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Bacterial networks and co-occurrence relationships in the lettuce root microbiota

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Summary

Lettuce is one of the most common raw foods worldwide, but occasionally also involved in pathogen outbreaks. To understand the correlative structure of the bacterial community as a network, we studied root microbiota of eight ancient and modern *Lactuca sativa* cultivars and the wild ancestor *Lactuca serriola* by pyrosequencing of 16S rRNA gene amplicon libraries. The lettuce microbiota was dominated by Proteobacteria and Bacteriodetes, as well as abundant Chloroflexi and Actinobacteria. Cultivar specificity comprised 12.5% of the species. Diversity indices were not different between lettuce cultivar groups but higher than in *L. serriola*, suggesting that domestication lead to bacterial diversification in lettuce root system. Spearman correlations between operational taxonomic units (OTUs) showed that co-occurrence prevailed over co-exclusion, and complementary fluorescence in situ hybridization-confocal laser scanning microscopy (FISH-CLSM) analyses revealed that this pattern results from both potential interactions and habitat sharing. Predominant taxa, such as *Pseudomonas*, *Flavobacterium* and *Sphingomonadaceae* rather suggested interactions, even though these are not necessarily part of significant modules in the co-occurrence networks. Without any need for complex interactions, single organisms are able to invade into this microbial network and to colonize lettuce plants, a fact that can influence the susceptibility to pathogens. The approach to combine co-occurrence analysis and FISH-CLSM allows reliably reconstructing and interpreting microbial interaction networks.

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Introduction

Plants are associated with diverse microbial communities and invest a substantial amount of energy in providing rhizodeposits to nurture their root microbiota, which is known for its importance on growth and health of their hosts (Mendes *et al.*, 2011; Philippot *et al.*, 2013). Because plants markedly vary in these parameters, specific microbiota were found associated with different host plants (Berg and Smalla, 2009; Bulgarelli *et al.*, 2012). One of the primary questions is to what level the specificity of microbiota is maintained in related plants. Several studies suggest a small but significant effect on the rhizosphere depending on the plant genotype (Smalla *et al.*, 2001; Schweitzer *et al.*, 2008; Weinert *et al.*, 2011; Bulgarelli *et al.*, 2012). Even the phylogenetically oldest land plants on Earth, the bryophytes, show an outstanding degree of plant specificity and diversity across closely related species (Opelt *et al.*, 2007; Bragina *et al.*, 2012), but it is still unclear at what level specific microbiota emerge. In maize, genetic differentiation through crop diversification was identified as a significant factor for plant–microbe interactions (Bouffaud *et al.*, 2012; Peiffer *et al.*, 2013). It still remains to be studied what ecological role these interactions have and what general function microbial diversity could have in the plant habitat (McGrady-Steed *et al.*, 1997). Recently, it was shown that plant-associated microbial diversity at both structural and functional levels is crucial to hamper pathogen invasions (Balint-Kurti *et al.*, 2010; Latz *et al.*, 2012; Van Elsas *et al.*, 2012) but the underlying principles are not well understood. To shed new light on this phenomenon, analysis of co-occurrence networks as developed by Barberán and colleagues (2012) could be utilized. They already helped to identify microbial co-occurrence relationships in the human microbiota (Faust *et al.*, 2012), but have not been applied to complex plant microbiota.

Cultivated lettuce is among the most popular raw-eaten vegetables in health-conscious societies because of its nutritional value. Lettuce-associated outbreaks of pathogens, however, are a recurrent health threat known throughout the world and primarily caused by enterics (Teplitski *et al.*, 2011; Ongeng *et al.*, 2013). Because

bacterial strains originating from soil can extend via the endosphere in the whole lettuce plant, it is therefore particularly important to know about the root microbiota and its implications on lettuce health and safety. Lettuce cultivars originated from different regions of the world and the complex breeding lines are hardly dated with precision (de Vries, 1997). Mediterranean and Chinese cultivars are regarded as the oldest, and tomb wall paintings of garden lettuce appear in numerous Old Kingdom and Middle Kingdom tombs in Egypt showing evidence for cultivation since 2680 BC (Křístková *et al*., 2008; Zohary *et al*., 2012). Today, lettuce cultivars are classified in 'cultivar groups'. Basically, these comprise phenotypes either forming either typically closed heads (convar. *incocta* Helm), or open forms (convar. *sativa* Helm). Closed heads are characteristic, for example, in the cultivar groups crisphead and butterhead lettuce, while open forms include the cultivar groups Cos and stalk lettuce. So far, studies of the lettuce microbiota demonstrated that this morphological difference is more influential on the structure of the phyllosphere microbiota than the lettuce genotype (Hunter *et al*., 2010; Rastogi *et al*., 2012). In contrast, the root-associated microbiota has not yet been studied at this level. We hypothesize that breeding of lettuce resulted in diversified microbiota at belowground level. In addition, we argue that the understanding of the indigenous lettuce network is imperative to limit pathogen outbreaks in the future.

The objective of this study was to investigate the specificity, diversity and interactions of the root microbiota associated with four main *Lactuca sativa* cultivar groups: Cos and stalk (subspecies. *longifolia* and *augustana*, respectively) belonging to the convar *sativa*; crisphead and butterhead (subspp. *crispa* and *capitata*, respectively) belonging to the convar. *incocta*. Additionally, we analysed the wild ancestor *Lactuca serriola*. All plants were intercropped for several years at a unique sampling site provided by the Seed Savers Association 'Arche Noah' (Austria) that preserves crop biodiversity in Europe. We combined 16S rRNA gene amplicon libraries of lettuce root microbiota and corresponding correlation analyses with fluorescence in situ hybridization-confocal laser scanning microscopy (FISH-CLSM) to (i) understand the impact of domestication on lettuce root microbiota, (ii) to assess the effect of host genotype on lettuce microbiota specificity and (iii) to detect potentially interacting bacterial taxa *in situ*. Finally, we discuss our results in relation to health issues.

Results

Structure and diversity of the lettuce root microbiota

A total of 709,021 reads were obtained using pyrosequencing of the 16S rRNA amplicon libraries from 27 root microbiota (24 representing *L. sativa* and three *L. serriola*; Fig. 1). After primer removal and length and quality filtering, 216 466 high-quality reads remained prior to denoising (mean sequence length 296.65 bp, mean GC content 54% ± 2.68; percentage of reads that were annotated at least at phylum level ranged from 98.37%, when calculated with 100% OTU cut-off level, to 99.06%, calculated with 90% OTU cut-off level). Sequences were grouped into OTUs with 90%, 95%, 97% and 100% similarity levels respectively. After removal of both plastid and mitochondrial OTUs, three *L. sativa* samples with a low number of reads were discarded and about 4000 ± 1690 sequences per sample were finally obtained. The properties of the four OTU tables are shown in Fig. S2. The taxonomic assignment of OTUs revealed 38 bacterial phyla, 10 of which exceeded 1% of relative abundance (Fig. S3). The most abundant phylum was Proteobacteria (39.5–40.00%), followed by Bacteroidetes and, surprisingly, Chloroflexi. Gammaproteobacteria was the most abundant class retrieved, irrespective of OTU cut-off level (Fig. S3). Most abundant OTUs (showing relative abundance > 0.5% of the total microbiota) included members of Gammaproteobacteria (Pseudomonadaceae, Xanthomonadaceae and *Cellvibrio*), Betaproteobacteria (Comamonadaceae), Bacteroidetes (*Flavobacterium*, Sphingomonadaceae and Chitinophagaceae), Chloroflexi (Anaerolineae), Acidobacteria-6 and Actinobacteria (*Streptomyces*) (Fig. S4A). These dominant taxa represented a rather high fraction of the microbiota ranging from 31.3% to 74.6% (at 100% and 90% OTU cut-off level, respectively; Fig. S4B).

