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IDEA AND PERSPECTIVE

Abiotic stress tolerance and competition-related traits underlie phylogenetic clustering in soil bacterial communities

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Abstract

Soil bacteria typically coexist with close relatives generating widespread phylogenetic clustering. This has been ascribed to the abiotic filtering of organisms with shared ecological tolerances. Recent theoretical developments suggest that competition can also explain the phylogenetic similarity of coexisting organisms by excluding large low-competitive clades. We propose that combining the environmental patterns of traits associated with abiotic stress tolerances or competitive abilities with phylogeny and abundance data, can help discern between abiotic and biotic mechanisms underlying the coexistence of phylogenetically related bacteria. We applied this framework in a model system composed of interspersed habitats of highly contrasted productivity and comparatively dominated by biotic and abiotic processes, i.e. the plant patch-gap mosaic typical of drylands. We examined the distribution of 15 traits and 3290 bacterial taxa in 28 plots. Communities showed a marked functional response to the environment. Conserved traits related to environmental stress tolerance (e.g. desiccation, formation of resistant structures) were differentially selected in either habitat, while competition related traits (e.g. organic C consumption, formation of nutrient-scavenging structures) prevailed under high resource availability. Phylogenetic clustering was stronger in habitats dominated by biotic filtering, suggesting that competitive exclusion of large clades might underlie the ecological similarity of co-occurring soil bacteria.

Keywords

Competitive abilities, environmental filtering, organic carbon consumption, phenotype, phylogenetic community structure, resistant structures, soil bacteria, stress tolerance.

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INTRODUCTION

Soil bacterial communities hold incommensurable levels of species diversity (Curtis *et al*. 2002). This contrasts with the low phylogenetic diversity detected in soil bacterial communities compared with those thriving in other terrestrial and marine ecosystems (Lozupone & Knight 2007). This observation relates to the notion that soil bacteria typically co-occur with evolutionarily related organisms more often than expected by chance, a process that results in phylogenetic clustering (Horner-Devine & Bohannan 2006; Bryant *et al*. 2008). Based on classical community phylogenetics, such clustered patterns have been assigned to the environmental selection of taxa sharing conserved traits that allow them to surpass an abiotic filter (Webb *et al*. 2002; Horner-Devine & Bohannan 2006; Costello *et al*. 2009; Ganz *et al*. 2012). Under this traditional framework, biotic interactions – mainly competition, the other major force driving community assembly – lead to the co-existence of phylogenetically distant organisms and hence create overdispersed patterns. These result from the competitive exclusion of ecologically (and phylogenetically) similar organisms based on their niche similarities (Webb *et al*. 2002; Horner-Devine & Bohannan 2006). Modern coexistence theory has refined this vision arguing that competition can generate phenotypic and phylogenetic clustering when it operates through environmentally mediated differences in competitive abilities among entire clades rather than through limiting similarity (Mayfield & Levine 2010; HilleRisLambers *et al*. 2012). We postulate that this mechanism might operate in soil bacterial communities, which are typically carbon-limited, driven by the superior competitive ability of the dominant Proteobacteria and Actinobacteria under the presence of carbon substrates such as those released by roots (Goldfarb *et al*. 2011). Recently, a method has been proposed to test whether phylogenetic clustering is determined by abiotic and/or biotic filters by comparing the fundamental and realised niches of co-occurring plants (de Bello *et al*. 2012). This framework seems currently impracticable for bacteria owing

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to the lack of basic information on the vast majority of organisms (Green *et al*. 2008). Here, we propose a trait-based approach to discern between abiotic and biotic environmental filters, which would differentially select traits associated with abiotic stress tolerance or competitive abilities, respectively. This approach requires assessing the existence of a phylogenetic signal (or anti-signal) in the traits so as to demonstrate that the phylogenetic community structure responds to the phenotypic patterns observed (Losos 2008).

Stressful water-limited ecosystems provide an appropriate setting to test which processes dominate the community assembly of soil bacteria. Gypsum soils developed in dry lands are frequently characterised by a patchy plant distribution, with sparse plant clumps surrounded by a low-cover matrix. Soil bacteria living in open spaces withstand high temperatures and desiccation, intense radiation, nutrient scarcity and elevated concentrations of sulphur compounds (Bochet *et al*. 1999; Goberna *et al*. 2007). Abiotic stress is partly alleviated within the plant patches, which filter the quantity and quality of light and allow preferential accumulation of water and resources (Bochet *et al*. 1999; Goberna *et al*. 2007). In the densely populated plant clumps, biotic interactions are magnified (Aguiar & Sala 1999). Particularly, soil bacterial communities underneath plant patches are denser, more active and show higher microbial quotients (respiration-to-biomass ratios) reflecting competitive stress (Goberna *et al*. 2007). Therefore, these ecosystems can be viewed as a mosaic of low-productive habitats (hereafter 'gaps') comparatively dominated by abiotic filtering interspersed with high-productive habitats (hereafter 'patches') comparatively driven by biotic interactions (Aguiar & Sala 1999). The balance between biotic and abiotic processes in both environments might have several outcomes on the phenotypic and phylogenetic patterns of community assembly of soil bacteria (Table 1). In plant gaps, we expect that the abiotic environment will filter traits conferring resistance to environmental stress (e.g. tolerance to desiccation, formation of resistant structures) leading to phenotypic clustering and, in case these traits are conserved, to phylogenetic clustering as well (Table 1). In plant patches, we expect the overrepresentation of traits related to the competition for resources (e.g. organic C consumption, nitrogen fixation). In case these traits show a significant phylogenetic signal, two outcomes can be expected depending on how competition operates. In our hypothetic scenario (A) in Table 1, competition operates by limiting similarity resulting in the competitive exclusion of closely related organisms, and hence in phenotypic and phylogenetic overdispersion (Webb *et al*. 2002). In this scenario (A), soil bacterial communities in gaps will necessarily be phylogenetically more clustered, i.e. will have a higher Net Relatedness Index (NRI) values (Webb *et al*. 2002), than those in patches. In our hypothetic scenario (B), competition operates by competitive ability differences, leading to the exclusion of distantly related taxa and thus to phenotypic and phylogenetic clustering (Mayfield & Levine 2010). In scenario (B), the relative magnitude of the biotic and abiotic environmental filters will determine that soil bacterial communities are comparatively more clustered in either habitat.

