1 **An ecological perspective on microbial genes of unknown function in soil**

4 Hannah Holland-Moritz*[1], Chiara Vanni[2], Antonio Fernandez-Guerra[3], Andrew Bissett[4], and Noah
5 Fierer[5]

7 [1] Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH,
8 USA
9 [2] Microbial Genomics and Bioinformatics Research Group, Max Planck Institute for Marine
10 Microbiology, Celsiusstraße 1, 28359, Bremen, Germany
11 Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany
12 [3] Lundbeck GeoGenetics Centre, The Globe Institute, University of Copenhagen, Copenhagen,
13 Denmark
14 [4] CSIRO, Oceans and Atmosphere, Hobart, Tasmania, Australia
15 [5] Department of Ecology and Evolutionary Biology, Cooperative Inst. for Research in Environmental
16 Sciences, University of Colorado, Boulder, USA

19 **Competing Interests Statement**
20 The authors declare no competing financial interests.

Abstract

Genes that remain hypothetical, uncharacterized, and unannotated comprise a substantial portion of metagenomic datasets and are likely to be particularly prevalent in soils where poorly characterized taxa predominate. Documenting the prevalence, distribution, and potential roles of these genes of unknown function is an important first step to understanding their functional contributions in soil communities. We identified genes of unknown function from 50 soil metagenomes and analyzed their environmental distributions and ecological associations. We found that genes of unknown function are prevalent in soils, particularly fine-textured, higher pH soils that harbor greater abundances of *Crenarchaeota, Gemmatimonadota, Nitrospirota,* and *Methylomirabilota*. We identified 43 dominant (abundant and ubiquitous) gene clusters of unknown function and determined their associations with soil microbial phyla and other "known" genes. We found that these dominant unknown genes were commonly associated with microbial phyla that are relatively uncharacterized, with the majority of these dominant unknown genes associated with mobile genetic elements. This work demonstrates a strategy for investigating genes of unknown function in soils, emphasizes the biological insights that can be learned by adopting this strategy, and highlights specific hypotheses that warrant further investigation regarding the functional roles of abundant and ubiquitous genes of unknown function in soil metagenomes.

Introduction

Soils are one of the largest reservoirs of genetic diversity on Earth (1,2). One gram of soil can harbor hundreds to thousands of distinct microbial taxa (3,4) and an estimated 1 000 Gbp of microbial genome sequences (5). Soil microbial communities are essential contributors to terrestrial nutrient cycling and carbon dynamics, with wide-ranging effects on plant and animal health (6). Unfortunately, soil microbial taxa are underrepresented in culture and genomic sequence databases (6,7). The adoption of high-throughput sequencing technologies has yielded new insights into the full-extent of soil

49 microbial diversity and provide glimpses into the genomes, transcriptomes, and functional attributes of

50 organisms that are as-yet uncharacterized. While these molecular approaches bring clear benefits to

51 understanding soil microbial communities, they also pose a fundamental challenge as a large fraction of

52 genomic data is composed of genes of unknown function.

53   Genes of unknown function are abundant in many habitats (40-60% of genes in a given

54 environment (8–11)). While this issue is not unique to soils, several aspects of soil microbiology

55 indicate that soil microbial communities harbor large proportions of genes of unknown function. First,

56 soils are one of the most phylogenetically diverse microbial habitats (12,13) and the genetic diversity

57 found within soils is equally impressive (2,14,15). Second, the majority of soil microbes are difficult to

58 isolate and study in the laboratory (16). There are many reasons for this, including the observation that

59 many soil microbes are likely slow-growing oligotrophs that can be difficult to culture using standard

60 approaches (17). Thus, soil environments often harbor a higher proportion of uncultivated taxa (18,19)

61 as compared to environments (like human skin and gut) where a larger fraction of the microbial

62 communities are represented in culture-based collections (16). Third, many physical and biological

63 aspects of soil, such as micro-site heterogeneity and high strain-level variation, make it a particularly

64 challenging environment to apply assembly-based metagenomic techniques (6,20). Despite these

65 limitations, the genes of unknown function found in soil may hold important ecological information

66 (9,21). From work with marine and human microbiomes (11), we know that these genes are enriched in

67 phyla with more genomes represented only in metagenome-assembled genomes (MAGs), have more

68 restricted distributions than known genes (i.e., less likely to be shared across distinct samples), and are

69 more common in marine environments than in host-associated environments. Metagenomic studies of

70 soil microbial communities typically focus only on "known" genes, i.e. genes that can be functionally

71 annotated with a reasonably high degree of confidence (with some notable exceptions, e.g. (19)),

72 effectively discarding the unannotated genes from downstream analyses and ignoring the insight that

73 these genes may provide about microbial life in soil. While these unannotated genes are often

74 acknowledged (2,11,12), they are typically not the explicit focus of study.

75   We set out to understand the genes of unknown function in 50 soil samples from a publicly

76 available dataset compiled by the Australian Microbiome Initiative (22). Specifically, we used a new

77 tool, AGNOSTOS (11), to identify gene clusters and group them into four categories: "Known with

78 Pfam Annotation", "Known without Pfam Annotation", "Genomic Unknowns" (found in cultured and

79 sequenced isolates and in MAGs of sufficient quality to be included in genome databases), and

80 "Environmental Unknowns" (found in metagenomes) – see Table 1. Using these data, we asked the

81 following questions: 1) How abundant are genes of unknown function? 2) Are there particular soil

82 conditions, or soils containing particular microbial taxa, that are more likely to harbor higher

83 abundances of these genes? 3) Which genes of unknown function are particularly abundant and

84 ubiquitous across soils? Broadly, we hypothesized that genes of unknown function would be

85 widespread in soils and the proportion of these genes found in metagenomes would vary along

86 ecological gradients, with higher proportions in soils where environmental conditions limit the

87 abundances of more copiotrophic taxa (19). We further hypothesized that the genes of unknown

88 function would be enriched in soil that contained higher abundances of under-studied microbial

89 lineages (i.e. lineages with few cultivated representatives). Finally, we compiled a "hit list" of abundant

90 and ubiquitous ("dominant") genes of unknown function in soils and used their associations with

91 microbial taxa and genes of known function to infer their potential ecological characteristics and direct

92 future research efforts to further explore the novel genetic diversity found in soil microbial

93 communities.

94

95 **Methods**

96 *Sample selection*

97   We used publicly available soil metagenomic data from the Australian Microbiome Initiative

98   Biomes of Australian Soil Environments (BASE) project (22). The project includes 449 soil samples

99   for which shotgun metagenomic data are available. From this subset of samples that had metagenomic

100 data, we selected a random sub-sample of 48 surface soil samples and viewed them on a map to make

101 sure that they represented a good spatial sampling of regions across Australia (Figure S1A). The goal of

102 this random sub-sampling was to reduce the number of samples to a computationally manageable size

103 and to select a set of samples that represented a wide range of environmental gradients across Australia.

104 The sample identifiers are listed in Table S1 and raw sequencing data are available at the Australian

105 Microbiome Initiative Data Portal (https://data.bioplatforms.com/organization/about/australian-

106 microbiome) as well as on the NCBI Sequence Read Archive (BioProject accession number

107 PRJNA317932).

108

109 *Sampling protocol, DNA extraction, and sequencing*

110 The Australian Microbiome Initiative uses a standard set of protocols for all samples. Full

111 details are available at https://www.australianmicrobiome.com/protocols/. Specific protocols used for

112 the samples and data included in this study are found under protocol headings labeled ("as per BASE").

113 To summarize, between 9-30 soil samples were taken from the top 10 cm of a 25×25 m plot with the

114 plot location selected to represent a visually homogeneous soil environment. Soil samples from the plot

115 were then homogenized to generate one representative surface sample per plot with the composited

116 sample frozen immediately after collection.

117 DNA was extracted from triplicate 250 mg sub-samples of each soil using the DNeasy

118 PowerLyzer PowerSoil Kit (Qiagen), following the Earth Microbiome protocol (23), and pooled prior

119 to downstream processing. Metagenomic sequencing libraries were prepared following the Illumina

120 TruSeqNano DNA Sample Preparation Guide. Briefly, 200 ng of DNA from each sample was sheared

121 to 550bp using Covaris sonic shearing. Clean-up of the sheared DNA, ligation of the adapters, and PCR

122   amplification of the DNA followed the TruSeq Nano DNA Sample Preparation Guide. The resulting

123   libraries were sequenced on an Illumina HiSeq with 2x150bp sequencing chemistry. The median

124   number of reads per sample was 30.2 million reads, with a range from 11.8 million reads to 154.9

125   million reads (Table S1).

