The Functional Order (FunOrder) tool – Identification of essential biosynthetic genes through computational molecular co-evolution

Gabriel A. Vignolle¹, Denise Schaffer¹, Robert L. Mach¹, Astrid R. Mach-Aigner¹ and Christian Derntl¹,*

¹ Institute of Chemical, Environmental and Bioscience Engineering, TU Wien, Gumpendorfer Strasse 1a, 1060 Wien, Austria

* To whom correspondence should be addressed. Email: christian.derntl@tuwien.ac.at

ABSTRACT

Secondary metabolites (SMs) are a vast group of compounds with different structures and properties. Humankind uses SMs as drugs, food additives, dyes, and as monomers for novel plastics. In many cases, the biosynthesis of SMs is catalysed by enzymes whose corresponding genes are co-localized in the genome in biosynthetic gene clusters (BGCs). Notably, BGCs may contain so-called gap genes, that are not involved in the biosynthesis of the SM. Current genome mining tools can identify BGCs but they have problems with distinguishing essential genes from gap genes and defining the borders of a BGC. This can and must be done by expensive, laborious, and time-consuming comparative genomic approaches or co-expression analyses. In this study, we developed a novel tool that allows automated identification of essential genes in a BGC based solely on genomic data. The Functional Order (FunOrder) tool – Identification of essential biosynthetic genes through computational molecular co-evolution – searches for co-evolutionary linked genes in the BGCs. In light of the growing number of genomic data available, this will contribute to the studies of BGCs in native hosts and facilitate heterologous expression in other organisms with the aim of the discovery of novel SMs, including antibiotics and other pharmaceuticals.

INTRODUCTION

Secondary metabolites (SMs) are a diverse group of compounds with a plethora of different chemical structures and properties. SMs are found in all domains of life, but are predominantly studied in bacteria, fungi, and plants (1). SMs are not necessary for the basic survival and growth of an organism but can be beneficial under certain conditions. For example, pigments help to sustain radiation, antibiotics help in competitive situations, and toxins can serve as defensive compounds or as virulence factors (2,3). Notably, many SMs are used by humankind as drugs and pharmaceuticals, pigments and dyes, sweeteners and flavours, and most recently also as precursors for the synthesis of plastics (4). The study of the secondary metabolism holds the promise for novel antibiotics, pharmaceuticals and other useful compounds (5).

A major hinderance in the discovery of yet undescribed SMs is the fact that most SMs are not produced under standard laboratory conditions, as they do not serve a purpose for the organisms then. Currently, different strategies are followed to circumvent this problem (6,7). Untargeted approaches aim to
induce the expression of any SM. To this end, biotic and abiotic stresses are applied, or global regulators and regulatory mechanisms are manipulated (8). These strategies may lead to the discovery of novel compounds, whose corresponding genes have to be identified subsequently by time-consuming and expensive methods (7). An extreme example are the aflatoxins, major food contaminants with serious toxicological effects (9). It took over 40 years from the discovery of the aflatoxins as the causal agent of “turkey X” disease in the 1950s (10) until the corresponding genes were finally described in 1995 (11). Targeted SM discovery approaches aim to induce the production of specific SMs by either overexpression genes in the native host or by heterologous expression in another organism (12). The targeted approaches, also called reverse strategy or bottom-up strategy allows a direct connection of SMs to the corresponding genes and does not rely on the inducibility of SM production in the native host. Inherently, the bottom-up approach is depending on modern genomics and accurate gene prediction tools (13).

In bacteria and fungi, the genes responsible for the biosynthesis of a certain SM are often co-localized in the genome, forming so called biosynthetic gene clusters (BGCs) (14). The BGCs consists of one or more core genes and several tailoring enzymes. The core genes are responsible for assembling the basic chemical scaffold, which is further modified by the tailoring enzymes yielding the final SM (15). Depending on the class of the produced SM, the core genes differ. In bacteria and fungi, the main SM classes are polyketides (e.g. the cholesterol-lowering drug lovastatin (16) and the mycotoxin aflatoxin (9)) and non-ribosomal peptides (e.g. the immunosuppressant cyclosporine (17) and the antibiotic penicillin (18)), with polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS) as core enzymes, respectively. Other SM classes are terpenoids, alkaloids, melanins (19,20), and ribosomally synthesized and posttranslationally modified peptides (RiPPs) (21,22), whose corresponding genes may also be organized in BGCs. Notably, BGCs contain not only genes necessary for the production of a SM, but also so-called gap genes that are not involved in the biosynthesis of the SM (Fig. 1).

