non-target sequences in database analyses, specific amplification was achieved for all primer combinations on the subgroup level. Anneal temperatures of 64–66 °C provided specific signals for the target strains only, both in pure culture (data not shown) and inoculated into soil (Table 4). DNA from non-inoculated soil used as template in similar concentrations as from inoculated soils did not result in any amplification (Table 2.2), confirming our previous results using the *nif*H gene of frankiae in SybrGreen-based *q*PCR [31]. The lack of amplification signals using the 23S rRNA gene as target indicates that DNA from indigenous organisms other than *Frankia* that might include the above-mentioned non-

target actinomycetes are likely not major constituents of this soil microbial community, and thus the low specificity of the primer pair targeting all nitrogen-fixing frankiae might not be an issue for quantification of *Frankia* in soils. While this speculation might hold for *Acidothermus cellulolyticus* that has been isolated from hot-springs [21], and been characterized as being thermo- and acidophilic [3,21] rather than mesophilic like its close relative *Frankia* [3], results might be different for non-nitrogen-fixing frankiae that have been commonly detected in waterlogged soils of natural alder stands together with nitrogen-fixing frankiae [36,38].

*q*PCR verification on indigenous *Frankia* populations in soil

Similar to studies on introduced strains, analyses of indigenous *Frankia* populations targeting all nitrogen-fixing frankiae resulted in comparable values for both the use of the *nif*H or the 23S rRNA gene as target (Table 5), again indicating that the low specificity of primer pair 23Fra1655f/23Fra1769r was not affecting quantitative analyses of nitrogen fixing frankiae, with non-target organisms including non-nitrogen-fixing frankiae likely not being prominent members of the indigenous microbial community even in waterlogged soils. Despite high variability in extraction efficiencies and DNA concentrations in replicate samples and between sites, both targets provided numbers corresponding to approx. 10^6 cells g^{-1} soil in surface samples from sites A-C, with numbers declining by approximately one order of magnitude with depth (Table 5). This decline with depth largely corresponded to lower organic matter contents in samples from lower depths (Fig. 2.); these data, however, did not allow us to establish a causal relationship because other variables potentially affecting *Frankia* populations such as fine root density or other environmental characteristics were not evaluated. Indications for a

more complex interaction of environmental characteristics and *Frankia* populations are observed in samples from water-logged site D that harbored frankiae in numbers approximately 1 order of magnitude lower than surface samples from sites A-C (Table 5), at all depths despite reductions in organic matter content with depth (Fig. 2.).

More specific analyses on the subgroup level revealed subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15 as the only subgroup detectable by *q*PCR. Numbers corresponded largely to those retrieved for nitrogen-fixing frankiae (Fig. 2. B) indicating the presence of one major *Frankia* population only, apparently independent of environmental characteristics such as organic matter content and matric potential. Both characteristics have been shown to affect the development of specific *Frankia* populations [18,19,23,24], with organic matter such as leaf litter supporting growth of one specific population, i.e. subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15, only [19]. Leaf litter amendments to soils consequently resulted in shifts of nodule-forming *Frankia* populations from subgroup I to subgroup II, both for introduced and indigenous populations [24]. Effects of matric potential are less well established even though drier conditions seem to favor root nodule formation of frankiae representing subgroup I over those of subgroup II [24]. The lack of detection of frankiae other than those representing subgroup II, however, makes it difficult to elucidate the potential effects of these environmental characteristics, especially because other potential factors affecting *Frankia* populations were not considered. These factors include rhizosphere effects since all populations grow in the rhizosphere of their host plants [19] but also potentially in that of other plants [30].

Subgroup II of the *Alnus* host infection group was also the only subgroup detected in lobes of nodules collected from the surface samples of all 4 sites, with increasing numbers in lobes obtained from locations A (1.9 (0.2) x 10^5), B (2.3 (0.0) x 10^5), C (3.7 (1.4) x 10^5) to D (3.9 (0.1) x 10^5). These results demonstrate that the major *Frankia* population present at all sites is also the one producing the root nodules. It is unknown, however, whether differences in *Frankia* cell densities in root nodules between sites are of any ecological significance, or might have been result of small samples size (n = 3 per site) or the sample preparation (i.e. the removal of the epidermis and potentially adhering material containing frankiae).

The basic observations on the establishment of specific frankiae in soils and root nodules warrants further studies on potential effects of environmental conditions such as organic matter content and matric potential on the fate of specific populations in soil and their interaction with host and non-host plant species. Our *q*PCR-based quantification approach provides the tool to address these questions and follow the dynamics of specific *Frankia* populations, with the 23S rRNA gene being an adequate target for both *q*PCR-based quantifications of all nitrogen-fixing frankiae as well as for specific subgroups of *Frankia*. Future developments, however, should consider the design and evaluation of a *q*PCR-based quantification approach for non-nitrogen fixing frankiae, and the inclusion of additional subgroups that were not covered in this study.

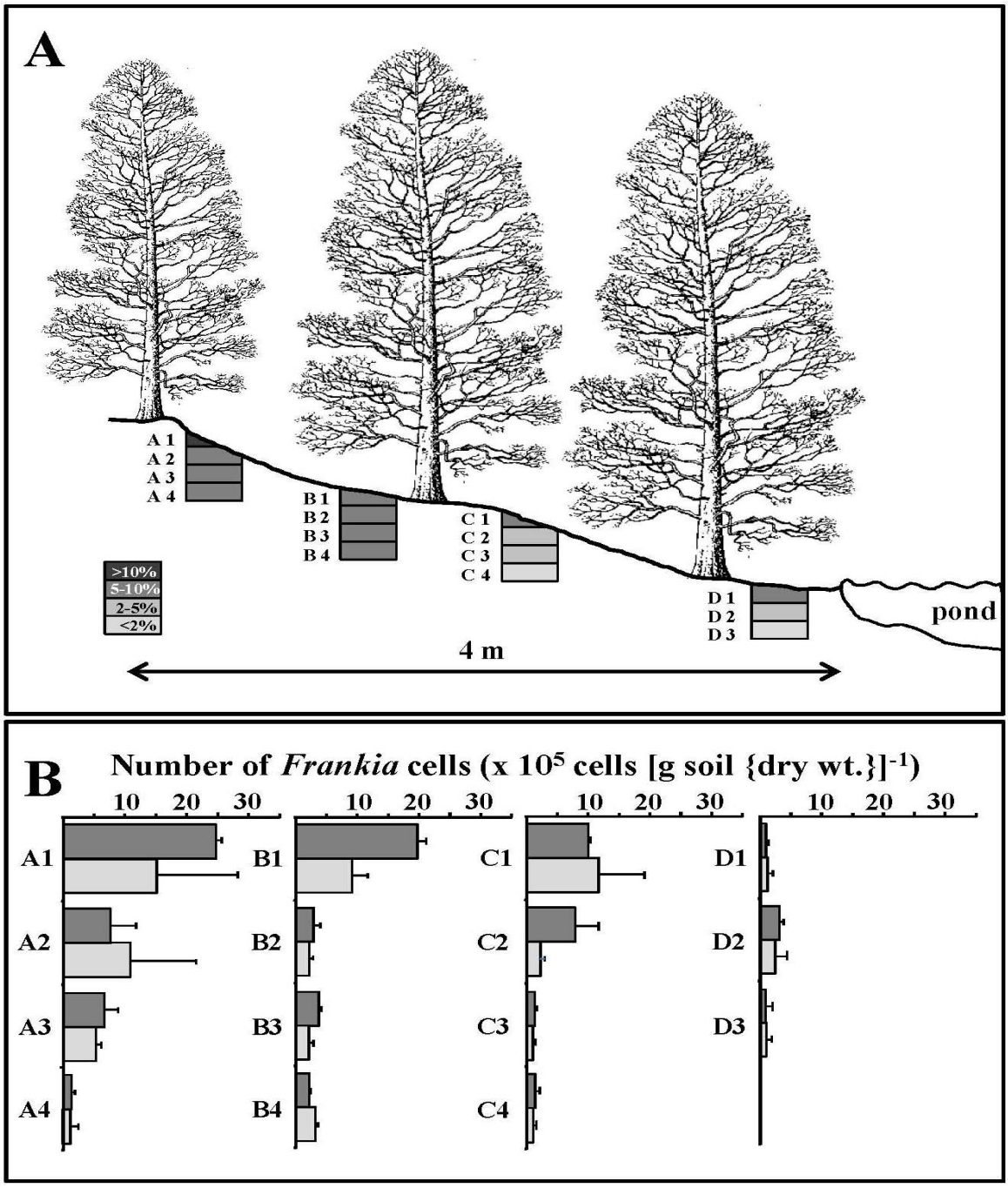

Fig. 2. Schematic presentation of the sampling site, an *Alnus glutinosa* stand near Boostedt, Schleswig-Holstein, Germany (53.9963N, 10.0257E) (A). Using a Purkhauer auger system (2 cm diameter), soil samples were taken from 4 locations (A-D) from the upper 20 cm in 5 cm intervals (1-4) on June 21, 2010. All samples from location D were water-saturated, while those from locations A-C were not. The lower panel (B) shows the corresponding analyses of *Frankia* populations in depth profiles at these locations, with the dark bars representing all nitrogen-fixing frankiae, and the light bars those represented by *Frankia* strain Ag45/Mut15 (subgroup II of the *Alnus* host infections group)

Table 3. Primer combinations targeting *nif*H or 23S rRNA gene sequences representing nitrogen-fixing members of the genus *Frankia* or subgroups within the genus

Target group	Primer combination (5' → 3')	Anneal Temperature (°C)	Fragment size (bp)	Reference
Target gene: *nif*H				
Nitrogen-fixing *Frankia* strains (clusters 1, and 3)	*nif*Hf1 ($^{5'}$GGC AAG TCC ACC ACC CAG C) *nif*Hr158 ($^{5'}$GAC GCA CTT GAT GCC CCA)	64	191	(1)
Target gene: 23S rRNA				
Nitrogen-fixing *Frankia* strains (clusters 1, 2, and 3)	23Fra1655f ($^{5'}$ CTG GTA GTA GGC AAG CGA TGG) 23Fra1769r ($^{5'}$ GGC TCG GCA TCA GGT CTC AG)	64	133	This study
Alnus host infection group (cluster 1)				
Subgroup I (ArI3)/*Casuarina*	23Ar/Cas1579f ($^{5'}$GTT GTG CTA ACC ATC TGA TCG GAT) 23Fra1769r ($^{5'}$ GGC TCG GCA TCA GGT CTC AG)	66	209	This study
Subgroup II (Ag45/Mut15)	23Mut1555f ($^{5'}$TTG ATG CGT CCA TGC TGA GG) 23Fra1769r ($^{5'}$ GGC TCG GCA TCA GGT CTC AG)	66	233	This study
Casuarina-infective strains	23Cas1610f ($^{5'}$TGTCTC TTC GGA GGT GTG TTC G) 23Fra1769r ($^{5'}$ GGC TCG GCA TCA GGT CTC AG)	66	178	This study
Elaeagnus host infection group (cluster 3)	23EAN1579f ($^{5'}$GTT TGT GCT AAC CGT TCT GGT) 23Fra1769r ($^{5'}$ GGC TCG GCA TCA GGT CTC AG)	64	209	This study

