Genomic, metabolic and literature oriented annotation of microbial co-occurrence networks enhances associations confidence level and hypothesis generation

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Abstract

Up to 350 words.
The abstract must include the following separate sections:
Background: the context and purpose of the study
Results: the main findings
Conclusions: a brief summary and potential implications

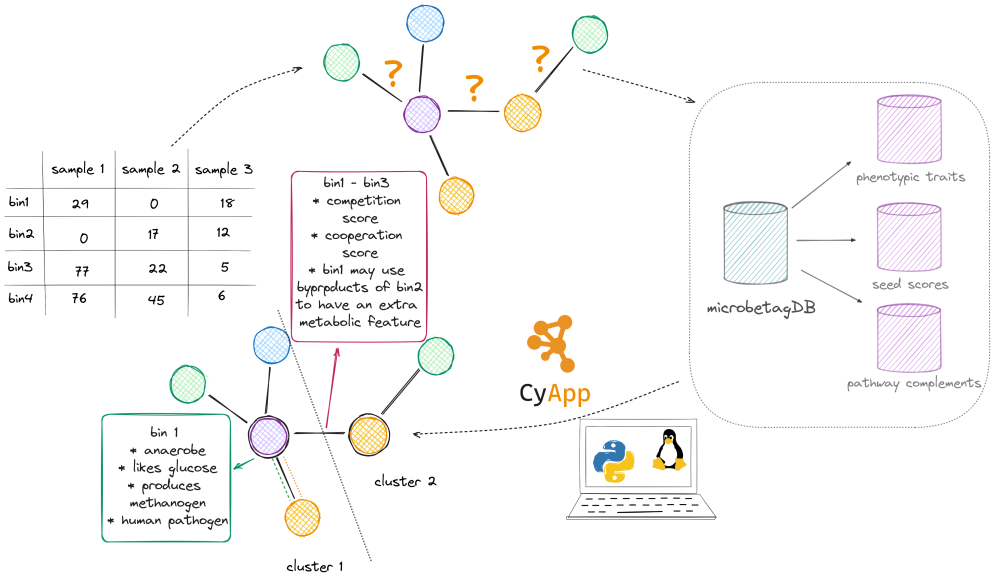

Figure abstract.

Keywords: microbial associations, enrichemnt analysis, data integration, pathway complementarity, seed set

1 Introduction

[1] [2]

- Role of microbial associations in the study of microbial communities
- HTP sequencing
- cooccurrence networks to analyse HTP data
- challenges of cooccurrence networks / refs: [1]: microbial network inference as a tool for interaction prediction has highlighted this tool's low accuracy and the biological implications of network properties are unclear / [2] : modules in microbial co-occurrence networks may be indicative of ecological processes governing community structure, such as niche filtering and habitat preference
- current approaches to deal with those challenges
- our contribution

It is well known that most microbial species live not in isolation but in communities [3]. Such communities play a crucial role in ecosystem functioning in almost

[1] We are to submit in the Microbiome journal as a "Software" manuscript, thus we follow these rules
[2] The introduction should not include subheadings.

any ecosystem type [4, 5]. High-throughput sequencing (HTP) has provided great insight into the diversity and composition of microbial communities [6]. Uncultivated species can now be detected and their featues can be inferred through their genomic information [7].

NGS facilitates culture-independent sampling of the microorganisms in an area with the potential for both taxonomic and functional annotation;

NGS not only provides information about the taxonomic composition of the microbial community but also enables the annotation of their functional capabilities. This combination of taxonomic and functional annotation provides a more detailed and holistic view of the role and activities of microorganisms.

Understanding the structure, composition, and dynamics of microbial communities is crucial for unraveling the intricate web of interactions that shape ecosystems. To come up with a comprehensive understanding of these interactions, we need to comprehend the relationships between microorganisms and thus their own interactions.

The notion of an interaction varies including cooperation, competition, parasitism, commensalism and ammensalism [5].

Microbial interactions play a crucial role in shaping ecosystems and influencing various biological processes.

