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Research Article

RESEARCH ARTICLE

Rhizosphere microbiomes of potato cultivated in the High Andes show stable and dynamic core microbiomes with different responses to plant development

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One sentence summary: The rhizosphere microflora of potato grown at distinct field locations in the Andean Altiplano show a core microbiome with stable and dynamic subsets responding differently to plant vegetation.

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ABSTRACT

The rhizosphere hosts a rich microflora supporting plant nutrition and health. We examined bacterial rhizosphere microbiota of *Solanum tuberosum* grown in its center of origin, the Central Andean Highlands, at different vegetation stages and sites at altitudes ranging from 3245 to 4070 m.a.s.l., differing in soil characteristics, climate and the agricultural practices by 454 sequence analysis of 16S rRNA genes. We observed that the taxonomic composition of bacteria repeatedly occurring at particular stages of plant development was almost unaffected by highly diverse environmental conditions. A detailed statistical analysis on the operational taxonomic unit (OTU) level, representing bacterial species, revealed a complex community structure of the rhizosphere. We identified an opportunistic microbiome which comprises OTUs that occur randomly or under specific environmental conditions. In contrast, core microbiome members were found at all sites. The 'stable' component of the core microbiome consisted of few ubiquitous OTUs that were continuously abundant in all samples and vegetation stages, whereas the 'dynamic' component comprised OTUs that were enriched at specific vegetation stages.

Keywords: rhizosphere; core microbiome; 16S rRNA; potato

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INTRODUCTION

The importance of the rhizosphere microbiome for plant growth and health is widely acknowledged (Berg and Smalla 2009). Of particular interest is the ectorhizosphere (Hiltner 1904), nowadays referred to as the rhizosphere, the soil layer that is under the direct influence of compounds released by the plant (Ziegler *et al.* 2013), which covers the root and represents the interface between plant and soil and plays a key role in biogeochemical cycling, nutrient flow and plant metabolism (Philippot *et al.* 2013). Rhizosphere microorganisms are influenced by multiple factors (Garbeva, van Veen and van Elsas 2004) including the soil type (Berg and Smalla 2009), environmental conditions (Rasche *et al.* 2006a), plant genotype (Raaijmakers *et al.* 2009) and plant development (İnceoğlu *et al.* 2011).

The definition of a 'core' root microbiome for *Arabidopsis thaliana* suggested that certain bacterial groups are consistently enriched in the rhizosphere compared to the surrounding bulk soil irrespective of the sampling site (Lundberg *et al.* 2012). These findings fueled the discussion to which extent the host plant shapes the rhizosphere microbiome (Philippot *et al.* 2013). A detailed analysis of root exudation patterns of phytochemicals over plant development of *A. thaliana* together with community profiling and transcriptome analysis of its associated rhizosphere microbiota surmised that certain plant beneficial functions are triggered by the plant through the exudation of particular phytochemicals caused by changing requirements during plant development (Chaparro *et al.* 2013; Chaparro, Badri and Vivanco 2014). Furthermore, plant exudates can additionally recruit a subset of soil microbes for specific needs (Rudrappa *et al.* 2008).

According to the Food and Agriculture Organization of the United Nations (http://faostat.fao.org), potato (*Solanum tuberosum*), with an annual production of more than 300 million metric tons, is the world´s fourth most produced staple crop, after maize, wheat and rice. Root exudation of *S. tuberosum* discerns among cultivar types and was observed to reach up to 20% of total carbon assimilation during tuber formation at the late vegetative stage before flowering (Gschwendtner *et al.* 2011). In the potato rhizosphere, Weinert *et al.* (2011) observed similarities in the relative abundance of the dominating bacterial phyla irrespective of the soil type and cultivar. A 3-year study at a Dutch field site on the microbial community of different potato genotypes, sampled at different time points of plant development, showed that certain bacterial genera were universally present at the plant flowering stage (İnceoğlu *et al.* 2013a). We therefore hypothesized that potato plants are associated with a core microbiome and that subsets follow specific patterns and are driven by distinct environmental parameters.

To test our hypotheses, we investigated potato plants grown in their center of origin, the Andean Highlands, where potatoes have been cultivated at high altitudes and under highly stressful conditions since ancient times. We characterized the rhizosphere microbiome of *S. tuberosum* var. Yungay, a cross-breed of native Peruvian and an exotic potato, which was the second most commonly cultivated potato variety of Peru in 2015 (Freshplaza.com). Sampling was performed across three vegetation stages (emergence, flowering and senescence) at three distinct traditional farming sites in Peru located at altitudes between 3245 and 4070 m.a.s.l. and exposed to different edaphoclimatic conditions. Rhizosphere microbiomes were analyzed by 454 pyrosequencing of 16S rRNA genes.

MATERIALS AND METHODS

Sample collection and storage conditions

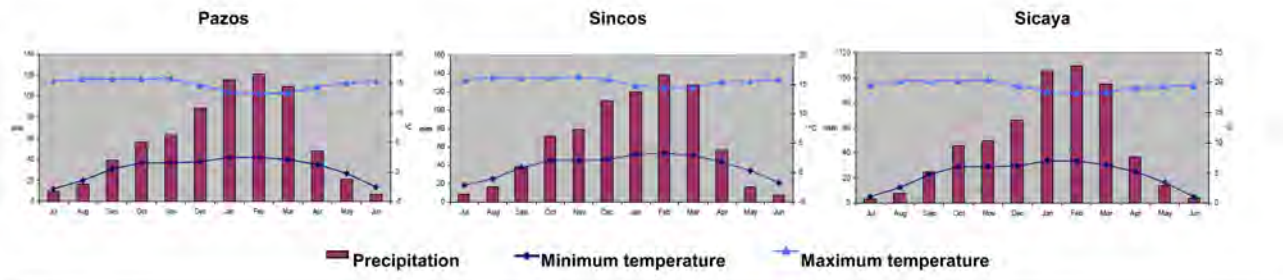
Samples were taken from three agricultural field sites in the Andes of Peru: Pazos—Huancavelica region, Tayacaja province, Pazos district (S12°14′40.6″; W75°03′03.9″; 4 075 m.a.s.l.), Sincos—Junin region, Huancayo province, Sincos district (S11°53′14.4″; W075°25′05.1″; 3 751 m.a.s.l.) and Sicaya—Junin region, Huancayo province, Sicaya district (S12°01′42.9″; W75°16′0.3″; 3 245 m.a.s.l.). All three sites were subsistence farms, and samples were taken during the 2009–2010 growth season, dating from October to May. At all three sites, the potato cultivar *Solanum tuberosum* var. Yungay was cultivated. Soil chemical and physical parameters of the sampling sites were analyzed after the tubers were planted by local farmers in October 2009 by collecting a representative bulk soil sample (at 30 cm depth) (Fig. 1). We assessed the physico-chemical differences between the sampling sites by performing principal component analysis using PAST (Hammer, Harper and Ryan 2001). Rhizospheres of five randomly selected plants in a 20 m × 20 m subplot were sampled at three plant growth stages: emergence (December 2009), flowering (January/February 2010) and senescence (April/May 2010), according to the description by Hack *et al.* (1992). Sampling was performed by pulling out the plants with the complete root system. Plants were gently shaken and soil adhering to the roots was recovered by using a sterile brush. The rhizosphere soil was sieved through a 1 mm sieve and mixed thoroughly. In a pre-experiment, we compared different preservation methods with regard to potential effects on microbial community structures, assessed via 16S rRNA gene-based terminal restriction length polymorphism (data not shown). Among those, we observed that drying of the soil samples in an oven for 2–3 h at 85°C had no effect on the bacterial community structure as compared to immediate analysis of fresh soil samples. Thus, we chose this method to preserve the rhizosphere soil samples before the transport from Peru to Austria. DNA extraction was based on the method by Pfeiffer *et al.* (2014). Briefly, we combined the FastDNA spin kit for soil (MP Biomedicals, LLC, Solon, OH, USA) with classical phenol-chloroform-isoamyl extraction and repeated bead beating, to avoid the preferential extraction of Gram-negative bacteria or bacteria with thinner cell walls (Feinstein, Sul and Blackwood 2009). We further aimed to dilute the amount of PCR-inhibiting low molecular contaminants such as humic acids by agarose plug diffusion (Moreira 1998).