Table 4. *q*PCR-based quantification of *Frankia* strains inoculated into soil using primer combinations targeting *nif*H or 23S rRNA gene sequences representing nitrogen-fixing members of the genus *Frankia* or subgroups within the genus

Target group	Number of *Frankia* cells ($x10^5$ [g soil {dry wt.}]$^{-1}$) inoculated into soil					
	Ag45/Mut15	ArI3	$EAN1_{pec}$	CcI3	Mix	None
Target gene: *nif*H						
Nitrogen-fixing *Frankia* strains (clusters 1, and 3)	15.2 (1.3)	6.2 (0.7)	8.7 (1.4)	21.2 (0.6)	46.6 (5.0)	0
Target gene: 23S rRNA						
Nitrogen-fixing *Frankia* strains (clusters 1, 2, and 3)	11.5 (5.6)	7.1 (0.6)	6.1 (0.6)	14.4 (1.2)	39.9 (1.0)	0
Alnus host infection group (cluster 1)						
Subgroup I (ArI3)/*Casuarina*	0	6.9 (0.0)	0	13.6 (1.2)	20.7 (3.7)	0
Subgroup II (Ag45/Mut15)	20.2 (3.0)	0	0	0	26.3 (0.1)	0
Casuarina-infective strains	0	0	0	12.4 (1.5)	12.8 (0.3)	0
Elaeagnus host infection group (cluster 3)	0	0	5.5 (0.4)	0	5.8 (0.5)	0

Table 5. *q*PCR-based quantification of nitrogen-fixing members of the genus *Frankia* in soil samples targeting *nif*H or 23S rRNA gene sequences

Soil sample	DNA concentration		Extraction	Number of *Frankia* cells (x 10^5)	
	µg g^{-1} soil	(ng in PCR)	efficiency (%)	*nif*H gene	23S rRNA gene
A1	7.0 (2.0)	17.5 (4.9)	4.5 (0.7)	24.8 (10.6)	24.0 (0.6)
A2	5.0 (1.4)	12.6 (3.6)	5.4 (1.1)	7.1 (0.4)	7.8 (4.5)
A3	5.8 (0.2)	14.6 (0.6)	15.0 (6.5)	6.9 (1.8)	6.9 (1.8)
A4	6.4 (0.8)	16.1 (2.0)	31.3 (1.6)	1.0 (0.3)	1.4 (0.3)
B1	18.9 (11.3)	47.1 (28.1)	13.0 (10.8)	16.4 (1.1)	19.8 (0.1)
B2	17.8 (8.7)	44.4 (21.6)	18.1 (7.3)	2.3 (0.8)	2.9 (0.5)
B3	13.1 (2.1)	32.7 (5.3)	21.8 (4.2)	3.8 (0.2)	3.4 (0.9)
B4	9.3 (0.1)	23.3 (0.1)	11.1 (7.1)	1.0 (0.2)	1.9 (0.0)
C1	10.6 (3.4)	26.4 (8.5)	22.4 (20.7)	12.2 (2.1)	10.1 (0.2)
C2	10.7 (10.6)	26.7 (26.5)	9.1 (6.9)	8.0 (6.2)	8.0 (3.3)
C3	12.2 (2.2)	30.5 (5.6)	13.1 (9.3)	1.4 (0.5)	1.1 (0.1)
C4	9.4 (0.2)	23.3 (0.6)	39.4 (1.3)	1.7 (0.6)	1.4 (0.9)
D1	3.4 (0.4)	8.6 (0.9)	22.0 (9.5)	1.2 (0.8)	1.2 (0.0)
D2	4.0 (0.1)	10.0 (0.3)	17.6 (7.1)	2.5 (0.7)	3.0 (0.9)
D3	4.0 (0.0)	10.0 (0.0)	35.0 (1.9)	1.0 (0.0)	1.0 (0.8)

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CHAPTER IV

ABUNDANCE AND DIVERSITY OF *FRANKIA* UNDER ACTINORHIZAL *ALNUS GLUTINOSA* AND NON-ACTINORHIZAL *BETULA NIGRA* TREES

Abstract

Quantitative polymerase chain reaction (*q*PCR) was used to assess the abundance and diversity of the root-nodule forming, nitrogen-fixing actinomycete *Frankia* in four soils with similar physicochemical characteristics, two of which were vegetated with a host plant, *Alnus glutinosa,* and two with a non-host plant, *Betula nigra*. Analyses of DAPI-stained cells at three locations, i.e. at a distance of less than 1 m (near stem), 2.5 m (middle crown) and 3-5 m (crown edge) from the stems of both tree species revealed no statistically significant differences in abundance. Frankiae generally accounted for 0.01 to 0.04% of these cells, with values between 4 and 36 x 10^5 cells (g soil)$^{-1}$. In three out of four soils, abundance of frankiae was significantly higher at locations "near stem" and/or "middle crown" compared to "crown edge", while numbers at these locations were not different in the fourth soil. Frankiae of the *Alnus* host infection group were dominant in all samples accounting for about 75% and more of the cells, with no obvious differences with distance to stem. In three of the soils, all of these cells were represented by strain Ag45/Mut15. In the fourth soil that was vegetated with older *A. glutinosa* trees, about half of these cells belonged to a different subgroup represented by strain ArI3. In all soils, the remaining cells belonged to the *Elaeagnus* host infection group represented by strain EAN1pec. *Casuarina*-infective frankiae were not found. Abundance and diversity of *Frankia* were similar in soils under the host plant *A. glutinosa* and the non-host plant *B. nigra*. Results did thus not reveal any specific effects of plant species on soil *Frankia*

populations shown to have differing nodulation capacities for *Alnus* in past studies.

Key words: alder, birch, *q*PCR, quantification, saprotrophic, soil

Introduction

Actinorhizal shrubs and trees represent a group of plant species capable of forming root nodules in symbiosis with nitrogen-fixing soil actinomycetes of the genus *Frankia* [1-3]. Actinorhizal plants are therefore found often on sandy and wet soils where low levels of available nitrogen may preclude the growth of other species, and thus usually occur as pioneer vegetation at early stages of plant secession [4]. The formation of root nodules is host plant-specific, with frankiae of clusters 1, 2, and 3 representing nitrogen-fixing frankiae of the *Alnus*, *Dryas* and *Elaeagnus* host infection groups [5], and those of cluster 4 representing non-nitrogen-fixing strains [6].

Frankia strains of the *Alnus* and *Elaeagnus* host infection groups have been shown to grow saprotrophically in the rhizosphere of both host and non-host plants, likely using root exudates that are easily available carbon (C) resources [7-10]. In the absence of plants, amendment of soils with slowly available C resources such as leaf litter affected the nodulation capacity of different *Frankia* strains [11] and allowed growth of a small number of strains only, all of which belonged to a distinct phylogenetic cluster within the *Alnus* host infection group [9, 10]. *Casuarina*-infective strains, another distinct phylogenetic cluster within the *Alnus* host infection group [12, 13], also grew saprotrophically, however, only in the rhizosphere of their host plant [10] or with leaf litter of *Casuarina* sp. [10, 14]. These results demonstrated that saprotrophic growth of frankiae is a common trait for the genus, but that the supporting factors for growth (i.e. C

utilization capabilities) varied with host infection group and phylogenetic affiliation of the strains.

Plant bioassays in which a quantification of the nodulation capacity on a specific host plant was used to describe the infective *Frankia* population in soils have demonstrated differential effects of vegetation on nodulation capacities. Soils vegetated with birch (*Betula sp.*), for example, were found to often [7, 15, 16], though not always [17], produce more nodules in bioassays than soils vegetated with alders or other non-host plant species such as pine, spruce or larch. It is tempting to use these results to imply larger populations of frankiae in soils vegetated with birch, however, any correlation of nodulation units with cell numbers is highly biased because a nodule can theoretically be induced by a single spore, a hyphal fragment, or a colony [18]. We recently developed a SYBR Green-based quantitative PCR (*q*PCR) that used either *nif*H or 23S rRNA gene sequences as target in DNA extracts from soil samples that allowed us to quantify nitrogen-fixing members of the genus *Frankia* directly in soil samples [19, 20]. Quantification results in different mineral soils from temperate regions using both targets were comparable, with cell density estimates for frankiae of up to more than 10^6 cells [g soil {dry wt.}]$^{-1}$ depending on the soil [19, 20]. In contrast to the *nif*H gene, the 23S rRNA gene also provided target sequences that allowed us to distinguish between the *Alnus* and *Elaeagnus* host infection groups, and subgroups within the *Alnus* host infection group, including the *Casuarina*-infective strains [19].

In this study, we have optimized primer combinations to better distinguish subgroups within the *Alnus* host infection group, and used them together with the

developed *q*PCR methods to investigate potential effects of the host plant *Alnus glutinosa* and the non-host plant *Betula nigra* on the abundance and diversity of *Frankia* populations in soil. These studies used four field sites with similar soil properties. Two of these sites were planted with European alder (*A. glutinosa*) and two others with River birch (*B. nigra*). Soil samples were taken at three distances from the stems of these plants (crown edge, middle crown and near stem) to assess potential effects of subcanopy location, hence root and litter influences along a gradient from main stem to canopy edge.

Materials and Methods

Primer design and evaluation Using the same alignment as in our previous study with target and non-target organisms [19], two new primers were designed, a specific forward primer for the subgroup represented by strain ArI3 (23Ar1607f), and a reverse primer targeting all frankiae (23Fra1715r). In addition, a previously designed primer was modified (23Cas1607f). Selected primers were checked for low potential of self- and hetero-dimer formation using OligoAnalyzer 3.1 (www.idtdna.com/analyzer/Applications/OligoAnalyzer) and for target specificity using TestPrime 1.0 [21] from the SILVA rRNA database project (www.arb-silva.de, accessed 06/04/2014) [22]. Annealing temperatures for all primer combinations were tested in *q*PCRs with DNA of representative *Frankia* strains of the *Elaeagnus* and *Alnus* host infection groups and of *Casuarina*-infective strains, and quantifications compared to results with established primers (i.e. reverse primer 23Fra1769r, and forward primers 23Ar/Cas1579f and 23Cas1610f) [19].

Sampling Soil samples were obtained from four sites with similar soil properties but planted with either European alder (*Alnus glutinosa*) (soils ABA and BAHF) or River birch (*Betula nigra*) (soils LWRB and RBW) (Table 6). Soils ABA, BAHF, and RBW formed under tallgrass prairie on post-glacial loess deposited on loamy Wisconsinan outwash plains, while soil LWRB formed under deciduous forest on coarse Wisconsinan outwash plains, all about 23,000 years before present. The planted tree species are not native to the study sites and the sites are at least 200 km from the nearest native *Alnus* stands. At each site, soil samples of about 1 kg were taken from the upper 10 cm from 3 trees at 3 distances from the stem. "Crown edge" samples were collected about 3-5 m from the stem, "middle crown" samples about 1.5-2.5 m from the stem, and "near stem" samples less than 1 m from the stem. All locations had little ground cover beneath tree canopies reducing potential impacts of grasses and other vegetation underneath the canopy on sampling. Samples were stored at 4°C until further processing.