Shannon, Equitability and Chao 1 indices were highest for the 100% cut-off level OTUs and lower at 97%, 95% and 90% OTU cut-off levels (paired samples *t*-test, $P < 0.001$). Shannon and Equitability indices were significantly higher in *L. sativa* than in *L. serriola* [analysis of variance (ANOVA) $F_{(4,19)} = 8.661$, $P = 0.0004$ and $F_{(4,19)} = 12.861$, $P = 0.00004$, respectively, calculated on the whole microbiota at 97% OTU cut-off level; Fig. 2], indicating that domestication of lettuce lead to the increase of bacterial diversity in the root system. No significant differences were found for Chao1 richness estimator (ANOVA $F_{(4,19)} = 1.338$, $P = 0.294$, calculated on the whole microbiota at 97% OTU cut-off level; Fig. 2). Similar results for both Shannon and Equitability indices were obtained with the other OTU cut-off levels, both on the whole microbiota and after removal of singletons or < 10 reads OTUs (Table S1). Removing singletons had the effect to reveal significant differences between *L. sativa* and *L. serriola* for the Chao 1 richness estimator. Removing the OTUs with < 10 reads resulted in significantly different Chao 1 values between *L. sativa* and *L. serriola* only at 100% and 97% OTU cut-off levels (Table S1). Among *L. sativa* samples, statistically significant differ-

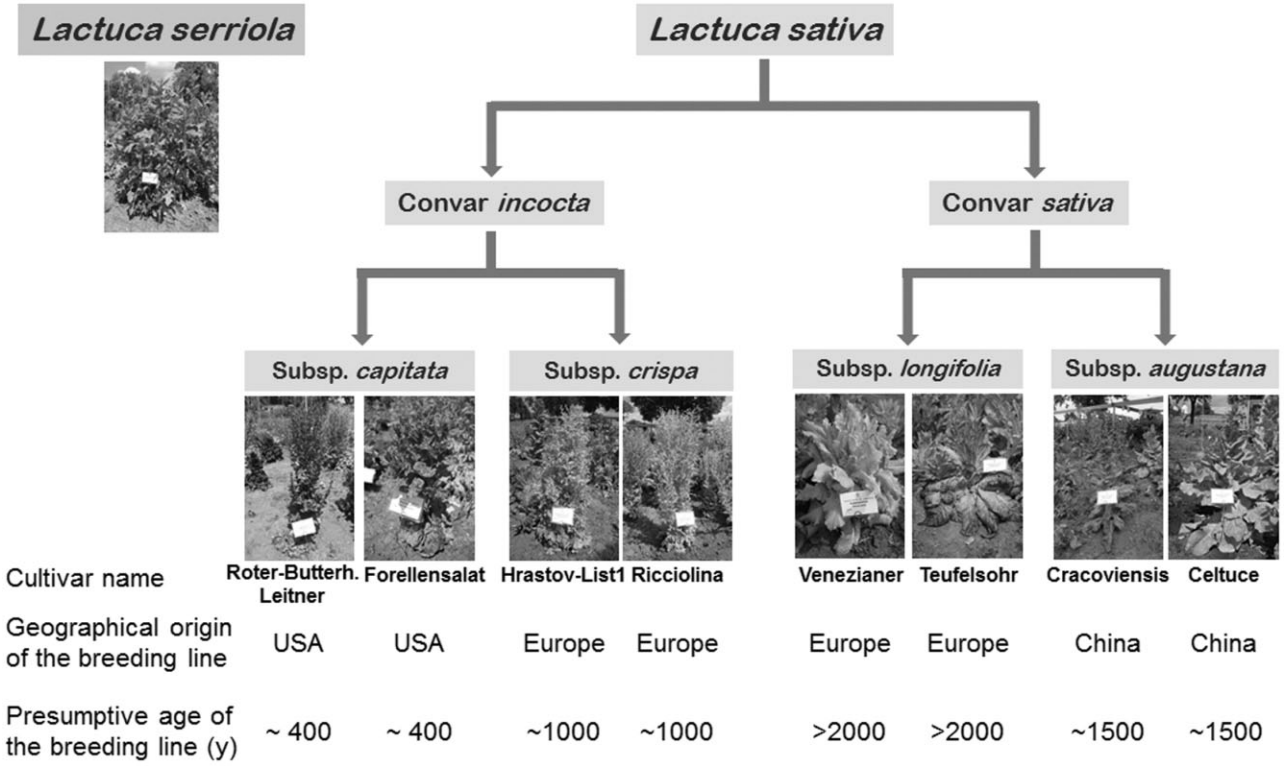

Fig. 1. Sampling scheme. Eight *Lactuca sativa* cultivars and *Lactuca serriola* were collected at the field of the Archae Noah (Schiltern, Austria). Four main cultivar groups (corresponding to subspecies) were sampled. Three replicates per sample were collected.

ences were found for both Shannon and Equitability indices and for Chao1 richness estimator only at cultivar level (Table S1), with the cultivars Hrastov Lst1 (subsp. *crispa*) and Venezianer (subsp. *longifolia*) belonging to the convars *incocta* and *sativa*, respectively, showing the highest diversity (Fig. S4). Since three *L. sativa* samples were removed from the analysis due to the low number or sequence reads (hence three cultivars had only two replicates), differences at cultivar level would require confirmation with more replicates per cultivar.

Root microbiota associated with lettuce were significantly different between subspecies, and more different than between convars (Adonis test on UniFrac weighted pairwise distances, Fig. 3A). UniFrac distances within subspecies were significantly lower than between subspecies (ANOVA $F_{(8,476)} = 3.451$, $P = 0.0007$) (Fig. 3B). Pairwise distances between subspecies were also significantly different: *L. sativa capitata* and *crispa* (belonging to the convar *incocta*) were more different to each other, whereas *longifolia* and *augustana* (belonging to the convar *sativa*) were more similar (Fig. 3B and Fig. S5). These results indicate a prevailing effect of the host genotype over the plant morphology on the structure of the root microbiota. The OTU cut-off level and removal of singletons had a negligible effect on the extent of beta-diversity (Fig. 3A). Moreover, UniFrac distance matrices obtained with different OTU cut-off levels were extremely similar to each other (Mantel test, $r = 0.960–0.973$), similar to the distance matrices obtained with and without singletons at each cut-off level ($r = 0.982–0.990$). Statistical differences between microbiota of lettuce cultivars were comparable or even higher than between those of subspecies (Adonis ≤ 0.001, Fig. S5), pending confirmation by more replicates per cultivar.

Core microbiota and cultivar-specific OTUs

Because beta-diversity was not significantly influenced by the OTU cut-off level (Fig. 3), both shared and cultivar specific fractions of the microbiota were computed only at the 97% OTU cut-off level. Sixty-eight OTUs, representing 48.79% of all reads in the rarefied OTU table, were found in all *L. sativa* microbiota ('core microbiome'), including members of Gammaproteobacteria (15 OTUs), Bacteroidetes (10), Actinobacteria (7), Alphaproteobacteria (7), Acidobacteria (6), Betaproteobacteria (6), Chloroflexi (6), Verrucomicrobia (5), Deltaproteobacteria (2), Saccharibacteria (2), Firmicutes (1) and Planctomycetes (1) (Fig. 4). Five of these shared OTUs, identified as Flammeovirgaceae, Bradyrhizobiaceae, Actinoplanes,