454 Sequencing

16S rRNA gene amplification was performed at 68°C annealing temperature using the universal bacterial primers 27f 5-AGAGTTTGATCCTGGCTCAG-3′ (Weisburg *et al.* 1991) and 518r 5′-ATTACCGCGGCTGCTGG-3′ (Muyzer, de Waal and Uitterlinden 1993) with amplicon adaptors and 12 different barcodes (Kuffner *et al.* 2012), on 45 samples in total. To reduce common biases of PCR amplification, we repeated PCR three times and pooled the technical replicates. The amplicons were quantified using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and purified using Ampure magnetic purification beads (Beckman-Coulter, Brea, CA). Cleaned amplicons were quantified using Pico Green (Life Technologies Corp., Carlsbad, CA). 454 sequencing was performed for one full picotiter plate on a GS FLX instrument with titanium reagents (Roche, Mannheim, Germany), following the manufacturers´ instructions. After filtering out genomic DNA and non-amplicon sequences, a total of 436 838 sequences were

Place	Elevation (m.a.s.l)	Slope %	P (ppm)	K (ppm)	N %	Salinity dS/m	Sand %	Clay %	Lime %	pH	SOM %	Manure (t)
Pazos	4075	30	64.5	418	0.41	0.62	46	46	8	4.36	7	6
Sincos	3751	15	50.5	236	0.14	0.82	32	54	14	7.36	2.28	3
Sicaya	3245	0	56	268	0.28	1.13	40	40	20	5.71	2.98	2.2

	Inorganic fertilization			Pesticide
Pazos	Diamoniumnitrate -110kg N/ha	Triplesuperphosphate 150kg K/ha	Potassiumchloride – 200kg K/ha	Regent (Fipronil)
Sincos	Urea, Diammoniumphosphate, Diammoniumnitrate 280kg N/ha	Diammoniumphosphate 164kg P/ha	Potassiumchloride – 214kg K/ha	Regent (Fipronil), Dithane (Mancozeb)
Sicaya	Diammoniumphosphate, Urea 228kg N/ha	Diammoniumphosphate 255kg P/ha	Potassiumchloride – 167kg K/ha	Decis (Deltrametrina)



Field history	
Pazos	Mixture of potato varieties (2009), fallow (2003-2008)
Sincos	Potato (variety Canchan; 2007-2009), fallow (2004-2007)
Sicaya	Carrot (2008-2009), lucerne, maize and pea (2005-2008)

Figure 1. Edaphoclimatic conditions of the soil samples used in this study.

obtained (8928 average/sample; for more information, see SI Dataset 1, Supporting Information). Sequences were deposited at the NCBI short read archive under the bioproject number SRP032503.

Sequence processing and OTU picking

We applied *de novo* OTU picking based on a ≥97% pairwise sequence identity, using the UPARSE pipeline (Edgar 2013; http://drive5.com/usearch/manual/uparse_pipeline.html). We further performed quality filtering of all individual sequences with a maximum expected error rate of 0.5, sequences below 300 bp length and singletons were removed, and the remaining sequences were truncated to an equal length of 300 bp. OTUs were chimera checked using reference filtering against the Greengenes-13-05 database, with a similarity threshold of 99%. Overall, the 436 838 sequence reads were clustered into 16 096 OTUs (5264 OTUs without singletons), on average 2977 non-singleton OTUs/sample (SI Dataset 1, Supporting Information).

Due to a different format of the UPARSE generated OTU sequence data and OTU table, we wrote several Perl scripts (available on request) to enable QIIME downstream data processing tools, including taxonomic assignment of the representative OTU sequences against the Greengenes-13-05 database, sequence alignment using MUSCLE (Edgar 2004) and tree calculation using FastTree (Price, Dehal and Arkin 2009). Following the taxonomic assignment, one sample of the Pazos flowering stage, that represented an outlier, was removed. For diversity estimates, we applied multiple rarefactions (100 bootstraps) in QIIME on the UPARSE generated OTU table. The OTU table was rarefied to 524 sequences per sample to provide a well-balanced number of samples included (41 of 44) and depth.

Statistical analyses

α-Diversity, which describes the diversity of an individual sample, was measured calculating total OTU richness, the Simpson similarity index and Shannon diversity as well as Faith's phylogenetic depth measurements (Faith 1992), which take into account the respective branch range of an OTU sequence in relation to the total 16S rRNA gene phylogenetic branch length. ANOVA using Tukey post-hoc analysis was performed to estimate the effects of sampling sites and vegetation stages on species richness and Faith´s phylogenetic depth measurements. Analysis of β-diversity, which describes the extent of diversity between the different samples, was performed using a jackknifed approach with 1000 bootstraps to calculate the Bray–Curtis dissimilarity matrix and weighted and unweighted UniFrac distance matrices based on UPMGA tree clustering (Lozupone and Knight 2005). Sample relationships were calculated using principal coordinate analysis (PCoA) and visualized as 2D projection using KING (Chen, Davis and Richardson 2009). We performed a constrained analysis of principle coordinates (CAP), which corresponds to a distance-based redundancy analysis, by applying the capscale function together with PERMANOVA using the vegan package (Oksanen *et al.* 2013) in R (R Core-Team 2013) as previously described (Hartmann *et al.* 2012). Thereby, we took the vegetation stage as environmental factor of the constrained analysis. CAP performance on the sampling site was done on all samples and also on individual vegetation stages, to reveal which vegetation stage had the largest influence on the bacterial community composition. Analysis of similarity (ANOSIM) (Clarke 1993) with 5000 permutations was applied to estimate the influence of sampling site or vegetation stage for all metrics calculated.

Multivariate analyses on taxonomic ranks were performed using GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E/) and LEfSe (Segata *et al.* 2011). Percentage shares of

classes were visualized in GENE-E. To assess significant taxonomic differences between the samples with regard to the sampling site or the vegetation stage, we used linear discriminant analysis (LDA) in LEfSe (Segata *et al.* 2011). LDA determines taxa determined by the Kruskal–Wallis sum-rank ($\alpha = 0.05$) and pairwise Wilcoxon test ($\alpha = 0.05$) to be significantly differently distributed across categories via LDA effect size (data were normalized to 1; threshold for logarithmic LDA score: 3.0). Cladograms were generated to display significant LDA effects of sampling sites and vegetation stages on the different taxonomic levels from kingdom to genus.

Determination of the core microbiome

The vast majority of the 5265 OTUs in the dataset was found to be unique at individual samples. To determine if OTUs were consistently found among the five replicates of a sampling collection, we defined 'reproducibly occurring OTUs', which were detected in at least 60% (3 of 5) of rhizosphere replicate samples (SI Dataset 2-1, Supporting Information). To avoid biases originating from uneven number of OTUs per sample (SI Dataset 1, Supporting Information), we only included OTUs that had a relative abundance of minimum 0.1% of the OTUs per sample. OTU tables were processed in QIIME by (i) splitting the OTUs according to the treatment <split˙otu_table.py>, (ii) collecting OTUs that were present in at least three of five replicates <filter_otus_from_otu_table.py> command and (iii) merging the resulting OTUs in one table <merge_otu_tables.py>. Proportions of OTUs were calculated in MS Office and filtered for OTUs that occurred in at least 0.1% of the respective sample.