Microbial analysis DNA was extracted from 250 mg soil samples (dry wt.) using the SurePrep^{TM} Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) with small modifications as described before [20]. DNA was extracted from triplicate samples, with 10-fold dilutions used as template in Sybr Green-based quantitative PCR (*q*PCR) analyses for nitrogen-fixing members of the genus *Frankia* using either *nif*H gene sequences [20] or 23S rRNA gene sequences [19] as target. More specific analyses focused on three subgroups of the *Alnus* host infection group (cluster 1), or the *Elaeagnus* host infection group (cluster 3) [19], that were represented by *Frankia* strains Ag45/Mut15, ArI3, CcI3 and EAN1pec, respectively (Table 7), again using 10-fold dilutions.

*q*PCR quantification of frankiae was performed for each sample in triplicate in a total volume of 10 µl containing 5 µl of SsoADV SybrGreen Mix (BioRad, Hercules, CA), 0.2 µl of each primer (100 nM each) and 1 µl of template in an Eco Real-time PCR system (Illumina, San Diego, CA) using an initial denaturation at 95°C for 5 minutes, and 40 cycles of denaturation at 95°C, annealing at 64 or 66°C depending on the primer combination (Table 7), and extension at 72°C, each for 30 seconds, as in our previous studies [19, 20]. The amplification was followed by a melting curve analysis. Quantification was based on standard curves generated from purified PCR products of *nif*H or 23S rRNA genes of strains Ag45/Mut15, ArI3, CcI3 and EAN1pec depending on the primer combination. Amplicons were generated using the genus-specific primers, and concentrations measured with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA). Copy numbers were calculated from concentrations (http://www.uri.edu/research/gsc/resources/cndna.html) and normalized after *q*PCR quantification with the primer combination targeting all nitrogen-fixing frankiae. Copy numbers were divided by copy numbers of the 23S rRNA gene per genome (2 or 3 for pure cultures, 2.5 for unknown populations) to relate copy numbers to *Frankia* cell numbers [19]. Results of all analyses were corrected for extraction efficiencies. These were determined as the ratio of inoculated *Salmonella* Typhimurium (ATCC14028) cells detected by *q*PCR-based quantification of a 268-bp *inv*A gene fragment before and after extraction as described previously [20].

These data were contrasted to numbers of all organisms quantified by epifluorescence microscopy (Eclipse 80i; Nikon, Lewisville, TX) after DAPI staining [23].

Statistical Analysis One way ANOVA and pairwise multiple comparison procedures (Holm-Sidak method) were used in SigmaPlot 13.0 (Systat Software Inc., San Jose, USA) was used to assess the effects of location (near stem, middle crown and crown edge) on abundance of DAPI-stained cells and frankiae, with a significance level $P<0.05$.

Results

Primer design and evaluation TestPrime 1.0 analyses revealed that all primers and primer combinations retrieved their respective target sequences available in the SILVA databases with no mismatches. The new forward primer 23Ar1607f was specific for the target (i.e. *Frankia* strains ArI3 and ACN14a), however, with 1 or 2 mismatches to sequences of non-target organisms (*Pseudocorynosoma* and *Corynesoma* sp.). The modified primer 23Cas1607f targeting strain CcI3 was also specific, with 4 mismatches to the sequence of a non-target organism (*Frankia* strain ACN14a). The reverse primer 23Fra1715r perfectly matched all *Frankia* sequences in the database, however, revealed 1 mismatch to sequences from uncultured organisms of marine or terrestrial origin, and 2 mismatches to many other actinomycetes (e.g. many *Streptomyces* sp., *Kitasatospora*). The combination of forward and reverse primers, however, was highly specific for the target organisms, with non-target sequences remaining undetected at the program search limit of 5 mismatches.

Annealing temperatures for all primer combinations were in the same range as those of our established primers (i.e. reverse primer 23Fra1769r, and forward primers 23Ar/Cas1579f and 23Cas1610f) with 64-66°C, allowing specific detection of representative *Frankia* strains of the *Elaeagnus* and *Alnus* host infection groups and of

Casuarina-infective strains by *q*PCR. The new forward primer 23Ar1607f instead of 23Ar/Cas1579f now also allowed us to distinguish *Alnus* host infection subgroup 1 represented by strain ArI3 from *Casuarina*-infective strains (i.e. CcI3).

Microbial analyses Abundance of DAPI-stained cells ranged from 4 to 14 x 10^9 cells (g soil)$^{-1}$, with up to twice as many cells at locations "middle crown" and/or "near stem" compared to "crown edge" (Table 8). Differences, however, were not statistically significant for any of the sites [ABA ($P = 0.149$), BAHF ($P = 0.338$), LWRB ($P = 0.101$), RBW ($P = 0.822$)].

Values of *q*PCR analyses of abundance of all nitrogen-fixing members of the genus *Frankia* were comparable independent of the use of *nif*H or 23S rRNA gene sequences as target (data not shown), and ranged between 4 and 36 x 10^5 cells (g soil)$^{-1}$ (Table 8). In 3 soils (ABA, BAHF and RBW) abundance varied with distance to stems, with values for frankiae at locations "middle crown" and/or "near stem" being significantly higher compared to "crown edge" [ABA ($P = 0.031$), BAHF ($P < 0.001$), RBW ($P < 0.001$)] (Table 8). These differences were not obtained for soil LWRB ($P = 0.324$). Frankiae account for 0.01 to 0.04% of the DAPI-stained cells, with generally higher percentages closer to the stem in soils ABA and BAHF, but not in soils LWRB and RBW.

Frankiae of the *Alnus* host infection group were dominant in all samples accounting for about 75% and more of the cells, with no obvious differences with distance to stem (Table 8, Fig. 3.). In three of the soils (i.e. BAHF, LWRB and RBW), all of these cells were represented by strain Ag45/Mut15 (subgroup II). In soil ABA that was

vegetated with *A. glutinosa*, about half of these cells belonged to a different subgroup represented by strain ArI3 (subgroup I). In all soils, the remaining cells belonged to the *Elaeagnus* host infection group represented by strain EAN1pec. *Casuarina*-infective frankiae were not found.

Discussion

Soils are highly heterogeneous environments that can support a large microbial community with often more than 10^9 cells (g soil)$^{-1}$ [24, 25]. Enumeration of DAPI-stained cells in our soils resulted in numbers at a similar range (i.e. 4 to 14 x 10^9 cells (g soil)$^{-1}$), with higher numbers usually found at locations closer to the tree stems (i.e. near stem and middle crown). These numbers, however, were not significantly different from those further away (i.e. crown edge), indicating no or only limited effects of tree roots or their leaf litter on microbial abundance. Microbial abundance is therefore most likely a function of the physicochemical characteristics and the high organic matter content of all soils sampled. Soils in this study are high in organic matter which sustains productivity for corn and soybeans [26].

Estimates for the abundance of frankiae in soil range between approx. 10^4 and 10^6 cells (g soil)$^{-1}$ depending on the method of detection [18, 20, 27, 28]. Plant bioassays usually provide data at the lower end of the range (i.e. nodulation units up to 4,600 units (g soil)$^{-1}$) [16, 29, 30], since they are highly selective, i.e. only nodule forming populations on a specific host plant are detected, and potentially biased because nodulation units can theoretically be represented by a single spore, a hyphal fragment, or a colony [18]. Plant bioassays with *A. glutinosa* as capture plant had been used previously

with one of the soils used in our study (i.e. LWRB) to assess potential effects of river birch, prairie grasses and other vegetation on nodule forming capacity [15]. Compared to other vegetation, river birch seemed to increase nodulation capacity even though nodulation units obtained were still low (i.e. 228 units (g soil)$^{-1}$) [15].

Compared to plant bioassays, molecular analyses such as end-point PCR detected numbers about one order of magnitude higher (e.g. 92,000 genomic units g^{-1} soil) [29], while *q*PCR analyses revealed cell density estimates for frankiae of up to 10^6 cells (g soil)$^{-1}$ [19, 20]. Numbers ranged from 10^5 to 10^6 cells (g soil)$^{-1}$ depending on the sampling depth, physicochemical conditions and the vegetation. Our current analyses that ranged from 4 to 36 x 10^5 cells (g soil)$^{-1}$ fit well into this range, with values for abundance of *Frankia* being significantly higher closer to the tree stems compared to crown edge samples at 3 out of 4 sites. While this indicates a possible effect of the trees on *Frankia* abundance, the lack of significant differences in abundance of *Frankia* between sites vegetated with *A. glutinosa* or *B. nigra* does not support the assumption of tree-specific effects on abundance. Since differences in abundance of frankiae in soils vegetated with either *A. glutinosa* or *B. nigra* are small, and numbers comparable to those in soils vegetated with other host and non-host plants such as grasses [19], abundance of frankiae in soils is apparently not the driving factor in the higher nodule forming capacity observed for soils vegetated with river birch and other birch species [7, 15, 16].

Soil characteristics were meant to be very similar between sites, thus allowing for the isolation of tree species as the most prominent variable. However, soils were not identical and thus small differences could have affected abundance estimates of frankiae.

Frankia abundance was shown to be lower in depth profiles related to lower organic matter content, and also to increasing water content, with water-logged soils supporting about 10-fold lower *Frankia* populations than adjacent soils (i.e. 10^5 versus 10^6 cells (g soil)$^{-1}$) [19]. Ground cover was low under tree canopies at all sites, however, not absent. *Frankia* has been shown to grow in the rhizosphere of grasses [8], and analyses of abundance in soils vegetated with grasses provided similar densities as in soils vegetated with *A. glutinosa*, with numbers of about 10^6 cells (g soil)$^{-1}$ [20]. Thus, small differences in physicochemical conditions or changes in vegetation might have large effects on abundance of *Frankia*.

Our *q*PCR procedure allowed us to analyze 4 subgroups within the genus *Frankia*, with cell density estimates for the sum of all specific groups accounting for cell density of all *Frankia* (Table 3). In our previous analyses only one group of frankiae, i.e. subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15, was detected by *q*PCR in both nodules on *A. glutinosa* and in soils, though with numbers in soils that corresponded largely to those retrieved for nitrogen-fixing frankiae [19]. This subgroup was dominant apparently independent of environmental characteristics such as organic matter content and matric potential that had been shown to affect the development of specific *Frankia* populations [9-11, 31]. Our current analyses detected three of our four target subgroups within the genus demonstrating the potential of our *q*PCR based quantification procedure for population analyses in soils. The fourth target group, i.e. the *Casuarina*-infective frankiae, was not expected to be present in these soils because they are not able to grow in the climate of the study region and their presence seems to be dependent on co-introduction with their exotic host plant species [32, 33].

Again, subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15 was most prominent in all soils and at all locations accounting for 75% and more of the cells of frankiae (Fig. 1). Pure cultures of this group had been shown to grow with organic matter such as leaf litter in addition to root exudates [10], and thus this group might have a growth advantage over others. Leaf litter amendments to soils, for example, resulted in shifts of nodule-forming *Frankia* populations from subgroup I represented by strain ArI3 to subgroup II, both for introduced and indigenous populations [11]. Pure cultures representing subgroup I or the *Elaeagnus* host infection group like strain EAN1pec did not grow with leaf litter but in the rhizosphere of host plants [10] and potentially in that of other plants [8]. Subgroup I of the *Alnus* host infection group represented by strain ArI3 could only be detected in one soil, and here locations affected by trees of *A. glutinosa* (i.e. soil ABA locations near stem and middle crown). At this location *A. glutinosa* trees were double the stem diameter at 1.4 m above the root collar (55 cm) and at least double the age (65 y) of trees planted at other locations. This older stand of alder trees would have had a higher probability of colonization by the less saprophytic and locally less common subgroup I *Frankia* strains, perhaps due to dispersal by migratory birds [34]. While it is tempting to assume host plant effects on the development of this population, the absence of this population in soil BAHF characterized by similar physicochemical conditions and vegetated with *A. glutinosa* does not support these speculations. Members of the *Elaeagnus* host infection group were present in all soils and at all locations, similar to subgroup II of the *Alnus* host infection group though with highly variable abundance patterns. These results do not allow us to resolve potential effects of the host plant *Alnus glutinosa* and the non-host plant *Betula*

nigra on the abundance and diversity of *Frankia* populations in soil.

