Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species

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Introduction

The genus Agave, native to the deserts and dry lands of central Mexico and the southwestern USA, includes a number of promising candidate biofuel crops for arid and semiarid climates (Somerville et al., 2010; Davis et al., 2011). There are >200 species of Agave, and several of these have been cultivated for centuries for the production of alcohols and fiber (Garcia-Moya et al., 2011). Recent research has highlighted the suitability of agaves as biofuel feedstocks as a consequence of their comparatively low lignin content, ease of decomposition, high yields with minimal resource inputs (Davis et al., 2014; Li et al., 2014). Additionally, agaves are capable of growing on otherwise nonarable rocky soils in regions characterized by prolonged drought and extreme temperatures owing in part to physiological and biochemical adaptations that prevent excess water loss (Davis et al., 2011; Shakeel et al., 2012; Campos et al., 2014). This ability to grow on subprime land reduces the competition for valuable and limited arable agricultural land between Agave and other food crops (Borland et al., 2009; Holtum et al., 2011).

However, the economic viability of Agave-based biofuels will hinge on the maximization of plant yield (Núñez et al., 2011). Conventional methods of crop improvement with agaves are possible, but are made more difficult by the long period plants take to reach maturity; transgenic approaches could accelerate this process but have been impeded by the limited tools for genetic transformation of Agave (Flores-Benítez et al., 2007; Escamilla-Treviño, 2012).

It is now well established that symbiotic bacteria and fungi have profound impacts on plant health and adaptation to stress

Summary

• Desert plants are hypothesized to survive the environmental stress inherent to these regions in part thanks to symbioses with microorganisms, and yet these microbial species, the communities they form, and the forces that influence them are poorly understood.

• Here we report the first comprehensive investigation of the microbial communities associated with species of Agave, which are native to semiarid and arid regions of Central and North America and are emerging as biofuel feedstocks. We examined prokaryotic and fungal communities in the rhizosphere, phyllosphere, leaf and root endosphere, as well as proximal and distal soil samples from cultivated and native agaves, through Illumina amplicon sequencing.

• Phylogenetic profiling revealed that the composition of prokaryotic communities was primarily determined by the plant compartment, whereas the composition of fungal communities was mainly influenced by the biogeography of the host species. Cultivated A. tequilana exhibited lower levels of prokaryotic diversity compared with native agaves, although no differences in microbial diversity were found in the endosphere.

• Agaves shared core prokaryotic and fungal taxa known to promote plant growth and confer tolerance to abiotic stress, which suggests common principles underpinning Agave–microbe interactions.
et al. (2013) and that manipulation of the plant microbiome has the potential to dramatically improve the yield of agronomically important crops (Turner et al., 2013). Indeed, it has been proposed that plants should be considered a collective ‘holobiont’ rather than standalone entities (Zilber-Rosenberg & Rosenberg, 2008; Vandenkruystyne et al., 2015). Recent research has demonstrated that plant-growth-promoting microbes (PGPM) harvested from plants grown in semiarid environments are capable of enhancing plant growth and stress tolerance in a variety of crop species (Marasco et al., 2012; Mengual et al., 2014; Rolli et al., 2015). In Agave, recent research highlighted the likely beneficial role of native associated diazotrophic bacteria, which may play a role not only in nutrition, but also in tolerance to drought (Desgarennes et al., 2014). Also, previous reports in which either mycorrhizal or endophytic fungi were found to promote plant growth in Agave species (Cui & Nobel, 1992; Obedo et al., 2003) suggest that the Agave microbiome plays an important role in the fitness and productivity of this genus. Thus, an alternative to improve Agave’s yield may be the rational use and manipulation of the microbiome.

Unlike many food crops, current commercially important Agave species and cultivars have undergone limited domestication efforts, despite having been grown under continuous cultivation for centuries in plantations that exist in proximity to related native populations (Nobel, 2003). Therefore, the genus also offers an opportunity to evaluate whether cultivation is associated with changes in the composition of microbial communities associated with a plant host, as has been suggested for other species by recent studies (Edwards et al., 2015). Importantly, studies in other plants and soil systems have provided divergent results, describing examples of both decreased (Fierer et al., 2013) and increased microbial diversity (Rodrigues et al., 2013) upon cultivation.

Previous studies investigating the microbial communities associated with diverse plant hosts have demonstrated that sample type (plant compartment), host species, geographic location, and season are all capable of influencing community composition, although the extent to which each plays a role has varied from study to study (DeAngelis et al., 2008; Lundberg et al., 2012; Lanconì et al., 2013; Peiffer et al., 2013; Mendes et al., 2014; Edwards et al., 2015; Zarraonaindia et al., 2015). Most previous studies have focused on prokaryotic communities, providing limited insights into the forces shaping plant-associated fungal communities. While recent comparative studies (Hilton et al., 2013; Shakya et al., 2013) indicate that distinct factors can drive fungal and prokaryotic rhizosphere communities, it is unclear to what extent these differences extend to other plant compartments, plant hosts and soil systems.

The primary goal of this research was to investigate the prokaryotic and fungal communities associated with the bulk and proximal soil, the rhizosphere, the phyllosphere, and the root and leaf endospheres, for three Agave species: the cultivated Agave tequilana and the native species, Agave salmiana and Agave deserti (Fig. 1a–c). These species were selected based on their natural geographic distribution (Table 1; Fig. 1a) (CONABIO, 2006; Gentry et al., 2013), their differentiated management status (cultivated versus native), and their relevance to both existing and prospective industries. Comparisons across these three Agave species would allow us to infer the impact of biogeography and cultivation status on microbial composition and diversity in the genus Agave. Additionally, we also evaluate the major biotic (i.e. plant species and plant compartment) and abiotic (i.e. season and site) factors shaping both prokaryotic and fungal communities, and identify prominent microbial players in order to develop rational microbiome-based strategies for improving the yield and productivity of Agave.