We applied functions of the phyloseq package (McMurdie and Holmes 2013) in R (R Core-Team 2013) to estimate the similarity between the samples consisting of reproducibly occurring OTUs only. Therefore, we applied a network analysis based on Jaccard distances and set a threshold for edges being displayed based on a maximum ecological distance <max.dist> between connected nodes of 0.8.

The 479 reproducibly occurring OTUs served as a basis to determine core microbiome members. The core microbiome comprises OTUs, which reproducibly occurred and which were constantly found at all field sites in at least one vegetation stage. The 40 OTUs which met these criteria were defined as 'core OTUs' (SI Dataset 2, Supporting Information). We analyzed the distribution and relative abundances of core OTUs using Microsoft Excel. Venn diagrams were created using JVenn (Bardou *et al.* 2014). Furthermore, the core OTU sequences were analyzed for highest similarities by using blastn of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE˙TYPE=BlastSearch). OTU correlation networks of the core OTUs were predicted using the Conet Application (Faust *et al.* 2012) in Cytoscape 3.0. (Shannon *et al.* 2003) using Pearson correlation, Spearman correlation, and Bray–Curtis and Kullback–Leibler dissimilarities for inference measurement of OTUs (1000 permutations). We searched for clusters in the resulting OTU inference network using MCODE (Bader and Hogue 2003), applying a node score cutoff of 0.2 and

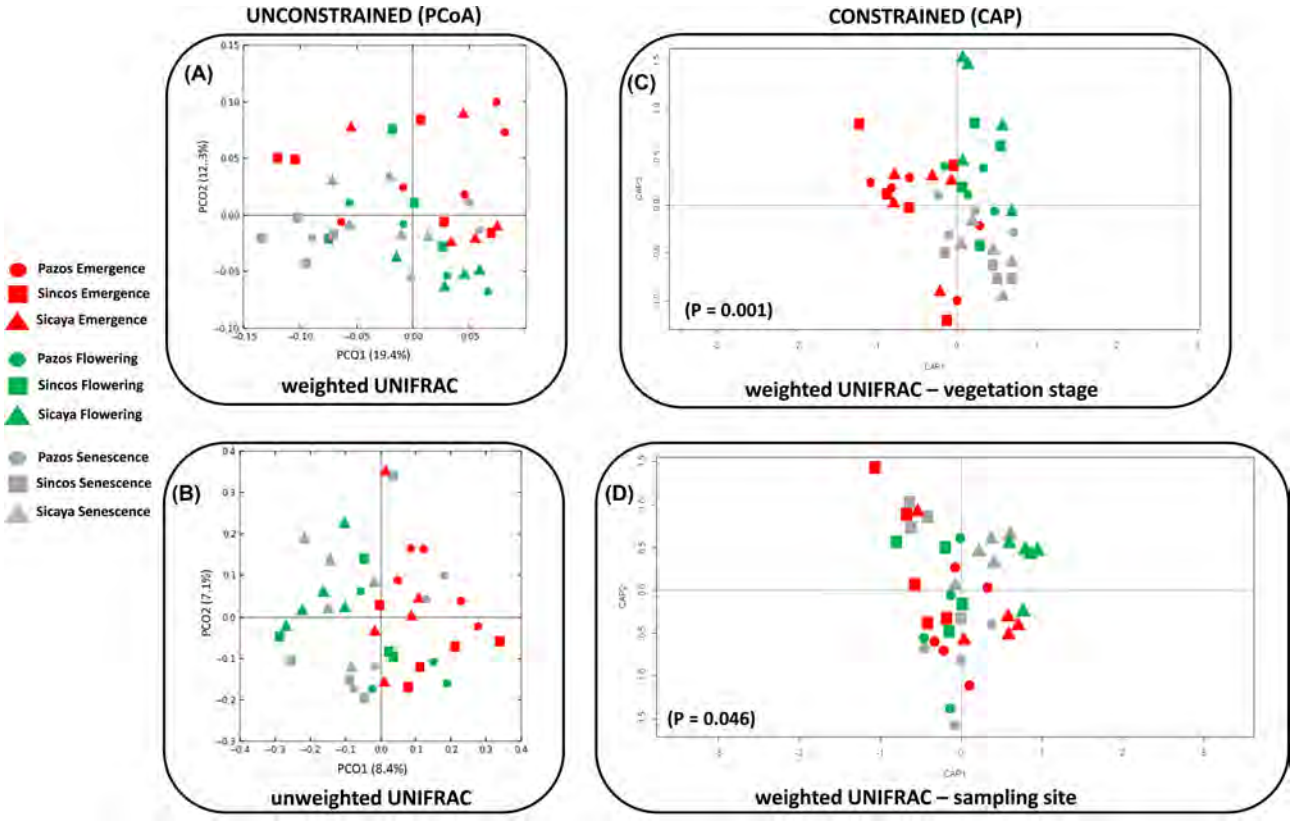


Figure 2. (**A** and **B**) Effects of the sampling sites and the vegetation stages on the bacterial community structures. Analyses were calculated on 41 samples. (A and B) Unconstrained PCoA. The variance explained by PCoA axis is given in parenthesis. (**A**) PCoA based on weighted UNIFRAC distances. Symbols display the three sampling sites, colors display the three vegetation stages as shown in the legend. (B) PCoA based on unweighted UNIFRAC distances. (**C** and **D**) CAP of bacterial communities based on weighted UNIFRAC distances (PERMANOVA with 999 permutations). The permutation-based level of significance is displayed on each plot in parentheses: (**C**) constrained dispersion maximized by vegetation stage (**D**) and constrained dispersion maximized by sampling site.