As mentioned, the bottom-up approach for SM discovery is depending on modern genomics and the accurate prediction of genes and BGCs. Each important gene missing in the prediction is detrimental for obvious reasons, whereas each unnecessarily considered gap gene and gene outside the BGC make the study of a BGC more complicated and complex, and the construction and transformation processes for heterologous expression more challenging. Currently, several BGC prediction tools are available for fungi and bacteria. For fungi, some prominent tools for genome mining are antiSMASH (23), Cassis (24) which has been integrated into antiSMASH, SMURF (25) and DeepBGC, a recently developed algorithm based on deep neural networks (26). For bacteria, the EvoMining tool predicts BGCs by searching for duplicates of primary metabolism enzymes (27). These tools are effective and successful in finding and predicting BCGs based solely on genomic data, but they do not predict gap genes. A further challenging task is the accurate determination of the borders of a BGC; the BGC prediction tools often contradict each other in this regard. These two problems can be solved by the analysis of transcriptome data, because the genes necessary for SM production within a BGC are co-expressed with each other but not with the
gap genes and genes outside of the borders of the BGC (28). Notably, such methods demand the knowledge of expression conditions. Alternatively, the BGC can be analysed for co-evolution of the contained genes if no transcriptome data are available or the BGC is completely shut-off in the native host. A co-evolution analysis is a laborious and time-consuming task because a phylogenetic tree has to be calculated for each gene and then the trees compared to each other manually (29). However, it is an obvious advantage to have as much information as possible about a BGC before studying a BGC in the native host or performing heterologous expression for a bottom-up approach for SM discovery.

In this study we describe the Functional Order (FunOrder) tool – Identification of essential biosynthetic genes through computational molecular co-evolution, which identifies the relevant genes within a BGC based on the principle of co-evolution. We describe the workflow and demonstrate the applicability by an exemplary analysis of a well characterised BGC. We tested the robustness and the applicability of FunOrder by analysing empirically validated BGCs as positive controls, and randomly generated BGCs as negative controls and evaluated the FunOrder tool using stringent statistical tests. To our knowledge, FunOrder is the first program giving a prediction about core genes in fungal BGCs based solely on genomic data. Notably, the underlying strategy and workflow of FunOrder can be used to search for co-evolution in other phyla and for other objectives. FunOrder is available as command line version tool running on Linux and MacOS platforms.

MATERIAL AND METHODS

FunOrder - Workflow

The Functional Order (FunOrder) tool is written in the BASH (Bourn Again Shell) environment and includes all necessary subprograms. As BASH is the default shell-language of all Linux distributions and MacOS, FunOrder can run on these two operation systems. The only two dependencies are RAxML (Randomized Axelerated Maximum Likelihood) (30) and the EMBOSS (The European Molecular Biology Open Software Suite) package (31). The overall workflow is depicted in Figure 2. The FunOrder tool is deposited in the GitHub repository https://github.com/gvignolle/FunOrder. Notably, FunOrder includes scripts adapted to the use on servers and for the integration in various pipelines.

The software input files are BGCs with gene translations in genbank file format or fasta format. In case a genbank file is provided, a python script (Genbank to FASTA by Cedar McKay and Gabrielle Rocap, University of Washington) is called to extract the amino acid sequence of the genes in the BGC and create a fasta file. The multi-fasta file is then split into individual fasta files each containing a single protein sequence. These are placed in a subfolder created for the analysis of the BGC. Each file is named either after the position of the gene in the BGC or after the respective protein sequence description. This varies from the input file and the varying annotations used. Each header of the query sequences is tagged with the identifier "query" at the beginning of the header. The individual sequences are compared to our manually curated database of fungal proteomes (Table 1) by a sequence similarity
search using blastp 2.8.1+ (Protein-Protein BLAST) (32). The output of this search is saved in a file with the ".tab" extension. Additionally, an optional remote search of the non-redundant National Center for Biotechnology Information (NCBI) protein database can be performed, yielding a file with the "ncbi.tab" extension. This allows a manual preliminary analysis of the input sequences and supports subsequent annotations of the BGCs.