Materials and Methods

Experimental design

Our study investigated three Agave species: Agave tequilana F.A.C. Weber, Agave salmiana Otto ex Salm subsp. crassispina (Trel.) Gentry, and Agave deserti Engel. These species are distributed in central Mexico and in southern California, but they are not sympatric (Fig. 1a). Agave tequilana belongs to the group Rigidae (Gentry, 1982) and exists only in cultivated populations, mainly in Jalisco (Fig. 1a). Agave salmiana belongs to the group Salmianae, whose natural populations extend from Sonora to Guerrero in western Mexico (Gentry, 1982; CONABIO, 2006). Agave deserti belongs to the group Deserticolae and it is native to California and can also be found in parts of Arizona and Baja California (Gentry, 1982). In 2012, samples were collected at two different times: in late May (the end of the 8 months of low or null precipitation in Mexico) and in early October (at the end of the 4 months of the most precipitation in Mexico). Agave tequilana samples were taken from two fields managed by tequila companies in Guanajuato and Jalisco, Mexico: Penjamo (Pe) and Amatitan (Am) (Fig. 1a), while Agave salmiana samples were collected from the natural populations of El Maguéyal (Ma) and San Felipe (SF) in Guanajuato, Mexico. Agave deserti samples were collected in September and April (in a year with little rainfall) from the natural populations within the Philip L. Boyd Deep Canyon Desert Reserve in California called Agave Hill (AH), Boyd Ridge (BR), and Pinyon Flats (PF). Soil characteristics as well as temperature and precipitation data for each site are presented in Table 1. For each species, and at each location and time, samples from three healthy plants were collected. Four samples per plant were collected to analyze six different communities (Fig. 1b): two leaves for leaf endosphere and phyllosphere; root tissue for root endosphere and rhizosphere; root zone soil, which was soil loosely attached to or adjacent to the roots, and bulk soil gathered 1 m away from the selected plant specimen. All samples were stored at 4°C until processing. We analyzed in total 252 samples; 72 from A. tequilana, 72 from A. salmiana and 108 from A. deserti.

Sample preparation, DNA extraction, PCR, and sequencing

Samples were prepared and total DNA was extracted as previously described (Desgarennes et al., 2014). For amplification of
the ITS2 and 16S regions, we used the ITS9F/ITS4R and 515F/816R primer pairs, respectively, and followed the PCR protocol described in the Supporting Information. For 16S amplification, peptide nucleic acid (PNA) clamps were used as in Lundberg et al. (2013), which substantially reduced overall chloroplast and mitochondrial contamination and increased the proportion of prokaryotic reads for the root endosphere, leaf endosphere, and phyllosphere samples. Paired-end 2 × 250 bp sequencing was performed on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA) operating with v2 chemistry. All quality sequences related to this project are available in the NCBI Sequence Read Archive (SRA) under project IDs SRA211411, SRA211420, SRA211416, SRA211422 and SRA211408.

Data processing and statistical analyses

The raw Fastq reads were processed using a custom pipeline developed at the Joint Genome Institute (Tremblay et al., 2015) (Supporting Information Methods S1). Raw reads were contaminant-filtered, quality trimmed, merged and clustered to produce 25 871 and 40 759 fungal and prokaryotic operational taxonomic units (OTUs), respectively, at 95% and 97% identity using the UPARSE pipeline (Edgar, 2013). Taxonomies were assigned to each OTU using the RDP Naïve Bayesian Classifier (Wang et al., 2007) with custom reference databases. OTUs whose RDP classifications did not match their expected taxonomic kingdoms (Fungi and Bacteria/Archaea, respectively) were removed. Average read counts varied by sample type for both data sets, and in particular the aerial endophytic samples had substantially fewer reads than the other sample types (Fig. S1). To reduce low-abundance and spurious OTUs, technical reproducibility thresholds determined empirically from technical replicates as in Lundberg et al. (2013) (Fig. S2) were set and OTUs kept only if they had at least two reads in at least five samples (ITS2 data) or at least seven reads in at least five samples (16S data).

For diversity analyses, all samples were randomly subsampled (rarefied) to 1000 reads per sample to account for differences in the number of reads across samples. We calculated the Shannon diversity ($H'$) index using the package ‘BiodiversityR’ in R. All other statistical analyses were performed in R using a variety of packages and custom scripts using the measurable OTUs, as
greater statistical support was achieved using this data set instead of the measurable rarefied data set. In brief, Venn diagrams were plotted with the function ‘venn.diagram’ using the package ‘vegan’. Distances were calculated using the ‘vegdist’ function of the package ‘vegan’ for Bray–Curtis. Nonmetric multidimensional scaling (NMDS) was performed using ‘Mass’ and ‘vegan’ packages. Permutational ANOVAs (PERMANOVAs) were performed with the function ‘adonis’ in the package ‘vegan’ as described in Desgarennes et al. (2014). The major microbial player analyses and graphics were performed using the average relative abundance and relative frequency of each OTU in each community (i.e. sample) across the three *Agave* species.