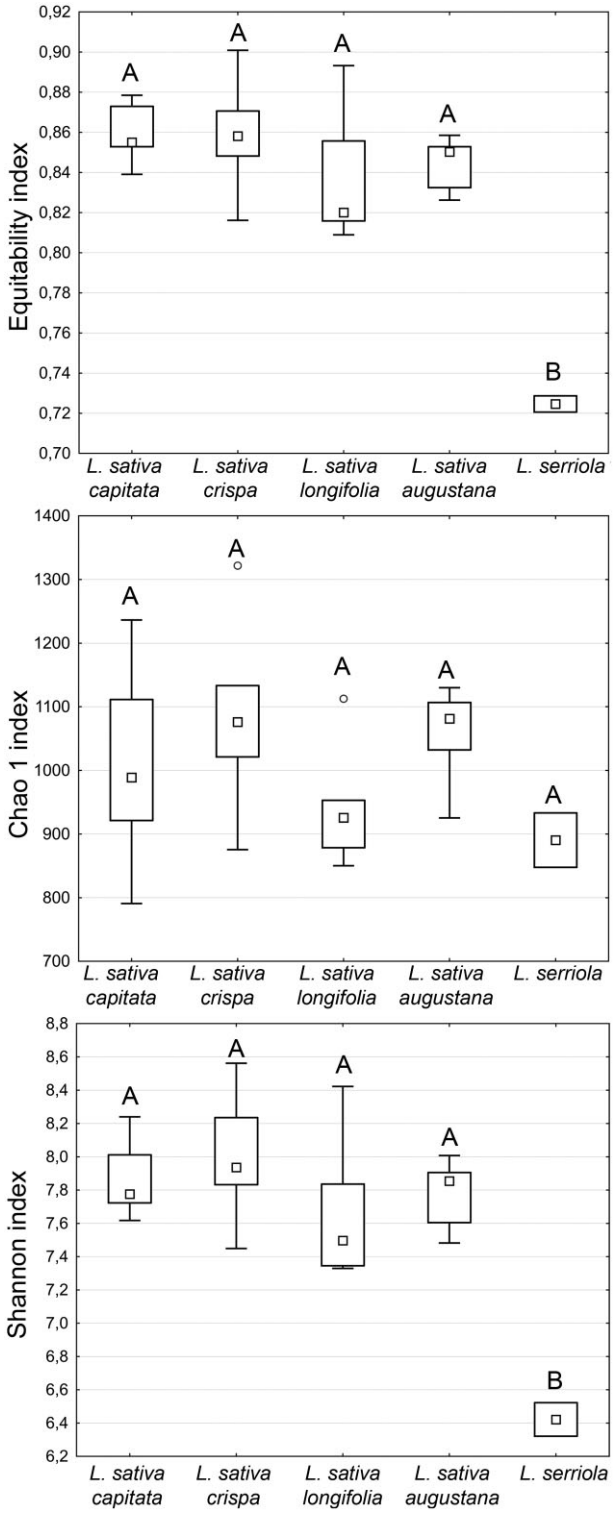

Fig. 2. Alpha-diversity analysis. Comparison of diversity indices between *L. sativa* subspecies and *L. serriola*, calculated on the whole microbiota grouped at 97% similarity level OTUs (including singletons). Different letters indicate significantly different means at $P < 0.05$.

Opitutaceae and Chthoniobacteraceae, respectively, were not detected in *L. serriola*, suggesting that a significant fraction (7.4%) of the shared root microbiota could be highly *L. sativa* specific. These OTUs were homogeneously distributed across the samples.

Seventy-one OTUs showed significantly different relative abundances between lettuce subspecies, as estimated with ANOVA after false discovery rate (FDR) correction (Fig. S6). These OTUs were responsible for significant UniFrac/Adonis differences, and included 12.86% of all reads in the rarefied OTU table. Among such OTUs, members of Planctomycetes (15), Chloroflexi (11), Bacteroidetes (10), Gammaproteobacteria (9), Actinobacteria (4), Betaproteobacteria (4), Verrucomicrobia (4), Acidobacteria (3), Gemmatimonadetes (3), Deltaproteobacteria (2), Alphaproteobacteria (2), Saccharibacteria (2), Elusimicrobia (1) and one unidentified bacterium were found (Fig. S5). The most abundant organisms occurring within such host-specific OTUs were identified as TM7-3 (Saccharibacteria), Acidobacteria-6 (Acidobacteria) and *Cellvibrio* (Gammaproteobacteria; Fig. S6). Interestingly, six shared OTUs showed a significantly different relative abundance between lettuce subspecies (labelled with 'C' in Fig. S6), which indicates a higher affinity of different lettuce cultivars for certain bacteria. These strains included three Gammaproteobacteria, one member of the Alphaproteobacteria, one of the Verrucomicrobia and one belonging to Chloroflexi (Fig. S6).

Correlation analysis of co-occurrence patterns

To reveal potential interactions between bacteria, Spearman correlations between OTUs were calculated based on their occurrence patterns across the 24 *L. sativa* and *L. serriola* samples. The analysis was performed with sufficiently abundant OTUs that were detectable by microscopy (> 0.5% of the total microbiota) because our aim was to detect the colocalization of correlated OTUs *in situ* by FISH-CLSM analyses. A total of 27, 37, 77 and 102 highly significant correlations were found between OTUs at 100%, 97%, 95% and 90% cut-off levels respectively. The decreasing OTU cut-off level revealed a higher number of abundant OTUs (Fig. S4B). The number of correlations per OTU increased, demonstrating that the OTU cut-off level had a strong impact on the analysis (Fig. S7). However, some traits remained unchanged, such as the central position of Gammaproteobacteria and the marginal involvement of Betaproteobacteria. Surprisingly, all correlations found were positive except one between *Streptomyces* and Acidobacteria-6. This negative correlation was present at 95%, 97% and 100% OTU cut-off levels, but not at 90% (Fig. S7). Since it was demonstrated that different organisms (with non-coherent occurrence patterns) can be