These interactions contribute to the overall stability and functioning of ecosystems by influencing nutrient cycling, disease dynamics, and the overall diversity and composition of microbial communities. For example, some microorganisms can produce and release certain compounds that benefit neighboring microbes or inhibit the growth of competing species. Other interactions involve symbiotic relationships, where different microbes rely on each other for survival and perform complementary functions. Understanding and studying microbial interactions is vital for unraveling the complexity of microbial communities and their impact on human health, agriculture, and environmental processes.

Physical Interactions: Microorganisms can interact physically by forming biofilms, which are communities of microorganisms attached to surfaces and enclosed in a matrix of extracellular substances. Biofilms provide protection and promote cooperation among the microorganisms within them.

Quorum Sensing: Many bacteria use quorum sensing to communicate with each other and coordinate their behavior. They release and detect signaling molecules called autoinducers, which allow them to sense the local population density. This mechanism enables bacteria to coordinate processes like biofilm formation, virulence factor expression, and nutrient acquisition.

Antibiosis: Antibiosis refers to the production of antimicrobial substances by microorganisms that inhibit the growth or survival of other microorganisms. This can be a competitive strategy to gain an advantage in a particular environment.

metabolic interactions specifically, microorganisms often engage in metabolic exchanges during their interactions. For instance, one microbe may produce metabolites that serve as nutrients or signaling molecules for another microbe. These metabolic interactions can involve the transfer of essential nutrients, the breakdown of complex compounds, or the production of secondary metabolites with antimicrobial properties. Overall, understanding the different types of microbial interactions,

including metabolic interactions, provides insights into the complexity and dynamics of microbial communities and their impact on various processes in nature.

A widely used approach is the creation of co-occurrence networks based on community data. To build such networks, there is a great number of approaches: Spearman and Pearson correlations, CoNet [8] SparCC [9] SpiecEasi [10], MAGMA [11] and FlashWeave [12] are just a few of them. However, the outcome is usually tool-dependent [3, 13, 14].

FlashWeave

microbeAnnotator [15]

Related literature: Karaoz U and Brodie EL (2022) microTrai [16] , a computational pipeline that infers and distills ecologically relevant traits from microbial genome sequences. It does not apply networks

2 Implementation

[3]

Genomes included

Using the GTDB v202 metadata files, we retrieved the NCBI genome accessions of the representative genomes of high quality, i.e. completeness $\geq 95\%$ and contamination $\leq 5\%$. That resulted a set of $26,778$ covering $22,009$ unique NCBI Taxonomy Ids. Using these accession numbers, we were able to download their corresponding `.faa` files when available (`get_gtdb_faa.py`) leading to a set of $16,900$ amino acid sequence files.

Taxonomy schemes

microbetag maps the taxonomy of each entry in the abundance table to its corresponding NCBI Taxonomy id and if available its closest GTDB representative genome(s). Two well established taxonomy schemes are supported. The Genome Taxonomy DataBase (GTDB) [17] that is being broadly used in bins and/or MAGs taxonomical classification and the Silva database [18] that has NCBI Taxonomy [19]. The primer links the representative genomes included to their corresponding NCBI Taxonomy ids too.

There is a great number of taxonomies that are being used in such studies, e.g. Silva [18], Ribosomal Database Project (RDP) [20], manually curated ones and more, As a consequence, there is not a standardised format of the taxonomies assigned, from bioinformatics pipelines used for the analysis of such data. microbetag makes use of the `fuzzywuzzy` library that implements the Levenshtein Distance Metric to get the closest NCBI taxon name and thus its corresponding NCBI Taxonomy id. ++ ncbi nodes dump A relatively high similarity score is used (90) to avoid false positives.

DADA2 formatted 16S rRNA gene sequences for both bacteria and archaea [21] were used to trained the TAXID classifier [22] of the DECIPHER package.

[3] This should include a description of the overall architecture of the software implementation, along with details of any critical issues and how they were addressed.