126

127   *Environmental data*

128   To better understand the relationships between genes of unknown function and environmental

129   gradients, we selected ten soil and site factors that are often important in structuring soil microbial

130   communities (6). The factors included four site-specific variables: mean annual temperature, mean

131   annual precipitation, aridity (as inferred from calculation of the aridity index, (24)), and net

132   aboveground primary productivity (NPP). We also included six soil-specific variables: soil pH, percent

133   organic carbon, extractable inorganic nitrogen, phosphorus, conductivity, and texture (% silt + % clay)

134   (Figure S1B). We calculated Pearson correlations between each of these environmental variables. Most

135   were weakly to moderately correlated in our samples, except site aridity index and mean annual

136   precipitation, which were understandably well-correlated as precipitation is used to calculate the aridity

137   index (Figure S1B). As is evident from Figure S1C, the 48 samples span broad gradients in soil and site

138   characteristics (Figure S1C).

139   Analyses of soil chemical and physical properties were conducted by a commercial laboratory

140   (CSBP Laboratories, Bibra Lake, Western Australia) using standard methods. Briefly, percent organic

141   carbon was measured according to the Walkley-Black method using concentrated sulfuric acid and

142   dichromate in a colorimetric test. For extractable inorganic nitrogen, we summed extractable nitrate and

143   ammonium together. Both nitrate and ammonium were extracted with 2M KCl and measured

144   colorimetrically. Available phosphorous was calculated using the Colwell method (25). We also

145   summarized the texture of each soil into a single metric by summing the silt and clay percentages.

146   For each soil, we also compiled data on climate and plant productivity (i.e., mean annual

147   temperature, mean annual precipitation, aridity index, and NPP) at the collection site from the Atlas of

148   Living Australia spatial portal (26). From this data portal, we selected the mean annual precipitation,

149   aridity index, and NPP standard environmental data products produced by the ecosystem sciences

150   division of Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO), and

151   the mean annual temperature product from the WorldClim database (27).

152

153   *Generation of metagenomic assemblies and taxonomic composition table*

154   We downloaded the raw sequence data from the Bioplatforms Australia Data Portal

155   (https://data.bioplatforms.com/). After downloading, we removed adapters and other library-preparation

156   contaminant sequences from the FASTQ files using Cutadapt (28) and filtered out sequences with low

157   average quality scores and short sequence length with Sickle (29) (-q 20 -l 50). We verified the read

158   quality of our filtered data with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

159   and MultiQC (30) before proceeding. To assemble the reads, we tried three different assemblers (IDBA

160   – settings: default (31), metaSPAdes – settings: default (32), and MEGAHIT – settings: "meta-large"

161   preset (33)) and ultimately used MEGAHIT as it represented a good balance between assembly quality

162   and computational burden. To minimize downstream computational costs, we filtered out small contigs

163   <1000 bp, since these were unlikely to contain whole genes. We assessed assembly quality with

164   QUAST (default settings) (34), and used Bowtie2 (default settings) (35) to map the filtered reads back

165   to the filtered assemblies.

166   As is common for soils, assembly rates were fairly low with an average assembly length of 123

167   Mbp (ranging from 7 – 954 Mbp), and a mean N50 of 0.79 Kbp (ranging from 0.6 – 1.3 Kbp) before

168   filtering. We note that, although the assemblies are fragmented, our analyses focused on dominant and

169   abundant gene clusters (see below), which are less likely to be affected by the low assembly rates.

170   Assembly, rather than read-based analysis, is required for downstream analysis with AGNOSTOS (see

171   below). Full assembly statistics and mapping rates can be found in Table S1.

172   We also determined the taxonomic composition of the microbial communities in each sample.

173   We used phyloFlash (36) in "almost everything" mode to identify and assign taxonomy to fragments of

174   the small-subunit (16S/18S) rRNA gene in each metagenome. Using the number of hits to fragments

175   identified in each sample, we created a taxon-by-sample table for downstream analyses. After creating

176   the table, we filtered the table to exclude taxa identified as Chloroplast, Mitochondria, or Eukaryotes,

177   with these categories typically representing <7.2% of recovered small-subunit rRNA gene reads. We

178   also removed any taxa that were unassigned at the phylum level (<3.5% of small-subunit rRNA gene

179   reads). After filtering the table, we controlled for differences in sequencing depth by rarefying the table.

180   We picked 1720 rRNA gene hits at random from each sample, which was the number of hits in the

181   sample with the lowest number of hits. Finally, we converted our taxon-by-sample table into

182   proportional abundances for downstream analyses.

183

184   *Identifying genes of unknown function*

185   To identify genes of unknown function in our samples we used AGNOSTOS (11). AGNOSTOS

186   is a tool for partitioning coding sequence space into known and unknown clusters of genes. It clusters

187   genes based on sequence homology and classifies each gene cluster into one of four categories, *Known*

188   *with Pfam Annotation, Known without Pfam Annotation, Genomic Unknown*, and *Environmental*

189   *Unknown* (Table 1). To integrate soil metagenomes with the original AGNOSTOS database

190   (seedDB+NCLDV), we used the "DB-update" module of AGNOSTOS described in (37) which

191   integrates new sequences into the original gene cluster database. We note that the AGNOSTOS

192   database is dominated by prokaryotic (bacterial and archaeal) genes, as genes from eukaryotes

193   (including fungi) and viruses represent only a small fraction of the total database (37). Briefly,

194   AGNOSTOS uses Prodigal (38) to identify open reading frames in assembled contigs and filters out

195 spurious open reading frames that overlap with true protein-coding open reading frames (commonly

196 called "shadow genes" (38) and those that are found in the Antifam database of spurious open reading

197 frames (40). Importantly for metagenomic analysis, partial and incomplete genes are retained.

198 AGNOSTOS then annotates these genes against Pfam (41) using hmmsearch from HMMER (42). After

199 the Pfam annotation, genes are clustered with MMseqs2 (43) to create a homology-based database that

200 is independent of a gene cluster's known or unknown status. AGNOSTOS then screens the gene

201 clusters for quality, ensuring that each cluster is made up of sufficiently homologous genes, and

202 discarding those clusters composed of insufficiently similar genes and those composed of a high

203 number of spurious genes. At this stage, clusters containing singletons are identified, but were not

204 further categorized as there is insufficient information in a singleton cluster for reliable categorization

205 into one of the four categories (Table 1). Although singleton clusters lack sufficient information to

206 rigorously categorize, it is likely that the majority of these clusters represent genes of unknown

207 function that are rarer in databases and in our samples. We note that Vanni and others (37) found that,

208 as new data is added to the AGNOSTOS database, many of the singleton gene clusters acquire more

209 representation and can be identified and characterized, suggesting that the majority of these sequences

210 are not sequencing artifacts or assembly errors, but simply rarer genes. After clusters are cleaned and

211 filtered (including removing singletons), a consensus sequence for each cluster is generated and

212 clusters are each assigned to one of the four categories (Table 1). Gene clusters with at least one

213 member assigned to a Pfam domain of known function are classified as "known with Pfam". Gene

214 clusters with a consensus sequence that matched sequences in the NCBI *nr* database (44), UniRef90

215 database (45) or the Uniclust database (46), are categorized as either "known without Pfam annotation"

216 or "genomic unknowns" depending on their similarity to characterized proteins or to proteins annotated

217 with terms typical of genes of unknown function (e.g. "hypothetical", "uncharacterized", etc.) or

218 another name used to classify unknown proteins in the databases' controlled vocabulary. Gene clusters

219 with Pfam annotations to domains of unknown function ("DUF") are then also added in the "genomic

220 unknown" category. Finally, any gene clusters whose consensus sequences do not align with any of the

221 databases are characterized as "environmental unknowns".

222   After running AGNOSTOS, we identified ~3.7 million genes overall with an average of about

223 63 000 genes per sample. Of the 3.7 million predicted genes, ~2.43 million (66%) were assigned to one

224 of the 574 173 original AGNOSTOS database gene clusters. The remaining genes (~1.24 million) were

225 clustered separately into 152 634 new gene clusters with more than one gene and 713 940 'clusters'

226 singletons. The new gene clusters (which contained only soil genes) and singletons from the original

227 database that recruited new genes when the soil samples were integrated, were processed through the

228 AGNOSTOS validation, refinement, and classification steps. The new validated set of high-quality

229 gene clusters was integrated with the original clusters to form a new database of ~598 000 gene clusters

230 containing ~126 million genes (including ~2.61 million soil genes). We generated a gene-cluster-by-

231 sample table of gene cluster abundances by using the average coverage depth of each cluster in each

232 sample. As with the taxon-by-sample table (see above), we combined duplicate samples at this point.

233

234 *Statistical Analyses*

235   To determine the soil or site characteristics that correlated with the observed variation in the

236 proportional abundances of gene clusters of unknown function in our samples we ran Pearson

237 correlations between the percentage of gene clusters of unknown function in each sample (defined as

238 the number of gene clusters assigned to "genomic unknown" and "environmental unknown" divided by

239 the total number of non-discarded gene clusters), and each of the ten environmental variables we had

240 selected for analysis. We used the same methodology to determine which taxonomic groups (phylum

241 level) were correlated with the proportion of gene clusters of unknown function, after filtering out low-

242 abundance phyla (relative abundance < 0.1 %) to reduce the chance of spurious correlations. For these

243 correlations, we used the false discovery rate method (47) to correct the p-values for multiple tests.