Future studies need to take advantage of the *q*PCR tools developed for the quantification of specific *Frankia* populations in soils in order to determine the variables that affect growth and abundance of specific populations. These include microcosm studies in which the development of both indigenous and inoculated *Frankia* populations in bulk and rhizosphere soil can be monitored over time as a function of plant species and organic matter supply. While our data suggest that our *q*PCR procedure retrieves data from the major populations of *Frankia* present in soils, additional assessments on overall diversity using next generation sequencing methods will be required to confirm these assumptions.

Legends

Fig. 3. Distribution (%) of subgroups of the genus *Frankia* in soils ABA and BAHF vegetated with *Alnus glutinosa*, and LWRB and RBW vegetated with *Betula nigra* at three distances from the stems of three plants each (crown edge, middle crown and near stem). Two subgroups are represented by strains of the *Alnus* host infection group, Ag45/Mut15 and ArI3, respectively, and a third by one strain of the *Elaeagnus* host infection group, EAN1pec. *Casuarina*-infective frankiae, represented by strain CcI3 were not detected.

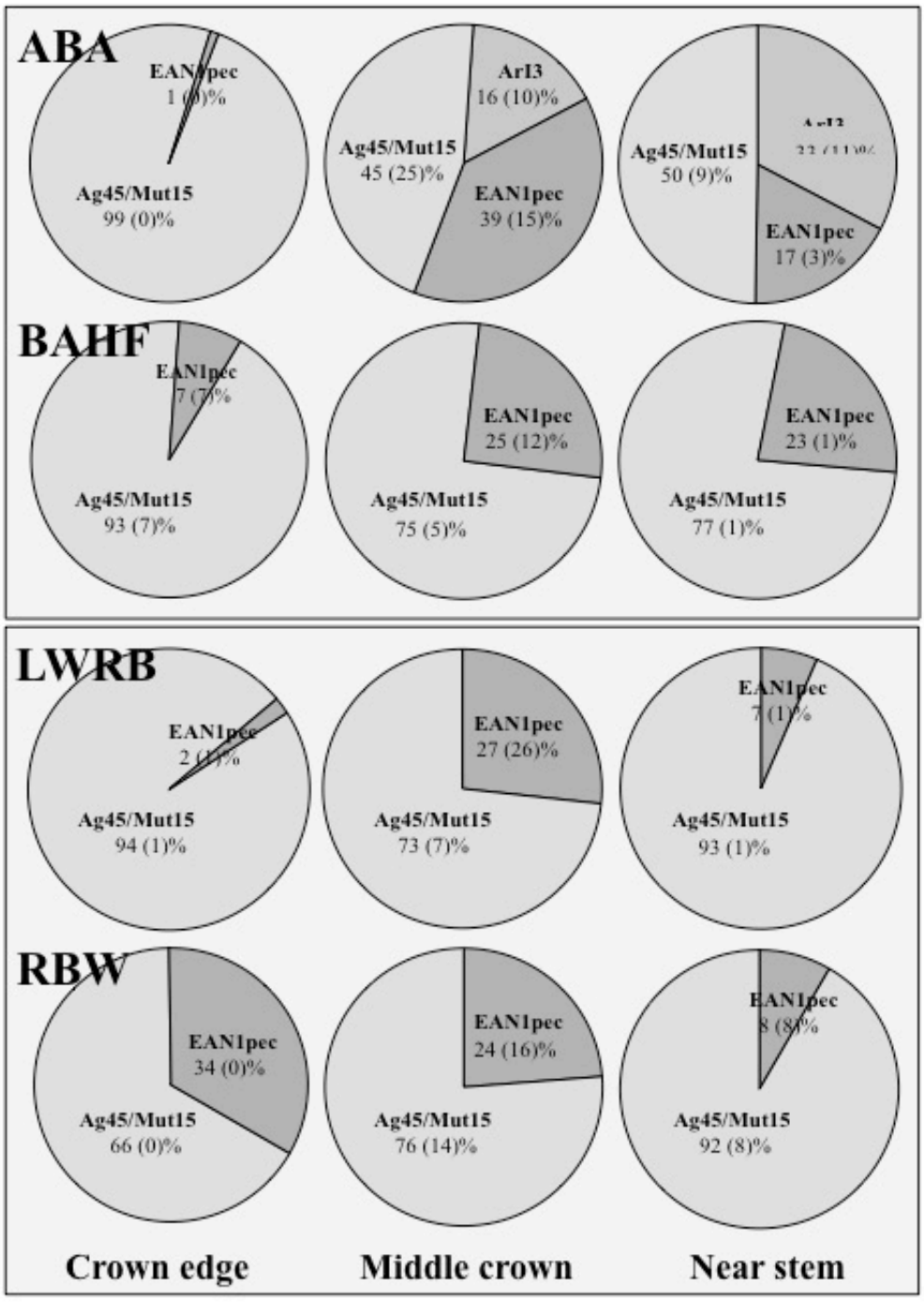
ABA
EAN1pec
1 (0)%
Ag45/Mut15
99 (0)%
Ag45/Mut15
45 (25)%
ArI3
16 (10)%
EAN1pec
39 (15)%
Ag45/Mut15
50 (9)%
EAN1pec
17 (3)%
BAHF
EAN1pec
7 (7)%
Ag45/Mut15
93 (7)%
EAN1pec
25 (12)%
Ag45/Mut15
75 (5)%
EAN1pec
23 (1)%
Ag45/Mut15
77 (1)%
LWRB
EAN1pec
2 (1)%
Ag45/Mut15
94 (1)%
EAN1pec
27 (26)%
Ag45/Mut15
73 (7)%
EAN1pec
7 (1)%
Ag45/Mut15
93 (1)%
RBW
EAN1pec
34 (0)%
Ag45/Mut15
66 (0)%
EAN1pec
24 (16)%
Ag45/Mut15
76 (14)%
EAN1pec
8 (8)%
Ag45/Mut15
92 (8)%
Crown edge
Middle crown
Near stem

Table 6. Location and characteristics of sampling sites ABA and BAHF vegetated with European alder, and RBW and LWRB vegetated with River birch

Acronym	Site (GPS location)	USDA soil series*	Soil taxonomic classification*	Soil pH**	Org. matter (%)***	Soil internal drainage*	Original vegetation*
European alder (*Alnus glutinosa*)							
ABA	Arboretum University of Illinois (40.093585 N, 88.218016°W)	Flanagan Silt Loam	Fine, smectic, mesic Aquic Argiudoll	6.8	4.5	Slow	Tallgrass prairie
BAHF	Horticulture Farm University of Illinois (40.079306 N, 88.190558°W)	Catlin Silt Loam	Fine-silty, mixed, superactive, mesic Oxyaquic Argiudoll	6.9	3.5	Medium	Tallgrass prairie
River birch (*Betula nigra*)							
LWRB	Lake of the Woods Park (40.203501 N, 88.387924°W)	Martinsville Silt Loam	Fine-loamy, mixed, active, mesic Typic Hapludalf	6.6	2.5	Well drained	Deciduous forest
RBW	Illinois State Water Survey Campus (40.083917 N, 88.242038 W)	Drummer Silty Clay Loam mixed with calcareous subsoil	Fine-silty, mixed, superactive, mesic Typic Endoaquoll	7.1	3.0	Slow	Tallgrass prairie

*https://soilseries.sc.egov.usda.gov/OSD_Docs

**from bulked sample

***combustion at 700 deg. C

Table 7. Primer combinations targeting *nif*H or 23S rRNA gene sequences representing nitrogen-fixing members of the genus *Frankia* or subgroups within the genus

Target group	Primer combination (5' → 3')	Anneal Temperature (°C)	Fragment size (bp)	Reference
Target gene: *nif*H				
Nitrogen-fixing *Frankia* strains (clusters 1, and 3)	*nif*Hf1 ($^{5'}$GGC AAG TCC ACC ACC CAG C) *nif*Hr158 ($^{5'}$GAC GCA CTT GAT GCC CCA)	64	191	(3)
Target gene: 23S rRNA				
Nitrogen-fixing *Frankia* strains (clusters 1, 2, and 3)	23Fra1655f ($^{5'}$CTG GTA GTA GGC AAG CGA TGG) 23Fra1769r ($^{5'}$GGC TCG GCA TCA GGT CTC AG)	64	133	(2)
***Alnus* host infection group (cluster 1)**				
Subgroup I (ArI3)	23Ar1607f ($^{5'}$GTG TCT TTT CGG AGA TGT GTC T) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	64	128	This study
Subgroup II (Ag45/Mut15)	23Mut1555f ($^{5'}$TTG ATG CGT CCA TGC TGA GG) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	66	170	(2)
***Casuarina*-infective strains**	23Cas1607f ($^{5'}$GTG TCT CTT CGG AGG TGT GTT C) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	66	128	(2)
***Elaeagnus* host infection group (cluster 3)**				
	23EAN1579f ($^{5'}$GTT TGT GCT AAC CGT TCT GGT) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	64	146	(2)

Table 8. *q*PCR-based quantification of members of the genus *Frankia* in three locations (crown edge, middle crown, near stem) of four soils vegetated with either *A. glutinosa* (ABA and BAHF) or *B. nigra* (LWRB and RBW) (cell numbers x 10^5) (mean [± standard error])

Soil	**ABA**			**BAHF**		
	Crown edge	Middle crown	Near stem	Crown edge	Middle crown	Near stem
DAPI	41226 (10050)	69778 (12063)	77202 (16675)	49612 (11075)	77244 (12418)	69220 (16024)
Frankia	4 (1)	12 (1)	27 (10)	4 (0)	10 (0)	12 (1)
	4 (0)	6 (3)	14 (2)	4 (1)	6 (0)	8 (2)
Ag45/Mut15						
ArI3	0	2 (1)	9 (4)	0	0	0
EAN1pec	0 (0)	5 (1)	5 (1)	1 (0)	3 (1)	4 (0)
CcI3	0	0	0	0	0	0
Soil	**LWRB**			**RBW**		
	Crown edge	Middle crown	Near stem	Crown edge	Middle crown	Near stem
DAPI	62800 (21226)	128866 (29766)	139136 (26513)	100480 (19843)	89930 (18992)	113794 (37631)
Frankia	15 (3)	18 (1)	14 (1)	12 (1)	36 (2)	10 (1)
	15 (1)	12 (1)	10 (1)	7 (0)	26 (9)	8 (4)
Ag45/Mut15						
ArI3	0	0	0	0	0	0
EAN1pec	1 (0)	5 (1)	7 (0)	3 (0)	8 (3)	1 (0)
CcI3	0	0	0	0	0	0