Network inference

FlashWeave [12]

a computational approach based on a flexible Probabilistic Graphical Model framework that integrates metadata and predicts direct microbial interactions from heterogeneous microbial abundance data sets with hundreds of thousands of samples.

A flexible Probabilistic Graphical Model framework is used in a computational approach that incorporates metadata and predicts direct microbial interactions. This is done using heterogeneous microbial abundance datasets consisting of hundreds of thousands of samples.

Literature oriented node annotation

Using a set of Tara Oceans samples [23] FAPROTAX [24] estimates the functional potential of the bacterial and archaeal communities, by classifying each taxonomic unit into functional group(s) based on current literature, announcements of cultured representatives and/or manuals of systematic microbiology. In this manually curated approach, a taxon is associated with a function if and only if all the cultured species within the taxon have been shown to exhibit that function. In its current version, FAPROTAX includes more than 80 functions based on 7600 functional annotations and covering more than 4600 taxa. Contrary to gene content based approaches, e.g. PICRUSt2, FAPROTAX estimates metabolic phenotypes based on experimental evidence.

microbetag invokes the accompanying script of FAPROTAX and converts the taxonomic microbial community profile of the samples included in the user's abundance table or of the taxa present in the provided network, into putative functional profiles. Then, it parses FAPROTAX's subtables to annotate each taxonomic unit present on the user's data with all the functions for which they had a hit. FAPROTAX annotations are not part of the microbetagDB but are computed on the fly.

Genomic oriented node annotation

phenDB [25] is a publicly available resource that supports the analysis of bacterial (meta)genomes to identify 47 distinct functional traits. It relies on support vector machines (SVM) trained with manually curated datasets based on gene presence/absence patterns for trait prediction. More specifically, the model for a particular trait is trained using a collection of EggNOG annotated genomes where the knowledge of whether that trait is present or absent among its members is available. The `compute-genotype` program of phenotrex supports the creation of such tabular *genotype* files. A *genotype* file can be used along with a *phenotype* one, i.e., a file containing true phenotypic trait values for each input genome on which to train the model, and the `train` program of phenotrex can then be performed. Last, the models can now be used to predict their corresponding traits; based on the completeness/contamination of the genomes, the accuracy varies.

In the frameowrk of microbetagDB, phenotrex classifiers were re-trained using the genomes provided by phenDB for each trait to sync with the latest version of eggNOG.

Genomes were downloaded from NCBI using the Batch Entrez program. Then, *genotype* files were produced for all the high quality GTDB representative genomes. Each model was then used against all the GTDB *genotype* files to annotate each with the presence or the absence of the trait.

Pathway complementarity

For the subset of the 16,900 high quality GTDB representative genomes that a `.faa` was available, *kofamscan* [26] was performed to annotate them with KEGG ORTHOLOGY terms (KOs) [27]. Their KOs were then mapped to their corresponding KEGG modules. A KEGG module is defined as a functional unit within the KEGG framework, that represents a set of enzymes and reactions involved in a specific biological process or pathway [28]. A module's definition is a logical expression and consists of KOs and the following symbols: a. the space, representing a connection in the pathway b. plus sign, representing a molecular complex, c. comma, representing alternatives and d. minus sign, designates an optional item in the complex. Both (a) and (b) cases should be considered as "AND" logical operators, while (c) would be the "OR".