244   We also identified "dominant" gene clusters of unknown function in soils. A gene cluster was

245   considered dominant based on its abundance and ubiquity across samples, following Delgado-

246   Baquerizo et al. (13). To identify gene clusters of unknown function that were abundant and ubiquitous

247   in soils, we filtered the 550 000 gene clusters in our 48 samples to remove those that were found in

248   fewer than five samples (< 10% of soil samples). This reduced the list of gene clusters to ~64 000

249   clusters. Using the Bowtie2 mapping results, we first normalized the per-base coverage by the length of

250   the gene and used this normalized coverage as a proxy for abundance. Then we calculated the median

251   normalized coverage for each gene cluster across all samples. Gene clusters with a median normalized

252   coverage across all samples >0 were considered abundant. After filtering for dominant gene clusters,

253   we identified a total of 43 genomic unknowns and 2461 knowns. We recognize that deeper sequencing

254   would likely have increased the number of dominant gene clusters identified here. However, our goal

255   was not to identify all genes (whether "known" or "unknown") in each soil, as that is arguably an

256   impossible task given the complexity of soil metagenomes. Rather, our goal was to identify the more

257   abundant and ubiquitous genes found across the set of 48 soils included here.

258   Because all of the dominant unknowns were classified as genomic unknowns, we wanted to

259   understand which taxa correlated with abundances of these unknowns. To determine this, we ran

260   Spearman correlations between the relative abundances of dominant unknown genes and the

261   abundances of microbial phyla in our soil samples. We used false discovery rate to correct for multiple

262   tests (47). To better understand the functions of the unknowns we ran correlations between each

263   dominant cluster of known function and each dominant unknown cluster using SparCC (48) as

264   implemented in the FastSpar software package (49). To minimize the chance of capturing spurious

265   correlations, we used a p-value cutoff of 0.05, and selected only the strongest correlations (correlation

266   > 0.5) for downstream analysis. Finally, we visualized the results of these gene cluster-to-gene cluster

267   correlations in a co-occurrence network. The network was created in R, using packages R packages

268   tidygraph (https://tidygraph.data-imaginist.com/), and ggraph (https://ggraph.data-imaginist.com/).

269

270 *GTDB-classification of dominant gene clusters*

271   To better understand the scope of genomic diversity represented by the dominant unknown gene

272 clusters, we used the Genome Taxonomy Database (GTDB) (50) to assign taxonomy to the contigs

273 containing at least one of each of the 43 dominant unknown gene clusters. We used MMSeqs2 (43) to

274 query sequences against GTDB and assign taxonomy using the MMSeqs2 taxonomic assignment

275 methodology. Briefly, MMSeqs2 uses a computationally efficient method to query each gene on a

276 genome fragment against a reference database and assigns taxonomy to genome fragments containing

277 those genes (i.e., the contigs assigned to one of the 43 dominant unknown genes clusters) based on the

278 2bLCA taxonomy assignment protocol (51). The 2bLCA method filters hits to a database for quality

279 based on an e-score cut-off (e < $1x10^{-12}$) and finds the lowest common ancestor of all hits that pass

280 filter. We used GTDB (version 95) as our taxonomy reference database and the following parameters:

281 "--tax-lineage 2 --majority 0.5 --vote-mode 1 --lca-mode 3 --orf-filter 1 --lca-ranks

282 superkingdom,phylum,class,order,family,genus" to run MMSeqs2. From these results, we were able to

283 calculate the number of genomes in GTDB with at least one dominant unknown gene cluster and the

284 most likely taxonomic assignment of each contig based on the taxonomy associated with those

285 genomes in GTDB.

286

287 **Results and Discussion**

288 *Abundance of genes of unknown function in soils*

289   We identified ~550 000 gene clusters (and ~607 000 singleton clusters for a total of ~1.16

290 million clusters) that met the established criteria across the 48 soil samples and calculated the

291 proportion of genes in each sample assigned to each of the four categories (Table 1). On average across

292 the samples, 73.6% of gene clusters were known with Pfam, 10.2% were known without Pfam, 15.8%

293 were genomic unknowns, and 0.4% were environmental unknowns (Figure 1A). The proportion of

294   unknowns varied from 11.9% to 21.7% (Figure 1A). Although not included in downstream analyses as

295   they could not be categorized into the four categories (Table 1), we note that singletons represented a

296   large proportion of identified genes in soils, with nearly twice as many singletons as unknowns (Figure

297   S2A). Singletons also varied from around 13% to 30% of gene clusters per sample, confirming that a

298   large fraction of the genes in soil metagenomics are likely of unknown function (Figure S2A).

299   Importantly, the majority of unknown gene clusters were classified as "genomic unknowns", suggesting

300   that most unknown gene clusters in these soils represent "low hanging fruit", that is genes that are

301   unknown (unannotated, hypothetical, or otherwise uncharacterized), but found in genomic databases of

302   sequenced organisms, and therefore more easily characterized than environmental unknowns (i.e., those

303   found exclusively in metagenomes or metagenome-assembled genomes) (11).

304   While the soil metagenomes were found to have a high proportion of genomic unknowns

305   (approximately 12-20% of non-singleton gene clusters), we note that quantifying the specific

306   proportion of genomic unknowns comes with important caveats. First, our ability to detect genes of

307   unknown function in soils is limited by our ability to detect genes in assembled soil metagenomic data.

308   Therefore, we are likely missing many genes from these soil metagenomes as a result of the well-

309   recognized challenges posed by soil metagenomic analyses, and evidenced by the low number of

310   sequences that mapped to our assembled contigs (Table S1) (14,52). The large proportion of singletons

311   we identified in the samples supports this explanation, as they likely represent rarer, genomic and

312   environmental unknowns that are insufficiently captured in our assemblies. These computational

313   challenges limit our ability to detect genes of unknown function that are less abundant or exhibit

314   attributes such as highly variable regions, that make assembly challenging (52–54). Second, the

315   methods we used to classify genes as having a known function yield estimates that are fundamentally

316   conservative. Although a gene may be identified as known, this does not mean that we know much

317   about its function across the wide breadth of microbial diversity (9). As evolution is inherently an

318   iterative process, many proteins from within the same family may exhibit different functions in

319   different organisms and ecological contexts. For example, ammonia monooxygenase and methane

320   monooxygenase genes are assigned to the same Pfam family (AMO), but are functionally distinct (55).

321   Likewise, the similarities between *nifH* genes (involved in nitrogen fixation) and *frxC* genes (involved

322   in light-independent chlorophyll biosynthesis) place them in the same Pfam family (Fer4_NifH),

323   despite coding for distinct proteins (56). Therefore, our estimate of the proportion of genes of unknown

324   function in soil metagenomes is likely an underestimate.

325   Despite these caveats, we next sought to understand how the proportion of genes of unknown

326   function in soils compared to other metagenomic datasets that were also processed with AGNOSTOS.

327   We compared the total percentages of gene clusters in each category in two additional environments:

328   marine waters and the human microbiome (see refs. (57–61) for assembly statistics). We compared the

329   total number of gene clusters in each category identified in our soil samples to the total number of gene

330   clusters in each category identified in the four marine and two human microbiome metagenomic

331   surveys used in Vanni et al. (11) (Figure 1B). We found that the percentages of gene clusters in each

332   category were consistent across the human microbiome, marine, and soil environments in three of the

333   four gene-cluster categories. On average, 66-74% of the gene clusters in each environment were known

334   with a Pfam designation, 10-13% were known without a Pfam designation, and 16-22% were genomic

335   unknowns. The proportion of environmental unknowns in soils was lower than in marine and human-

336   associated environments, with 0.93% and 1.6% of gene clusters assigned to this category in human and

337   marine environments, respectively, and only 0.32% of soil gene clusters found as environmental

338   unknowns. However, given the diversity of soil metagenomes and the aforementioned challenges

339   associated with assembling genes from soil metagenomes (52,53), this comparatively small proportion

340   of environmental unknowns is likely to be a product of under-sampling the less-abundant genes in soil

341   metagenomes. In support of this explanation, we note the high proportion of singletons in soils (Figure

342   S2B) which are likely composed of a majority of real genes that are rare rather than sequencing

343   artifacts or assembly errors (37).