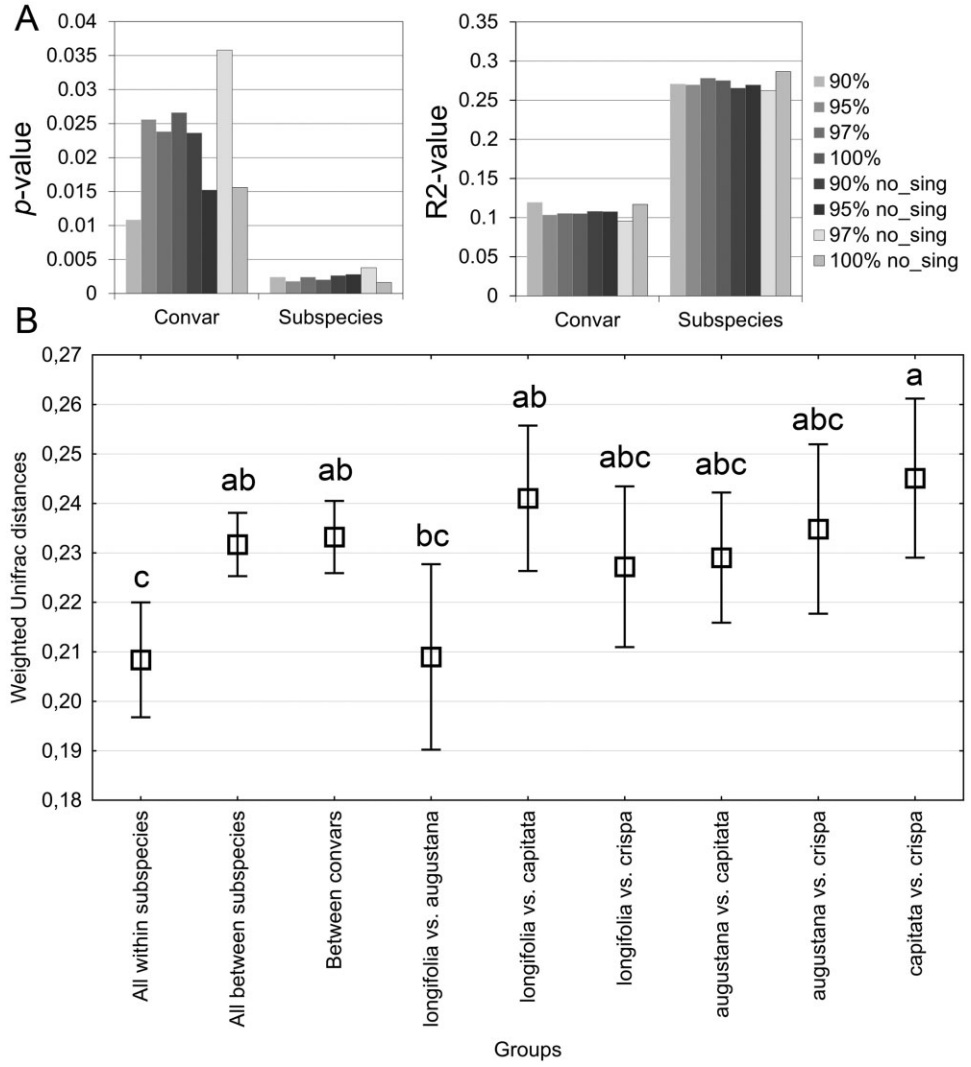

Fig. 3. Beta diversity analysis.
A. Significance of differences (left plot) and fraction of variability explained (right plot) for UniFrac pairwise distances between lettuce convars and between subspecies at different cut-off levels (90 to 100), with and without singletons (no_sing).
B. Pairwise UniFrac distances between groups of both lettuce convars and subspecies. Means with 95% confidence interval are shown. Different letters indicate different means at $P < 0.05$ (Tukey honest significant difference (HSD) test).

grouped into the same OTU even at 99% cut-off level (Patin *et al*., 2013), we avoided potential bias by focusing on the correlations detected with 100% OTU cut-off level (Fig. 5). The correlation network was organized in fully connected subunits (such as Ps-Ps-Fs-Spm and Xa-Xa-An-Spb) (Fig. 5). Correlated out pairs among either Pseudomonadaceae or Xanthomonadaceae (Ps-Ps and Xa-Xa), respectively, could be interpreted as similar organisms (possibly the same species) sharing ecological traits, when they share connections with other OTUs (as suggested by Barberán *et al*., 2012). For example, representative sequences of V4 variable rRNA gene region of two correlated Pseudomonadaceae OTUs (Ps-Ps) showed similarity of 98.35%. The two connected Xanthomonadaceae OTUs (Xa-Xa) were 93.05% similar, and might represent different species but with similar ecology. The two connected Anaerolinae OTUs (An-An) of 86.18% similarity did not share all further connections (Fig. 5), suggesting different species with differential ecological relations in the microbiota. Two OTUs in Sphingomonadaceae of 95.05% similarity were not correlated with each other and share only one connection with *Flavobacterium succinicans* (Fig. 5). We regard these as phylogenetically close species with slightly different ecology. These results demonstrate that phylogenetically close OTUs are often positively correlated, as also shown also by Faust and colleagues (2012) in the human habitat.

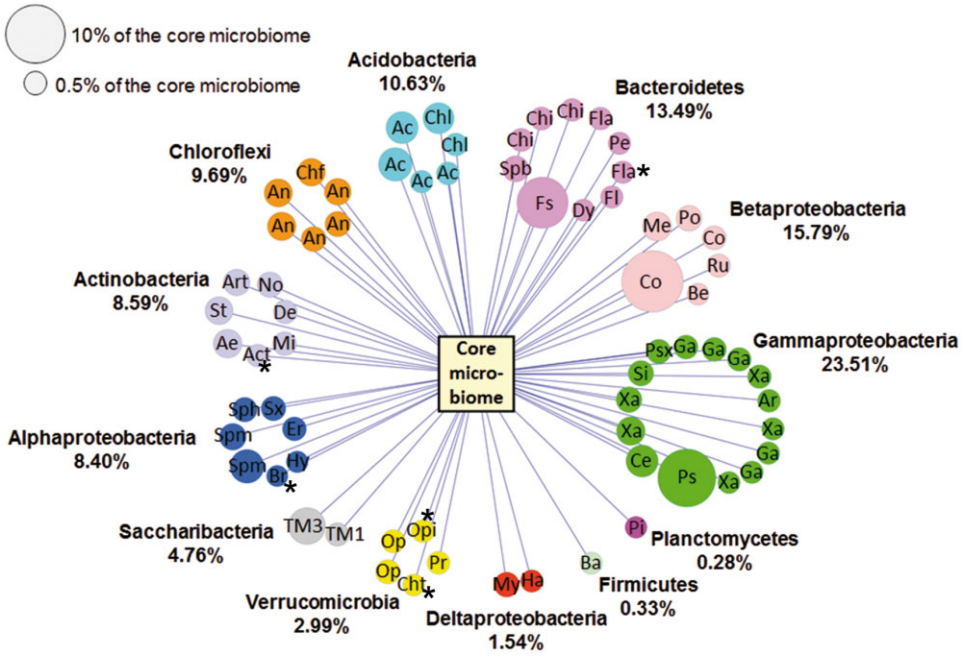

Fig. 4. Core root microbiota of lettuce. Percentage after the phylum/class indicates the respective contribution to the core microbiota. Node labels indicate the taxonomic identification of the OTUs: Ac, Acidobacteria-6; Act, *Actinoplanes*; Ae, *Aeromicrobium*; An, Anaerolineae; Ar, *Arenimonas*; Art, *Arthrobacter*; Ba, Bacillales; Be, Betaproteobacteria; Br, Bradyrhizobiaceae; Ce, *Cellvibrio*; Chf, Chloroflexi; Chi, Chitinophagaceae Chl; Chloracidobacteria, Cht – [Chthoniobacteraceae]; Co, Comamonadaceae; De, *Demequina*; Dy, *Dyadobacter*; Er, Erythrobacteraceae; Fl, *Flavobacterium*; Fla, Flammeovirgaceae A4; Fs, *Flavobacterium succinicans*; Ga, Gammaproteobacteria; Ha, Haliangiaceae; Hy, *Hyphomicrobium*; Me, *Metylotenera*; Mi, Micromonosporaceae; My, Myxococcales; No, Nocardioidaceae; Op, Opitutaceae; Opi, *Opitutus*; Pe, *Pedobacter*; Pi – Pirellulaceae A17, Po; *Polaromonas*, Pr – *Prosthecobacter debontii*; Psx, *Pseudoxanthomonas*; Ps, Pseudomonadaceae; Ru, *Rubrivivax*; Si, Sinobacteraceae; Spb, Sphingobacteriaceae; Sph, *Sphingomonas*; Spm, Sphingomonadaceae; St, *Streptomyces*; Sx – *Sphingobium xenophagum*, TM1; TM7-1 (Saccharibacteria), TM3 – TM7-3 (Saccharibacteria); Xa, Xanthomonadaceae. Asterisks indicate OTUs not detected in the intercropped *L. serriola*.

The relative abundance of the OTUs did not significantly influence the number of co-occurrences (Spearman $R = 0.214$, $P = 0.338$, $n = 22$) which suggests that the most dominant organisms (such as Comamonadaceae) are not necessarily involved in intricate relationships. OTUs with highest numbers of connections in the correlation networks belonged to Chloracidobacteria, Anaerolineae and Sphingobacteriaceae (six correlations each), followed by *Flavobacterium succinicans* and Pseudomonadaceae (five correlations each) (Fig. 5). When grouped by phylum or class, Gammaproteobacteria showed the highest number of total correlations, followed by Bacteroidetes and Chloroflexi (16, 11 and 8 correlations, respectively, among 5, 2 and 3 OTUs, respectively, at 100% OTU cut-off level; Fig. 5 and Fig. S7D). Consequently, the highest connectivity was shown by Bacteroidetes (5.5 average correlations per OTUs), followed by Gammaproteobacteria (3.2 average correlations per OTUs) (Fig. 5 and Fig. S7D). Betaproteobacteria, however, were the less connected (only one correlation among four OTUs; Fig. 5 and Fig. S7D). The correlation network for the genotype-specific OTUs, which excludes any effects resulting from soil, revealed that 51 of these OTUs (out of 71) were involved in a total of 76 correlations (65 positive and 11 negative), organized in a core module with 'branches' and three small correlated modules (Fig. S8). Similar to the general correlation network, the Gammaproteobacteria and the Anaerolinae seem to play a key role for the establishment of the microbiota sociability. Moreover, the marginal involvement of Betaproteobacteria was also confirmed. As an important new feature compared with the general network, the Planctomycetes OTUs appear highly involved in co-occurrences, also in the smaller modules.

