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Incorporating phylogenetic metrics to microbial co-occurrence networks based on amplicon sequences to discern community assembly processes

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Abstract

Co-occurrence network analysis based on amplicon sequences is increasingly used to study microbial communities. Patterns of co-existence or mutual exclusion between pairs of taxa are often interpreted as reflecting positive or negative biological interactions. However, other assembly processes can underlie these patterns, including species failure to reach distant areas (dispersal limitation) and tolerate local environmental conditions (habitat filtering). We provide a tool to quantify the relative contribution of community assembly processes to microbial co-occurrence patterns, which we applied to explore soil bacterial communities in two dry ecosystems. First, we sequenced a bacterial phylogenetic marker in soils collected across multiple plots. Second, we inferred co-occurrence networks to identify pairs of significantly associated taxa, either co-existing more (aggregated) or less often (segregated) than expected at random. Third, we assigned assembly processes to each pair: patterns explained based on spatial or environmental distance were ascribed to dispersal limitation (2%–4%) or habitat filtering (55%–77%), and the remaining to biological interactions. Finally, we calculated the phylogenetic distance between taxon pairs to test theoretical expectations on the linkages between phylogenetic patterns and assembly processes. Aggregated pairs were more closely related than segregated pairs. Furthermore, habitat-filtered aggregated pairs were closer relatives than those assigned to positive interactions, consistent with phylogenetic niche conservatism and cooperativism among distantly related taxa. Negative interactions resulted in equivocal phylogenetic signatures, probably because different competitive processes leave opposing signals. We show that microbial co-occurrence networks mainly reflect environmental tolerances and propose that incorporating measures of phylogenetic relatedness to networks might help elucidate ecologically meaningful patterns.

KEYWORDS
biological interactions, co-ocurrence patterns, dry ecosystems, habitat filtering, microbial networks, phylogenetic distance, soil bacteria

1 | INTRODUCTION

Ecological communities are assembled by a plethora of processes that operate at a wide range of scales (HilleRisLambers, Adler, Harpole, Levine, & Mayfield, 2012). The composition of local communities is constrained by the evolutionary history of the global species pool. Community composition is further influenced by stochastic processes such as dispersal and demographic events, as well as niche-based processes including species interactions between them and with their abiotic environment (HilleRisLambers et al., 2012). Since Diamond first used the patterns of species co-occurrence to infer biological interactions between pairs of species (Diamond, 1975), the study of assembly mechanisms rapidly incorporated the need to discard random species associations (Connor & Simberloff, 1979). Nowadays, most studies identify those species pairs that are significantly associated, either aggregated (i.e. copresent or co-absent) or segregated (i.e. mutually excluding each other) across multiple assemblages. Species aggregation is often assigned to positive biological interactions (e.g. mutualism and commensalism) and segregation to negative biological interactions (e.g. competition and predation) (Freilich, Wieters, Broitman, Marquet, & Navarrete, 2018). This approach disregards that co-occurrence patterns may be substantially determined by the failure of certain species to reach an available site (dispersal limitation) and by their shared (or unshared) tolerances to the local set of abiotic conditions (habitat filtering) (Freilich et al., 2018). An unambiguous interpretation of the mechanisms that structure ecological communities, therefore, requires adding spatial and environmental information to traditional co-occurrence analyses (Blois et al., 2014; D'Amen, Mod, Gotelli, & Guisan, 2018).

Microbial communities are extremely diverse, adding further complexity to the challenge of understanding the processes that structure ecological communities. Dispersal has been traditionally assumed not to be limiting for terrestrial microorganisms, with their minute size, enormous population numbers and high dispersal rates underlying the cosmopolitan distribution of many taxa (Finlay, 2002; Ramette & Tiedje, 2007). However, spatial features such as latitude or geographic distance (even at the metre scale) impact the structure and diversity of soil bacterial communities (Horner-Devine, Lage, Hughes, & Bohannan, 2004; Martiny et al., 2006; Meyer et al., 2018). Such spatial patterns, in which neighbouring communities resemble each other more than distant communities, might be attributed to dispersal intensity decaying with distance (Bahn, Krohn, & O'Connor, 2008). Environmental characteristics, including salinity, acidity, humidity or fertility, have been repeatedly reported as relevant factors, leading to the general conception that habitat filters play a key role in shaping microbial communities (Fierer, 2017; Martiny et al., 2006; Schimel, Balser, & Wallenstein, 2007). Biological interactions have received less attention, probably due to the difficulty of demonstrating their effect at the community level (but see e.g. Goldfarb et al., 2011). However, multiple biological interactions among bacteria have been documented in the laboratory. Evidence for positive interactions includes cell–cell communication, division of labour in biofilms, exchange of electron donors and metabolites, sharing of public goods or coordinated motility (Jousset, Eisenhauer, Materne, & Scheu, 2013; Morris, Lenski, & Zinser, 2012; Zengler & Zaramela, 2018), among others. Main negative interactions include competition by interference (e.g. through toxins, antibiotics or direct lysis using nanoneedles) and resource exploitation (e.g. phosphorous sequestration or iron scavenging), predation, disruption of communication or social cheating by the capitalization of public goods (Hibbing, Fuqua, Parsek, & Peterson, 2010; Russell, Peterson, & Mougous, 2014).

Network analysis based on amplicon sequences is increasingly used to study co-occurrence patterns in complex microbial communities (Barberán, Bates, Casamayor, & Fierer, 2012; Faust & Raes, 2012). Networks can provide relevant insights into biological interactions, particularly within well-known microbial guilds (e.g. Ho et al., 2016). However, as several authors note (Brisson, Schmidt, Northen, Vogel, & Gaudin, 2019; Faust & Raes, 2012; Pérez-Valera et al., 2017), significant associations between pairs of microbial taxa might respond to stochastic or niche-based processes other than biological interactions. We propose incorporating spatial and environmental information to soil bacterial co-occurrence networks to quantify the relative contribution of assembly mechanisms including dispersal limitation, habitat filtering and biological interactions. Barner, Coblentz, Hacker, and Menge (2018), in their recent criticism on the application of co-occurrence methods to predict nontrophic species interactions, highlight the necessity to associate the results at the community level (i.e. number of interacting species, number of interactions and proportion of positive and negative interactions) to functions or mechanisms. In a step forward, with the aim to link the observed co-occurrence patterns with their putative driving mechanisms, we propose to study the phylogenetic signal left by each of these processes. Previous approaches have used the phylogenetic composition of the whole community to elucidate the relative contribution of stochastic and niche-based assembly processes (Stegen, Lin, Konopka, & Fredrickson, 2012). Instead of calculating the phylogenetic distance across all possible pairwise comparisons or between each taxon and its closest relative in the community (Stegen et al., 2012), we compute the phylogenetic distance between pairs of significantly associated taxa that emerge from co-occurrence network analysis. A series of expectations can be drawn, based on the ecological theory, to link the resulting phylogenetic patterns to their ecological assembly processes. The following expectations assume that evolutionarily related taxa tend to be ecologically more similar than distant taxa, a scenario that has been found to be widespread in bacterial lineages (Goberna & Verdú, 2016; Martiny, Treseder, & Pusch, 2013; Stegen et al., 2012). First, habitat filtering favours the co-existence of closely related species based on their shared niche preferences (Webb, Ackerley, McPeek, & Donoghue, 2002). Second, competition, the most widespread negative interaction, limits the similarity of co-existing lineages and favours the co-existence of distantly related taxa (Webb et al., 2002). This pattern can be not so straightforward when traits conferring fitness to competing species are