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CHAPTER V

EFFECT OF *ALNUS* AND *CASUARINA* PLANTS ON ABUNDANCE AND DIVERSITY OF INDIGENOUS *FRANKIA* POPULATIONS IN SOIL

Abstract

Quantitative PCR (*q*PCR) was used to follow population dynamics of indigenous *Frankia* populations in bulk soil and the rhizosphere of *Alnus glutinosa* or *Casuarina equisetifolia* at 2 matric potentials representing dry and wet conditions in soil microcosms. Indigenous populations of *Frankia* in bulk soil that was originally vegetated with *Elaeagnus umbellata* and had been stored at 4°C for about half a year, increased between 10- and 100-fold within the incubation period of 12 weeks, with usually higher numbers obtained under dry conditions. Abundance of *Frankia* in the rhizosphere and in bulk soil amended with leaf litter showed a similar pattern, though values for abundance were generally higher, with highest values obtained for *Frankia* in the rhizosphere of *C. equisetifolia*. More specific analyses revealed that all frankiae detected at any time and treatment belonged to either subgroup I of the *Alnus* host infection group or the *Elaeagnus* host infection group. In bulk soil, growth of frankiae representing the *Elaeagnus* host infection group was usually faster than that of frankiae of *Alnus* subgroup I, resulting in higher density increases (i.e. up to 100-fold) than those of *Alnus* subgroup I (10-fold) during the incubation time. This was different in the rhizosphere of both *Alnus* and *Casuarina* plants where effects of matric potential were obtained, with more than 100-fold increases of frankiae of *Alnus* subgroup I under dry conditions compared to bulk soil at t_0, and less than 10-fold increases under wet conditions. The opposite pattern was obtained for frankiae of the *Elaeagnus* host infection group. Consequently, under dry conditions the

genus *Frankia* in the rhizosphere was to a large extent (i.e. up to 95% depending on the plant species) represented by subgroup I of the *Alnus* host infection group, while under wet conditions a similar percentage of the genus in the rhizosphere of both plant species was represented by the *Elaeagnus* host infection group. Leaf litter amendment resulted in growth of frankiae of the *Elaeagnus* host infection group only, essentially matching the values obtained for genus-specific analyses.

Introduction

Members of the bacterial genus *Frankia* are generally characterized as nitrogen-fixing actinomycetes that form root nodules in symbiosis with a variety of woody plants [2,5]. While a considerable amount of information is available on nodule-forming populations, and the interaction of frankiae with host plant species [2,5], information on frankiae in soil, its second ecological niche, is relatively scarce [4].

Most of the information on soil *Frankia* populations focuses on their potential to form root nodules[12,13,23,24] and the effects of environmental characteristics on nodule-forming capacity [10,26]. Soil organic matter and matric potential, for example, have been shown to affect the development of specific *Frankia* populations [11,14,15,16], with organic matter such as leaf litter supporting growth of one specific population, i.e., subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15, only [11]. Leaf litter amendments to soils consequently resulted in shifts of nodule-forming *Frankia* populations from subgroup I to subgroup II, both for introduced and indigenous populations [15]. Effects of matric potential are less well established even though drier conditions seem to favor root nodule formation of frankiae representing

subgroup I over those of subgroup II [16]. While these effects focus on nodule-forming frankiae, their impact on population dynamics of frankiae in soil is still unknown.

The aim of this study was to take advantage of *q*PCR tools recently developed for the quantification of specific *Frankia* populations in soils [19-21] in order to determine the variables that affect growth and abundance of specific populations. These studies include microcosm setups in which the development of both indigenous and inoculated *Frankia* populations in bulk and rhizosphere soil could be monitored over time as a function of plant species and carbon resource supply.

Materials and Methods

Experimental setup Microcosms were established in 50-ml falcon tubes using 40 g of soil (dry wt.) per tube at a density of approximately 1 g cm^{-3}. The soil was a Xenia silt loam, a mesic Aquic Hapludalf with about 1% organic material from a natural stand of Autumn Olive (*Elaeagnus umbellata*) (PIATT, IL). Half of the microcosms were adjusted to and maintained at a matric potential of -0.001 MPa (hereafter referred to as "wet"), the other half at a matric potential of -0.005 MPa (hereafter referred to as "dry") using round-bottom ceramic suction tubes (8 cm long with an outer diameter of 6 mm [#0652X02-B01M1], Soilmoisture Equipment Corp., Santa Barbara, CA) and 10 or 50 cm water columns, respectively [16].

For studies on indigenous *Frankia* populations, half of the tubes with PIATT soil at each matric potential were planted with seedlings (n=3 per tube) of either *Alnus glutinosa* or *Casuarina equisetifolia.* Seeds of these plant species had been surface sterilized in 3% H_2O_2 for 10 min., been washed twice with sterilized distilled water for 10

min. each, and then been germinated on sterile water agar. Plantlets were transferred to soil in tubes 2 weeks after germination. The remaining half of tubes stayed non-vegetated, but the soil was mixed with 2% (wt/wt) leaf litter of either *A. glutinosa* or *C. equisetifolia* ground to a particle size of about 0.1 mm. This setup resulted in 4 treatments per plant species: treatment 1 (plants, dry), treatment 2 (plants, wet), treatment 3 (organic matter, dry), and treatment 4 (organic matter, wet).

All tubes were kept at 23°C with a photoperiod of 16/8 h (day/night, respectively) for 3 months. Microcosms were sampled destructively at 3 time steps, i.e. directly after planting (PIATT soil) (t_0), and 6 or 12 weeks after planting or inoculation (t_6 and t_{12}). For each soil, microcosms that were neither vegetated nor amended with leaf litter were used as controls.

Sampling and DNA extraction For each time step and treatment, 2 microcosms were analyzed. Soils in leaf litter-amended microcosms were mixed, and triplicate 250 mg soil samples used for DNA extraction. In vegetated microcosms, samples were separated into bulk and rhizosphere soil before DNA extraction. Rhizosphere soil was defined as the soil adhering to plant roots when the plants were being removed from the remaining soil (i.e. bulk soil). DNA was extracted from both bulk and rhizosphere samples in triplicate 250 mg soil samples using the SurePrep™ Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) with small modifications as described before [21].

***q*PCR analyses** Ten-fold dilutions of the DNA extracts were used as template in Sybr Green-based quantitative PCR (*q*PCR) analyses for nitrogen-fixing members of the genus *Frankia* using 23S rRNA gene sequences as target [19]. More specific analyses focused

on four subgroups of the genus, i.e. subgroups I and II of the *Alnus* host infection group (cluster 1), *Casuarina*-infective strains and members of the *Elaeagnus* host infection group (cluster 3), that were represented by *Frankia* strains ArI3, Ag45/Mut15, CcI3 and EAN1pec, respectively. *q*PCR quantification of frankiae was performed for each sample in triplicate in a total volume of 10 µl containing 5 µl of SsoADV SybrGreen Mix (BioRad, Hercules, CA), 0.2 µl of each primer (100 nM each) and 1 µl of template in an Eco Real-time PCR system (Illumina, San Diego, CA) using an initial denaturation at 95°C for 5 minutes, and 40 cycles of denaturation at 95°C, annealing at 64 or 66°C depending on the primer combination (Table 9), and extension at 72°C, each for 30 seconds [20]. The amplification was followed by a melting curve analysis. Quantification was based on standard curves generated from purified PCR products of 23S rRNA gene fragments of strains ArI3, Ag45/Mut15, CcI3 and EAN1pec depending on the primer combination. Amplicons were generated using genus-specific primers 23Fra1533f/23Fra1769r, and concentrations measured with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA). Copy numbers were calculated from concentrations (http://www.uri.edu/research/gsc/ resources/cndna.html). Copy numbers were divided by copy numbers of the 23S rRNA gene per genome (2 or 3 for pure cultures, 2.5 for unknown populations) to relate copy numbers to *Frankia* cell numbers [19]. Results of all analyses were corrected for extraction efficiencies. These were determined as the ratio of inoculated *Salmonella* Typhimurium (ATCC14028) cells detected by *q*PCR-based quantification of a 268-bp *inv*A gene fragment before and after extraction as described previously [21].

Statistical Analysis One way ANOVA and pairwise multiple comparison procedures (Holm-Sidak method) were used in SigmaPlot 13.0 (Systat Software Inc., San Jose, USA) to assess the effects of either host plants (*Alnus* and *Casuarina*) or it's organic matter and water potential on abundance of frankiae, with a significance level $P<0.05$.

Results

Microcosms with soil PIATT analyzed for *Frankia* at t_0, revealed *Frankia* cell densities of $3 \pm 1 \times 10^4$ cells $(g\ soil)^{-1}$ (Fig. 4, 5). These cells belonged to subgroup I of the *Alnus* host infection group represented by strain ArI3 ($1 \pm 0 \times 10^4$ cells $(g\ soil)^{-1}$), and the *Elaeagnus* host infection group represented by strain EAN1pec ($1 \pm 0 \times 10^4$ cells $(g\ soil)^{-1}$). Cells of subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15 and of *Casuarina*-infective strains represented by strain CcI3 were not detected at this time.

Incubation increased *Frankia* populations in bulk soil, independent of plant species or the addition of organic material, between 10- and 100-fold (i.e. 2.0 and 28.4×10^5 cells $(g\ soil)^{-1}$), with usually higher numbers obtained in dry microcosms compared to those in wet microcosms (Fig. 4, 5). The increase in *Frankia* populations was usually most prominent between t_0 and t_6 with about 10-fold increases representing 3-5 generations assuming exponential growth and doubling times between 8 and 15 days. Except for treatments with *Casuarina* plants only, where additional 10-fold increases were obtained, numbers of *Frankia* remained similar or increased only slightly between t_6 and t_{12} with 0-1 generations and doubling times of about 25 days and higher (Fig. 4, 5).

Values for abundance of *Frankia* in the rhizosphere followed a similar pattern as in bulk soil, though they were generally higher compared to those in bulk soil at each time point (t_6 or t_{12}), and irrespective of matric potential (dry, wet) and plant species (*Casuarina*, *Alnus*)[$Casuarina_{wet.}$ (t_6, P=0.037; t_{12}, P<0.001), $Casuarina_{dry}$ (t_6, P<0.001; t_{12}, P<0.001) and $Alnus_{dry}$ (t_6, P=0.007; t_{12}, P<0.001)], except for soil vegetated with *Alnus* kept under wet conditions [$Alnus_{wet}$ (t_6, P=0.260; t_{12}, P<0.095)]. Doubling times were generally slightly shorter in the rhizosphere than in bulk soil (Fig. 1, 2). In PIATT soils amended with organic matter, a significant increase in frankiae abundance was observed over time as well [($Alnus_{wet}$, P=0.062; $Alnus_{dry}$, P=0.013) and ($Casuarina_{dry}$, P<0.001)], except for soils amended with *Casuarina* organic matter and maintained wet conditions ($Casuarina_{wet}$, P<0.760).

