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Abstract

Functional, physiological traits are the underlying drivers of niche differentiation. A common framework related to niches occupied by terrestrial prokaryotes is based on copiotrophy or oligotrophy, where resource investment is primarily in either rapid growth or stress tolerance, respectively. A quantitative trait-based approach sought relationships between taxa, traits and niche in terrestrial prokaryotes. With 175 taxa from 11 Phyla and 35 Families (*n* = 5 per Family), traits were considered as discrete counts of shared genome-encoded proteins. Trait composition strongly supported non-random functional distributions as preferential clustering of related taxa via unweighted pair-group method with arithmetic mean. Trait similarity between taxa increased as taxonomic rank decreased. A suite of Random Forest models identified traits significantly enriched or depleted in taxonomic groups. These traits conveyed functions

related to rapid growth, nutrient acquisition and stress tolerance consistent with their presence in copiotroph-oligotroph niches. Hierarchical clustering of traits identified a clade of competitive, copiotrophic Families resilient to oxidative stress *versus* glycosyltransferase-enriched oligotrophic Families resistant to antimicrobials and environmental stress. However, the formation of five clades suggested a more nuanced view to describe niche differentiation in terrestrial systems is necessary. We suggest considering traits involved in both resource investment and acquisition when predicting niche.

1. Introduction

Niche differentiation, the process of physiologically distinct organisms adapting better to certain conditions, is a contributing factor to the high biodiversity inherent in microbial communities (Prosser, 2012). Such differentiation is likely an inevitable consequence of the principles of competitive exclusion and natural selection working in tandem – no two organisms can theoretically occupy the same niche, as the poorer competitor must either adapt to a unique niche or be driven to extinction in that system (Gause, 1932, Hutchinson, 1957, Leibold, 1995). The physiological traits driving niche differentiation must have the capacity to convey an advantage to the organism's ability to survive and reproduce (*i.e.* fitness) and be inherited by successive generations (McGill *et al.*, 2006). Importantly, this implies that microbial communities are not only diverse in terms of individual 16S rRNA gene sequences, commonly used to assess community diversity, but also diverse in regard to their physiological traits.

Explaining niche differentiation through the functional, physiological traits present in ecological community members has a long history in macroecology. For example, differences between beak size and shape in Galápagos finches was instrumental in Darwin's hypothesis that a common ancestor had differentiated into multiple, island-specific species. Within the past century, trait-based analyses have been particularly predominant in plant ecology, with seed germination in submerged soil, salt tolerance, carbon to nitrogen biomass stoichiometry, and leaf mass per unit area acting as examples of traits linked to niche differentiation (Gleason, 1926, Grime, 1979, Keddy, 1992). In contrast, trait-based approaches to explain microbial ecology have only been performed in few instances, such as conceptualizing niches of methanotrophs based on abundance in high *versus* low methane environments or disturbed *versus*

undisturbed soils (Ho *et al.*, 2013), correlating increasing growth rate with increasing ribosomal gene and ribosome-associated gene copy number (Vieira-Silva & Rocha, 2010), deterministic modelling of nitrification rate based on ammonia and oxygen uptake rate, temperature sensitivity and growth rate (Bouskill *et al.*, 2012), defining distinct niches of 32 marine microorganisms based on clustering of genome-encoded functional proteins (Lauro *et al.*, 2009), identifying habitat generalists and specialists based on taxon co-occurrence patterns (Barbéran *et al.*, 2012) and recently comparisons of 23 'core' traits (*e.g.* motility, carbon metabolism, optimal pH for growth) across 15 000 diverse host-associated and environmental genomes (Madin *et al.*, 2020).

A consistent trend noted in macroecology is that traits linked to how carbon and energy is processed and allocated to biomass can describe separate niches (Brown *et al.*, 2004). The canonical example are *r* and *K* strategists, where carbon and energy are primarily invested in reproduction, or alternatively invested in tolerating biotic and/or abiotic stressors, respectively (Grime, 1977). These dichotomous strategies have been observed in microbial ecology: copiotrophs are considered as microorganisms with relatively high growth rates that have relatively poor growth efficiency (as carbon incorporated to biomass per unit resource), relatively high cell maintenance energy costs, dependence on relatively high concentrations of organic carbon in their environment, demonstrate rapid population blooms upon the addition of organic matter and are not overly tolerant of abiotic stress (Semenov, 1991, Koch, 2001, Roller & Schmidt, 2015, Ho *et al.*, 2017). Oligotrophs are considered as the inverse – low growth rate, high growth efficiency, low cell maintenance energy requirements, high substrate uptake affinity, slow growth yet at a consistent rate and are resilient to abiotic stress. Although the niche concept in macroecology has a formalized definition founded on where a taxon can maintain a stable population within multi-dimensional environmental space (Leibold, 1995), in this study, niche is used simply to distinguish between prokaryotes being relatively more copiotrophic *versus* oligotrophic.

These distinct niches became associated with specific terrestrial taxa at high taxonomic rank based on recent molecular analyses. In complex microbial communities, the relative abundance of Gammaproteobacteria, Bacteroidetes and Actinobacteria were correlated with rapid growth in response to the addition of labile organic matter or nitrogen (copiotrophs) (Fierer *et al.*, 2007, Goldfarb *et al.*, 2011,

Fierer *et al.*, 2012, Leff *et al.*, 2015). Conversely, the Deltaproteobacteria, Acidobacteria, Verrucomicrobia and Planctomycetes were negatively correlated with the addition of organic matter or nitrogen (oligotrophs) (Fierer *et al.*, 2007, Fierer *et al.*, 2012, Leff *et al.*, 2015, Bastida *et al.*, 2016). Conflicting reports exist of Beta- and Alphaproteobacteria, with some studies describing them as copiotrophic and others as oligotrophic highlighting that a consistent niche may not necessarily exist across species within a large taxonomic group (Ho *et al.*, 2017). A genomic basis for traits associated with soils dominated by putative copiotrophs and oligotrophs has been expertly reviewed elsewhere, and interested readers are referred to Trivedi *et al.*, (2013) and references therein. Importantly, these observations suggest that specific traits that allow terrestrial prokaryotes to occupy these two niches should (generally) be associated with taxonomy. This is an example of ecological coherence at high taxonomic rank, whereby members within a taxon tend to have similar life strategies, niches and possess common traits compared to members of other taxa (Philippot *et al.*, 2010). While ecological coherence of taxa has been considered previously, the shared, specific traits that drive niche differentiation in terrestrial prokaryotes remains an open question.

To identify the traits that differ between terrestrial prokaryote taxonomic groups, and whether these traits could describe the niches they occupy, a functional trait-based approach was adopted here. We posited that a trait must: a) be associated with a physiological process that conveys a fitness advantage under certain environmental conditions; b) be measurable in well-defined units; and c) vary more between taxonomic groups than within a taxonomic group (McGill *et al.*, 2006, Kearney *et al.*, 2010). Traits were measured as discrete counts of chromosome-encoded proteins shared between at least two of 175 terrestrial prokaryotes. Markov Chain clustering (MCL) was used to group proteins as traits based on amino acid sequence similarity (%) akin to a previous approach that confirmed close taxonomic relatives tend to share functional traits in 1 374 genomes (Zhu *et al.*, 2015). This was necessary to compare highly similar (but non-identical) proteins from separate genomes that carry out the same biological function. To better identify important, distinguishing traits of terrestrial prokaryotes, this study differed from Zhu *et al.*, by: a) comparing 175 publicly available terrestrial prokaryote genomes from 35 Families (n = 5 each), from 11 Phyla and two Kingdoms; b) selecting prokaryotes involved in terrestrial ecosystem processes of interest, including organic matter decomposition, nitrogen fixation, nitrification,

denitrification, methane oxidation, plant-growth promotion, bioremediation of pollutants, pathogenesis and methanogenesis; c) selecting taxa isolated from a wide range of terrestrial environments, such as nutrient rich decomposing plant material and rhizosphere, submerged wetland and rice paddy soils, polluted soils, and nutrient poor hot and cold arid environments; and d) avoiding the inclusion of multiple subspecies and/or strains of a single species to prevent biases in analyses where highly over-represented species are compared with species that have fewer cultured representatives. The taxonomic system used here is from the NCBI, which is built upon a historical array of culture-dependent, physiological observations and genetic similarity to cultured isolates as average nucleotide identity, DNA-DNA hybridisation or 16S rRNA gene homology (ncbi.nlm.nih.gov/Taxonomy/Browser). The use of this classification system and comparison to others is discussed further below.

We hypothesised that: 1) traits are non-randomly distributed, with relatively closely related taxa demonstrating greater similarity than unrelated taxa (ecological coherence); 2) traits that are differentially enriched between taxonomic groups would primarily be involved in metabolism, nutrient acquisition and/or tolerating environmental stress; and 3) copiotrophic and oligotrophic taxonomic groups would emerge based on collective trait enrichment.

2. Methodology

2.1 Collection of terrestrial prokaryote genomes

A collection of 175 sequenced and annotated genomes was collated (Supplementary Table 1). Listed are the genome ID, phylogenetic lineage, role in an ecosystem process if known, and isolation or genome sequencing reference. These genomes were sourced from the National Centre for Biotechnology Information (NCBI) and Joint Genome Institute (JGI) databases. Genomes were chosen based on several criteria: a) five isolates per Family were chosen to have an equal minimum sample size per group, with this sample size being constrained by sequenced genomes of under-represented groups in public databases; b) only a single subspecies/strain per species was included to avoid bias due to over-representation of some species in public databases; c) an emphasis was placed to include isolates from diverse taxonomic lineages involved in terrestrial ecosystem processes of interest, such as ammonia oxidation and methanogenesis; and d) there was an emphasis to include taxonomic

groups frequently stated to be either copiotrophic (*e.g.* Actinobacteria, Gammaproteobacteria) or oligotrophic (*e.g.* Acidobacteria, Planctomycetes) based on observations from soil nutrient addition studies (Ho *et al.*, 2017). Taxonomic annotations for Phyla, Class, Order etc. were based on NCBI taxonomy as most genomes were sourced there. The authors recognise that taxonomy is constantly shifting, particularly so with the recent development of the Genome Taxonomy Database (GTDB) (Parks *et al.*, 2018). Of note is that the vast majority of taxa here have the same taxonomy in NCBI as in GTDB, with the exceptions that GTDB considers the Sporomusaceae as split into three separate Families, the Leuconostocaceae to be Lactobacillaceae, the Promicromonosporaceae to be Cellulomonadaceae, and the Bradyrhizobiaceae and Methylococcaceae have been renamed as Xanthobacteraceae and Methylomonadaceae, respectively. Taxon selection was constrained by availability of genomes for under-represented groups, such as the Chloroflexi, Verrucomicrobia, Planctomycetes, Thaumarchaeota and Euryarchaeota. To meet the $n = 5$ requirement for balanced statistical analyses, it was necessary to consider these under-represented groups as 'Families'. Furthermore, due to the great diversity inherent within Proteobacterial Classes, Gamma-, Alpha-, Beta- and Deltaproteobacteria were considered as independent 'Phyla' for statistical analyses here. Indeed, GTDB now defines Deltaproteobacteria as its own Phylum, while Betaproteobacteria are considered as the Burkholderiales Order within the Gammaproteobacteria. The total of 175 genomes analysed here falls within the upper range of previous hypothesis-driven trait-based studies which varies from 11 isolates (Bouskill *et al.*, 2012) to 214 genomes (Vieira-Silva & Rocha, 2010).

2.2 Functional trait clustering

A step-by-step walkthrough of reproducible code to perform the following analyses on a subset of 12 genomes is available at: https://github.com/DamienFinn/Trait-based_analyses. Firstly, a pairwise similarity comparison of all amino acid sequences (964 951 sequences) across the 175 genomes was performed with the all *versus* all basic local alignment tool function for proteins, BLAST-P (Altschul *et al.*, 1990). Amino acid sequences were subsequently clustered as traits via MCL weighted by pairwise amino acid similarity (Enright *et al.*, 2002). Functional traits were grouped at a cluster value of 90.2, whereby > 65 is considered 'fair' and confidence in accurately separating

clusters cannot be higher than 100. The value of 90.2 is not chosen by the user but rather is a reflection of the quality of clustering in a given dataset. The MCL identified a total of 220 664 traits shared between at least two genomes. A random subset of 1700 amino acid sequences were selected and the similarity of each sequence within its trait group (as determined by MCL) *versus* between other trait groups was visualised as a box and whisker plot (Supplementary Figure 1) in R version 4.0.0 (R Core Team, 2013). 1 700 sequences were chosen to maximise comparisons between trait groups under technical limitations, as increasing sequences led to exponential increases in trait combinations. A Student's T test was applied to determine whether sequences were more similar within their trait group relative to between trait groups in R. Finally, a matrix of genome *x* functional trait was generated in a two-step process by first associating genome IDs to the MCL output with a novel script 'MCLtoReshape2.py' (available at the above Github address) and secondly by casting the long data format to a wide data matrix with the 'reshape2' package in R (Wickham, 2007). Box and whisker plots comparing counts of proteins per genome (input) and counts of functional traits shared by at least two genomes (output of computational workflow), for the 35 Families, is presented as Figure 1.

2.3 UPGMA dendrogram of trait similarity between genomes

The unweighted pair group method with arithmetic mean (UPGMA) was chosen to compare distance-based similarity between taxa based on discrete counts of individual traits per genome. This method is more robust for comparing similarity between sample units (*i.e.* taxa) based on discrete counts of variables (*i.e.* individual traits per taxon) rather than neighbour joining or maximum likelihood methods better suited for DNA or amino acid sequence comparisons (Weins, 1998). The UPGMA was performed in R with the 'phangorn' package as described (Schliep *et al.*, 2017) on a Bray-Curtis transformed dissimilarity functional trait matrix, generated with the 'vegdist' function in the 'vegan' package (Oksanen *et al.*, 2013). To measure ecological coherence (*C*) of taxa within shared Super Groups, Phyla and Families, a similarity index was adapted from Levins' Overlap (Finn *et al.*, 2020a), which measures pairwise similarity in distributions of taxa, as the following:

$$C = 1 - \left(\frac{\sum b_{ij}}{n^2} \right) \qquad \text{Equation 1.}$$

Where b_{ij} is the pairwise branch length between taxon *i* and *j* in the UPGMA tree, measured here as Bray-Curtis dissimilarity, which is summed for each taxon and its relatives within a shared Super Group, Phylum or Family, and where *n* is the number of taxa being compared within a shared Super Group, Phylum or Family.

Furthermore, the full length 16S rRNA gene of each taxon was collated from NCBI. Genes were aligned with MUSCLE (Edgar, 2004) and a neighbour joining phylogenetic tree was constructed with the 'phangorn' package in R. Phylogenetic distance present in taxonomic groups (*P*) was measured as per Equation 1., excepting that branch length was in units of DNA sequence similarity as opposed to Bray-Curtis dissimilarity. Finally, simple linear regression was used to test a relationship between *P* and *C*.

2.4 Functional trait annotation

To inform the biological process a functional trait facilitated, traits were annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This was performed in five steps: 1) a representative amino acid sequence from each trait was extracted with the novel script 'IdentifyTraits.py'; 2) these sequences were annotated with KEGG Orthology (KO) terms using the BlastKOALA database algorithm with a bit score cut-off value of 75 (Kanehisa *et al.*, 2016); 3) BRITE functional heirarchies associated with each KO term (*e.g.* KO1179 gene: endoglucanase, BRITE 1: Metabolism, BRITE 2: Carbohydrate Metabolism, BRITE 3: Starch and Sucrose Metabolism) were collected with the novel script 'GetBRITEinfo.py'; 4) Genome ID, trait ID, KO term and BRITE metadata were all collated with the novel script 'MatchFCs.py'; and 5) the 'reshape2' package in R was used to create matrices of genome *x* BRITE hierarchy. Where KEGG was unable to annotate a trait, it was considered as 'Uncharacterised'. As above, all novel scripts and a step-by-step walkthrough of reproducible code is available at: https://github.com/DamienFinn/Trait-based_analyses.

2.5 Identifying traits differentially enriched in taxonomic groups

Random Forest classification was chosen as a non-linear, multivariate cluster-based method capable of identifying numerous predictor variables (*i.e.* traits) that define

different classes of a response variable (*i.e.* taxonomic group). This was performed with the 'randomForest' package as described (Liaw & Weiner, 2002). Discrete counts of traits at BRITE level 3 were used (*e.g.* Starch and Sucrose Metabolism) as this level had the most accurate resolution of biological processes facilitated by traits. A total of six Random Forest models were optimised to classify taxonomic groups at the level of: a) Phylum, with Proteobacteria Classes separated due to their extensive diversity (n = 14); b) Family (n = 35); c) specifically for Families in the Proteobacteria (n = 13); d) Families in the Actinobacteria (n = 7); e) Families in the Firmicutes (n = 5); and f) Families from 'Under-represented' groups, which were all other Families (n = 10). Optimal numbers of trees grown for each model were: 300, 400, 320, 300, 300 and 400, respectively. Six traits were randomly selected at each branch. As the Random Forest only identifies traits that best explain separation of taxonomic groups, and does not show whether traits have positive or negative associations with groups, box and whisker plots and Fisher's Least Significant Difference (LSD) *post hoc* tests were performed with the 'agricolae' package (de Mendiburu, 2014) to definitively state which taxonomic groups were significantly enriched or depleted in traits identified via Random Forest.

2.6 Hierarchical clustering of Families by defining traits

Finally, the relationship between Families based on similarity in counts of 60 traits was assessed via hierarchical clustering. Traits were chosen based on being selected via the above Random Forest models in this study, and from previous studies that identified traits associated with copiotroph-oligotroph growth strategies in single species or mixed communities (Lauro *et al.*, 2009, Vieira-Silva & Rocha, 2010, Roller & Schmidt, 2015, Pascual-Garcia & Bell, 2020). The mean of trait discrete counts in the five Family members was used as representative of each Family. Comparing trait means between Families was considered acceptable as prior LSD *post hoc* tests had demonstrated significant differences between Families. As traits had highly variable copy numbers per Family (*e.g.* ABC transporter trait copies ranged from 10 – 350, while bacterial chemotaxis traits ranged from 0 – 15 copies) the trait copies were normalised for more appropriate comparisons. Normalised variance was calculated across the 35 Families for all traits with the 'decostand' function in the 'vegan' package (Oksanen *et al.*, 2013). Hierarchical clustering of Families based on normalised trait

counts was visualised with the 'heatmap.2' function in the 'gplots' package (Warnes *et al.*, 2019).

3. Results

3.1 Trait clustering and UPGMA

The 964 951 amino acid sequences encoded by the 175 genomes were clustered as 220 664 traits by MCL. A random subset of 1 700 traits showed that amino acid sequence similarity within traits ranged from 62.4, 82.5 and 100% for the 1st quartile, mean and 3rd quartile, respectively (Supplementary Figure 1). Amino acid sequence similarity between traits ranged from 27.6, 34 and 37.8% for the 1st quartile, mean and 3rd quartile, respectively. A Student's T test found that sequences grouped together as a trait were significantly more similar to each other than to sequences grouped as different traits (t value = 61.3, p = 2 x 10^{-16}). Manual comparisons of amino acid sequences within several traits supported clustering of proteins with identical biological function based on KEGG annotation. Thus, the MCL was considered to perform well. However, the minimum amino acid sequence similarity within traits was 23.27% and maximum similarity between traits was 85.24%, indicating that across the 220 664 traits, a small proportion of dissimilar amino acid sequences were grouped as a trait incorrectly, while some amino acid sequences that were highly similar were considered different traits. This small number of incorrectly clustered sequences can be explained by the MCL clustering efficiency being 90.2, out of a possible 100.

A comparison of the number of proteins per genome at the Family level (mean = 5 533), found that there were fewer functional traits per genome (mean = 4 240) (Figure 1). These represent the input number of proteins per genome before trait clustering and the output number of traits after clustering, respectively. As only functional traits shared between at least two genomes were considered here, the loss of highly genome-specific traits that could not be compared between genomes was expected. Despite this drop in average traits per genome, this initial approach serves as a proof of concept to demonstrate that numbers of traits per genome at the Family level reflect trends in proteins per Family, and thus the MCL was not distorting trait clustering (Figure 1).

The UPGMA dendrogram comparing the 220 664 traits per genome showed that trait compositions were non-randomly distributed (Figure 2a). Specifically,

Thaumarchaeota, Euryarchaeota, Acidobacteria, Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Cyanobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes, Actinobacteria, Firmicutes and Deltaproteobacteria clustered together preferentially. The Chloroflexi were split into two clusters: one *Dehalococcoides* and one *Ktedonobacter/Herpetosiphon/Anaerolinea* cluster. Several prokaryotes did not cluster with their high taxonomic rank, including a Planctomycetes bacterium, *Polyangium brachysporum* (Deltaproteobacteria), *Agreia pratensis* (Actinobacteria) and *Sporomusa ovata* (Firmicutes). Also of interest was that, in regard to distance between terminal nodes (as noted by the scale bar), the Betaproteobacteria and Gammaproteobacteria were more similar to each other than the Alphaproteobacteria, which formed its own large, diverse clade. A neighbour joining tree of full length 16S rRNA genes showed that all taxa clustered preferentially based on their taxonomic nomenclature at high taxonomic rank, including the Chloroflexi, indicating that the discrepancies in the UPGMA were not due to misclassification of the individual taxa (Supplementary Figure 2a).

A simple index to measure trait similarity, as ecological coherence (C), within groups was devised (Equation 1). C increased as taxonomic rank decreased: Super Group < Phylum < Family (Figure 2b). C was lowest for the larger, more diverse Proteobacteria and Terrabacteria (Super Group), and Firmicutes and Actinobacteria (Phylum). As the number of taxa being compared at the Super Group (*e.g.* Terrabacteria = 70 *versus* Acidobacteria = 10) and Phylum (*e.g.* Actinobacteria = 35 *versus* Thaumarchaeota = 5) were variable, the most meaningful comparisons between groups are at the Family level (n = 5 each). With the exception of the highly divergent 'Acidobacteria Lineage', all Families had a C greater than 0.3, with certain groups in the Alphaproteobacteria (Beijerinckiaceae), Firmicutes (Bacillaceae and Leuconostocaceae) and Actinobacteria (Promicromonosporaceae) being highly coherent ($C > 0.55$). Indeed, all individual Proteobacterial Families had $C > 0.4$, indicating that all five taxa within each of these Families had similar trait compositions. Despite not truly belonging to the same Family as per NCBI taxonomy, the $C > 0.33$ of the five Cyanobacteria, Thaumarchaeota and the Methanogen Lineage taxa was similar to other Families from the Bacteroidetes and Firmicutes. Thus, the UPGMA demonstrated that taxonomic relatives at the Phylum level tended to cluster with each other preferentially based on trait composition, and secondly that while similarity was broadly highest at low taxonomic rank, some Families were more coherent than others.

Phylogenetic distance (*P*) of each taxonomic group increased with decreasing taxonomic rank, and was highest in Proteobacteria, Actinobacteria and Firmicutes Families (Supplementary Figure 2b). There was a strong positive linear relationship between *P* and *C* ($y = 0.86x - 0.25$, $R^2 = 0.39$, $p < 0.001$) supporting the result that taxonomic groups of closer related taxa tended to share more similar compositions of traits.

3.2 Random Forest trait identification

The KEGG annotated traits belonged to 260 different BRITE 3 categories. The percentage of traits that could not be annotated (and were termed 'Uncharacterised') ranged from 28 – 65% per genome, being particularly high in the Archaea. On average, 47% of traits per genome were Uncharacterised with a standard deviation of 9.5%.

Of the 260 BRITE 3 categories, the 60 most important traits in separating all Phyla and Families are ranked by importance measured as Mean Decrease in Accuracy (MDA) of the Random Forest models (Figure 3). This is a measure of the average increase in classification error during permutation of trees ($n = 300 - 400$) when that particular trait is missing from the tree. For example, the accuracy of classifying Families was most improved by inclusion of the ABC transporters trait. Based on the identified traits, the Phylum model was capable of successfully classifying 81.14% of individual taxa. The Family model was capable of successfully classifying 71.43% of individual taxa. Confusion matrices for both models are presented as Supplementary Tables 2 – 4, and show that classification was particularly difficult for Chloroflexi and Planctomycetes (classification error > 80%) in the Phylum model and for Cellulomonadaceae and the divergent Acidobacteria Lineage (classification error > 80%) in the Family model. Random Forest models were robust against variation in *P* within Families, for example the nine families with all taxa perfectly classified ranged in *P* from the lowest (0.68) to highest (0.9).

The important traits in classifying the taxonomic groups were involved in: a) metabolism and nutrient acquisition (oxidative phosphorylation, tricarboxylic acid (TCA) cycle, glyoxylate/decarboxylate, thermogenesis, propanoate, starch/sucrose, nitrogen, methane metabolism, synthesis of antioxidants such as glutathione, ATP-binding cassette (ABC) transporters, sugar uptake via phosphotransferase systems (PTS)); b) responding to environmental cues and stressors (protein kinases, two-

component systems, transcription factors, proteasome, protein chaperones, RNA transport, chromosome repair via non-homologous DNA end joining); c) core cell physiology (flagella assembly, chemotaxis, sporulation, lipopolysaccharide (LPS), peptidoglycan, glycerolipid, sphingolipid and lipoarabinomannan (LAM) biosynthesis); and d) cell-cell interactions (beta-Lactam resistance, general secretion systems and Type IV secretion systems). Box and whisker plots of discrete counts of identified traits, and LSD results, are provided as Supplementary Figures 4 – 7. The Families significantly enriched and depleted in these traits are listed in Table 1. The Phyla significantly enriched and depleted in identified traits are listed in Supplementary Table 9.

To better identify the more subtle differences between Families in the Proteobacteria, Actinobacteria, Firmicutes and the 'Under-represented' Phyla, individual Random Forest models were constructed for each of the four groups. The successful classification rates were 72.31, 74.29, 76 and 72%, respectively. Confusion matrices for each model are presented as Supplementary Tables 5 – 8. The models were unable to reliably classify Bradyrhizobiaceae and the divergent Acidobacteria Lineage (classification error > 80%).

Supplementary Figure 8 shows the most important traits in classifying the four groups. Unique traits not identified in the prior Phylum and Family models were: a) for Proteobacteria, glycosyltransferases, butanoate metabolism, aminotransferases, ribosome biogenesis, mRNA biogenesis and degradation; b) for Actinobacteria, porphyrin and chlorophyll synthesis, pyruvate metabolism, aminotransferases, fatty acid and aliphatic hydrocarbon metabolism, polyketide and Type II polyketide biosynthesis, antimicrobial resistance genes; c) for Firmicutes, lysine, folate and varied amino acid synthesis, porphyrin and chlorophyll synthesis, DNA replication, bacterial toxins, penicillin and cephalosporin synthesis; and d) for the 'Under-represented' taxa, glycosyltransferases, peptidases and inhibitors, photosynthesis and AMP-activated protein kinases. Box and whisker plots of discrete counts of identified traits, and LSD results, are provided as Supplementary Figures 9 – 12. Tables summarising Families enriched and depleted in these traits are included as Supplementary Tables 10 and 11.

3.3 Hierarchical clustering of defining traits

Hierarchical clustering based on 60 traits, identified from Random Forest in this study and by previous copiotroph-oligotroph studies, indicated five general clades. The dendrogram on the y axis of Figure 4 shows clustering of taxa as these five clades. The dendrogram on the x axis shows clustering of co-occurring traits. Clade I consisted of Proteobacteria, specifically the Pseudomonadaceae, Burkholderiaceae, Rhodospirillaceae, Bradyrhizobiaceae and Rhizobiaceae. These Families were uniquely enriched in flagellar assembly, chemotaxis, pyruvate metabolism, glutathione metabolism, ABC transporters, benzoate metabolism, transcription factors, glyoxylate and fatty acid metabolism. Clade II, also Proteobacteria, included Nitrosomonadaceae, Neisseriales Lineage, Methylocystaceae, Beijerinckiaceae, Methylococcaceae and Moraxellaceae. These Families clustered based on being enriched in Clade I traits, but to a lesser degree than the Pseudomonadaceae, Burkholderiaceae, Rhodospirillaceae, Bradyrhizobiaceae and Rhizobiaceae. Exceptions included the absence of benzoate metabolism and enrichment of methane metabolism in several Clade II Families.

Clade III, a diverse collection of Bacteroidetes (Chitinophagaceae, Cytophagaceae), Verrucomicrobia, Planctomycetes, divergent Acidobacteria Lineage and the Deltaproteobacteria (Polyangiaceae, Myxococcaceae), shared enrichment of sphingolipid metabolism, beta-Lactam resistance, penicillin and cephalosporin biosynthesis, LPS biosynthesis, glycosyltransferases and starch/sucrose metabolism. Many of these Families shared Clade I and II traits, including Type IV secretion system, oxidative phosphorylation, TCA cycle, PTS, nitrogen and glycerophospholipid metabolism. The absence of glutathione in non-Deltaproteobacterial Clade III Families was notable.

The three Actinobacteria Families in Clade IV, Mycobacteriaceae, Frankiaceae and Streptomycetaceae, were highly similar to each other because they were enriched in Type II polyketide biosynthesis. They also shared some Clade I traits (ABC transporters, transcription factors, pyruvate, benzoate and fatty acid metabolism) and Clade III traits (membrane trafficking, transcription machinery, polyketide biosynthesis and starch/sucrose metabolism). Similar to Clade III, these Actinobacteria were also depleted in glutathione traits.

Finally, taxa within Clade V were similar to each other due to being depleted in traits shared among the other clades. Cyanobacteria were the only taxa that possessed photosynthesis traits. Lactic acid bacteria (Lactobacillaceae and Leuconostocaceae)

were enriched in Unclassified nucleotide metabolism. The Archaea (Thaumarchaeota and Methanogen Lineage) shared eukaryote-like traits, proteasome, basal transcription factors and RNA transport. The Archaea were enriched in carbon fixation traits. Methanogens were also enriched in methane metabolism. Ammonia oxidising Thaumarchaeota were not enriched in nitrogen metabolism, however they were enriched in traits annotated by KEGG as Global maps only (unclassified metabolism). Further analysis found this to be the *fpr* gene, encoding a ferredoxin-flavodoxin $NADP^+$ reductase (K00528). Non-lactic acid bacteria of the Firmicutes (Bacillaceae, Sporomusaceae and Clostridiaceae) were enriched in sporulation and motility traits.

4. Discussion

4.1 Non-random trait clustering demonstrates ecological coherence of taxa

A trait-based approach to investigate taxonomic relationships and potential biological function was carried out with a collection of 175 terrestrial prokaryotes. We hypothesised that traits would be non-randomly distributed amongst taxonomic groups, supported by previous observations that noted closely related taxa are isolated from similar habitats (Philippot *et al.*, 2010). Similarity in the composition of 220 664 traits, within 175 taxa, demonstrated strong agreement with established taxonomy at high (Phyla) and low (Family) rank (Figure 2a). The exceptions to this ecological coherence at high rank were the division of the Chloroflexi and an individual from each of the Planctomycetes, Deltaproteobacteria, Actinobacteria and Firmicutes clustering with unrelated Phyla. These taxa were not mischaracterised, as based on phylogenetics of the full length 16S rRNA gene (Supplementary Figure 2a). Trait similarity between related taxa, measured as *C*, tended to be highest at low rank (Figure 2b). Uneven sample sizes between groups within Super Group and Phyla make comparisons at this level difficult – the inclusion of many diverse Firmicutes and Actinobacteria likely drove *C* to be lower here than in Thaumarchaeota and Euryarchaeota. However, equal comparisons at the Family level demonstrated interesting variability in coherence. All Proteobacterial Families had relatively high coherence (C = 0.4 – 0.6). The high *C* in Beijerinckiaceae is of particular interest as this group contained both specialist methanotroph (*Methylocapsa*, *Methylocella*), methylotroph (*Methyloferula*) and generalist heterotroph (*Beijerinckia* spp.) taxa. With such varied metabolic traits, one could reasonably expect *C* to be relatively low within this Family. The Beijerinckiaceae

appear to have evolved from a common methylotrophic ancestor and still share traits for nitrogen fixation and tolerance for low pH soils (Tamas *et al.*, 2014), and the high *C* measured here indicates that many additional shared traits remain. Both the relatively recent divergence of Families from a common ancestor and the higher number of shared traits are likely causes of the higher *C* observed at low taxonomic rank. The differing values of *C* for Methanogen and photosynthetic Cyanobacteria functional groups (0.33 and 0.44, respectively) is also worthy of note. Despite all five taxa in each group performing the same core role in a community, the individual isolates came from varying environments. The methanogens were isolated from a range of geographically separate wetlands, rice paddy soil and farm slurry and, while the Cyanobacteria were also isolated from geographically separate environments, they were all from sandy deserts or other nutrient poor, arid soils (Supplementary Table 1 and references therein). Ultimately a taxon's trait composition will be affected by its functional role in a community, its evolutionary life-history (*e.g.* Beijerinckiaceae described above) and its local environment.