Next, the top 20 results of the database search of our manually curated fungal proteome database are extracted and combined with the query sequence for each gene. A custom Perl script removes potential duplicate entries. Using emma, a multiple sequence alignment of these protein sequences is calculated based on the ClustalW (33) algorithm, and a dendrogram computed. Based on the multiple sequence alignment 100 rapid Bootstraps and a subsequent search for the best-scoring maximum likelihood (ML) tree are performed using RAxML (Randomized Axelerated Maximum Likelihood) (30). The phylogenetic trees are computed using the LG amino acid substitution model. Furthermore, a standard ascertainment bias correction by Paul O. Lewis is performed. At this stage, we have obtained a phylogenetic tree (within the context of our manually curated database) for each gene of the input BGC.

To estimate if and to what extent the different genes within a BGC are co-evolved, the strict distance and speciation distance among the ML trees of the individual genes are calculated using the TreeKO algorithm (34). This tool was designed for automated tree comparison and was already suggested to be used for the detection of co-evolution in protein families (34). The tool compares the topology of different trees; a distance of 0 in both distance measures represents identical trees. In this context, a higher similarity between the different trees of the individual genes points towards a shared evolution. The strict distance is a weighted Robinson-Foulds (RF) distance measure that penalizes dissimilarities in evolutionarily important events such as gene losses and gene duplications. Furthermore, the strict distance has been suggested to be more significant in the detection of co-evolution (34). In contrast, the speciation or evolutionary distance is computed without taking evolutionary exceptions, such as duplication events or different species content of the two compared trees, into account and infers shared "speciation history" based solely on topology without considering branch lengths and only considering shared species of the compared trees. Therefore, an evolutionary distance of 0 does not necessarily describe identical trees but shared "speciation history" of shared species. The comparison and analysis of the two distance measures is made possible through a custom BASH script and a special identifier introduced in the fungal proteome database. This enables a more comprehensive picture of the potential co-evolution within the analysed BGC. All pairwise strict and evolutionary distances are combined into matrices which are used as input for an R script (35-39).

First, the strict and evolutionary distances are summed up to a third combined distance measure. Then, the three distance matrices are visualized as heatmaps with a dendrogram computed with the complete linkage method, to find similar clusters. Next, the Euclidean distance within the matrices is computed and clustered using Ward’s minimum variance method aiming at finding compact spherical
clusters, with the implemented squaring of the dissimilarities before cluster updating, for each of the three distance matrices separately, with scaled and unscaled input data (40). Lastly, a principal component analysis (PCA) is performed on each distance matrix and the score plot of the first two principal components visualized, respectively. These outputs enable the adoption of a larger view on the distance measures and thereby allow the analysis of co-evolution within the BGC from different perspectives.

**Manual curated fungal proteome database**

The manual curated database used for sequence similarity search consists of 134 fungal proteomes, containing mainly ascomycetes and two basidiomycetes as potential outgroup (Table 1). The sequences were downloaded from the National Center for Biotechnology Information (NCBI) database and the Joint Genome Institute (JGI) (41). A short identifier, unique in the database for each proteome, was introduced to enable multiple pairwise tree comparisons by the treeKO application. A custom Perl script was used for removing duplicated entries in the database. The database is deposited in the GitHub repository https://github.com/gvignolle/FunOrder.

**Table 1.** Fungal proteomes included in the manually curated database. The sequences were downloaded from the National Center for Biotechnology Information (NCBI) database or the Joint Genome Institute (JGI). The identifiers were used in the FunOrder tool.