344 With the exception of environmental unknowns, the proportions of each gene cluster category

345 were similar across environments. This observation runs counter to our hypothesis that soils would

346 have a higher proportion of unknown genes than other environments. Although it is likely that we are

347 missing many environmental unknowns due to insufficient sequencing and fragmented assemblies, the

348 consistency of our results across environments is supported by their similarity to proportions of each

349 gene category found by Vanni et al. (11) within the GTDB which contains genomes and high quality

350 MAGs from a wide variety of environments. These general similarities in the proportions of genes

351 across environments and genomes suggest that the proportions of unknown and known genes is fairly

352 consistent even across distinct habitat types.

353 Interestingly, although the proportions of unknown genes in soils were similar to those in

354 marine and human microbiome surveys, this result was not necessarily a product of the same gene

355 clusters being found across all three environments. Of the gene clusters found in the soil samples, ~

356 377 000 (68%) of the clusters were not found in either the human or the marine data sets. Those soil-

357 specific gene clusters were slightly enriched in genomic unknowns (29 %) and environmental

358 unknowns (1.1%) when compared to the set of gene clusters that included human and marine clusters

359 (22.8 % and 0.01 %). These results suggest that although proportions of unknown genes are similar

360 across environments, the soil-specific gene clusters may represent a source of unknown genetic

361 potential that is distinct from that found in either marine or human-associated environments.

362

363 *Environmental and microbial groups and genes of unknown functions*

364 Although the majority of gene clusters from assembled contigs in our samples were known, the

365 proportion of unknown non-singleton gene clusters in each soil sample varied from 12-20%. To better

366 understand which environmental factors explained variation in the percentage of unknown genes in our

367 samples, we ran correlations between ten environmental factors (Figure S1C) and the proportion of

368 gene clusters of unknown function in our samples. Of the ten environmental factors, only soil pH (cor =

369 0.42, p = 0.003) and texture (cor = 0.49, p = 0.001) were significantly correlated with the proportion of

370 gene clusters of unknown function (Figure S3). Importantly, these results were unlikely to be a product

371 of differences in sequencing depth across our samples as the proportion of unknown genes in our

372 samples was not strongly correlated with either sequencing depth (Pearson correlation = 0.26, p =

373 0.08), or with the total number of genes per sample (Pearson correlation = 0.32, p = 0.03).

374 Unknown gene clusters were enriched in high-pH soils and finer-textured soils. It is notable that

375 of the ten environmental factors tested, only pH and texture were found to be significant. The most

376 likely explanation for this pattern is that higher pH and finer-textured soils are more likely to harbor a

377 greater fraction of microbes with high proportions of genes of unknown function. This explanation is

378 supported by the analysis of correlations between individual microbial phyla and the proportion of

379 unknown genes in the metagenomes (Figure S4). We identified five phyla that were significantly

380 positively correlated with the percentage of unknowns, and four phyla that were negatively correlated

381 with unknowns. *Crenarchaeota, Gemmatimonadota, Nitrospirota, Entotheonellaeota,* and

382 *Methylomirabilota* were all positively correlated with the proportion of unknown genes.

383 *Bdellovibrionota*, WPS-2 (Eremiobacterota), *Proteobacteri*a, and *Patescibacteria* were all negatively

384 correlated with the percentage of unknowns (Figure S4). Many of the positively correlated phyla are

385 also positively correlated with pH or texture in our dataset (*Gemmatimonadota*: pH – rho = 0.66,

386 *Crenarchaeota*: pH – rho = 0.60, *Methylomirabilota*: texture – rho = 0.47, *Nitrospirota*: pH – rho =

387 0.47, *Entotheonellaeota*: pH – rho = 0.46, p < 0.05, Spearman correlations) and some of the negatively

388 correlated phyla are negatively correlated with soil pH (WPS-2/Eremiobacterota: pH – rho = -0.55,

389 *Proteobacteria*: pH – rho = -0.50, p < 0.05, Spearman correlations). While correlations do not imply

390 causal relationships, the most parsimonious explanation for these results is that higher pH and finer

391 textured soils are more likely to harbor certain phyla that have higher proportions of genes of unknown

392 function and serve as ripe targets for explorations of novel genetic diversity.

393

A "hit list" of ubiquitous and abundant genes of unknown function in soils

To identify the dominant gene clusters found in our samples, we filtered our data to select only the most abundant and ubiquitous gene clusters in soil samples (see Methods). Applying these criteria yielded 43 gene clusters of unknown function, all of which fell into the genomic unknown category, and 2461 gene clusters of known function (Figure 2). For simplicity, we hereafter refer to the 43 abundant and ubiquitous gene clusters as "dominant" unknown gene clusters.

To better understand the genomic and ecological context of the dominant unknown gene clusters, we ran correlations between the clusters and phyla in our samples (Figure 3). Positive correlations between the dominant gene clusters and taxa ranged from 0.41-0.77. While some of the gene clusters were positively correlated with many taxa (e.g., GC34767203 correlated with 8 phyla), others were only significantly correlated with one or two phyla (e.g., GC17275105). The phyla that had the largest numbers of positive correlations to the dominant unknown gene clusters included the Unclassified Phylum RCP2-54, *Verrucomicrobiota, Proteobacteria, Acidobacteriota,* and *Planctomycetota* (Figure 3). Many of the phyla that were positively correlated with dominant unknown gene clusters were lineages that are typically found in high abundances in soils (13). While many of these lineages, such *Acidobacteria* or *Verrucomicrobia*, contain large numbers of uncultivated taxa (62) some of these phyla come from lineages that have been reasonably well-studied (e.g. *Proteobacteria*). While positive correlations do not necessarily imply the presence of these genes in genomes from members of these phyla, these well-studied taxa potentially represent a straightforward and simple route to discovering the functions of these gene clusters as many of the members of these phyla are reasonably well-represented in culture collections and genomic databases. By searching for these dominant unknowns in genome databases, and cross-referencing the results with strains that are available in culture collections, it may be feasible characterize the functions of dominant unknown genes in soils using targeted experiments, as demonstrated by Price et al. (9).

418   To further understand the genomic context of the dominant unknown gene clusters, we searched

419   for the contigs associated with our 43 dominant unknown gene clusters in the GTDB (50) and used a

420   consensus taxonomy assignment approach to assign taxonomy to each contig. Our goal was to try to

421   identify which taxa might harbor these dominant unknown genes by searching for these genes in a

422   curated genome database, versus simply assessing correlations between taxa and cluster abundances

423   directly from shotgun data (Figure 3). The median number of genomes with matches to each dominant

424   unknown gene cluster was 175, ranging from 38 (GC36965309) to 796 (GC38645763) genomes per

425   dominant cluster and, in general, clusters that were unique to our soil data set (i.e., not found in either

426   the marine or human microbiome datasets) had fewer hits to genomes in the database (Figure 4).

427   Confirming the correlation-based results described above and in Figure 3, the most well-represented

428   phyla across the dominant unknown gene clusters were those that are typically abundant in soils (e.g.,

429   *Proteobacteria*, *Acidobacteria*, and *Actinobacteria*) (Figure 4). However, there are some important

430   discrepancies between the taxonomic results from the GTDB-database and the correlation results

431   (Figure 3). Many of the less well-studied lineages that were correlated with the abundances of

432   particular phyla in our soil dataset (Figure 3) are rare or absent from the GTDB analysis (e.g.,

433   Unclassified Phylum RCP2-54 and *Verrucomicrobia*). This is likely due to the fact that some of these

434   lineages are not well represented in GTDB. For example, there are no sequenced genomes or MAGs for

435   RCP2-52 and, even other phyla for which far more genomes are available in GTDB, genomes from

436   representative soil lineages may not be well-represented in GTDB. For example, the phylum

437   Actinobacteria, one of the best-represented lineages in GTDB and one of the most abundant phyla in

438   soils globally (13) has 24 602 entries in GTDB, but only 427 (1.7%) are representatives isolated from

439   soil. Thus, we expect that some of these dominant unknown genes in soil may be associated with

440   lineages that are reasonably common in soil, but under-represented in GTDB and comparable genomic

441   databases. Alternatively, the observed discrepancies may be the result of spurious correlations with the

442   correlation-based analyses (Figure 3). However, the overall consistency between the two results

443   suggests that the majority of these relationships are likely robust, and that a paired approach using

444   multiple methods of taxonomic assignment can be a productive method to generate hypotheses about

445   the taxa associated with genes of unknown function.

446   In addition to determining the potential taxonomic affiliation of the dominant genes of unknown

447   function, we also sought to determine their potential functions. We did so by assessing relationships

448   between known (annotated) gene clusters and the dominant "unknown" gene clusters across the 48

449   metagenomes, following the working assumption that annotated and unknown genes which are

450   correlated may have related functions (63,64). In total, 16 of the 43 gene clusters had at least one

451   positive correlation with known gene clusters above the established threshold. All 16 of these genomic

452   unknowns were correlated with mobile genetic elements such as transposases, reverse transcriptases, or

453   phage integrases (Table 2). These mobile genetic element-associated genes made up the majority of

454   "known" genes correlated with each of the 16 dominant genes of unknown function with (Table 2).