**Results**

Microbial community diversity associated with *Agave* species

We analyzed the prokaryotic and fungal communities associated with six sample types taken from three *Agave* species through Illumina iTag sequencing on the MiSeq platform. We obtained 35 770 987 and 27 596 665 total high-quality reads (Fig. S1), which resulted in 3923 and 3173 OTUs after applying technical reproducibility thresholds for the prokaryotic and fungal data sets (Fig. S2), respectively. The majority of all prokaryotic and fungal OTUs discovered in the endospheres (leaf and root) in this analysis were also present in the episphere (rhizosphere and phyllosphere) and surrounding soils (Fig. 1d). Similarly, the majority of OTUs associated with the aboveground portions of the plant were also present in the belowground plant tissues and soil-derived samples, although there was a considerably larger portion of fungal OTUs than prokaryotic OTUs (16.8 and 2.2%, respectively) detected solely in the aboveground tissues (Fig. S3). Intriguingly, the percentage of OTUs shared between sampling sites in Mexico and California was considerably smaller for fungal data sets than for the prokaryotic data sets (18.2 and 72.2%, respectively; Fig. S3), suggesting that fungal communities were perhaps more shaped by geographic distance. We also determined that *A. salmiana* shared a larger fraction of prokaryotic OTUs with the other native species, *A. deserti*, than with the other agave collected in Mexico, the cultivated *A. tequilana*, while the opposite trend was observed for fungal OTUs (Fig. 1e).

The levels of microbial diversity differed significantly among the sample types and across *Agave* species, except for the root and leaf endosphere and the prokaryotic bulk soil (Fig. 2a; Table S1). In general, alpha diversity as measured by the Shannon (H) index decreased in value from soil to episphere to endosphere (Fig. 2a, b). Alpha diversity was marginally higher in the root zone soil than in the bulk soil for both prokaryotic and fungal communities across all three *Agave* species, suggesting that root exudates or other plant-associated factors may be responsible for increasing local microbial diversity. Remarkably, the prokaryotic alpha diversity associated with both the rhizosphere and phyllosphere of the cultivated *A. tequilana* was substantially lower than that of the two wild agaves, suggesting a loss of natural microbial diversity (Fig. 2a; Table S1). Furthermore, these levels were even lower than the average values for the respective endophytic compartments of *A. tequilana*. However, it was surprising that microbial diversity in the phyllosphere for all three agaves was almost as

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**Table 1** Geographic location, annual mean temperature, precipitation and soil characteristics of study sites

| Sites             | Mexico          | Guanajuato | USA          |
|-------------------|-----------------|------------|--------------|
|                   | Jalisco (Am)    | Guanajuato | California   |
|                   |                 | A. salmiana | Boyd (BR)    |
|                   |                 | A. deserti | Agave Hill (AH) |
|                   |                 |            | Pinyon Flats (PF) |
| Latitude          | 21.053          | 20.686     | 33.714       |
| Longitude         | -103.902        | -101.875   | -116.524     |
| Altitude (m above sea level) | 1260 | 1714 | 2175 | 2089 | 452 | 814 | 1225 |
| Annual mean temperature (°C) | 26.4 | 19 | 17.9 | 17 | 23.7 | 20.6 | 18.0 |
| Annual precipitation (mm) | 558 | 790 | 485 | 204.5 | 136.0 | 183.0 | 238.3 |
| Precipitation during dry season (mm) | 4.2 | 147 | 130 | 68 | 51.7 | 69.4 | 95.5 |
| Precipitation during rainy season (mm) | 553.8 | 643 | 355 | 136.5 | 83.9 | 113.6 | 142.8 |
| Soil edaphic factors | Texture | Clay loam | Sandy loam | Sandy loam | Sandy loam | Sandy loam | Sandy loam |
| pH                | 6.8             | 6.68       | 5.79        | 6.25        | 8.02        | 7.48        | 7.95 |
| Organic matter (%)| 1.1             | 0.96       | 3.97        | 0.71        | nd          | nd          | nd |
| Nitrogen (µg g⁻¹) | 19.7            | 9.14       | 8.76        | 12.65       | nd          | nd          | nd |
| Phosphorus (µg g⁻¹) | 3.64 | 27.1 | 18.11 | 4.53 | 42.17 | 87 | 74 |
| Potassium (µg g⁻¹) | 193             | 482        | 64.35       | 251.7       | 155.5       | 131        | 91.33 |

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*For sites in Mexico, data were provided by Comisión Nacional del Agua (CONAGUA). Data from sites in California were obtained from DRI Weather Station Data Collection (http://www.wrcc.dri.edu/weather/ucde.html). nd, not determined.

*Statistically significant difference between species after Kruskal–Wallis test (H₂,₁₅ = 10.763; P = 0.0046).*
high as diversity in the rhizosphere, despite the harsher environmental conditions that prevail for aboveground plant surfaces than for underground surfaces.

Across all samples, we detected a total of 33 distinct prokaryotic phyla, although just four of these (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes) comprised on average >80% of each plant-associated sample type (rhizosphere, root endosphere, leaf endosphere, and phyllosphere; Fig. S4). The plant-associated sample types (epispheres and endospheres) were enriched for Proteobacteria (Kruskal–Wallis $\chi^2 = 50.84$; $P < 1.00 \times 10^{-12}$) and Actinobacteria (Kruskal–Wallis $\chi^2 = 11.07$; $P < 8.790 \times 10^{-6}$) and depleted for Acidobacteria (Kruskal–Wallis $\chi^2 = 125.51$; $P < 2.20 \times 10^{-16}$) with respect to the surrounding soils, consistent with previous observations for Agave and other plants (Bulgarelli et al., 2012; Lundberg et al., 2012; Shakya et al., 2013; Desgarennes et al., 2014). While the prokaryotic relative abundance profile seemed to be similar in the bulk and root zone soil samples, community composition differed substantially between the rhizosphere, root endosphere, leaf endosphere, and phyllosphere (Fig. 3). By contrast, the transition in relative abundance profiles between sample types for fungal communities was more gradual than discrete (Fig. 3), with several lineages exhibiting proportional increases or decreases in abundance across bulk soil, root zone, rhizosphere and root endosphere compartments. Fungal communities across all six sample types were dominated by the phylum Ascomycota (average 91.2% of total relative abundance), while Basidiomycota represented a much smaller portion of the communities (7.7%) (Fig. S4). Strikingly, very few members of the known arbuscular mycorrhizal fungi (AMF) phylum Glomeromycota (36 OTUs; 1.1%) were detected in the soil, root zone soil, rhizosphere and root endosphere of the native agaves. Those that were detected were members of Entrophospora and Glomus spp., and were predominantly associated with the two wild agaves (Kruskal–Wallis $\chi^2 = 23.79$; $P < 1.01 \times 10^{-10}$). Mycorrhizal species were virtually absent from all samples from A. tequilana, including their associated soils.