highly conserved. In this case, particularly superior lineages with high relative fitness can outcompete entire clades, resulting in the co-existence of closely related taxa (Goberna, García, & Verdú, 2014; Goberna, Navarro-Cano, Valiente-Banuet, García, & Verdú, 2014; Mayfield & Levine, 2010). Finally, positive biological interactions can be reasonably thought to occur between organisms that are distant enough not to compete with each other and therefore promote the co-existence of functionally (and phylogenetically) different species (Valiente-Banuet & Verdú, 2013).

In order to show that microbial co-occurrence patterns based on network analysis should not be uncritically assigned to biological interactions, we: (a) computed microbial co-occurrence networks across multiple assemblages, (b) used spatial and environmental data to ascribe co-occurrence patterns to assembly processes and (c) calculated phylogenetic distances between pairs of co-occurring taxa and analysed them under the light of the expectations above. In search for generalizable patterns, we applied this analysis in two ecosystems located in different continents and with contrasting conditions regarding lithology, soil properties and plant community composition. Both sites share, however, a common feature: they are water-limited environments, in which facilitation between plant species structures plant communities in multispecific patches (hereafter 'patches') surrounded by low-cover areas or open spaces (hereafter 'gaps') (Aguiar & Sala, 1999). The patchy structure of the vegetation strongly determines the assembly of soil bacterial communities (Goberna, Navarro-Cano, et al., 2014; Hortal et al., 2013; Roy et al., 2013). Soil bacteria living in gaps cope with intense radiation, high temperatures, desiccation and low levels of resources, resulting in the overrepresentation of bacterial functional traits conferring tolerance to abiotic stress (Goberna, Navarro-Cano, et al., 2014; Goberna, Pascual, García, & Sánchez, 2007). Plant patches relieve abiotic stress by reducing temperature and radiation, while fostering the accumulation of water and resources (Navarro-Cano, Verdú, García, & Goberna, 2015). Underneath plant patches, soil microbial communities are denser, more active and withstand higher competitive stress, as reflected by their larger respiration-to-biomass ratios and the overrepresentation of competition-related traits (Goberna, Navarro-Cano, et al., 2014; Goberna et al., 2007). Therefore, abiotically stressful ecosystems generate a spatial mosaic of low-productive habitats comparatively dominated by abiotic filtering interspersed with high-productive habitats with magnified biotic interactions. Such a mosaic brings an excellent opportunity to tease out the abiotic and biotic processes determining the phylogenetic patterns of the community assembly of soil bacteria. We show, in both study systems, that soil bacterial co-occurrence networks mainly reflect (un)shared environmental preferences and discuss their limitations for detecting biological interactions when analysing extremely complex communities. We provide a tool to the following: (a) quantify the relative contribution of ecological mechanisms—including dispersal limitation, habitat filtering, positive and negative biological interactions—that underlie co-occurrence patterns in microbial communities and (b) estimate the phylogenetic signature of each assembly mechanism.

2 | MATERIALS AND METHODS

We developed a four-step workflow to quantify the relative contribution of the main assembly mechanisms of soil bacterial communities: (a) sampling soils across plots (i.e. plant patches and gaps) and constructing a bacterial OTU (operational taxonomic unit) per plot abundance matrix; (b) identifying pairs of bacterial OTUs that are significantly associated across plots through co-occurrence network analysis and calculating their (pairwise) phylogenetic distances; (c) assigning community assembly processes to pairs of significantly associated OTUs by adding spatial and environmental information to co-occurrence patterns; and (d) testing for statistical differences in the phylogenetic distance of OTU pairs assembled through different ecological mechanisms. This workflow is schematized in Figure 1 and subsequently described. All analyses were performed separately for each study site, as our purpose was not to compare both ecosystems but to apply our analytical tool and seek for generalizable patterns.

2.1 | Study sites and soil sampling

We studied two dry ecosystems in Spain and Mexico. The study site in Crevillent Mountain Range (SE Spain; 38°16′N, 0°50′W) has a mean annual rainfall of 240 mm, an average temperature of 20°C and is located at 350 m a.s.l. Soils are loamey–clayey, calcaric and saline have neutral pH and developed from gypsum outcrops, with 25% plant cover of a shrubland dominated by *Ononis tridentata* (Goberna et al., 2007; Navarro-Cano et al., 2014; Supplementary Information S1). The Valley of Zapotitlán (18°19′45.44″N; 97°27′20.95″W), a local basin of the Tehuacán-Cuicatlán Valley in Mexico, has an annual average rainfall of 380 mm, an annual mean temperature of 21°C and is located at 1,500 m a.s.l. Soils are loamey–clayey, calcaric and nonsaline, have neutral to basic pH and developed from lutites, with 55% plant cover of a shrubland dominated by *Mimosa luisana* (Valiente-Banuet et al., 2000; Valiente-Banuet & Verdú, 2007; Supplementary Information S1).