455   These patterns are evident from a co-occurrence network visualization of the associations between

456   genes of known function and those of unknown function (Figure 5, see Figure S5 for a version of this

457   network with labeled known gene clusters). Importantly, many of the gene clusters associated with

458   mobile genetic elements were also positively correlated with more bacterial and archaeal phyla (Figure

459   3), and their positive correlations with mobile genetic elements may suggest a mechanism as to why

460   they are so taxonomically widespread.

461   Mobile genetic elements represent one of the main categories of unexplored genetic diversity

462   (65) and are thought to be one of the most abundant functional gene categories across the tree of life

463   (66). Mobile elements are associated with elevated genomic plasticity and accelerated biological

464   diversification (66). While traditionally associated with antibiotic resistance genes, one study of

465   integrons across a broad diversity of metagenomes ranging from oceans, to guts, to soils, found that

466   many are likely to be toxin/anti-toxin genes, but that most integron genes are of unknown function (65).

467   Their results are consistent with the findings from this study, which suggest that many of the most

468 dominant genes of unknown function in soils are correlated with the abundances of transposases,

469 recombinases, resolvases, phage integrases, and other mobile genetic elements. Clearly further

470 investigations into the ecology, function and genomic contexts of mobile genetic elements are

471 warranted, considering that many of the dominant unknown genes we identified from soil tend to co-

472 occur with genes coding for mobile genetic elements.

473

474 **Conclusions**

475 We have demonstrated that genes of unknown function comprise a substantial portion of the

476 genes in soils and we are likely underestimating their true abundances due to challenges associated

477 with adequately capturing the full extent of soil metagenomic diversity. Most notably, genes of

478 unknown function were more abundant in some soils than others and this variance was, in part,

479 predictable from soil edaphic properties and the taxonomic composition of the soil microbial

480 communities. Finally, we were able to identify the most dominant genes of unknown function in soil,

481 defined as those genes that are abundant and ubiquitous across a wide range of soil types. We find that

482 these dominant genes of unknown function are primarily found in typically abundant soil lineages that

483 are less-well studied such as *Verrucomicrobia* and *Acidobacteria,* with the majority of these dominant

484 genes associated with mobile genetic elements. Our work demonstrates the utility of investigating

485 genes of unknown function and represents a first step towards "ecological annotation" of the dominant

486 genes of unknown function in soils to elucidate what taxa are likely to have these genes, what

487 environments they are most likely to be found in, and the putative functions of the unknown genes that

488 represent an important fraction of soil metagenomes.

489

490 **Acknowledgments**

503   **Competing Interests Statement**

504   The authors declare no competing financial interests.

Bibliography

1. Alteio LV, Schulz F, Seshadri R, Varghese N, Rodriguez-Reillo W, Ryan E, et al. Complementary Metagenomic Approaches Improve Reconstruction of Microbial Diversity in a Forest Soil. mSystems. 2020;5(2):1–18.

2. Bahram M, Hildebrand F, Forslund SK, Anderson JL, Soudzilovskaia NA, Bodegom PM, et al. Structure and function of the global topsoil microbiome. Nature. 2018;560(7717):233–7.

3. Prosser JI. Dispersing misconceptions and identifying opportunities for the use of "omics" in soil microbial ecology. Nature Reviews Microbiology. 2015;13(7):439–46.

4. Schloss PD, Girard RA, Martin T, Edwards J, Thrash JC. Status of the Archaeal and Bacterial Census: an Update. Delong EF, McFall-Ngai MJ, editors. mBio [Internet]. 2016;7(3). Available from: https://mbio.asm.org/content/7/3/e00201-16

5. Vogel TM, Simonet P, Jansson JK, Hirsch PR, Tiedje JM, van Elsas JD, et al. TerraGenome: a consortium for the sequencing of a soil metagenome. Nature Reviews Microbiology. 2009;7(4):252.

6. Fierer N. Embracing the unknown: disentangling the complexities of the soil microbiome. Nature Reviews Microbiology. 2017;15(10):579–90.

7. Choi J, Yang F, Stepanauskas R, Cardenas E, Garoutte A, Williams R, et al. Strategies to improve reference databases for soil microbiomes. The ISME Journal. 2017;11(4):829–34.

8. Bernard G, Pathmanathan JS, Lannes R, Lopez P, Bapteste E. Microbial dark matter investigations: How microbial studies transform biological knowledge and empirically sketch a logic of scientific discovery. Genome Biology and Evolution. 2018;10(3):707–15.

9. Price MN, Wetmore KM, Waters RJ, Callaghan M, Ray J, Liu H, et al. Mutant phenotypes for thousands of bacterial genes of unknown function. Nature. 2018;557(7706):503–9.

10. Chen LX, Anantharaman K, Shaiber A, Murat Eren A, Banfield JF. Accurate and complete genomes from metagenomes. Genome Research. 2020;30(3):315–33.

11. Vanni C, Schechter MS, Acinas SG, Barberán A, Buttigieg PL, Casamayor EO, et al. Light into the darkness: Unifying the known and unknown coding sequence space in microbiome analyses. bioRxiv [Internet]. 2020; Available from: https://www.biorxiv.org/content/early/2020/08/11/2020.06.30.180448

12. Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey KJ, et al. A communal catalogue reveals Earth's multiscale microbial diversity. Nature. 2017;551(7681):457–63.

13. Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge DJ, Bardgett RD, et al. A global atlas of the dominant bacteria found in soil. Science. 2018;359(6373):320–5.

14. Howe AC, Jansson JK, Malfatti SA, Tringe SG, Tiedje JM, Brown CT. Tackling soil diversity with the assembly of large, complex metagenomes. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(13):4904–9.

15. Jansson JK, Hofmockel KS. The soil microbiome—from metagenomics to metaphenomics. Current opinion in microbiology. 2018;43:162–8.

16. Lloyd KG, Steen AD, Ladau J, Yin J, Crosby L. Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes. Neufeld JD, editor. mSystems [Internet]. 2018;3(5). Available from: https://msystems.asm.org/content/3/5/e00055-18

17. Carini P. A "Cultural" Renaissance: Genomics Breathes New Life into an Old Craft. mSystems [Internet]. 2019;4(3). Available from: https://msystems.asm.org/content/4/3/e00092-19

18. Brewer TE, Aronson EL, Arogyaswamy K, Billings SA, Botthoff JK, Campbell AN, et al. Ecological and Genomic Attributes of Novel Bacterial Taxa That Thrive in Subsurface Soil Horizons. Martiny J, editor. mBio [Internet]. 2019;10(5). Available from: https://mbio.asm.org/content/10/5/e01318-19

19. Chen Y, Neilson JW, Kushwaha P, Maier RM, Barberán A. Life-history strategies of soil microbial communities in an arid ecosystem. ISME Journal [Internet]. 2020; Available from: http://dx.doi.org/10.1038/s41396-020-00803-y

20. Lombard N, Prestat E, van Elsas JD, Simonet P. Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. FEMS Microbiology Ecology. 2011;78(1):31–49.

21. Berini F, Casciello C, Marcone GL, Marinelli F. Metagenomics: Novel enzymes from non-culturable microbes. FEMS Microbiology Letters. 2017;364(21):1–19.

22. Bissett A, Fitzgerald A, Meintjes T, Mele PM, Reith F, Dennis PG, et al. Introducing BASE: the Biomes of Australian Soil Environments soil microbial diversity database. GigaScience. 2016;5(1):21.

23. Marotz C, Amir A, Humphrey G, Gaffney J, Gogul G, Knight R. DNA extraction for streamlined metagenomics of diverse environmental samples. BioTechniques. 2017;62(6):290–3.

24. Middleton Nick, Thomas D. World atlas of desertification /. 2nd ed. London ; Arnold :; 1997.

25. Colwell J. An automatic procedure for the determination of phosphorus in sodium hydrogen carbonate extracts of soils. Chemical Industry. 1965;22:893–5.

26. Belbin L. The Atlas of Living Australia's spatial portal. In: Proceedings of the environmental information management conference. 2011. p. 28–9.

27. Fick SE, Hijmans RJ. WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. International Journal of Climatology. 2017;37(12):4302–15.

28. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011;17(1):10–2.

29. Joshi NA, Fass JN. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33) [Software]. 2011.

30. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32(19):3047–8.

31. Peng Y, Leung HCM, Yiu SM, Chin FYL. IDBA-UD: A de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics. 2012;28(11):1420–8.

32. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new versatile metagenomic assembler. Genome research. 2017/03/15 ed. 2017 May;27(5):824–34.

33. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2014;31(10):1674–6.

34. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. Bioinformatics. 2013;29(8):1072–5.

35. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature methods. 2013;9(4):357–9.