However, these results are dependent on accurate taxonomic classification, and the *C* of Sporomusaceae, relatively low compared to other Families here, supports splitting this group into Sporomusaceae, Anaeromusaceae and Pelosinaceae by GTDB (Parks *et al.*, 2018). Finally worth noting, some groups at high rank were considered as 'Families' here due to the number of available terrestrial genomes, *e.g.* Cyanobacteria and Chloroflexi. Even so, Cyanobacteria demonstrated a higher *C* than many taxonomically-defined Families, perhaps due to their common role as primary colonisers of nutrient-poor soils (Garcia-Pichel *et al.*, 2001). The number of Families are too numerous to discuss each at length here, but *C* was an effective means of measuring and comparing coherence between groups in the UPGMA tree.

While the method of comparing taxa here differs from other studies, the results were not surprising as many 16S rRNA gene surveys of terrestrial systems consistently demonstrate ecological coherence at high rank. For example, independent studies of increasing agricultural intensity in soils show reductions in Actinobacteria abundance (Philippot *et al.*, 2009, Jangid *et al.*, 2011). Nitrogen addition to soils frequently enriches numerous taxa within the Actinobacteria and Proteobacterial Classes while negatively affecting taxa within the Verrucomicrobia and Planctomycetes (Wessen *et al.*, 2010, Fierer *et al.*, 2012, Leff *et al.*, 2015, Bastida *et al.*, 2016). Arid, nutrient poor environments select for Actinobacteria-dominated communities (Cary *et al.*, 2010,

Crits-Christoph *et al.*, 2013) and, in the absence of other primary producers, allow biological soil crust forming Cyanobacterial taxa to establish (Garcia-Pichel *et al.*, 2001). Anoxic wetland and rice paddy environments support diverse communities of anaerobic Firmicutes, Chloroflexi and methanogenic Archaea (He *et al.*, 2019, Finn *et al.*, 2020b). These trends were noted prior to bioinformatic advances of metagenome assembled genomes (MAGs) that allow for the specific comparison of individual traits between uncultured environmental prokaryote genomes (Hug *et al.*, 2013). The generation of MAGs has emerged as a useful tool for identifying traits necessary for life in such environments, and particularly for expanding knowledge of severely under-represented, difficult to culture taxonomic groups. For example, the recent reconstruction of 52 515 MAGs from a wide range of host-associated and environmental metagenomes was able to increase genomic information of Planctomycetes and Verrucomicrobia by 79% and 68%, respectively (Nayfach *et al.*, 2021). Importantly, both 16S rRNA gene surveys and MAGs demonstrate that some functional traits that facilitate life under certain environmental conditions are intrinsically linked to taxonomy.

The ecological coherence observed in Figure 2 does not imply that closely related taxa (*e.g. Bacillus velezensis* LS69 and *Bacillus amyloliquefaciens plantarum* FZB42) have identical phenotypes. Close relatives possess a combination of core and accessory genes (traits) and the presence of even a single accessory gene is sufficient to dramatically alter a strain's phenotype (van Rossum *et al.*, 2020). Rather, our results (Figure 2) demonstrate that the composition of core and accessory traits in alphaproteobacterial Beijerinckiaceae are most similar to each other, relative to alphaproteobacterial Rhizobiaceae or to Actinobacteria, Firmicutes etc.

4.2 What can the Random Forests tell us?

The Random Forest works by identifying the traits that are most reliable in classifying individual Phyla and Families. It selects traits that tend to be: a) of equal copy number per genome within a taxonomic group; and b) that differ markedly in copy number between taxonomic groups, since distinct separation of copies will maximise successful classification. A clear example of this is the consistent identification of eukaryote-like basal transcription factors, proteasome and RNA transport present in the Thaumarchaeota and Euryarchaeota, since they are absent from the majority of

Bacterial Phyla. The binary nature of these traits (yes Archaea, no Bacteria) make them strong indicators. The presence of these and more eukaryote-like vesicular trafficking and actin traits have been noted in the Archaeal TACK super-phylum previously, and lend credence to the hypothesis that eukaryotes are descended from Archaea (Embley & Martin, 2006, Spang *et al.*, 2015). However, the Random Forest will not identify a trait unique to *Can.* Nitrosotalea devanterra that is absent from other Thaumarchaeota, as this single trait will not improve classification of the group as a whole. Consequently, the traits identified via Random Forest all tended to be core, fundamental traits shared by other members of a taxon's Phylum/Family.

Many of the best traits for distinguishing taxa have been historically used by microbiologists to do exactly that. These included fundamental cell physiology traits, such as oxidative phosphorylation, LPS biosynthesis, sporulation, flagellar assembly and chemotaxis. The Phylum model separated Betaproteobacteria, Actinobacteria and Bacteroidetes as taxa with the highest copies of oxidative phosphorylation traits. Firmicutes, Chloroflexi and Methanogens were identified as anaerobes depleted in oxidative phosphorylation, and all other taxa as sitting in between (Supplementary Figure 4 and Supplementary Table 9). Some Gram-negative Families were significantly enriched in LPS biosynthesis compared to others. These were the Pseudomonadaceae, Burkholderiaceae, Chitinophagaceae, divergent Acidobacteria and Verrucomicrobia (Table 1). This has been noted in soil communities previously. The extensive repertoire of LPS-associated genes in Chitinophagaceae, Acidobacteria and Verrucomicrobia likely play a critical role in enhancing soil aggregation (Cania *et al.*, 2019) potentially through high LPS production and/or biofilm formation (discussed further below). In a demonstration of the robustness of the methods used here, the highly unusual Firmicute Sporomusaceae were shown to possess similar counts of LPS biosynthesis traits relative to most Gram-negative Families (Supplementary Table 4) in addition to sharing heat-resistant spore formation with Bacillaceae and Clostridiaceae (Table 1). The presence of both traits in a single Family have been remarked upon previously and used to conceptualise the evolution of Gram-negative *versus* Gram-positive lineages (Stackebrandt *et al.*, 1985). The Sporomusaceae were also shown to have high Porphyrin and Chlorophyll metabolism traits in the Firmicutes model (Supplementary Table 10). The capacity to dechlorinate the soil pollutant perchloroethene to trichloroethylene via a porphyrin-based corrinoid is yet another interesting trait of this Family (Terzenbach & Blaut, 1994).

Finally, flagella assembly and chemotaxis traits identified Alphaproteobacteria, Betaproteobacteria and Acidobacteria as Phyla that were particularly enriched with this mechanism of motility, while bacterial Actinobacteria, Bacteroidetes, Cyanobacteria, Chloroflexi and Verrucomicrobia were depleted (Supplementary Table 9). The Proteobacteria, Firmicutes and Under-represented models were better suited for identifying specific Families homogenously enriched or depleted in bacterial flagella and chemotaxis (Supplementary Tables 10 and 11). Enriched Families included the Rhodospirillaceae, Nitrosomonadaceae, Neisseriales lineage, divergent Acidobacteria lineage, Sporomusaceae, Bacillaceae, Clostridiaceae and Planctomycetes. Other forms of motility such as twitching and gliding have been noted in the Pseudomonadaceae, Myxococcaceae and Cyanobacteria (McBride, 2001) but these traits were not identified by the Random Forest as being homogenously enriched in any Families. Furthermore, while Thaumarchaeota and Methanogens were both identified as being depleted in bacterial flagella assembly and chemotaxis traits (Supplementary Table 9 and 11), Archaea possess a structurally distinct flagellum more similar to the Type IV bacterial pilus (Jarrell and Albers, 2012). These taxa were not enriched with Type IV pilus, either, and it is possible that archaeal flagella may have failed proper characterisation by KEGG. Thus, while some Families were relatively enriched/depleted in bacterial flagella and chemotaxis traits, specific taxa depleted in these are not necessarily non-motile.

In summary, while the Random Forests may overlook certain traits in individual taxa, the models were highly robust in detecting conserved, shared traits within a Phylum/Family. Here the 'depth' of shared traits is limited by the number of taxa that could be considered as Phylum or Family. In future, if five (or more) taxa belonging to the same Genus or even Species could be compared, unique traits would be observed to explain how these subgroups have evolved from their respective Families to occupy distinct niches. Ideally the selection of individual taxa within groups for such future comparative analyses would also be standardised based on phylogenetic distance, either with *P* or a similar method, that would improve the robustness of trait-based comparisons at such a fine taxonomic level.

4.3 Plant-derived carbon metabolism and nutrient acquisition

Secondly, we hypothesised that the traits differentially enriched between taxonomic groups would largely reflect those associated with copiotrophs or oligotrophs, namely metabolism, nutrient acquisition and environmental stress response and tolerance. Of fundamental interest to soil microbiologists is the decomposition of plant biomass. This is the primary source of organic carbon to non-arid terrestrial systems (Kögel-Knabner, 2002) and the transformation of plant material to substrates bioavailable for microorganisms is essential for community growth and activity. The traits involved in plant material catabolism belonged to the BRITE categories 'Starch and Sucrose Metabolism' (*e.g.* extracellular cellobiosidases, endoglucanases, glucosidases, trehalases, amylases) and 'Glycosyltransferases', all of which are carbohydrate activated enzymes (CAZy). The Families particularly enriched in these traits were the Polyangiaceae, Myxococcaceae, Rhizobiaceae, Streptomycetaceae, Mycobacteriaceae, Frankiaceae and Verrucomicrobia (Table 1, Supplementary Table 11). Genomic and culture-dependent analyses support *Sorangium cellulosum* (Polyangiaceae), *Streptomyces coelicolor* A3(2) (Streptomycetaceae) and *Chthoniobacter flavus* (Verrucomicrobia) as having particularly large genomes with extensive repertoires for cellulose, hemicellulose, pectin and lignin degradation (Bentley *et al.*, 2002, Sangwan *et al.*, 2004, Schneiker *et al.*, 2007). Comparative genomics analyses have also identified Actinobacteria, Acidobacteria and Verrucomicrobia as being enriched in numerous enzymes for cellulose, hemicellulose and starch catabolism (Trivedi *et al*., 2013). *In situ* these Families likely play a critical role in making organic carbon bioavailable as di- and monosaccharides for the microbial community.

The Families equipped with many copies of high-affinity sugar uptake 'Phosphotransferase systems' (3 – 14 copies) did not necessarily correspond to those enriched with CAZy – only the Myxococcaceae and Verrucomicrobia were enriched in both. Pseudomonadaceae, Rhodospirillaceae, Neisseriales lineage and Clostridiaceae were only enriched in PTS. Despite being enriched in CAZy, the Frankiaceae were simultaneously depleted in PTS. The complex interplay between taxa capable of producing extracellular CAZy and competitors that rapidly scavenge available di- and monosaccharides has been well described by models that predict such competitive interactions exert important controls on the growth rate of the community as a whole (Freilich *et al.*, 2011) and may even act to aid terrestrial carbon storage and limit carbon dioxide emissions from microbial respiration (Kaiser *et al.*,

2015). Here, we identified the 'specialist' Families enriched in CAZy traits *versus* the 'opportunists' scavenging for sugars via PTS (Table 1, Supplementary Table 11).

ABC transporters facilitate the ATP-dependent uptake of soluble compounds across membranes or export waste metabolites, extracellular enzymes and toxins (Young & Holland, 1999, Higgins, 2001). This means of active transport allows microorganisms to acquire nutrients with high affinity at concentrations of 5 – 500 μg carbon L^{-1} *versus* the less efficient diffusion of nutrients across membranes, dependent on extracellular concentrations of 0.5 – 5 mg carbon L^{-1} (Kuznetsov *et al.*, 1979). In the spatially heterogenous soil environment where the concentration of bioavailable carbon substrate often limits growth (Blagodatsky & Richter, 1998), possession of high affinity transporters likely provides a competitive advantage. The rhizosphere-associated Rhodospirillaceae, Rhizobiaceae and Burkholderiaceae tended to have the highest trait copies of ABC transporters (100 – 350 copies per genome, Supplementary Figure 4). The diverse, non-rhizospheric Deltaproteobacteria, Actinobacteria, Firmicutes, Cyanobacteria and Verrucomicrobia all had greater than 50 copies per genome, highlighting the importance of these traits in soil. The particularly high gene copy number in rhizosphere-associated taxa from presumably nutrient-rich environments contrasts the assumption that ABC transporters are considered to play a greater role in nutrient-poor environments (Lauro *et al.*, 2009). Comparative genomics analyses of soil bacteria have also found putatively copiotrophic Proteobacteria and Firmicutes to be particularly enriched in PTS and ABC transporters (Trivedi *et al.*, 2013). In this study, the transporters enriched in rhizosphere-associated taxa were primarily aimed at scavenging maltose, phosphate, amino acids, oligopeptides and export of LPS, and these results suggest that these traits are not only for survival in nutrient-poor environments but also likely confer a competitive advantage in the rhizosphere. As prokaryotes compete simultaneously with other prokaryotes and plants for nitrogen and phosphorus in the rhizosphere, the high affinity acquisition of such nutrients is likely critical for growth.

4.4 Nitrogen and methane metabolism

The BRITE category 'Nitrogen metabolism' encompasses nitrogen fixation, denitrification, ammonia oxidation and synthesis of glutamate/glutamine which are critical amino acids for peptide synthesis. Since nitrogen limitation acts as an important

control on soil microbial activity, these traits are also of interest to soil microbiologists. Three Proteobacterial Families, Rhodospirillaceae, Bradyrhizobiaceae and Burkholderiaceae, were particularly enriched in these traits. Genomic and culture-dependent analyses show these Families to be free-living or symbiotic diazotrophs in soil and freshwater environments (Madigan *et al.*, 1984, Itakura *et al.*, 2009, de los Santos *et al.*, 2018). Given their significantly greater copies of nitrogen-fixing genes, these Families may be a particularly important source of organic nitrogen for soil communities. Saprotrophic Mycobacteriaceae genomes, also identified as nitrogen cyclers, tend to have many copies of genes involved in ammonia uptake and glutamate synthesis (Amon *et al.*, 2010). This taxon may play an alternative role in converting mineral nitrogen to biomass where organic nitrogen as protein in excreted products or necromass can undergo proteolysis and uptake between other community members. The identification of Sporomusaceae as enriched in 'Nitrogen metabolism' traits is unusual as these obligate anaerobic fermenters cannot use nitrate as an electron acceptor (Möller *et al.*, 1984). Nor were the Sporomusaceae enriched in ammonia uptake or glutamate synthesis genes (data not shown), and so it is uncertain what role this Family plays in nitrogen cycling. Thaumarchaeota and Nitrosomonadaceae, known ammonia oxidisers, were not enriched in 'Nitrogen metabolism' traits relative to other Families (Supplementary Figure 5) despite Nitrosomonadaceae possessing multiple copies of the operon responsible for ammonia oxidation (Klotz & Norton, 1998). Specific traits may be overlooked here if the BRITE category includes many diverse KOs (*e.g.* ammonia oxidation, nitrogen fixation, glutamate synthesis etc).

Another specialised metabolic pathway of interest involves 'Methane metabolism' that includes production and oxidation of a potent greenhouse gas. Unsurprisingly, the Methanogens and methanotrophic Methylococcaceae, Beijerinckiaceae, Methylocystaceae were all enriched in traits involved in methane metabolism. While methane oxidation can be present in some taxa from the Verrucomicrobia (Op den Camp *et al.*, 2009) the above proteobacterial representatives act as the primary terrestrial methane sink (Dunfield, 2007, Conrad, 2009).

4.5 Sensing, responding and tolerating the environment

A particularly interesting divergence of traits were involved in how taxa detect and respond to environmental stimuli. Gram-negative Pseudomonadaceae,

Rhodospirillaceae, Bradyrhizobiaceae and Myxococcaceae were enriched in two-component systems. These membrane-bound histidine kinases respond rapidly to extracellular stimuli (Galperin, 2005) and these traits were primarily involved in nitrogen, potassium, initiating chemotaxis and C_4-dicarboxylate responses. Families enriched in transcription factors were the Myxococcaceae, Polyangiaceae, Streptomycetaceae, Mycobacteriaceae and Frankiaceae. These factors regulate transcription in response to intracellular cues and here these factors were primarily *rpoD* (housekeeping), *rpoH* (heat-shock/protein damage), *rpoE* (extra-cellular cytoplasmic stress) and *rpoS* (starvation) responses (Shimada *et al.*, 2017). The genomes of these taxa are also heavily enriched in regulatory genes for complex developmental stages, fruiting bodies and/or filamentous branching growth in soils (Bentley *et al.*, 2002, Gao *et al.*, 2006, Schneiker *et al.*, 2007). Thus, certain taxa may respond primarily to extracellular cues while others strictly monitor and respond to changes in cell homeostasis. This trend has been noted previously – in 167 genomes across various Bacteria and Archaea, Proteobacteria had a higher ratio of sensors for external *versus* internal stimuli and were considered 'extroverts', while Cyanobacteria were considered strong 'introverts' focussed on responding to internal stimuli (Galperin, 2005).

As mentioned above, Archaea exhibited unique traits in basal transcription and protein regulation via proteasome. These transcription factors were primarily involved in identifying DNA damage and excision repair: TFII-B, TFII-D, ERCC-2 and ERCC-3. DNA repair differs markedly between Bacteria and Archaea/eukaryotes. Specifically, Bacteria excise 12 nucleotides around a damaged site with a 3 polypeptide system whereas Archaea excise 24 – 32 nucleotides with a 13 – 16 polypeptide system (Sancar, 1996). The use of ubiquitin-labelling and proteasome degradation of misfolded or 'old' proteins is arguably a more efficient system for recycling amino acids and regulating the 'lifespan' of a protein in eukaryotes, however, Bacteria are still fully capable of regulating protein misfolding or proteolysis with RpoH (and others) induced upon environmental stress (Goldberg, 2003). From an ecological perspective, it is difficult to discern if these eukaryote-like traits confer any sort of competitive advantage to Archaea. They may simply be examples of convergent evolution for dealing with environmental stress.

Finally, most microbial cells likely exist within complex biofilms and/or assemblages adhered to surfaces with excreted exopolysaccharides, DNA and protein that serve to

protect from adverse environmental factors (Flemming & Wingender, 2010). Families with high copy numbers of exopolysaccharide biosynthesis and secretion systems may act as integral members of soil communities by predominantly contributing to biofilm/aggregate formation. The 'LPS biosynthesis' and 'Starch and sucrose metabolism' BRITE categories can synthesise N-acetyl glucosamine-based and cellulose-based exopolysaccharides, respectively. Taxa enriched in both these categories and secretion systems were the Polyangiaceae and Burkholderiaceae (Table 1), and in the refined 'Under-represented' model, Acidobacteria and Verrucomicrobia (Supplementary Table 11).

4.6 Direct cell-cell interactions

Type IV secretion systems were another important trait identified in the Random Forest models. These were enriched in Pseudomonadaceae and Myxococcaceae (Table 1) and Acidobacteria, Planctomycetes and Verrucomicrobia (Supplementary Table 11). These are highly specialised exporters that deliver DNA and/or toxins directly to other bacterial or plant cells, however, their role in ecology is poorly understood beyond root galls induced by *Agrobacterium tumefaciens* (Christie & Vogel, 2000). These taxa should be explored for whether they utilise these traits for horizontal gene transfer or to inject toxins directly into other prokaryotes, and thus potentially provide a selective advantage for colonisation and competition.

Another archetypal trait for interactions between community members are production of antimicrobials and antimicrobial resistance genes. Penicillin and cephalosporin synthesis were enriched in the Sporomusaceae and Bacillaceae relative to other Firmicutes. Polyketide and Type II polyketide syntheses were important for separating Frankiaceae and Streptomycetaceae from other Actinobacteria (Supplementary Table 10). The Streptomycetaceae have a long history of use in biotechnology as prolific antimicrobial producers (Bentley *et al.*, 2002). Bacillaceae (in particular *Bacillus subtilis* species) are also well known producers of a wide variety of antimicrobials (Caulier *et al.*, 2019), but we noted that Sporomusaceae have an even greater number of these traits (Supplementary Figure 9). To the authors' knowledge, antibiotic production in Sporomusaceae has not been investigated thoroughly and this may be a consequence of its obligate anaerobic nature and difficulties in culturing. In addition to prolific Type II polyketide producers, Streptomycetaceae were also enriched

in antimicrobial resistance genes, while Bacteroidetes, Planctomycetes and Verrucomicrobia were specifically enriched in beta-Lactam resistance (Supplementary Tables 9 and 10).

4.7 Life strategies emerge from differentially enriched traits

We hypothesised that taxa would emerge as being inherently copiotrophic or oligotrophic based on trends in their enriched traits. Traits were chosen based on identification via Random Forest and identification as associated with copiotroph-oligotroph species or in mixed communities as described previously (Lauro *et al.*, 2009, Vieira-Silva & Rocha, 2010, Roller & Schmidt, 2015, Pascual-Garcia & Bell, 2020). Rhizosphere-associated Gamma-, Alpha- and Betaproteobacteria in Clade I fit the assumptions of a copiotrophic niche that invests in high metabolic rate – these taxa were uniquely enriched in competing for nutrient uptake via high-affinity ABC transporters, and energy generation from pyruvate, fatty acids, benzoate and glyoxylate carbon sources. Clade I was also enriched in glutathione metabolism, which acts as the major antioxidant for reducing intracellular free radicals produced during central carbon metabolism (Smirnova & Oktyabrsky, 2005). Antioxidants have been hypothesised as an essential function for copiotrophs to survive their high metabolic rates (Koch, 2001). All five Clade I Families were enriched in oxidative phosphorylation. The oxidative phosphorylation traits encompass a wide variety of electron transport chain proteins (oxidoreductases, dehydrogenases, cytochromes and ATPases) and are crucial for efficient energy production (Brochier-Armanet *et al.*, 2009). All five Families were also enriched in nitrogen metabolism, which included both nitrogen fixation and glutamate (*i.e.* protein) synthesis. Nitrogen fixation is an energy intensive process requiring 20 – 30 ATP per reduced N_2 (Burris & Roberts, 1993) and may be intrinsically linked to taxa with high oxidative phosphorylation. Finally, Clade I also shared motility and chemotaxis, which are also energy intensive traits. Clade II consisted of the remaining Gamma-, Alpha- and Betaproteobacteria, yet these were relatively less enriched in Clade I 'copiotroph' traits. These particular taxa may be responsible for the lack of a consistent copiotrophic response upon nutrient addition in Proteobacteria (Ho *et al.*, 2017).

Clade III was comprised of taxa generally considered as oligotrophs (Ho *et al.*, 2017) with the exception of Bacteroidetes (Fierer *et al.*, 2007). These taxa possessed high

LPS and sphingolipid synthesis that can defend against desiccation and antimicrobials through biofilm and capsule/slime production (Flemming & Wingender, 2010), beta-Lactam resistance, penicillin biosynthesis and several members had high pentose phosphate pathway for efficient carbon metabolism under starvation (Hodgson, 2000). Clade III also possessed high CAZy traits, which Clade I largely lacked, and is consistent with observations of oligotrophs being primarily responsible for catabolising relatively recalcitrant plant material (Goldfarb *et al.*, 2011). While Clade III were equally enriched in oxidative phosphorylation as Clade I, with the exception of the Deltaproteobacteria, these taxa were depleted in glutathione metabolism. The low copies per genome of this trait would explain why the abundance of oligotrophs drop rapidly in nutrient addition studies as they would be either out-competed by glutathione-rich taxa capable of exploiting plentiful nutrients or will lyse if their metabolic rate exceeds capacity to reduce free radicals (Koch, 2001). Taken together, all of these traits indicate Clade III lead oligotrophic lifestyles whereby they are tolerant to adverse environmental conditions, can acquire carbon from recalcitrant plant material, and are incapable of rapid growth rates.

These results support previous observations that Rhodospirillaceae, Bradyrhizobiaceae, Burkholderiaceae, Pseudomonadaceae and Rhizobiaceae are copiotrophic, while Planctomycetes, Verrucomicrobia, Myxococcaceae, Polyangiaceae and Acidobacteria are oligotrophic (Ho *et al.*, 2017 and references therein). As has been proposed previously, the dominance of these groups in certain soils can provide inferences for ecosystem processes in that system, for example soils dominated by Verrucomicrobia, Planctomycetes and Acidobacteria will have greater capacity to degrade complex plant material while retaining most catabolised carbon in biomass (*i.e.* high growth or carbon use efficiency) or excreted byproducts that assist in soil aggregation (*e.g.* high LPS production) (Trivedi *et a*l., 2013). Conversely, soils dominated by copiotrophic Proteobacteria Families will be systems primarily dependent on labile di- and monosaccharides that demonstrate low carbon use efficiency.

Streptomycetaceae, Mycobacteriaceae and Frankiaceae in Clade IV shared enrichment of several Clade I copiotroph traits. As mentioned above in Section 4.5, these Actinobacteria invest carbon and energy into complex filamentous growth and developmental cycles. They demonstrate classic copiotrophic responses to nutrient addition (Goldfarb *et al.*, 2011, Leff *et al.*, 2015) and their enriched ABC transporters,

pyruvate, glyoxylate, benzoate and fatty acid metabolism all likely contribute to generating energy for complex lifecycles. Simultaneously, their enrichment of Clade III oligotroph traits for CAZy metabolism in addition to many traits for producing and resisting antimicrobials indicate a unique niche for these Actinobacteria that does not necessarily fall within the classical copiotroph-oligotroph framework.

Clade V differed markedly from all other clades and mostly consisted of 'specialist' metabolic functional groups involved in photosynthesis, ammonia oxidation, methanogenesis, lactic acid production and other fermentation. Similar to Clade III, these taxa would also be expected to have relatively low metabolic rates due to depletion of copiotroph traits associated with rapid metabolism and energy generation. Unlike Clade III, these taxa seemed to lack consistent mechanisms for stress tolerance. Thus, while certain taxa did invest in traits for rapid metabolic rate (Clades I and IV) and others primarily in stress tolerance (Clades III and IV), some taxa lacked these approaches altogether and pursued entirely distinct niches (Clade V). As an average of 47% of traits within each genome were Uncharacterised, Clade V is an over-simplification and that if novel, currently uncharacterised proteins and the traits they fulfil were incorporated into hierarchical clustering, this clade would separate more meaningfully.

If one were to consider taxa within the one-dimensional copiotroph-oligotroph spectrum, Clade I would represent one extreme, Clades III and V another, with Clades II and IV falling in between. Figure 5a is a conceptual diagram where these Clades have been placed on a singular axis of 'resource investment', with the niche space of Clades enriched in traits associated with rapid growth (*e.g.* glutathione) toward the 'copiotrophy' pole while Clades enriched in stress tolerance traits (*e.g.* LPS production) are placed toward the 'oligotrophy' pole. However, this approach overlooks the diverse functional potentials (and distinct niches) for carbon and energy metabolism associated with the various Clades. Furthermore, the large overlaps in niche space between Clades would suggest taxa from each group could not co-exist if 'resource investment' was the only important consideration (Gause, 1932, Hutchinson, 1957, Leibold, 1995). A more meaningful perspective would be to consider the additional role of 'resource acquisition' that incorporates multiple axes for the life strategies identified via hierarchical clustering of traits. Figure 5b is a conceptual diagram of niche space where clades have been further separated along additional dimensions based on their enrichment of traits involved in competition, degradation or specialised metabolic

pathways. The BRITE categories listed on the various axes are chosen to be useful markers in predicting the niche of a taxon. This expanded niche space would suggest taxa in Clade I are well equipped for nutrient acquisition (primarily, but not limited to, di- and monosaccharides), rapid growth and oxidative stress regulation as 'copiotrophic competitors'. Clade II, which may not be capable of competing directly with Clade I for carbon and energy, may thus occupy the niche space of specialist Proteobacterial methylotrophs, methane and ammonia oxidisers so as to be 'copiotrophic metabolic specialists'. The strategy of Clade III would be to decompose plant material via diverse CAZy and possess a variety of environmental stress tolerance traits as 'oligotrophic degraders'. Clade IV, which include for example Actinobacteria that share traits for competition, degradation and oligotrophy, could thus occupy niche space between Clades I and III. Finally, the strategy of Clade V would be to fill highly specialised, unrelated metabolic niches reliant on completely distinct carbon sources to other taxa. The large space conceptualised for Clade V in Figure 5b, which does not imply either copiotrophic or oligotrophic resource investment, is an oversimplification and with improved understanding of traits in these taxa it may be possible to fracture and further separate them into more detailed groups. This would prove particularly beneficial for the poorly characterised Archaea.

Moving beyond a one-dimensional *r-K* spectrum to accommodate additional trait-driven life strategies has been proposed in plant ecology (Grime, 1977). Specifically, Grime argued that plant taxa fall within a multi-dimensional space defined by extremes on three axes: 'competitors' that acquire nutrients, light, water etc. more effectively than neighbouring taxa in the same environment, 'stress tolerators' that are long-lived, slow growing taxa that resist desiccation, alkaline soils etc., and 'ruderals' that have very brief lifecycles between periods of disturbance and invest in environmentally hardy seeds. Despite these varied strategies for resource investment, plants are unified in that photosynthesis is their primary form of acquiring carbon and energy. The diversity of microbial strategies for acquiring carbon and energy enables them to explore a greater range of potential niche space, and in addition to growth traits that allow for a relatively more copiotrophic or oligotrophic investment of those resources, likely contributes to the high diversity of co-existing taxa observed in soil microbial communities.

However, to truly unravel differentiated niches and general microbial life strategies, two limitations must be overcome. Firstly, a better understanding of the many

'Uncharacterised' traits in environmental isolates is required. For example, the recent large-scale MAG study by Nayfach *et al*., (2021) identified 5.8 million protein clusters (traits), of which over 75% could not be annotated meaningfully by current protein databases. Secondly, robust trait-based analyses down to the finer scale of distinct genomes will likely be necessary to consider how individual taxonomic members of a community have either differentiated in order to co-exist or are in the throes of competition that will ultimately exclude one of the competitors.

5. Conclusion

In a collection of 175 terrestrial prokaryotes that possess 220 664 traits shared between at least two taxa, concepts in niche differentiation were explored. Non-random trait distributions were shown as preferential clustering of related taxa within most Phyla with a general trend of highest similarity at the level of Family. This strongly supported ecological coherence of shared traits within close relatives. Random Forest models successfully identified BRITE 3 categories that best explained differing traits between taxonomic groups. These traits were involved in a wide range of biological functions, including core physiological traits used historically to categorise taxa. Many traits were also involved in functions often associated with copiotrophs and oligotrophs, namely metabolism, nutrient acquisition and environmental stress tolerance. Hierarchical clustering of differential traits formed five distinct clusters, with Clade I representing the classical copiotrophic niche, Clades III and V as oligotrophic, and Clades II and IV in between. A more refined perspective would be to consider each Clade as its own life strategy in a niche space that considers both resource investment and acquisition simultaneously; for example, the strategy of Clade I is to invest in competition and rapid growth, while Clade V pursue highly distinct, specialised metabolic functions. The trait-based analyses here were effective in identifying general trends in potential function of terrestrial microbial taxa at the Phylum and Family level. Further investigation will be necessary to identify traits that give rise to niche differentiation at lower taxonomic ranks and, ultimately, the importance of this for ecosystem processes of interest.

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Conflict of Interest

The authors declare no conflict of interest regarding this study or its outcomes.

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Figure Legends:

Figure 1. Box and whisker plots of the raw counts of proteins per genome beside the counts of shared functional traits per genome derived from MCL. Taxa *n* = 5 per Family.

Figure 2. a) Unweighted pair group method with arithmetic mean (UPGMA) dendrogram comparing similarity in composition of 220 664 traits across the 175 terrestrial microbial taxa. The Phylum of each taxon is highlighted. The scale bar units represent Bray-Curtis dissimilarity. b) Comparisons of ecological coherence (*C*) between taxa belonging to the same Super Group, Phylum and Family. *C*, which varies between 0 and 1, was measured from branch lengths in a). Values of *C* approaching 1 indicate an ecologically coherent group with a similar composition of traits. Super groups were as follows: Proteobacteria, all Gamma-, Alpha-, Beta- and Deltaproteobacteria; Terrabacteria, all Actinobacteria, Firmicutes, Chloroflexi and Cyanobacteria; FCB were Bacteroidetes; Acidobacteria, all Acidobacteria; PVC, Verrucomicrobia and Planctomycetes; TACK were Thaumarchaeota; Euryarchaeota were Euryarchaeota.

Figure 3. Random Forest identified traits that best explain separation of taxa groups at Phylum and Family taxonomic rank. Mean Decrease in Accuracy (MDA) is a measure of the average increase in classification error during permutation of trees if the trait is not included in the model. For example, the Family Random Forest was most accurate when ABC transporters were included in the model.