We observed a number of differences in prokaryotic and fungal relative abundance patterns between the three Agave species. For example, in both A. tequilana and A. deserti, the fungal orders Pleosporales and Eurotiales decreased and increased in average relative abundance, respectively, across the transition from bulk soil to root endosphere (Fig. 3b,f). In A. salmiana, however, relative abundances of both these taxonomic groups were relatively stable between these sample types. By contrast, the fungal order Capnodiales represented a significant portion of the aerial microbiomes of both Mexican agaves (Fig. 3b,d), but was nearly absent from all sample types in A. deserti (Kruskal–Wallis $\chi^2 = 28.64$; $P < 8.70 \times 10^{-7}$). Similarly, while the prokaryotic order Bacillales represented a substantial portion (>20%) of the leaf endosphere of both Mexican agaves, it represented <5% of the leaf endosphere community for A. deserti (Kruskal–Wallis $\chi^2 = 12.65$; $P < 3.77 \times 10^{-4}$).

The rhizospheres of the cultivated A. tequilana were enriched for the bacterial orders Pseudomonadales (Kruskal–Wallis $\chi^2 = 6.91$; $P < 9.97 \times 10^{-3}$) and Enterobacteriales (Kruskal–Wallis $\chi^2 = 21.37$; $P < 3.79 \times 10^{-6}$) with respect to the native rhizospheres (Figs 3a, S5). Combined, the two orders comprised as
much as 92% of a single *A. tequilana* rhizosphere sample, and on average represented 66% of the rhizosphere communities of the cultivated plants. By comparison, the rhizospheres of the wild *A. deserti* and *A. salmiana* showed considerably more diversity, with the most abundant orders *Actinomycetales* and *Bacillales* representing on average only 14 and 10%, respectively, of the native rhizosphere communities. The prokaryotic communities of the bulk soil were more similar across all sampling locations, and *Enterobacteriales* or *Pseudomonadales* comprised <1% of total community abundance in all cases. Interestingly, the phyllosphere of the cultivated agave had high average levels of the bacterial orders *Pseudomonadales*, *Enterobacteriales* and also *Flavobacteriales* (Fig. 3a), which together comprised on average 58% of the phyllosphere community. The abundance of these few lineages in the epiphytic of the cultivated *A. tequilana* came at the expense of many other depleted orders found to be significantly more abundant in the native rhizospheres, including *Pirellulales* (Kruskal–Wallis $\chi^2 = 8.19$; $P < 4.20 \times 10^{-2}$), *Solibacteriales* (Kruskal–Wallis $\chi^2 = 8.23$; $P < 4.05 \times 10^{-2}$), *Acidimicrobiales* (Kruskal–Wallis $\chi^2 = 12.85$; $P < 3.39 \times 10^{-5}$) and *Rhizobiales* (Kruskal–Wallis $\chi^2 = 13.25$; $P < 2.73 \times 10^{-4}$).

Factors driving microbial communities in *Agave* at the global scale

PERMANOVAs of both prokaryotic and fungal communities evaluating all the factors considered in the experimental design and their interactions revealed that sample type, the biogeography of the host plant species and the interaction of these two factors had the most influence on microbial communities, although in the prokaryotic communities sample type accounted for most of the variance (63%; Table 2), while in fungal communities the biogeography of the host plant species played the largest role (52.7% of variance; Table 2). Season and the interaction between sample type and season also had significant, albeit small, influences on the fungal communities, which were not observed at the global scale in the prokaryotic assemblages. It is worth remembering that in these global analyses the factor site could not be evaluated independently of plant species, as the *Agave* species used in this study do not grow in sympatry. PERMANOVA results also confirmed that variation among the three independent replicate samples of the same sampling time/condition in each species was generally low (small residuals in each F test; data not shown).