| Organism                         | Source Database | Identifier | Reference |
|----------------------------------|-----------------|------------|-----------|
| Acremonium chrysogenum           | JGI             | AcCh       | (42)      |
| Alternaria alternata             | NCBI            | AlAl       | (43)      |
| Alternaria arborescens           | NCBI            | AlAr       | (44)      |
| Alternaria gaisen                | NCBI            | AlGa       | (45)      |
| Alternaria sp. MG1               | NCBI            | AlSp       | (46)      |
| Alternaria tenuissima            | NCBI            | AlTe       | (45)      |
| Amanita muscaria                 | NCBI            | AmMu       | (47)      |
| Amorphotheca resinae             | JGI             | AmRe       | (48)      |
| Arthrotrypa oligospora           | JGI             | ArOl       | (49)      |
| Arthroderma benhamiae            | JGI             | ArBe       | (50)      |
| Ascobolus immersus               | JGI             | AsIm       | (51)      |
| Aspergillus costaricaensis       | NCBI            | AsCo       | (52)      |
| Aspergillus fijensis             | NCBI            | AsFi       | (52)      |
| Aspergillus flavus               | NCBI            | AsFl       | (53)      |
| Aspergillus fumigatus            | NCBI            | AsFu       | (54)      |
| Aspergillus homomorphus          | NCBI            | AsHo       | (52)      |
| Aspergillus ibericus             | NCBI            | AsIb       | (52)      |
| Species                        | Database | Code | Page |
|-------------------------------|----------|------|------|
| Aspergillus japonicus         | NCBI     | AsJa | (52) |
| Aspergillus niger             | NCBI     | AsNi | (55) |
| Aspergillus oryzae            | NCBI     | AsOr | (56) |
| Aspergillus phoenicis         | NCBI     | AsPh | (57) |
| Aspergillus terreus           | NCBI     | AsTe | (58) |
| Blumeria graminis             | JGI      | BlGr | (59) |
| Botryosphaeria dothidea       | JGI      | BoDo | (60) |
| Botrytis cinerea              | NCBI     | BoCi | (61) |
| Botrytis elliptica            | NCBI     | BoEl | (62) |
| Botrytis galanthina           | NCBI     | BoGa | (62) |
| Botrytis hyacinthi            | NCBI     | BoHy | (62) |
| Botrytis paoniae              | NCBI     | BoPa | (62) |
| Botrytis porri                | NCBI     | BoPo | (62) |
| Botrytis tulipae              | NCBI     | BoTu | (62) |
| Cadophora sp.                 | JGI      | CaSp | (63) |
| Capronia semiimmersa          | JGI      | CaSe | (64) |
| Chaetomium globosum           | JGI      | ChGl | (65) |
| Choiromyces venosus           | JGI      | ChVe | (51) |
| Cladonia grayi                | JGI      | ClGr | (66) |
| Cladophialophora bantiana     | JGI      | ClBa | (64) |
| Cladophialophora carrionii    | JGI      | ClCa | (64) |
| Cladophialophora immunda      | JGI      | ClIm | (64) |
| Cochliobolus heterostrophus   | JGI      | CoHe | (67) |
| Cochliobolus victoriae        | JGI      | CoVi | (68) |
| Colletotrichum nymphaeae      | JGI      | CoNy | (69) |
| Colletotrichum orchidophilum  | JGI      | CoOr | (70) |
| Colletotrichum salicis        | JGI      | CoSa | (69) |
| Colletotrichum simmondsii     | JGI      | CoSi | (69) |
| Colletotrichum tofieldiae     | JGI      | CoTo | (71) |
| Coniosporium apollinis        | JGI      | CoAp | (64) |
| Coniosporium apollinis CBS 100218 | JGI | Capo | (64) |
| Corynespora cassicola         | JGI      | CoCa | (72) |
| Daldinia eschscholzii         | JGI      | DaEs | (73) |
| Diaporthe ampelina            | JGI      | DiAm | (74) |
| Diplodia seriata              | JGI      | DiSe | (74) |
| Erysiphe necator              | JGI      | ErNe | (75) |
| Species                          | Database | Code | Reference |
|---------------------------------|----------|------|-----------|
| Eutypa lata                     | NCBI     | EuLa | (76)      |
| Exophiala aquamarina            | JGI      | ExAq | (64)      |
| Exophiala dermatitidis          | JGI      | ExDe | (64)      |
| Exophiala oligosperma           | JGI      | ExOl | (64)      |
| Exophiala spinifera             | JGI      | ExSp | (64)      |
| Exophiala xenobiotica           | JGI      | ExXe | (64)      |
| Fonsecaea monophora             | JGI      | FoMo | (77)      |
| Fusarium fujikuroi              | NCBI     | FuFu | (78)      |
| Fusarium graminearum            | NCBI     | FuGr | (79)      |
| Fusarium oxysporum              | NCBI     | FuOx | (80)      |
| Fusarium proliferatum           | NCBI     | FuPr | (81)      |
| Fusarium pseudograminearum      | NCBI     | FuPs | (82)      |