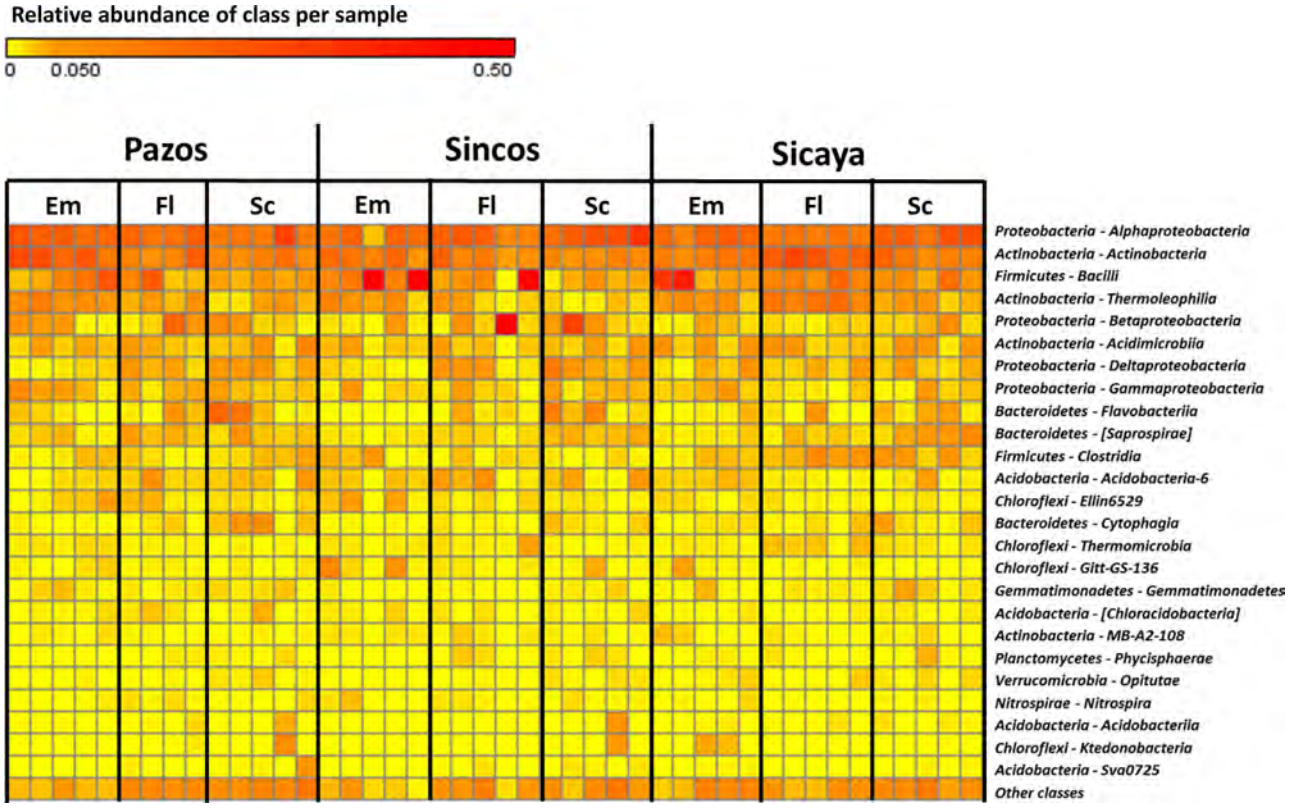


Figure 3. The heat map shows the proportion of classes at individual rhizosphere samples of each field site at the three vegetation stages: (Em) emergence, (Fl) flowering, (Sc) Senescence. The relative abundance of each class per sample is colored in shades of yellow (0% relative abundance) and red (0.5 = 50% relative abundance) as shown in the color key. Classes that represent more than 1%of the microbiome are listed individually; the remaining classes are grouped into 'Other classes'.

filtering out clusters that lack a minimum of two edges per interconnected core. Using GENE-E, a heatmap was generated to show the proportions of individual core microbiome OTUs on the full quantity of OTUs for each sample.

RESULTS

Plant development versus site-dependent effects on rhizosphere microbial diversity

We focused on the particular effects of the plant vegetation stage and sampling site on the rhizosphere microbiome of the potato cultivar Yungay grown in its original habitat of *Solanum tuberosum*, the Central Andean Highlands. Principal component analysis showed differences between the physicochemical and geographical parameters of the three investigated sites: Pazos, Sincos and Sicaya (Fig. 1, Fig. S1, Supporting Information).

OTU richness and phylogenetic depth depended prevailingly on the sampling site, probably due to the vast differences in soil properties, altitudes and climatic conditions but did not differ significantly between particular sampling sites or vegetation stages (Fig. S2, Supporting Information). Simpson's Index of Diversity 1–D and Shannon diversity index showed a high level of diversity in all sample collections (Table S1, Supporting Information).

Unconstrained PCoA of weighted UNIFRAC distances could not distinguish between field sites. On the second axis, a slight effect of the vegetation stage could be observed (Fig. 2A). PCoA ordinations of unweighted UNIFRAC distances showed a tendency to distinguish between emergence and later vegetation stages on the first axis (Fig. 2B). CAP revealed the effects of vegetation stage and sampling site which were insufficient to separate the samples in the unconstrained analysis. CAP on weighted UNIFRAC distances showed a clear discrimination between samples belonging to different vegetation stages (Fig. 2C), which was confirmed by PERMANOVA on the factors of the models (vegetation stage effect $P = 0.001$). The CAP showed less discrimination between samples belonging to different sampling sites (PERMANOVA: $P = 0.046$; Fig. 2D). ANOSIM on weighted UNIFRAC distances (vegetation stage effect $P = 0.005$; sampling site effect $P = 0.097$) confirmed the results of the CAP and PERMANOVA. CAP discriminating between field sites at individual vegetation stages showed no significant influence at the emergence ($P = 0.883$) and flowering stages ($P = 0.128$) but a clear discrimination between sites at the senescence stage ($P = 0.019$) following PERMANOVA (Fig. S3, Supporting Information). ANOSIM on qualitative measures (unweighted UNIFRAC) showed no significant differences between the sampling sites ($P = 0.165$), indicating that the rhizosphere community composition between field sites is more influenced by quantitative aspects rather than qualitative ones. This was underlined by ANOSIM of calculated Bray–Curtis dissimilarity matrix, which displayed a significant influence of both factors (vegetation stage $P = 0.006$; sampling site $P = 0.008$).

The bacterial community was dominated by *Proteobacteria*, *Firmicutes* and *Actinobacteria*. The proportions of the two most abundant classes *Alphaproteobacteria* and *Actinobacteria* on the whole bacterial microbiome were relatively stable, while proportions of *Firmicutes* and *Betaproteobacteria* strongly fluctuated (Fig. 3, Table S2, Supporting Information). LDA (Segata *et al.* 2011) revealed that the vegetation stages caused significant effects on different taxonomic levels of phylogenetic groups (Fig. S4, Supporting Information). Significantly higher abundances were

observed for *Actinobacteria*, in particular at the flowering stage. *Proteobacteria*, particularly *Sphingomonadacae* and *Bacteroidetes*, were enriched at the senescence stage and *Bacilli* at the emergence stage (Fig. S4). In contrast to the effect of the vegetation stage, the field environment exhibited its effects mostly on less abundant phyla/classes. In particular, the phylum *Gemmatimonadetes* and the classes *Sphingobacteriia* and *Acidobacteriia* were significantly more abundant at Pazos, while *Clostridia* had significantly higher LeFSe abundance values at the Sicaya field site (Fig. S5, Supporting Informatin).

Identification of different microbiome components

We tried to narrow down the soil-borne bacterial community to those OTUs (≥ 97% sequence similarity), which reproducibly occurred and showed a minimum abundance of 0.1% in each sample. The resulting 479 OTUs belonged to seven phyla (Supplementary Dataset 2-1) and comprised only 8.9% of distinct OTUs but accounted for 36.3% of the total OTU abundance. Based on the dataset of reproducibly occurring OTUs, differences were visualized by a network analysis of individual samples, showing stronger association between the flowering and senescence stages compared to the emergence stage, independent of the sampling site (Fig. 4A). The diversity of reproducibly occurring OTUs was largest at the flowering stage at all three field sites, especially at the Pazos field site (Supplementary Dataset 2-1). We further observed that the majority of reproducibly occurring OTUs were vegetation stage specific (Fig. 4B–D). This specificity was least pronounced for the senescence stage, which was also least diverse in reproducibly occurring OTUs (Fig. 4B–D; Supplementary Dataset 2-1).