Fig. 3 Order-level relative abundance plots of prokaryotic (a, c, e) and fungal (b, d, f) communities by sample type for *A. tequilana* (a, b), *A. salmiana* (c, d), and *A. deserti* (e, f). Asterisks in the legend indicate taxonomic bins containing operational taxonomic units that could not be resolved to the order level.
PERMANOVA results were corroborated by NMDS plots using the Bray–Curtis distance, where both the prokaryotic and fungal data sets displayed clustering by sample type, geography and host species, but the relative contributions of each factor differed across the two data sets (Fig. 4). Similar patterns of clustering were observable for the 16S data set using unweighted UniFrac distances (Fig. S6); weighted UniFrac distances exhibited less distinct clustering by any of the factors. NMDS ordination for the ITS data set was not performed with UniFrac because of known issues with generating accurate phylogenetic trees from the hypervariable ITS2 sequence data (Lindahl et al., 2013). In the case of the prokaryotic communities, the clusters that formed corresponded mainly to the different sample types, starting from the soils at one end (soil and root zone soil), followed by the rhizosphere and phyllosphere, and the root and leaf endospheres at the far end (Fig. 4a). The NMDS plots revealed that endosphere samples exhibited the greatest between-sample variation (Fig. 4a,b), while soil samples clustered more closely together. Similarly, variation in levels of alpha diversity for endospheres in the prokaryotic communities was larger than for soil samples (Fig. 2a). The prokaryotic samples formed subclusters within each sample type based on the biogeography of each host species, confirming the PERMANOVA results shown in Table 2. In the case of the fungal communities, the global NMDS plot (Fig. 4b) revealed that there was a clear distinction between samples from California (left) and Mexico (right), and that soils clustered close to the rhizosphere and phyllosphere, while endophytic communities were grouped separately from them (Fig. 4b). As sample type was a major factor contributing to data clustering, we evaluated the impact of all factors in the epiphytic (Fig. 4c,d) and endophytic communities (Fig. 4e,f) separately. While both prokaryotic and fungal epiphytic data sets were mainly influenced by the biogeography of the Agave species, the influence of this factor was greater in the fungal than in the prokaryotic communities. This trend is visually supported by the NMDS and numerically reflected by the PERMANOVA results (Table 2). In the epiphytic, sample type and the interaction sample type–species explained together 15.6 and 17.6% of the variance in prokaryotes and fungi, respectively. Season played only a minor role in the epiphytic fungal communities (Table 2). Statistical analyses of the endosphere of agaves suggested that these prokaryotic and fungal communities were influenced not only by the sample type, plant species and their interaction (which accounted for 65 and 62.4% of the total variance in prokaryotes and fungi, respectively), but also by the factor season and the interaction of this factor with the others, which explained 7.9% of the variance in prokaryotes and 6.1% in fungi (Fig. 4e,f; Table 2). NMDS results also confirmed that the three independent samples taken at each sampling time/condition generally clustered close to each other, as depicted in Fig. S7(a–d), indicating that intrasample variation between replicates was generally low.

Factors driving microbial communities in Agave at the species scale

We were also able to investigate the effect of all factors and their interactions, including the effect of geographic location, within each Agave species, as our experimental design considered at least two locations for each species (two 240 km apart for A. tequilana, two 130 km apart for A. salmiana, and three within 50 km for A. deserti; Table 1). From these analyses, in which we performed statistical tests similar to those described above (Fig. S5; Tables

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**Table 2** PERMANOVA analyses of the microbial communities associated with Agave species considering all factors and their interactions (only significant factors are displayed; \( P \leq 0.05 \))

| Prokaryotes Factora | \( F \)   | \( R^2 \)  | \( P \)  |
|----------------------|---------|------------|--------|
| Global               |         |            |        |
| Sample type \( 5,199 \) | 183.247 | 0.631      | 0.001  |
| Species \( 5,199 \)    | 107.096 | 0.418      | 0.001  |
| Sample type: species \( 10,199 \) | 10.517  | 0.072      | 0.001  |
| Fungi Factora         |         |            |        |
| Species \( 1,190 \)    | 350.97  | 0.527      | 0.001  |
| Sample type \( 1,190 \) | 49.04   | 0.184      | 0.001  |
| Sample type: species \( 10,190 \) | 16.83   | 0.126      | 0.001  |
| Sample type: species \( 1,190 \) | 1.94    | 0.007      | 0.016  |
| Season \( 1,190 \)     | 2.54    | 0.002      | 0.050  |

| Bulk soil and root zone soil |         |            |        |
| Species \( 5,71 \)     | 88.056  | 0.667      | 0.001  |
| Rhizosphere and phyllosphere |         |            |        |
| Species \( 2,70 \)    | 88.016  | 0.591      | 0.001  |
| Sample type \( 1,70 \) | 32.056  | 0.108      | 0.001  |
| Sample type: species \( 2,70 \) | 7.142   | 0.048      | 0.001  |
| Root and leaf endosphere |         |            |        |
| Sample type \( 1,58 \) | 110.965 | 0.479      | 0.001  |
| Species \( 2,58 \)     | 12.546  | 0.108      | 0.001  |
| Sample type: species \( 2,58 \) | 7.274   | 0.063      | 0.001  |
| Species: season \( 1,58 \) | 5.228   | 0.045      | 0.001  |
| Season \( 2,58 \)     | 4.624   | 0.020      | 0.014  |
| Sample type: season \( 2,58 \) | 3.364   | 0.014      | 0.027  |

|                     |         |            |        |
| Sample type \( 1,49 \) | 57.269  | 0.351      | 0.001  |
| Species \( 2,49 \)     | 17.771  | 0.218      | 0.001  |
| Sample type: species \( 2,49 \) | 4.451   | 0.055      | 0.001  |
| Season \( 2,49 \)     | 3.756   | 0.023      | 0.008  |
| Sample type: season \( 2,49 \) | 3.513   | 0.022      | 0.014  |
| Sample type: species: season \( 2,49 \) | 2.617   | 0.016      | 0.049  |

\( ^a \)Subscript numbers indicate the degrees of freedom and residuals of each \( F \) test.
S1–S3), we are able to conclude that the microbial communities associated with the two native agaves behaved similarly for the factors evaluated. In both native species, sample type explained most of the variance across samples, although its influence was greater in the prokaryotic communities than in the fungal ones (85–81% in prokaryotes; 74–62% in fungi for *A. salmiana* and *A. deserti*, respectively). Site played a minor role in the prokaryotic communities of *A. salmiana* (2.2%; Table S2) and none in those of *A. deserti* (Table S3). Fungal communities of *A. deserti* were more influenced by the site and the interaction of site with the sample type (explaining together 15% of the variance), while in *A. salmiana* the factor site only accounted for 1.5% of the variance in the fungal data set. By contrast, in the cultivated agave the factors site and season did influence both prokaryotic and fungal communities (Table S1), suggesting that abiotic factors played a more substantial role in shaping the microbiome of *A. tequilana*.