| Fusarium verticillioides        | NCBI     | FuVe | (79)      |
| Gaeumannomyces graminis         | JGI      | GaGr | (83)      |
| Glonium stellatum               | JGI      | GlSt | (84)      |
| Hypoxylon sp. EC38              | JGI      | HyEC | (73)      |
| Hypoxylon sp. CO27              | JGI      | Hysp | (73)      |
| Magnaporthe grisea              | JGI      | MaGr | (85)      |
| Magnaportheiopsis poae          | JGI      | MaPo | (83)      |
| Meliniozymes bicolor            | JGI      | MeBi | (48)      |
| Meliniomyces variabilis         | JGI      | MeVa | (48)      |
| Metarhizium acridum             | NCBI     | MeAc | (86)      |
| Metarhizium album               | NCBI     | MeAl | (87)      |
| Metarhizium anisopliae          | NCBI     | MeAn | (87)      |
| Metarhizium brunneum            | NCBI     | MeBr | (87)      |
| Metarhizium guizhouense         | NCBI     | MeGu | (87)      |
| Metarhizium majus               | NCBI     | MeMa | (87)      |
| Metarhizium rileyi              | NCBI     | MeRi | (88)      |
| Metarhizium robertsii           | NCBI     | MeRo | (86)      |
| Monacrosporium haptotylum       | JGI      | MoHa | (89)      |
| Morchella importuna             | JGI      | MoIm | (90)      |
| [Nectria ] haematococca         | NCBI     | NeHa | (91)      |
| Nectria haematococca            | JGI      | NeHa | (91)      |
| Neurospora crassa               | JGI      | NeCr | (92)      |
| Neurospora crassa FGSC          | JGI      | NeCr | (93)      |
| Neurospora tetrasperma          | JGI      | NeTe | (94)      |
| Species Name                             | Database | Accession | Entry Count |
|-----------------------------------------|----------|-----------|-------------|
| Oidiodendron maius                       | JGI      | OiMa      | (47)        |
| Ophiostoma piceae                        | JGI      | OpPi      | (95)        |
| Paecilomyces variotii                    | JGI      | PaVa      | (96)        |
| Panaeolus cyaneascens                    | NCBI     | PaCy      | (97)        |
| Paracoccidioides brasiliensis            | JGI      | PaBr      | (98)        |
| Penicillium camemberti                   | NCBI     | PeCa      | (99)        |
| Penicillium chrysogenum                  | NCBI     | PeCh      | (100)       |
| Penicillium digitatum                    | NCBI     | PeDi      | (101)       |
| Penicillium expansum                     | NCBI     | PeEx      | (102)       |
| Penicillium nalgiovense                  | NCBI     | PeNa      | (103)       |
| Penicillium oxalicum                     | NCBI     | PeOx      | (104)       |
| Penicillium roqueforti                   | NCBI     | PeRo      | (99)        |
| Penicillium rubens Wisconsin             | NCBI     | PeRu      | (105)       |
| Penicillium vulpinum                     | JGI      | PeVu      | (103)       |
| Periconia macropinosa                    | JGI      | PeMa      | (63)        |
| Pestalotiopsis fici                      | NCBI     | PeFi      | (106)       |
| Phaeoacremonium aleophilum               | JGI      | PhAl      | (107)       |
| Phaeomoniella chlamydomyspora            | JGI      | PhCh      | (74)        |
| Phialocephala scoiiformis                | JGI      | PhSc      | (108)       |
| Pneumocystis jirovecii                   | JGI      | PnJi      | (109)       |
| Pseudogymnoascus destructans             | JGI      | PsDe      | (110)       |
| Pseudomassariella vexata                 | JGI      | PsVe      | (111)       |
| Rhizoctonia solani                       | NCBI     | RhSo      | (112)       |
| Saccharomyces arboricola                 | NCBI     | SaAr      | (113)       |
| Saccharomyces cerevisiae                 | NCBI     | SaCe      | (114)       |
| Terfezia boudieri                         | JGI      | TeBo      | (51)        |
| Tolypocladium ophioglossoides            | NCBI     | ToOp      | (115)       |
| Tolypocladium paradoxum                  | NCBI     | ToPa      | (116)       |
| Trichoderma arundinaceum                 | NCBI     | TrAr      | (117)       |
| Trichoderma asperellum                   | NCBI     | TrAs      | (118)       |
| Trichoderma atroviride                   | NCBI     | TrAt      | (119)       |
| Trichoderma citrinoviride                | NCBI     | TrCi      | (118)       |
| Trichoderma harzianum                    | NCBI     | TrHa      | (120)       |
| Trichoderma longibrachiatum              | NCBI     | TrLo      | (121)       |
| Trichoderma reesei                       | NCBI     | TrRe      | (122)       |
| Trichoderma virens                       | NCBI     | TrVi      | (119)       |
Compilation of benchmark biosynthetic gene clusters