Based on the reproducibly occurring OTUs, we defined different microbiome components. A total of 40 OTUs were defined as 'core OTUs' (Table S3) being continuously present among replicates at all sites in at least one vegetation stage. The reproducibly occurring OTUs failing these criteria plus the non-reproducibly occurring OTUs were termed as 'opportunistic microbiome'. The core OTUs belonged to six phyla and 26 distinct taxa and constituted 16.9% of the microbiome (Table S3 and S4, Supplementary Dataset 2-2, Supporting Information). The majority of core OTUs were reproducibly detected only at particular vegetation stages, e.g. 22 OTUs were constantly present only at the emergence stage, 6 OTUs at the flowering stage and 1 OTU at the senescence stage (Fig. 5A). Interestingly, there were few OTUs that were constantly present at two out of three developmental stages (e.g. four OTUs that were constantly present at the emergence and the flowering stage, two at the emergence and senescence stage and none at the flowering and senescence stage), whereas five OTUs were consistently present at all developmental stages. These five OTUs were among the most abundant OTUs in all sample collections, encompassing 5.8% of the overall OTU abundance (Fig. 5B, Table S4,

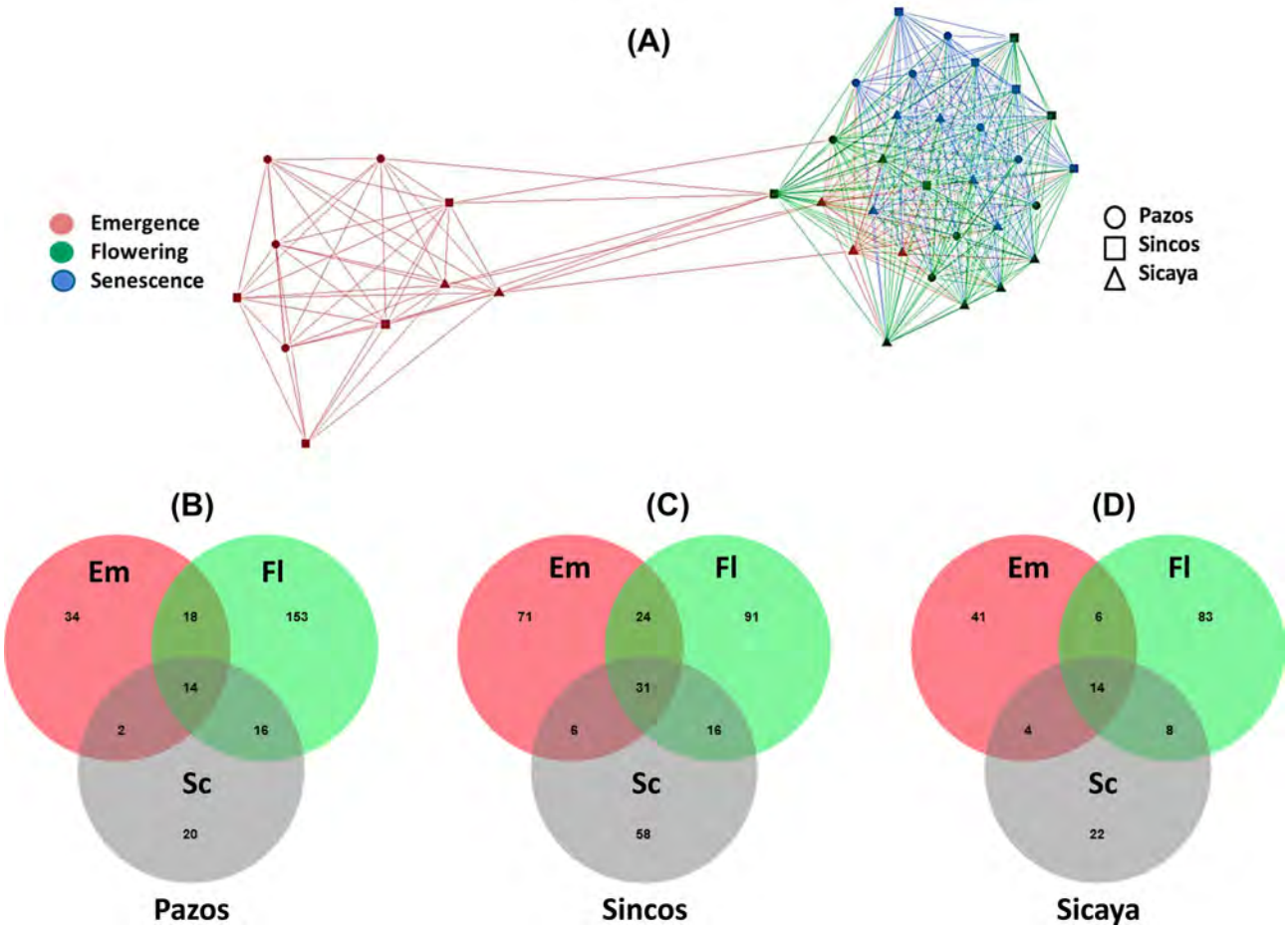


Figure 4. (**A**) Network analysis based on Jaccard distances of the samples based on reproducibly occurring OTUs. Colors of symbols and lines represent different vegetation stages, symbols represent different field sites; (**B–D**) venn diagrams of reproducibly occurring OTUs at the three different field sites Pazos (**B**), Sincos (**C**) and Sicaya (**D**). Red circle—emergence stage; green circle—flowering stage; gray circle—senescence stage.

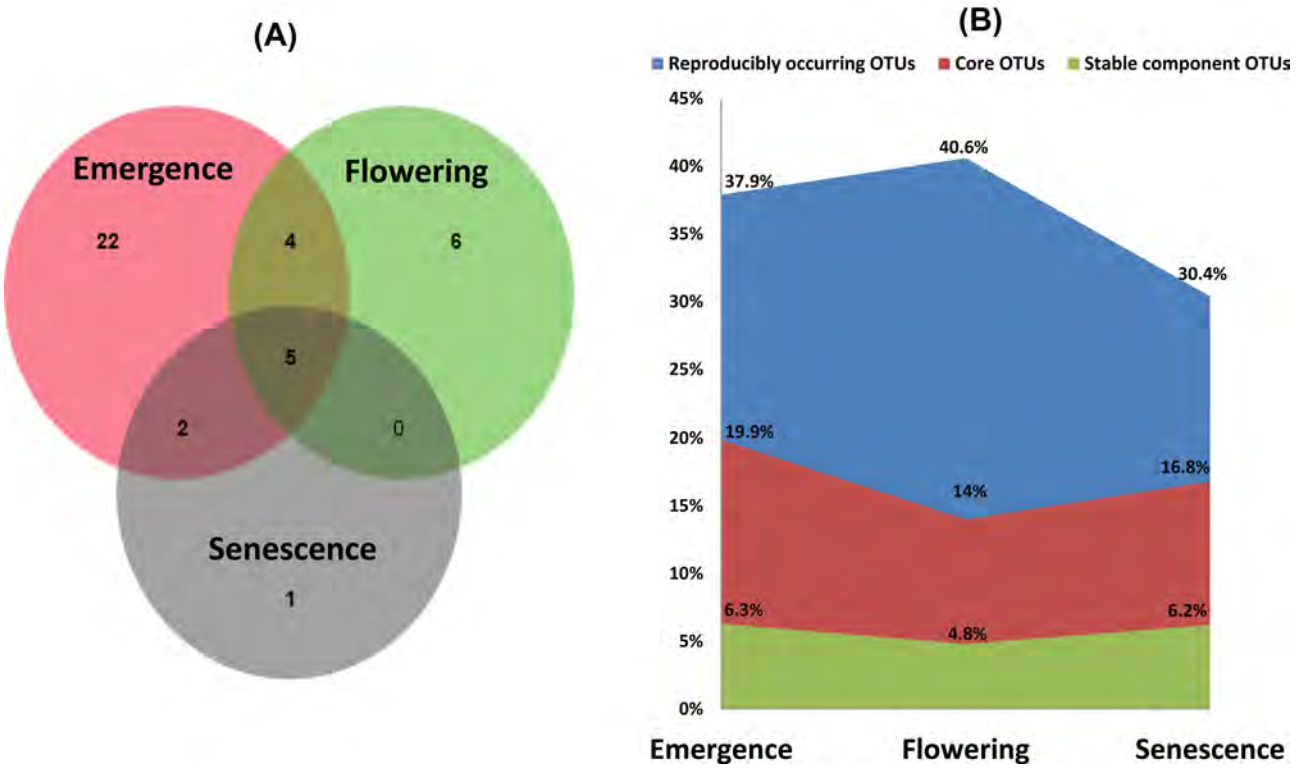


Figure 5. (**A**) Venn diagram of reproducibly occurring OTUs that occur reproducibly at all three sampling sites in at least one vegetation stage, representing the core microbiome. (**B**) Proportion of reproducibly occurring OTUs, dynamic component core OTUs and stable component core OTUs on the total OTU abundance at three vegetation stages.