Colonization patterns analysed by FISH-CLSM

All major taxa (Gammaproteobacteria, Bacteroidetes, Chloroflexi, Betaproteobacteria, Actinobacteria, Acidobacteria, Alphaproteobacteria and Saccharibacteria) were detected by FISH-CLSM either on the root surface and/or in the endorhiza of lettuce. Alphaproteobacteria were found in 100% of confocal stacks where the alpha-specific probe was used, Chloroflexi in 95.2%, Betaproteobacteria in 88.9%, Gammaproteobacteria in 82%, Actinobacteria in 68.75%, Bacteroidetes in 62.9%, Acidobacteria in 42.9%.

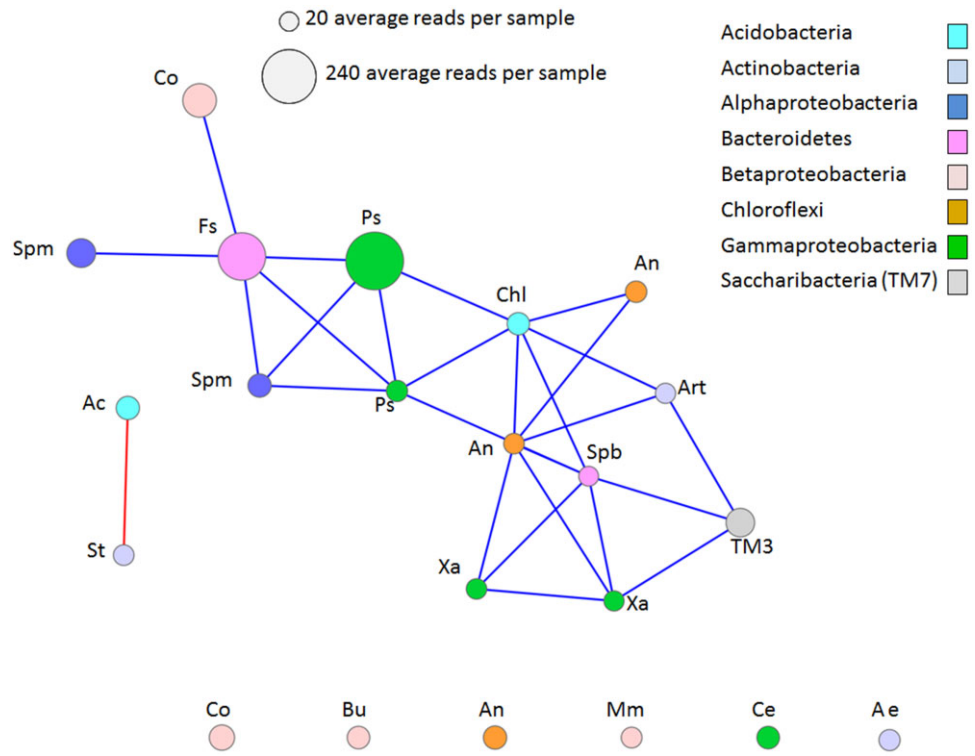

Fig. 5. Correlation of occurrence patterns between OTUs of the lettuce root microbiota, coloured by phylum/class. The network analysis shows correlations between 100% cut-off level OTUs (only abundant: > 0.5% of the total microbiota). Nodes represent OTUs and edges represent strong positive ($R > 0.6$, $P < 0.01$) or negative ($R < -0.6$, $P < 0.01$) Spearman correlations (blue and red lines respectively). OTU abundance (average number of reads per sample) and taxonomic affiliation (at phylum or class level) are indicated by node size and node colour respectively. Node labels indicate the taxonomic identification of the OTUs: Ac, Acidobacteria-6, Ae, *Aeromicrobium*, An, Anaerolineae, Art, *Arthrobacter*, Bu, *Burkholderia*, Ce, *Cellvibrio*, Ch, Chloracidobacteria, Co, Comamonadaceae, Fs, *Flavobacterium succinicans*, Me, *Metylotenera mobilis*, Ps, Pseudomonadaceae, St, *Streptomyces*, TM3, TM7-3 (Saccharibacteria), Spb, Sphingobacteriaceae, Spm, Sphingomonadaceae, Xa, Xanthomonadaceae.

Alphaproteobacteria and Gammaproteobacteria developed either as single cells or small clusters up to 50–60 cells. They colonized both the outer root surface as well as the endorhiza (Fig. S9). Alphaproteobacteria were detected in mixed colonies with Bacteroidetes (Fig. 6A), especially near the root tips where Bacteroidetes were more abundant. This colocalization indicates that the correlation *Flavobacterium succinicans*–Sphingomonadaceae (Fs-Spm, Fig. 5) agrees with potential interaction among these OTUs (cell size and morphology were also consistent with the genus *Flavobacterium* forming relatively long and flexible rods, Bernadet *et al*., 1996; Fig. S10). Bacteroidetes were heterogeneously distributed across the root system, but building dense colonies preferentially on the root tips. Otherwise, they occurred as single cells or small clusters (Fig. S11). The vicinity of Chloroflexi (filamentous Anaerolinae) within areas poorly colonized by Bacteroidetes is indicative of local enrichment (Fig. 6B, circles), and suggests commensalism for the correlation Anaerolinae-Sphingobacteriaceae (An-Spb, Fig. 5). The Chloroflexi was the most unexpected phylum among those dominating the lettuce. The OTU assignment to the class of the Anaerolineae was confirmed by microscopy (Fig. 6B and Fig. S12). They were ubiquitous at the outer root surface and often displayed cell-to-cell adhesion as well as involvement in mixed colonies (Fig. 6B, square, and Fig. S12, circles). Betaproteobacteria formed large colonies and often represented the great majority of total cell counts. Hence, the microscopically observed abundance exceeds the expectation of approximately 10% relative abundance according to pyrosequencing results (Fig. 6C,D). Both filamentous and non-filamentous Actinobacteria were detected, which is coherent with the pyrosequencing results (Fig. S4). Filamentous Actinobacteria seemed to preferentially colonize damaged root areas (Fig. S13, rectangle), which agrees with the wide catabolic activities typical of Actinobacteria. It might be of positive effect for the plant, when antibiotics-producing Streptomycetes could assist in protection against wound infections by plant pathogens. Non-filamentous Actinobacteria were ubiquitously distributed, and occurred also