36. Gruber-Vodicka HR, Seah BKB, Pruesse E. phyloFlash: Rapid Small-Subunit rRNA Profiling and Targeted Assembly from Metagenomes. Arumugam M, Kato S, editors. mSystems [Internet]. 2020;5(5). Available from: https://msystems.asm.org/content/5/5/e00920-20

37. Vanni C, Schechter MS, Delmont TO, Eren AM, Steinegger M, Glöckner FO, et al. AGNOSTOS-DB: a resource to unlock the uncharted regions of the coding sequence space. bioRxiv [Internet]. 2021; Available from: https://www.biorxiv.org/content/early/2021/06/07/2021.06.07.447314

38. Hyatt D, Chen GL, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: Prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010;11.

39. Yooseph S, Li W, Sutton G. Gene identification and protein classification in microbial metagenomic sequence data via incremental clustering. BMC Bioinformatics. 2008;9(1):182.

40. Eberhardt RY, Haft DH, Punta M, Martin M, O'Donovan C, Bateman A. AntiFam: a tool to help identify spurious ORFs in protein annotation. Database [Internet]. 2012;2012. Available from: https://doi.org/10.1093/database/bas003

41. El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, et al. The Pfam protein families database in 2019. Nucleic acids research. 2019 Jan;47(D1):D427–32.

42. Finn RD, Clements J, Eddy SR. HMMER web server: interactive sequence similarity searching. Nucleic Acids Research. 2011;39(suppl_2):W29–37.

43. Steinegger M, Söding J. MMseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. Nature Biotechnology. 2017;35(11):1026–8.

44. NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research. 2017;46(D1):D8–13.

45. The UniProt Consortium. UniProt: the universal protein knowledgebase. Nucleic Acids Research. 2016;45(D1):D158–69.

46. Mirdita M, von den Driesch L, Galiez C, Martin MJ, Söding J, Steinegger M. Uniclust databases of clustered and deeply annotated protein sequences and alignments. Nucleic Acids Research. 2016;45(D1):D170–6.

47. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289–300.

48. Friedman J, Alm EJ. Inferring Correlation Networks from Genomic Survey Data. PLOS Computational Biology. 2012;8(9):1–11.

49. Watts SC, Ritchie SC, Inouye M, Holt KE. FastSpar: rapid and scalable correlation estimation for compositional data. Bioinformatics. 2018;35(6):1064–6.

50. Parks DH, Chuvochina M, Chaumeil P-A, Rinke C, Mussig AJ, Hugenholtz P. A complete domain-to-species taxonomy for Bacteria and Archaea. Nature Biotechnology. 2020;38(9):1079–86.

51. Hingamp P, Grimsley N, Acinas SG, Clerissi C, Subirana L, Poulain J, et al. Exploring nucleo-cytoplasmic large DNA viruses in Tara Oceans microbial metagenomes. The ISME Journal. 2013;7(9):1678–95.

52. Vollmers J, Wiegand S, Kaster A-K. Comparing and Evaluating Metagenome Assembly Tools from a Microbiologist's Perspective - Not Only Size Matters! PLOS ONE. 2017;12(1):1–31.

53. Quince C, Walker AW, Simpson JT, Loman NJ, Segata N. Shotgun metagenomics, from sampling to analysis. Nature Biotechnology. 2017;35(9):833–44.

54. Sczyrba A, Hofmann P, Belmann P, Koslicki D, Janssen S, Dröge J, et al. Critical Assessment of Metagenome Interpretation—a benchmark of metagenomics software. Nature Methods. 2017;14(11):1063–71.

55. Holmes AJ, Costello A, Lidstrom ME, Murrell JC. Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiology Letters. 1995;132(3):203–8.

56. Fujita Y, Takahashi Y, Chuganji M, Matsubara H. The nifH-Like (frxC) Gene Is Involved in the Biosynthesis of Chlorophyll in the Filamentous Cyanobacterium Plectonema boryanum. Plant and cell physiology. 1992 Jan;33(1):81–92.

57. Sunagawa S, Coelho LP, Chaffron S, Kultima JR, Labadie K, Salazar G, et al. Structure and function of the global ocean microbiome. Science. 2015;348(6237):1–10.

58. Kopf A, Bicak M, Kottmann R, Schnetzer J, Kostadinov I, Lehmann K, et al. The ocean sampling day consortium. Gigascience. 2015;4(1):s13742-015.

59. Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S, et al. The Sorcerer II global ocean sampling expedition: northwest Atlantic through eastern tropical Pacific. PLoS biology. 2007;5(3):e77.

60. Duarte CM. Seafaring in the 21st century: the Malaspina 2010 circumnavigation expedition. Limnology and Oceanography Bulletin. 2015;24(1):11–4.

61. Lloyd-Price J, Mahurkar A, Rahnavard G, Crabtree J, Orvis J, Hall AB, et al. Strains, functions and dynamics in the expanded Human Microbiome Project. Nature. 2017;550(7674):61–6.

62. Solden L, Lloyd K, Wrighton K. The bright side of microbial dark matter: Lessons learned from the uncultivated majority. Current Opinion in Microbiology. 2016;31:217–26.

63. Yi G, Sze SH, Thon MR. Identifying clusters of functionally related genes in genomes. Bioinformatics. 2007;23(9):1053–60.

64. Rogozin IB, Makarova KS, Wolf YI, Koonin EV. Computational approaches for the analysis of gene neighbourhoods in prokaryotic genomes. Briefings in bioinformatics. 2004;5(2):131–49.

65. Buongermino Pereira M, Österlund T, Eriksson KM, Backhaus T, Axelson-Fisk M, Kristiansson E. A comprehensive survey of integron-associated genes present in metagenomes. BMC Genomics. 2020;21(1):1–14.

66. Aziz RK, Breitbart M, Edwards RA. Transposases are the most abundant, most ubiquitous genes in nature. Nucleic Acids Research. 2010;38(13):4207–17.

507

Tables and Figures for:

An ecological perspective on microbial genes of unknown function in soil

Hannah Holland-Moritz*[1], Chiara Vanni[2], Antonio Fernandez-Guerra[3], Andrew Bissett[4], and Noah Fierer[5]

[1] Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH, USA

[2] Microbial Genomics and Bioinformatics Research Group, Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359, Bremen, Germany

Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany

[3] Lundbeck GeoGenetics Centre, The Globe Institute, University of Copenhagen, Copenhagen, Denmark