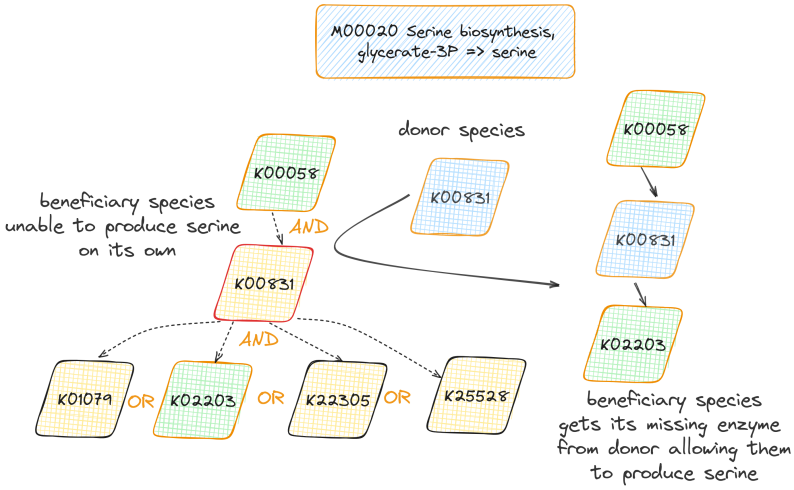

Fig. 1: Pathway complementarity approach. The high quality GTDB genomes were annotated with KEGG ORTHOLOGY (KO) terms. The various ways of getting a KEGG module complete were enumerated and all the possible ways a donor species could "fill" a beneficiary's non-complete module were calculated. In this case, there are 4 unique ways for having the serine biosynthesis module complete; in all of them K00831 is required. However, it is missing from the beneficiary species that supports the 2 out of the 3 steps of the module's definition. A donor species having and potentially sharing the corresponding enzyme of K00831 may enable the beneficiary species to produce serine.

We define a genome as having a "complete" module if and only if all of the KOs present in any of the module's alternatives are also found among the annotated KOs of the genome. All modules definitions were retrieved using the KEGG API and parsed (`parse_module_definitions.py`). A dictionary was built with all the alternatives, i.e. alternative sets of KOs, for a module to be complete (module_definition_map.json). Each pair of the KEGG annotated genomes was then investigated for potential pathway complementarities, i.e. whether a genome lacking a number of KOs ($genome_A$) to have a complete module ($module_x$) could benefit from another's species genome(s) ($genome_B$). In that case, $genome_B$ does not necessarily have a complete alternative of $module_x$; as long as it has the missing KOs that $genome_A$ needs to complete an alternative of it, $genome_B$ potentially complements $genome_A$ with respect to $module_x$. In total, 341,568 unique complementarities were exported (`pathway_complementarity.py`). Thanks to the graphical user interface (GUI) of the KEGG pathway map viewer [29, 30], each complementarity can be visualised as part of the closest KEGG metabolic map; where the KOs coming from the donor are shown with a blue-green colour, while those from the beneficiary's genome itself with rose.

As several GTDB representative genomes might map to the same NCBI Taxonomy Id, all the possible genomes' combinations are annotated in the edge of a pair of species level taxonomically annotated OTUs/ASVs/bins. On top of that, as co-occurrence networks are undirected, both nodes of a suggested association are considered as potential donors and beneficiary species.

Seed scores using genome scale metabolic reconstructions

A metabolic network's "seed set" is the set of compounds that, based on the network topology, need to be acquired exogenously [31]. Such nodes might be independent, i.e. they cannot be activated by any other node in the network, or they can be interdependent forming groups of seed nodes.

Based on the seed concept, several graph theory-based metrics have been described to predict species interactions directly from their networks' topologies. The Metabolic Complementarity Index ($MI_{Complementarity}$) measures the degree to which two microbial species can mutually assist each other by complementing each other's biosynthetic capabilities. As described in [32], it is defined as the proportion of seed compounds of a species that can be synthesized by the metabolic network of another, but are not included in the seed set of the latter. $MI_{Complementarity}$ offers an upper bound assessment of the potential for syntrophic interactions between two species. Further, the Metabolic Competition Index ($MI_{Competition}$) represents the similarity in two species' nutritional profiles. This index establishes an upper limit on the level of competition that one species may face from another.

Those indices have been thoroughly described and implemented in the NetCooperate [33] and NetCompt [34] tools correspondingly. We will be referring to those two indices as "seed scores". Most recently, the PhyloMint Python package [32] was released supporting the calculation of the seed scores of genome scale metabolic network reconstructions (GENREs) in SBML format.