### Major microbial players associated with Agave – the endosphere

Inspired by the possibility of approaching ecological relationships as microbial markets (Werner *et al.*, 2012), we made use of the Pareto concept (the 80–20 rule) to identify those prokaryotic and fungal taxa accounting for 80% of *Agave’s* microbial communities. After their identification, we plotted their average relative abundance. We found that the endosphere had a significant influence on the microbial communities, with the endosphere species contributing more to the total microbial community than the rhizosphere or phyllosphere species. The results suggest that the endosphere plays a crucial role in shaping the microbiome of *Agave*.

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**Fig. 4** Nonmetric multidimensional scaling (NMDS) plots for Bray–Curtis distances of prokaryotic and fungal communities associated with *Agave* species. *(a, b) All six sample types; (c, d) only rhizospheres and phyllospheres; (e, f) only root and leaf endospheres.*
abundance and frequency across each sample type in each *Agave* species to infer functional relationships and ecological relevance (Figs S8–S13).

In general, our analyses confirmed low levels of microbial diversity associated with cultivated *A. tequilana*, as we identified a reduced number of microbial taxa playing a major role in this agave in comparison with the native species (Figs 5, S8, S11). Despite this reduction, our analyses also revealed that there were a number of shared major players at the phylum/class level across the three *Agave* species studied. At the taxonomic level of individual OTUs, we observed different microbes playing major roles between native and cultivated agaves.

We found that *A. tequilana*'s prokaryotic leaf endosphere was dominated by OTUs identified as *Acinetobacter* and *Bacillus* (12% each), while *Leclercia* (8–30%) was the dominant OTU for this compartment in the native agaves (Figs S8–S10). We observed that in *A. tequilana*'s root endosphere the major players were OTUs identified as *Stenotrophomonas* (33%) and *Agrobacterium* (17%) (both Proteobacteria). By contrast, the root endosphere of native agaves was dominated by *Actinosynnemataceae* and *Promicromonospora* (16% for each, both belonging to *Actinobacteria*), plus *Rhizobiales* (11%) in *A. salmiana* and *Leclercia* (15%) in *A. deserti* (Fig. 6a). Notably, the differences observed in the major bacterial players in the endophytic compartment were not evident in the soil communities across the three plant species (Figs S8–S10).

In the fungal communities, we found that in the leaf endosphere of *A. tequilana* and *A. salmiana* three of the most abundant OTUs belonged to *Alternaria* (9 and 14%, respectively), *Penicillium* (7% in *A. tequilana*) and *Cladosporium* (20% in *A. salmiana*) (Figs S11, S13). By contrast, in *A. deserti*'s leaf endosphere the dominant OTUs were identified as *Paathryrella* (17%) and *Cyphellophora* (13%) (Fig. S13). Likewise, we observed that native agaves’ root endospheres were dominated by *Lophiostoma* (6–11%), plus *Cladosporium* (6% in *A. salmiana*) and *Cyphellophora* (11% in *A. deserti*), while *A. tequilana*'s root endosphere was dominated by *Penicillium* (28%) and *Fusarium* (11%) in *A. salmiana* and *Leclercia* (15%) in *A. deserti* (Fig. 6b). Finally, we observed that the number of fungal OTUs and taxa detected in the proximal and distal soil communities in the native agaves was greater than those observed in *A. tequilana* (Figs S9–S11).

Is there a dry core endophytic microbiome in *Agave*?

Our experimental design allowed us to evaluate the impact of naturally occurring dry periods on the microbial communities associated with *Agave*. Our *Agave* samples from Mexico were collected at the highest peak of drought (dry season) and after 4 months of variable levels of precipitation (rainy season) (Table 1). Based on results from NMDS and PERMANOVA, which suggested that season influenced prokaryotic and fungal endophytic communities (Fig. 3e,f; Table 2), we investigated whether root and leaf endophytic major microbial players were affected by these seasonal changes (Fig. S14; Tables S4, S5). In the case of the endophytic prokaryotic communities, we observed that a core group formed by OTUs of *Actinobacteria*, *Bacilli*, *Alpha-*, *Beta-*, and *Gammaproteobacteria* was enriched during the dry season in comparison to the rainy season for leaf endospheres of *A. tequilana* and *A. salmiana*, as well as for the root endosphere of *A. tequilana*, but not in either endosphere of *A. deserti* (Figs 6a,b,
did in fact share a core of eighty OTUs representing another (Fig. 4f) based on total relative abundance patterns, they of the three (Fig. S15). In general, while fungal root endosphere communities similar to one another across both dry and rainy seasons. At higher taxonomic levels, the leaf endospheres associated with A. tequilana and A. salmiana were similar to one another across both dry and rainy seasons (Fig. S15). In general, while fungal root endosphere communities of the three Agave species could be differentiated from one another (Fig. 4f) based on total relative abundance patterns, they did in fact share a core of eighty OTUs representing c. 40% or more of the total community in each species (Figs S14, S15).