In both sites, plant communities are characterized by individuals of multiple species that are spatially associated forming discrete vegetation clumps (Supplementary Information S2). Plant patches range from 1 to 5 m^2. Gaps, that is the open space between patches located at least 1 m beyond the vertical projection of the canopy of the patch, were defined with an area and geometric shape that corresponded to that of the adjacent patch. We selected patches and adjacent gaps in a paired design, making a total of 28 plots (i.e. patches and gaps) in Spain and 64 in Mexico. We recorded the spatial coordinates of all plots, which were distributed in a total area of 0.2 and 1 ha. in Spain and Mexico, respectively. We collected one surface soil sample (0–2 cm) per plot, making 92 soil samples. Each sample was composed by five subsamples (ca. 100 g) that were randomly taken from the area of each patch (or gap), transported to the laboratory on ice and sieved through a <2 mm mesh. We measured physical and chemical soil parameters using standard protocols (Supplementary Information S3). To test for spatial autocorrelation in soil physical and chemical

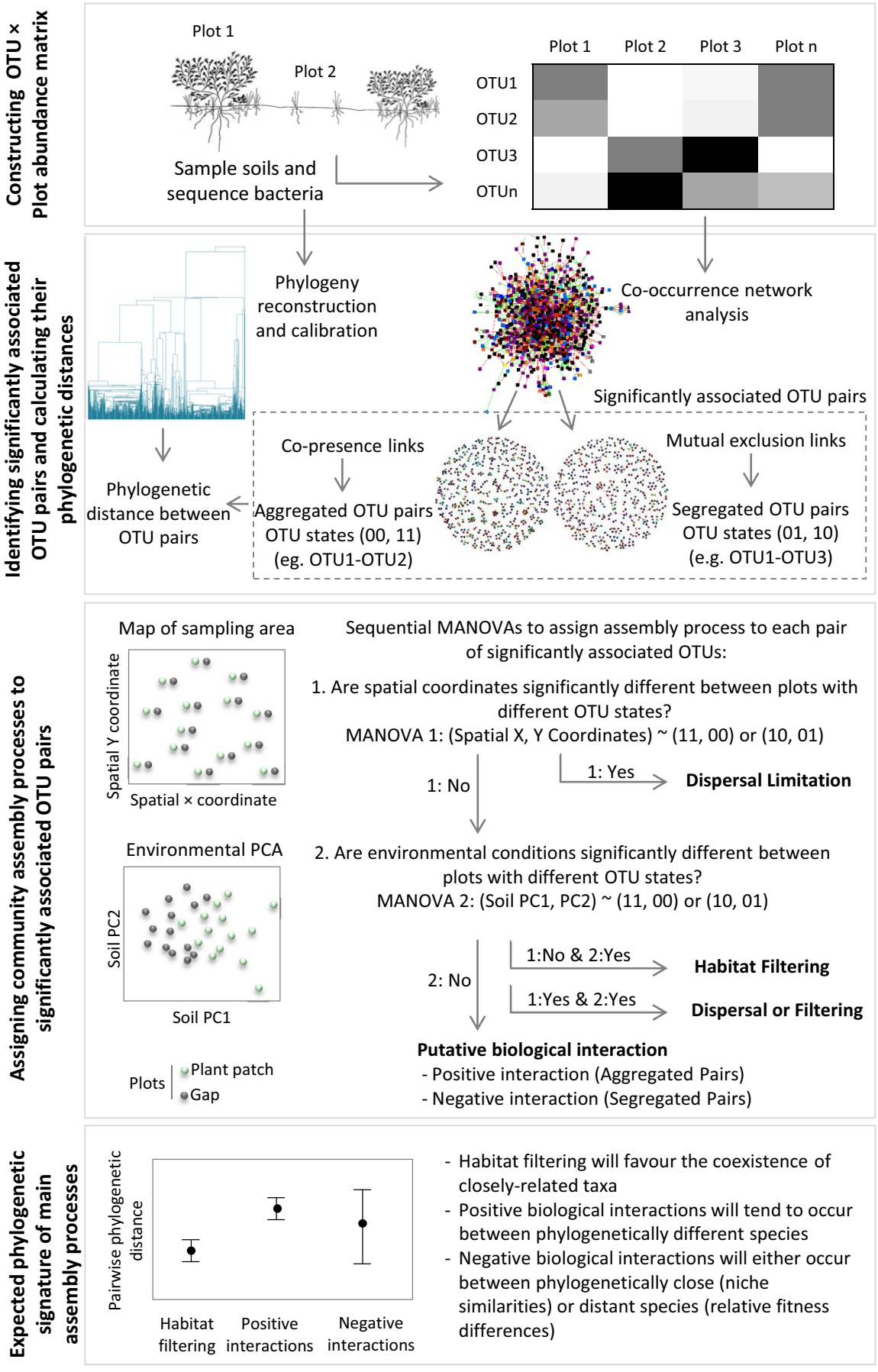
Constructing OTU × Plot abundance matrix
Plot 1
Plot 2
Sample soils and sequence bacteria
Plot 1
Plot 2
Plot 3
Plot n
OTU1
OTU2
OTU3
OTUn
Identifying significantly associated OTU pairs and calculating their phylogenetic distances
Phylogeny reconstruction and calibration
Co-occurrence network analysis
Significantly associated OTU pairs
Co-presence links
Mutual exclusion links
Phylogenetic distance between OTU pairs
Aggregated OTU pairs OTU states (00, 11) (eg. OTU1-OTU2)
Segregated OTU pairs OTU states (01, 10) (e.g. OTU1-OTU3)
Assigning community assembly processes to significantly associated OTU pairs
Map of sampling area
Spatial Y coordinate
Spatial × coordinate
Environmental PCA
Soil PC2
Soil PC1
Plots
Plant patch
Gap
Sequential MANOVAs to assign assembly process to each pair of significantly associated OTUs:
1. Are spatial coordinates significantly different between plots with different OTU states?
MANOVA 1: (Spatial X, Y Coordinates) ~ (11, 00) or (10, 01)
1: No
1: Yes
Dispersal Limitation
2. Are environmental conditions significantly different between plots with different OTU states?
MANOVA 2: (Soil PC1, PC2) ~ (11, 00) or (10, 01)
2: No
1:No & 2:Yes
Habitat Filtering
1:Yes & 2:Yes
Dispersal or Filtering
Putative biological interaction
- Positive interaction (Aggregated Pairs)
- Negative interaction (Segregated Pairs)
Expected phylogenetic signature of main assembly processes
Pairwise phylogenetic distance
Habitat filtering
Positive interactions
Negative interactions
- Habitat filtering will favour the coexistence of closely-related taxa
- Positive biological interactions will tend to occur between phylogenetically different species
- Negative biological interactions will either occur between phylogenetically close (niche similarities) or distant species (relative fitness differences)

FIGURE 1 Methodological framework used to calculate the relative contribution of dispersal limitation, habitat filtering and biological interactions in the assembly of soil bacterial communities [Colour figure can be viewed at wileyonlinelibrary.com]

parameters, we performed Mantel tests to correlate geographic and environmental distance matrices, based on Euclidean distances, in the *vegan* package for R v 3.5.1 (Oksanen et al., 2018; R Core Team, 2018).