Supplementary Dataset 2-2). Consequently, we defined different fractions of the 40 core OTUs—a stable component comprising (five) OTUs that were consistently present in high abundance in all samples irrespective of the vegetation stage, and a dynamic component comprising those (35) core OTUs present at all sites, but not throughout all stages of plant development (Fig. 5B, Table S4, Supplementary Dataset 2-2). Using classification and nucleotide blast, the stable component OTUs were assigned to *Microvirga zambiensis* (OTU11), *Bradyrhizobium* sp. (OTU13), *Sphingobium vermicomposti* (OTU4), the genus SMB53 of the *Clostridiaceae* (OTU33) and the actinobacterial species *Blastococcus* sp. (OTU28). Several OTUs showed 100% sequence identity to characterized bacterial isolates (Table S3), e.g. the stable component OTUs OTU11 (Ardley *et al.* 2012), OTU28 (Buerger *et al.* 2012), OTU4 (Vaz-Moreira *et al.* 2009) or OTU13 (Ramírez-Bahena *et al.* 2013) (Table S3).

A detailed network analysis of the 40 core OTUs indicated tight associations between the members of the core microbiome that could be distinguished into three OTU clusters (Fig. 6). One cluster (MCode score = 5.4) consisted of OTUs prevalently found at the early growth stage (Fig. 6A). Another cluster (MCode score = 5.3) indicated a correlation of stable core OTUs of the *Alphaproteobacteria* and associated OTUs (Fig. 6B). A third cluster (MCode score = 3.2) indicated a specific correlation of the stable core OTUs, OTU33 and OTU28, and four OTUs that were constantly present at the emergence and flowering stages and taxonomically affiliated to the *Bacillales* (Fig. 6C). The heat map of rank-normalized core OTUs confirmed our observations. Stable core component OTUs were relatively highly abundant in most samples independent of the vegetation stage, while most dynamic core component OTUs showed a high affinity to particular vegetation stages or sample collections, while they were almost absent in others (Fig. 7).

DISCUSSION

The analysis of potato plants cultivated in their center of origin, i.e. the Andean highlands, at high altitudes and under low-input farming revealed that the rhizosphere microbiome is differently affected by field/sampling site and plant development. Previously, the same samples were used to analyze the composition of arbuscular mycorrhizal fungi (AMF) communities (Senés-Guerrero *et al.* 2013). The study showed that the AMF species richness was highest at the Pazos field site and that the diversity of AMF species increased throughout the stages of plant development. For Bacteria, which we analyzed in this study, richness (α-diversity) was influenced by the sampling site representing different soil properties, altitudes and climatic conditions, while effects of plant development on α-diversity were less pronounced. Diversity metrics could not detect significant differences between the sampling sites and vegetation stages. The climatic and soil physical characteristics, such as pH, influence soil bacterial diversity, the interaction between plants and microbes in the rhizosphere as well as the development of plant roots (Lauber *et al.* 2009; Neumann *et al.* 2014; Schreiter *et al.* 2014). Thus, higher richness found in our study, e.g. at the Pazos field site, might be due to its higher acidity and nutrient content compared to the other field sites. However, we measured soil characteristics of bulk soil, which might differ from rhizosphere soil characteristics.

In contrast, bacterial β-diversity was significantly affected by the vegetation stage, taking into account the phylogenetic

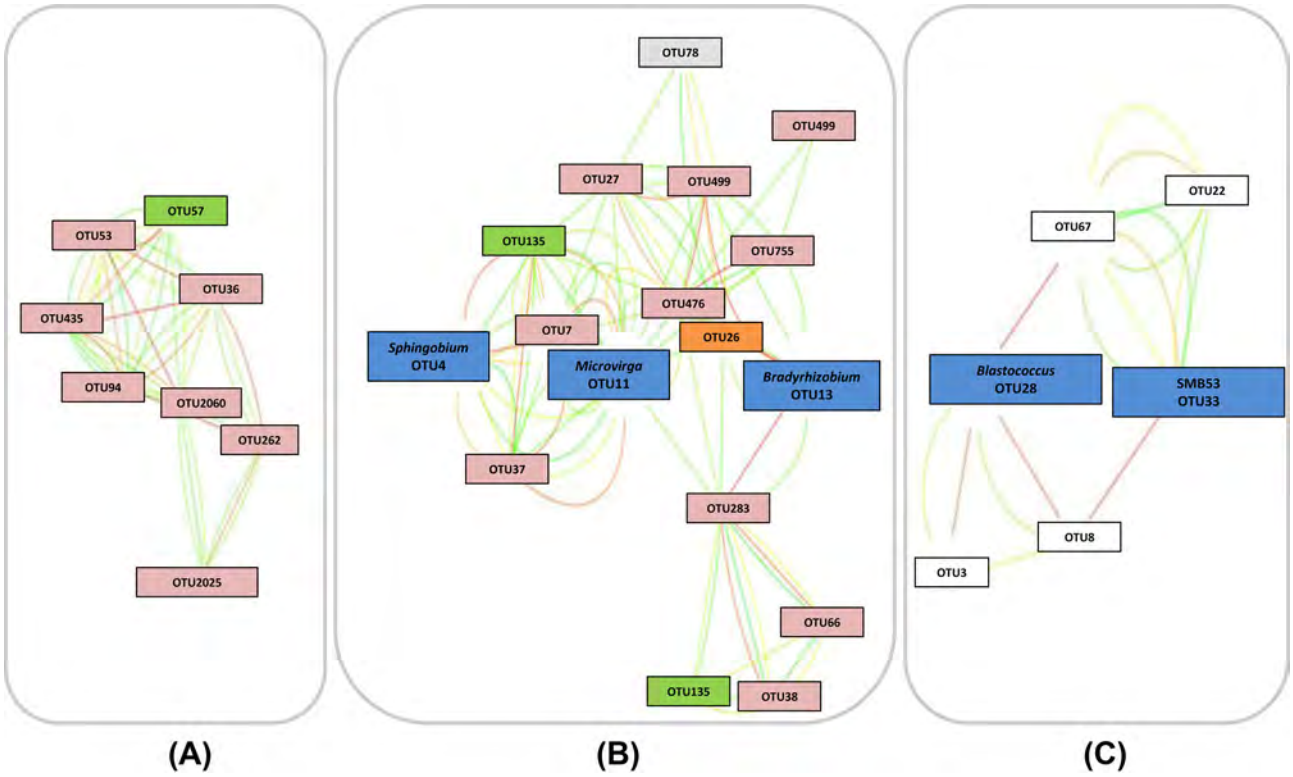


Figure 6. CoNet generated OTU inference network of the 40 core OTUs, based on Spearman correlation, Pearson correlation and Bray–Curtis and Kullback-Leibler distances (1000 permutations). Highly connected subnetworks clustered via MCODE (MCODE scores > 2) are displayed. Line color: green>yellow>red indicates the weight of the correlation between two OTUs (number of connections + OTU abundance). **(A)** OTUs prevalently found at the early growth stage. Box colors: red—dynamic core OTUs of the emergence stage; green—dynamic core OTUs of the flowering stage (Fl); **(B)** stable core OTUs of the *Alphaproteobacteria* and associated OTUs. Box colors: blue—stable core component OTUs; gray—dynamic core OTUs of the senescence stage (Sc); orange—dynamic core OTUs constantly detected at Em and Sc stage. **(C)** Stable core OTUs—four OTUs belonging to the *Bacillales*, constantly present at the Em and Fl stage. Box colors: white—dynamic core OTUs constantly detected at Em and Fl stage.