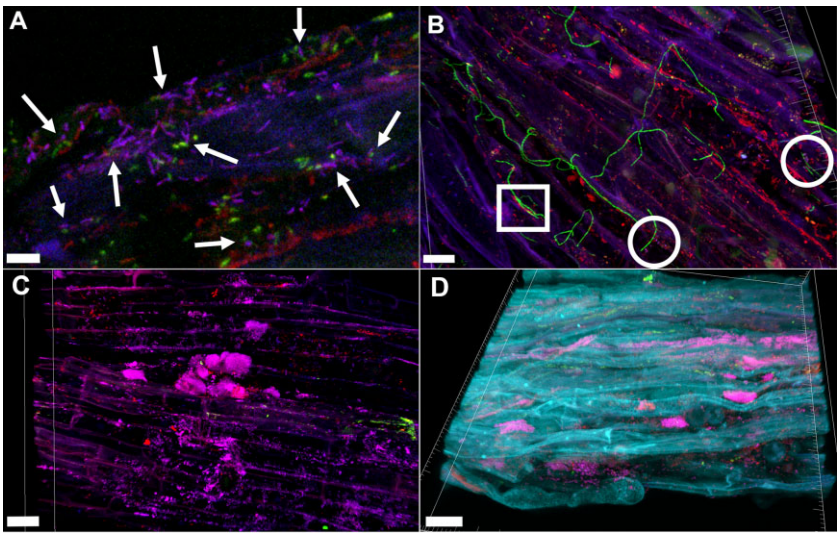

Fig. 6. FISH-CLSM analysis of root-associated bacteria in lettuce.
A. Colocalization and cell–cell interactions between Bacteroidetes (pink/purple) and Alphaproteobacteria (yellow). Red: other bacteria. Scale bar: 5 μm.
B. Filamentous Chloroflexi (green) colonize the root surface of lettuce ubiquitously. Interestingly, within areas low colonized by Bacteroidetes (pink/purple), vicinity of Chloroflexi seems to contribute to their local enrichment (circles). Chloroflexi also showed frequent cell–cell interactions with other bacteria (red) (square; see also Fig. S11). Scale bar: 20 μm.
C–D. Dominance of Betaproteobacteria in the lettuce root system. The relative abundance of Betaproteobacteria appeared much higher than the ~10% expected from the pyrosequencing results. Pink/purple: Betaproteobacteria; Green/yellow: Gammaproteobacteria; Red: other bacteria; Cyan (only in panel D): lettuce root stained with calcofluor. Scale bar: 20 μm.

endophytically inside root hairs (Fig. S14, arrows). When Gammaproteobacteria and Bacteroidetes were co-detected in the same field of view, no colocalization could be observed (Fig. S15), suggesting that the correlation *Flavobacterium succinicans*–Pseudomonadaceae (Fs-Ps, Fig. 5) is more likely to be the effect of habitat sharing.

Discussion

In this work, we present new insights into the bacterial microbiota associated with the root system of lettuce (*Lactuca sativa* L.) as represented by eight cultivars and their wild relative prickly lettuce (*L. serriola* L.). Our approach was based on a step-by-step analysis from basic alpha- and beta-diversity to more sophisticated network analysis and integration of FISH-CLSM. The conditions of our sampling site provided an 'open laboratory' for testing the effect of the host genotype on the root microbiota. *Lactuca serriola* showed lower diversity than *L. sativa* cultivars, which indicates that the domestication led to a bacterial diversification within the root system. Also in case of lettuce, crop domestication can be compared with a human-driven evolutionary radiation with positive selection of heritable phenotypes displaying appealing qualities for consumers. We show that domestication of lettuce is supported by a diversified spectrum of associated microbiota and an overall increase of bacterial diversity.

Microbial diversity on plants is crucial to prevent pathogen invasion and a higher abundance of rare species also seems to represents a barrier against pathogens (Chapin *et al.*, 2000; Gonzalez *et al.*, 2011; Latz *et al.*, 2012; Van Elsas *et al.*, 2012). Interestingly, in the rare fraction of the microbiota (1 up to 10 reads) of our analysed root microbiota, we found a high number of potentially plant-associated taxa known for their beneficial effect, e.g. Rhizobiales (including Bradyrhizobiaceae), Actinobacteria, Bacillales (including *Paenibacillus*), Burkholderiales, *Pseudomonas*, *Stenotrophomonas*, methylotrophic bacteria and Planctomycetales. In contrast, no potential plant pathogens specific for lettuce were identified (e.g. *Pseudomonas cichorii*, *Rhizomonas suberifaciens* or *Xanthomonas campestris* pv. *vitians*), but Legionellales and Enterobacteriaceae, known to include some dreadful human pathogens (Brandl, 2006), were surprisingly frequent. Due to the presence of beneficials and the absence of plant pathogens in the lettuce root microbiota, we conclude that the high diversity along with a higher abundance of beneficial rare species could enhance the barrier effect against plant pathogens. FISH-CLSM also showed higher numbers of *Streptomyces* at damaged areas of the root, but we could not test whether these could also protect against invasive intruders by production of antimicrobials.

All lettuce cultivar groups displayed diverse root communities with 38 phyla detected (10 phyla > 1% relative

abundance) and an overall similarity in primary profiles and variations at lower taxonomic levels. Beta-diversity measures revealed that statistically significant differences between root microbiota are more pronounced at subspecies (and also cultivar) level than at convar level, which primarily denotes morphological types. Thus, host genetic variation shapes root associated bacterial communities in lettuce, similarly as in other crops such as potatoes (Weinert *et al*., 2011) and cottonwood (Schweitzer *et al*., 2008). Consequently, specific root microbiota not only could influence soil parameters, with effects on soil microbial community, ecosystem functioning and phytosociology, but also potentially modulate the metabolism of the host plant (and hence the nutrient values of different lettuce varieties). Such impact on the host has been demonstrated already for *Arabidopsis* (Badri *et al*., 2013). The influence of the cultivar groups exceeds that of the morphology (i.e. the convar level) which suggests that the host genotype rather than morphology is of influence on the root microbiota. We achieved insights into the structure of the core microbiota of lettuce roots, which comprises 68 OTUs, six of which showed significantly variable relative abundance across subspecies. Using *L. serriola* as a control for specificity, we found five core OTUs occurring exclusively in all lettuce samples, thereby demonstrating that at least a part of this core microbiota remains highly specific even after many years of co-cultivation. So far, only the root core microbiota of the model plant *Arabidopsis thaliana* has been unraveled (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012). Because the main difference of our lettuce microbiota analysis is that we assessed shared OTUs between plants grown in the same soil since more than two decades. A direct comparison with the *Arabidopsis* core microbiota would therefore not be meaningful. Nonetheless, certain interesting similarities are noteworthy: (i) the observations of Comamonadaceae as one of the most abundant taxon, (ii) the ubiquitous occurrence of Sphingobacteriales and *Streptomyces*, (iii) the higher abundance of Pseudomonadeceae *versus* Xanthomonadaceae among the Gammaproteobacteria and (iv) the high abundance of Flavobacteriaceae. In comparison to *Arabidopsis*, the analysed lettuce roots harboured abundant Chloroflexi. They were discarded by Bulgarelli and colleagues (2012) due to both unreliable, extreme abundances in adjacent soil samples and failure to detect them through specific fluorescence in situ hybridization (FISH) staining. In our study, we did not analyse soil samples, but rather confirmed the presence of Chloroflexi in the root system through specific FISH staining and their morphology coincided with the typical filamentous Anaerolineae (Yamada *et al*., 2006). This class of environmental bacteria, typical in aquatic habitats and also found in agricultural soil but never reported as plant inhabitants (Shrestha *et al*., 2009; Yamada and Sekiguchi, 2009), was found here as one of the dominant lettuce-associated microorganisms. Previous works on several *L. sativa* cultivars using 16S rRNA gene clone libraries did not find any Chloroflexi member in the phyllosphere (Hunter *et al*., 2010). Recently, Podosokorskaya and colleagues (2013) described a new Anaerolineae bacterium able to degrade complex polysaccharides such as cellulose, thus suggesting a possible role as degraders of senescing root tissues. However, both the ubiquity and frequent cell–cell interactions observed here suggest more prominent and interactive functions within the root microbiota system.