2.2 | Constructing bacterial OTU × plot abundance matrices

We extracted total DNA from each soil sample, PCR amplified and sequenced a phylogenetic marker using primers specific for bacteria. Procedures used for the Spanish site were described in Goberna, Navarro-Cano, et al. (2014), and similar methods were used in Mexico as follows:

We extracted soil DNA with the PowerSoil® DNA isolation kit (MO BIO Laboratories) and amplified the 16S rRNA gene with the universal bacterial primers 27Fmod (5′-AGRGTTTGATCMTGGCTCAG; Kuske, Barns, Grow, Merrill, & Dunbar, 2006) and 519Rmod (5′-GTNTTACNGCGGCKGCTG; Frank, Rogers, Olins, Vidoudez, & Girguis, 2013). We sequenced amplicons with the Roche 454 FLX titanium technology. After denoising, and removal of short (<200 base pairs), low-quality (average quality score <25, including Ns or homopolymers >6 base pairs) and chimeric sequences with QIIME (Caporaso, Kuczynski, et al., 2010), we delimited OTUs at 97% similarity and removed singleton OTUs (details in Supplementary Information S3). This process yielded a total 3,290 bacterial OTUs in Spain and 5,689 in Mexico. For each site, we constructed a matrix including the number of sequences for every OTU in each plot. To account for the differential sampling depth across plots, we transformed the number of sequences into relative abundances by dividing the number of sequences of each OTU in each plot by the total number of sequences in the same plot. Finally, we corrected the relative abundance of each OTU by the number of estimated 16S rRNA gene copies using the procedure by Kembel, Wu, Eisen, and Green (2012). To describe overall patterns in bacterial community variation, we calculated metrics of alpha diversity (Shannon index) in the *vegan* package (Oksanen et al., 2018) and beta diversity with the *beta.multi.abund* function in the *betapart* package for R based on Bray–Curtis multiple-site dissimilarity (Baselga, Orme, Villeger, De Bortoli, & Leprieur, 2018). We used this function to compute the nestedness and turnover components of beta diversity, in order to examine whether poorer communities contain a subset of the species present in richer communities or whether there is spatial species replacement across plots (Baselga, 2010).

2.3 | Identifying significantly associated OTU pairs

Co-occurrence network analysis was used to detect significant associations between pairs of OTUs across plots including the following: (a) aggregated pairs, that is to say, pairs of OTUs that co-occur more frequently than expected at random, and thus share a positive or copresence link and (b) segregated pairs, that is pairs of OTUs that co-occur less frequently than expected by chance, and thus share a negative or mutual exclusion link. We performed network analysis using CoNet 1.0b6 (Faust & Raes, 2012, 2016; Faust et al., 2012). One network was reconstructed per study system with the script available at http://psbweb05.psb.ugent.be/conet/cmdline.php, as detailed in Pérez-Valera et al. (2017). Prior to network construction, low-abundance OTUs were removed to reduce artefactual associations (Faust et al., 2012). Specifically, our original matrices contained the relative abundance of 3,290 OTUs in 28 plots (Spain) and 5,689 OTUs in 64 plots (Mexico). First, we removed those OTUs showing less than 0.05% relative abundance on average across plots, leaving 441 and 370 OTUs in the filtered matrices in Spain and Mexico, respectively. We further excluded those OTUs that were present in less than 1/3 of the plots, making a total of 229 (Spain) and 298 OTUs (Mexico) in the final filtered matrices. The sum of the filtered OTUs was kept to preserve taxon proportions. Network links were identified including two measures of correlation (Pearson and Spearman) and dissimilarity (Bray–Curtis and Kullback–Leibler) to increase the robustness of the analysis (Faust & Raes, 2016). Links were considered as undirected, and their sign was used to distinguish between copresence (positive) and mutual exclusion (negative) links. Statistical significance was tested by obtaining the link- and measure-specific *p*-value as the mean of the permutation distribution under the bootstrap distribution, with 1,000 iterations each. Probability values of different correlation/dissimilarity measures supporting the same link were merged using Brown's method and corrected for multiple testing using Benjamini–Hochberg's procedure, which helps controlling the number of false-positive associations (Faust & Raes, 2012). To reduce the detection of spurious associations, only those links supported by at least two measures of correlation/dissimilarity and having an adjusted merged *p*-value below .05 were included in downstream analyses.

2.4 | Calculating phylogenetic distances between OTU pairs

To calculate the phylogenetic distance between all pairs of aggregated or segregated OTUs, we aligned sequences representative of each OTU using PyNAST (Caporaso, Bittinger, et al., 2010) and manually curated the alignments. The reconstruction and calibration of bacterial phylogenetic trees in the Spanish site were performed in Goberna and Verdú (2018). We used the same procedure to reconstruct phylogenies in the Mexican site using RAxML 8.2.4 (Stamatakis, 2014; details in Supplementary Information S3). Then, we calibrated the tree so that branch lengths represent evolutionary time (in Myr) instead of nucleotidic changes to facilitate comparisons of the results derived from both study sites. We calibrated the trees by using eight dated nodes based on a penalized likelihood approach with treePL (Sanderson, 2002; Smith & O'Meara, 2012;

Supplementary Information S3). We finally calculated the phylogenetic distance in calibrated trees for all pairs of aggregated or segregated OTUs with the *cophenetic* function in the *APE* package for R (Paradis & Schliep, 2018).

2.5 | Assigning community assembly processes to OTU pairs

To infer which assembly process underlies the association between each OTU pair, we adapted the methodological framework by Blois et al. (2014). To do so, we first transformed the OTU × Plot relative abundance matrices into incidence matrices by assigning 1 to present (relative abundance >0) and 0 to absent OTUs. Then, for every pair of significantly associated OTUs, we registered the state (present/absent) of each OTU across plots: (a) for aggregated pairs, we recorded whether both OTUs were copresent (state 11) or co-absent (state 00) in each plot; (b) for segregated pairs, we recorded whether one (state 10) or the other OTU (state 01) was present in each plot.