Here, we characterised the environment in 28 plant patches and gaps in semi-arid Mediterranean gypsum soils, pyrosequenced a phylogenetic marker (16S rRNA gene) to identify bacterial taxa and characterised their phenotypes on the basis of fifteen functional traits potentially relevant to the survival in either landscape component. We tested whether: (1) soil bacterial communities are phenotypically clustered due to the overrepresentation of competition-related traits in patches and environmental tolerance traits in gaps, (2) traits relevant to phenotypic clustering in either habitat are evolutionarily conserved, and thus may underlie the phylogenetic structure of soil bacterial communities and (3) soil bacterial communities are phylogenetically more clustered in patches than gaps, due to the exclusion of distantly related deeply branching bacterial clades expected under carbon-enriched environments (Goldfarb *et al*. 2011). We suggest that the overrepresentation of phylogenetically conserved traits conferring environmental stress tolerance or competitive abilities can be used to discern the relevance of abiotic and biotic processes in driving the phylogenetic community assembly of soil bacteria.

MATERIALS AND METHODS

Study area and characterisation of patches and gaps

The study site was located in Algepsar dels Burutaus (Serra de Crevillent, Alacant, SE Spain; UTM 30 N 689062, 4238201). Climate is semi-arid Mediterranean (240 mm mean

Table 1 Expected phenotypic and phylogenetic patterns of community assembly of soil bacteria depending on the relevant structuring force (abiotic vs. biotic) under two scenarios based on: (A) the classical framework by Webb *et al*. (2002), and (B) the assumptions by Mayfield & Levine (2010)

		Phenotypic and phylogenetic structure	
Structuring force	Trait type	(A) Webb *et al*. scenario	(B) Mayfield & Levine scenario
Abiotic	Environmental tolerance traits	Clustering due to abiotic filtering NRI > 0	Clustering due to abiotic filtering NRI > 0
Biotic	Competition-related traits	Overdispersion due to niche similarities NRI < 0	Clustering due to competitive ability differences NRI > 0

Abiotic filtering results in the overrepresentation of environmental tolerance traits leading to phenotypically clustered communities in both scenarios. Biotic filtering results in the overrepresentation of competition-related traits generating either phenotypic overdispersion if competition proceeds through niche similarities (scenario A) or phenotypic clustering if it proceeds through competitive ability differences (scenario B). If traits are conserved, phylogenetic community structure is expected to reflect the phenotypic community structure. Positive Net Relatedness Index (NRI) values indicate phenotypic and phylogenetic clustering, while negative NRI indicate phenotypic and phylogenetic overdispersion.

annual rainfall, 20 °C mean annual temperature). Soils are Typic Xerorthents (Soil Survey Staff 1998) developed on gypsum hills (40% slope, 350 m a.s.l.) and are covered with a patchy shrub steppe dominated by the leguminous shrub *Ononis tridentata* L. subsp. *tridentata*. This gypsophyte legume is able to colonise bare gypsum and ameliorate the stressful abiotic conditions, thus facilitating the establishment of other plant species (Navarro-Cano *et al*. 2014). Plant patches founded by *O*. *tridentata* cover 25% of the landscape and open spaces are mostly covered by sealing crusts (Goberna *et al*. 2007; Appendix S1).

Patches were defined as groups of plants growing underneath the canopy of an *O*. *tridentata* individual. Fifteen patches were selected along two parallel 100 m long transects located roughly 20 m apart. Patch area averaged (mean ± SD) 2.4 ± 1.1 m^2. Gaps were defined as the open spaces between patches, and fifteen gaps were systematically located one metre west beyond the vertical projection of the canopy of each patch. Gaps were located at 0.5–1.5 m to any other neighbouring plant patches. The sampling area of each gap was equivalent to that of its adjacent patch. Soil samples were collected on May 2010, which is the growing season in the study area. This sampling season reflects the differences that are found throughout the year between patches and gaps with regard to soil chemical variables, as well as microbial biomass and activity (Goberna *et al*. 2007). Surface soil samples (0–2 cm) were collected from patches and gaps after removing the litter layer when present. Five sub-samples (*c*.100 g) were collected randomly from the area of each patch or gap, and then bulked into a single composite sample. Soil samples were transported to the laboratory on ice, immediately sieved through a < 1 mm mesh and stored at 4 °C. Eleven soil variables were determined to characterise the microbial environment in a previous study (Navarro-Cano *et al*. 2014). Soils underneath plant patches were remarkably more productive than those in gaps as indicated by their contrasted contents in total organic carbon, water-soluble carbon and carbohydrates, total nitrogen, or ammonium nitrogen (Navarro-Cano *et al*. 2014).

Soil DNA extraction and tag-encoded FLX-titanium amplicon pyrosequencing

Soil DNA was extracted within 48 h after sampling and DNA extracts were stored at −20 °C. Extractions were performed from 1 g soil using the UltraClean® Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). Extracted DNA was electrophoresed in 1% agarose gels run in 0.5 × TAE buffer (Tris-acetate-EDTA; 100 V, 15 min) and quantified with the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, Carlsbad, CA, USA).