Correlations between bacterial strains are likely of functional relevance (Van Elsas *et al*., 2012), and were here identified in the lettuce root microbiota. Such correlations are also thought to influence the sequential community assemblages, since communities rather aggregate in deterministic manner than at random (Horner-Devine *et al*., 2007; Barberán *et al*., 2012). However, the predominant organisms are not necessarily involved in modules comprising highly correlated strains in our study. This observation somehow differs from an analysis of plant flower microbiota successional patterns (Shade *et al*., 2013), where the most-prominent strains persisted and co-occurred with minor strains. On the other hand, it might explain why allochthonous species, including human pathogens, can successfully invade the native lettuce microbiota, replacing its dominating, harmless bacteria. The correlation network reconstructed with the genotype-specific OTUs showed that minor, specific OTUs are also involved in frequent co-occurrence. What extent these OTUs directly influence the host plant remains unclear so far. With their low relative abundance, it is tempting to speculate that they may not be important in shaping plant features. Nevertheless, highly correlated OTUs may belong to the same taxonomical group, such as Anaerolinae and Pilellulaceae, respectively (Fig. S8), and the potential role of their joint occurrences merits further study.

Microbiota-mediated colonization resistance against intestinal pathogens was first shown for the human microbiota by Buffie and Pamer (2013). The phenomenon that human pathogens can colonize lettuce plants as well as cause serious outbreaks was often described (Brandl, 2006; Teplitski *et al*., 2011; Ongeng *et al*., 2013) but it can be explained by linking this potential with the bacterial co-occurrence network structure. In addition, there are several reports showing exceptional biocontrol of soil-borne pathogens on lettuce (Scherwinski *et al*., 2008; Adesina *et al*., 2009), which can be also linked with the loose bacterial network, which was identified. According to our results, co-occurrence characterized the relationships among members of the root microbiota as opposed to co-exclusion. Presumably, the distance between

colonies of different organisms reduces competition throughout the whole root system. We were also able to largely reconcile the abundances assessed by 16S rRNA amplicon sequencing with FISH-CLSM results. Such a combined approach is useful, since variation in copy numbers at ribosomal RNA gene loci may bias sequence-based interpretations of abundances (Kembel *et al*., 2012). According to pyrosequencing results, Gammaproteobacteria (dominated by the genus *Pseudomonas*) was the prominent group, whereas Betaproteobacteria (mainly Comamonadaceae) were less frequent, whereas the FISH-CLSM approach suggested the alternative picture. A possible reason can thus be the higher average ribosomal operon copy number found in *Pseudomonas* spp. (5.04) with respect to Comamonadaceae (2.8; data obtained from the Ribosomal RNA Operon Copy Number Database, Klappenbach *et al*., 2001).

In this study, we developed an integrative approach based on co-occurrence analysis and FISH-CLSM, which results in a reliable reconstruction and interpretation of interaction networks in complex microbial communities. This approach thus serves to open new opportunities for future targeted studies on pathogen suppression but also on biocontrol on plants.

Experimental procedures

Sampling site and strategy

Root systems of 24 fully matured lettuce plants (*Lactuca sativa* L.) in the final growing stage (leaf development and partly flowering) and 3 *Lactuca serriola* L. (the wild ancestor) were collected in Schiltern, Austria (+48°31′ +15°37′) at the same time. Sampling was from the field of the Arche Noah: a non-profit association devoted to the preservation of plant cultivars in Europe including those which are no longer used in horticulture (http://www.arche-noah.at). The sampling site has been a field parcel since 1990, where numerous lettuce cultivars, representing all lettuce subspecies, along with the wild ancestor, are conserved and planted intermingled (distance between individuals: 30–50 cm) (Fig. S1). This special location represented a field laboratory for investigating the long-term effect of the plant genotype on the root-associated microbiota, under field conditions and under completely levelled environmental/pedological factors, and has the characteristic of an organic managed 'common garden'. At the sampling time, all plants were at the same growth stage (Fig. S1). Three plants per lettuce cultivar and three *L. serriola* plants were available for sampling and were collected. Two cultivars per subspecies were selected (thus, each subspecies included six samples). Since two subspecies belongs to the convar incocta and the other two ones to the convar sativa (Fig. 1), this sampling strategy allowed us to compare lettuce microbiota at convar, subspecies and cultivar level. Moreover, we used *L. serriola* to understand the effect of lettuce domestication on bacterial diversity and as a control for the specificity of the *L. sativa* core microbiota.

DNA extraction

The microbial fraction associated with lettuce roots was extracted according to Bragina and colleagues (2012). Briefly, after gentle removal of the adhering soil and washing into 0.85% NaCl to remove rhizosphere soil and loosely adhering microorganisms, ~ 5 g of roots were physically disrupted with a sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl. Two millilitre of suspension were then centrifuged (16 500 *g*, 20 min, 4°C) and the obtained pellet was used for isolation of the total community DNA with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenized twice in a FastPrep® FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30 s at a speed of 5.0 m s^{-1} and treated according to the manufacturer's protocol. Crude DNA extracts were purified with Geneclean turbokit (MP Biomedicals, Illkirch, France) to improve quality for downstream PCR reactions.

454-amplicon sequencing and data analysis

The V4 region of the 16S rRNA genes were amplified with multiplex identifier (MID) tagged universal bacterial primers F515 and R806 (Caporaso *et al*., 2011). The PCR reaction mixture (25 µl final volume) contained 1 × Taq&Go, 0.25 µM of each primer, 2 mM MgCl2 and 1 µl of template corresponding to 40–70 ng of DNA (96°C, 4 min; 32 cycles of 96°C, 1 min; 64°C, 1 min; 74°C, 1 min; and final elongation at 74°C, 10 min). The products of three independent PCR reactions per sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified PCR products were pooled (200 ng each) and sequenced using the Roche GS FLX+ 454 Titanium platform by Macrogen Korea (Seoul, South Korea). The nucleotide sequences obtained in this work were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) and are available under the accession number PRJEB5101.

Sequences ('reads') were analysed with the QIIME software version 6.0 (Caporaso *et al*., 2010). MID, primer and adapter sequences were removed prior to length and quality filtering (including option '-w') of sequences with final denoising. Four different OTU tables were created with similarity levels ('cut-off levels') of 90%, 95%, 97% and 100%. We decided to investigate the effect of different OTU similarity cut-off levels on the taxonomical structure and correlations because of discordant opinions about sequence similarity thresholds for species and genus delimitation (e.g. Stackebrandt and Ebers, 2006, Yarza *et al*., 2008).

For each OTU table, sequences of plastidal or mitochondrial origin, as well as chimeras (detected with chimera slayer; as part of QIIME) were removed. The dataset was normalized to 2200, 2150 or 2023) reads per sample (whole microbiota, no singletons and > 10 reads only respectively).

Statistical comparison of alpha diversity between samples was performed with the software SPSS 20 (IBM Corporation, Armonk, NY, USA), using *t*-test, ANOVA or non-parametric tests depending on both the number of groups (2 versus > 2) and the distribution of the variable (normal versus non-normal). Sample-specific OTUs (showing significantly different relative abundances between samples) were assessed

by ANOVA after FDR correction of the *P*-value (q-value), while the core microbiota comprises OTUs occurring in all *L. sativa* root microbiota.

Correlation and network analysis

Spearman correlation between OTU occurrence patterns was calculated in order to identify potential interactions between OTUs, similar as in other studies (Barberán *et al*., 2012; Faust *et al*., 2012), but here we also used the 100% cut-off level OTUs. This rigorous approach avoided apocryphal occurrence patterns which may occur already at 99% cut-off level OTUs (Patin *et al*., 2013). Only strong correlations ($R > 0.6$ or < -0.6, and $P < 0.01$) were considered and visualized through network analysis using Cytoscape 2.8 (Smoot *et al*., 2011) by applying the spring embedded layout. Only abundant OTUs (> 0.5% of the total microbiota) were included due to the fact that our aim was to complement this analysis with FISH-CLSM whose detection limit ranges between 0.2% (Cardinale *et al*., 2012) and 1% (Mogge *et al*., 2000). Moreover, not including rare OTUs also eliminates PCR artefacts generated by the typical PCR error rates (Patin *et al*., 2013). In addition, we calculated the correlation of occurrence patterns between genotype-specific OTUs of the lettuce root microbiota (at 97% OUT cut-off level).