Similar to analyses at t_0, cells of subgroup II of the *Alnus* host infection group and of *Casuarina*-infective strains were not detected after incubation for 6 (t_6) or 12 (t_{12}) weeks, i.e. neither in bulk no in rhizosphere soil for any of the treatments (Fig. 4, 5). In bulk soil, abundance patters of frankiae of subgroup I of the *Alnus* host infection group and those of the *Elaeagnus* host infection group followed patterns obtained for the genus, i.e. with largest increases usually obtained between t_0 and t_6, and no or little increases in cell numbers between t_6 and t_{12}, except again for treatments with *Casuarina* plants only (Fig. 4, 5). Growth of frankiae representing the *Elaeagnus* host infection group was usually faster between t_0 and t_6 than that of frankiae of *Alnus* subgroup I with doubling times between 6 and 11 days compared to 10 and 15 days, and thus frankiae of the *Elaeagnus* host infection group represented higher density increases (i.e. up to 100-fold) than those of *Alnus* subgroup I (10-fold) compared to numbers at t_0.

In the rhizosphere of both *Alnus* and *Casuarina* plants, effects of matric potential were obtained for abundance of specific *Frankia* populations, with more than 100-fold increases of frankiae of *Alnus* subgroup I under dry conditions compared to bulk soil at t_0, and less than 10-fold increases under wet conditions. The opposite pattern was obtained for frankiae of the *Elaeagnus* host infection group (Fig. 4, 5). Consequently, under dry conditions the genus *Frankia* in the rhizosphere was to a large extent (i.e. up to 95% depending on the plant species) represented by subgroup I of the *Alnus* host infection group, while under wet conditions a similar percentage of the genus in the rhizosphere of both plant species was represented by the *Elaeagnus* host infection group.

Discussion

Although many soils contain significant amounts of organic material, carbon resources are usually not meant to be easily available. Considering that microbial growth in bulk soil might therefore be significantly affected by carbon limitation [7], the 10- to 100-fold increase in indigenous *Frankia* populations in bulk PIATT soil during the 6 to 12 week incubation period is quite surprising. However, densities at the beginning of the incubation were very low with about 10^4 cells (g soil)$^{-1}$ and increased to final abundance values of about 10^6 cells (g soil)$^{-1}$ which represented numbers normally encountered in soils in our previous studies using *q*PCR-based analyses [19,21]. The increase might therefore reflect changing environmental conditions in our experimental set up, with low numbers obtained after storage of soils at 4°C, and increasing numbers as a function of increasing temperature (25°C), enhanced water availability and consequently better nutrient availability. Incubation increased *Frankia* populations in bulk soil, independent of plant species or the addition of organic material. Increases were also independent of

matric potential that had been shown to affect the development of specific *Frankia* populations [15,16], with drier conditions favoring root nodule formation of frankiae representing subgroup I over those of subgroup II [16].

These frankiae were represented by two populations, i.e. *Alnus* host infection subgroup I represented by strain ArI3 and the *Elaeagnus* host infection group represented by strain EAN1pec. None of these populations were shown to be able to grow on leaf litter [11], and thus the presence of leaf litter did not significantly affect their abundance. The indigenous populations of soil PIATT might therefore use more complex carbon resources such as humic acids for growth. We can also not exclude some effects of plant roots and their exudates since all microcosms were vegetated and members of both groups had been shown to grow in the rhizosphere of host and non-host plants [11]. Both groups had been detected in previous studies as well [20], even though they did not represent the major *Frankia* populations [19,20]. In those studies, frankiae of *Alnus* host infection subgroup II represented by strain Ag45/Mut15 were the most prominent group, with this subgroup exclusively found in young, wet and dry sandy soils under *Alnus glutinosa*, and representing up to 75% of frankiae in silty loams vegetated with *A. glutinosa* or *Betula nigra*. *Alnus* subgroup I was only found in the oldest plantation (65 yrs), together with subgroup II, suggesting environmental changes in time that might have promoted *Frankia* population changes from subgroup II to include subgroup I. *Frankia* strains representing *Alnus* host infection subgroup II were shown to grow with leaf litter of host and/or non-host plants [11,14], and thus could be adapted to carbon resources provided by the decomposition of plant material. Leaf litter amendments to soils have resulted in shifts of nodule-forming *Frankia* populations from subgroup I to

subgroup II, both for introduced and indigenous populations [15]. Members of subgroup I could rely on transformed components such as humic acids that might be present in higher abundance in more established and thus older sites. Soil extract media that potentially contain more complex carbon resources including humic acids, than leaf litter have been used to increase detection of growing organisms from soils [17]. These media have also successfully been used to isolate bacteria from soil samples that were related to *Frankia* of *Alnus* host infection subgroup I [8]. *Casuarina*-infective frankiae were not detected at all in this study, nor in the previous studies [19,20], supporting the assumption that the presence of members of the *Casuarina*-infective frankiae seems to be dependent on co-introduction with their exotic host plant species [6,25].

Rhizodeposition, i.e. the shedding of root cells and the exudation of sugars, organic acids, and amino acids from roots into the soil can overcome carbon limitation and allow microbes to proliferate [1,3]. The composition of root exudates depends on the plant species, the developmental stage, the plant growth substrate, and on stress factors [22]. Root exudate composition can also be influenced by the rhizosphere microflora itself [9]. As a consequence, availability and composition of the nutrients for microorganisms in the rhizosphere are highly dynamic. All *Frankia* strains tested so far grow in the rhizosphere of their host plants [12] but also potentially in that of other plants [18]. It is thus not surprising that abundance of *Frankia* is generally higher in the rhizosphere compared to bulk soil, independent of plant species and matric potential. For members of *Alnus* host infection subgroup I, matric potential seems to be important since growth in the rhizosphere of either plant species occurs under dry conditions (i.e. a matric potential of -0.005 MPa) only, but not under wet conditions (i.e. matric potential of -

0.001 MPa). This result relates to those of previous studies in which drier conditions were meant to favor root nodule formation of frankiae representing subgroup I over those of subgroup II [16]. Members of the *Elaeagnus* host infection group grow in bulk soil, the rhizosphere and with leaf litter, independent of matric potential and plant species, and thus represent a group with broad physiological adaptations.

Our results demonstrate the usefulness of the *q*PCR methodology developed for ecological studies on frankiae in soils. However, definite conclusions about population dynamics of frankiae in general and individual groups as a function of environmental characteristics require the further reduction of variables (e.g. assessments of population dynamics of individual strains introduced into soil), and the inclusion of additional resources (e.g. soil extracts) in the analyses of population dynamics under more controlled conditions, i.e. microcosms with introduced strains). Future analyses should also include next generation sequencing techniques using either *nif*H or rRNA gene fragments as target that should provide insight on overall diversity of frankiae in terrestrial systems, and confirm coverage of our *q*PCR based analyses of all *Frankia* or specific subgroups.

Table 9. Primer combinations targeting 23S rRNA gene sequences representing nitrogen-fixing members of the genus *Frankia* or subgroups within the genus

Target group	Primer combination (5' → 3')	Anneal Temperature (°C)	Fragment size (bp)	Reference
Nitrogen-fixing *Frankia* strains (clusters 1, 2, and 3)	23Fra1655f ($^{5'}$CTG GTA GTA GGC AAG CGA TGG) 23Fra1769r ($^{5'}$GGC TCG GCA TCA GGT CTC AG)	64	133	(20)
***Alnus* host infection group (cluster 1)**				
Subgroup I (ArI3)	23Ar1607f ($^{5'}$GTG TCT TTT CGG AGA TGT GTC T) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	64	128	(21)
Subgroup II (Ag45/Mut15)	23Mut1555f ($^{5'}$TTG ATG CGT CCA TGC TGA GG) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	66	170	(21)
***Casuarina*-infective strains**	23Cas1607f ($^{5'}$GTG TCT CTT CGG AGG TGT GTT C) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	66	128	(21)
***Elaeagnus* host infection group (cluster 3)**				
	23EAN1579f ($^{5'}$GTT TGT GCT AAC CGT TCT GGT) 23Fra1715r ($^{5'}$CCT ACA CCC TTG CCC CAG GA)	64	146	(21)

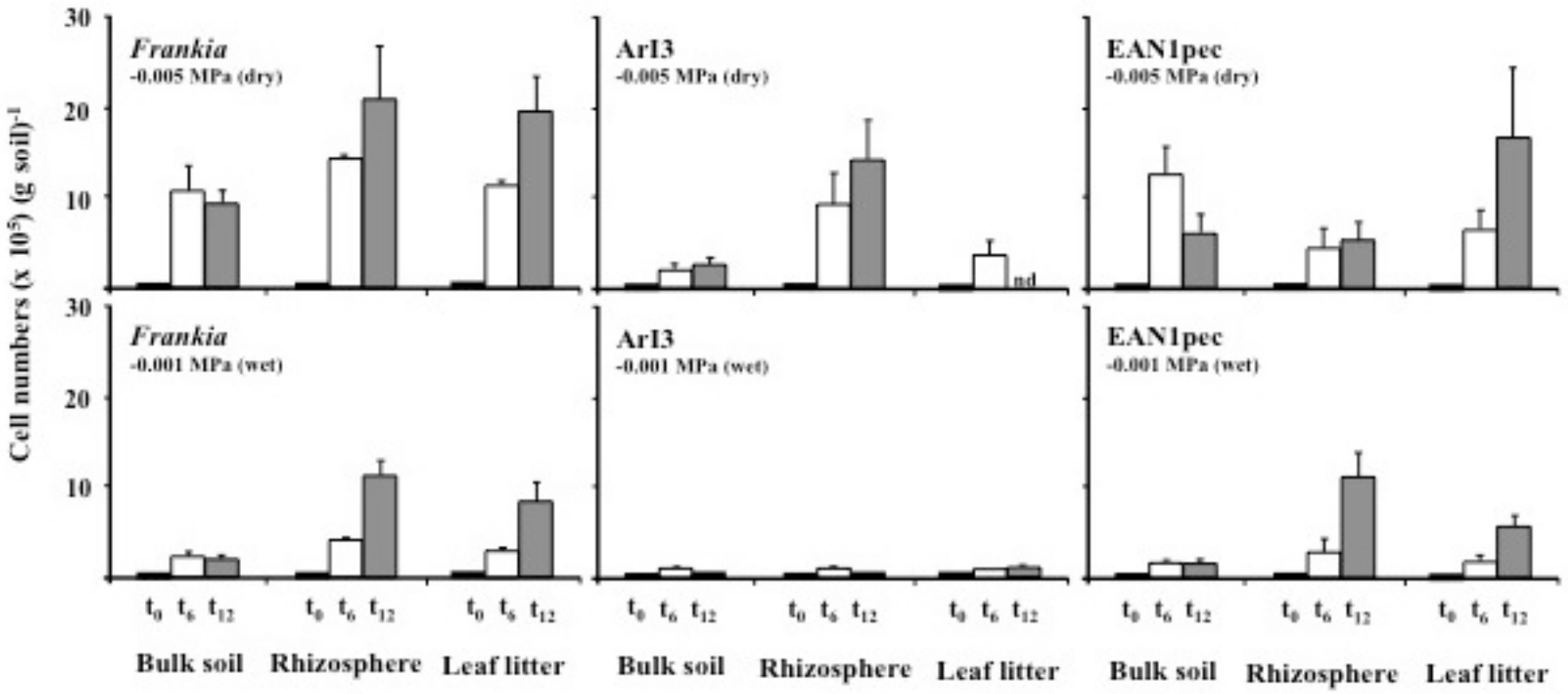

Fig. 4. Population dynamics of indigenous frankiae in soil PIATT in bulk soil, the rhizosphere of *Alnus glutinosa* or bulk soil amended with leaf litter of *A. glutinosa* analyzed after storage at 4°C (t_0) and incubated at 25°C for 6 weeks (t_6) or 12 weeks (t_{12}) at two matric potentials [-0.005 MPa (dry) or -0.001 MPa (wet)]. Population analyses included assessments on the genus level (*Frankia*), and 4 subgroups of which only subgroup I of the *Alnus* host infection group represented by strain ArI3, and the *Elaeagnus* host infection group represented by strain EAN1pec were detected. Members of *Alnus* subgroup II represented by strain Ag45/Mut15 and *Casuarina*-infective strains represented by strain CcI3 were not detected.