Soil DNA was submitted to Polymerase Chain Reaction (PCR) amplification of the 16S rRNA gene, using the universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′; Turner *et al*. 1999) and 534R (5′-ATTACCGCGGCTGCTG GC-3′; Muyzer *et al*. 1993). One forward primer was synthesised per sample, including a 454 sequencing adaptor (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and a unique 8-nucleotide barcode in their 5′-end which was randomly selected from those published by Hamady *et al*. (2008). The reverse primer had a 454 sequencing adaptor in its 5′-end (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′). PCR amplifications were performed in a Flexcycler (Analytik Jena, Jena, Germany) in 50 μL volumes, with each reaction containing a final concentration of 1× Platinum® PCR SuperMix High Fidelity (Invitrogen), 0.3 μM of each primer and 0.4 mg mL^{-1} bovine serum albumin. A volume of 1.5 μL DNA was directly applied to the reaction mix. Thermal cycling was initiated with 5 min at 94 °C, followed by 20 amplification cycles consisting of 45 s at 94 °C, 45 s at 54 °C and 90 s at 72 °C, and terminated with 10 min at 72 °C. PCR products (100 μL) were purified with the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany), eluted in 50 μL DNAase free 1 × TE (Tris-EDTA) buffer and checked for size and quality in 2% agarose gels run in 1 × TAE buffer (80 V, 45 min). Non-template controls followed the same procedure. Purified tagged amplicons were quantified in duplicate using the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen) and pooled in equimolar amounts. Pyrosequencing was performed by GATC Biotech (Konstanz, Germany) with the Roche 454 GS-FLX system using Titanium chemistry.

Sequences were sorted out according to their tags and trimmed to remove sequencing adaptors and primers with the RDP 10.26 pyrosequencing pipeline (Cole *et al*. 2009). After removal of low quality sequences and artefacts, 24 486 sequences were aligned using the Infernal aligner (Nawrocki & Eddy 2007) and visually inspected. Originally, the sequencing depth did not vary significantly between patches and gaps, with (mean ± SE) 1624 ± 66 and 1818 ± 392 reads, respectively. After initial processing, patches had 1175 ± 53 and gaps 835 ± 91 sequences. Operational taxonomic units (OTUs) were defined using the complete-linkage clustering method in RDP 10.26 (Cole *et al*. 2009) at a maximum identity level of 97% to avoid an overestimation of diversity (Kunin *et al*. 2010). The total 24 486 bacterial sequences were assigned to 6823 OTUs. After removal of 3209 singletons and 324 chimeric sequences detected by ChimeraSlayer, using QIIME (Caporaso *et al*. 2010), we obtained a final 3290 OTUs. Sequences representative of each OTU were assigned to bacterial taxa using the Naïve Bayesian Classifier at a confidence threshold of 80%. This algorithm can classify 400-base pair 16S rRNA sequences (in this study, mean ± SD sequence length was 410 ± 4 bp) down to the genus level (Wang *et al*. 2007) into the taxonomy proposed by Garrity *et al*. (2007). A community matrix (OTU × plot) was constructed using RDP 10.26, showing the abundance of the total 3290 OTUs in patches and gaps. The relative abundance of each OTU in each plot was calculated based on the total number of sequences in the same plot, and subsequently corrected by the number of 16S rRNA gene copies as proposed by Kembel *et al*. (2012) (Appendix S2). A total 3290 sequences, one representative of each OTU, was deposited in EMBL within the study with accession number PRJEB4887 (http://www.embl.de/).

Bayesian generalised linear models (GLM) were used to assess the overrepresentation of bacterial phyla in patches or gaps using the patch-gap block as a random grouping factor with the MCMCglmm package for R (Hadfield 2010). We

used the default priors and ran 13.000 MCMC iterations with a burn-in period of 3.000 iterations. Convergence of the chain was tested by means of an autocorrelation statistic. The statistical significance of the factors in the model was estimated by calculating the 95% credible interval of their posterior distribution.

Bacterial functional traits

Fifteen traits that potentially determine the differential survival of soil bacteria in plant patches and gaps were binary coded (1: trait has been reported; 0: trait has not been reported). We categorised these traits as conferring either competitive abilities or environmental tolerance as follows. We considered that traits confer competitive abilities if they allow organisms to consume resources (organic substances, mineral ions, light, etc.), which might potentially become restricted to be consumed by others (Tilman 1982). This implies the existence of a biological interaction, particularly competition by exploitation of limiting resources (Birch 1957). Alternatively, we considered that traits confer tolerance to environmental stress if they allow organisms to tolerate abiotic factors that determine their survival and adaptation to the environment (e.g. pH, desiccation, salinity, etc.) (Odum 1959). In contrast to resources, the latter abiotic factors cannot be consumed and do not imply biological interactions.

We specifically considered eight traits that confer competitive abilities either by providing the ability to consume limiting resources in the study soils, obtain them from alternative sources (e.g. the atmosphere), intensify their acquisition from the environment, or store them in the cell. We also considered that if the possession of these traits implies a superior competitive ability, this should be ultimately reflected in a trait indicating higher bacterial growth rates. In particular, we considered two limiting resources in the study soils, i.e. carbon and nitrogen (Navarro-Cano *et al.* 2014). We coded: (1) organic C consumption, that allows growth on organic molecules through aerobic oxidation or anaerobic fermentation, either facultative or obligate, detected under laboratory conditions (e.g. Reddy *et al.* 2006), (2) phototrophic C fixation, that allows aerobic or anaerobic growth either using CO_2 as a sole carbon source or through the light-stimulated consumption of reduced organic compounds, either facultative or obligate detected under laboratory conditions (e.g. Overmann & Garcia-Pichel 2006; Madigan & Jung 2009), (3) N fixation, that is, the bio-assimilation of atmospheric N_2 in the form of ammonium, either due to the possession of *nif* genes (coding for nitrogenase reductases) or detected under laboratory conditions (e.g. Martinez-Romero 2006), (4) ammonia oxidation to hydroxylamine, which is the first step of nitrification, either due to the possession of *amo* genes (coding for ammonia monooxygenases) or detected under laboratory conditions (e.g. Wessén 2011), (5) nitrate reduction to nitrite, which is the first step of denitrification but is not unique to denitrifying organisms (Jones *et al.* 2008), either due to the possession of *nap* genes (coding for nitrate reductases) or detected under laboratory conditions (e.g. Kurahashi *et al.* 2009), (6) denitrification, involvement in any step from the reduction of nitrite to the production of molecular nitrogen, either due to the possession of *nir*, *nor* and/or *nos* genes (respectively, coding for nitrite, nitric oxide and nitrous oxide reductases) or detected under laboratory conditions (e.g. Shapleigh 2006; Jones *et al.* 2008), (7) formation of prosthecae, which act as nutrient-scavenging antennas that become more effective under nutrient limitation (McAdams 2006) and (8) formation of polyhydroxyalkanoate (PHA) inclusions, including granules composed by polyhydroxyvalerate, polyhydroxybutyrate or undetermined polysaccharides, which serve as carbon and energy storage materials that are formed in response to nutritional imbalances (e.g. Kanso & Patel 2003). Finally, we used the number of 16S rRNA gene copies as a proxy for growth rate (Klappenbach *et al.* 2000), as a competition-related trait which eventually determines the overrepresentation of competitively superior clades.