Figure 4. Hierarchical clustering of Families based on 60 traits selected from Random Forest and previously identified copiotroph-oligotroph traits. Families are clustered along the *y* axis, and specific co-occurring traits are clustered on the *x* axis. Five general Clades were identified based on Family clustering. Units are normalised variance in mean counts of traits per Family.

Figure 5: A conceptual diagram of overlapping life strategies between the five functional clades identified in this study. a) the classical copiotroph-oligotroph dichotomy whereby the five clades are placed on a singular axis of 'resource investment', where growth strategies are either relatively more targeted toward rapid growth (copiotroph) or toward stress tolerance (oligotroph). BRITE categories are given as examples of functional traits contributing to either axis pole. The space occupied by clades along this axis is dependent on enrichment or depletion of these traits. All clades exhibit a great deal of overlap and certain clades, such as IV (including, for example, Actinobacteria), are hard to identify as either copiotroph or oligotroph. b) a multidimensional concept where three axes for 'resource acquisition' are added, further separating taxa as either 'competitors', 'degraders' or 'metabolic specialists'. Again, BRITE categories are provided as examples of traits contributing to the additional axes and space occupied by clades is dependent on enrichment or depletion of these traits. These extra dimensions would suggest the niche space of clade IV exists between 'copiotrophic competitors' (I) and 'oligotrophic degraders' (III), potentially allowing IV to co-exist alongside both.

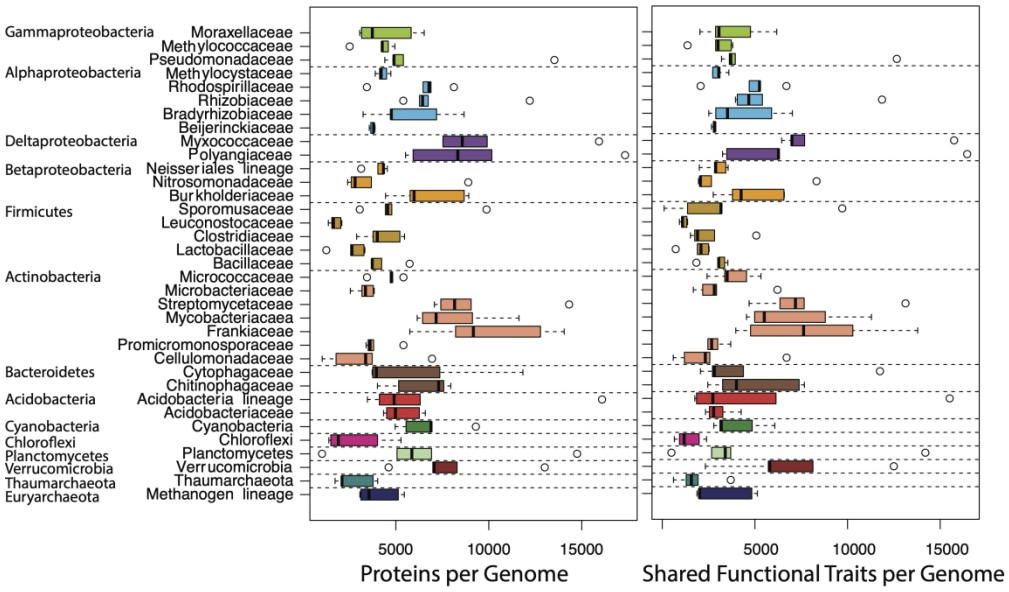

Figure 1

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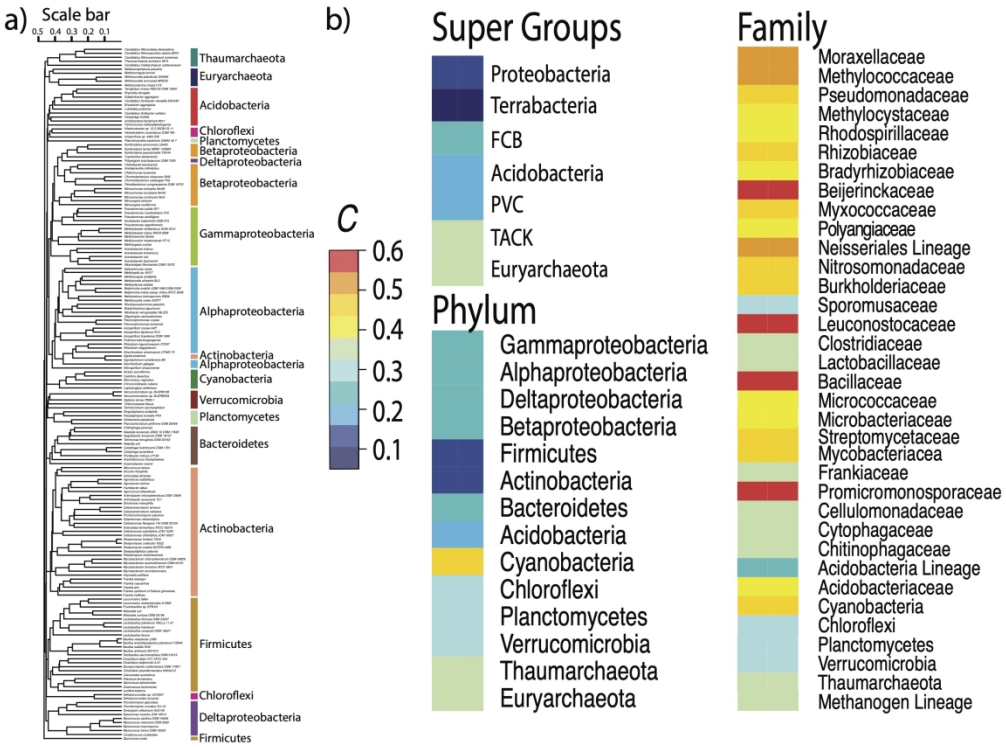

Figure 2

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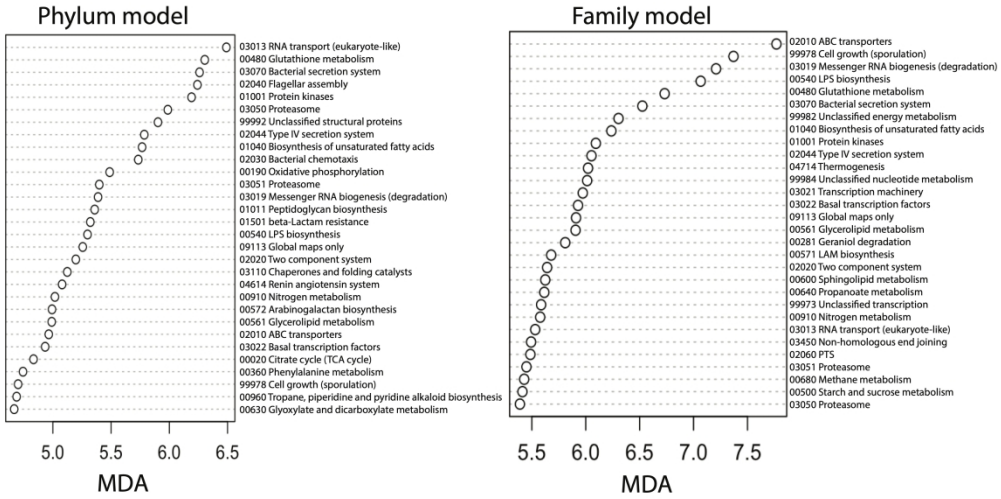

Figure 3

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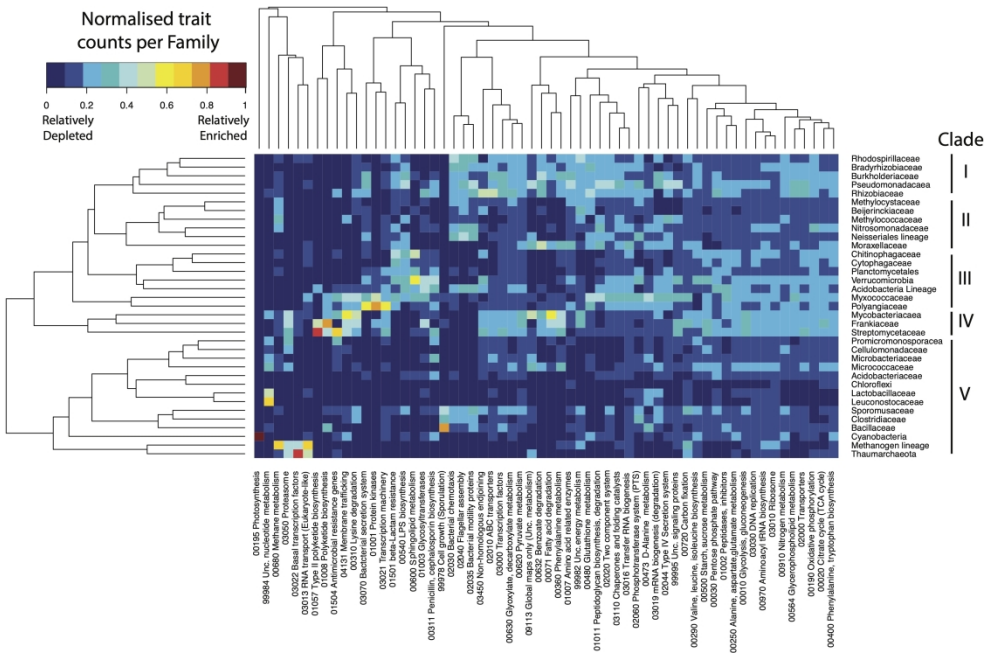

Figure 4

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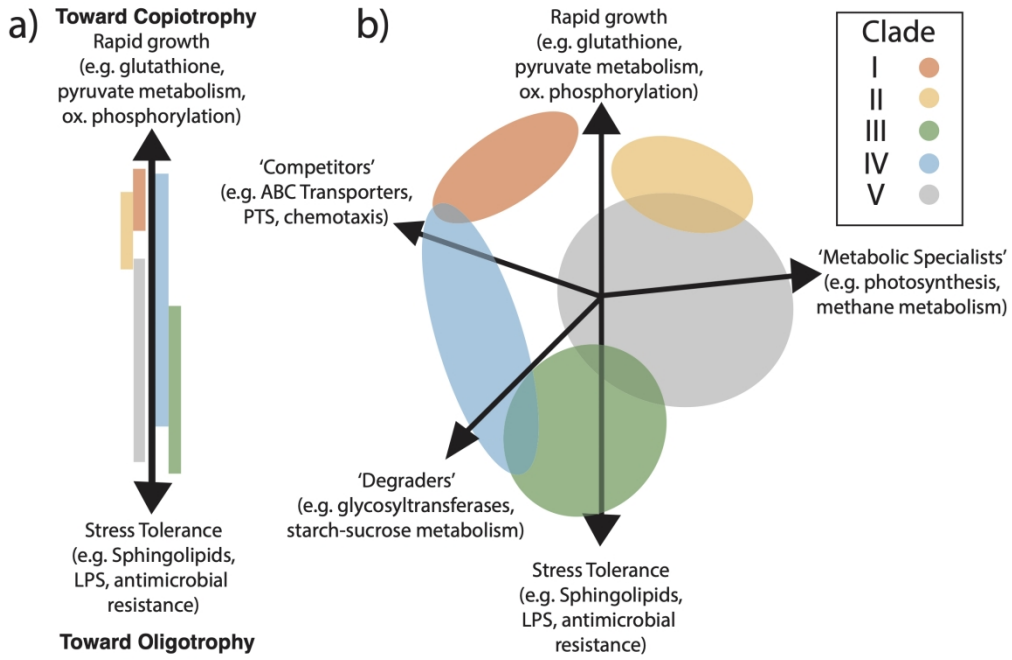


Figure 5

203x134mm (300 x 300 DPI)

Table 1: Summary of traits significantly enriched and depleted in the Family Random Forest model.

BRITE 1	BRITE 3	Significantly enriched	Significantly depleted
Metabolism	00540 LPS biosynthesis	Pseudomonadaceae, Burkholderiaceae, Chitinophagaceae, Acidobacteria Lineage, Verrucomicrobia	Leuconostocaceae, Clostridiaceae, Lactobacillaceae, Bacillaceae, all Actinobacteria, Chloroflexi, Thaumarchaeota, Methanogen Lineage
	01040 Biosynthesis of unsaturated fatty acids	Mycobacteriaceae, Frankiaceae, Pseudomonadaceae	Leuconostocaceae, Clostridiaceae, Lactobacillaceae, Bacillaceae, Cyanobacteria, Chloroflexi, Thaumarchaeota, Methanogen lineage
	00561 Glycerolipid metabolism	Myxococcaceae, Mycobacteriaceae, Frankiaceae	Thaumarchaeota, Methanogen Lineage
	00571 LAM biosynthesis	Streptomycetaceae, Mycobacteriaceae, Frankiaceae	All other Families
	00600 Sphingolipid metabolism	Myxococcaceae, Chitinophagaceae, Planctomycetaceae, Verrucomicrobia	All Gammaproteobacteria, Rhodospirillaceae, Bradyrhizobiaceae, Neisseriales Lineage, Nitrosomonadaceae, Sporomusaceae, Leuconostocaceae, Lactobacillaceae, Cellulomonadaceae, Chloroflexi, Thaumarchaeota, Methanogen Lineage
	00500 Starch and sucrose metabolism	Polyangiaceae, Streptomycetaceae, Mycobacteriaceae, Frankiaceae, Verrucomicrobia	Moraxcellaceae, Thaumarchaeota, Methanogen Lineage
	04714 Thermogenesis	Methylocystaceae, Rhodospirillaceae, Bradyrhizobiaceae, Beijerinckaceae, Mycobacteriaceae	All other Families
	00640 Propanoate metabolism	Burkholderiaceae, Mycobacteriaceae, Frankiaceae	Methylococcaceae, Leuconostocaceae, Lactobacillaceae, Thaumarchaeota
	00910 Nitrogen metabolism	Rhodospirillaceae, Bradyrhizobiaceae, Burkholderiaceae, Sporomusaceae, Mycobacteriaceae	Leuconostocaceae, Lactobacillaceae, Chloroflexi
	00680 Methane metabolism	Methylococcaceae, Methylocystaceae, Methanogen Lineage	Leuconostocaceae, Lactobacillacaceae
	99982 Unclassified energy metabolism	Rhodospirillaceae, Beijerinckiaceae, Mycobacteriaceae, Frankiaceae	Leuconostocaceae, Lactobacillaceae, Microbacteriaceae, Promicromonosporaceae, Cellulomonadaceae, Chloroflexi, Planctomycetales, Thaumarchaeota
	09113 Global maps only (unclassified metabolism)	Pseudomonadaceae, Mycobacteriaceae	Methylococcaceae, Beijerinckiaceae, Nitrosomonadaceae, Leuconostocaceae, Bacillaceae, Cytophagaceae, Acidobacteriaceae, Cyanobacteria, Verrucomicrobia
	00480 Glutathione metabolism	Rhizobiaceae, Bradyrhizobiaceae, Myxococcaceae, Polyangiaceae, Burkholderiaceae	All Firmicutes, Acidobacteriaceae, Chloroflexi, Planctomycetales, Verrucomicrobia, Thaumarchaeota, Methanogen Lineage
	00281 Geraniol degradation	Moraxellaceae, Myxococcaceae, Mycobacteriaceae	Methylococcaceae, Pseudomonadaceae, Rhizobiaceae, Beijerinckiaceae, Neisseriales Lineage, Nitrosomonadaceae, all Firmicutes, Streptomycetaceae, Promicromonosporaceae, Cellulomonadaceae, Acidobacteriaceae, Cyanobacteria, Chloroflexi, Planctomycetaceae, Verrucomicrobia, Thaumarchaeota, Methanogen Lineage
Environmental Information Processing	01001 Protein kinases	Myxococcaceae, Polyangiaceae, Frankiaceae	Lactobacillaceae
	02010 ABC transporters	Rhodospirillaceae, Rhizobiaceae, Burkholderiaceae	Acidobacteriaceae, Thaumarchaeota, Methanogen Lineage
	02020 Two component system	Pseudomonadaceae, Rhodospirillaceae, Bradyrhizobiaceae, Myxococcaceae	Leuconostocaceae, Lactobacillaceae, Bacillaceae, Promicromonosporaceae, Cellulomonadaceae, Chloroflexi, Thaumarchaeota
	02060 Phosphotransferase system (PTS)	Pseudomonadaceae, Rhodospirillaceae, Myxococcaceae, Neisseriales lineage, Clostridiaceae, Verrucomicrobia	Methylococcaceae, Frankiaceae, Acidobacteria Lineage, Thaumarchaeota, Methanogen Lineage

	03070 Bacterial secretion system	Polyangiaceae, Myxococcaceae, Burkholderiaceae	Leuconostocaceae, Lactobacillaceae, Bacillaceae, Streptomycetaceae, Mycobacteriaceae, Chloroflexi, Thaumarchaeota, Methanogen lineage
	02044 Type IV secretion system	Pseudomonadaceae, Myxococcaceae	Leuconostocaceae, all Bacteroidetes, Thaumarchaeota
Genetic Information Processing	03021 Transcription machinery	Myxococcaceae, Polyangiaceae, Streptomycetaceae, Mycobacteriaceae, Frankiaceae	Methylocystaceae, Rhizobiaceae, Bradyrhizobiaceae, Beijerinckiaceae, Leuconostocaceae, Lactobacillaceae, Thaumarchaeota, Methanogen lineage
	99973 Unclassified transcription factors	Pseudomonadaceae, Rhizobiaceae, Myxococcaceae, Streptomycetaceae	Chitinophagaceae, Cyanobacteria, Chloroflexi, Planctomycetaceae, Verrucomicrobia
	03022 Basal transcription factors	Thaumarchaeota, Methanogen Lineage	All other Families
	03013 RNA transport (eukaryote-like)	Myxococcaceae, Thaumarchaeota, Methanogen Lineage	All other Families
	03019 Messenger RNA biogenesis (degradation)	Myxococcaceae, Burkholderiaceae	Methylocystaceae, Rhizobiaceae, Bradyrhizobiaceae, Beijerinckiaceae, Cyanobacteria, Chloroflexi, Thaumarchaeota
	03450 Non-homologous end joining	Rhizobiaceae, Streptomycetaceae, Verrucomicrobia	Moraxellaceae, Methylococcaceae, Neisseriales Lineage, Leuconostocaceae, Clostridiaceae, Lactobacillaceae, Cyanobacteria
	03050 Proteasome	Micrococcaceae, Streptomycetaceae, Mycobacteriaceae, Frankiaceae, Promicromonosporaceae, Cellulomonadaceae, Thaumarchaeota, Methanogen Lineage	All other families
	03051 Prokaryote 20S Proteasome	Micrococcaceae, Streptomycetaceae, Mycobacteriaceae, Frankiaceae	All other Families
Signalling and Cellular Processes	99978 Cell growth (sporulation)	Sporomusaceae, Clostridiaceae, Bacillaceae	All other families
Not included in pathway or BRITE	99984 Unclassified nucleotide metabolism	Leuconostocaceae, Lactobacillaceae, Micrococcaceae	Moraxellaceae, Methylococcaceae, Methylocystaceae, Rhodospirillaceae, Bradyrhizobiaceae, Beijerinckiaceae, all Deltaproteobacteria, all Betaproteobacteria, Sporomusaceae, Clostridiaceae, Streptomycetaceae, Frankiaceae, all Bacteroidetes, all Acidobacteria, Chloroflexi, Planctomycetes, Thaumarchaeota, Methanogen Lineage

Supplementary Material

Supplementary Table 1: List of microorganisms utilised in this study.

ID	Phylum	Class	Order	Family	Genus	Species	Strain	Ecosystem Process[a]	Reference
Ga0074846_101	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Terribacillus*	*saccharophilus*	DSM21619	ND	(An et al 2007b)
Ga0067102_11	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*amyloliquefaciens*	Plantarum FZB42	Rhizosphere	(Chen et al 2007)
Ga0077930_11	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*anthracis*	BA1015	Pathogen	(Helgason et al 2000)
Ga0175433_11	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*subtilis*	KH2	ND	(Graham and Istock 1978)
CP015911	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*velezensis*	LS69	Rhizosphere	(Liu et al 2017)
LAC_LBA_DRAFT__contig00001	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*plantarum*		Rhizosphere	(Chen et al 2005)
Ga0106160_101	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*composti*	DSM18527	Rhizosphere	(Endo and Okada 2007)
Ga0106062_101	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*floricola*	DSM23037	Rhizosphere	(Kawasaki et al 2011)
1423745	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*florum*	F91	Rhizosphere	(Endo et al 2010)
1670446	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*herbarum*		ND	(Mao et al 2015)
Ga0126468_101	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	*mesenteroides*	213MO	Rhizosphere	(Chen et al 2005)
Ga0106620_101	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Weissella*	*confusa*	DSM20196	Rhizosphere	(Chen et al 2005)
Ga0114202_101	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Fructobacillus*	*sp*	EFBN1	Decomposition	(Endo et al 2009)
155866	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Weissella*	*soli*		Rhizosphere	(Magnusson et al 2002)
907931	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	*fallax*	KCTC3537	Rhizosphere	(Barrangou et al 2002)
BO10DRAFT_scf7180000000004_quiver.1	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*phytofermentans*	KNHs212	Decomposition	(Warnick et al 2002)
CLOBI_contig000001	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*beijerinckii*	A21	Rhizosphere	(Johnson et al 1997)

ct454_454454contig00001	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*tetani*	ATCC454	Pathogen	(Cohen et al 2017)
1121919	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Geosporobacter*	*subterraneus*	DSM17957	ND	(Klouche et al 2007)
857293	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Caloramator*	*australicus*	RC3	ND	(Ogg and Patel 2009)
GCA_900476375.1	Firmicutes	Negativicutes	Selenomonadales	Sporomusaceae	*Lucifera*	*butyrica*		ND	(Sanchez-Andrea et al 2018)
NZ_CP010978.1	Firmicutes	Negativicutes	Selenomonadales	Sporomusaceae	*Pelosinus*	*fermentans*	JBW45	Decomposition	(de Leon et al 2012)
16187	Firmicutes	Negativicutes	Selenomonadales	Sporomusaceae	*Sporomusa*	*ovata*	DSM2662	ND	(Möller et al 1984)
51809	Firmicutes	Negativicutes	Selenomonadales	Sporomusaceae	*Sporomusa*	*sphaeroides*	DSM2875	ND	(Möller et al 1984)
GCA_000430605.1	Firmicutes	Negativicutes	Selenomonadales	Sporomusaceae	*Anaeroarcus*	*burkinensis*	DSM6283	Rhizosphere	(Strömpl et al 1999)
Ga0074713_11	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Arthrobacter*	*chlorophenolicus*	DSM12829	Bioremediation	(Westerberg et al 2000)
Ga0067230_11	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Arthrobacter*	*aurescens*	TC1	Bioremediation	(Mongodin et al 2006)
1531955	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Sinomonas*	*mesophila*	MPKL26	ND	(Prabhu et al 2015)
378753	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Kocuria*	*rhizophila*	DC2201	Rhizosphere	(Takarada et al 2008)
574650	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Micrococcus*	*terreus*		ND	(Zhang et al 2010)
Ga0080924_101	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriacaea	*Mycobacterium*	*chlorophenolicum*	DSM43826	Bioremediation	(Das et al 2015)
Ga0124153_1001	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriacaea	*Mycobacterium*	*fortuitum*	ATCC6841	Bioremediation	(Nohynek et al 1993)
Ga0056865_101	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriacaea	*Mycobacterium*	*austroafricanum*	DSM44191	Bioremediation	(Leys et al 2005)
443218	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriacaea	*Hoyosella*	*subflava*	DQS39A1	Bioremediation	(Hamada et al 2016)
1440774	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriacaea	*Mycobacterium*	*aromaticivorans*	JCM16368	Bioremediation	(Hennessee et al 2009)
NC_014151	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Cellulomonas*	*flavigena*	DSM20109	Decomposition	(Abt et al 2010)
Ga0128466_10001	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Cellulomonas*	*chitinilytica*	JCM16927	Decomposition	(Yoon et al 2008)
Ga0128457_10001	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Cellulomonas*	*xylanilytica*	JCM14281	Decomposition	(Rivas et al 2004[a])
862422	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Actinotalea*	*fermentans*	DSM3133	Decomposition	(Bagnara et al 1985)

948458	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	*Actinotalea*	*ferrariae*	CF54	ND	(Li et al 2013)
Ga0057460_1001	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*	*scabiei*	NCPPB4086	Pathogen	(Dees et al 2013)
Ga0057466_gi672367150.1	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*	*lividans*	TK24	Decomposition	(Ruckert et al 2015)
Ga0076575_11	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptomyces*	*coelicolor*	A3(2)	Decomposition	(Bentley et al 2002)
105422	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Streptacidiphilus*	*carbonis*	DSM41754	Decomposition	(Kim et al 2003)
1348663	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	*Kitasatospora*	*cheerisanensis*	KCTC2395	ND	(Hwang et al 2015)
326424	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Frankia*	*alni*	ACN14a	Nitrogen fixation	(Normand and Lalonde 1982)
106370	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Frankia*	*casuarinae*	Ccl3	Nitrogen fixation	(Normand et al 2007)
298654	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Frankia*	*inefficax*	EuI1c	ND	(Nouioui et al 2017)
656024	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Frankia*	*sp*	Datisca symbiont	Nitrogen fixation	(Persson et al 2015)
222534	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Frankia*	*elaeagni*	BMG512	Nitrogen fixation	(Nouioui et al 2016)
15565	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	*Cellulosimicrobium*	*cellulans*	J36	Decomposition	(Schumann et al 2001)
75317	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	*Cellulosimicrobium*	*terreum*		Decomposition	(Yoon et al 2007)
NC_013530.1	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	*Xylanimonas*	*cellulosilytica*	DSM15894	Decomposition	(Rivas et al 2003)
16475	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	*Promicromonospora*	*sukumoe*	327MFSha3.1	Decomposition	(Takahashi et al 1987)
76147	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	*Xylanibacterium*	*ulmi*		Decomposition	(Rivas et al 2004[b])
50964	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Agromyces*	*cerinus*		ND	(Zgurskaya et al 1992)
16552	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Agromyces*	*subbeticus*	DSM16689	ND	(Jurado et al 2005)
54323	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Agreia*	*pratensis*		Rhizosphere	(Schumann et al 2003)
16541	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Agrococcus*	*lahaulensis*	DSM17612	ND	(Mayilraj et al 2006)
16623	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Humibacter*	*albus*	DSM18994	Decomposition	(Vaz-Moreira et al 2008)
Ga0065637_11	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	Candidatus *Koribacter*	*versatilis*	Ellin345	Decomposition	(Ward et al 2009)

Terro_Contig51.1	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Terriglobus*	*roseus*	DSM18391	Decomposition	(Eichorst et al 2007)
Ga0133452_11	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Luteitalea*	*pratensis*	DSM100886	Decomposition	(Vieira et al 2017)
863522	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Bryocella*	*elongata*	DSM22489	Decomposition	(Dedysh et al 2012)
1121860	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Edaphobacter*	*aggregans*	DSM19364	Decomposition	(Koch et al 2008)
234267	Acidobacteria	Acidobacteria	Solibacteriales	Divergent Acidobacteria lineage	Candidatus *Solibacter*	*usitatus*	Ellin6076	Decomposition	(Ward et al 2009)
1340493	Acidobacteria	Acidobacteria	Solibacteriales	Divergent Acidobacteria lineage	*Bryobacter*	*aggregatus*	MPL3	Decomposition	(Kulichevskaya et al 2010)
454194	Acidobacteria	Acidobacteria	Blastocatellales	Divergent Acidobacteria lineage	*Pyrinomonas*	*methylaliphatogenes*	K22	Decomposition	(Crowe et al 2014)
903818	Acidobacteria	Acidobacteria	Holophagales	Divergent Acidobacteria lineage	*Holophaga*	*foetida*	DSM6591	ND	(Liesack et al 1994)
1660251	Acidobacteria	Acidobacteria	Unclassified Acidobacterium	Divergent Acidobacteria lineage	*Acidobacteria*	*bacterium*	Mor1	Rhizosphere	(Tanaka et al 2017)
Ga0077356_1001	Proteobacteria	Beta	Burkholderiales	Burkholderiaceae	*Burkholderia*	*pseudomallei*	TSV44	Pathogen	(Brook et al 1997)
Ga0065534_11	Proteobacteria	Beta	Burkholderiales	Burkholderiaceae	*Burkholderia*	*xenovorans*	LB400	Rhizosphere	(Chain et al 2006)
Ga0056711_1001	Proteobacteria	Beta	Burkholderiales	Burkholderiaceae	*Burkholderia*	*terrae*	NBRC100964	Rhizosphere	(Yang et al 2006)
1121278	Proteobacteria	Beta	Burkholderiales	Burkholderiaceae	*Chitinimonas*	*koreensis*	DSM17726	ND	(Kim et al 2006)
1040978	Proteobacteria	Beta	Burkholderiales	Burkholderiaceae	*Cupriavidus*	*taiwanensis*	STM6018	Nitrogen fixation	(Chen et al 2001)
Ga0105865_101	Proteobacteria	Beta	Nitrosomonadales	Nitrosomonadaceae	*Nitrosomonas*	*europaea*	Nm50	Nitrification	(Koops et al 1991)
Ga0105862_1001	Proteobacteria	Beta	Nitrosomonadales	Nitrosomonadaceae	*Nitrosomonas*	*communis*	Nm2	Nitrification	(Koops et al 1991)
Ga0105869_101	Proteobacteria	Beta	Nitrosomonadales	Nitrosomonadaceae	*Nitrosomonas*	*eutropha*	Nm56	Nitrification	(Koops et al 1991)
1266925	Proteobacteria	Beta	Nitrosomonadales	Nitrosomonadaceae	*Nitrosospira*	*briensis*	C128	Nitrification	(Rice et al 2016)
323848	Proteobacteria	Beta	Nitrosomonadales	Nitrosomonadaceae	*Nitrosospira*	*multiformis*	ATCC25196	Nitrification	(Norton et al 2008)

G520DRAFT_scaffold00001.1	Proteobacteria	Beta	Neisseriales	Neisseriales_lineage	*Paludibacterium*	*yongneupense*	DSM19731	ND	(Kwon et al 2008)
Ga0077161_101	Proteobacteria	Beta	Neisseriales	Neisseriales_lineage	*Chromobacterium*	*subtsugae*	F49	ND	(Soby et al 2013)
Ga0080895_101	Proteobacteria	Beta	Neisseriales	Neisseriales_lineage	*Chromobacterium*	*violaceum*	GN5	Pathogen	(Koburger and May 1982)
1121274	Proteobacteria	Beta	Neisseriales	Neisseriales_lineage	*Chitinibacter*	*tainanensis*	DSM15459	Decomposition	(Chern et al 2004)
1120999	Proteobacteria	Beta	Neisseriales	Neisseriales_lineage	*Andreprevotia*	*chitinilytica*	DSM18519	Decomposition	(Weon et al 2007)
Ga0077767_1001	Proteobacteria	Gamma	Pseudomonadales	Pseudomonadacaea	*Pseudomonas*	*putida*	SF1	Rhizosphere	(Tikariha et al 2016)
Ga0061119_scaffold00001.1	Proteobacteria	Gamma	Pseudomonadales	Pseudomonadacaea	*Azotobacter*	*beijerinckii*	DSM373	Nitrogen fixation	(Lipman 1904)
Ga0129289_101	Proteobacteria	Gamma	Pseudomonadales	Pseudomonadacaea	*Pseudomonas*	*oryzihabitans*	H72	Rhizosphere	(Lymperopoulou et al 2017)
289370	Proteobacteria	Gamma	Pseudomonadales	Pseudomonadacaea	*Pseudomonas*	*argentinensis*	CECT7010	Rhizosphere	(Peix et al 2005)
1292027	Proteobacteria	Gamma	Pseudomonadales	Pseudomonadacaea	*Pseudomonas*	*azotifigens*	DSM17556	Nitrogen fixation	(Hatayama et al 2005)
Ga0044516_101	Proteobacteria	Gamma	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*soli*		ND	(Kim et al 2008)
Ga0080829_1001	Proteobacteria	Gamma	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*baumannii*		Pathogen	(Davenport et al 2014)
Q337DRAFT_scaffold00010.10	Proteobacteria	Gamma	Pseudomonadales	Moraxellaceae	*Alkanindiges*	*illinoisensis*	DSM15370	Bioremediation	(Bogan et al 2003)
1217715	Proteobacteria	Gamma	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*bohemicus*	ANC3994	ND	(Krizova et al 2014)
1217660	Proteobacteria	Gamma	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*indicus*	ANC4215	Bioremediation	(Malhotra et al 2012)
GY38DRAFT_scf7180000000021_quiver.1	Proteobacteria	Gamma	Methylococcales	Methylococcaceae	*Methylobacter*	*whittenburyi*	ACM3310	Methane oxidation	(Whittenbury et al 1970)
MetmiDRAFT_scaffold1.1	Proteobacteria	Gamma	Methylococcales	Methylococcaceae	*Methylovulum*	*miyakonense*	HT12	Methane oxidation	(Iguchi et al 2011)
MetluDRAFT_scaffold1.1	Proteobacteria	Gamma	Methylococcales	Methylococcaceae	*Methylobacter*	*luteus*	IMVB3098	Methane oxidation	(Whittenbury et al 1970)
1132443	Proteobacteria	Gamma	Methylococcales	Methylococcaceae	*Methylosarcina*	*fibrata*	AMLC10	Methane oxidation	(Wise et al 2001)