In order to detect whether the spatial and/or environmental distance across plots significantly explains the co-occurrence patterns of significantly associated OTU pairs, we performed a series of one-way multivariate analysis of variance (MANOVA) as follows (Figure 1). First, we tested whether there is a relationship between spatial distance and OTU state for every OTU pair. The dependent variable in each MANOVA was a two-element vector including the *X* and *Y* spatial coordinates of each plot. The independent variable was the OTU state across plots, that is 11 versus 00 states for aggregated pairs and 10 versus 01 for segregated pairs. In these MANOVAs, the rejection of the null hypothesis—that is the detection of significant differences in the geographic distance between plots with different OTU states—can be interpreted as an indication of dispersal limitation (Blois et al., 2014). That is to say, if plots where two aggregated OTUs are copresent are spatially distant from plots where the same OTUs are co-absent, this association might be based on limitations to reach distant plots. Similarly, if the presence (or absence) of each OTU conforming a segregated pair can be explained based on the spatial distance across plots, then dispersal limitation might underlie this pattern.

Second, we tested whether there is a relationship between environmental distance, in terms of soil abiotic conditions, and OTU state for every OTU pair. To reduce the dimensionality of soil abiotic parameters we used principal component (PC) analysis, with the *prcomp* function in R based on a correlation matrix. The dependent variable in each MANOVA was a two-element vector including the scores of the first two PCs, and the independent variable was the OTU state across plots as above. In these MANOVAs, the rejection of the null hypothesis—that is the detection of significant differences in the environmental distance between plots with different OTU states—can be interpreted as an indication of habitat filtering (Blois et al., 2014). That is, if plots where two aggregated OTUs are copresent tend to be similar in their abiotic conditions and significantly different from plots where the same OTUs are co-absent, this association might be based on their shared abiotic tolerances. A similar argumentative line can be applied to the mutual exclusion of two OTUs based on their differential abiotic requirements. When both the spatial and the environmental distances across plots are significantly different between OTU states, both dispersal limitation and habitat filtering might be operating (Figure 1). Note that the spatial autocorrelation of abiotic parameters could also lead to an ambiguous ascription to dispersal limitation or habitat filtering.

Following the rationale by Blois et al. (2014), only significant associations between pairs of taxa that are neither based on spatial distance nor on environmental factors can be interpreted as putative biological interactions (Figure 1). We ascribed pairs of aggregated OTUs meeting such conditions to positive biological interactions and those of segregated OTUs to negative interactions. Since the variation that cannot be accounted for by space or environment might not be exclusively capturing biological interactions, we introduced a final validation step in our workflow. We analysed the phylogenetic signal left by each assembly mechanism to verify whether they match the expectations based on the ecological theory (as elaborated above).

2.6 | Phylogenetic distance of OTU pairs assembled through different mechanisms

We tested whether the phylogenetic distance among OTU pairs differs significantly depending on the underlying assembly mechanism by using permutation one-way ANOVA. Since dispersal limitation was negligible in our study systems (see Results section), we performed this analysis to compare phylogenetic distances between pairs of OTUs assembled through habitat filtering and putative biological interactions. Specifically, the dependent variable was the phylogenetic distance for all significantly associated OTU pairs and the independent variable was a categorical factor coding the assembly mechanism with four levels: habitat filtering for aggregated pairs, positive biological interactions, habitat filtering for segregated pairs and negative biological interactions. Post hoc comparisons to determine significant differences between assembly mechanisms were run through pairwise permutation *t* tests corrected by false discovery rates. We ran 999 permutations to obtain model significance and post hoc tests in the *RVAIDEMEMOIRE* package for R (Hervé, 2018). Appendix 1 contains a R script and example data (from the Spanish site) to allow reproducing the assignment of assembly processes to OTU pairs and testing for differences in phylogenetic distances among OTU pairs assembled through different processes.

3 | RESULTS

Soil physical and chemical parameters did not show spatial autocorrelation in the Spanish site for any of the 11 variables measured (Mantel $r \approx 0$, $p > .05$). Only three out of fifteen parameters measured in Mexico were significantly correlated with spatial distance, namely, total organic carbon (Mantel $r = .13$, $p < .01$),

pyrophosphate extractable carbon (Mantel r = .61, p < .01) and ammonium nitrogen (Mantel r = .17, p < .01). Soil bacterial communities in Spain and Mexico were highly dominated by Proteobacteria and Actinobacteria, which accounted for 51%–80% of the community both underneath plant patches and gaps, but included up to 11–15 other phyla at detectable levels (Supplementary Information S4). Both sites showed (mean ± *SE*) 430 ± 24 (Spain) and 544 ± 16 (Mexico) OTUs per plot (i.e. averaging patches and gaps). Alpha-diversity values averaged 5.70 ± 0.08 and 5.98 ± 0.03 and beta diversity 0.949 and 0.973 in Spain and Mexico, respectively. In both cases, beta diversity mostly originated from a high turnover across plots (≥99.98% of beta-diversity values), while nestedness was negligible.

We used co-occurrence network analysis to identify significant associations between pairs of bacterial taxa, including OTUs that co-occur more (aggregated pairs) and less (segregated pairs) frequently than expected at random (Supplementary Information S5–S8). Aggregated pairs were more abundant than segregated pairs in Spain (4,691 vs. 1,866) and Mexico (787 vs. 201). We used the spatial and environmental distance across plots to quantify the relative contribution of dispersal limitation, habitat filtering and biological interactions in determining the observed co-occurrence patterns. Habitat filtering was the main assembly mechanism explaining significant species associations in both sites: up to 71% of the aggregated and 76% of segregated pairs of bacterial OTUs in Spain and 57% and 66% in Mexico responded to abiotic conditions (Figure 2). Dispersal limitation explained less than 2% and 4% of all significant pairs in Spain and Mexico, respectively (Figure 2). Ambiguous assignation to dispersal limitation or habitat filtering occurred on average for 4.3% pairs in Spain and 8.5% in Mexico (Figure 2). We interpret that the higher proportion of associations with an ambiguous ascription in Mexico could be caused by the existence of abiotic variables showing spatial autocorrelation. Positive biological interactions represented 21% of the aggregated pairs in Spain and 31% in Mexico, while negative interactions occurred, respectively, in 20% and 23% of segregated pairs (Figure 2).