Seven traits were considered to confer tolerance to environmental stress, namely: (1) formation of resistant structures, which are triggered by (and can tolerate) environmental stress and germinate under favourable conditions (Dworkin 2006); in particular, we coded independently the formation of endospores, which are the most resistant structures to extremes of temperature, desiccation, radiation, physical disruption, chemical agents (Dworkin 2006), exospores, other spores and spore-like elements, cysts and akinetes, (2) tolerance to desiccation and/or radiation, either known to be due to the presence of a particular morphological feature (i.e. resistant cell walls, formation of capsules, sheaths, or extracellular polymers) or observed under laboratory conditions (e.g. Alburquerque *et al.* 2005; Buczolits *et al.* 2006) and (3) tolerance to salts, including from slightly halotolerant to highly halophilic organisms, either known to be due to a particular adaptation (i.e. production of salt-stress proteins, accumulation of osmoprotective compounds) or observed under laboratory conditions (e.g. Lau *et al.* 2005; Oren 2006).

Coding of each trait was based on an extensive review of both published literature and databases of functional genes involved in biogeochemical cycling up to February 2012 (http://fungene.cme.msu.edu). Trait value assignment (presence/absence) to each OTU was based on the features described for its nearest known taxon according to the Naïve Bayesian Classifier (Wang *et al.* 2007). This trait assignment was performed at the genus level in 85% of the cases on average for all traits. Trait values for each OTU, the taxonomic level at which each trait value was assigned to each OTU and the references used to generate the information for each OTU are given in Appendix S2. We detected 632 bacterial OTUs belonging to taxa known to form resistant structures (14 endospore-, 5 exospore-, 570 spore-, 37 cyst- and 7 akinete-formers), 450 tolerant to desiccation, 1850 tolerant to salts, 3136 organic C consumers, 334 phototrophic C fixers, 360 N fixers, 7 ammonia oxidisers, 1489 nitrate reducers, 343 denitrifiers, 115 that form prosthecae, and 468 that form PHA inclusions (Appendix S2). The number of 16S rRNA gene copies for each OTU was estimated using the procedure by Kembel *et al.* (2012) (Appendix S2). The average number of 16S rRNA gene copies of all the organisms in each patch or gap was calculated as the weighted average of the number of copies of each OTU by its relative abundance in

the community. Relative abundance was calculated as the proportion of sequences of each OTU in patch or gap. Candidate divisions, for which no trait information is available in the literature, constituted only 1.0 and 0.6% of the total community in patches and gaps, respectively.

Trait assignment to environmental sequences was based on the state of the nearest taxon rather than in the ancestral trait state reconstruction since non-random taxon sampling seemed to bias character reconstruction towards the most frequent state in our data set (data not shown), likely due to community assembly processes favouring certain trait states (Ackerly 2000; Hearn & Huber 2006). In order to account for the uncertainty associated with the assignment of trait values to environmental sequences based on their taxonomic affiliation, we explored the consistency of our results by repeating all analyses with the subset of OTUs that were classified at the finest taxonomic level with confidence thresholds equal or over 80% according to the Naïve Bayesian Classifier (Wang *et al*. 2007). The conclusions reached with this second data set (including 1387 OTUs) were identical to those obtained with the original data set (including 3290 OTUs) (Appendix S3).

Phenotypic community structure

Multiple traits selected by simultaneously operating ecological filters determine community membership (Mayfield *et al*. 2009). Increasing the number of traits to define the phenotype increases the biological realism and the statistical power to detect community assembly processes (Kraft *et al*. 2007). For this reason, we evaluated the phenotypic community structure by computing a single metric, the NRI, on the basis of multiple traits. To test for the co-existence of similar phenotypes, we computed phenotypic NRI values based on trait distance matrices (de Bello *et al*. 2012). Phenotypic NRI was calculated separately for traits conferring environmental tolerance or competitive abilities in patches and gaps using the picante package for R (Kembel *et al*. 2010). This computes NRI = $-(\mathrm{MPD}_{obs} - \mathrm{MPD}_{rand})/\mathrm{sd\_MPD}_{rand}$, where MPD_{obs} is the average of all pairwise phenotypic distances between the taxa in a local community weighed by their abundances, MPD_{rand} is the average of MPD calculated in n randomly constructed communities considering the regional pool of taxa (in our case, the sum of all taxa identified in all plots), and $\mathrm{sd\_MPD}_{rand}$ is the standard deviation of MPD_{rand} (Webb *et al*. 2002). The Jaccard Distance index was used to calculate phenotypic distances between binary trait matrices using the stats package in R (R Core Team 2013). Phenotypic NRI allows examining whether co-occurring taxa are more (positive NRI) or less (negative NRI) phenotypically similar than expected by chance. Thus, positive NRIs are indicative of phenotypic clustering while negative NRIs indicate phenotypic overdispersion. To test for the existence of a significant phenotypic community structure, i.e. phenotypic NRI departure from zero, we calculated the mean phenotypic structure of the community as the average NRI of all patches or gaps. If the mean NRI for all plots statistically differs from zero, it can be concluded that the community is significantly phylogenetically clustered or overdispersed on average, since both NRIs are standardised effect sizes whose expected values are zero for randomly structured communities (see Kembel & Hubbel 2006 for a similar procedure). This test was performed independently for NRI values calculated from the set of traits conferring competitive abilities or environmental tolerance by means of Bayesian GLMs using the MCMCglmm package for R (Hadfield 2010).