Discussion

Distinct factors shape prokaryotic and fungal communities in Agave

The microbial communities associated with plant hosts are probably shaped by a wide variety of environmental and host-related factors, including geographic location, plant phenotype and genotype, soil chemistry, and seasonal effects. This study determined that prokaryotic communities associated with agaves were chiefly influenced by the sample type or plant compartment, where the rhizosphere, the phyllosphere and the root and leaf endosphere were clearly distinct from one another and also from the surrounding soils. These results were previously suggested for agaves (Desgarennes et al., 2014), but by using next-generation sequencing (NGS), these differences were made more clear even among epiphytic groups. Moreover, by including a Californian species, A. deserti, living >2000 km apart from A. tequilana and A. salmiana, our data revealed the convergence of prokaryotic communities in Agave, regardless of cultivation status and biogeography. Interestingly, fungal communities presented a different pattern, in which the biogeography of the plant host species played the dominant role. Recent studies in several different experimental systems have similarly reported that fungal communities appear to be differentiated more by geographic distance than are prokaryotic communities (Peay et al., 2007; Shakya et al., 2013; Meiser et al., 2014), which suggests that fungal endemism may be a community-shaping force operating at multiple scales and in multiple habitats. One hypothesis suggests that dispersal limitation is the root cause of this phenomenon (Taylor et al., 2006), and that fungi behave more like plants and animals than do bacteria in this respect. Our analyses and comparisons across sampling sites within individual species substantiate the suggestion that geography plays a larger role in driving fungal than prokaryotic communities. The considerably smaller proportion of shared fungal OTUs than prokaryotic OTUs between samples collected in California and Mexico lends further support to this hypothesis. Nevertheless, the six microhabitats or sample types investigated represent, in both prokaryotic and fungal data sets, a source of major selection. Prokaryotic and fungal epiphytic communities in Agave revealed a strong influence of the plant species’ biogeography (Fig. 4c,d), which can be related to a plant selection and/or niche effect as previously discussed (Desgarennes et al., 2014; Mendes et al., 2014). To what extent this phenomenon depends not only on the biogeography of the plant host species, but also on the host genotype must still be
investigated by comparing microbial communities of agaves with those associated with other sympatric Agave or non-Agave species in these arid environments. Recent studies have demonstrated that plant host-specific traits, including broad morphological characteristics (Kembel et al., 2011) and specific genetic pathways and gene products (Horton et al., 2014; Lebeis et al., 2015), can have significant effects on microbiome composition and diversity. In the endosphere, an influence of the season was observed in the Mexican agaves (Figs 6, S13; Table S4), especially in the prokaryotic communities, as detected previously (Desgarennes et al., 2014). Leaf endophytic fungal communities were similar in A. tequilana and A. salmiana, which suggests common mechanisms of plant–fungus interaction in this habitat.

Low levels of microbial diversity in the cultivated A. tequilana compared with native agaves

Cultivated agaves are susceptible to a number of diseases, many of which cause significant losses in yield and revenue each year (Dalton, 2005). One of the most costly of these diseases, called ‘soft rot’, was previously estimated to have cost the tequila industry $200 million yr$^{-1}$ (Jiménez-Hidalgo et al., 2004). A putative causative agent of ‘soft rot’ has been identified as a member of the bacterial family Enterobacteriaceae (Pantoea agglomerans) (Jiménez-Hidalgo et al., 2004). In this study, we observed significantly lower levels of prokaryotic diversity in the rhizosphere and phyllosphere of the cultivated A. tequilana compared with the native A. salmiana and A. deserti. Furthermore, this lower alpha diversity was the direct result of the dominance in the epiphytes of A. tequilana of a few bacterial families, including genera belonging to Enterobacteriaceae (Pantoea, Leclercia, and Trabusiella). The lack of enrichment for Enterobacteriaceae in the surrounding bulk soils suggests that these bacterial species have adapted to specific associations with their plant host. A number of agronomic and regulatory practices within the tequila industry may be potentially influencing prokaryotic alpha diversity in A. tequilana. First, continuous monocultures, as is typically the case on agave plantations, have been shown to decrease microbial community diversity (Hilton et al., 2013). Second, tequila plantations typically grow young agave plants indoors for a period of time before transplanting to the field, and, before transplant, the roots are sterilized in formaldehyde and left to dry for a period of time (Davis et al., 2011). As rhizosphere microbial communities have been shown to assemble very early in plant development (Chaparro et al., 2014), root sterilization probably alters the progression and development of a natural microbial community. Third, governmental regulations that limit the available genetic pool and propagation techniques (vegetative reproduction via bulbils and rhizome offshoots preferred) result in agave plantlets that are genetically identical to the parental line (Valenzuela, 2011). High genetic homogeneity of the A. tequilana cultivar used in the tequila industry may have allowed the pathogenic microbes to evolve strategies to evade the limited arsenal of available host defenses. Current research aimed at adapting agave as a biofuels feedstock is exploring the use of a variety of Agave species, including the main cultivars from both the fiber and alcohol industries as well as traditionally uncultivated species (García-Moya et al., 2011; Escamilla-Treviño, 2012; Li et al., 2014), thereby potentially increasing the cultivated agave’s resilience to pathogen attack by introducing additional defensive capabilities into the collective gene pool. Moreover, vegetative reproduction of Agave species either in agricultural settings or in natural environments may also have an influence on the endophytic communities, as microorganisms residing inside the mother plant could be potentially inherited by the offshoots and remain present in internal tissues despite surface sterilization. Our observation that endosphere samples exhibited greater between-sample variability, compared with episphere and soil samples, could be explained in part by a combination of the stochastic nature of endosphere colonization and this cross-generational propagation of endophytic microbes. Further research is required to evaluate the heritability of the plant microbiome both in the episphere and in the endosphere compartments of agaves.

Major microbial players highlight convergence across the genus Agave

In accordance with our global NMDS and PERMANOVA analyses, an investigation of the major microbial players demonstrated that the prokaryotic and fungal epiphytic and endophytic communities had a reduced number of OTUs playing a major role in cultivated A. tequilana in comparison with the native species A. salmiana and A. deserti. This analysis also revealed that a group of prokaryotic and fungal taxa was conserved across the three Agave species, although the relative distribution of the common taxa varied across the three hosts. This result is consistent with findings from recent studies in which the microbiomes of closely related species or cultivars exhibit both specific microbial lineages with host-specific abundance patterns and a conserved core microbiome (Schlaeppi et al., 2014; Bulgarelli et al., 2015; Haney et al., 2015; Lebeis et al., 2015). The variation we observed can be explained by the fact that these agaves are native to different habitats and represent different genotypes, as microbial communities are assembled on niche-based processes, as a result of the plant selection effect and environmental factors (Mendes et al., 2013). Interestingly, we found that differences in bulk soil prokaryotic communities seemed not to be significantly correlated with species management status. By contrast, we found a reduced number of major fungal players associated with A. tequilana soils in comparison with those of native agaves.