[4] CSIRO, Oceans and Atmosphere, Hobart, Tasmania, Australia

[5] Department of Ecology and Evolutionary Biology, Cooperative Inst. for Research in Environmental Sciences, University of Colorado, Boulder, USA
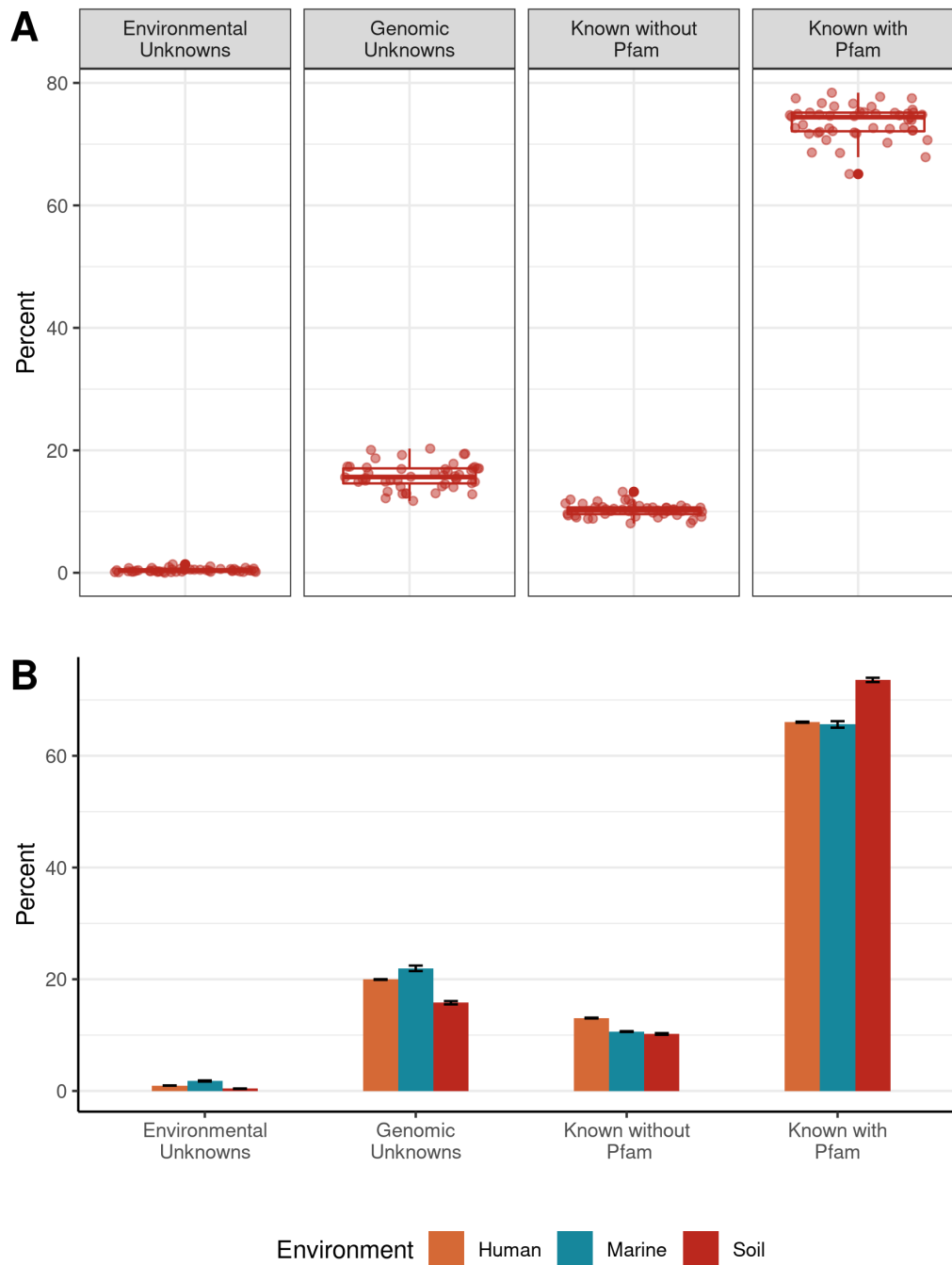
Figure 1: A) The percentage of each gene cluster category in our soil samples. B) A comparison between the percentages of each gene cluster category across metagenomes from three environments: soil, marine, and human-associated. Error bars represent mean standard error across samples.
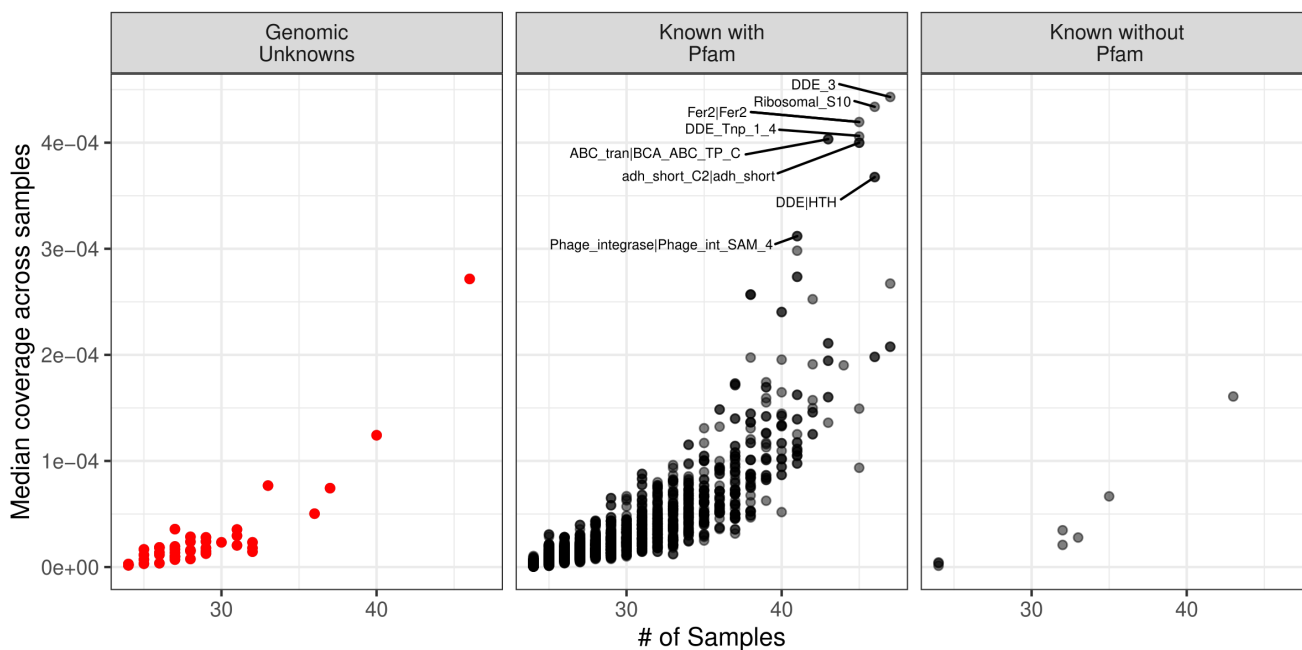
Figure 2: The "dominant" gene clusters in our samples. Each point represents one gene cluster. The x-axis shows the number of soil samples in which each gene cluster was found ("ubiquity") and the y-axis shows the median coverage of each gene cluster across those samples ("abundance"). The most dominant clusters are in the top right corner of each plot, while less dominant clusters are in the bottom left corner of each plot. We identified 43 dominant unknowns in our samples (red) and 2461 knowns (black). Environmental unknowns are omitted from this figure, as no environmental unknowns were sufficiently abundant (mean coverage > 0) and ubiquitous (present in > 10% of samples) to be considered a "dominant" gene cluster. Pfam designations for some of the most abundant and ubiquitous known genes are indicated in the "Known with Pfam" panel.
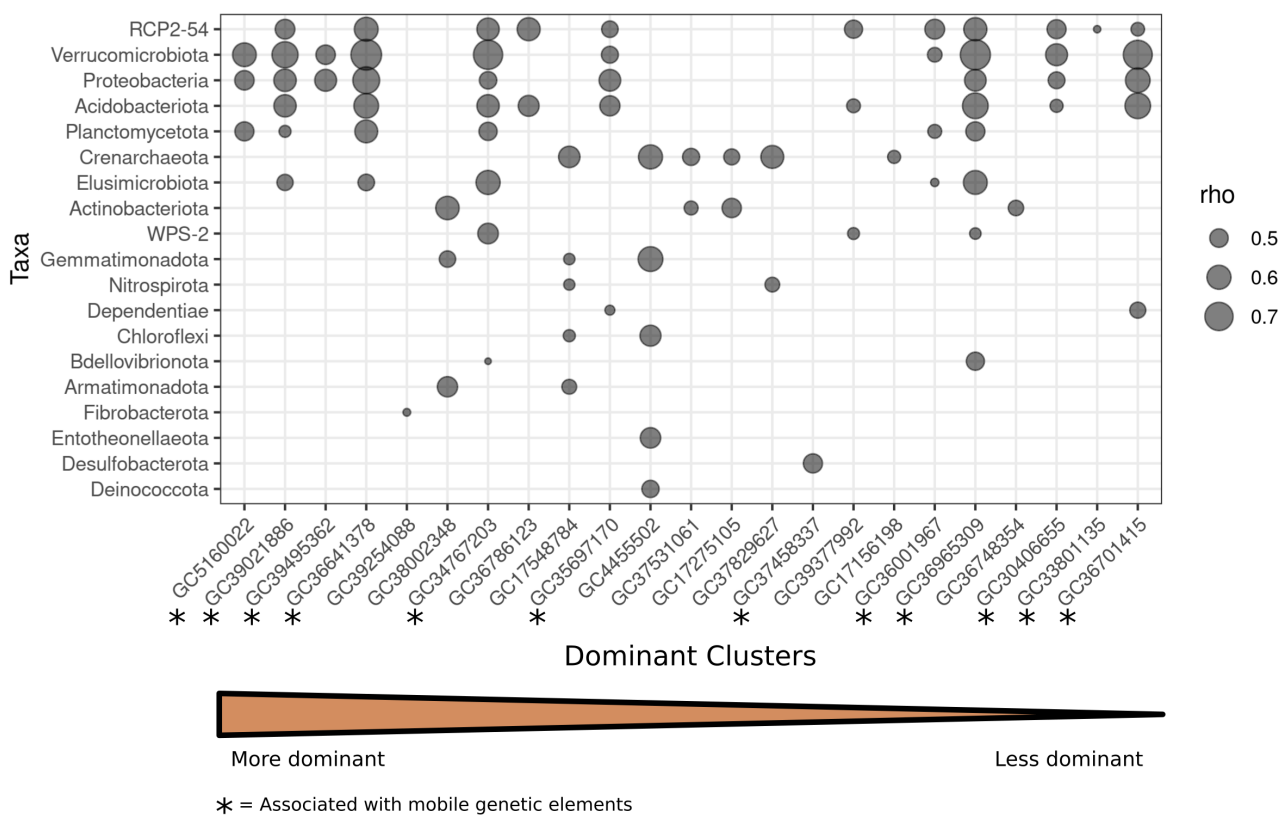
Figure 3: Positive correlations between dominant unknown gene clusters and specific bacterial and archaeal phyla across the 48 soil samples included in this study. Each point represents a significant Spearman correlation ($p < 0.05$) between one of the 43 unknown clusters (x-axis) and a microbial phylum (y-axis). Phyla are arranged in order of increasing number of significant correlations to dominant clusters (bottom to top). Clusters are arranged in decreasing order of dominance (left to right). The size of each point represents the strength of the rho value. Gene clusters that were also found to be strongly associated with many mobile genetic elements (see Figure 5) are indicated with asterisks.
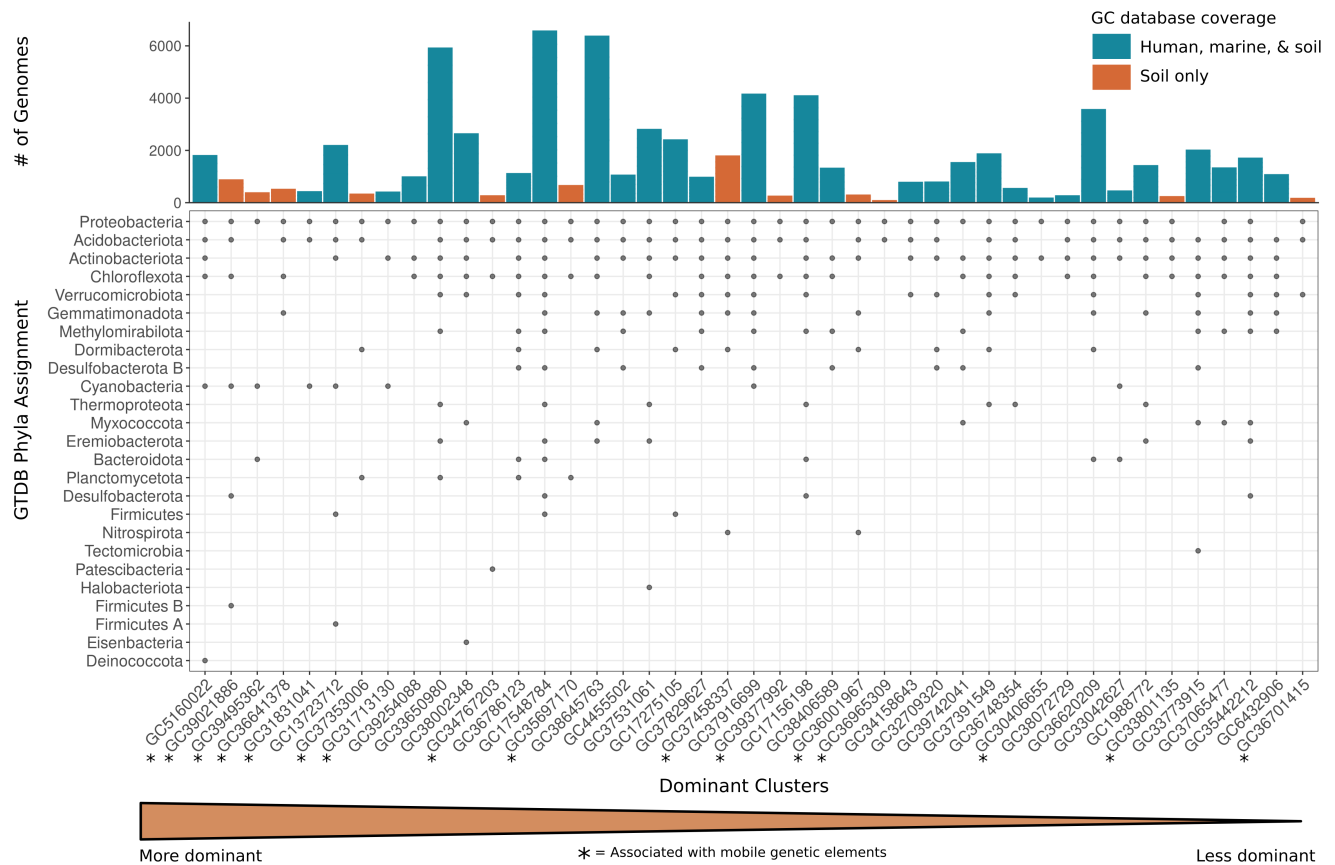