To assess the individual traits underlying the phenotypic community structure, we used Bayesian GLMs to explore the overrepresentation of bacterial taxa possessing each trait in patches or gaps using the patch-gap block as a random grouping factor. The same test was used to explore differences in the average number of 16S rRNA gene copies in patches and gaps.

Phylogenetic trait conservatism

Testing for the evolutionary conservatism of traits requires combining information on trait distribution across taxa and their phylogenetic relationships (Pausas & Verdú 2010). To assess the phylogenetic conservatism of all fifteen traits, we used a reference bacterial tree based on full 16S rRNA sequences contained in the Silva Database (Release 111, Quast *et al*. 2013). In particular, we selected 385 reference organisms, covering the diversity of genera to which our 3290 OTUs resembled with the highest probability according to the Naïve Bayesian Classifier (Wang *et al*. 2007) by using ARB software (Ludwig *et al*. 2004). A total 99% of these reference sequences corresponded to cultured organisms, 90% of which were type strains. Trait values (presence/absence) were assigned to each organism at the genus level as above. Trait values for the reference organisms, the literature reviewed to generate the information for each genus, and the accession numbers of the reference organism are given in Appendix S4.

Phylogenetic trait conservatism was assessed by testing the existence of a phylogenetic signal for each trait by calculating the statistic D (Fritz & Purvis 2010). This metrics allows testing the existence of a phylogenetic signal in binary traits and provides a measure of its strength (Fritz & Purvis 2010). It has been previously applied to the study of the evolutionary conservatism of bacterial traits (Martiny *et al*. 2013). The statistic D is defined as:

$$D = \left[\sum d_{obs} - \mathrm{mean}\left(\sum d_b\right)\right] \Big/ \left[\mathrm{mean}\left(\sum d_r\right) - \mathrm{mean}\left(\sum d_b\right)\right],$$

$\sum d_{obs}$ being the observed sum of sister-clade differences, $\sum d_b$ the distribution of sums expected under Brownian evolution and $\sum d_r$ the distribution of sums of sister-clade differences expected for a random phylogenetic pattern. We tested the departure of D values from those estimated after a random shuffle of trait values in the tree by using the caper package for R (Orme *et al*. 2012).

We recalculated all phylogenetic signals by using a second approach in which traits were assigned to OTUs based on their taxonomic affiliation. These phylogenetic signals were highly consistent with those calculated based on the reference organisms (Appendix S5).

Phylogenetic community structure

Bacterial richness in patches and gaps was measured as the number of OTUs. Phylogenetic community structure was quantified as the phylogenetic NRI values, with MPD_{obs} being the average of all pairwise phylogenetic distances between the taxa in a local community weighed by their abundances (Webb *et al*. 2002), using the picante package for R as above. This allows examining whether co-occurring taxa are more (positive NRI) or less (negative NRI) closely related than expected by chance. Thus, positive NRIs are indicative of phylogenetic clustering while negative NRIs indicate phylogenetic overdispersion. To reconstruct the phylogenetic relationships of soil bacteria in our data set, we calculated three independent maximum likelihood trees using RAxML 7.3.0 (Stamatakis 2006) with the GTRGAMMA substitution model in the CIPRES portal (Miller *et al*. 2011; Appendix S6). Prior to phylogenetic inference, hypervariable regions were screened out using the Lane mask (Lane 1991) to improve downstream analyses (Capella-Gutiérrez *et al*. 2009). To avoid high phylogenetic uncertainty resulting from the usage of short sequences, tree topology was constrained to match that of the megatree built from the Silva database (Quast *et al*. 2013). *Archaeoglobus profundus* was used as the outgroup. Each tree was selected among the best of 1000 iterations.

The existence of a phylogenetic community structure significantly differing from randomness, i.e. phylogenetic NRI departure from zero, was tested by means of Bayesian GLMs using the MCMCglmm package for R (Hadfield 2010). The same tests were performed for comparison of bacterial richness and phylogenetic community structure between patches and gaps using the patch-gap block as a random grouping factor. The use of Bayesian GLMs allowed us to accommodate the uncertainty associated with phylogenetic reconstruction (Huelsenbeck *et al*. 2000). In particular, we ran three Bayesian GLM models with the NRIs calculated from the three independent phylogenetic trees using the MCMCglmm package for R (Hadfield 2010). Then, we integrated over the posterior samples by drawing 1000 random samples across models.

Metrics of phenotypic and phylogenetic community structure other than NRI, specifically the Nearest Taxon Index (NTI; Webb *et al*. 2002), Faith's Phylogenetic Diversity (PD; Faith 1992), the Phylogenetic Species Variability (PSV) and the Phylogenetic Species Evenness (PSE; Helmus *et al*. 2007), were calculated using the picante package for R (Kembel *et al*. 2010). Absolute correlation coefficients between NRI and these other metrics ranged from 0.60 (NTI, PD) to 0.88 (PSV, PSE), with $P \leq 0.001$. All Bayesian GLM models were repeated using NTI instead of NRI and yielded the same results (Appendix S7). All analyses were performed using the R 3.0.1 software package (R Core Team 2013).