Interestingly, prokaryotic communities in the desert environments surveyed here do not differ markedly at the phylum level from other soil environments described previously in other phytobiome studies (Lundberg et al., 2012; Bulgarelli et al., 2015; Edwards et al., 2015; Zarraonaindia et al., 2015). Similarly, the increased abundance of Proteobacteria and decreased presence of Acidobacteria in the plant-associated samples with respect to the surrounding soil has been observed for other plant hosts (Edwards et al., 2015; Zarraonaindia et al., 2015). Taken together, these findings suggest the presence of a set of conserved forces acting to shape the structure of both the plant-associated and soil
prokaryotic microorganisms across a wide array of environments and host species. For fungi, however, most temperate forests are dominated by Basidiomycota (O’Brien et al., 2005); the fungal communities associated with agaves described here were dominated by members of Ascomycota. This is consistent with studies of other semiarid plants such as perennial grasses, which are predominantly colonized by Ascomycota (Porras-Alfaro et al., 2011), and is also consistent with the trend we observed within our study that differences in fungal communities are correlated with differences in geographic distance.

The presence of Glomeromycota sequences in our study was low, but we detected a preferential association of the Entrophospora genus with A. salmiana and Glomus with A. deserti. This apparent lack of Glomeromycota may in part be attributable to primer bias, as it is well known that ‘universal fungal’ primers often fail to amplify these basal organisms (Martin & Rygiewicz, 2005). Ongoing metagenomic studies on the rhizosphere of agaves will help elucidate whether members of the Glomeromycota were underrepresented in this study.

The endophytic core of Agave

Our extensive analyses on the Agave-associated microbial communities suggested that the endospheres were less influenced by the biogeography of the host species, but more by the plant compartment and seasonal changes. We corroborated that indeed there is a group of prokaryotic organisms living inside agaves which increased in abundance under natural drought conditions, as already suggested (Desgarennes et al., 2014). We discovered that fungal leaf endophytic communities were similar across the Mexican agaves, and that these fungal taxa were not affected by season. Importantly, a broader group of taxa were shared across Agave species in the root endosphere, representing in all cases >40% of the community. As previously mentioned, Agave species reproduce in nature mostly through vegetative offshoots that remain connected to the mother through their lives (Gentry, 1982), probably enhancing the heritability of the endophytic microbial symbionts. As recent studies have shown that plant microbial communities can change significantly across the life span of a single plant for even annual species (Copeland et al., 2015), it will be interesting to investigate successional dynamics in perennial species with asexually reproducing lifestyles. Some of the microorganisms shared among the three Agave species, among them several previously reported diazotrophic strains (Desgarennes et al., 2014), have been isolated in our laboratories and will be sequenced in the near future. These efforts will provide a genomic baseline to further deepen our understanding of the complex plant–microbe interactions in arid and semiarid ecosystems.

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Author contributions

S.G., D.C-D., G.N., T.W., A.V., L.P.P-M. and S.G.T. planned and designed research; D.C-D., D.D., C.F-G. and S.G. performed experiments; S.G., G.N., D.D., C.F-G. and L.P.P-M. conducted fieldwork; D.C-D. and S.C. prepared libraries and processed sequencing data; D.C-D., D.D., C.F-G. and L.P.P-M. analyzed the data; and D.C-D., D.D., A.V., L.P.P-M. and S.G.T. wrote the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Raw read counts across all samples grouped by sample type.

Fig. S2 Established cut-off thresholds for technical reproducibility across OTUs for 16S and ITS2 data sets.

Fig. S3 OTU distributions across sample subsets.

Fig. S4 Phylum-level relative abundance plots of prokaryotic and fungal communities associated with each Agave species by sample type.

Fig. S5 Order-level relative abundance plots of prokaryotic communities associated with all rhizosphere and bulk soil samples.

Fig. S6 NMDS clustering for prokaryotic data of all samples using unweighted and weighted UniFrac distances.

Fig. S7 NMDS clustering of all samples by sample type, host species and location according to the legend.

Fig. S8 Major prokaryotic taxa associated with Agave tequilana based on the 80–20 rule (Pareto).

Fig. S9 Major prokaryotic taxa associated with Agave salmiana based on the 80–20 rule (Pareto).

Fig. S10 Major prokaryotic taxa associated with Agave deserti based on the 80–20 rule (Pareto).

Fig. S11 Major fungal taxa associated with Agave tequilana based on the 80–20 rule (Pareto).

Fig. S12 Major fungal taxa associated with Agave salmiana based on the 80–20 rule (Pareto).

Fig. S13 Major fungal taxa associated with Agave deserti based on the 80–20 rule (Pareto).

Fig. S14 OTU distributions across Agave species in the endosphere.

Fig. S15 Fungal taxa associated with the endosphere of all three Agave species and their relative abundance at the dry and rainy seasons.

Table S1 Estimated Shannon diversity in the prokaryotic and fungal communities associated with each Agave species

Table S2 PERMANOVA of the microbial communities associated with Agave tequilana considering all factors and their interactions

Table S3 PERMANOVA of the microbial communities associated with Agave salmiana considering all factors and their interactions

Table S4 PERMANOVA of the microbial communities associated with Agave deserti considering all factors and their interactions

Table S5 PERMANOVA of the microbial communities associated with the endosphere of Agave tequilana and A. salmiana considering all factors and their interactions

Methods S1 Methods for sample collection, DNA extraction, PCR amplification, sequencing, and data processing, including statistical analyses.

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