Habitat filtering in both study sites responded to different soil abiotic variables. In Spain, the two main axes extracted from a PCA on abiotic variables explained 82% of the total variance. PC1 markedly segregated plant patches and gaps based on their differential fertility and pH, patches showing higher contents in all oxidizable forms of carbon and nitrogen as well as soil moisture, and lower pH values (Supplementary Information S9). PC2 captured the variation across patches in the mineral forms of nitrogen (nitrate and ammonium) and water-soluble carbohydrates (Supplementary Information S9). In Mexico, both PCs explained 40% of the variance. As in Spain, PC1 discriminated patches and gaps mainly along a gradient of soil fertility and pH. PC2, however, recorded the across-plot variability (both for patches and gaps) in the forms of mineral nitrogen and granulometric fractions (clay, silt and sand).

Bacterial OTUs from aggregated pairs were evolutionarily more related than segregated pairs in the Spanish (3,744 ± 35 vs. 4,417 ± 51 My; $F_{1, 6555}$ = 109.2; p < .001) and Mexican communities (5,001 ± 65 vs. 5,971 ± 44 My; $F_{1, 986}$ = 55.6; p < .001). The phylogenetic distance of associated OTU pairs was significantly different depending on the mechanism involved in their assembly both in Spain ($F_{3, 6004}$ = 42.5; p < .001) and Mexico ($F_{3, 851}$ = 18.9; p < .001) as follows. In the two study systems, OTUs aggregated due to habitat filtering were phylogenetically closer than OTUs segregated by the same process (left and right panels, Figure 3). Aggregated OTUs co-existing due to habitat filtering were phylogenetically closer than those co-existing through positive interactions (left panel, Figure 3). OTUs living segregated due to habitat filtering had a phylogenetic distance that did not differ statistically from OTUs mutually excluding each other due to negative interactions (right panel, Figure 3). The phylogenetic patterns described held despite the fact that the taxa involved in aggregated and segregated pairs differed between study sites (Supplementary Information S10 and S11).

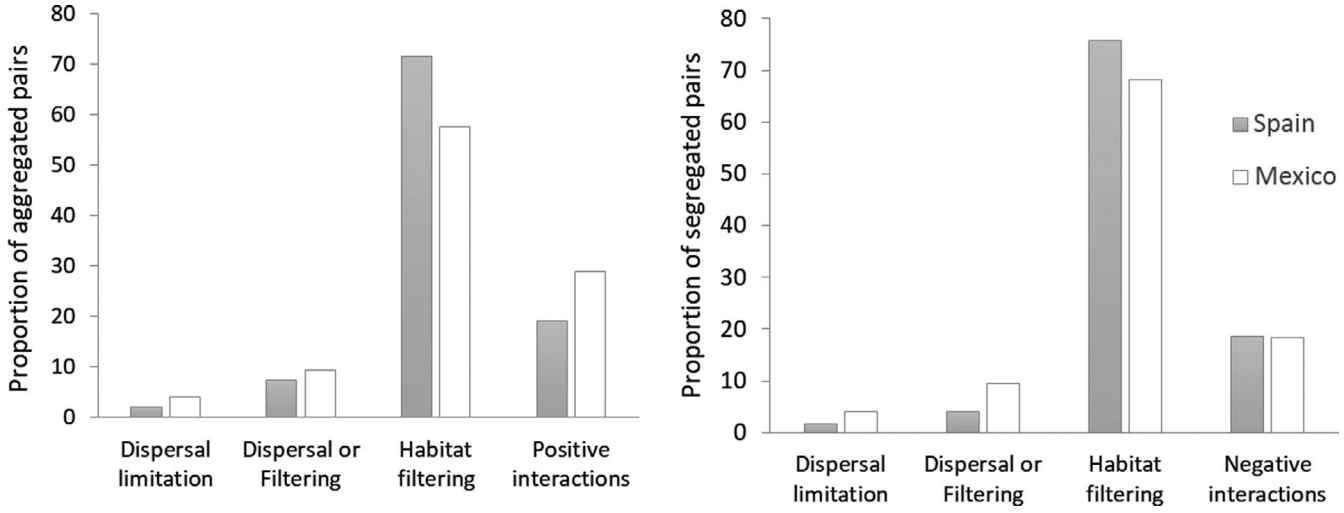

FIGURE 2 Frequency distribution of assembly mechanisms explaining aggregated and segregated bacterial OTU pairs in two dry ecosystems in Spain and Mexico

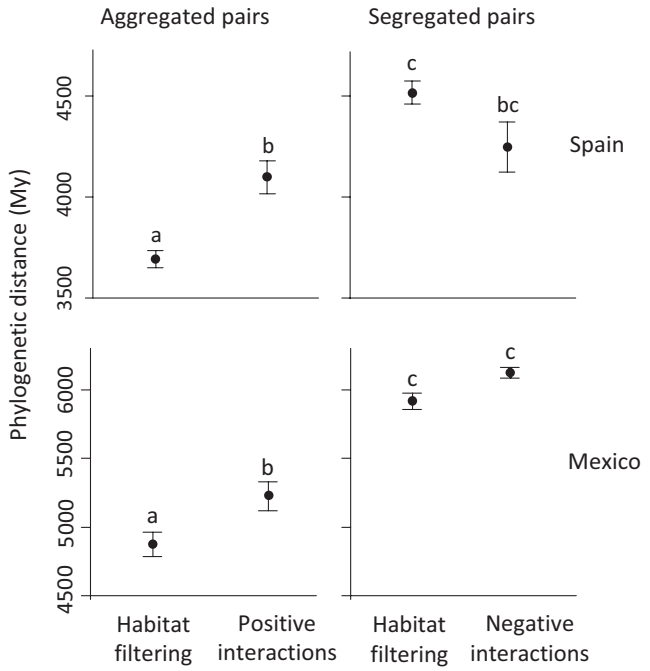

FIGURE 3 Phylogenetic distance, in million years, between the bacterial OTUs involved in pairs assembled through habitat filtering and (positive or negative) biological interactions. Left panels refer to aggregated pairs and right panels refer to segregated pairs in Spain (top plots) and Mexico (bottom plots). Different letters denote significant differences according to permutation one-way ANOVA ($p < .05$) within each study site