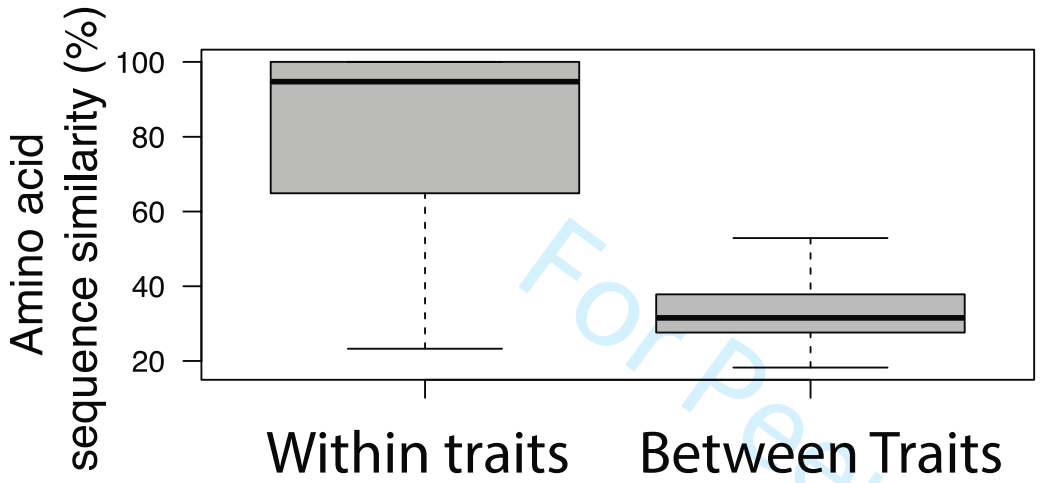
493385	Proteobacteria	Gamma	Methylococcales	Methylococcaceae	*Methylogaea*	*oryzae*	JCM16910	Methane oxidation	(Geymonat et al 2011)
Ga0061138_scaffold00001.1	Proteobacteria	Alpha	Rhodospirillales	Rhodospirillaceae	*Azospirillum*	*brasiliense*	DSM1690	Nitrogen fixation	(Kuklinsky-Sobral et al 2004)
Ga0048939_110	Proteobacteria	Alpha	Rhodospirillales	Rhodospirillaceae	*Azospirillum*	*oryzae*	A2P	Nitrogen fixation	(Xie and Yokota 2005a)
Ga0048941_11	Proteobacteria	Alpha	Rhodospirillales	Rhodospirillaceae	*Azospirillum*	*lipoferum*	R1C	Nitrogen fixation	(Van et al 1997)
570967	Proteobacteria	Alpha	Rhodospirillales	Rhodospirillaceae	*Fodinicurvata*	*fenggangensis*	DSM21160	ND	(Wang et al 2009)
1003237	Proteobacteria	Alpha	Rhodospirillales	Rhodospirillaceae	*Nitrospirillum*	*amazonense*	Y2	Nitrogen fixation	(Magalhaes et al 1983)
DL88DRAFT_scaffold00001.1	Proteobacteria	Alpha	Rhizobiales	Beijerinckiaceae	*Beijerinckia*	*mobilis*	DSM2326	Nitrogen fixation	(Derx 1950)
182677003	Proteobacteria	Alpha	Rhizobiales	Beijerinckiaceae	*Beijerinckia*	*indica*	ATCC9039	Nitrogen fixation	(Derx 1950)
217976201	Proteobacteria	Alpha	Rhizobiales	Beijerinckiaceae	*Methylocella*	*silvestris*	BL2	Methane oxidation	(Dunfield et al 2003)
395964	Proteobacteria	Alpha	Rhizobiales	Beijerinckiaceae	*Methylocapsa*	*acidiphila*	B2	Methane oxidation	(Dedysh et al 2002)
876269	Proteobacteria	Alpha	Rhizobiales	Beijerinckiaceae	*Methyloferula*	*stellata*	AR4	Methane oxidation	(Vorobev et al 2011)
A3OODRAFT_scaffold1.1	Proteobacteria	Alpha	Rhizobiales	Methylocystaceae	*Methylocystis*	*rosea*	SV97T	Methane oxidation	(Wartiainen et al 2006)
MettrDRAFT_Contig106	Proteobacteria	Alpha	Rhizobiales	Methylocystaceae	*Methylosinus*	*trichosporium*	OB3b	Methane oxidation	(Whittenbury et al 1970)
A3OUDRAFT_chromosome1.1	Proteobacteria	Alpha	Rhizobiales	Methylocystaceae	*Methylopila*	*sp*	M107	Methane oxidation	(Chistoserdova 2011)
1122963	Proteobacteria	Alpha	Rhizobiales	Methylocystaceae	*Pleomorphomonas*	*oryzae*	DSM16300	Nitrogen fixation	(Xie and Yokota 2005b)
1122962	Proteobacteria	Alpha	Rhizobiales	Methylocystaceae	*Pleomorphomonas*	*koreensis*	DSM23070	Nitrogen fixation	(Im et al 2006)
Ga0065594_11	Proteobacteria	Alpha	Rhizobiales	Bradyrhizobiaceae	*Nitrobacter*	*winogradskyi*	Nb255	Nitrification	(Starkenburg et al 2006)
Ga0098245_11	Proteobacteria	Alpha	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	*japonicum*	E109	Nitrogen fixation	(Brunel et al 1988)
Ga0077765_1001	Proteobacteria	Alpha	Rhizobiales	Bradyrhizobiaceae	*Rhodopseudomonas*	*palustris*	42OL	Bioremediation	(Larimer et al 2004)
1031710	Proteobacteria	Alpha	Rhizobiales	Bradyrhizobiaceae	*Oligotropha*	*carboxidovorans*	OM4	Decomposition	(Volland et al 2011)

1123229	Proteobacteria	Alpha	Rhizobiales	Bradyrhizobiaceae	*Salinarimonas*	*rosea*	DSM21201	ND	(Liu et al 2010)
Ga0127029_101	Proteobacteria	Alpha	Rhizobiales	Rhizobiaceae	*Agrobacterium*	*tumefaciens*	B6	Rhizosphere	(Levanon 1978)
EW91DRAFT_scaffold00001.1	Proteobacteria	Alpha	Rhizobiales	Rhizobiaceae	*Rhizobium*	*leguminosarum*	CF307	Nitrogen fixation	(Naeem et al 2004)
Ga0174993_11	Proteobacteria	Alpha	Rhizobiales	Rhizobiaceae	*Sinorhizobium*	*americanum*	CFNEI73	Rhizosphere	(Toledo et al 2003)
1028800	Proteobacteria	Alpha	Rhizobiales	Rhizobiaceae	*Neorhizobium*	*galegae*	HAMBI540	Nitrogen fixation	(Osterman et al 2014)
1764550	Proteobacteria	Alpha	Rhizobiales	Rhizobiaceae	*Rhizobium*	*aegyptiacum*	1010	Nitrogen fixation	(Shamseldin et al 2016)
Ga0128794_10470	Proteobacteria	Delta	Myxococcales	Polyangiaceae	*Sorangium*	*cellulosum*	So0149	Decomposition	(Li et al 2014)
Ga0098230_11	Proteobacteria	Delta	Myxococcales	Polyangiaceae	*Chondromyces*	*crocatus*	Cmc5	ND	(Zaburannyi et al 2016)
Ga0081738_11	Proteobacteria	Delta	Myxococcales	Polyangiaceae	*Polyangium*	*brachysporum*	DSM7029	ND	(Tang et al 2015)
1192034	Proteobacteria	Delta	Myxococcales	Polyangiaceae	*Chondromyces*	*apiculatus*	DSM436	ND	(Kleinig and Reichenbach 1973)
Ga0128396_10001	Proteobacteria	Delta	Myxococcales	Polyangiaceae	*Byssovorax*	*cruenta*		ND	(Reichenbach et al 2006)
Ga0131205_101	Proteobacteria	Delta	Myxococcales	Myxococcaceae	*Myxococcus*	*xanthus*	DSM16526	ND	(Voelz and Dworkin 1962)
Ga0070493_101	Proteobacteria	Delta	Myxococcales	Myxococcaceae	*Myxococcus*	*virescens*	DSM2260	ND	(Lang et al 2008)
Ga0131203_101	Proteobacteria	Delta	Myxococcales	Myxococcaceae	*Myxococcus*	*fulvus*	DSM16525	ND	(Jahn 1911)
1144275	Proteobacteria	Delta	Myxococcales	Myxococcaceae	*Corallococcus*	*coralloides*	DSM2259	ND	(Huntley et al 2012)
1189310	Proteobacteria	Delta	Myxococcales	Myxococcaceae	*Myxococcus*	*macrosporus*	DSM14697	ND	(Lang and Stackebrandt 2009)
NC_013132	Bacteroidetes	Bacteroidetes	Chitinophagales	Chitinophagaceae	*Chitinophaga*	*pinensis*	DSM2588	Decomposition	(Sangkhobol and Skerman 1981)
B154DRAFT_scaffold_0.1	Bacteroidetes	Bacteroidetes	Chitinophagales	Chitinophagaceae	*Segetibacter*	*koreensis*	DSM18137	ND	(An et al 2007a)
H608DRAFT_scaffold00001.1	Bacteroidetes	Bacteroidetes	Chitinophagales	Chitinophagaceae	*Terrimonas*	*ferruginea*	DSM30193	ND	(Xie and Yokota 2006)
551991	Bacteroidetes	Bacteroidetes	Chitinophagales	Chitinophagaceae	*Arachidicoccus*	*rhizosphaerae*	Vu144	Rhizosphere	(Madhaiyan et al 2015)
929713	Bacteroidetes	Bacteroidetes	Chitinophagales	Chitinophagaceae	*Niabella*	*soli*	DSM19437	ND	(Weon et al 2008)

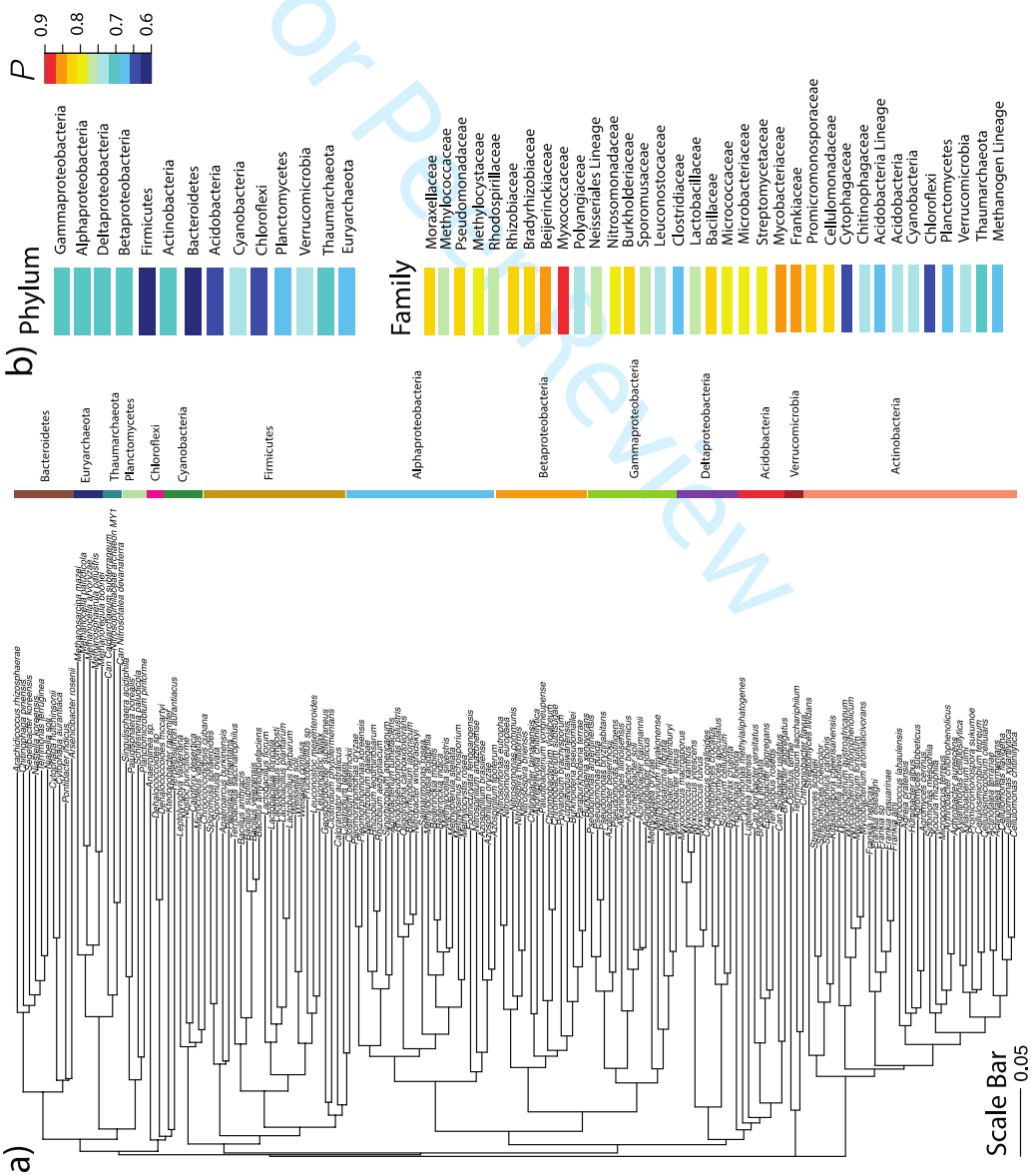
2506615776	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Niastella*	*koreensis*	GR2010	ND	(Weon et al 2006)
Ga0066359_101	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Cytophaga*	*hutchinsonii*	DSM1761	Decomposition	(Xie et al 2007)
Ga0116983_11	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Pontibacter*	*indicus*	LP100	Bioremediation	(Singh et al 2014)
1750698	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Arsenicibacter*	*rosenii*	SM1	Bioremediation	(Huang et al 2017)
1121373	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Cytophaga*	*aurantiaca*	DSM3654	Decomposition	(Winogradsky 1929)
Ga0056857_1001	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobia lineage	*Verrucomicrobium*	*sp*	BvORR106	Rhizosphere	Unpublished
Ga0056855_1001	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobia lineage	*Verrucomicrobium*	*sp*	BvORR034	Rhizosphere	Unpublished
182411827	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobia lineage	*Opitutus*	*terrae*	PB90	ND	(Chin et al 2001)
690879	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobia lineage	*Terrimicrobium*	*sacchariphilum*	NM5	ND	(Qiu et al 2014)
497964	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobia lineage	*Chthoniobacter*	*flavus*	Ellin428	ND	(Sangwan et al 2004)
Ga0154327_1001	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetales lineage	*Planctomyces*	*sp*	GWA2407	ND	Unpublished
Ga0111584_101	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetales lineage	*Planctomicrobium*	*piriforme*	DSM26348	ND	(Kulichevskaya et al 2015)
Ga0198709_11	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetales lineage	*Paludisphaera*	*borealis*	PX4	ND	(Kulichevskaya et al 2016)
1123242	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetales lineage	*Schlesneria*	*paludicola*	DSM18645	ND	(Kulichevskaya et al 2007)
886293	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetales lineage	*Singulisphaera*	*acidiphila*	DSM18658	ND	(Kulichevskaya et al 2008)
GCA_000214075.2	Cyanobacteria	Cyanobacteria	Oscillatoriales	Microcoleaceae	*Microcoleus*	*vaginatus*	FGP-2	Phototroph	(Starkenburg et al 2011)
	Cyanobacteria	Cyanobacteria	Nostocales	Nostocaceae	*Nostoc*	*punctiforme*	PCC73102	Phototroph	(Rippka and Herdman, 1992)
67533	Cyanobacteria	Cyanobacteria	Chroococcidiopsidales	Chroococcidiopsidaceae	*Chroococcidiopsis*	*cubana*		Phototroph	(Komarek and Hindak, 1975)
74975	Cyanobacteria	Cyanobacteria	Nostocales	Calotrichaceae	*Calothrix*	*desertica*		Phototroph	(Schwabe, 1960)
GCA_001637395.1	Cyanobacteria	Cyanobacteria	Synechoccales	Leptolyngbyaceae	*Leptolyngbya*	*valderiana*	BDU20041	Phototroph	(Anagnostidis and Komarek, 1988)
NC-002936.3	Chloroflexi	Dehalococcoidia	Dehalococcoidales	Dehalococcoidaceae	*Dehalococcoides*	*mccartyi*	CG5	Bioremediation	(He et al 2003)
50999	Chloroflexi	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	*Ktedonobacter*	*Sp.*		ND	Unpublished

46725	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	*Anaerolinea*	*Sp.*		Decomposition	Unpublished
13657	Chloroflexi	Dehalococcoidia	Dehalococcoidales	Dehalococcoidaceae	*Dehalococcoides*	*Sp.*		Bioremediation	Unpublished
GCA_000018565.1	Chloroflexi	Chloroflexia	Herpetosiphonales	Herpetosiphonaceae	*Herpetosiphon*	*aurantiacus*		ND	(Kiss et al 2011)
LRC50LV__640427159standard	Euryarchaeota	Methanomicrobia	Methanocellales	Methanogen lineage	*Methanocella*	*arvoryzae*	MRE50	Methanogenesis	(Sakai et al 2010)
MCPLV__646311984standard	Euryarchaeota	Methanomicrobia	Methanocellales	Methanogen lineage	*Methanocella*	*paludicola*	SANAE	Methanogenesis	(Sakai et al 2008)
Ga0072472_11	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanogen lineage	*Methanosarcina*	*mazei*	C16	Methanogenesis	(Maestrojuán et al 1992)
456442	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanogen lineage	*Methanoregula*	*boonei*	6A8	Methanogenesis	(Brauer et al 2011)
521011	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanogen lineage	*Methanosphaerula*	*palustris*	E19c	Methanogenesis	(Cadillo-Quiroz et al 2009)
Ga0073019_101	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Thaumarchaeota lineage	*Nitrocosmicus*	*oleophilus*	MY3	Nitrification	(Jung et al 2016)
CCsub_BA000048_1bd31_gi386807657.1	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Thaumarchaeota lineage	Candidatus *Caldiarchaeum*	*subterraneum*		Nitrification	(Takami et al 2015)
	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Thaumarchaeota lineage	Candidatus *Nitrosopumilus*	*salaria*	BD31	Nitrification	(Mosier et al 2012)
1078905	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Thaumarchaeota lineage	Candidatus *Nitrosotalea*	*devanterra*		Nitrification	(Lehtovirta-Morley et al 2014)
1001994	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Thaumarchaeota lineage	Candidatus *Nitrosoarchaeum*	*koreensis*	MY1	Nitrification	(Kim et al 2011)

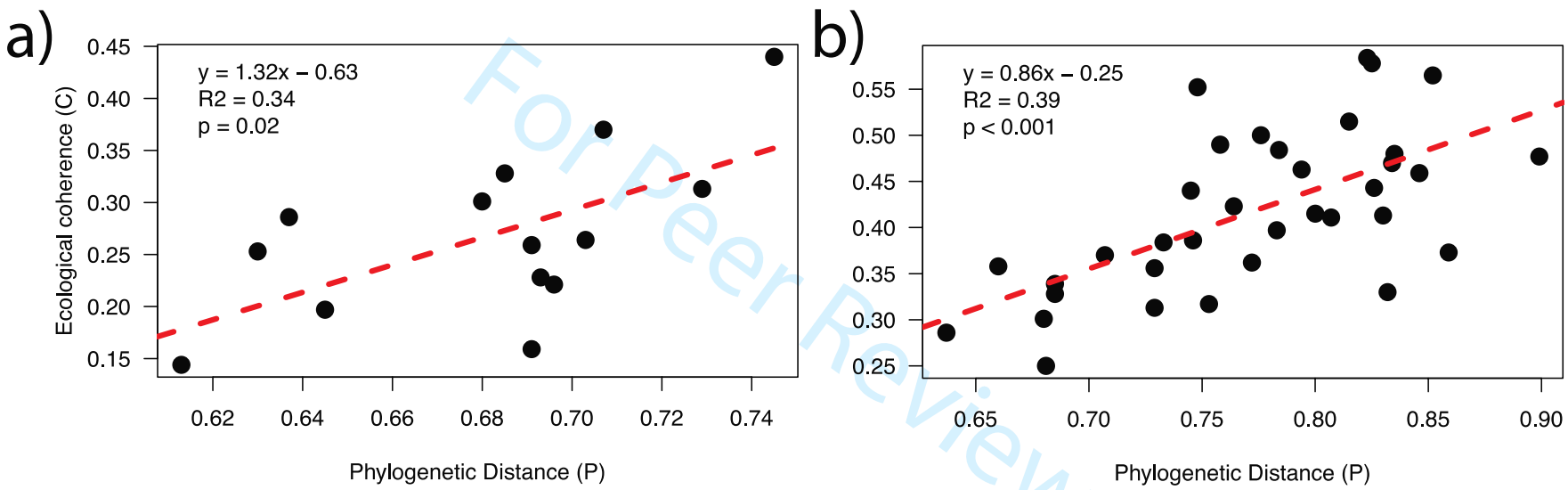
[a]ND: Not defined.



Supplementary Figure 1: Box and whisker plot comparing amino acid sequence similarity (%) within traits versus between traits, as determined by MCL. Amino acid sequences grouped together as traits were significantly more similar than those in different traits (Student's T test, $p = 2 \times 10^{-16}$).



Supplementary Figure 2: a) Neighbour joining tree of full length 16S rRNA genes of taxa analysed in this study. b) Phylogenetic distance (*P*) of each taxonomic group, ranging between 0.6 (dissimilar) and 0.9 (similar) determined from branch lengths of 16S rRNA gene analysis in a). *P* was calculated as ecological coherence (Eq 1.) excepting that branch lengths are in units of gene sequence similarity and not Bray-Curtis distance (*C*).



Supplementary Figure 3: a) Linear correlation between Phylogenetic Distance (*P*) derived from 16S rRNA genes and ecological coherence (*C*) of functional traits present in Phyla analysed in this study. b) Linear correlation at the level of Family.

Supplementary Table 2: Confusion matrix of Phylum level predictions based on Random Forest selection of important traits.

	Successful classifications														
	Acid.	Actino.	Alpha.	Bacter.	Beta.	Chloro.	Cyano.	Delta.	Eury.	Firm.	Gamma.	Planc.	Thaum.	Verruc.	Success rate (%)
Acidobacteria	6			1				1		2					60
Actinobacteria		33	1							1					95
Alphaproteobacteria			25												100
Bacteroidetes				9						1					90
Betaproteobacteria			2		11						2				74
Chloroflexi		1				0				3			1		0
Cyanobacteria		1					3			1					60
Deltaproteobacteria	1				1			8							80
Euryarchaeota									5						100
Firmicutes						2				23					92
Gammaproteobacteria			1		1					1	12				80
Planctomycetes	2							1		1		1			20
Thaumarchaeota										1			4		80
Verrucomicrobia	1			2										2	40

Supplementary Table 3: Confusion matrix of Family level predictions.

Phylum	Family	Mor.	Methyloc.	Pseu.	Methylocy.	Rhod.	Rhiz.	Brad.	Beij.	Myxo.	Poly.	Neis.	Nitr.	Burk.	Spor.	Leuc.	Clos.	Lact.	Bac.	Micrococ.	Microbac.	Success Rate (%)
Gammaproteobacteria	Moraxellaceae	5																				100
	Methylococcaceae		4																			80
	Pseudomonadaceae			4									1									80
Alphaproteobacteria	Methylocystaceae				3				2													60
	Rhodospirillaceae				1	4																80
	Rhizobiaceae						4	1														80
	Bradyrhizobiaceae				2		1	2														40
	Beijerinckiaceae				1				4													80
Deltaproteobacteria	Myxococcaceae									5												100
	Polyangiaceae									1	3			1								60
Betaproteobacteria	Neisseriales Lineage											5										100
	Nitrosomonadaceae			1									4									80
	Burkholderiaceae			1								1		3								60
Firmicutes	Sporomusaceae														2							40
	Leuconostocaceae															5						100
	Clostridiaceae																4		1			80
	Lactobacillaceae															2		3				60
	Bacillaceae																1		4			80
Actinobacteria	Micrococcaceae																			3		60
	Microbacteriaceae						1														4	80
	Streptomycetaceae																			1		60
	Mycobacteriaceae																					100
	Frankiaceae																					60
	Promicromonosporaceae																					100

	Cellulomonadaceae														1		20
Bacteroidetes	Cytophagaceae																40
	Chitinophagaceae																40
Acidobacteria	Divergent Acidobacteria							1		1		1					0
	Acidobacteriaceae																100
Cyanobacteria	Cyanobacteria																100
Chloroflexi	Chloroflexi										1						80
Planctomycetes	Planctomycetales Lineage										1						40
Verrucomicrobia	Verrucomicrobia Lineage																80
Thaumarchaeota	Thaumarchaeota Lineage																80
Euryarchaeota	Methanogen Lineage																100

Supplementary Table 4: Confusion matrix of Family level predictions.

Phylum	Family	Strep.	Myco.	Fran.	Prom.	Cell.	Cyto.	Chitin.	Acido.Lin.	Acido.	Cyano.	Chloro.	Planc.	Verru.	Thaum.	Methan.	Success Rate (%)
Gammaproteobacteria	Moraxellaceae																100
	Methylococcaceae											1					80
	Pseudomonadaceae																80
Alphaproteobacteria	Methylocystaceae																60
	Rhodospirillaceae																80
	Rhizobiaceae																80
	Bradyrhizobiaceae																40
	Beijerinckiaceae																80
Deltaproteobacteria	Myxococcaceae																100
	Polyangiaceae																60
Betaproteobacteria	Neisseriales Lineage																100

	Nitrosomonadaceae															80
	Burkholderiaceae															60
Firmicutes	Sporomusaceae								1			1	1			40
	Leuconostocaceae															100
	Clostridiaceae															80
	Lactobacillaceae															60
	Bacillaceae															80
Actinobacteria	Micrococcaceae		1		1											60
	Microbacteriaceae															80
	Streptomycetaceae	3		1												60
	Mycobacteriaceae		5													100
	Frankiaceae	1	1	3												60
	Promicromonosporaceae				5											100
	Cellulomonadaceae				2	1							1			20
Bacteroidetes	Cytophagaceae						2	3								40
	Chitinophagaceae						3	2								40
Acidobacteria	Divergent Acidobacteria								0	1		1				0
	Acidobacteriaceae									5						100
Cyanobacteria	Cyanobacteria										5					100
Chloroflexi	Chloroflexi											4				80
Planctomycetes	Planctomycetales Lineage								1	1			2			40
Verrucomicrobia	Verrucomicrobia Lineage									1				4		80
Thaumarchaeota	Thaumarchaeota Lineage					1									4	80
Euryarchaeota	Methanogen Lineage														5	100

Supplementary Table 5: Confusion matrix of Proteobacterial Family predictions.

	Successful classifications													
	Beij.	Brady.	Burkh.	Methylococ.	Methylocys.	Morax.	Myxo.	Neiss.	Nitroso.	Polyan.	Pseudo.	Rhizo.	Rhodo.	Success rate (%)
Beijerinckiaceae	5													100
Bradyrhizobiaceae		1			2						1	1		20
Burkholderiaceae			4					1						80
Methylococcaceae				4					1					80
Methylocystaceae	3				2									40
Moraxellaceae						4		1						80
Myxococcaceae							5							100
Neisseriales lineage								4	1					80
Nitrosomonadaceae									4		1			80
Polyangiaceae			1				1			3				60
Pseudomonadaceae						1					3	1		60
Rhizobiaceae											1	4		80
Rhodospirillaceae					1								4	80

Supplementary Table 6: Confusion matrix of Actinobacterial Family predictions.

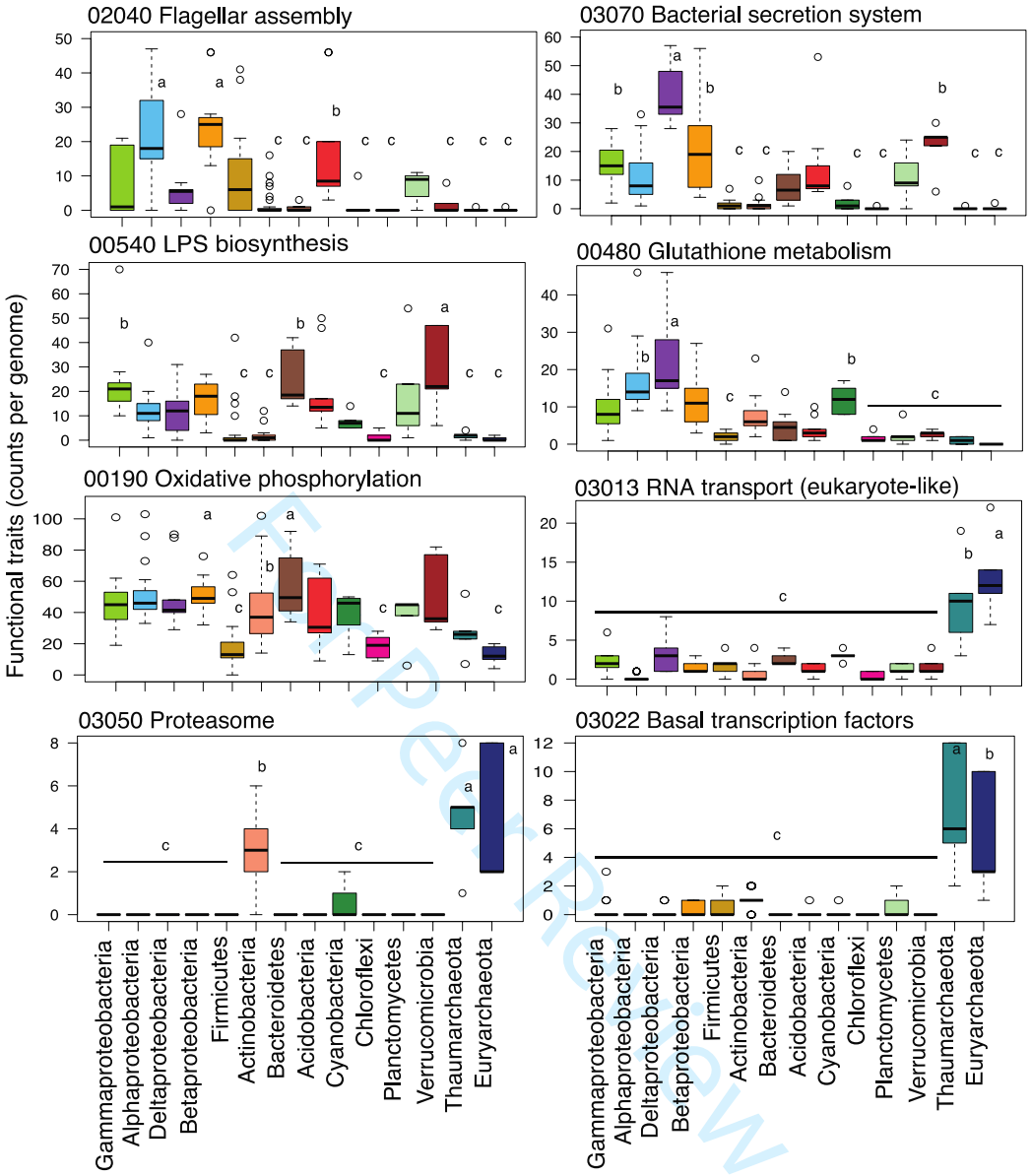
	Successful classifications							
	Cellu.	Frank.	Microbac.	Micrococ.	Mycobac.	Promicro.	Strepto.	Success rate (%)
Cellulomonadaceae	2		1	1		1		40
Frankiaceae		3		1	1			60
Microbacteriaceae	1		3				1	60
Micrococcaceae				4			1	80
Mycobacteriaceae					5			100
Promicromonosporaceae						5		100
Streptomycetaceae				1			4	80

Supplementary Table 7: Confusion matrix of Firmicutes Family predictions.