RESULTS

Soil bacterial communities in patches had (mean ± SE) 506 ± 19 OTUs compared with 334 ± 31 OTUs in gaps. Thus, bacterial communities were significantly richer in patches than in gaps [MCMCglmm; number of OTUs posterior mean-estimate = 180, (108, 252) 95% credible interval]. Richness trends were consistent at several rarefaction levels, regardless the inclusion or not of singleton OTUs in the analysis (Appendix S8). The two dominant phyla, Proteobacteria and Actinobacteria, accounted for 80% of the community in patches compared to 60% in gaps (Fig. 1). Proteobacteria was the dominant phylum in both environments, but was significantly more abundant in patches than gaps (Fig. 1). Four out of ten of the subdominant phyla were significantly more abundant in the gaps (Acidobacteria,

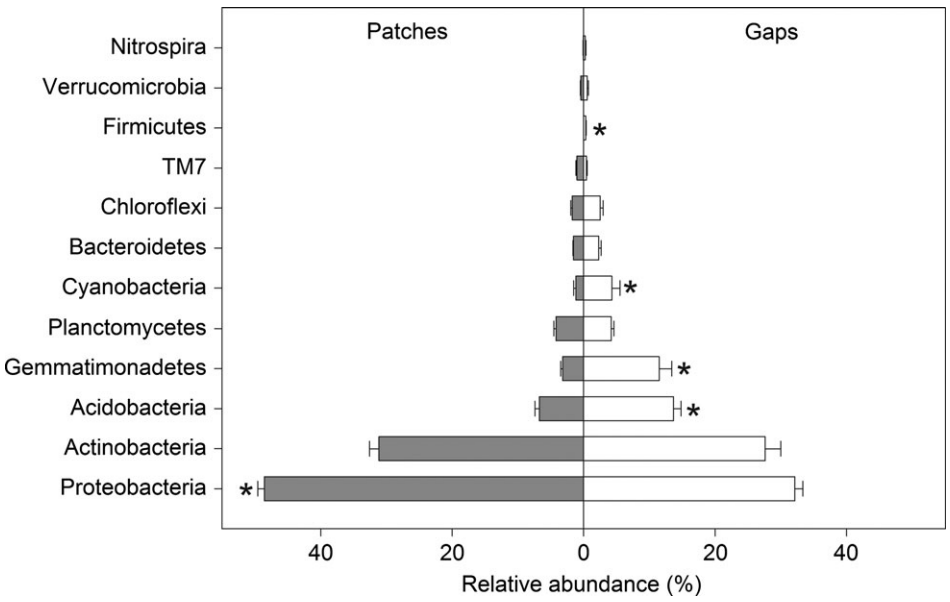

Figure 1 Relative abundance of the bacterial phyla that represented > 0.2% of the total community in plant patches and gaps in semi-arid gypsic soils. Asterisks indicate significant differences ($P < 0.05$). Bars represent standard errors.

Gemmatimonadetes, Cyanobacteria, and Firmicutes), while seven phyla were equally abundant in patches and gaps (Actinobacteria, Planctomycetes, Bacteroidetes, Chloroflexi, TM7, Verrucomicrobia and Nitrospira; Fig. 1).

Soil bacterial communities in patches were phenotypically clustered, as shown by their phenotypic NRI values significantly larger than zero, both for traits conferring tolerance to environmental stress and competitive abilities (Fig. 2a). Also in gaps, bacteria that were phenotypically similar in terms of environmental tolerance tended to co-exist, but this result did not apply to competition-related traits which showed a random structure (Fig. 2a). Thus, phenotypic clustering was driven by traits conferring tolerance to environmental stress both in patches and gaps, whereas traits conferring competitive abilities were relevant exclusively to patches. Among these sets of traits determining phenotypic community structure, we detected those individual traits being overrepresented in each habitat (Fig. 3). Specifically, traits conferring environmental tolerance that were overrepresented in gaps were tolerance to desiccation and formation of endospores, while the same type of traits overrepresented in patches were tolerance to salts, formation of other spores, and cysts (Fig. 3). Relevant traits conferring competitive abilities, which only determined a significant phenotypic community structure in patches, were organic C consumption, nitrogen fixation, nitrate reduction and formation of prosthecae (Fig. 3). Finally, the number of 16S rRNA gene copies, which was used as a proxy of growth rate ultimately determining the overrepresentation of competitively superior clades, was 2.80 ± 0.01 gene copies in patches and 2.70 ± 0.05 gene copies in gaps. Therefore, the number of 16S rRNA gene copies was significantly higher in patches than gaps [Log (Average 16S rRNA gene copies) = 0.037 (0.002, 0.079)].

All traits analysed (except one) were phylogenetically conserved, although the magnitude of the signal varied across traits (D value; Table 2). Formation of endospores, other spores, organic C consumption, and formation of prosthecae showed the highest phylogenetic signals indicated by their negative D values.

Soil bacterial communities were phylogenetically clustered in both environments, that is, NRI was significantly larger than zero in patches and gaps (Fig. 2b). Phylogenetic clustering was significantly stronger in bacterial communities thriving in patches compared to gaps [NRI = 2.45 (1.33, 3.68)]. This result was consistent when the number of sequences per plot was introduced as a factor in the model (data not shown).