Figure 4: The taxonomic representation (phylum level) of contigs in dominant unknown gene clusters mapped against the GTDB genome database. Contigs within each dominant unknown gene cluster were assigned to a particular phylum based on best-hit consensus taxonomy assignments of the genes within that contig using the 2blca method as implemented in mmseqs2. In the lower panel, a point indicates the presence of at least one contig from the gene cluster (x-axis) being assigned to a particular phylum (y-axis). In upper panel, bars indicate the number of genomes that contigs within a given gene cluster mapped to (a hit to a genome was counted only for hits passing the filter threshold e-score $< 1 \times 10^{-12}$). The colors of the bars indicate whether or not a particular gene cluster was also found in marine or human microbiome datasets (blue) or if the cluster was only found in soils (orange). Dominant clusters that were also found to be strongly associated with many mobile genetic elements (see Figure 5) are indicated with asterisks.
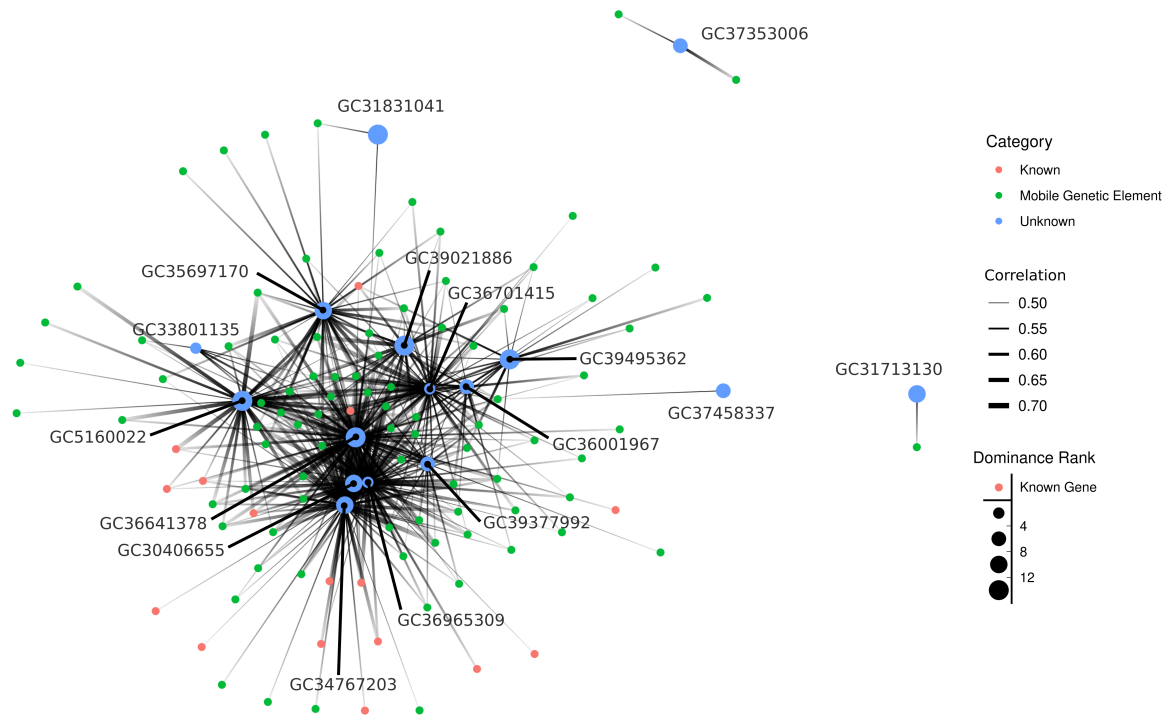
Figure 5: A co-occurrence network showing significant positive Spearman correlations (rho > 0.6) between dominant unknown gene clusters and dominant known gene clusters. Unknown genes are indicated with the blue points, and the size of the points indicates how dominant they are relative to other dominant unknowns, with larger circles indicating a greater level of dominance. Known genes are displayed in green. Green points represent known genes that are mobile elements while all other known genes are displayed in red. The thickness of the edges represents the strength of the rho value. A version of this figure with all points labeled with their Pfam designations can be found in the supplementary material (Figure S5)**.**

Category	Description
Knowns	
Known with Pfam annotation	Gene cluster contains at least one Pfam domain of known function
Known without Pfam annotation	Gene cluster contains genes that are without Pfam domain of known function, but are annotated with a function in the NCBI *nr* or UniRef90 databases
Unknowns	
Genomic unknown	Gene cluster contains at least one gene that is found in sequenced and draft genomes but are hypothetical, uncharacterized, or otherwise unannotated
Environmental unknown	Gene cluster contains genes that have no known sequence homology in sequenced or draft genomes and only found in metagenomes and MAGs

575 **Table 1:** Description of each gene cluster category.