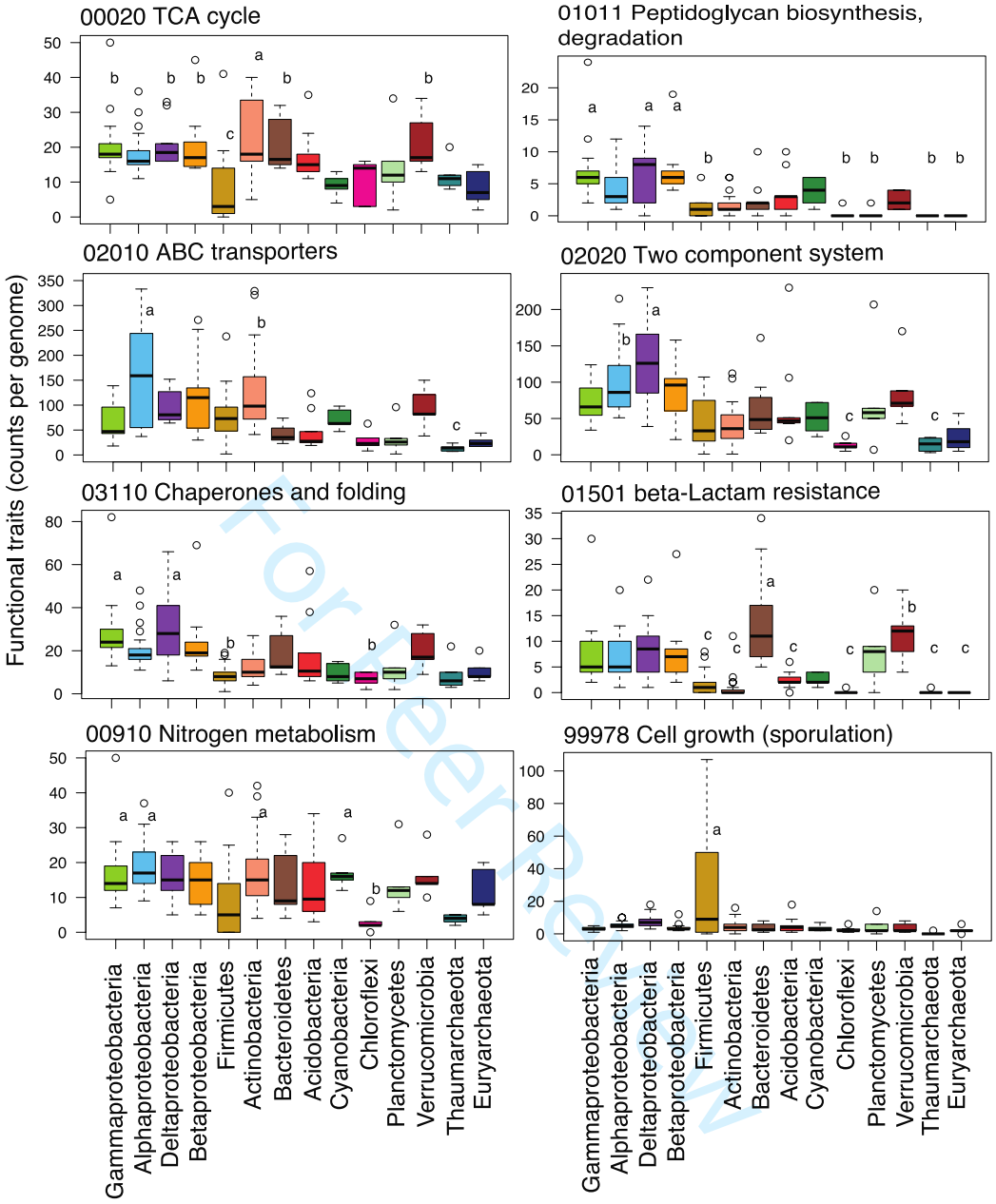
	Successful classifications					
	Bacill.	Clostrid.	Lacto.	Leuco.	Sporo.	Success rate (%)
Bacillaceae	4	1				80
Clostridiaceae	1	3	1			60
Lactobacillaceae			4	1		80
Leuconostocaceae				5		100
Sporomusaceae		1		1	3	60

Supplementary Table 8: Confusion matrix of 'Under-represented' Family predictions.

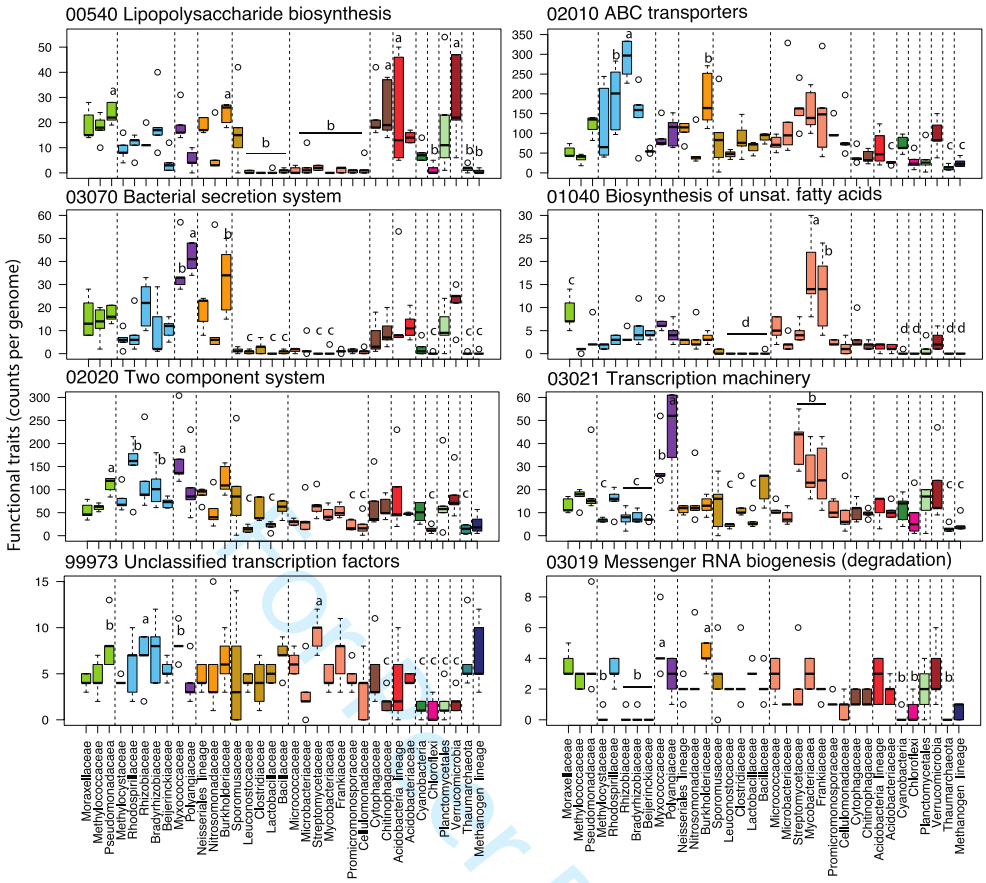
	Successful classifications										
	Acid.	Chitin.	Chloro.	Cyano.	Cytoph.	Acido. Lin.	Methan.	Plancto.	Thaum.	Verruc.	Success rate (%)
Acidobacteriaceae	5										100
Chitinophagaceae		3			2						60
Chloroflexi			4					1			80
Cyanobacteria				5							100
Cytophagaceae		2			3						60
Acidobacteria Lineage	1		2							2	0
Methanogen Lineage							5				100
Planctomycetales			1			1		3			60
Thaumarchaeota			1						4		80
Verrucomicrobia	1									4	80



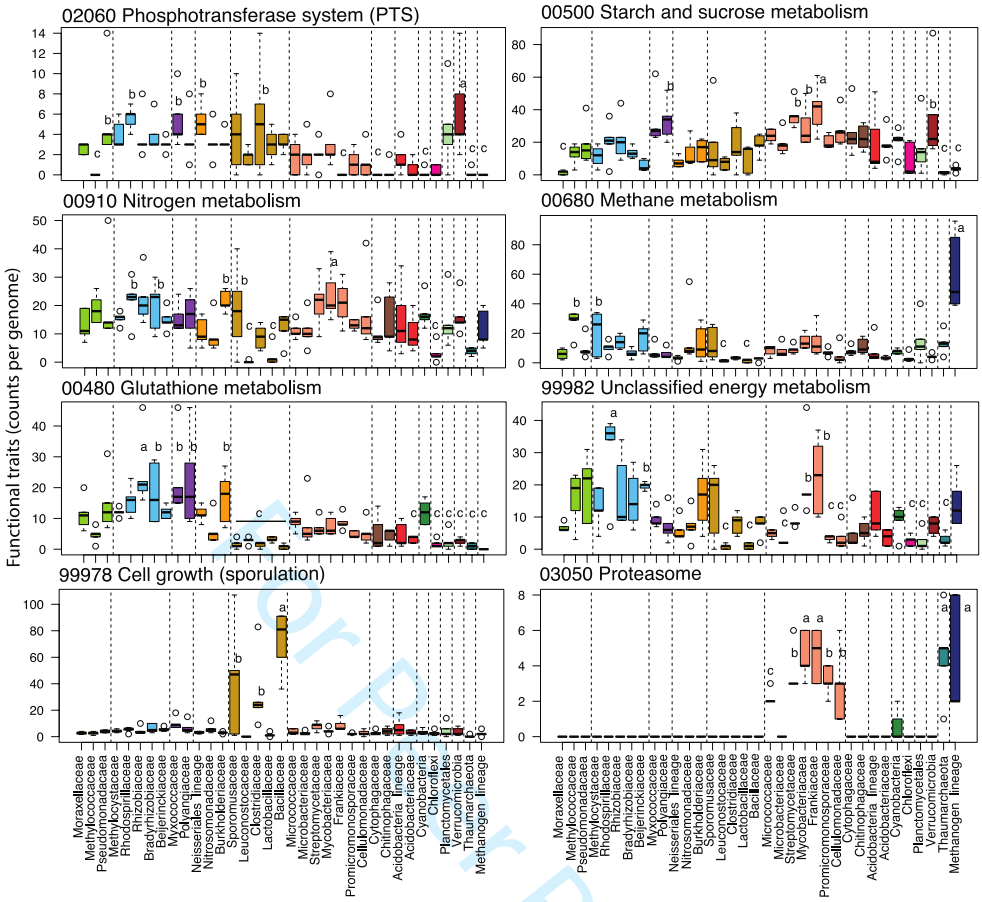
Supplementary Figure 4: Box and whisker plots of functional traits present in Phyla. Traits were selected based on being selected by the Phylum Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



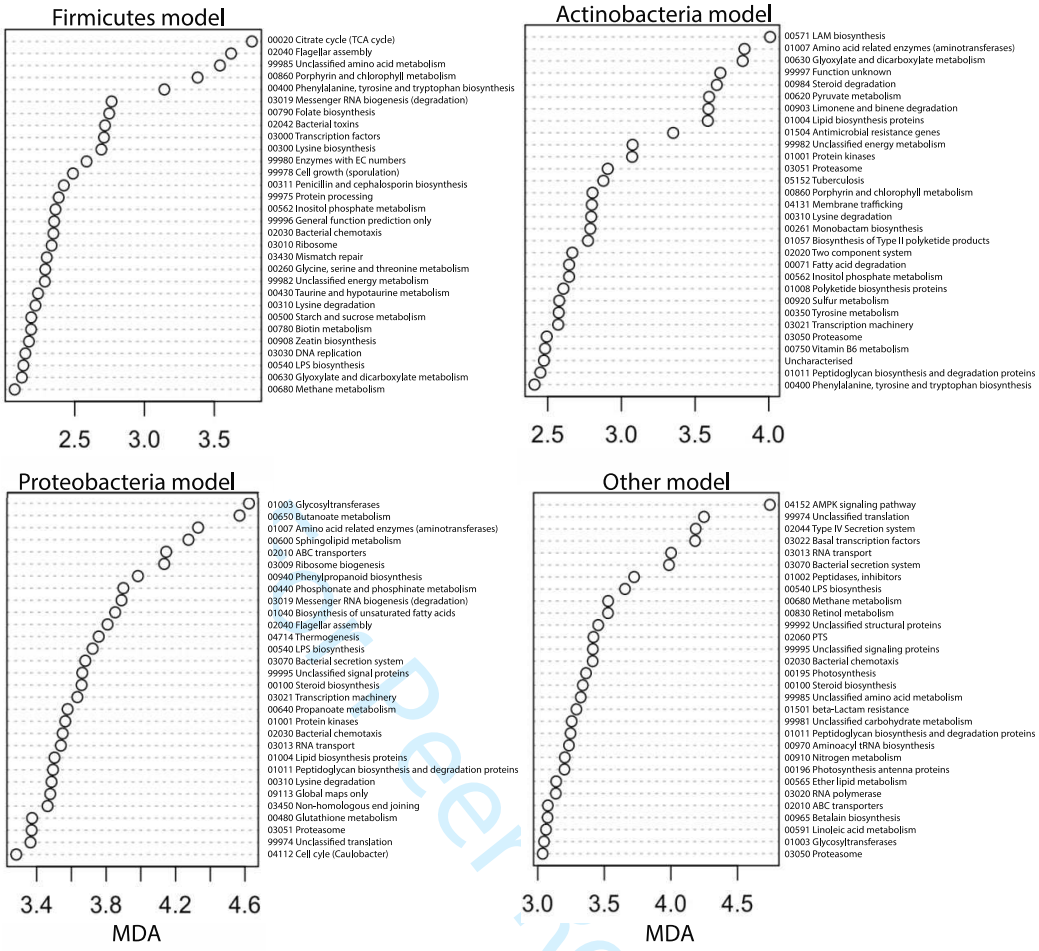
Supplementary Figure 5: Box and whisker plots of functional traits present in Phyla. Traits were selected based on being selected by the Phylum Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



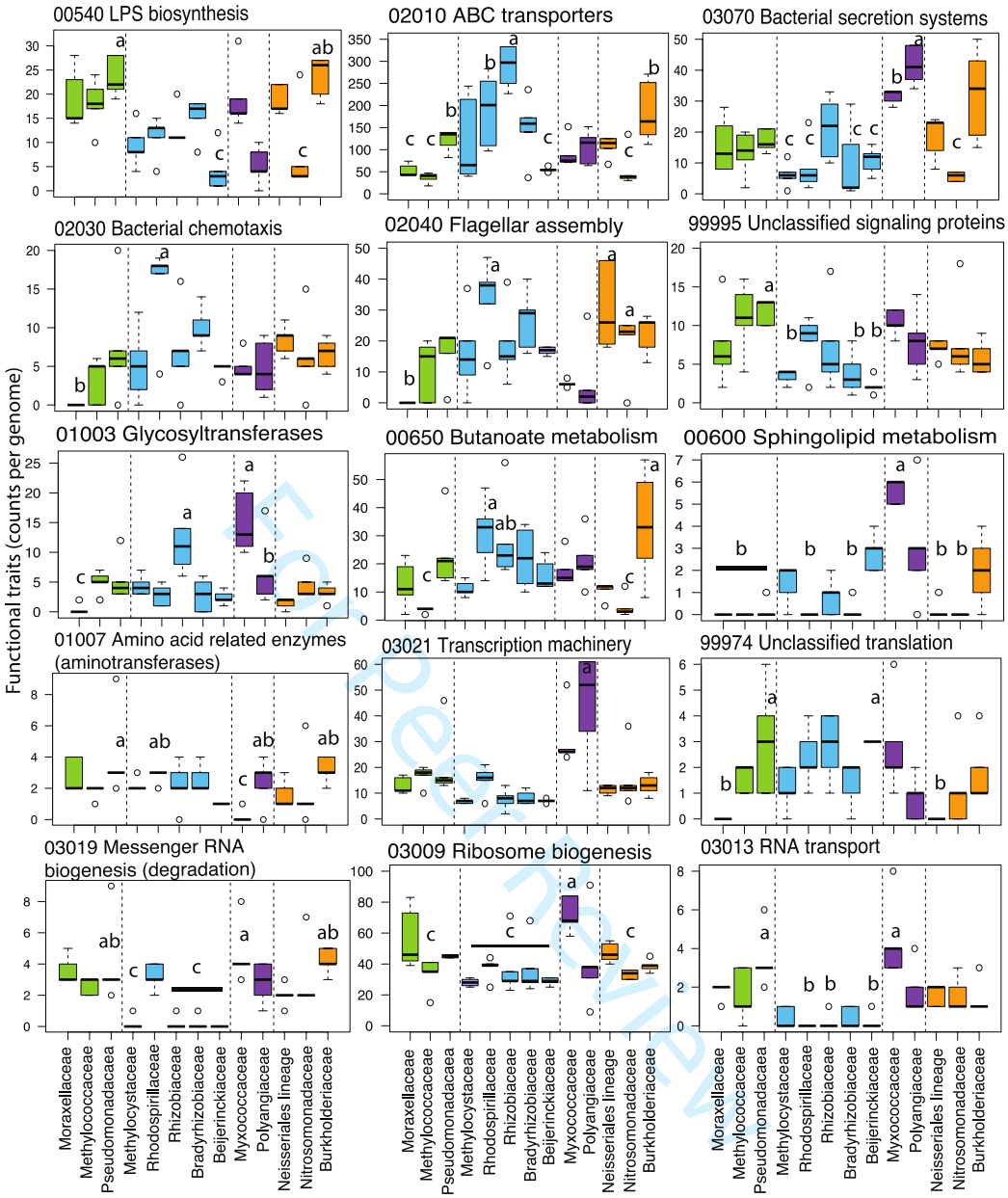
Supplementary Figure 6: Box and whisker plots of functional traits present in Families. Traits were selected based on being selected by the Family Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



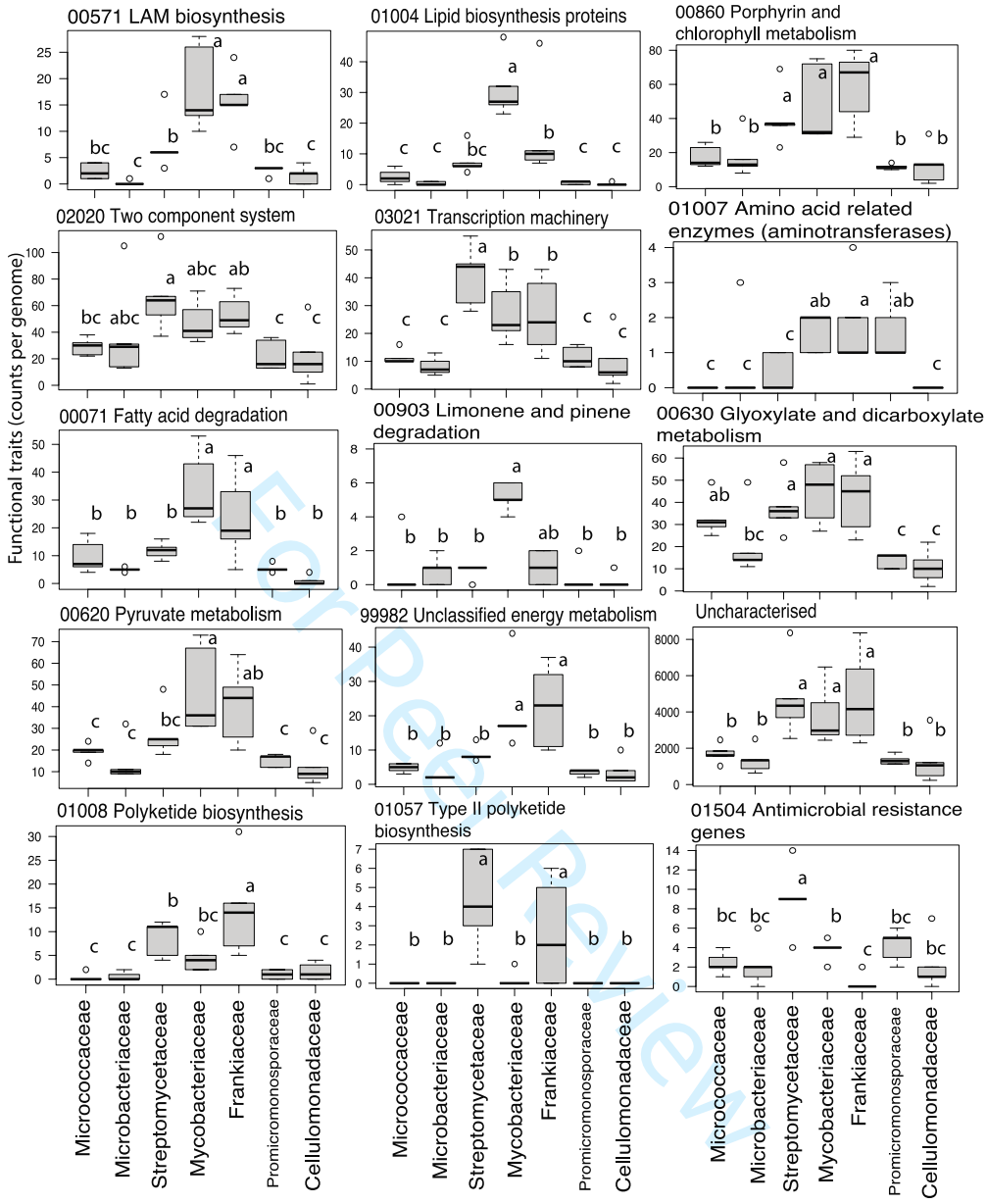
Supplementary Figure 7: Box and whisker plots of functional traits present in Families. Traits were selected based on being selected by the Family Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



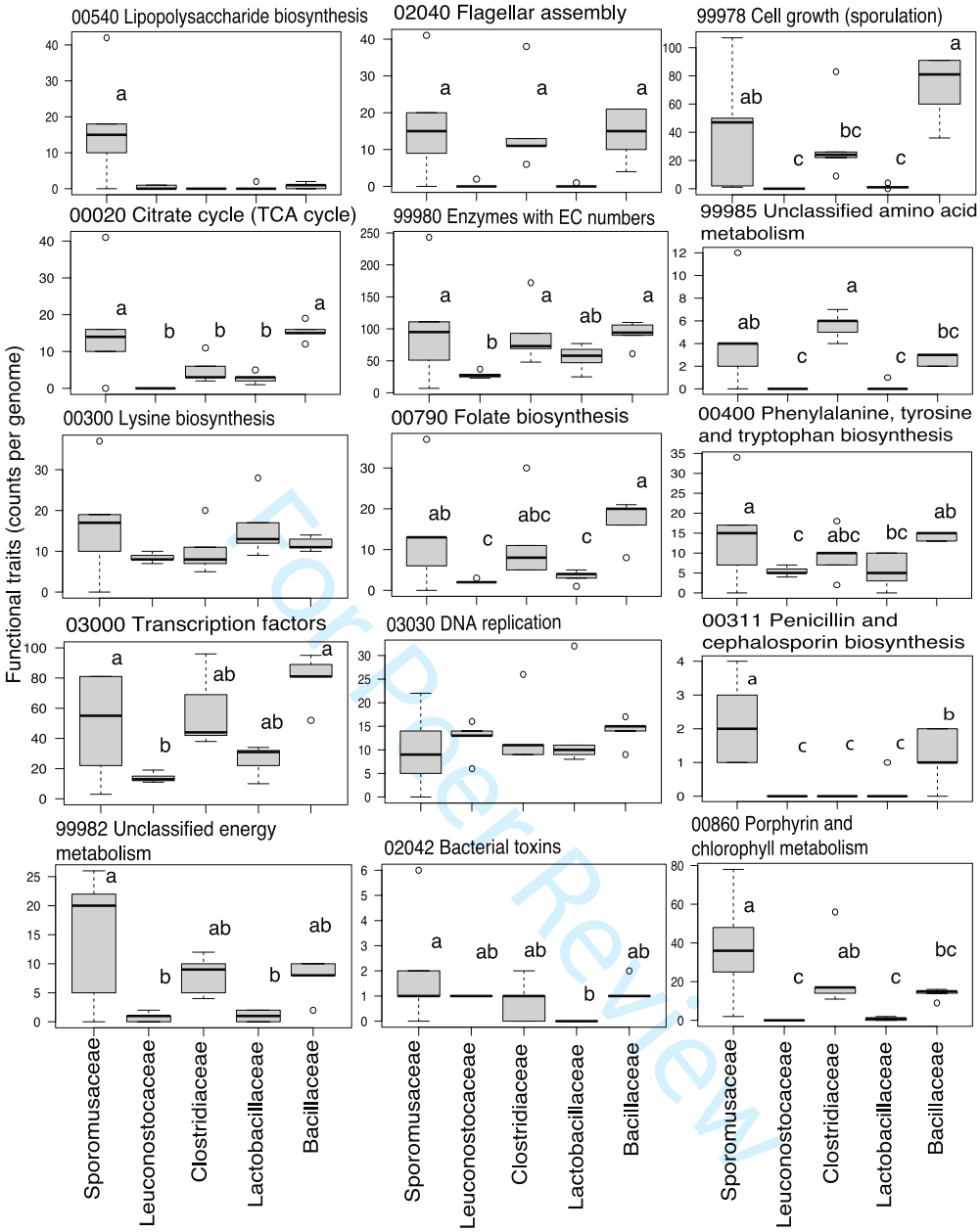
Supplementary Figure 8: Important predictor traits in classifying subgroups of Families as selected by the Proteobacteria, Actinobacteria, Firmicutes and Others model. Traits are ranked in order of decreasing Mean Decrease in Accuracy (MDA) of the four models.



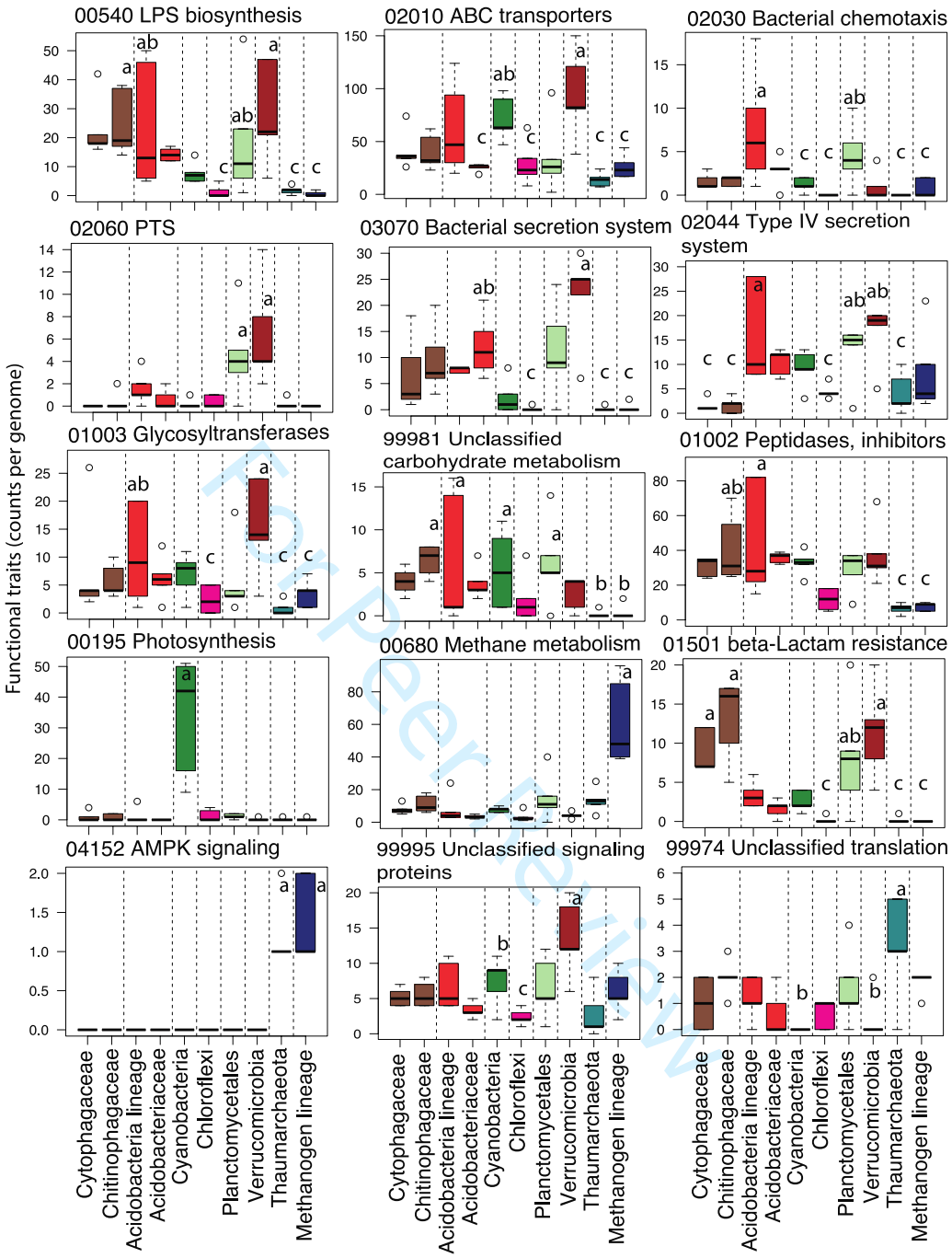
Supplementary Figure 9: Box and whisker plots of functional traits present in Proteobacterial Families. Traits were selected based on being selected by the Proteobacteria Family Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



Supplementary Figure 10: Box and whisker plots of functional traits present in Actinobacterial Families. Traits were selected based on being selected by the Actinobacterial Family Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



Supplementary Figure 11: Box and whisker plots of functional traits present in Firmicutes Families. Traits were selected based on being selected by the Firmicutes Family Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.



Supplementary Figure 12: Box and whisker plots of functional traits present in Under-represented Families. Traits were selected based on being selected by the Under-represented Family Random Forest model. Letters indicate significantly high or low ($p < 0.05$) counts based on LSD *post hoc* tests.

Supplementary Table 9: Summary of traits significantly enriched and depleted in the Phylum Random Forest model.

Model	BRITE 1	BRITE 3	Significantly enriched	Significantly depleted
Phylum	Metabolism	00020 TCA cycle	Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia	Firmicutes, Euryarchaeota
		00190 Oxidative phosphorylation	Betaproteobacteria, Actinobacteria, Bacteroidetes	Firmicutes, Chloroflexi, Euryarchaeota
		00480 Glutathione metabolism	Alphaproteobacteria, Deltaproteobacteria, Cyanobacteria	Firmicutes, Chloroflexi, Planctomycetales, Verrucomicrobia, Thaumarchaeota, Euryarchaeota
		00910 Nitrogen metabolism	Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, Cyanobacteria	Chloroflexi
		00540 LPS biosynthesis	Gammaproteobacteria, Bacteroidetes, Verrucomicrobia	Firmicutes, Actinobacteria, Chloroflexi, Thaumarchaeota, Euryarchaeota
		01011 Peptidoglycan biosynthesis, degradation	Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria	Firmicutes, Chloroflexi, Planctomycetales, Thaumarchaeota, Euryarchaeota
		01001 Protein kinases	Deltaproteobacteria, Planctomycetales	Alphaproteobacteria, Firmicutes, Thaumarchaeota, Euryarchaeota
		01040 Biosynthesis of unsaturated fatty acids	Deltaproteobacteria, Actinobacteria	Firmicutes, Chloroflexi, Thaumarchaeota, Euryarchaeota
		09113 Global maps only (unclassified metabolism)	Gammaproteobacteria, Actinobacteria, Thaumarchaeota	Firmicutes, Bacteroidetes, Acidobacteria, Cyanobacteria, Chloroflexi, Planctomycetales, Verrucomicrobia
		04614 Renin-angiotensin system	Deltaproteobacteria, Bacteroidetes	Gammaproteobacteria, Firmicutes, Thaumarchaeota
		00572 Arabinogalactan biosynthesis	Actinobacteria, Planctomycetales	Gammaproteobacteria, Alphaproteobacteria, Chloroflexi, Euryarchaeota
		00561 Glycerolipid metabolism	Deltaproteobacteria, Actinobacteria, Verrucomicrobia	Thaumarchaeota, Euryarchaeota
		00360 Phenylalanine metabolism	Gammaproteobacteria, Actinobacteria	Firmicutes, Acidobacteria, Cyanobacteria, Chloroflexi
		00960 Tropane, piperidine and pyridine alkaloid biosynthesis	Alphaproteobacteria, Betaproteobacteria, Euryarchaeota	Firmicutes, Planctomycetales
		00630 Glyoxylate and dicarboxylate metabolism	Alphaproteobacteria, Acidobacteria	Firmicutes, Chloroflexi, Thaumarchaeota, Euryarchaeota
	Environmental Information Processing	02010 ABC transporters	Alphaproteobacteria, Actinobacteria	Thaumarchaeota
		02020 Two component system	Alphaproteobacteria, Deltaproteobacteria	Chloroflexi, Thaumarchaeota
		03070 Bacterial secretion system	Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria, Verrucomicrobia	Firmicutes, Actinobacteria, Cyanobacteria, Chloroflexi, Thaumarchaeota, Euryarchaeota
		02044 Type IV secretion system	Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria, Verrucomicrobia	Firmicutes, Bacteroidetes, Chloroflexi, Thaumarchaeota
	Genetic Information Processing	03013 RNA transport (eukaryote-like)	Thaumarchaeota, Euryarchaeota	All other phyla

		03022 Basal transcription factors	Thaumarchaeota, Euryarchaeota	All other phyla
		03050 Proteasome	Actinobacteria, Thaumarchaeota, Euryarchaeota	All other phyla
		03110 Chaperones and folding	Gammaproteobacteria, Deltaproteobacteria	Firmicutes, Chloroflexi
		03051 proteasome 20S subunit	Deltaproteobacteria, Actinobacteria	All other phyla
		03019 mRNA biogenesis (degradation)	Gammaproteobacteria, Deltaproteobacteria	Alphaproteobacteria, Cyanobacteria, Chloroflexi, Thaumarchaeota, Euryarchaeota
	Signaling and Cellular Processes	99978 Cell growth (sporulation)	Firmicutes	All other phyla
		01501 beta-Lactam resistance	Bacteroidetes, Verrucomicrobia	Firmicutes, Actinobacteria, Acidobacteria, Chloroflexi, Thaumarchaeota, Euryarchaeota
		02040 Flagellar assembly	Alphaproteobacteria, Betaproteobacteria, Acidobacteria	Actinobacteria, Bacteroidetes, Cyanobacteria, Chloroflexi, Verrucomicrobia, Thaumarchaeota, Euryarchaeota
		02030 bacterial chemotaxis	Alphaproteobacteria, Betaproteobacteria	Actinobacteria, Chloroflexi, Thaumarchaeota
	Not included in pathway or BRITE	99992 Unclassified structural proteins	Gammaproteobacteria, Betaproteobacteria	Firmicutes, Actinobacteria, Chloroflexi, Thaumarchaeota, Euryarchaeota

Supplementary Table 10: Summary of traits significantly enriched and depleted in Firmicutes and Actinobacteria models.