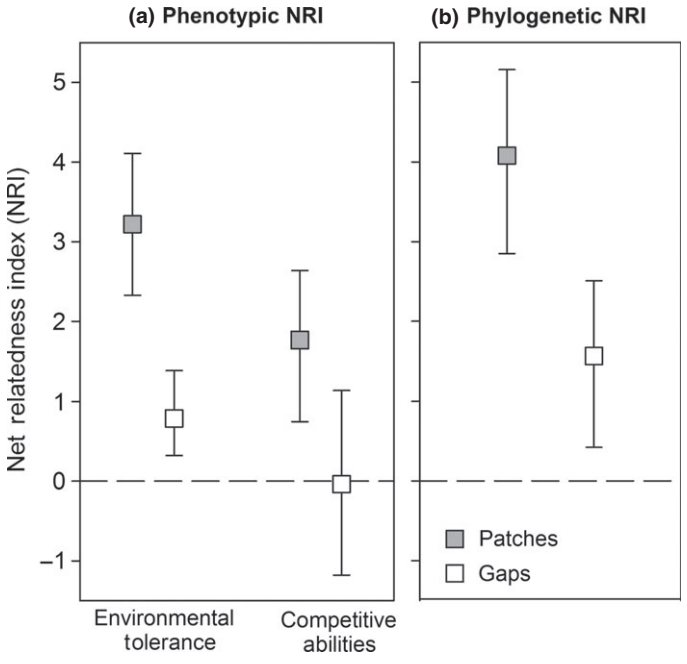

Figure 2 Phenotypic and phylogenetic bacterial community structure in plant patches and gaps in semi-arid gypsic soils. Bayesian post mean estimates and 95% credible intervals are shown for the Net Relatedness Index (NRI) values calculated based on (a) phenotypic distance matrices including traits conferring either environmental tolerance or competitive abilities, and (b) phylogenetic distance matrices. Soil bacterial communities in patches were phenotypically clustered for both trait types (i.e. effects with positive intervals not including zero). Bacterial communities in gaps were phenotypically clustered for traits associated to environmental tolerance (as above), but showed a random structure for competition-related traits (i.e. effects with intervals including zero). Soil bacterial communities were phylogenetically clustered in both environments (i.e. effects with positive intervals not including zero).

DISCUSSION

Soil bacterial communities were phylogenetically clustered, as has been repeatedly reported in the literature across biomes (Horner-Devine & Bohannan 2006; Bryant *et al*. 2008; Costello *et al*. 2009; Chong *et al*. 2012; Ganz *et al*. 2012). Using a trait-based approach, here we provide evidence supporting that phylogenetic clustering in soil bacteria can arise from the environmental selection of conserved functional traits either conferring tolerance to environmental stress or competitive abilities.

Bacterial communities presented a clear taxonomic and functional response to the soil environment. Soil bacterial communities underneath plant patches were richer, in terms of OTU numbers, but were composed of phenotypically and phylogenetically more closely related microbes than those in gaps. Proteobacteria dominated both environments as is common for soils worldwide (Janssen 2006), but were significantly overrepresented in patches with their high resource availability. This correlates well with the observation that Proteobacterial abundance increases with total organic carbon from the local to the global scales (Fierer *et al*. 2007; Ganz *et al*. 2012). Furthermore, the overrepresentation of Proteobacteria in carbon-rich soils was associated with the underrepresentation of four major phyla (Fig. 1). This is consistent with the experimental increase in Proteobacterial abundance induced by organic carbon additions to soil, which have been shown to generate asymmetric competition among bacterial phyla (Fierer *et al*. 2007; Goldfarb *et al*. 2011). Under such carbon-enriched conditions Proteobacteria are superior competitors which exclude other bacterial lineages, and this process results in intense phylogenetic clustering of soil bacterial communities (Goldfarb *et al*. 2011). This suggests that Proteobacteria competitively excluded deeply branching bacterial clades more strongly in the fertile plant patches than in inter-patch areas.

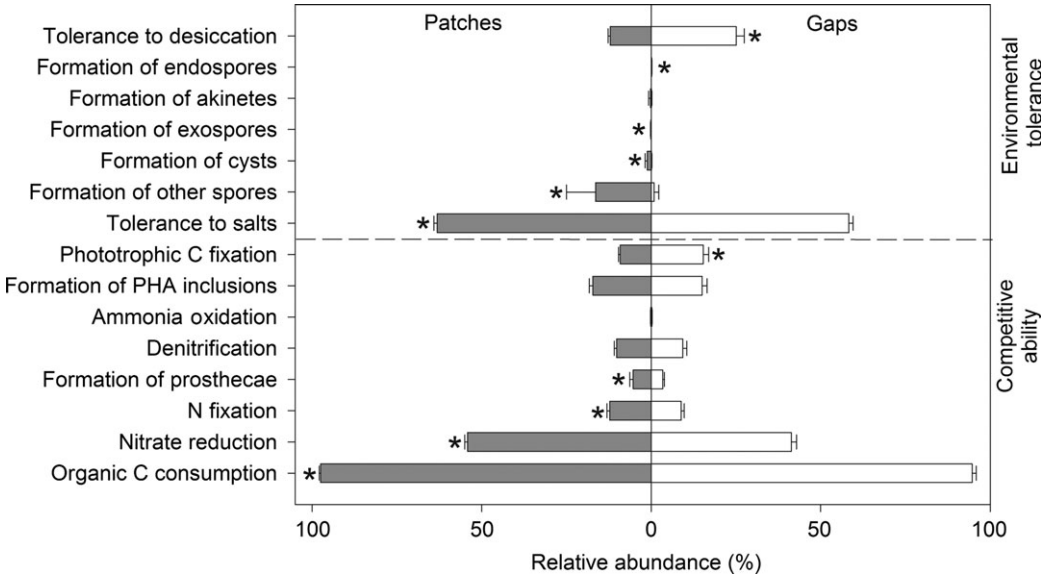

Figure 3 Relative abundance of seven traits conferring environmental tolerance and eight traits conferring competitive abilities to bacteria in plant patches and gaps in semi-arid gypsic soils. Asterisks indicate significant differences ($P \leq 0.05$). Bars represent standard errors.

Table 2 Phylogenetic conservatism of bacterial traits calculated based on 385 reference organisms

Traits	D value	$N = 0$	$N = 1$
Environmental tolerance			
Tolerance to desiccation	**0.608**	345	40
Formation of endospores	**−0.748**	379	6
Formation of akinetes	NA	383	2
Formation of exospores	NA	383	2
Formation of cysts	0.678 ns	377	8
Formation of other spores	**−0.565**	323	62
Tolerance to salts	**0.521**	190	195
Competitive abilities			
Phototrophic C fixation	**0.342**	347	38
Formation of PHA inclusions	**0.438**	320	65
Ammonia oxidation	NA	383	2
Denitrification	**0.597**	332	53
Formation of prosthecae	**−0.160**	374	11
N fixation	**0.507**	341	44
Nitrate reduction	**0.807**	223	162
Organic C consumption	**−0.239**	10	375

Traits with D values smaller than 1 show a phylogenetic signal, whose intensity increases as D value decreases. Traits with significant phylogenetic signals are marked in bold ($P < 0.001$). For each trait, the number of taxa with each trait value (absence: $N = 0$, presence: $N = 1$) are given. NA indicates that the test was not performed due to extremely low variability in the trait.