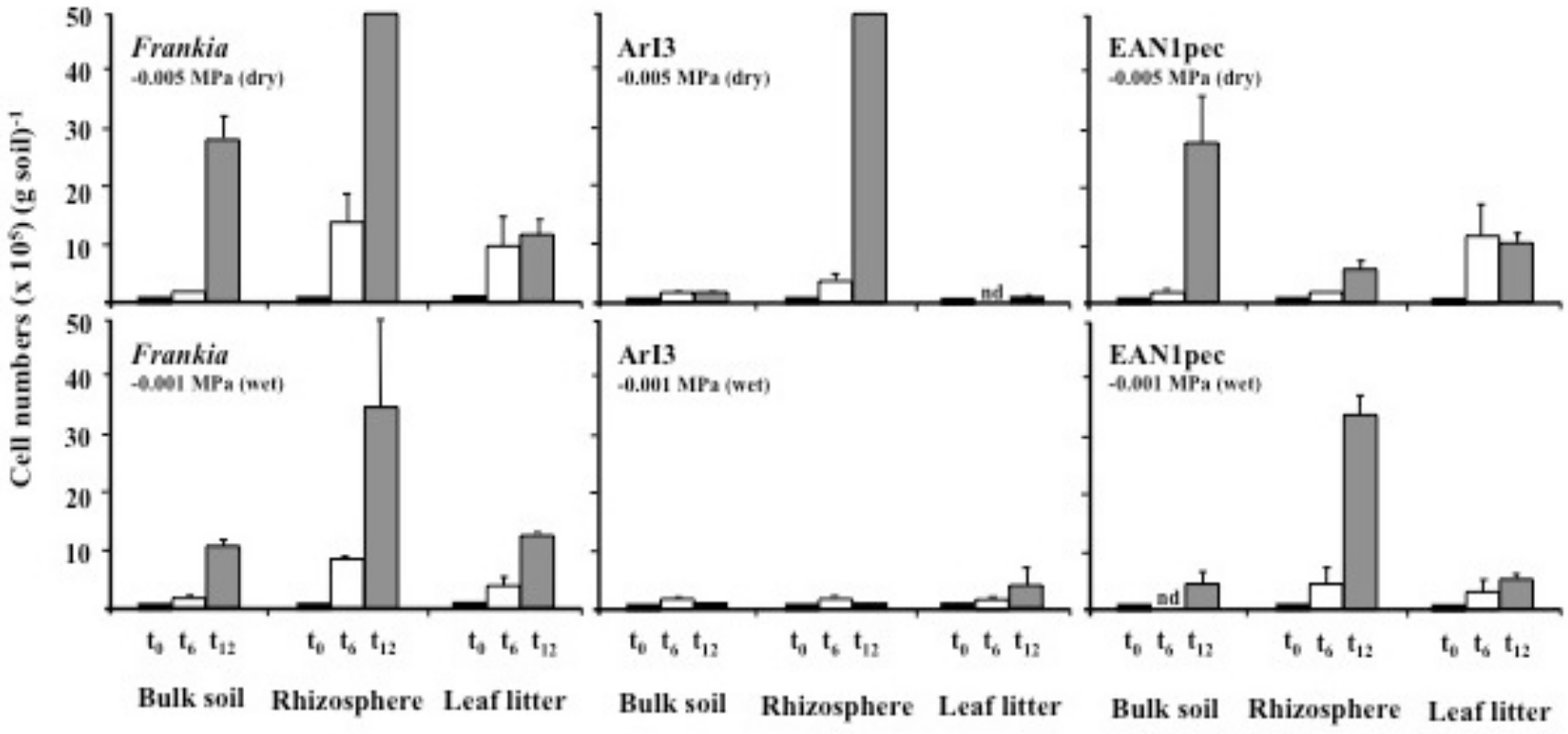

Fig. 5. Population dynamics of indigenous frankiae in soil PIATT in bulk soil, the rhizosphere of *Casuarina equisetifolia* or bulk soil amended with leaf litter of *C. equisetifolia* analyzed after storage at 4°C (t_0) and incubated at 25°C for 6 weeks (t_6) or 12 weeks (t_{12}) at two matric potentials [-0.005 MPa (dry) or -0.001 MPa (wet)]. Population analyses included assessments on the genus level (*Frankia*), and 4 subgroups of which only subgroup I of the *Alnus* host infection group represented by strain ArI3, and the *Elaeagnus* host infection group represented by strain EAN1pec were detected. Members of *Alnus* subgroup II represented by strain Ag45/Mut15 and *Casuarina*-infective strains represented by strain CcI3 were not detected.

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CHAPTER VI

GENERAL DISCUSSION

The studies presented in this dissertation focused on two basic objectives: 1) to develop detection and quantification methods that allowed us to analyze *Frankia* populations in soil, and 2) to address questions on the fate of indigenous frankiae in soils by employing the methods developed and evaluated.

Work on the first objective resulted in the development of a SYBR Green based *q*PCR method that quantified clusters 1 and 3 of the actinomycete *Frankia* in soils by targeting *nif*H gene sequences (chapter II). *Nif*H gene sequences encode for the structural gene for nitrogenase reductase [37], and have been used extensively in comparative sequence analyses to unravel phylogenetic relationships of nitrogen-fixing bacteria in the past [32,33,62]. Thus, elaborated databases of *nif*H gene sequences are available. As a consequence, *nif*H gene sequences are the preferred genes characterizing nitrogen-fixing bacteria in phylogenetic studies compared to other *nif* genes such as *nif*D and *nif*K genes. *Nif*H gene sequences are now also used as targets in ecological studies using molecular methods. PCR-based detection of nitrogen-fixing microorganisms in natural environments, for example, use *nif*H gene-specific primers to detect different groups of nitrogen-fixing bacteria [19,39,72,102]. Sequence variation between *nif*H genes of nitrogen-fixing bacteria is sufficient to allow assessments of shifts in the community structure of nitrogen fixing bacteria in ecosystems under changing conditions [4], and also of shifts in community structure over time and related to soil type and chemical properties [68].

Comparative sequence analyses of *nif*H genes or gene fragments have allowed researchers to differentiate members of the genus *Frankia* from other nitrogen-fixing organisms [5,25,56,62,83,84,97,98]. Alternative methods using genes related to nitrogen fixation used the size or the sequences of the intergenic spacer (IGS) between the *nif*H and *nif*D (*nif*H-D) genes [86] or the *nif*D and *nif*K (*nif*D-K) genes [41]. In addition to structural genes like nitrogenase, the glutamine synthetase II (*gln*II) gene has been used as target for PCR based identification and differentiation of frankiae from other actinomycetes [16].

Comparative sequence analyses of genes involved in nitrogen fixation like *nif*H have increased our awareness of the genetic diversity and distribution of *Frankia*. It has also helped elaborate on endophyte-host specificities [55,97]. However, the focus on *nif*H gene sequences deals with some drawbacks that might affect the reliability of the analyses. First, several organisms are known to have multiple copies of *nif*H that can be very similar [59] but can also display considerable sequence differences [11,46,95]. Multiple copies of the *nif*H gene or the presence of alternative nitrogenase systems can in some cases confuse the placement of an organism within the *nif*H phylogeny [12]. Second, genes involved in nitrogen fixation such as *nif*H have not only been localized on chromosomal DNA, but also been detected on plasmids in several organisms [6,89]. Thus, there is a possibility of a certain degree of lateral gene transfer, especially between related organisms, which may account for some of the 'stray' sequences in the *Frankia* *nif*H phylogeny [79]. The phylogeny of *nif*H thus provides a reasonable, although imperfect framework for sequence interpretation, which potentially results in a limited phylogenetic resolution and higher ambiguity as compared to rRNA phylogenies.

Using *nif*H gene sequence information, PCR-based approaches have been developed to unravel the phylogenetic relationships of isolates, as well as of uncultured endophytes in root nodules of many actinorhizal plants from which no isolates have been obtained (reviewed in [26]). Quantitative PCR (*q*PCR) is another sensitive, robust technique that is used to estimate the abundance of specific genes or microbial groups in different environmental habitats [47,64]. It has also been used to estimate the abundance and diversity of *nif*H **s**equences in order to monitor the microbial potential for nitrogen fixation, against a variable background of environmental genomic DNA [19,66,69,90]. Primers targeting *nif*H gene sequences previously designed for other techniques, like T-RFLP and conventional PCR, were used in SYBR Green-based *q*PCR [69,77], as were newly designed primers targeting larger phylogenetic groups [50].

Our study (chapter II) used large *nif*H gene sequence databases established from pure cultures and uncultured *Frankia* populations in root nodules of different host plants to design primers targeting the *nif*H gene in nitrogen-fixing frankiae [79]. The database of 454 sequences was highly variable with 178 unique sequences, and only few regions within the 522 bp fragment were sufficiently conserved to provide signature sequences present in the entire set of sequences. One of these signature sequences was used to design primer *nif*Hr 158 that was 100% complementary to *Frankia* of the *Elaeagnus* (cluster 3), and the *Alnus* and *Casuarina* host infection groups (cluster 1) in our database. Together with forward primer *nif*Hf1 it was used to specifically amplify a 191-bp fragment of the *nif*H gene of frankiae of cluster 1 and 3. Cluster 2 representing as yet uncultured members of the *Dryas* host infection group was not covered though only 1 sequence for this group was available. Our SYBR Green based *q*PCR method provided a

fast, specific and direct analysis method for *Frankia* in both habitats, i.e. root nodules and soil, with cell density estimates of up to 10^6 *Frankia* cells [g soil {dry wt.}]$^{-1}$ depending on the soils.

The study, however, also revealed some problems with using *nif*H genes as a target for the quantification of frankiae. First, primers developed only detected frankiae of the *Alnus* and *Elaeagnus* host infection groups (clusters 1 and 3, respectively), but not frankiae of the *Dryas* host infection group (cluster 2) or non-nitrogen-fixing strains (cluster 4). Second, even though variation in *nif*H gene sequences allowed us to identify signature sequences for subgroups, differences in signature sequences were too small to be used as target for molecular detection methods. Third, indications for *nif*H gene transfer were observed with up to 99.3% sequence similarity of *nif*H gene fragments of confirmed members of the genus *Frankia* to those of *Micromonospora lupini* that was isolated from root nodules of *Lupinus angustifolius* [91]. These issues prompted us to look for other genes that could be used as target in *q*PCR applications to quantify all members of the genus *Frankia* but also to distinguish clusters or specific subgroups within the genus.