Model	BRITE 1	BRITE 3	Significantly high	Significantly low
Firmicutes	Metabolism	00540 LPS biosynthesis	Sporomusaceae	All other families
		00020 Citrate cycle (TCA cycle)	Sporomusaceae, Bacillaceae	All other families
		99985 Unclassified amino acid metabolism	Clostridiaceae	Leuconostocaceae, Lactobacillaceae
		00300 Lysine biosynthesis	All equal	All equal
		00790 Folate biosynthesis	Bacillaceae	Leuconostocaceae, Lactobacillaceae
		00400 Phenylalanine, tyrosine and tryptophan biosynthesis	Sporomusaceae	Leuconostocaceae
		99982 Unclassified energy metabolism	Sporomusaceae	Leuconostocaceae, Lactobacillaceae
		00860 Porphyrin and chlorophyll metabolism	Sporomusaceae	Leuconostocaceae, Lactobacillaceae
	Genetic Information Processing	03000 Transcription factors	Sporomusaceae, Bacillaceae	Leuconostocaceae
		03030 DNA replication	All equal	All equal
	Signaling and Cellular Processes	02040 Flagellar assembly	Sporomusaceae, Clostridiaceae, Bacillaceae	Leuconostocaceae, Lactobacillaceae
		99978 Cell growth (sporulation)	Bacillaceae	Leuconostocaceae, Lactobacillaceae
		02042 Bacterial toxins	Sporomusaceae	Lactobacillaceae
		00311 Penicillin and cephalosporin biosynthesis	Sporomusaceae, Bacillaceae	Leuconostocaceae, Lactobacillaceae, Clostridiaceae
	Enzymes	99980 Enzymes with EC numbers	Sporomusaceae, Clostridiaceae, Bacillaceae	Leuconostocaceae
Actinobacteria	Metabolism	00571 LAM biosynthesis	Mycobacteriaceae, Frankiaceae	Microbacteriaceae, Cellulomonadaceae
		01004 Lipid biosynthesis proteins	Mycobacteriaceae	Micrococcaceae, Microbacteriaceae, Promicromonosporaceae, Cellulomonadaceae
		00860 Porphyrin and chlorophyll metabolism	Streptomycetaceae, Mycobacteriaceae, Frankiaceae	All other families
		01007 Amino acid related enzymes (aminotransferases)	Frankiaceae	Micrococcaceae, Microbacteriaceae, Streptomycetaceae, Cellulomonadaceae
		00071 Fatty acid degradation	Mycobacteriaceae, Frankiaceae	All other families
		00903 Limonene and pinene degradation	Mycobacteriaceae	Micrococcaceae, Microbacteriaceae, Streptomycetaceae, Promicromonosporaceae, Cellulomonadaceae
		00630 Glyoxylate and dicarboxylate metabolism	Streptomycetaceae, Mycobacteriaceae, Frankiaceae	Promicromonosporaceae, Cellulomonadaceae

		00620 Pyruvate metabolism	Mycobacteriaceae	Micrococcaceae, Microbacteriaceae, Promicromonosporaceae, Cellulomonadaceae
		99982 Unclassified energy metabolism	Mycobacteriaceae, Frankiaceae	All other families
		01008 Polyketide biosynthesis	Frankiaceae	Micrococcaceae, Microbacteriaceae, Promicromonosporaceae, Cellulomonadaceae
		01057 Type II polyketide biosynthesis	Streptomycetaceae, Frankiaceae	All other families
	Environmental Information Processing	02020 Two component system	Streptomycetaceae	Promicromonosporaceae, Cellulomonadaceae
	Genetic Information Processing	03021 Transcription machinery	Streptomycetaceae	Micrococcaceae, Microbacteriaceae, Promicromonosporaceae, Cellulomonadaceae
	Signaling and Cellular Processes	01504 Antimicrobial resistance genes	Streptomycetaceae	Frankiaceae
	Uncharacterised	Uncharacterised	Streptomycetaceae, Mycobacteriaceae, Frankiaceae	All other families

Supplementary Table 11: Summary of traits significantly enriched and depleted in Proteobacteria and Others models.

Model	BRITE 1	BRITE 3	Significantly high	Significantly low
Proteobacteria	Metabolism	00540 LPS biosynthesis	Pseudomonadaceae, Burkholderiaceae	Beijerinckiaceae, Nitrosomonadaceae
		01003 Glycosyltransferases	Rhizobiaceae, Myxococcaceae, Polyangiaceae	Moraxellaceae
		00650 Butanoate metabolism	Rhodospirillaceae, Rhizobiaceae, Burkholderiaceae	Methylococcaceae, Nitrosomonadaceae
		00600 Sphingolipid metabolism	Myxococcaceae	All Gammaproteobacteria, Rhodospirillaceae, Bradyrhizobiaceae, Neisseriales Lineage, Nitrosomonadaceae
		01007 Amino acid related enzymes (aminotransferases)	Pseudomonadaceae, Rhodospirillaceae, Polyangiaceae, Burkholderiaceae	Myxococcaceae
	Environmental Information Processing	02010 ABC transporters	Pseudomonadaceae, Rhizobiaceae, Burkholderiaceae	Moraxellaceae, Methylococcaceae, Beijerinckiaceae, Nitrosomonadaceae
		03070 Bacterial secretion systems	Myxococcaceae, Polyangiaceae	Methylocystaceae, Rhodospirillaceae, Bradyrhizobiaceae, Beijerinckiaceae, Nitrosomonadaceae
	Genetic Information Processing	03021 Transcription machinery	Polyangiaceae	All other families
		99974 Unclassified translation	Pseudomonadaceae, Beijerinckiaceae	Moraxellaceae, Neisseriales Lineage
		03019 Messenger RNA biogenesis (degradation)	Pseudomonadaceae, Myxococcaceae, Burkholderiaceae	Methylocystaceae, Rhizobiaceae, Bradyrhizobiaceae, Beijerinckiaceae
		03009 Ribosome biogenesis	Myxococcaceae	Methylococcaceae, all Alphaproteobacteria, Nitrosomonadaceae
		03013 RNA transport	Pseudomonadaceae, Myxococcaceae	Rhodospirillaceae, Rhizobiaceae, Beijerinckiaceae
	Signaling and Cellular Processes	02030 Bacterial chemotaxis	Rhodospirillaceae	Moraxellaceae
		02040 Flagellar assembly	Rhodospirillaceae, Neisseriales Lineage, Nitrosomonadaceae	
		99995 Unclassified signaling proteins	Pseudomonadaceae	Methylocystaceae, Bradyrhizobiaceae, Beijerinckiaceae
Other	Metabolism	00540 LPS biosynthesis	Chitinophagaceae, Acidobacteria Lineage, Planctomycetales, Verrucomicrobia	Chloroflexi, Thaumarchaeota, Methanogen Lineage
		01003 Glycosyltransferases	Acidobacteria Lineage, Verrucomicrobia	Chloroflexi, Thaumarchaeota, Methanogen Lineage
		99981 Unclassified carbohydrate metabolism	Chitinophagaceae, Acidobacteria Lineage, Planctomycetales	Thaumarchaeota, Methanogen Lineage
		01002 Peptidases, inhibitors	Chitinophagaceae, Acidobacteria Lineage	Thaumarchaeota, Methanogen Lineage
		00195 Photosynthesis	Cyanobacteria	All other families
		00680 Methane metabolism	Methanogen Lineage	All other families
	Environmental Information Processing	02010 ABC transporters	Cyanobacteria, Verrucomicrobia	Acidobacteriaceae, Chloroflexi, Thaumarchaeota, Methanogen Lineage

		02060 PTS	Planctomycetales, Verrucomicrobia	All other families
		04152 AMPK signaling	Thaumarchaeota, Methanogen Lineage	All other families
		03070 Bacterial secretion system	Acidobacteriaceae, Verrucomicrobia	Chloroflexi, Thaumarchaeota, Methanogen Lineage
		02044 Type IV Secretion system	Acidobacteria Lineage, Planctomycetales, Verrucomicrobia	Cytophagaceae, Chitinophagaceae, Chloroflexi, Thaumarchaeota
	Genetic Information Processing	99974 Unclassified translation	Thaumarchaeota	Cyanobacteria, Verrucomicrobia
	Signaling and Cellular Processes	02030 Bacterial chemotaxis	Acidobacteria Lineage, Planctomycetales	Cyanobacteria, Chloroflexi, Thaumarchaeota, Methanogen Lineage
		01501 beta-Lactam resistance	Cytophagaceae, Chitinophagaceae, Planctomycetales, Verrucomicrobia	Chloroflexi, Thaumarchaeota, Methanogen Lineage
		99995 Unclassified signaling proteins	Cyanobacteria	Chloroflexi

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Manuscript id: FEMSEC-21-01-0026
Title: Functional trait relationships demonstrate generalisable life strategies in terrestrial prokaryotes.
Authors: Finn, Damien; Bergk-Pinto, Benoit; Hazard, Christina; Nicol, Graeme; Tebbe, Christoph; Vogel, Tim

Reviewers' Comments to Authors:

Reviewer: 1
Comments to the Author
Review of the paper: Functional trait relationships demonstrate generalisable life strategies in terrestrial prokaryotes.
This paper aims at identifying copiotrophic vs oligotrophic strategies among 170 species of bacteria and archea by comparing their similarity in their traits (based on clustering of functionally similar amino acid sequences). Instead of finding two clear cluster reflecting copiotrophic-oligotrophic framework, they identify 5 clusters. Only one of these clusters can be fit as copiotrophic. All other ones, reflect, as the author suggest, unique life-history strategies on their own.
I really like the paper and I think it is worth publication for two reasons: 1) It challenges simplistic views that entire kingdoms (or other higher taxa) of microorganisms can placed along a unique axis of energy use. 2) the method they use will surely be used by other researchers. I also send my kudos to the authors to develop a step-by-step repo explaining their method. Doing so, not only ensures reproducibility but also encourage other scientist to test similar questions with their approach.
I like it as it is. However, I want to acknowledge that I cannot judge on the information of the natural history and ecology of many of the taxa included.

Comment:
My only major suggestion is to make more explicit the way they test hypothesis 3: “copiotrophic and oligotrophic groups would emerge based on trait enrichment”. (L149.150)
From what I understood in the method section (283-287) that hypothesis was tested by subsetting 60 traits that emerge from their random forest clustering that are “associated with copiotroph-oligotroph growth strategies”. This association is based “previous studies”. Since this is a major hypothesis in the paper, I suggest to summarize the traits as a table (at least in the supplementary) where it is indicated which ones are expected to belong in copiotroph-oligotroph axis. Even better, it would be great to have them grouped as a figure. A way to simplify this large number of traits in a figure would be to group them according the Brite categories. Ultimately, I am talking about some conceptual figure that shows the idealized clustering of copiotroph-oligotroph based exactly on the 60 traits you use.

Response:

The authors would like to thank the reviewer for their incredibly generous comments and the suggestion to create a conceptual figure summarising the Brite category clustering per Family. Indeed, by trying to consider the best way to communicate our results it has helped us to further synthesise them. Figure 5 is now a conceptual diagram that attempts to show how the five identified clades overlap based on their trait composition. This is either as a) a one-dimensional axis of 'resource investment', analogous to the classical dichotomy of copiotroph-oligotroph, and b) a multi-dimensional space where clades are further separated based on traits for both 'resource investment' and different means of 'resource acquisition'. By considering both investment and acquisition together, we hope to show that the clades show less overlap in space, which would theoretically allow them to occupy separate niches and co-exist. We do not include all 60 Brite categories in this figure, but rather have chosen some select traits (14 in total) that may be useful in demonstrating our point e.g. if a taxon possesses many glycosyltransferases in addition to spore formation or antimicrobial resistance genes it could be considered an 'oligotrophic degrader'. The 14 traits were partially chosen based on a comment from Reviewer 2, which suggested to name major functional markers that could be used to predict a taxon's niche. Section 4.7 has been updated to incorporate and explain Figure 5 in the context of our results.

We are acutely aware that this conceptual figure may be met with criticism from the scientific community, and we are more than happy to modify, adjust or otherwise take on board any further constructive comments from the reviewer.

Minor comment

L40-42. I think you can be bolder. I think your study shows that the copio-oligotroph view is to simplistic and that more life-history strategies emerge once you compare species based on their traits and not pre-conceived ideas of how microorganisms should behave. I think that stating that the study "warrants a more nuanced view of life-histories" is too soft and vague.

Response:

The concluding sentences of the abstract have been amended to emphasise that the copiotroph-oligotroph framework is insufficient to describe prokaryote niche, and that we suggest considering traits involved in growth and resource acquisition in tandem when predicting niche:

"However, the formation of five clades suggested a more nuanced view to describe niche differentiation in terrestrial systems is necessary. We suggest considering traits involved both in resource investment and acquisition when predicting niche."

Reviewer: 2

Comments to the Author

I reviewed the manuscript "Functional trait relationships demonstrate generalisable

life strategies in terrestrial prokaryotes" by Finn et al. The objective of the manuscript is to identify the traits within soil bacterial and archaeal species that can distinguish their lifestyle and niche occupancy. Genome sequence of 175 taxa from 11 Phyla and 35 Families were used for analysis. The findings strongly suggested a non-random distribution of traits across phylogenetic lineages. Random forest analysis further identified traits that were directly related to niche colonization, substrate use efficiency and metabolism within phylogenetic lineages.
I enjoyed reading the manuscript. The manuscript fits very well within the scope of journal. Proper statistical tests have been performed to reach the conclusions. Manuscript is very well written and presented. I have few suggestions that will further strengthen the manuscript.

Comment:
1. There has been some work that has explored the genomic basis of tropic lifestyle in soil bacteria. This work should be cited and discussed appropriately. e.g. Trivedi et al. 2013. Trends in Microbiology.

Response:
The authors would like to thank the reviewer for their generous comments. In regard to this specific comment, references and discussions of Trivedi et al., 2013 have been incorporated several times throughout the manuscript, for example:

Ln 121: "A genomic basis for traits associated with soils dominated by putative copiotrophs and oligotrophs has been expertly reviewed elsewhere, and interested readers are referred to Trivedi *et al*., 2013 and references therein."

Ln 639: "Comparative genomics analyses have also identified Actinobacteria, Acidobacteria and Verrucomicrobia as being enriched in numerous enzymes for cellulose, hemicellulose and starch catabolism (Trivedi *et al*., 2013)."

And Ln 674: "Comparative genomics analyses of soil bacteria have also found putatively copiotrophic Proteobacteria and Firmicutes to be particularly enriched in PTS and ABC transporters (Trivedi *et al*., 2013)."

Furthermore, the study by Barbéran et al., 2012 to define 'generalist' and 'specialist' soil taxa based on co-occurrence has been incorporated in the Introducion:

Ln 86: "… identifying habitat generalists and specialists based on taxon co-occurrence patterns (Barbéran *et al*., 2012) …"

Comment:
2. Only five strains from family level classification were selected. Authors have mentioned multiple times that some families will have a significant phylogenetic

diversity within strains, while for others, it will be restricted. How do these differences impact overall results? What was the criteria of selecting only 5 and not more?

Response:
A minimum of five genomes per taxonomic group were selected to accommodate Thaumarchaeota, Verrucomicrobia Chloroflexi and some others, which had the least number of publicly available sequenced genomes of soil taxa. We specifically wanted to avoid generating a dataset which favoured frequently isolated and sequenced soil organisms (e.g. Bacilli, Rhizobia) and thus five genomes per group were used to balance statistical analyses. This is mentioned at line 177 in the Methods section, under Collection of terrestrial prokaryote genomes.

Questions 2 and 3 from the reviewer have prompted additional analyses to quantify the phylogenetic (dis)similarity of taxonomic groups based on full length 16S rRNA genes, and to compare this to trait similarity (*C*). The 16S genes were aligned with MUSCLE, simple Neighbour Joining trees were constructed in R with ape/phangorn packages, and phylogenetic distance (*P*) calculated as per Equation 1, excepting that *P* is in units of gene sequence similarity as opposed to Bray-Curtis distance for trait similarity (*C*). Important considerations between *P* and *C* are as follows:

1) Taxa were originally chosen in the hope of maximising *P* (and presumably *C*) per Family by choosing different Genera where possible. This was not always possible, and ultimately choices were constrained by what genomes were publicly available. However, looking at values of *P* per Phylum and Family (Supplementary Figure 2), there does not appear to be a consistent relationship between Families where different Genera were included and *P*. For example, Lactobacillaceae consisted of 5 *Lactobacillus* spp. (*P* of 0.772) whereas the Beijerinckiaceae consisted of 4 different genera that appear to be actually more similar to each other in regard to the 16S rRNA gene (*P* of 0.852). In retrospect this lack of consistent variation in phylogenetic distance between taxa based on pre-existing nomenclature should not be a surprise at it is the reason for the efforts of the researchers involved with the Genome Taxonomy Database who seek to standardise the current state of microbial taxonomy.

2) There is a very nice linear relationship between *P* and *C* when considering taxa at both the Phyla and Family levels (Supplementary Figure 3). As implied by the reviewer, this will mean any Families that consist of more closely related taxa (*i.e.* high P) will be more likely to be similar in regard to their traits.

3) The importance of this underlying relationship between the variation in phylogenetic distance between taxa in a group and the results of the Random Forest models is, however, uncertain. Supplementary Table 3 shows the Confusion Matrix for the Family level Random Forest. Some Families with the poorest predictive capacity (< 50% taxa correctly predicted) had quite high *P* (> 0.8), e.g. Bradyrhizobiaceae, Cellulomonadaceae. Some Families with low *P* (< 0.7) had poor predictive capacity,

e.g. Divergent Acidobacteria. Conversely, Families that had the highest predictive capacity (100%) ranged in *P* from 0.68 – 0.9. It is the opinion of the authors that the presence of specific, unique and consistent traits in a taxonomic group is far more important for the RF results than the underlying relationship between phylogenetic distance and trait composition. For example, the five Methanogen taxa had very low phylogenetic distance (0.68) yet the RF was perfectly accurate in classifying them, probably due to their methanogen metabolism and Eukaryote-like genetic traits.

Nevertheless, we feel the reviewer raises a very important point when considering taxon selection for these types of comparative genomic analyses, and in future studies we shall place emphasis on standardising phylogenetic distance within groups. The following has been added to the manuscript to highlight this:

In the Methods section 2.3 discussing UPGMA of trait similarity analysis:
Ln 291: “Furthermore, the full length 16S rRNA gene of each taxon was collated from NCBI. Genes were aligned with MUSCLE (Edgar, 2004) and a Neighbour-Joining phylogenetic tree was constructed with the ‘phangorn’ package in R. Phylogenetic distance present in taxonomic groups (*P*) was measured as per Equation 1., excepting that branch length was in units of DNA sequence similarity as opposed to Bray-Curtis dissimilarity. Finally, simple linear regression was used to test a relationship between *P* and *C*.”

In the Results section discussing the UPGMA trait tree and RF models:
Ln 364: “A Neighbour-Joining tree of full length 16S rRNA genes showed that all taxa clustered preferentially based on their taxonomic nomenclature at high taxonomic rank, including the Chloroflexi, indicating that the discrepancies in the UPGMA were not due to misclassification of the individual taxa (Supplementary Figure 2a).”

Ln 388: “Phylogenetic distance (*P*) of each taxonomic group increased with decreasing taxonomic rank, and was highest in Proteobacteria, Actinobacteria and Firmicutes Families (Supplementary Figure 2b). There was a strong positive linear relationship between *P* and *C* ($y = 0.86x – 0.25$, $R^2 = 0.39$, $p < 0.001$) demonstrating that taxonomic groups of closer related taxa tended to share more similar compositions of traits.”

Ln 412: “Random Forest models were robust against variation in *P* within Families, for example the nine families with 0% classification error ranged in *P* from the lowest (0.68) to highest (0.9).”

And finally in the Discussion section in regard to the robustness and interpretation of the Random Forest approach:

Ln 643: “Ideally the selection of individual taxa within groups for such future comparative analyses would also be standardised based on phylogenetic distance,

either with *P* or a similar method, which would improve the robustness of trait-based comparisons at such a fine taxonomic level."

Comment:

3. What were the criteria of selection of 5 species within a family e.g., phylogenetic distance?

Response:

As mentioned above, the selection criteria of the five taxa per Family was based primarily on public availability of genomes. However, the authors acknowledge and fully agree with the reviewers point that phylogenetic distance should be considered as a means to standardise selection of taxa. While we are confident that our methods are robust to the underlying relationship between *P* and *C* in the coarse Phyla and Family taxonomic groups analysed here, we will incorporate a method for standardising taxon selection in future studies that consider a finer detail of niche differentiation between closely related Species and Strains.

Comment:

4. Lines 165-166: I like the idea of choosing strains that perform particular functions. However, these functions are performed by specific bacterial lineages. My question is whether these biased selections for traits inflate the closeness within a group. Or in other words, if the other members within the families of the selected functional groups were selected, do the results would have been the same?

Response:

This is an interesting question, and fortunately our results have several functional groups that can be compared to address it. For example, to our knowledge, all characterised members of Nitrosomonadaceae are chemolithoautotrophic nitrifiers while the Cyanobacteria perform photosynthesis. Thus, the relatively high value of *C* measured here for these groups that share common functions is not surprising. However, trait variation certainly exists within other functional groups, for example the five Methanogens (isolated from diverse anaerobic habitats including rice rhizosphere, tropical and boreal wetlands) had an intermediate value of *C*. Perhaps the best example is that the inclusion of functionally distinct methanotrophs, methylotrophs and heterotrophs within the Beijerinckiaceae did not have a negative effect on its *C*, which yielded one of the highest values (discussed at Ln 524). Ultimately trait composition will be influenced by a taxon's functional group (e.g. ammonia oxidiser, heterotroph, etc), its evolutionary life-history (as a very general example Bacteria, Archaea) and the environment from where the taxon was isolated.

We have included a statement this effect in the Discussion, which is an interesting and important concept to consider:

Ln 569: “The differing values of *C* for Methanogen and photosynthetic Cyanobacteria functional groups (0.33 and 0.44, respectively) is also worthy of note. Despite all five taxa in each group performing the same core role in a community, the individual isolates came from varying environments. The methanogens were isolated from a range of geographically separate wetlands, rice paddy soil and farm slurry and, while the Cyanobacteria were also isolated from geographically separate environments, they were all from sandy deserts or other nutrient poor, arid soils (Supplementary Table 1 and references therein). Ultimately a taxon’s trait composition will be affected by its functional role in a community, its evolutionary life-history (*e.g*. Beijerinckiaceae described above) and its local environment.”

Comment:

5. Like Lauro et al. 2009, is there a way to do a self-organizing map or a constrained analysis showing the separation of different phylogenetic groups according to the traits?

Response:

The constrained analyses of Lauro *et al*., 2009 revolved around the *a priori* classification of one genome as ‘copiotrophic’ and one genome as ‘oligotrophic’. Unknown genomes were then placed somewhere between these two based on similarity of shared traits. In this study we specifically sought to avoid any preconceived *a priori* assumptions of ‘taxon A is a copiotroph’, and instead opted for unconstrained, exploratory cluster-based statistical techniques (*e.g.* dendrograms, Random Forest) that would let the data speak for itself. We hoped that the UPGMA dendrogram (Figure 2a) would be the simplest and most flexible way to demonstrate relationships between different phylogenetic groups and their trait composition, while the clustering of taxonomic groups based on the counts of specific copiotroph-oligotroph traits would validate whether certain taxa could be considered copiotrophic (Figure 4).

Comment:

6. Based on the results, can you substantiate the phylogenetic grouping of copiotroph/oligotroph groups? I think yes, there is much information, but I would like to have a strong statement suggesting that based on the results, the inclusion of X, Y, and Z phylum as copiotroph was validated. Then can you resolve the debate about the inclusion of groups such as Alpha and betaproteobacteria as copiotroph/oligotroph (Lines 104-105). See 5 as the analysis suggested there can help you to address this question. This information will help the researchers that want to link microbial community composition to soil processes, particularly soil C cycling.

Response:

A definitive statement outlining which groups were confirmed as copiotrophs and oligotrophs has been included in the Discussion. This also explicitly links to how these results can give insight into soil processes such as C cycling:

Ln 872: “These results support previous observations that Rhodospirillaceae, Bradyrhizobiaceae, Burkholderiaceae, Pseudomonadaceae and Rhizobiaceae are copiotrophic, while Planctomycetes, Verrucomicrobia, Myxococcaceae, Polyangiaceae and Acidobacteria are oligotrophic (Ho *et al*., 2017 and references therein). As has been proposed previously, the dominance of these groups in certain soils can provide inferences for ecosystem processes in that system, for example soils dominated by Verrucomicrobia, Planctomycetes and Acidobacteria will have greater capacity to degrade complex plant material while retaining most catabolised carbon in biomass (*i.e.* high growth or carbon use efficiency) or excreted byproducts that assist in soil aggregation (*e.g.* high LPS production) (Trivedi *et a*l., 2013). Conversely, soils dominated by copiotrophic Proteobacteria Families will be systems primarily dependent on labile di- and monosaccharides that demonstrate low carbon use efficiency.”

Resolving the outstanding question of taxonomic groups that do not fit into the classical copiotroph-oligotroph framework is addressed further below as a conceptual figure (Comment 11).

Comment:
7. One important trait that distinguish copiotrophs/oligotrophs is the carbon use efficiency (CUE). There have been some efforts to incorporate microbial CUE to parameterize models for soil carbon. While CUE's topic is touched briefly in Introduction, it has not been explicitly discussed in later sections.

Response:
Carbon use efficiency has now been mentioned in regard to the specific copiotrophic/oligotrophic taxonomic groups that will have a high CUE (please see the above response to Comment 6).

Comment:
8. Authors talk about MAGs in the discussion section. It will be worth discussing how MAGs will help to elaborate similar studies that is reported. Where we are and what needs to be done further?

Response:
The authors agree that this is worth expanding upon. The following two statements have been added to the Discussion to better incorporate the value of MAGs and current gaps for trait-based analyses:

Ln 560: “The generation of MAGs has emerged as a useful tool for identifying traits necessary for life in such environments, and particularly for expanding knowledge of severely under-represented, difficult to culture taxonomic groups. For example, the recent reconstruction of 52, 515 MAGs from a wide range of host-associated and environmental metagenomes was able to increase genomic information of Planctomycetes and Verrucomicrobia by 79% and 68%, respectively (Nayfach et al., 2021). Importantly, both 16S rRNA gene surveys and MAGs demonstrate that some functional traits that facilitate life under certain environmental conditions are intrinsically linked to taxonomy.”

Ln 970: “However, to truly unravel differentiated niches and general microbial life strategies, two limitations must be overcome. Firstly, a better understanding of the many ‘Uncharacterised’ traits in environmental isolates is required. For example, the recent large-scale MAG study by Nayfach *et al*., (2021) identified 5.8 million protein clusters (traits), of which over 75% could not be annotated meaningfully by current protein databases. Secondly, robust trait-based analyses down to the finer scale of distinct genomes will likely be necessary to consider how individual members of a community have either differentiated in order to co-exist or are in the throes of competition that will ultimately exclude one of the competitors.”

Comment:
9. Based on the results, where will you fit archaea (copiotrophs or oligotrophs)?

Response:
The authors are hesitant to place Archaea as either copiotrophs or oligotrophs as we consider the one-dimensional framework to be insufficient for describing niche. Please see further below for a detailed response (Comment 11).

Comment:
10. How this type of data will be used to distill the complexity within the soil microbiome functions? Can we use the functional signatures to determine the metabolic potential of microbial communities from metagenome data? How can this information help us to incorporate microbial activities into process models? I suggest touching on these in conclusions.

Response:
Again, this will be addressed further in Comment 11.

Comment:
11. One of the hallmarks of impactful papers is to move beyond information to

knowledge. Can you think of making a conceptual figure showing different genes and how they are responsible for different lifestyles or niche colonization? This is just a thought as I understand how difficult it can be.

Response:
The authors particularly appreciated this comment, and in particular the expression 'moving beyond information to knowledge'! Indeed, our attempts to consider a conceptual figure that conveys our results in a simple manner has been extremely helpful in further synthesising them.

Figure 5 is now a conceptual diagram that attempts to show how the five identified clades overlap based on their trait composition. This is either as a) a one-dimensional axis of 'resource investment', analogous to the classical dichotomy of copiotroph-oligotroph, and b) a multi-dimensional space where clades are further separated based on traits for both 'resource investment' and different means of 'resource acquisition'. By considering both investment and acquisition together, we hope to show that the clades show less overlap in space, which would theoretically allow them to occupy separate niches and co-exist. The final section of the Discussion, from Ln 916, has been expanded to discuss this figure and its significance for interpreting microbial niche.

In regard to Comment 10, which asks how our results may provide signatures for process-based models or linking to community function, we have chosen the BRITE categories in Figure 5 to act as signatures for defining, for example, Rhizobiaceae as 'copiotrophic competitors'. This is explicitly stated:

Ln 929: "Figure 5b is a conceptual diagram of niche space where clades have been further separated along additional axes based on their enrichment of traits involved in competition, degradation or specialised metabolic pathways. The BRITE categories listed on the various axes are chosen to be useful markers in predicting the niche of a taxon. This expanded niche space would suggest taxa in Clade I are well equipped for nutrient acquisition (primarily, but not limited to, di- and monosaccharides), rapid growth and oxidative stress regulation as 'copiotrophic competitors'. …"

And finally, specifically in regard to whether Archaea should be copiotrophs or oligotrophs:

Ln 956: "The large space conceptualised for Clade V in Figure 5b, which does not imply either copiotrophic or oligotrophic resource investment, is an oversimplification and with improved understanding of traits in these taxa it may be possible to fracture and further separate them into more detailed groups. This would prove particularly beneficial for the poorly characterised Archaea."

Comment:
12. I appreciate the authors of acknowledging the tedious work of other researchers on culturing. This is very important.

Response:
Of course we are happy to do so, as it is essential work.

Functional trait relationships demonstrate ~~generalisable~~ life strategies in terrestrial prokaryotes.