(UNIFRAC) distance of samples, and this effect was more pronounced than that of soil environment and altitude. It is well known that plants alter their exudation pattern from carbohydrates to phenolic and nitrogen-based secretion (Gschwendtner *et al.* 2011). Our results are in accordance with other studies documenting an influence of the vegetation stage on potato rhizosphere microbiota (Rasche *et al.* 2006b; İnceoğlu *et al.* 2011, 2013a). Similar to our observations, it was previously shown that the relative abundance of *Alphaproteobacteria* and *Bacteroidetes* increase in the rhizosphere throughout plant development of the common grass *Avena fatua* (Shi *et al.* 2015).

We were able to identify different components of the rhizosphere microbiome (Fig. 8), which follow distinct patterns with regard to their persistence and response to site characteristics or plant development. Most OTUs, the members of 'the opportunistic microbiome', consist of occasionally occurring OTUs and many of them were found in low abundance. This component may include weaker competitors or might be part of specific plant–microbe interactions. The latter might particularly apply to OTUs, which were found in high abundance only in solitary or few rhizosphere samples, which could be recruited upon specific needs (Rudrappa *et al.* 2008). For example, we found several OTUs belonging to the *Betaproteobacteria* that were highly abundant only in few replicate samples. Similarly, OTU 14, classified as *Bacillus safensis*, was found to be highly abundant in only few samples in Sicaya and Sincos.

We identified a core microbiome comprising 40 OTUs, which were found at all sampling sites despite huge differences in soil characteristics, cropping history, climate and altitude. Strikingly, we identified a 'stable' and a 'dynamic' component of the core microbiome (Fig. 6), the latter being highly influenced by the plant developmental stage, whereas members of the stable component were found to be highly abundant irrespective of the vegetation stage or the sampling site (Fig. 5B, Tables S3 and S4). Moreover, the stable component contained genera, which are known for their plant growth-promoting abilities, even though here we cannot conclude from OTUs on functional characteristics. *Bradyrhizobium*, which comprised more than 1.5% of total reads, is well known for its N_2-fixing symbiosis with legumes, but was also reported to be involved in disease suppression of non-leguminous plants such as reddish (Antoun *et al.* 1998) and linked with possible N_2-fixation activity in non-leguminous plants (Terakado-Tonooka, Fujihara and Ohwaki 2013). *Sphingobium* was reported to be highly abundant in the rhizosphere of various maize cultivars (Peiffer *et al.* 2013). A *Sphingobium* isolate effectively colonized and showed disease suppression in *Arabidopsis thaliana* (Innerebner, Knief and Vorholt 2011). Another representative of the stable component, *Microvirga zambiensis*, was previously shown to fix N_2 (Ardley *et al.* 2012), and to play a role in triggering defense mechanisms of plants against larvae feeding (Badri *et al.* 2013). The dynamic component of the core microbiome, however, depended on the plant development, most likely due to specific selection by root exudation. Most strikingly, several actinobacterial OTUs were constantly found in high abundance at the early plant development stage. Generally, OTUs of the core microbiome belong to taxa that are usually

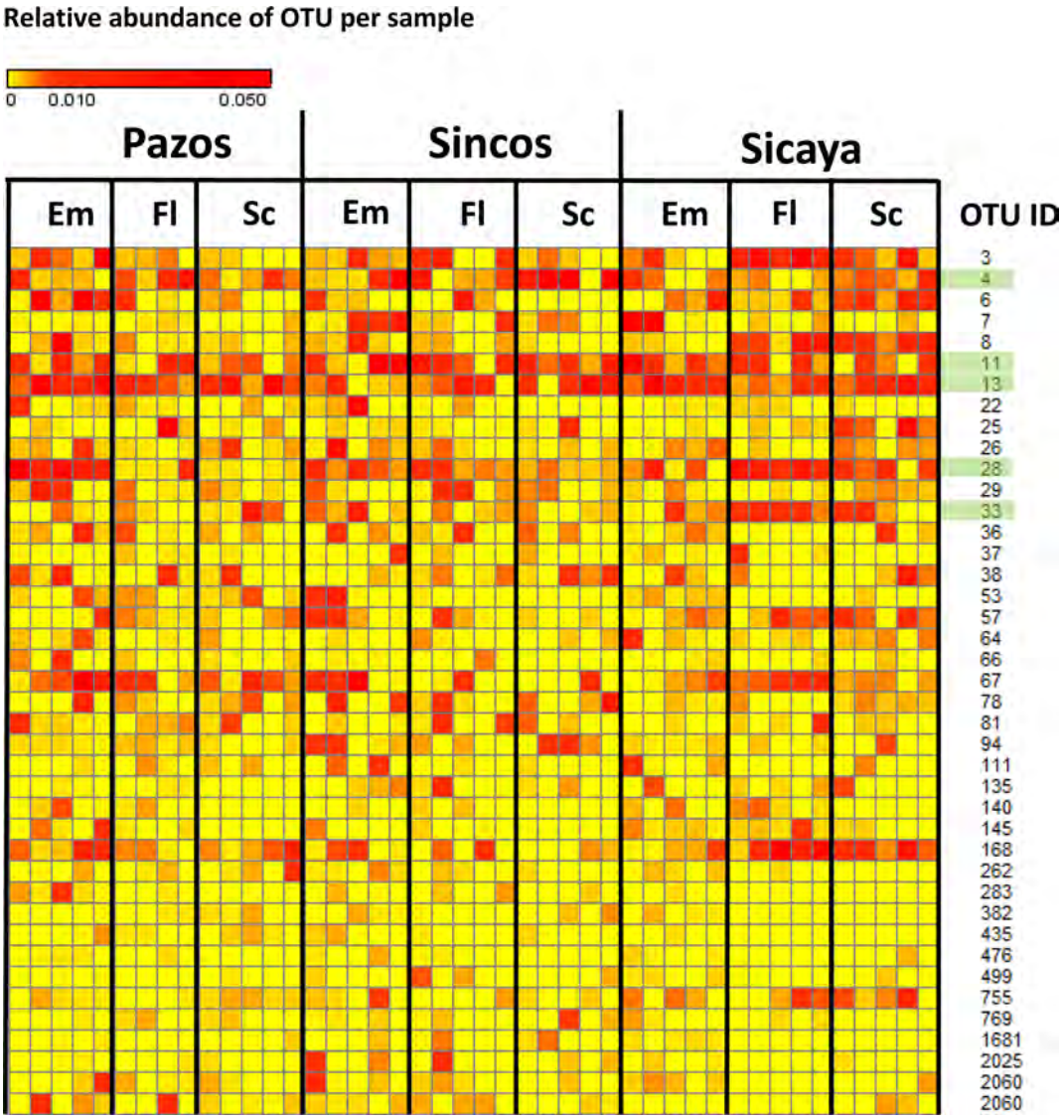


Figure 7. The heat map shows the proportion of each core OTU on the full quantity of OTUs at individual rhizosphere samples of each field site at the three vegetation stages: (Em) emergence, (Fl) flowering, (Sc) senescence. The relative abundance for each OTU is colored in shades of yellow (0% relative abundance) and red (0.050 = 5% relative abundance) as shown in the color key. OTUs are listed by their numeric identifier (ID). OTU IDs displayed on a green background represent stable component core OTUs.