FISH-CLSM

Root subsamples were fixed with paraformaldehyde for 6 h, washed three times with cold PBS and then stored at –20°C in 1:1 (vol : vol) 96% ethanol: PBS until FISH-CLSM analysis. FISH was performed using the phylum- and class-specific probes listed in (Table S2) according to Cardinale and colleagues (2012). Briefly, after pre-treatment with Lysozyme, Cy3-, Cy5- and ATTO488-labelled FISH probes were applied to confirm the presence of all dominant phyla/classes detected by pyrosequencing. Additionally, we combined the FISH probes to attempt visualizing in situ the sequence-based correlated taxa. FISH-stained samples were mounted with SlowFade Gold Antifadent (Molecular Probes, Eugene, OR, USA) and stored at 4°C until observation with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany). For each field of view, an appropriate number of optical slices were acquired with a Z-step of 0.15 – 0.5 μm ('confocal stacks'), and the software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for visualization and post-processing. For each bacterial phylum/class, at least two independent FISH experiments were performed on two different lettuce subspecies, and a minimum of 20 confocal stacks were compared. Adobe Photoshop CS2 version 10.0.1 (Adobe Systems, USA) was used to assemble and label the figures.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Sampling site. Field plot of Arche Noah seed savers association (www.arche-noah.at, Schiltern, Austria; N48°31′, E15°37′) where numerous *Lactuca sativa* cultivars, representing all subspecies, along with the wild ancestor *Lactuca serriola*, are conserved and planted intermingled since 1990 (distance between individuals: 30–50 cm).

Fig. S2. Properties of four OTU tables created with different cut-off levels and corresponding rarefaction curves.

Fig. S3. Taxonomic structure of the lettuce root microbiota ($n = 24$, all lettuce samples including also *L. serriola*) as retrieved at different OTU cut-off levels.

Fig. S4. (A) Abundant OTUs (> 0.5% relative abundance to the whole microbiota) at different OTU cut-off levels. (B) Number of abundant OTUs at different OTU cut-off levels and corresponding number of sequences. Fraction (percentage) with respect to both the total OTUs and the total sequences are indicated into parenthesis.

Fig. S5. Beta-diversity plot created with weighted UniFrac distances. Nodes represent whole lettuce root microbiota at 97% OTU cut-off level. Different node colours represents different lettuce subspecies (also delimited by circles), whereas white edges connect samples belonging to the same cultivars. For this plot, Adonis values were $P = 0.0238$, $P = 0.0024$ and $P = 0.00002$ for convar, subspecies and cultivars respectively.

Fig. S6. OTUs showing a significant difference in their relative abundance between lettuce subspecies. Bars indicate the relative abundance to the whole microbiota (left Y-axis) and dots indicate significance values obtained by ANOVA after FDR correction (right Y-axis). OTUs labelled with 'C' are also occurring in the shared microbiota (Fig. 4).

Fig. S7. Network analysis showing the correlations between abundant OTUs (> 0.5% of the total microbiota) at different cut-off levels; coloured by phylum/class. Nodes represent phyla/classes and edges represent strong positive ($R > 0.6$, $P < 0.01$) or negative ($R < -0.6$, $P < 0.01$) Spearman correlations (blue and red lines respectively). Edge thickness represents the number of the interactions between OTUs belonging to the respective phylum/class. Node size indicates phylum/class abundance (percentage of the total microbiota) and node label indicates number of OTUs within the phylum/class. (A) 90% cut-off level OTUs; (B) 95%; (C) 97%; (D) 100%.

Fig. S8. Correlation of occurrence patterns between genotype-specific OTUs of the lettuce root microbiota, calculated at 97% cut-off level. Nodes represent OTUs and edges represent strong positive ($R > 0.6$, $P < 0.01$) or negative ($R < -0.6$, $P < 0.01$) Spearman correlations (blue and red lines respectively). OTU abundance (number of reads) and taxonomic affiliation (at phylum or class level) are indicated by node size and node colour respectively. Node labels indicate the taxonomic identification of the OTUs: Ac, Acidobacteria; Ac6, Acidobacteria-6; Acb, Actinobacteria; An, Anaerolineae; B, Bacteria; Bu, Burkholderia; Chi, Chitinophagaceae; Ch, Chloracidobacteria; Dy, Dyadobacter; El, Elusimicrobia; Fla, Flammeovirgaceae; Flb, Flavobacteria; Fle, Flexibacteraceae; Ga, Gammaproteobacteria; Ge, Gemmatimonadetes; Ge, Gemmata; Kr, Kribella; Meb, Methylobacillus; My, Myxococcales; No, Nocardioidaceae; Op, Opitutales; Ph, Phycispherae; Pi, Pirellulaceae; Pl, Planctomycetes; Pr, Pirellula; Ps, Pseudomonadaceae; Rh, Rhodobacteraceae; Ro, Roseiflexaceae; Si, Sinobacteraceae; Spb, Sphingobacteriales; Ste, Steroidobacter; Sx, *Sphingobium xenophagum*; TM1, TM7-1 (Saccharibacteria); Ve, Verrucomicrobiaceae; Xa, Xanthomonadaceae.

Fig. S9. FISH-CLSM analysis of root-associated bacteria in lettuce. Alphaproteobacteria (yellow) and Gammaproteobacteria (pink/purple) ubiquitously colonize the lettuce root both endophytically and on the surface. Red: other bacteria. A: volume rendering; B: 3D model; C: 3D model with transparent root signal (grey), to allow visualizing the endophytic bacteria. Scale bars: 20 μm.

Fig. S10. Pure culture of a *Flavobacterium* sp. stained by FISH using the CF319a/b FISH probe (Table S2). Scale bar: 5 μm.

Fig. S11. FISH-CLSM analysis of root-associated bacteria in lettuce. Bacteroidetes (pink/purple) were abundant on root tips (A) and less diffused on other parts of the root (B). Red: other bacteria. Scale bars: 20 μm.

Fig. S12. FISH-CLSM analysis of root-associated bacteria in lettuce. Anaerolinae (green) colonized the lettuce root ubiquitously and showed frequent cell–cell interactions with other bacteria (red). Scale bars: A, B and D, 5 μm; C, 10 μm.

Fig. S13. FISH-CLSM analysis of root-associated bacteria in lettuce. Filamentous Actinobacteria (likely *Streptomyces* spp.) colonize the lettuce root, forming more dense colonies on the damaged areas. This could determine a protection from plant pathogens due to the antimicrobial substances usually produced by Streptomycetes. A–D: individual confocal channels showing Actinobacteria (A), root tissues stained by calcofluor white (B), root autofluorescence (C) and all bacteria (D); E: overlap of the channels A–D. Here, the Actinobacteria appears yellow. Scale bar: 20 μm.

Fig. S14. FISH-CLSM analysis of root-associated bacteria in lettuce. A: XY-XZ-YZ projections of a confocal stack showing non-filamentous Actinobacteria (yellow) growing endophytically inside a root hair (arrows). B: volume rendering. C: three-dimensional model. Red: other bacteria. Grey: root tissues. Scale bars: A, 20 μm; B–C: 5 μm.

Fig. S15. FISH-CLSM analysis of root-associated bacteria in lettuce. Although abundant, Gammaproteobacteria and Bacteroidetes did not tend to be colocalized when detected in the same field of view. Yellow and purple circles delimitate the area of the root colonized by Gammaproteobacteria (yellow cells) and Bacteroidetes (pink/purple cells) respectively. Scale bars: A, 15 μm; B and C, 10 μm.

Table S1. Statistical significance of differences for Shannon, Equitability and Chao1 indices between samples (from species to cultivar level) at different cut-off levels, with and without singletons. Significant differences ($P < 0.05$) are in bold and marked with an asterisk.

Table S2. FISH probes used in this work.