Therefore, our results indicate that more bacterial ecological strategies coexist in the harsh gaps, while the milder patches increase the ecological and phylogenetic similarities among coexisting bacteria via competitive dominance.

We also detected differential bacterial trait selection based on the soil environment. Traits conferring tolerance to environmental stress determined the phenotypic community structure of soil bacteria in both environments, indicating that abiotic filtering was a generally relevant process in this eco-system affected by water-limitation and high soil electrical conductivity (Navarro-Cano *et al.* 2014). Open spaces exposed to high radiation and temperature variation (Goberna *et al.* 2007) had more bacterial traits associated with tolerance to desiccation and formation of endospores, which are the most resistant structures to environmental extremes (Dworkin 2006). Plant patches filtered bacteria able to form other resistant structures (exospores, other spores, and cysts) and a wide diversity of salt-tolerant organisms. Competition-related traits, however, exclusively generated a significant phenotypic community structure in plant patches, i.e. in the high productive environments of intensified biotic interactions (Aguiar & Sala 1999; Goberna *et al.* 2007). Phototrophic C fixing bacteria were overrepresented in gaps, but this pattern did not suffice to generate a phenotypic community structure significantly differing from random. Plant patches promoted organic C consumers, N fixers, nitrate reducers and prosthecate nutrient scavengers (McAdams 2006). This was eventually reflected in the higher growth rates of bacteria thriving in patches compared to gaps, as broadly indicated by their larger average number of 16S rRNA gene copies (Klappenbach *et al.* 2000), which is the necessary determinant leading to the overrepresentation of competitively superior clades.

Phylogenetic community structure reflects phenotypic community structure only if traits are phylogenetically conserved (Kraft *et al.* 2007; Pausas & Verdú 2010). This was the case of the majority of traits analysed, as derived from their significant phylogenetic signals across a reference phylogeny (Fritz & Purvis 2010). Thus, our results support the notion that bacterial traits tend to be phylogenetically conserved, as reported by Martiny *et al.* (2013) who detected significant phylogenetic signals in 16 out of 19 genomic traits and in 56 out of 57 organic C consumption-related traits. Therefore, in our study, phylogenetic clustering was likely mediated by the selection of conserved traits conferring environmental tolerance in both environments, and it was further strengthened in patches by the filtering of conserved traits conferring competitive abilities.

These observations fit well to our hypothetic scenario (B) in Table 1, according to which there is a predominant abiotic filter under harsh low productive conditions while the competitive exclusion of low competitive clades becomes significant under high resource availability (Mayfield & Levine 2010). In both cases, the overrepresentation of bacteria bearing conserved traits results in phylogenetic clustering, but the mechanisms underlying such a pattern are radically distinct in either environment. Most importantly ours is the first evidence of phylogenetic clustering in bacterial communities being partly driven by the promotion of conserved traits that confer competitive abilities. Thus, our results support the theoretical framework by Mayfield & Levine (2010) and the experimental demonstration by Goldfarb *et al*. (2011), and lead us to the conclusion that this process is realistic under natural conditions. This is necessarily a simplified version of the balance of forces that might structure the soil bacterial communities, since various ecological filters (e.g. predation, facilitation, competition based on niche similarity, competition by interference) operate simultaneously on the multiple traits that condition the survival and adaptation of the myriad of clades shaping the community (Mayfield *et al*. 2009).

Bacterial phylogenies and phenotypes are ongoing hypotheses, and hence we necessarily assume uncertainty associated with our work. The inherent difficulty related to the reconstruction of bacterial phylogenetic trees is further complicated by the usage of short sequences of a single phylogenetic marker, as is our case and that of most microbial ecology studies. We have tried to reduce the uncertainty of phylogenetic reconstruction by assuming the topology of the deep relationships between bacterial lineages of a well resolved tree based on over 285.000 full 16S rRNA sequences (Quast *et al*. 2013). Furthermore, we have used Bayesian inference to obtain the probabilistic distribution of the phylogenetic community structure based on replicated phylogenetic trees (de Villemereuil *et al*. 2012). Similarly, assigning phenotypes to bacterial OTUs bears high complexity for several reasons (Green *et al*. 2008). The most obvious is that only a fraction of the microbiota has yet been cultured, and thus many physiological, morphological and ecological characters remain unexplored (Green *et al*. 2008). Therefore, using high-throughput sequencing allows a deeper exploration of microbial diversity, but still trait definition is constrained to the closest cultured representative to each query sequence. In order to account for the uncertainty associated with assigning traits to environmental sequences, we explored the consistency of our results by using a subset of OTUs classified at a high confidence threshold at the finest taxonomic level. Trait assignment to closely related bacteria is supported by the widespread phylogenetic conservatism of functional traits in prokaryotes (Martiny *et al*. 2013). Similar approaches to ours based on trait assignment to close relatives have been recently proposed for the prediction of gene contents (Kembel *et al*. 2012; Langille *et al*. 2013). Further efforts to enlarge our functional trait database for reference organisms will hopefully help understanding the community assembly processes of prokaryotes.

In summary, by combining abundance data, phylogeny and traits capturing functional responses to the abiotic and biotic environment, we conclude that the phylogenetic clustering of soil bacteria can be mediated by the filtering of traits either conferring resistance to abiotic stress or competitive abilities (Mayfield & Levine 2010; HilleRisLambers *et al*. 2012). Together with other lines of evidence (Goldfarb *et al*. 2011), our results suggest that the competitive exclusion of large bacterial clades based on their low (environmentally mediated) competitive abilities might help explain the widespread coexistence of evolutionarily related soil bacteria.

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AUTHORSHIP

MG and MV designed research and all authors discussed it; MG and JANC generated data; MG and MV performed analyses; MG and MV wrote the first draft of the manuscript and all authors contributed substantially to revisions.

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