To test and evaluate the applicability of FunOrder, we compiled negative and positive control BGCs (sequences deposited in the GitHub repository https://github.com/gvignolle/FunOrder). The first set of negative controls were 42 completely randomly generated synthetic BGCs, which were created with a custom BASH script. Therein, ATGC strings of random composition and length were translated to an amino acid string using transeq from the EMBOSS package and the asterisks were removed. The second set of negative controls were 60 random BGCs which were created by subsampling randomly the fungal proteome database with a Perl script from the MEME suit (129). For each random BGC a different seed number was given to guarantee non repetitive BGCs, each random BGC contained 3-10 randomly chosen protein sequences in a random order.

We used a set of 30 empirically well characterized BGCs from a broad range of different genera as positive controls (Table 2). The sequences were downloaded from NCBI or the MIBiG (Minimum information about a biosynthetic gene cluster) database (130). All BGCs were manually inspected for correctness and completeness based on the respective literature.

Table 2. Empirically characterized biosynthetic gene clusters used as positive controls.

| Product - BGC                        | Organism                     | MIBiG id     | Reference(s) |
|--------------------------------------|------------------------------|--------------|--------------|
| 2-Pyridon-Desmethylbassianin (dmb)   | *Beauveria bassiana*         | BGC0001136   | (131)        |
| Aflatoxin (afl)                      | *Aspergillus flavus*         | BGC00000008  | (132,133)    |
| Botrydial (bot)                      | *Botrytis cinera*            | BGC0000631   | (134,135)    |
| Cephalosporin (cef)                  | *Acremonium chrysogenum*     | BGC00000317  | (136)        |
| Compactin (mlc)                      | *Penicillium citrinum*       | BGC00000039  | (137,138)    |
| Cyclosporin (cyc2)                   | *Beauveria felina*           | BGC0001565   | (17,139-141) |
| Destruxin (dtxs)                     | *Metarhizium robertsii*      | BGC00000337  | (142)        |
| Fumagillin (fma)                     | *Aspergillus fumigatus*      | BGC0001067   | (143)        |
| Chemical          | Producing Organism              | Accession  | (Gene ID) |
|-------------------|---------------------------------|------------|-----------|
| Fumitremorgin (ftm) | *Aspergillus fumigatus*              | -          | (144-147) |
| Fumonisin (fum1)   | *Fusarium oxysporum*                 | BGC0000063 | (148)     |
| Fumonisin (fum2)   | *Fusarium verticilloides*            | BGC0000062 | (149-156) |
| Fumonisin (FUB)    | *Fusarium fujikuroi*                 | -          | (157)     |
| Illicicolin H (ili)| *Neonectaria sp. DH2*               | BGC0002035 | (158)     |
| Leporin (lep)      | *Aspergillus flavus*                 | BGC0001445 | (159)     |
| Lovastatin (lov)   | *Aspergillus terreus*                | -          | (16,58,160)|
| Mycophenolic acid (mpa1) | *Penicillium brevicaelum*         | BGC0000104 | (161-166) |
| Mycophenolic acid (mpa2) | *Penicillium roqueforti*           | BGC0001360 | (167)     |
| Mycophenolic acid (mpa3) | *Penicillium roqueforti*           | BGC0001677 | (168)     |
| Paxillin (pax)     | *Penicillium paxilli*               | BGC0001082 | (169)     |
| Penicillin (pen1)  | *Penicillium chrysogenum*           | BGC0000404 | (170)     |
| Penicillin (pen2)  | *Penicillium chrysogenum*           | BGC0000405 | (18)      |
| Pestheic acid (pta)| *Pestalotiopsis fici*              | BGC0000121 | (171)     |
| Pneumocandin (GL)  | *Glaera lozoyensis*                 | BGC0001035 | (172-174) |
| Sorbicillinol (sor1)| *Penicillium rubens*               | BGC0001404 | (175,176) |
| Sorbicillinol (sor2)| *Trichoderma reesei*              | -          | (177)     |
| Tenellin (ten)     | *Beauveria bassiana*                | BGC0001049 | (178,179) |
| Terrein (ter)      | *Aspergillus terreus*               | BGC0000161 | (180)     |
| Tetramic acid (tas)| *Hapsidospora irregularis*         | -          | (181)     |
| Ustiloxin B (ust)  | *Aspergillus flavus*                | -          | (182)     |
| Xanthocillin (xan) | *Aspergillus fumigatus*             | BGC0001990 | (183)     |