4 | DISCUSSION

An essential question in understanding co-occurrence patterns is identifying the ecological mechanisms that underlie species co-existence and mutual exclusion. Microbial co-occurrence networks, mainly reconstructed from amplicon sequencing data, are being increasingly used to infer significant associations between pairs of co-occurring taxa and often ascribed to biological interactions (Faust & Raes, 2012; Fuhrman, Cram, & Needham, 2015; Ho et al., 2016; Pérez-Valera et al., 2017). Critical voices, however, call for caution when analysing and interpreting co-occurrence networks in order to avoid the description of ecologically meaningless interactions (Barner et al., 2018; Connor, Barberán, & Clauset, 2017; Freilich et al., 2018). Here, we use co-occurrence networks to identify associations between pairs of bacterial taxa across multiple assemblages and test their significance against a null model. We complement this classical approach with statistical tests on the spatial distribution and environmental features of plots occupied by pairs of associated taxa in order to discern their assembly mechanism, either dispersal limitation, habitat filtering, positive or negative biological interactions (Blois et al., 2014). By computing the phylogenetic distance between pairs of taxa involved in each significant association, we intend to validate the ecological significance of our results based on theoretical expectations that link phylogenetic patterns to assembly processes (Figure 1). We applied this framework in two contrasting water-limited sites (in terms of plant communities, lithology and soil properties) that are located in different continents, thousands of kilometres apart, and found consistent patterns in the relative contribution of assembly processes and their phylogenetic signatures.

The number of significant nonrandom associations that we detected between pairs of bacterial taxa represented, taking both data sets together, 11% of the potential number of pairwise associations (7,545 significant out of 70,359 potential associations). Other authors who analysed soil bacterial co-occurrence networks across different biomes and land uses systematically detected that a small portion of OTUs shows significant associations with other community members (Lupatini et al., 2014). In our case, this result was not unexpected since we used stringent methodological settings for network reconstruction in order to reduce the detection of artefactual associations following the recommendations by Faust et al. (2012) and Faust and Raes (2012). Our conservative approach substantially restricts the proportion of taxa that are included in the analysis, but avoids falsely attributing biological mechanisms to spurious patterns as strongly suggested by Knight et al. (2018). Beyond methodological considerations, these data support a high contribution of stochasticity to the assembly of soil bacterial communities, a pattern that has been globally attributed to soils with pH values close to neutrality given their low niche-based lineage exclusion (Tripathi et al., 2018). In addition, the low proportion of significantly associated pairs is coherent with the high levels of beta diversity in both study sites, which mostly originate from a large species turnover across plots. This finding is common in studies across a set of local communities (Soininen, Heino, & Wang, 2018). Contrary to nestedness, which indicates an orderly species loss in poorer compared with richer communities, spatial turnover reflects species replacement theoretically due to spatial constraints or environmental controls (Baselga, 2010). Dispersal limitation was negligible in our study areas, as in only 2%–4% of all cases did the spatial distance across plots significantly differ between states for each OTU pair (e.g. plots where two aggregated OTUs are copresent are spatially distant from those plots where the same OTUs are co-absent). Such a low effect of dispersal limitation is typical for organisms with large populations, small body size and high rates of passive dispersal, particularly in local sampling areas (Finlay, 2002; Martiny et al., 2006; Ramette & Tiedje, 2007). However, in heterogeneous ecosystems (as is the case of our patchy landscapes) microbial communities, in spite of their efficient dispersal abilities, can show a high turnover across plots due to local environmental filtering (Soininen et al., 2018).

Habitat filtering was the key mechanism behind the co-occurrence patterns of soil bacterial communities in both ecosystems. Soil abiotic factors are main drivers of bacterial community structure and composition (Fierer, 2017; Martiny et al., 2006; Meyer et al., 2018) and topological features of co-occurrence networks at wide biogeographic scales (Ma et al., 2016). At a local scale, we could explain 57%–77% of all nonrandom pairwise associations based on the environmental distance (i.e. dissimilarity in soil abiotic conditions) between plots bearing different states for each OTU pair. That is, plots where two aggregated OTUs were copresent were environmentally different from plots where the

same OTUs were co-absent. Similarly, the presence (or absence) across plots of each OTU conforming a segregated pair could be explained based on local environmental features. Therefore, our results indicate that both copresence and mutual exclusion patterns mainly reflect shared or differential niche requirements of spatially associated taxa. This evidence, showing an essential role of environmental tolerances in determining the distribution of soil bacteria across space, adds up to recent criticism on equating co-occurrences with biological interactions (Barner et al., 2018; Freilich et al., 2018). Our methodological framework, based on Blois et al. (2014), helped us identify which soil parameters constitute the abiotic filter in the study systems. In both cases, soil fertility (several forms of oxidizable C and total N), electrical conductivity, moisture and pH were the main abiotic factors that underlay environmental variability across plots, mostly due to differences between plant patches and gaps. Variation within patches and gaps, which further helped explain the bacterial co-occurrence patterns, mainly responded to the levels of mineral N (ammonium and nitrate) and, in the case of the Mexican site, to the distribution of granulometric fractions (clay, silt and sand). It is essential to notice that this methodological approach requires previous knowledge on the abiotic parameters that underlie the environmental heterogeneity relevant to the structure and composition of the microbial communities. Otherwise, high levels of unexplained variation could be falsely attributed to biological interactions. In our case, we selected an assortment of 11–13 soil abiotic parameters that show variability at the metre scale in the study sites according to our previous studies (Goberna, Navarro-Cano, et al., 2014; Goberna et al., 2007; Navarro-Cano et al., 2014, 2015; Sortibrán, Verdú, & Valiente-Banuet, 2014). Admittedly, however, other unmeasured abiotic factors, such as the partial pressure of oxygen or the content in certain micronutrients, might be relevant at the microscale level (reviewed in Fierer, 2017). Considering a larger set of abiotic parameters would most probably reinforce the message that environmental preferences are the main determinants of soil bacterial co-occurrence networks.