In the framework of microbetag, seed scores were computed using PhyloMint and draft GENREs for all pair-wised combinations of GTDB representative genomes that have been RAST annotated in the framework of the PATRIC database [35]. GENREs were reconstructed using the Model SEED pipeline [36] through its Python interface ModelSEEDpy.

Clustering network

manta is a heuristic network clustering algorithm that clusters nodes within weighted networks effectively, leveraging the presence of negative edges and discerning between weak and microbetag invokes manta [37] to infer clusters from the microbial network. A taxonomically-informed layout is

strong cluster assignments. ++ taxonomy layout

Groups of annotations

Biologically meaningful groups were built using the micrO ontology [38].

Building the CytoscapeApp

The microbetag CytoscapeApp was build based on the source code of the scVizNet [39]. Java @Ermis to add

Enrichment analysis is supported. Hypergeometric distribution FDR +++

Dependencies, Web server and API

The microbetag web service is container - based and consists of three Docker [40] (v24.0.2) images: a. the MySQL database b. an nginx [41] web server and c. the app itself. The latter uses Gunicorn (20.1.0) to build an application server which communicates with the web server using the Web Server Gateway Interface (WSGI) protocol and handles incoming HTTP requests. microbetag is implemented as a Flask application (v2.3.2); Flask is a micro web framework for developing Python web applications and RESTful APIs. A thorough description of microbetag's API is available at the ReadTheDocs web site. The source code of the microbetag web service is available on GitHub.

python 3.11 slim docker image julia 1.7.1 for flashweave mysql.connector 8.0.27 python library pandas 2.1.1. numpy 1.26.0 multiprocessing

text processing using awk

KEGG API

2.1 Running large datasets

3 Results

[4]

[4] Significant advance over previously published software (usually demonstrated by direct comparison with available related software) This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures. This section may be combined with the Discussion section for Software articles.