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Theoretical ecology, niche differentiation, copiotroph-oligotroph, Random Forest modelling

Abstract

Functional, physiological traits are the underlying drivers of niche differentiation. A common framework related to niches occupied by terrestrial prokaryotes is based on copiotrophy or oligotrophy, where ~~genomic~~ resource investment is primarily in either ~~metabolism~~ rapid growth or stress tolerance, respectively. A quantitative trait-based approach ~~was employed to demonstrate quantitative~~sought relationships between taxa, traits and niche in terrestrial prokaryotes. With 175 taxa from 11 Phyla and 35 Families (n = 5 per Family), traits were considered as discrete counts of shared genome-encoded proteins. ~~The composition of 220 664 traits~~Trait composition strongly supported non-random ~~trait~~ functional distributions as preferential clustering of related taxa via unweighted pair-group method with arithmetic mean. Trait similarity between

taxa increased as taxonomic rank decreased. A suite of Random Forest models identified traits significantly enriched or depleted in taxonomic groups. ~~Many traits~~These traits conveyed functions related to ~~metabolism~~rapid growth, nutrient acquisition and stress tolerance consistent with their presence in copiotroph-oligotroph niches. ~~Finally, h~~Hierarchical clustering of ~~these~~ traits identified a clade of competitive, copiotrophic Families resilient to oxidative stress *versus* glycosyltransferase-enriched oligotrophic Families resistant to antimicrobials and environmental stress. However, the formation of five clades~~, including an Actinobacteria and 'specialised metabolic function' clade, warrants~~ suggested a more nuanced view ~~of 'life strategies'~~ to describe niche differentiation in terrestrial systems is necessary. We suggest considering traits involved in both resource investment and acquisition when predicting niche.

1. Introduction

Niche differentiation, the process of physiologically distinct organisms adapting better to certain conditions, is a contributing factor to the high biodiversity inherent in microbial communities (Prosser, 2012). Such differentiation is likely an inevitable consequence of the principles of competitive exclusion and natural selection working in tandem – no two organisms can theoretically occupy the same niche, as the poorer competitor must either adapt to a unique niche or be driven to extinction in that system (Gause, 1932, Hutchinson, 1957, Leibold, 1995). The physiological traits driving niche differentiation must have the capacity to convey an advantage to the organism's ability to survive and reproduce (*i.e.* fitness) and be inherited by successive generations (McGill *et al.*, 2006). Importantly, this implies that microbial communities are not only diverse in terms of individual 16S rRNA gene sequences, commonly used to assess community diversity, but also diverse in regard to their physiological traits.

Explaining niche differentiation through the functional, physiological traits present in ecological community members has a long history in macroecology. For example, differences between beak size and shape ~~differed between~~in Galápagos finches was instrumental in Darwin's hypothesis that a common ancestor had differentiated into multiple, island-specific species. Within the past century, trait-based analyses have been particularly predominant in plant ecology, with seed germination in submerged soil, salt tolerance, carbon to nitrogen biomass stoichiometry, and leaf mass per unit area acting as examples of traits linked to niche differentiation (Gleason, 1926, Grime,

1979, Keddy, 1992). In contrast, trait-based approaches to explain microbial ecology have only been performed in few instances, such as conceptualizing niches of methanotrophs based on abundance in high *versus* low methane environments or disturbed *versus* undisturbed soils (Ho *et al.*, 2013), correlating increasing growth rate with increasing ribosomal gene and ribosome-associated gene copy number (Vieira-Silva & Rocha, 2010), deterministic modelling of nitrification rate based on ammonia and oxygen uptake rate, temperature sensitivity and growth rate (Bouskill *et al.*, 2012), defining distinct niches of 32 marine microorganisms based on clustering of genome-encoded functional proteins (Lauro *et al.*, 2009), identifying habitat generalists and specialists based on taxon co-occurrence patterns (Barbéran *et al.*, 2012) and recently comparisons of 23 'core' traits (*e.g.* motility, carbon metabolism, optimal pH for growth) across 15 000 diverse host-associated and environmental genomes (Madin *et al.*, 2020).

A consistent trend noted in macroecology is that traits linked to how carbon and energy is processed and allocated to biomass can describe separate niches (Brown *et al.*, 2004). The canonical example are *r* and *K* strategists, where carbon and energy are primarily invested in reproduction, or alternatively invested in tolerating biotic and/or abiotic stressors, respectively (Grime, 1977). These dichotomous strategies have been observed in microbial ecology: copiotrophs are considered as microorganisms with relatively high growth rates that have relatively poor growth efficiency (as carbon incorporated to biomass per unit resource), relatively high cell maintenance energy costs, dependence on relatively high concentrations of organic carbon in their environment, demonstrate rapid population blooms upon the addition of organic matter and are not overly tolerant of abiotic stress (Semenov, 1991, Koch, 2001, Roller & Schmidt, 2015, Ho *et al.*, 2017). Oligotrophs are considered as the inverse – low growth rate, high growth efficiency, low cell maintenance energy requirements, high substrate uptake affinity, slow growth ~~at a slow~~ yet at a consistent rate and are resilient to abiotic stress. Although the niche concept in macroecology has a formalized definition founded on where a taxon can maintain a stable population within multi-dimensional environmental space (Leibold, 1995), in this study, niche is used simply to distinguish between prokaryotes being relatively more copiotrophic *versus* oligotrophic.

These distinct niches became associated with specific terrestrial taxa at high taxonomic rank based on recent molecular analyses. In complex microbial

communities, the relative abundance of Gammaproteobacteria, Bacteroidetes and Actinobacteria were correlated with rapid growth in response to the addition of labile organic matter or nitrogen (copiotrophs) (Fierer *et al.*, 2007, Goldfarb *et al.*, 2011, Fierer *et al.*, 2012, Leff *et al.*, 2015). Conversely, the Deltaproteobacteria, Acidobacteria, Verrucomicrobia and Planctomycetes were negatively correlated with the addition of organic matter or nitrogen (oligotrophs) (Fierer *et al.*, 2007, Fierer *et al.*, 2012, Leff *et al.*, 2015, Bastida *et al.*, 2016). Conflicting reports exist of Beta- and Alphaproteobacteria, with some studies describing them as copiotrophic and others as oligotrophic highlighting that a consistent niche may not necessarily exist across species within a large taxonomic group (Ho *et al.*, 2017). A genomic basis for traits associated with soils dominated by putative copiotrophs and oligotrophs has been expertly reviewed elsewhere, and interested readers are referred to Trivedi *et al.*, (2013) and references therein. Importantly, these observations suggest that specific traits that allow terrestrial prokaryotes to occupy these two niches should (generally) be associated with taxonomy. This is an example of ecological coherence at high taxonomic rank, whereby members within a taxon tend to have similar life strategies, niches and possess common traits compared to members of other taxa (Philippot *et al.*, 2010). While ecological coherence of taxa has been considered previously, the shared, specific traits that drive niche differentiation in terrestrial prokaryotes remains an open question.

To identify the traits that differ between terrestrial prokaryote taxonomic groups, and whether these traits could describe the niches they occupy, a functional trait-based approach was adopted here. We posited that a trait must: a) be associated with a physiological process that conveys a fitness advantage under certain environmental conditions; b) be measurable in well-defined units; and c) vary more between taxonomic groups than within a taxonomic group (McGill *et al.*, 2006, Kearney *et al.*, 2010). Traits were measured as discrete counts of chromosome-encoded proteins shared between at least two of 175 terrestrial prokaryotes. Markov Chain clustering (MCL) was used to group proteins as traits based on amino acid sequence similarity (%) akin to a previous approach that confirmed close taxonomic relatives tend to share functional traits in 1~~,~~ 374 genomes (Zhu *et al.*, 2015). This was necessary to compare highly similar (but non-identical) proteins from separate genomes that carry out the same biological function. To better identify important, distinguishing traits of terrestrial prokaryotes, this study differed from Zhu *et al.*, by: a) comparing 175 publicly available

terrestrial prokaryote genomes from 35 Families (*n* = 5 each), from 11 Phyla and two Kingdoms; b) ~~the~~ selecting prokaryotes ~~selected are all~~ involved in terrestrial ecosystem processes of interest, including organic matter decomposition, nitrogen fixation, nitrification, denitrification, methane oxidation, plant-growth promotion, bioremediation of pollutants, pathogenesis and methanogenesis; c) ~~selected~~ selecting taxa ~~were~~ isolated from a wide range of terrestrial environments, such as nutrient rich decomposing plant material and rhizosphere, submerged wetland and rice paddy soils, polluted soils, and nutrient poor hot and cold arid environments; and d) avoiding the inclusion of multiple subspecies and/or strains of a single species ~~was specifically avoided~~ to prevent biases in analyses where highly over-represented species are compared with species that have fewer cultured representatives. The taxonomic system used here is from the NCBI, which is built upon a historical array of culture-dependent, physiological observations and genetic similarity to cultured isolates as average nucleotide identity, DNA-DNA hybridisation or 16S rRNA gene homology (ncbi.nlm.nih.gov/Taxonomy/Browser). The use of this classification system and comparison to others is discussed further below.

We hypothesised that: 1) traits are non-randomly distributed, with relatively closely related taxa demonstrating greater similarity than unrelated taxa (ecological coherence); 2) traits that are differentially enriched between taxonomic groups would primarily be involved in metabolism, nutrient acquisition and/or tolerating environmental stress; and 3) copiotrophic and oligotrophic taxonomic groups would emerge based on collective trait enrichment.

2. Methodology

2.1 Collection of terrestrial prokaryote genomes

A collection of 175 sequenced and annotated genomes was collated (Supplementary Table 1). Listed are the genome ID, phylogenetic lineage, role in an ecosystem process if known, and isolation or genome sequencing reference. These genomes were sourced from the National Centre for Biotechnology Information (NCBI) and Joint Genome Institute (JGI) databases. Genomes were chosen based on several criteria: a) five isolates per Family were chosen to have an equal minimum sample size per group, with this sample size being constrained by sequenced genomes of under-represented groups in public databases; b) only a single subspecies/strain per species

was included to avoid bias due to over-representation of some species in public databases; c) an emphasis was placed to include isolates from diverse taxonomic lineages involved in terrestrial ecosystem processes of interest, such as ammonia oxidation and methanogenesis; and d) there was an emphasis to include taxonomic groups frequently stated to be either copiotrophic (*e.g.* Actinobacteria, Gammaproteobacteria) or oligotrophic (*e.g.* Acidobacteria, Planctomycetes) based on observations from soil nutrient addition studies (Ho *et al.*, 2017). Taxonomic annotations for Phyla, Class, Order etc. were based on NCBI taxonomy as most genomes were sourced there. The authors recognise that taxonomy is constantly shifting, particularly so with the recent development of the Genome Taxonomy Database (GTDB) (Parks *et al.*, 2018). Of note is that the vast majority of taxa here have the same taxonomy in NCBI as in GTDB, with the exceptions that GTDB considers the Sporomusaceae as split into three separate Families, the Leuconostocaceae to be Lactobacillaceae, the Promicromonosporaceae to be Cellulomonadaceae, and the Bradyrhizobiaceae and Methylococcaceae have been renamed as Xanthobacteraceae and Methylomonadaceae, respectively. Taxon selection was constrained by availability of genomes for under-represented groups, such as the Chloroflexi, Verrucomicrobia, Planctomycetes, Thaumarchaeota and Euryarchaeota. To meet the $n = 5$ requirement for balanced statistical analyses, it was necessary to consider these under-represented groups as 'Families'. Furthermore, due to the great diversity inherent within Proteobacterial Classes, Gamma-, Alpha-, Beta- and Deltaproteobacteria were considered as independent 'Phyla' for statistical analyses here. Indeed, GTDB now defines Deltaproteobacteria as its own Phylum, while Betaproteobacteria are considered as the Burkholderiales Order within the Gammaproteobacteria. The total of 175 genomes analysed here falls within the upper range of previous hypothesis-driven trait-based studies which varies from 11 isolates (Bouskill *et al.*, 2012) to 214 genomes (Vieira-Silva & Rocha, 2010).

2.2 Functional trait clustering

A step-by-step walkthrough of reproducible code to perform the following analyses on a subset of 12 genomes is available at: https://github.com/DamienFinn/Trait-based_analyses. Firstly, a pairwise similarity comparison of all amino acid sequences (964 951 sequences) across the 175 genomes was performed with the all *versus* all

basic local alignment tool function for proteins, BLAST-P (Altschul *et al.*, 1990). Amino acid sequences were subsequently clustered as traits via MCL weighted by pairwise amino acid similarity (Enright *et al.*, 2002). Functional traits were grouped at a cluster value of 90.2, whereby > 65 is considered 'fair' and confidence in accurately separating clusters cannot be higher than 100. The value of 90.2 is not chosen by the user but rather is a reflection of the quality of clustering in a given dataset. The MCL identified a total of 220 664 traits shared between at least two genomes. A random subset of 1700 amino acid sequences were selected and the similarity of each sequence within its trait group (as determined by MCL) *versus* between other trait groups was visualised as a box and whisker plot (Supplementary Figure 1) in R version 4.0.0 (R Core Team, 2013). 1 700 sequences were chosen to maximise comparisons between trait groups under technical limitations, as increasing sequences led to exponential increases in trait combinations. A Student's T test was applied to determine whether sequences were more similar within their trait group relative to between trait groups in R. Finally, a matrix of genome *x* functional trait was generated in a two-step process by first associating genome IDs to the MCL output with a novel script 'MCLtoReshape2.py' (available at the above Github address) and secondly by casting the long data format to a wide data matrix with the 'reshape2' package in R (Wickham, 2007). Box and whisker plots comparing counts of proteins per genome (input) and counts of functional traits shared by at least two genomes (output of computational workflow), for the 35 Families, is presented as Figure 1.

2.3 UPGMA dendrogram of trait similarity between genomes

The unweighted pair group method with arithmetic mean (UPGMA) was chosen to compare distance-based similarity between taxa based on discrete counts of individual traits per genome. This method is more robust for comparing similarity between sample units (*i.e.* taxa) based on discrete counts of variables (*i.e.* individual traits per taxon) rather than neighbour joining or maximum likelihood methods better suited for DNA or amino acid sequence comparisons (Weins, 1998). The UPGMA was performed in R with the 'phangorn' package as described (Schliep *et al.*, 2017) on a Bray-Curtis transformed dissimilarity functional trait matrix, generated with the 'vegdist' function in the 'vegan' package (Oksanen *et al.*, 2013). To measure ecological coherence (*C*) of taxa within shared Super Groups, Phyla and Families, a similarity index was adapted

from Levins' Overlap (Finn *et al.*, 2020a), which measures pairwise similarity in distributions of taxa, as the following:

$$C = 1 - \left(\frac{\sum b_{ij}}{n^2} \right) \qquad \text{Equation 1.}$$

Where b_{ij} is the pairwise branch length between taxon *i* and *j* in the UPGMA tree, measured here as Bray-Curtis dissimilarity, which is summed for each taxon and its relatives within a shared Super Group, Phylum or Family, and where *n* is the number of taxa being compared within a shared Super Group, Phylum or Family.

Furthermore, the full length 16S rRNA gene of each taxon was collated from NCBI. Genes were aligned with MUSCLE (Edgar, 2004) and a neighbour joining phylogenetic tree was constructed with the 'phangorn' package in R. Phylogenetic distance present in taxonomic groups (*P*) was measured as per Equation 1., excepting that branch length was in units of DNA sequence similarity as opposed to Bray-Curtis dissimilarity. Finally, simple linear regression was used to test a relationship between *P* and *C*.

2.4 Functional trait annotation

To inform the biological process a functional trait facilitated, traits were annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This was performed in five steps: 1) a representative amino acid sequence from each trait was extracted with the novel script 'IdentifyTraits.py'; 2) these sequences were annotated with KEGG Orthology (KO) terms using the BlastKOALA database algorithm with a bit score cut-off value of 75 (Kanehisa *et al.*, 2016); 3) BRITE functional heirarchies associated with each KO term (*e.g.* KO1179 gene: endoglucanase, BRITE 1: Metabolism, BRITE 2: Carbohydrate Metabolism, BRITE 3: Starch and Sucrose Metabolism) were collected with the novel script 'GetBRITEinfo.py'; 4) Genome ID, trait ID, KO term and BRITE metadata were all collated with the novel script 'MatchFCs.py'; and 5) the 'reshape2' package in R was used to create matrices of genome *x* BRITE hierarchy. Where KEGG was unable to annotate a trait, it was considered as 'Uncharacterised'. As above, all novel scripts and a step-by-step walkthrough of reproducible code is available at: https://github.com/DamienFinn/Trait-based_analyses.

2.5 Identifying traits differentially enriched in taxonomic groups

Random Forest classification was chosen as a non-linear, multivariate cluster-based method capable of identifying numerous predictor variables (*i.e.* traits) that define different classes of a response variable (*i.e.* taxonomic group). This was performed with the 'randomForest' package as described (Liaw & Weiner, 2002). Discrete counts of traits at BRITE level 3 were used (*e.g.* Starch and Sucrose Metabolism) as this level had the most accurate resolution of biological processes facilitated by traits. A total of six Random Forest models were optimised to classify taxonomic groups at the level of: a) Phylum, with Proteobacteria Classes separated due to their extensive diversity (n = 14); b) Family (n = 35); c) specifically for Families in the Proteobacteria (n = 13); d) Families in the Actinobacteria (n = 7); e) Families in the Firmicutes (n = 5); and f) Families from 'Under-represented' groups, which were all other Families (n = 10). Optimal numbers of trees grown for each model were: 300, 400, 320, 300, 300 and 400, respectively. Six traits were randomly selected at each branch. As the Random Forest only identifies traits that best explain separation of taxonomic groups, and does not show whether traits have positive or negative associations with groups, box and whisker plots and Fisher's Least Significant Difference (LSD) *post hoc* tests were performed with the 'agricolae' package (de Mendiburu, 2014) to definitively state which taxonomic groups were significantly enriched or depleted in traits identified via Random Forest.

2.6 Hierarchical clustering of Families by defining traits

Finally, the relationship between Families based on similarity in counts of 60 traits was assessed via hierarchical clustering. Traits were chosen based on being selected via the above Random Forest models in this study, and from previous studies that identified traits associated with copiotroph-oligotroph growth strategies in single species or mixed communities (Lauro *et al.*, 2009, Vieira-Silva & Rocha, 2010, Roller & Schmidt, 2015, Pascual-Garcia & Bell, 2020). The mean of trait discrete counts in the five Family members was used as representative of each Family. Comparing trait means between Families was considered acceptable as prior LSD *post hoc* tests had demonstrated significant differences between Families. As traits had highly variable copy numbers per Family (*e.g.* ABC transporter trait copies ranged from 10 – 350,

while bacterial chemotaxis traits ranged from 0 – 15 copies) the trait copies were normalised for more appropriate comparisons. Normalised variance was calculated across the 35 Families for all traits with the 'decostand' function in the 'vegan' package (Oksanen *et al.*, 2013). Hierarchical clustering of Families based on normalised trait counts was visualised with the 'heatmap.2' function in the 'gplots' package (Warnes *et al.*, 2019).

3. Results

3.1 Trait clustering and UPGMA

The 964 951 amino acid sequences encoded by the 175 genomes were clustered as 220 664 traits by MCL. A random subset of 1 700 traits showed that amino acid sequence similarity within traits ranged from 62.4, 82.5 and 100% for the 1st quartile, mean and 3rd quartile, respectively (Supplementary Figure 1). Amino acid sequence similarity between traits ranged from 27.6, 34 and 37.8% for the 1st quartile, mean and 3rd quartile, respectively. A Student's T test found that sequences grouped together as a trait were significantly more similar to each other than to sequences grouped as different traits (t value = 61.3, p = 2 x 10^{-16}). Manual comparisons of amino acid sequences within several traits supported clustering of proteins with identical biological function based on KEGG annotation. Thus, the MCL was considered to perform well. However, the minimum amino acid sequence similarity within traits was 23.27% and maximum similarity between traits was 85.24%, indicating that across the 220 664 traits, a small proportion of dissimilar amino acid sequences were grouped as a trait incorrectly, while some amino acid sequences that were highly similar were considered different traits. This small number of incorrectly clustered sequences can be explained by the MCL clustering efficiency being 90.2, out of a possible 100.

A comparison of the number of proteins per genome~~, per Family~~ at the Family level (mean = 5 533), found that there were fewer functional traits per genome (mean = 4 240) (Figure 1). These represent the input number of proteins per genome before trait clustering and the output number of traits after clustering, respectively. As only functional traits shared between at least two genomes were considered here, the loss of highly genome-specific traits that could not be compared between genomes was expected. Despite this drop in average traits per genome, this initial approach serves as a proof of concept to demonstrate that numbers of traits per genome at the Family

level reflect trends in proteins per Family, and thus the MCL was not distorting trait clustering (Figure 1).

The UPGMA dendrogram comparing the 220 664 traits per genome showed that trait compositions were non-randomly distributed (Figure 2a). Specifically, Thaumarchaeota, Euryarchaeota, Acidobacteria, Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Cyanobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes, Actinobacteria, Firmicutes and Deltaproteobacteria clustered together preferentially. The Chloroflexi were split into two clusters: one *Dehalococcoides* and one *Ktedonobacter/Herpetosiphon/Anaerolinea* cluster. Several prokaryotes did not cluster with their high taxonomic rank, including a Planctomycetes bacterium, *Polyangium brachysporum* (Deltaproteobacteria), *Agreia pratensis* (Actinobacteria) and *Sporomusa ovata* (Firmicutes). Also of interest was that, in regard to distance between terminal nodes (as noted by the scale bar), the Betaproteobacteria and Gammaproteobacteria were more similar to each other than the Alphaproteobacteria, which formed its own large, diverse clade. A neighbour joining tree of full length 16S rRNA genes showed that all taxa clustered preferentially based on their taxonomic nomenclature at high taxonomic rank, including the Chloroflexi, indicating that the discrepancies in the UPGMA were not due to misclassification of the individual taxa (Supplementary Figure 2a).

A simple index to measure trait similarity, as ecological coherence (*C*), within groups was devised (Equation 1). *C* increased as taxonomic rank decreased: Super Group < Phylum < Family (Figure 2b). *C* was lowest for the larger, more diverse Proteobacteria and Terrabacteria (Super Group), and Firmicutes and Actinobacteria (Phylum). As the number of taxa being compared at the Super Group (*e.g.* Terrabacteria = 70 *versus* Acidobacteria = 10) and Phylum (*e.g.* Actinobacteria = 35 *versus* Thaumarchaeota = 5) were variable, the most meaningful comparisons between groups are at the Family level (n = 5 each). With the exception of the highly divergent 'Acidobacteria Lineage', all Families had a *C* greater than 0.3, with certain groups in the Alphaproteobacteria (Beijerinckiaceae), Firmicutes (Bacillaceae and Leuconostocaceae) and Actinobacteria (Promicromonosporaceae) being highly coherent ($C > 0.55$). Indeed, all individual Proteobacterial Families had $C > 0.4$, indicating that all five taxa within each of these Families had similar trait compositions. Despite not truly belonging to the same Family as per NCBI taxonomy, the $C > 0.335$ of the five Cyanobacteria, Thaumarchaeota and the Methanogen Lineage taxa was similar to other Families from

the Bacteroidetes and Firmicutes. Thus, the UPGMA demonstrated that taxonomic relatives at the Phylum level tended to cluster with each other preferentially based on trait composition, and secondly that while similarity was broadly highest at low taxonomic rank, some Families were more coherent than others.

Phylogenetic distance (*P*) of each taxonomic group increased with decreasing taxonomic rank, and was highest in Proteobacteria, Actinobacteria and Firmicutes Families (Supplementary Figure 2b). There was a strong positive linear relationship between *P* and *C* ($y = 0.86x - 0.25$, $R^2 = 0.39$, $p < 0.001$) supporting the result that taxonomic groups of closer related taxa tended to share more similar compositions of traits.

3.2 Random Forest trait identification

The KEGG annotated traits belonged to 260 different BRITE 3 categories. The percentage of traits that could not be annotated (and were termed 'Uncharacterised') ranged from 28 – 65% per genome, being particularly high in the Archaea. On average, 47% of traits per genome were Uncharacterised with a standard deviation of 9.5%.

Of the 260 BRITE 3 categories, the 60 most important traits in separating all Phyla and Families are ranked by importance measured as Mean Decrease in Accuracy (MDA) of the Random Forest models (Figure 3). This is a measure of the average increase in classification error during permutation of trees ($n = 300 – 400$) when that particular trait is missing from the tree. For example, the accuracy of classifying Families was most improved by inclusion of the ABC transporters trait. Based on the identified traits, the Phylum model was capable of successfully classifying 81.14% of individual taxa. The Family model was capable of successfully classifying 71.43% of individual taxa. Confusion matrices for both models are presented as Supplementary Tables 2 – 4, and show that classification was particularly difficult for Chloroflexi and Planctomycetes (classification error > 80%) in the Phylum model and for Cellulomonadaceae and the divergent Acidobacteria Lineage (classification error > 80%) in the Family model. Random Forest models were robust against variation in *P* within Families, for example the nine families with all taxa perfectly classified ranged in *P* from the lowest (0.68) to highest (0.9).

The important traits in classifying the taxonomic groups were involved in: a) metabolism and nutrient acquisition (oxidative phosphorylation, tricarboxylic acid

(TCA) cycle, glyoxylate/decarboxylate, thermogenesis, propanoate, starch/sucrose, nitrogen, methane metabolism, synthesis of antioxidants such as glutathione, ATP-binding cassette (ABC) transporters, sugar uptake via phosphotransferase systems (PTS)); b) responding to environmental cues and stressors (protein kinases, two-component systems, transcription factors, proteasome, protein chaperones, RNA transport, chromosome repair via non-homologous DNA end joining); c) core cell physiology (flagella assembly, chemotaxis, sporulation, lipopolysaccharide (LPS), peptidoglycan, glycerolipid, sphingolipid and lipoarabinomannan (LAM) biosynthesis); and d) cell-cell interactions (beta-Lactam resistance, general secretion systems and Type IV secretion systems). Box and whisker plots of discrete counts of identified traits, and LSD results, are provided as Supplementary Figures 42 – 75. The Families significantly enriched and depleted in these traits are listed in Table 1. The Phyla significantly enriched and depleted in identified traits are listed in Supplementary Table 9.

To better identify the more subtle differences between Families in the Proteobacteria, Actinobacteria, Firmicutes and the 'Under-represented' Phyla, individual Random Forest models were constructed for each of the four groups. The successful classification rates were 72.31, 74.29, 76 and 72%, respectively. Confusion matrices for each model are presented as Supplementary Tables 5 – 8. The models were unable to reliably classify Bradyrhizobiaceae and the divergent Acidobacteria Lineage (classification error > 80%).

Supplementary Figure 86 shows the most important traits in classifying the four groups. Unique traits not identified in the prior Phylum and Family models were: a) for Proteobacteria, glycosyltransferases, butanoate metabolism, aminotransferases, ribosome biogenesis, mRNA biogenesis and degradation; b) for Actinobacteria, porphyrin and chlorophyll synthesis, pyruvate metabolism, aminotransferases, fatty acid and aliphatic hydrocarbon metabolism, polyketide and Type II polyketide biosynthesis, antimicrobial resistance genes; c) for Firmicutes, lysine, folate and varied amino acid synthesis, porphyrin and chlorophyll synthesis, DNA replication, bacterial toxins, penicillin and cephalosporin synthesis; and d) for the 'Under-represented' taxa, glycosyltransferases, peptidases and inhibitors, photosynthesis and AMP-activated protein kinases. Box and whisker plots of discrete counts of identified traits, and LSD results, are provided as Supplementary Figures 97 – 120. Tables summarising

Families enriched and depleted in these traits are included as Supplementary Tables 10 and 11.

3.3 Hierarchical clustering of defining traits

Hierarchical clustering based on 60 traits, identified from Random Forest in this study and by previous copiotroph-oligotroph studies, indicated five general clades. The dendrogram on the y axis of Figure 4 shows clustering of taxa as these five clades. The dendrogram on the x axis shows clustering of co-occurring traits. Clade I consisted of Proteobacteria, specifically the Pseudomonadaceae, Burkholderiaceae, Rhodospirillaceae, Bradyrhizobiaceae and Rhizobiaceae. These Families were uniquely enriched in flagellar assembly, chemotaxis, pyruvate metabolism, glutathione metabolism, ABC transporters, benzoate metabolism, transcription factors, glyoxylate and fatty acid metabolism. Clade II, also Proteobacteria, included Nitrosomonadaceae, Neisseriales Lineage, Methylocystaceae, Beijerinckiaceae, Methylococcaceae and Moraxellaceae. These Families clustered based on being enriched in Clade I traits, but to a lesser degree than the Pseudomonadaceae, Burkholderiaceae, Rhodospirillaceae, Bradyrhizobiaceae and Rhizobiaceae. Exceptions included the absence of benzoate metabolism and enrichment of methane metabolism in several Clade II Families.

Clade III, a diverse collection of Bacteroidetes (Chitinophagaceae, Cytophagaceae), Verrucomicrobia, Planctomycetes, divergent Acidobacteria Lineage and the Deltaproteobacteria (Polyangiaceae, Myxococcaceae), shared enrichment of sphingolipid metabolism, beta-Lactam resistance, penicillin and cephalosporin biosynthesis, LPS biosynthesis, glycosyltransferases and starch/sucrose metabolism. Many of these Families shared Clade I and II traits, including Type IV secretion system, oxidative phosphorylation, TCA cycle, PTS, nitrogen and glycerophospholipid metabolism. The absence of glutathione in non-Deltaproteobacterial Clade III Families was notable.

The three Actinobacteria Families in Clade IV, Mycobacteriaceae, Frankiaceae and Streptomycetaceae, were highly similar to each other because they were enriched in Type II polyketide biosynthesis. They also shared some Clade I traits (ABC transporters, transcription factors, pyruvate, benzoate and fatty acid metabolism) and Clade III traits (membrane trafficking, transcription machinery, polyketide biosynthesis

and starch/sucrose metabolism). Similar to Clade III, these Actinobacteria were also depleted in glutathione traits.

Finally, taxa within Clade V were similar to each other due to being depleted in traits shared among the other clades. Cyanobacteria were the only taxa that possessed photosynthesis traits. Lactic acid bacteria (Lactobacillaceae and Leuconostocaceae) were enriched in Unclassified nucleotide metabolism. The Archaea (Thaumarchaeota and Methanogen Lineage) shared eukaryote-like traits, proteasome, basal transcription factors and RNA transport. The Archaea were enriched in carbon fixation traits. Methanogens were also enriched in methane metabolism. Ammonia oxidising Thaumarchaeota were not enriched in nitrogen metabolism, however they were enriched in traits annotated by KEGG as Global maps only (unclassified metabolism). Further analysis found this to be the *fpr* gene, encoding a ferredoxin-flavodoxin $NADP^+$ reductase (K00528). Non-lactic acid bacteria of the Firmicutes (Bacillaceae, Sporomusaceae and Clostridiaceae) were enriched in sporulation and motility traits.

4. Discussion

4.1 Non-random trait clustering demonstrates ecological coherence of taxa

A trait-based approach to investigate taxonomic relationships and potential biological function was carried out with a collection of 175 terrestrial prokaryotes. We hypothesised that traits would be non-randomly distributed amongst taxonomic groups, ~~supportive of~~supported by previous observations that noted closely related taxa are isolated from similar habitats (Philippot *et al.*, 2010). Similarity in the composition of 220 664 traits, within 175 taxa, demonstrated strong agreement with established taxonomy at high (Phyla) and low (Family) rank (Figure 2a). The exceptions to this ecological coherence at high rank were the division of the Chloroflexi and an individual from each of the Planctomycetes, Deltaproteobacteria, Actinobacteria and Firmicutes clustering with unrelated Phyla. ~~It is possible that these four individual taxa have been~~These taxa were not mischaracterised, as based on phylogenetics of the full length 16S rRNA gene (Supplementary Figure 2a). Trait similarity between related taxa, measured as *C*, tended to be highest at low rank (Figure 2b). Uneven sample sizes between groups within Super Group and Phyla make comparisons at this level difficult – the inclusion of many diverse Firmicutes and Actinobacteria likely drove *C* to be lower here than in Thaumarchaeota and Euryarchaeota. However, equal

comparisons at the Family level demonstrated interesting variability in coherence. All Proteobacterial Families had relatively high coherence (*C* = 0.4 – 0.6). The high *C* in Beijerinckiaceae is of particular interest as this group contained both specialist methanotroph (*Methylocapsa*, *Methylocella*), methylotroph (*Methyloferula*) and generalist heterotroph (*Beijerinckia* spp.) taxa. With such varied metabolic traits, one could reasonably expect *C* to be relatively low within this Family. The Beijerinckiaceae appear to have evolved from a common methylotrophic ancestor and still share traits for nitrogen fixation and tolerance for low pH soils (Tamas *et al.*, 2014), and the high *C* measured here indicates that many additional shared traits remain. Both the relatively recent divergence of Families from a common ancestor and the higher number of shared traits are likely causes of the higher *C* observed at low taxonomic rank. The differing values of *C* for Methanogen and photosynthetic Cyanobacteria functional groups (0.33 and 0.44, respectively) is also worthy of note. Despite all five taxa in each group performing the same core role in a community, the individual isolates came from varying environments. The methanogens were isolated from a range of geographically separate wetlands, rice paddy soil and farm slurry and, while the Cyanobacteria were also isolated from geographically separate environments, they were all from sandy deserts or other nutrient poor, arid soils (Supplementary Table 1 and references therein). Ultimately a taxon's trait composition will be affected by its functional role in a community, its evolutionary life-history (*e.g.* Beijerinckiaceae described above) and its local environment.