Biological interactions were the second assembly force influencing the co-occurrence patterns in our study systems. Microorganisms in extremely complex communities establish intricate networks of positive and negative biological interactions (Hibbing et al., 2010; Morris et al., 2012; Zengler & Zaramela, 2018). Based on Darwin's ideas, competition for space and resources has been classically considered as the key interaction between co-occurring species. Foster and Bell (2012) experimentally demonstrated this hypothesis for culturable bacteria. Using co-occurrence networks, however, we detected a larger number of positive than negative interactions (1,240 aggregated vs. 420 segregated pairs), confirming a pattern that has been reported before for other organisms (Freilich et al., 2018). These authors defend that nontrophic positive interactions, and particularly those involving habitat engineers, might leave a more detectable signal because they expand the niche for second (beneficiary) species. This argument could well apply to Cyanobacteria, Planctomycetes or Chloroflexi which take part in biofilms (Bengtsson & Øvreås, 2010; Kushumi et al., 2013), and we detected as sharing positive interactions. On the other hand, mutual exclusion patterns based on competitive interactions are difficult to detect, particularly for organisms with high dispersal rates, since dispersal by competing species between habitat patches tends to erase checkerboard patterns (Dallas, Melbourne, & Hastings, 2019). In addition, the study of complex networks based on pairwise associations can mask the effect of higher order interactions leading to misinterpretations of the interaction sign. As an illustration, nontransitive competition networks between three species can lead to the co-existence (thus to aggregated spatial patterns) of competing organisms. An example of this type of interaction involves a toxin-producing species, which outcompetes a sensitive species that can further outcompete a third resistant species since it does not incur in the cost of resistance. In turn, the resistant species closes the loop by outcompeting the toxin-producing species since it does not incur in the cost of toxin production (Hibbing et al., 2010). Despite these and other limitations that need to be taken into account when analysing co-occurrence patterns (Connor et al., 2017; Freilich et al., 2018), we detected a phylogenetic imprint that depended on the assembly mechanism and was consistent across sites.

Soil bacteria living spatially aggregated across multiple assemblages were evolutionarily more closely related than those living segregated. This general pattern was modulated by each particular assembly mechanism underlying species copresence and mutual exclusion. Specifically, taxa that co-existed based on habitat filtering were closer relatives than those that did not co-exist based on the same process. These results are consistent with the expectation of habitat filtering favouring the co-existence of closely related species with shared ecological tolerances (Webb et al., 2002). Functional traits conferring tolerance to environmental stress are conserved across the prokaryotic phylogeny, that is, evolutionarily related prokaryotes tend to have more similar trait values than distant taxa (Goberna & Verdú, 2016). This is the case, for instance, of the bacterial ability (a) to form resistant structures, (b) tolerate desiccation and/or radiation based on the formation of resistant cell walls, capsules, sheaths or extracellular polymers, or (c) tolerate salinity based on the production of salt-stress proteins or accumulation of osmoprotective compounds (Goberna, Navarro-Cano, et al., 2014). For this reason, soil bacteria living in abiotically stressful environments are not only functionally but also phylogenetically similar (Goberna, Navarro-Cano, et al., 2014). In addition, bacteria living spatially aggregated based on habitat filtering were phylogenetically more closely related than those sharing a positive biological interaction. Positive interactions include a myriad of processes—for example biofilm formation, quorum sensing, metabolic dependencies and sharing of goods (Morris et al., 2012; Zengler & Zaramela, 2018)—each of them probably yielding differential phylogenetic signatures. Despite this variability, our results suggest that cooperation among soil bacteria (or at least interactions that

benefit one species without harming the other) tends to occur between phylogenetically dissimilar organisms. This observation fits well to the notion that mutualistic (or commensalistic) interactions predominantly occur between evolutionarily distant species (Valiente-Banuet & Verdú, 2013). There are many examples of convolute networks involving distant microorganisms that exchange electron donors and metabolites (Zengler & Zaramela, 2018). This is not to say that positive interactions do not take place among close relatives, for example synchronized crosstalk-induced gene expression (Ng & Bassler, 2009). There is also evidence of mechanisms that set an upper threshold to the phylogenetic distance of bacterial cooperators. For instance, maintenance of cooperation via sharing of public goods is more stable among close cooperators, as they inhibit more efficiently spontaneous selfish mutants (Jousset et al., 2013).

Bacteria excluding each other owing to negative biological interactions had levels of phylogenetic relatedness that were not significantly different to those living segregated based on their differential environmental preferences. The phylogenetic outcome of negative interactions is difficult to predict because opposite patterns are expected depending on whether competitive exclusion occurs through niche similarities or relative fitness differences (Mayfield & Levine, 2010). Competitive exclusion through niche similarities tends to limit the functional (and phylogenetic) similarity of competing lineages, thus favouring the co-existence of distantly related taxa (Webb et al., 2002). In support of this idea, competition by interference among pairs of 148 soil Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes revealed that antagonism increases with phylogenetic proximity (Russel, Roder, Madsen, Burmolle, & Sorensen, 2017). In contrast, relative fitness differences associated with particular superior clades tend to outcompete entire distant lineages resulting in the co-existence of closely related taxa (Mayfield & Levine, 2010). Also this pattern has been found to be widespread in soils and attributed to the high competitive abilities of several clades of Proteobacteria and Actinobacteria able to outcompete Acidobacteria, Planctomycetes or Verrucomicrobia (Fierer, Bradford, & Jackson, 2007; Goberna, García, et al., 2014; Goberna, Navarro-Cano, et al., 2014; Goldfarb et al., 2011). Niche and relative fitness differences indeed concurrently determine the outcome of competitive interactions. In laboratory communities, the success of (outcompetition by) invasion depends on niche differences between invader and native species, whereas the impact of the interaction depends on their relative fitness differences (Shao-peng, Tan, Yang, Ma, & Jiang, 2019). The simultaneous operation of negative interactions, whose phylogenetic signatures can cancel out, might underlie the difficulties in detecting unequivocal evolutionary signals of competitive interactions (e.g. Foster & Bell, 2012).

In conclusion, informing co-occurrence networks with spatial and environmental data allows quantifying the relative contribution of community assembly processes. By doing so, we detected a prevailing role of environmental preferences (rather than biological interactions) in determining the co-existence and mutual exclusion patterns of soil bacteria across multiples assemblages. We propose that the phylogenetic signal left by each assembly process might help elucidate ecologically meaningful co-occurrence patterns in microbial networks.

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AUTHOR CONTRIBUTION

The research was designed by M.G. and M.V.; data were collected by M.G., A.M.N., A.V.B. and J.A.N.C.; data were analysed by M.G., Y.C., A.G.F., S.D. and M.V.; manuscript was written by M.G. and M.V.; revision was contributed by all authors.

DATA AVAILABILITY STATEMENT

DNA sequences were deposited in the European Nucleotide Archive for the Spanish (https://www.ebi.ac.uk/ena/data/view/PRJEB4887; Goberna, Navarro-Cano, et al., 2014) and Mexican data sets (https://www.ebi.ac.uk/ena/data/view/PRJEB27091).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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