microbetag and microbetagDB

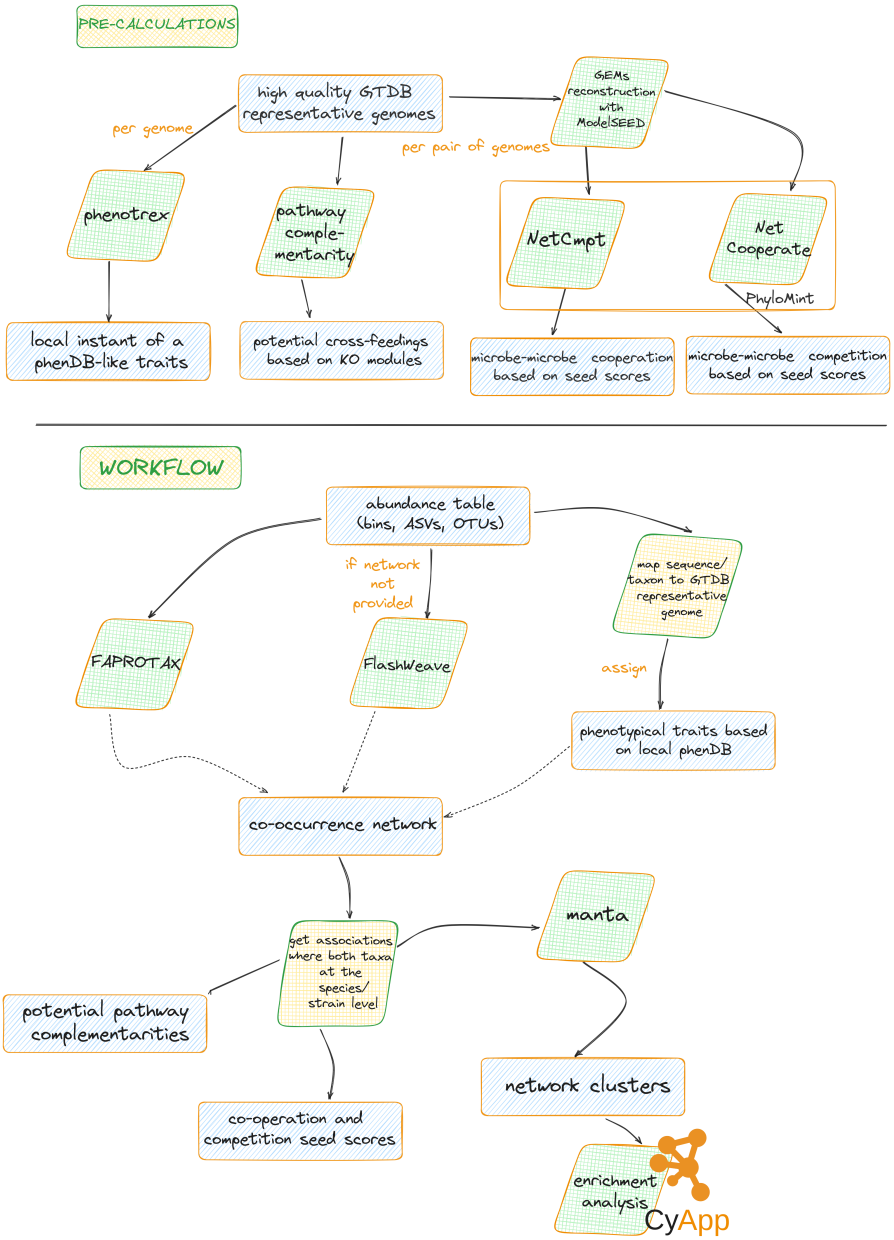

Fig. 2: Diagram of the microbetag pre - calculations and the on the fly workflow. GTDB v207 representative genomes were filtered and for those of high-quality 33 phenotypic traits were predicted using `phenotrex` [25]. To this end, models were re-trained to sync with recent version of eggNOG [42].

microbetag in numbers: 34,608 GTDB representative genomes 32 phen-model-oriented metabolic functions 92 FAPROTAX functions 341,568 unique complements involved in > 184 million beneficiary - donor pairs' complementarities 30,755 GENREs leading to 1 billion competition and complementarity scores

annotated network returned in `.cyjs` format

For a computationally efficient way to annotate large networks, a Docker image is provided so the user runs a taxonomy assignment using the IDTAXA algorithm [22] of the DECIPHER R package [43]. A co-occurrence network is also built using FlashWeave [12], as microbetag also does.

microbetag CytoscapeApp

Overall comment, the CytoscapeApp returns averages and s.d. for example in seed scores. If you want the exact values, go through the API.

A. GTDB-tk: 480 bins

Step	Time(sec)	Notes
Taxonomy mapping	Cell 1,2	on the fly
Network inference	Cell 2,2	on the fly
microbetag annotations	Cell 3,2	on the fly
manta clustering	Cell 4,2	on the fly

B. GTDB 16S: 3000 ASVs

Step	Time(sec)	Notes
Taxonomy assignment		Docker image on HP[5]
Taxonomy mapping	Cell 1,2	Cell 1,3
Network inference	Cell 2,2	Cell 2,3
microbetag annotations	Cell 3,2	Cell 3,3
manta clustering	Cell 4,2	Cell 4,3

C. Silva:

Step	Time(sec)	Notes
Taxonomy mapping	Cell 1,2	Cell 1,3
Network inference	Cell 2,2	Cell 2,3
microbetag annotations	Cell 3,2	Cell 3,3
manta clustering	Cell 4,2	Cell 4,3

D. fuzzywuzzy:

Step	Time(sec)	Notes
Taxonomy mapping	Cell 1,2	Cell 1,3
Network inference	Cell 2,2	Cell 2,3
microbetag annotations	Cell 3,2	Cell 3,3
manta clustering	Cell 4,2	Cell 4,3

Table 1: Computating times per step using an abundance table of 400 taxa with taxonomy: A. taxonomy scheme B. C. D. [5] specs of the laptop used.