However, these results are dependent on accurate taxonomic classification, and the *C* of Sporomusaceae, relatively low compared to other Families here, supports splitting this group into Sporomusaceae, Anaeromusaceae and Pelosinaceae by GTDB (Parks *et al.*, 2018). Finally worth noting, some groups at high rank were considered as 'Families' here due to the number of available terrestrial genomes, *e.g.* Cyanobacteria and Chloroflexi. Even so, Cyanobacteria demonstrated a higher *C* than many taxonomically-defined Families, perhaps due to their common role as primary colonisers of nutrient-poor soils (Garcia-Pichel *et al.*, 2001). The number of Families are too numerous to discuss each at length here, but *C* was an effective means of measuring and comparing coherence between groups in the UPGMA tree.

While the method of comparing taxa here differs from other studies, the results were not surprising as many 16S rRNA gene surveys of terrestrial systems consistently demonstrate ecological coherence at high rank. For example, independent studies of

increasing agricultural intensity in soils show reductions in Actinobacteria abundance (Philippot *et al.*, 2009, Jangid *et al.*, 2011). Nitrogen addition to soils frequently enriches numerous taxa within the Actinobacteria and Proteobacterial Classes while negatively affecting taxa within the Verrucomicrobia and Planctomycetes (Wessen *et al.*, 2010, Fierer *et al.*, 2012, Leff *et al.*, 2015, Bastida *et al.*, 2016). Arid, nutrient poor environments select for Actinobacteria-dominated communities (Cary *et al.*, 2010, Crits-Christoph *et al.*, 2013) and, in the absence of other primary producers, allow biological soil crust forming Cyanobacterial taxa to establish (Garcia-Pichel *et al.*, 2001). Anoxic wetland and rice paddy environments support diverse communities of anaerobic Firmicutes, Chloroflexi and methanogenic Archaea (He *et al.*, 2019, Finn *et al.*, 2020b). These trends were noted prior to bioinformatic advances of metagenome assembled genomes (MAGs) that allow for the specific comparison of individual traits between uncultured environmental prokaryote genomes (Hug *et al.*, 2013). The generation of MAGs has emerged as a useful tool for identifying traits necessary for life in such environments, and particularly for expanding knowledge of severely under-represented, difficult to culture taxonomic groups. For example, the recent reconstruction of 52 515 MAGs from a wide range of host-associated and environmental metagenomes was able to increase genomic information of Planctomycetes and Verrucomicrobia by 79% and 68%, respectively (Nayfach *et al.*, 2021). I~~ but i~~mportantly, both 16S rRNA gene surveys and MAGs ~~the earlier studies~~ demonstrate~~d~~ that some functional traits that facilitate life under certain environmental conditions are intrinsically linked to taxonomy.

The ecological coherence observed in Figure 2 does not imply that closely related taxa (*e.g. Bacillus velezensis* LS69 and *Bacillus amyloliquefaciens plantarum* FZB42) have identical phenotypes. Close relatives possess a combination of core and accessory genes (traits) and the presence of even a single accessory gene is sufficient to dramatically alter a strain's phenotype (van Rossum *et al.*, 2020). Rather, our results (Figure 2) demonstrate that the composition of core and accessory traits in alphaproteobacterial Beijerinckiaceae are most similar to each other, relative to alphaproteobacterial Rhizobiaceae or to Actinobacteria, Firmicutes etc.

4.2 What can the Random Forests tell us?

The Random Forest works by identifying the traits that are most reliable in classifying individual Phyla and Families. It selects traits that tend to be: a) of equal copy number per genome within a taxonomic group; and b) that differ markedly in copy number between taxonomic groups, since distinct separation of copies will maximise successful classification. A clear example of this is the consistent identification of eukaryote-like basal transcription factors, proteasome and RNA transport present in the Thaumarchaeota and Euryarchaeota, since they are absent from the majority of Bacterial Phyla. The binary nature of these traits (yes Archaea, no Bacteria) make them strong indicators. The presence of these and more eukaryote-like vesicular trafficking and actin traits have been noted in the Archaeal TACK super-phylum previously, and lend credence to the hypothesis that eukaryotes are descended from Archaea (Embley & Martin, 2006, Spang *et al.*, 2015). However, ~~it must be noted that~~ the Random Forest will not identify a trait unique to *Can.* Nitrosotalea devanterra~~, and~~ that is absent from other Thaumarchaeota, as this single trait will not improve classification of the group as a whole. Consequently, the traits identified via Random Forest all tended to be core, fundamental traits shared by other members of a taxon's Phylum/Family.

Many of the best traits for distinguishing taxa have been historically used by microbiologists to do exactly that. These included fundamental cell physiology traits, such as oxidative phosphorylation, LPS biosynthesis, sporulation, flagellar assembly and chemotaxis. The Phylum model separated Betaproteobacteria, Actinobacteria and Bacteroidetes as taxa with the highest copies of oxidative phosphorylation traits. Firmicutes, Chloroflexi and Methanogens were identified as anaerobes depleted in oxidative phosphorylation, and all other taxa as sitting in between (Supplementary Figure ~~4~~2 and Supplementary Table 9). Some Gram-negative Families were significantly enriched in LPS biosynthesis compared to others. These were the Pseudomonadaceae, Burkholderiaceae, Chitinophagaceae, divergent Acidobacteria and Verrucomicrobia (Table 1). This has been noted in soil communities previously. The extensive repertoire of LPS-associated genes in Chitinophagaceae, Acidobacteria and Verrucomicrobia likely play a critical role in enhancing soil aggregation (Cania *et al.*, 2019) potentially through high LPS production and/or biofilm formation (discussed further below). In a demonstration of the robustness of the methods used here, the highly unusual Firmicute Sporomusaceae were shown to possess similar counts of LPS biosynthesis traits relative to most Gram-negative Families (Supplementary Table

4) in addition to sharing heat-resistant spore formation with Bacillaceae and Clostridiaceae (Table 1). The presence of both traits in a single Family have been remarked upon previously and used to conceptualise the evolution of Gram-negative *versus* Gram-positive lineages (Stackebrandt *et al.*, 1985). The Sporomusaceae were also shown to have high Porphyrin and Chlorophyll metabolism traits in the Firmicutes model (Supplementary Table 10). The capacity to dechlorinate the soil pollutant perchloroethene to trichloroethylene via a porphyrin-based corrinoid is yet another interesting trait of this Family (Terzenbach & Blaut, 1994).

Finally, flagella assembly and chemotaxis traits identified Alphaproteobacteria, Betaproteobacteria and Acidobacteria as Phyla that were particularly enriched with this mechanism of motility, while bacterial Actinobacteria, Bacteroidetes, Cyanobacteria, Chloroflexi and Verrucomicrobia were depleted (Supplementary Table 9). The Proteobacteria, Firmicutes and Under-represented models were better suited for identifying specific Families homogenously enriched or depleted in bacterial flagella and chemotaxis (Supplementary Tables 10 and 11). Enriched Families included the Rhodospirillaceae, Nitrosomonadaceae, Neisseriales lineage, divergent Acidobacteria lineage, Sporomusaceae, Bacillaceae, Clostridiaceae and Planctomycetes. Other forms of motility such as twitching and gliding have been noted in the Pseudomonadaceae, Myxococcaceae and Cyanobacteria (McBride, 2001) but these traits were not identified by the Random Forest as being homogenously enriched in any Families. Furthermore, while Thaumarchaeota and Methanogens were both identified as being depleted in bacterial flagella assembly and chemotaxis traits (Supplementary Table 9 and 11), Archaea possess a structurally distinct flagellum more similar to the Type IV bacterial pilus (Jarrell and Albers, 2012). These taxa were not enriched with Type IV pilus, either, and it is possible that archaeal flagella may have failed proper characterisation by KEGG. Thus, while some Families were relatively enriched/depleted in bacterial flagella and chemotaxis traits, specific taxa depleted in these are not necessarily non-motile.

In summary, while the Random Forests may overlook certain traits in individual taxa, the models were highly robust in detecting conserved, shared traits within a Phylum/Family. Here the 'depth' of shared traits is limited by the number of taxa that could be considered as Phylum or Family. In future, if five (or more) taxa belonging to the same Genus or even Species could be compared, unique traits would be observed to explain how these subgroups have evolved from their respective Families to occupy

distinct niches. Ideally the selection of individual taxa within groups for such future comparative analyses would also be standardised based on phylogenetic distance, either with *P* or a similar method, that would improve the robustness of trait-based comparisons at such a fine taxonomic level.

4.3 Plant-derived carbon metabolism and nutrient acquisition

Secondly, we hypothesised that the traits differentially enriched between taxonomic groups would largely reflect those associated with copiotrophs or oligotrophs, namely metabolism, nutrient acquisition and environmental stress response and tolerance. Of fundamental interest to soil microbiologists is the decomposition of plant biomass. This is the primary source of organic carbon to non-arid terrestrial systems (Kögel-Knabner, 2002) and the transformation of plant material to substrates bioavailable for microorganisms is essential for community growth and activity. The traits involved in plant material catabolism belonged to the BRITE categories 'Starch and Sucrose Metabolism' (*e.g.* extracellular cellobiosidases, endoglucanases, glucosidases, trehalases, amylases) and 'Glycosyltransferases', all of which are carbohydrate activated enzymes (CAZy). The Families particularly enriched in these traits were the Polyangiaceae, Myxococcaceae, Rhizobiaceae, Streptomycetaceae, Mycobacteriaceae, Frankiaceae and Verrucomicrobia (Table 1, Supplementary Table 11). Genomic and culture-dependent analyses support *Sorangium cellulosum* (Polyangiaceae), *Streptomyces coelicolor* A3(2) (Streptomycetaceae) and *Chthoniobacter flavus* (Verrucomicrobia) as having particularly large genomes with extensive repertoires for cellulose, hemicellulose, pectin and lignin degradation (Bentley *et al.*, 2002, Sangwan *et al.*, 2004, Schneiker *et al.*, 2007). Comparative genomics analyses have also identified Actinobacteria, Acidobacteria and Verrucomicrobia as being enriched in numerous enzymes for cellulose, hemicellulose and starch catabolism (Trivedi *et al.*, 2013). *In situ* these Families likely play a critical role in making organic carbon bioavailable as di- and monosaccharides for the microbial community.

The Families equipped with many copies of high-affinity sugar uptake 'Phosphotransferase systems' (3 – 14 copies) did not necessarily correspond to those enriched with CAZy – only the Myxococcaceae and Verrucomicrobia were enriched in both. Pseudomonadaceae, Rhodospirillaceae, Neisseriales lineage and

Clostridiaceae were only enriched in PTS. Despite being enriched in CAZy, the Frankiaceae were simultaneously depleted in PTS. The complex interplay between taxa capable of producing extracellular CAZy and competitors that rapidly scavenge available di- and monosaccharides has been well described by models that predict such competitive interactions exert important controls on the growth rate of the community as a whole (Freilich *et al.*, 2011) and may even act to aid terrestrial carbon storage and limit carbon dioxide emissions from microbial respiration (Kaiser *et al.*, 2015). Here, we identified the 'specialist' Families enriched in CAZy traits *versus* the 'opportunists' scavenging for sugars via PTS (Table 1, Supplementary Table 11).

ABC transporters facilitate the ATP-dependent uptake of soluble compounds across membranes or export waste metabolites, extracellular enzymes and toxins (Young & Holland, 1999, Higgins, 2001). This means of active transport allows microorganisms to acquire nutrients with high affinity at concentrations of 5 – 500 μg carbon L^{-1} *versus* the less efficient diffusion of nutrients across membranes, dependent on extracellular concentrations of 0.5 – 5 mg carbon L^{-1} (Kuznetsov *et al.*, 1979). In the spatially heterogenous soil environment where the concentration of bioavailable carbon substrate often limits growth (Blagodatsky & Richter, 1998), possession of high affinity transporters likely provides a competitive advantage. The rhizosphere-associated Rhodospirillaceae, Rhizobiaceae and Burkholderiaceae tended to have the highest trait copies of ABC transporters (100 – 350 copies per genome, Supplementary Figure 4). The diverse, non-rhizospheric Deltaproteobacteria, Actinobacteria, Firmicutes, Cyanobacteria and Verrucomicrobia all had greater than 50 copies per genome, highlighting the importance of these traits in soil. The particularly high gene copy number in rhizosphere-associated taxa from presumably nutrient-rich environments contrasts the assumption that ABC transporters are considered to play a greater role in nutrient-poor environments (Lauro *et al.*, 2009). Comparative genomics analyses of soil bacteria have also found putatively copiotrophic Proteobacteria and Firmicutes to be particularly enriched in PTS and ABC transporters (Trivedi *et al.*, 2013). In this study, t~~T~~he transporters enriched in ~~these~~ rhizosphere-associated taxa were primarily aimed at scavenging maltose, phosphate, amino acids, oligopeptides and export of LPS, and these results suggest that these traits are not only for survival in nutrient-poor environments but also likely confer a competitive advantage in the rhizosphere. As prokaryotes compete simultaneously with other prokaryotes and plants for nitrogen

and phosphorus in the rhizosphere, the high affinity acquisition of such nutrients is likely critical for growth.

4.4 Nitrogen and methane metabolism

The BRITE category 'Nitrogen metabolism' encompasses nitrogen fixation, denitrification, ammonia oxidation and synthesis of glutamate/glutamine which are critical amino acids for peptide synthesis. Since nitrogen limitation acts as an important control on soil microbial activity, these traits are also of interest to soil microbiologists. Three Proteobacterial Families, Rhodospirillaceae, Bradyrhizobiaceae and Burkholderiaceae, were particularly enriched in these traits. Genomic and culture-dependent analyses show these Families to be free-living or symbiotic diazotrophs in soil and freshwater environments (Madigan *et al.*, 1984, Itakura *et al.*, 2009, de los Santos *et al.*, 2018). Given their significantly greater copies of nitrogen-fixing genes, these Families may be a particularly important source of organic nitrogen for soil communities. Saprotrophic Mycobacteriaceae genomes, also identified as nitrogen cyclers, tend to have many copies of genes involved in ammonia uptake and glutamate synthesis (Amon *et al.*, 2010). This taxon may play an alternative role in converting mineral nitrogen to biomass where organic nitrogen as protein in excreted products or necromass can undergo proteolysis and uptake between other community members. The identification of Sporomusaceae as enriched in 'Nitrogen metabolism' traits is unusual as these obligate anaerobic fermenters cannot use nitrate as an electron acceptor (Möller *et al.*, 1984). Nor were the Sporomusaceae enriched in ammonia uptake or glutamate synthesis genes (data not shown), and so it is uncertain what role this Family plays in nitrogen cycling. Thaumarchaeota and Nitrosomonadaceae, known ammonia oxidisers, were not enriched in 'Nitrogen metabolism' traits relative to other Families (Supplementary Figure 5) despite Nitrosomonadaceae possessing multiple copies of the operon responsible for ammonia oxidation (Klotz & Norton, 1998). Specific traits may be overlooked here if the BRITE category includes many diverse KOs (*e.g.* ammonia oxidation, nitrogen fixation, glutamate synthesis etc).

Another specialised metabolic pathway of interest involves 'Methane metabolism' that includes production and oxidation of a potent greenhouse gas. Unsurprisingly, the Methanogens and methanotrophic Methylococcaceae, Beijerinckiaceae, Methylocystaceae were all enriched in traits involved in methane metabolism. While

methane oxidation can be present in some taxa from the Verrucomicrobia (Op den Camp *et al.*, 2009) the above proteobacterial representatives act as the primary terrestrial methane sink (Dunfield, 2007, Conrad, 2009).

4.5 Sensing, responding and tolerating the environment

A particularly interesting divergence of traits were involved in how taxa detect and respond to environmental stimuli. Gram-negative Pseudomonadaceae, Rhodospirillaceae, Bradyrhizobiaceae and Myxococcaceae were enriched in two-component systems. These membrane-bound histidine kinases respond rapidly to extracellular stimuli (Galperin, 2005) and these traits were primarily involved in nitrogen, potassium, initiating chemotaxis and C_4-dicarboxylate responses. Families enriched in transcription factors were the Myxococcaceae, Polyangiaceae, Streptomycetaceae, Mycobacteriaceae and Frankiaceae. These factors regulate transcription in response to intracellular cues and here these factors were primarily *rpoD* (housekeeping), *rpoH* (heat-shock/protein damage), *rpoE* (extra-cellular cytoplasmic stress) and *rpoS* (starvation) responses (Shimada *et al.*, 2017). The genomes of these taxa are also heavily enriched in regulatory genes for complex developmental stages, fruiting bodies and/or filamentous branching growth in soils (Bentley *et al.*, 2002, Gao *et al.*, 2006, Schneiker *et al.*, 2007). Thus, certain taxa may respond primarily to extracellular cues while others strictly monitor and respond to changes in cell homeostasis. This trend has been noted previously – in 167 genomes across various Bacteria and Archaea, Proteobacteria had a higher ratio of sensors for external *versus* internal stimuli and were considered 'extroverts', while Cyanobacteria were considered strong 'introverts' focussed on responding to internal stimuli (Galperin, 2005).

As mentioned above, Archaea exhibited unique traits in basal transcription and protein regulation via proteasome. These transcription factors were primarily involved in identifying DNA damage and excision repair: TFII-B, TFII-D, ERCC-2 and ERCC-3. DNA repair differs markedly between Bacteria and Archaea/eukaryotes. Specifically, Bacteria excise 12 nucleotides around a damaged site with a 3 polypeptide system whereas Archaea excise 24 – 32 nucleotides with a 13 – 16 polypeptide system (Sancar, 1996). The use of ubiquitin-labelling and proteasome degradation of misfolded or 'old' proteins is arguably a more efficient system for recycling amino acids

and regulating the 'lifespan' of a protein in eukaryotes, however, Bacteria are still fully capable of regulating protein misfolding or proteolysis with RpoH (and others) induced upon environmental stress (Goldberg, 2003). From an ecological perspective, it is difficult to discern if these eukaryote-like traits confer any sort of competitive advantage to Archaea. They may simply be examples of convergent evolution for dealing with environmental stress.

Finally, most microbial cells likely exist within complex biofilms and/or assemblages adhered to surfaces with excreted exopolysaccharides, DNA and protein that serve to protect from adverse environmental factors (Flemming & Wingender, 2010). Families with high copy numbers of exopolysaccharide biosynthesis and secretion systems may act as integral members of soil communities by predominantly contributing to biofilm/aggregate formation. The 'LPS biosynthesis' and 'Starch and sucrose metabolism' BRITE categories can synthesise N-acetyl glucosamine-based and cellulose-based exopolysaccharides, respectively. Taxa enriched in both these categories and secretion systems were the Polyangiaceae and Burkholderiaceae (Table 1), and in the refined 'Under-represented' model, Acidobacteria and Verrucomicrobia (Supplementary Table 11).

4.6 Direct cell-cell interactions

Type IV secretion systems were another important trait identified in the Random Forest models. These were enriched in Pseudomonadaceae and Myxococcaceae (Table 1) and Acidobacteria, Planctomycetes and Verrucomicrobia (Supplementary Table 11). These are highly specialised exporters that deliver DNA and/or toxins directly to other bacterial or plant cells, however, their role in ecology is poorly understood beyond root galls induced by *Agrobacterium tumefaciens* (Christie & Vogel, 2000). These taxa should be explored for whether they utilise these traits for horizontal gene transfer or to inject toxins directly into other prokaryotes, and thus potentially provide a selective advantage for colonisation and competition.

Another archetypal trait for interactions between community members are production of antimicrobials and antimicrobial resistance genes. Penicillin and cephalosporin synthesis were enriched in the Sporomusaceae and Bacillaceae relative to other Firmicutes. Polyketide and Type II polyketide syntheses were important for separating Frankiaceae and Streptomycetaceae from other Actinobacteria

(Supplementary Table 10). The Streptomycetaceae have a long history of use in biotechnology as prolific antimicrobial producers (Bentley *et al.*, 2002). Bacillaceae (in particular *Bacillus subtilis* species) are also well known producers of a wide variety of antimicrobials (Caulier *et al.*, 2019), but we noted that Sporomusaceae have an even greater number of these traits (Supplementary Figure 9). To the authors' knowledge, antibiotic production in Sporomusaceae has not been investigated thoroughly and this may be a consequence of its obligate anaerobic nature and difficulties in culturing. In addition to prolific Type II polyketide producers, Streptomycetaceae were also enriched in antimicrobial resistance genes, while Bacteroidetes, Planctomycetes and Verrucomicrobia were specifically enriched in beta-Lactam resistance (Supplementary Tables 9 and 10).

4.7 L~~Generalisable l~~ife strategies emerge from differentially enriched traits

We hypothesised that taxa would emerge as being inherently copiotrophic or oligotrophic based on trends in their enriched traits. Traits were chosen based on identification via Random Forest and identification as associated with copiotroph-oligotroph species or in mixed communities as described previously (Lauro *et al.*, 2009, Vieira-Silva & Rocha, 2010, Roller & Schmidt, 2015, Pascual-Garcia & Bell, 2020). Rhizosphere-associated Gamma-, Alpha- and Betaproteobacteria in Clade I fit the assumptions of a copiotrophic niche that invests in high metabolic rate – these taxa were uniquely enriched in competing for nutrient uptake via high-affinity ABC transporters, and energy generation from pyruvate, fatty acids, benzoate and glyoxylate carbon sources. Clade I was also enriched in glutathione metabolism, which acts as the major antioxidant for reducing intracellular free radicals produced during central carbon metabolism (Smirnova & Oktyabrsky, 2005). Antioxidants have been hypothesised as an essential function for copiotrophs to survive their high metabolic rates (Koch, 2001). All five Clade I Families were enriched in oxidative phosphorylation. The oxidative phosphorylation traits encompass a wide variety of electron transport chain proteins (oxidoreductases, dehydrogenases, cytochromes and ATPases) and are crucial for efficient energy production (Brochier-Armanet *et al.*, 2009). All five Families were also enriched in nitrogen metabolism, which included both nitrogen fixation and glutamate (*i.e.* protein) synthesis. Nitrogen fixation is an energy intensive process requiring 20 – 30 ATP per reduced N_2 (Burris & Roberts, 1993) and may be

intrinsically linked to taxa with high oxidative phosphorylation. Finally, Clade I also shared motility and chemotaxis, which are also energy intensive traits. Clade II consisted of the remaining Gamma-, Alpha- and Betaproteobacteria, yet these were relatively less enriched in Clade I 'copiotroph' traits. These particular taxa may be responsible for the lack of a consistent copiotrophic response upon nutrient addition in Proteobacteria (Ho *et al.*, 2017).

Clade III was comprised of taxa generally considered as oligotrophs (Ho *et al.*, 2017) with the exception of Bacteroidetes (Fierer *et al.*, 2007). These taxa possessed high LPS and sphingolipid synthesis that can defend against desiccation and antimicrobials through biofilm and capsule/slime production (Flemming & Wingender, 2010), beta-Lactam resistance, penicillin biosynthesis and several members had high pentose phosphate pathway for efficient carbon metabolism under starvation (Hodgson, 2000). Clade III also possessed high CAZy traits, which Clade I largely lacked, and is consistent with observations of oligotrophs being primarily responsible for catabolising relatively recalcitrant plant material (Goldfarb *et al.*, 2011). While Clade III were equally enriched in oxidative phosphorylation as Clade I, with the exception of the Deltaproteobacteria, these taxa were depleted in glutathione metabolism. The low copies per genome of this trait would explain why the abundance of oligotrophs drop rapidly in nutrient addition studies as they would be either out-competed by glutathione-rich taxa capable of exploiting plentiful nutrients or will lyse if their metabolic rate exceeds capacity to reduce free radicals (Koch, 2001). Taken together, all of these traits indicate Clade III lead oligotrophic lifestyles whereby they are tolerant to adverse environmental conditions, can acquire carbon from recalcitrant plant material, and are incapable of rapid growth rates.

These results support previous observations that Rhodospirillaceae, Bradyrhizobiaceae, Burkholderiaceae, Pseudomonadaceae and Rhizobiaceae are copiotrophic, while Planctomycetes, Verrucomicrobia, Myxococcaceae, Polyangiaceae and Acidobacteria are oligotrophic (Ho *et al.*, 2017 and references therein). As has been proposed previously, the dominance of these groups in certain soils can provide inferences for ecosystem processes in that system, for example soils dominated by Verrucomicrobia, Planctomycetes and Acidobacteria will have greater capacity to degrade complex plant material while retaining most catabolised carbon in biomass (*i.e.* high growth or carbon use efficiency) or excreted byproducts that assist in soil aggregation (*e.g.* high LPS production) (Trivedi *et al.*, 2013). Conversely, soils

dominated by copiotrophic Proteobacteria Families will be systems primarily dependent on labile di- and monosaccharides that demonstrate low carbon use efficiency.

Streptomycetaceae, Mycobacteriaceae and Frankiaceae in Clade IV shared enrichment of several Clade I copiotroph traits. As mentioned above in Section 4.5, these Actinobacteria invest carbon and energy into complex filamentous growth and developmental cycles. They demonstrate classic copiotrophic responses to nutrient addition (Goldfarb *et al.*, 2011, Leff *et al.*, 2015) and their enriched ABC transporters, pyruvate, glyoxylate, benzoate and fatty acid metabolism all likely contribute to generating energy for complex lifecycles. Simultaneously, their enrichment of Clade III oligotroph traits for CAZy metabolism in addition to many traits for producing and resisting antimicrobials indicate a unique niche for these Actinobacteria that does not necessarily fall within the classical copiotroph-oligotroph framework.

Clade V differed markedly from all other clades and mostly consisted of 'specialist' metabolic functional groups involved in photosynthesis, ammonia oxidation, methanogenesis, lactic acid production and other fermentation. Similar to Clade III, these taxa would also be expected to have relatively low metabolic rates due to depletion of copiotroph traits associated with rapid metabolism and energy generation. Unlike Clade III, these taxa seemed to lack consistent mechanisms for stress tolerance. Thus, while certain taxa did invest in traits for rapid metabolic rate (Clades I and IV) and others primarily in stress tolerance (Clades III and IV), some taxa lacked these approaches altogether and pursued entirely distinct niches (Clade V). As an average of 47% of traits within each genome were Uncharacterised, Clade V is an over-simplification and that if novel, currently uncharacterised proteins and the traits they fulfil were incorporated into hierarchical clustering, this clade would separate more meaningfully.

If one were to consider taxa within the one-dimensional copiotroph-oligotroph spectrum, Clade I would represent one extreme, Clades III and V another, with Clades II and IV falling in between. Figure 5a is a conceptual diagram where these Clades have been placed on a singular axis of 'resource investment', with the niche space of Clades enriched in traits associated with rapid growth (*e.g.* glutathione) toward the 'copiotrophy' pole while Clades enriched in stress tolerance traits (*e.g.* LPS production) are placed toward the 'oligotrophy' pole. However, this approach overlooks the diverse functional potentials (and distinct niches) for carbon and energy metabolism ~~in Clade~~

~~V~~associated with the various Clades. Furthermore, the large overlaps in niche space between Clades would suggest taxa from each group could not co-exist if 'resource investment' was the only important consideration (Gause, 1932, Hutchinson, 1957, Leibold, 1995). A more meaningful perspective would be to consider the additional role of 'resource acquisition' that incorporates multiple axes for the life strategies identified via hierarchical clustering of traits. Figure 5b is a conceptual diagram of niche space where clades have been further separated along additional dimensions based on their enrichment of traits involved in competition, degradation or specialised metabolic pathways. The BRITE categories listed on the various axes are chosen to be useful markers in predicting the niche of a taxon. ~~-~~This expanded niche space would suggest ~~hierarchical clustering in Figure 4 with each clade as its own 'life strategy'. The strategy of~~ taxa in Clade ~~I follow that of classical 'copiotrophs' and~~I are well equipped for nutrient acquisition (primarily, but not limited to, di- and monosaccharides), rapid growth and oxidative stress regulation as 'copiotrophic competitors'. Clade II, which may not be capable of competing directly with Clade I for carbon and energy, ~~and perhaps the evolution of~~may thus occupy the niche space of specialist Proteobacterial methylotrophs, methane and ammonia oxidisers ~~may be a response~~so as to ~~avoid competition over saccharides~~be 'copiotrophic metabolic specialists'. The strategy of Clade III ~~is~~ would be to ~~specialise in decomposing~~decompose plant material via diverse CAZy and possess a variety of environmental stress tolerance traits as 'oligotrophic degraders'. Clade IV, which include for example ~~are unique~~ Actinobacteria that share traits for competition, degradation and oligotrophy, could thus occupy niche space between Clades I and III ~~traits, yet likely fill a distinct niche due to complex developmental cycles and filamentous growth~~. Finally, the strategy of Clade V ~~is~~ would be to fill highly specialised, unrelated metabolic niches reliant on completely distinct carbon sources to other taxa. The large space conceptualised for Clade V in Figure 5b, which does not imply either copiotrophic or oligotrophic resource investment, is an oversimplification and with improved understanding of traits in these taxa it may be possible to fracture and further separate them into more detailed groups. This would prove particularly beneficial for the poorly characterised Archaea. ~~and are unified by an absence of classical copiotroph and oligotroph traits.~~

Moving beyond a one-dimensional *r-K* spectrum to accommodate additional trait-driven life strategies has been proposed in plant ecology (Grime, 1977). Specifically, Grime argued that plant taxa fall within a multi-dimensional space defined by extremes

on three axes: 'competitors' that acquire nutrients, light, water etc. more effectively than neighbouring taxa in the same environment, 'stress tolerators' that are long-lived, slow growing taxa that resist desiccation, alkaline soils etc., and 'ruderals' that have very brief lifecycles between periods of disturbance and invest in environmentally hardy seeds. Despite these varied strategies for resource investment, plants are unified in that photosynthesis is their primary form of acquiring carbon and energy. The diversity of microbial strategies for acquiring carbon and energy enables them to explore a greater range of potential niche space, and in addition to growth traits that allow for a relatively more copiotrophic or oligotrophic investment of those resources, likely contributes to the high diversity of co-existing taxa observed in soil microbial communities~~complexity of co-occurring traits across microbial Families indicate more generalisable life strategies than the copiotroph-oligotroph spectrum are needed~~.

However, to truly unravel differentiated niches~~—~~ and general microbial life strategies~~within these generalisable life strategies~~, two limitations must be overcome. Firstly, ~~will require~~ a better understanding of the many 'Uncharacterised' traits in environmental isolates~~,~~ ~~in addition to robust trait-based analyses at the Species or even Strain taxonomic level~~is required. For example, the recent large-scale MAG study by Nayfach *et al*., (2021) identified 5.8 million protein clusters (traits), of which over 75% could not be annotated meaningfully by current protein databases. Secondly, robust trait-based analyses down to the finer scale of distinct genomes will likely be necessary to consider how individual taxonomic members of a community have either differentiated in order to co-exist or are in the throes of competition that will ultimately exclude one of the competitors.

5. Conclusion

In a collection of 175 terrestrial prokaryotes that possess 220 664 traits shared between at least two taxa, concepts in niche differentiation were explored. Non-random trait distributions were shown as preferential clustering of related taxa within most Phyla with a general trend of highest similarity at the level of Family. This strongly supported ecological coherence of shared traits within close relatives. Random Forest models successfully identified BRITE 3 categories that best explained differing traits between taxonomic groups. These traits were involved in a wide range of biological functions, including core physiological traits used historically to categorise taxa. Many

traits were also involved in functions often associated with copiotrophs and oligotrophs, namely metabolism, nutrient acquisition and environmental stress tolerance. Hierarchical clustering of differential traits formed five distinct clusters, with Clade I representing the classical copiotrophic niche, Clades III and V as oligotrophic, and Clades II and IV in between. A more refined perspective would be to consider each Clade as its own ~~generalisable~~ life strategy in a niche space that considers both resource investment and acquisition simultaneously; for example, the strategy of Clade I is to invest in competition and rapid growth, while Clade V pursue highly distinct, specialised metabolic functions. The trait-based analyses here were effective in identifying general trends in potential function of terrestrial microbial taxa at the Phylum and Family level. Further investigation will be necessary to identify traits that give rise to niche differentiation at lower taxonomic ranks and, ultimately, the importance of this for ecosystem processes of interest.

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Conflict of Interest

The authors declare no conflict of interest regarding this study or its outcomes.

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