In chapter III, we report on the evaluation of ribosomal RNA gene sequences as potential target for the detection of all members of the genus *Frankia* and specific subgroups within the genus. Ribosomal RNAs (rRNAs) have been shown to be almost perfect phylogenetic clocks, and thus comparative sequence analyses of rRNA genes or gene fragments have been used as powerful tools to assess the phylogenetic relationships of cultured and uncultured microbes in numerous studies [21,22,45,48,65,99-101]. Large

databases of both small subunit rRNA with currently (i.e. July 24, 2014) 1,583,868 aligned sequences of at least 900 bp, and large subunit rRNA with 57,546 aligned sequences of at least 1,900 bp allow for accurate analyses of phylogenetic relationships, but also for the assessment of specific primers or probes targeting organisms at different levels of phylogenetic resolution (i.e. from Domain to genus level) [70]. Signature sequences on rRNA have been used as targets in ecological studies using different molecular tools. The most promising applications included *in situ* hybridization assays on uncultured microbes in environmental samples that allowed researchers to visualize target organisms on different levels of phylogenetic resolution, and to retrieve information on their abundance in environmental samples (see [2] for review, [20,23,42,49]. PCR-based approaches were initially used to detect organisms in specific environments [7,14,54,96], but more recently also to quantify them [18,24]. Quantification of broad phylogenetic groups (e.g. on the Domain level) can be achieved by *q*PCR methods, however, accurate enumeration is hampered by the need for a conversion factor required to relate a signal to cell numbers. Thus, the number of gene copies per cell effects accurate quantification. While this might not be a big problem for highly specific targets (e.g. members of the species *Salmonella enterica* or *E. coli* that all are supposed to have 7 copies of the 16S rRNA gene) [67], it definitely is for large groups of organisms. For all bacteria, for example, rRNA operons with copy numbers from one to as many as 15 copies have been detected in currently available whole genome sequences [43,93]. Quantification of all bacteria by *q*PCR uses an average 16S rRNA gene copy number per bacterial cell of 4.2 [93]. Therefore, depending on the bacterial community composition in any environmental sample, the average 16S rRNA gene copy number per bacterial cell might be different

from the 4.2 copies. Bacterial communities developing from the same source under different environmental conditions, for example, produced different average copy numbers, i.e. average copy numbers of 1.4, 2.7, 5.4, or 5.5 [43].

Comparative sequence analyses of ribosomal RNAs have been used in several studies to assess the phylogenetic relationship of *Frankia* isolates or uncultured populations in root nodules to other bacteria [30,35,103]. These databases were subsequently used to detect signature sequences for the genus *Frankia* or subgroups within the genus, which were then used as targets for molecular detection methods [9,15,29,58,61,73,82,103,104]. Ribosomal RNAs have been used as target in dot blot analyses [57], though most studies employed the detection of PCR-amplified partial 16S rRNA gene sequences from isolates or uncultured *Frankia* populations in root nodules [56,82,83]. While these studies enabled researchers to detect members of the genus or retrieve sequence information from pure cultures or uncultured nodule populations, a differentiation of *Frankia* strains from different host infection groups or between *Frankia* strains within the same host infection group was difficult since sequence variation was not sufficient for the application of reliable molecular detection methods [31,58].

Compared to 16S rRNA, 23S rRNA provides longer sequences and more length variations [69]. In high G+C gram-positive bacteria, a highly variable insertion is present in domain III of the 23S rRNA and its gene [75]. Sequences on this insertion have been used as a target for oligonucleotide probing [74] and for *in situ* hybridization to analyze bacterial cells from various terrestrial systems including alder root nodules [27,103,104], sediments [71], soils [13,28,34], and aquatic systems [51,53,94]. Sequence variation

within this insertion in three *Frankia* strains belonging to the *Alnus* host infection group suggested that the domain could be used as a target in the differentiation of *Frankia* subgroups [75]. Comparative sequence analysis of PCR amplified and cloned 23S rRNA gene fragments containing this insertion from *Frankia* strains belonging to different host infection groups demonstrated that *Frankia* strains of the *Casuarina* and *Elaeagnus* groups show limited sequence variation whereas the *Alnus* group could be separated into four subgroups, three (IIIa, IIIb and IV) containing typical nitrogen-fixing strains and a fourth (I) containing only non-nitrogen-fixing strains [35]. Thus, this actinomycetes-specific insertion in Domain III of the 23S rRNA and its gene is a promising target for the differentiation of groups of *Frankia* [75]**,** as demonstrated by oligonucleotide probing methods before [74]**.**

These 23S rRNA gene sequences were thus evaluated as an alternative to *nif*H gene sequences for the analyses of nitrogen-fixing members of the genus (i.e. clusters 1, 2 and 3), as well as for subgroups within genus (chapter III). A small database of about 60 sequences of this insertion from different *Frankia* strains was available from previous taxonomic studies that demonstrated sufficient sequence variation to distinguish several subgroups within the genus *Frankia* [35,52]**.** This database was amended with published and unpublished sequences obtained from whole genome sequencing projects for *Frankia* [60]. The rRNA database SILVA, www.arb-silva.de) also provided information on copy numbers of the 23S rRNA gene per genome, with two copies in strains CcI3, ACN14a and the endophyte in *D. glomerata*, and three copies in strains EAN1pec, EUN1f and EuI1c. Although differences in copy numbers between members of different target groups are small, accurate quantification is effected by the number of gene copies per

cell, and thus the conversion factor of 2.5 used to quantify unknown populations in environmental samples [78,80].

Our study allowed us to quantify members of the genus *Frankia* (i.e., clusters 1, 2 and 3) with similar numbers as obtained with *nif*H as target [78]. Since *nif*H as target only allowed to quantify members of clusters 1 and 3, the result indicated that frankiae of cluster 2 (*Dryas* host infection group) do not play a major role in the soils that were analyzed for this purpose. We also demonstrated the usefulness of this insertion as a target for the quantification of specific subgroups, though targets needed further refinement (Chapter IV) to allow the analyses of members of the *Elaeagnus* host infection group (represented by strain EAN1pec), subgroups I (strain ArI3) and II (strain Ag45/Mut15) of the *Alnus* host infection group, and the *Casuarina*-infective frankiae (strain CcI3) [80].

Results of studies presented in chapter III provided valuable information on the usefulness of 23S rRNA gene sequences for the quantification of the genus *Frankia* as well as of subgroups within the genus. However, quantification of indigenous *Frankia* populations on the subgroup level in different soil samples from one location resulted in the detection of one population only, though with numbers similar to those obtained for genus-level analyses with both *nif*H and 23S rRNA gene as target. While these results might correctly reflect the abundances of *Frankia* subgroups in these soils, with one dominant population and the remaining ones close to or below the detection limit, the failure to detect more subgroups required additional studies on indigenous *Frankia* populations in different soil environments to assess the usefulness of our primer

combinations for the quantification of different subgroups of *Frankia* in soils.

Studies presented in chapter IV do not only refine primer/target systems for specific subgroups of *Frankia*, but also use a set of soils with potentially different conditions that might favor growth of specific subgroups. *Frankia* can be found in soils vegetated with host plants, but has also been detected in soils lacking host plants [1,3,8,10,36,40,87,88,92]. In Finland, Smolander and Sundman (1987) found high numbers of *Frankia* in the soil under stands of the non-host plant, *Betula pendula*. Saprophytic growth of *Frankia* was found in the rhizosphere of host and non-host plants in the field [55,88] as well as under axenic conditions, which could be attributed to the carbon sources provided by root exudates [76,86]. Carbon sources available from root exudates have been shown to support growth of frankiae in the rhizosphere, due to availability of high water soluble fractions, which decreases with increasing distance from the roots [38]. Birch root exudates are known to provide carbohydrates and contain organic acids such as succinic, adipic, citric, acetic and propionic acid [81,85]. Propionic acid is generally used as a universal C source in the growth medium of *Frankia*.

In our study presented here, samples from 4 soils with similar physicochemical conditions, two of which were vegetated with the host plant *Alnus glutinosa* and two with the non-host plant *Betula nigra*, were obtained from close locations in Illinois. Samples were obtained at the same time, and analyzed for different *Frankia* populations at different distances from the stem. Different *Frankia* populations were detected, with no obvious differences in population structure as a function of plant species or distance to stem. *Frankia* populations of the *Elaeagnus* and *Alnus* host infection groups, not only

appears to occur in the rhizosphere of host plant (*Alnus glutinosa*), but also to be present in similar numbers in the rhizosphere of the non-host plants (*Betula nigra*). The lack of significant differences in the abundance of *Frankia* obtained in our study with *Alnus* soil compared to *Betula* soil, could be attributed to small differences in soil properties, depth profile and water content [78], or to potentially unusual characteristics of the indigenous *Frankia* strains. Numbers ranged from 4 to 36 x 10^5 cells (g soil)$^{-1}$ with values for abundance of *Frankia* being significantly higher closer to the tree stems compared to crown edge samples at 3 out of 4 sites. These results indicated potential effects of root density and thus root exudates, however, such effects could not be substantiated in this study.

Studies presented in chapter V were meant to further assess the usefulness of our developed *q*PCR in the quantification of frankiae and subgroups in soils. *q*PCR was used to follow population dynamics of indigenous *Frankia* populations in bulk soil and the rhizosphere of *Alnus glutinosa* or *Casuarina equisetifolia* at 2 matric potentials representing dry and wet conditions. The methodology allowed us to quantify increases in *Frankia* populations over time, and to discover different subgroups being abundant than those in our previous studies. For example, under dry conditions the genus *Frankia* in the rhizosphere was to a large extent (i.e. up to 95% depending on the plant species) represented by subgroup I of the *Alnus* host infection group, while under wet conditions a similar percentage of the genus in the rhizosphere of both plant species was represented by the *Elaeagnus* host infection group. Leaf litter amendment resulted in growth of frankiae of the *Elaeagnus* host infection group only, essentially matching the values obtained for genus-specific analyses. These results indicated complex interactions

between physicochemical characteristics (i.e. matric potential, plant species), nutrient resource availability (rhizosphere, leaf litter and autochthonous carbon sources such as humic acids) and competition (strains Ag45/Mut15, EAN1pec, CcI3 or population density effects) that all together affect population dynamics of individual *Frankia* strains.

In future studies, physicochemical and nutrient resource factors need to be singled out in microcosm setups as sole variables, however, competition and nutrient resources other than those studied before (i.e. autochthonous carbon sources) need to be considered as well. Definite conclusions about population dynamics of frankiae in general and individual groups as a function of environmental characteristics therefore require the further reduction of variables (e.g. assessments of population dynamics of individual strains), and the inclusion of additional resources (e.g. soil extracts) in the analyses of population dynamics. *q*PCR based analyses could also be matched by next generation sequencing data which would also provide additional evidence about the accuracy and specificity of our quantification methodology. Ultimately, the results obtained in these short-term microcosm experiments should be evaluated on the same soil in a field setting. Long-term field studies are important and necessary in order to extrapolate laboratory data to real environmental situations. For instance, the *Frankia* strains that were used in the present studies could be inoculated directly into the soil on the field site. Thus, the effect of the inoculation on host plant seedlings growing at the site could be assessed. It is also important to know for how many years the inoculated plants maintain an advantage over non-inoculated control plants and whether this time could be extended by any means. Such long-term studies should also focus on the performance of introduced *Frankia* strains compared to the indigenous population and also the persistence of

introduced strains in this soil. Priority should be given to not only identifying the strains showing the best "long-term-performance" regarding persistence in the soil, infectious capacity, and N_2-fixation rates, but also to determining the optimal environmental conditions for the activity of these strains.

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