Validation of microbetag potential

vitamin dataset [44]

Interpetating a real-world network with microbetag

Annelies' dataset.

4 Discussion

[6]

[6]The user interface should be described and a discussion of the intended uses of the software, and the benefits that are envisioned, should be included, together with data on how its performance and functionality

5 Conclusions

[7]

Data integration

Supplementary information. [8]

Declarations

- **Availability of data and materials**
  - Raw sequences for the use case:
  - Raw data for the validations case:
- **Funding**
  This work was initiated thanks to an EMBO Scientific Exchange Grant to HZ. It was then supported by the 3D'omics Horizon project (101000309). We would also like to thank the National Resource for Network Biology (NRNB) and the Google Summer of Code 2023 for the support of E.I.M.D.
- **Conflict of interest/Competing interests**
  The authors declare that they have no other competing interests.
- **Authors' contributions** [9]
  Conceptualization: K.F. Methodology: K.F. and H.Z. Software: H.Z., E.I.M.D. and J.M Validation: H.Z. and K.F. Formal analysis: H.Z. and K.F. Investigation: H.Z. Resources: K.F., A.E. and A.G. Data Curation: H.Z. Writing - Original Draft: H.Z. and K.F. Writing - Review & Editing: all Visualization: H.Z. Supervision: K.F., H.Z. and S.M. Project administration: K.F. Funding acquisition: K.F., A.E.
- **Acknowledgements**
  We would like to thank Dr Christina Pavloudi and ++ for the insight on how to organise the trait groups.
- **Ethics approval**
  Not applicable
- **Consent to participate**
  Not applicable.
- **Code availability:**
  - microbetagDB related scripts: https://github.com/hariszaf/microbetag
  - microbetagApp and webserver: https://github.com/msysbio/microbetagApp.
  - CytoscapeApp: https://github.com/ermismd/MGG/
  - Validation and use case: ¡think of having that under the 3D'omics organization¿
  - Documentation web-site: https://hariszaf.github.io/microbetag/

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compare with, and improve, on functionally similar existing software. A case study of the use of the software may be presented. The planned future development of new features, if any, should be mentioned.

[7] This should state clearly the main conclusions and provide an explanation of the importance and relevance of the case, data, opinion, database or software reported.

[8] If your article has accompanying supplementary file(s) please state so here. E.g. supplementary figures and tables captions.

[9] Based on the CRediT system. Current list is indicative.

Appendix A Background on seed scores and complementarities

A.1 Background on seed scores

In that case, once a seed is assured, it activates all the rest of that group. Therefore, a confidence level (C) ranging from 0 to 1, has been previously described to quantify the relevance of each seed:

$$C_i = 1/seed's\ group\ with\ i\ size \tag{A1}$$

$C = 0$ corresponds to a non-seed node, while $C = 1$ represents an independent node.

$$MI_{Complementarity} = \frac{|SeedSet_A \cap \neg SeedSet_B|}{|SeedSet_A \cap (SeedSet_B \cup \neg SeedSet_B)|} \tag{A2}$$

As also described in [32], it is calculated as the proportion of compounds in a species' seed set that coincide with those in an other's, while also factoring in the confidence scores associated with seed compounds.

$$MI_{Competition} = \frac{\sum C(SeedSet_A \cap SeedSet_B)}{\sum C(SeedSet_A)} \tag{A3}$$

A.2 Background on pathway complementarity

For example, the definition of the D-Galacturonate degradation in Bacteria (M00631) is:

K01812 K00041 (K01685,K16849+K16850) K00874 (K01625,K17463)
that once breaking down, it leads to 4 alternative sets of KOs (pathways):

K01812 K00041 K01685 K00874 K01625

K01812 K00041 K16849+K16850 K00874 K01625

K01812 K00041 K01685 K00874 K17463

K01812 K00041 K16849+K16850 K00874 K17463

A.3 Complementarities

KEGG compound ModelSEED compounds ModelSEED compounds mapped to KEGG compounds and kept only those related to KEGG modules.

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