motile and a majority has been previously found in rhizosphere environments. For instance, many *Bacillus* species are known plant growth promoters (Vacheron *et al.* 2013). *Ramlibacter* was previously reported to be enriched in the rhizosphere of potatoes (İnceoğlu *et al.* 2013b). *Variovorax paradoxus* 5C-2 showed multiple plant growth-promoting effects on pea (Jiang *et al.* 2012), whereas many *Actinobacteria* are known for their biosynthetic potential (Hopwood 2007; Azman *et al.* 2015; Corretto *et al.* 2015; Parrot *et al.* 2015). Most members of the dynamic component were found in most of the nine individual sample collections. Thus, the reason why many of the 'dynamic component' OTUs fail the 'stable component' criteria might be that the sequencing depth was not sufficient to detect the OTUs constantly under all conditions, bearing in mind the high level of diversity in rhizosphere soils (Roesch *et al.* 2007; Sugiyama *et al.* 2014). Thus, a higher sequencing depth might identify some of the current dynamic core microbiome members as stable component taxa.

It could be argued that the rhizosphere microbiome and fractions of it, such as the 'dynamic core microbiome', do not (only) change with plant development, but that also bulk soil microbiomes change with time. This question was not addressed in this study, but it has been reported in several studies that rhizosphere and bulk soil microbiota are only distantly related. For example, a study focusing on the effect of vegetation stage and six different cultivars on the potato-associated rhizosphere microflora at one field site in the Netherlands showed a clear difference of the bacterial community composition and the bacterial community dynamics between bulk soil and the rhizosphere (İnceoğlu *et al.* 2011). Furthermore, it was shown for other crops, such as soybean (Sugiyama *et al.* 2014), wheat (Donn *et al.* 2015) or grass (Shi *et al.* 2015), that the community changes of rhizosphere bacteria over the growth season stand only in a loose relationship with seasonal changes in the soil microbial community.

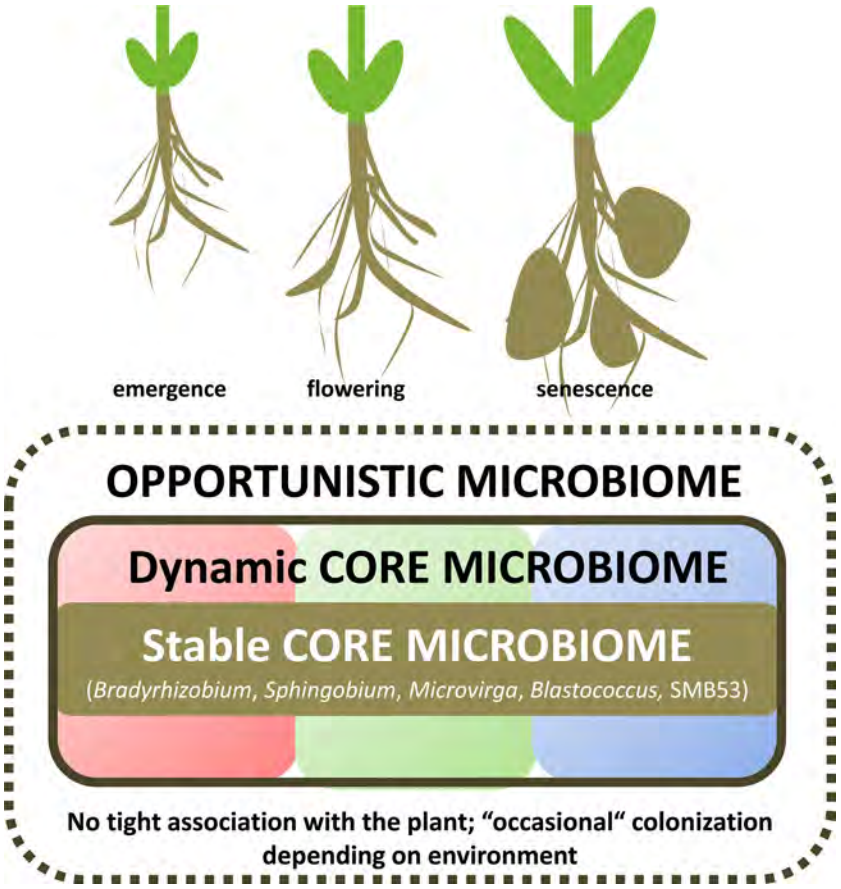


Figure 8. Scheme showing the proposed rhizosphere microbiome components. The opportunistic microbiome comprises occasionally occurring or specifically enriched OTUs, whereas core microbiome OTUs are found at all sites. The dynamic component of the core microbiome includes OTUs colonizing only at specific vegetation stages, while the stable core OTUs are found throughout plant development. Red—emergence stage; green—flowering stage; blue—senescence stage.

An interesting question is, whether the members of the core microbiome also associate with other potato varieties and under even more different environmental conditions, which could point toward an even closer association between plant and core OTUs. İnceoğlu *et al.* (2011) performed a study on potato rhizosphere microbial communities using high-throughput 16S rRNA gene amplicon sequencing. They analyzed the effects of vegetation stage and six different cultivars on the potato-associated rhizosphere microflora at one field site in the Netherlands. Analysis of the 10 most abundant genera revealed that the genus *Sphingobium* was present in large numbers in most samples. Weinert *et al.* (2011) observed co-occurrence of several distinct OTUs via Phylochip analysis on rhizosphere microbial communities at two potato field sites. Furthermore, in a recent study on potato at different potato field sites in the USA, Barnett *et al.* (2015) reported the continuous presence (≥ 50% samples) of root-associated alphaproteobacterial taxa (e.g. *Sphingobium*, *Bradyrhizobium*), which were among the members of the stable component core microbiome shown in our study.. However, more investigations are needed to elucidate whether specific microbial taxa associate with different potato genotypes cropped under distinct field conditions.

It is well known that the rhizosphere microbiome or plant-associated microbial communities are generally influenced by root exudates and thereby change throughout the plant development (Gschwendtner *et al.* 2011; Chaparro *et al.* 2013; Chaparro, Badri and Vivanco 2014). However, it is striking to find stable and highly abundant core microbiome OTUs, which seem to have a very tight association with potato. These OTUs are likely versatile in function, competitive, able to metabolize different types of root exudates and potentially associated with potato at planting (e.g. as endophytes). The finding of distinct microbiome fractions could also indicate that root exudation follows a similar pattern with plant development, i.e. that certain root exudates are constantly secreted by roots and feed or attract stable core microbiome members, whereas dynamic core microbiome members respond to root exudates changing with plant development. In future, experiments testing different cultivars and environmental conditions could help to reveal the degree at which the rhizosphere microbiome composition can be attributed to species-specific ecological host adaptation or the phylogenetic distance of the host. Similarly, isolation of bacteria of core and dynamic microbiome components could open new paths in studying plant–microbe interactions. Recently, a study on *A. thaliana* showed that synthetic bacterial communities following the isolation of the majority of the root and leaf bacteria can be reestablished in a gnotobiotic plant system (Bai *et al.* 2015). Another perspective could be opened by combining microbiome data with transcriptomics or stable-isotope probing to gain further insight into the role of rhizosphere bacteria throughout plant development.

In conclusion, the results from our study support the idea that the rhizosphere of potato plants cultivated under highly different conditions shares a number of core bacterial taxa. Those particular taxa seem to be tightly associated with potato irrespective of site and vegetation stage.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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