**Performance evaluation**

First, we performed a manual comparison of the phylogenetic trees for each control BGC and calculated a manual similarity measurement (manual evaluation measure - MEM) for each gene pair. Branch lengths, number of nodes, topology and shared leafs were considered (see Supplementary data Table S1 and S2). The measure ranges from 3 (same) to 0 (no shared leafs) (see Supplementary data “MEM_values.xlsx”). The MEM values were evaluate using thresholds of 1.2 and 2.0 for the negative control BGCs and for the positive control BGCs, respectively. These two thresholds were determined to reflect the biologically relevant evolution and to circumvent the selection bias introduced in the positive control BGCs (see Table S3). The MEM values were then compared to distance measures calculated by the treeKO algorithm (i.e., the strict distance, the evolutionary distance, and the combined distance measure) by exploratory data analysis. For the positive control BGCs, only those genes that had previously been described as necessary for the SM production were analysed. Based on these comparisons, we set threshold values for the treeKO analysis. Two genes are considered as co-evolved
Next, we calculated three measures (two measures for the positive control BGCs and one for the negative control BGCs), to evaluate the performance of FunOrder regarding its capability to find co-evolved genes (necessary for the biosynthesis of an SM), to distinguish them from not co-evolved (gap) genes, and to test FunOrder for its robustness, respectively. The previously set threshold values for the treeKO analysis was used to define which genes were detected as co-evolved by the FunOrder tool; genes had to have low distance values to each other (< 0.7 for strict and evolutionary distance and ≤ (0.6 * max value) for the combined distance) and/or cluster together in the PCAs, in the Ward’s minimum distance dendrograms and/or the heatmaps. Notably, the three measures are linked, meaning a single threshold for their subsequent evaluation can be used.

The measure to find the correct genes (find correct genes measure, FCGM) is a relative value that expresses how well the FunOrder tool identified genes that are necessary for the biosynthesis of a SM within the positive control BGCs. The FCGM was calculated using Equation 1, resulting in values between 0 and 1, with 1 representing a complete success in finding all necessary genes in the tested positive control BGC.

\[
FCGM = 1 - \left( \frac{a}{c} \right)
\]

**Equation 1.** \(FCGM = \) find correct genes measure; \(a = \) number of genes necessary for the biosynthesis of a SM, that did not cluster with the other necessary genes in the FunOrder analysis; \(c = \) total number of genes necessary for the SM production in the BGC.

The error rate measure (ERM) is a relative value that expresses how well the FunOrder tool performed in finding the correct genes and differentiating them from the genes in a BGC not needed for the biosynthesis of a SM. The ERM was calculated using Equation 2, resulting in values between 0 and 1, with 1 representing no false classifications in the tested positive control BGC.

\[
ERM = 1 - \left[ \left( \frac{a}{d} \right) + \left( \frac{b}{d} \right) \right]
\]

**Equation 2.** \(ERM = \) error rate measure; \(a = \) number of genes necessary for the biosynthesis of a SM, that did not cluster with the other necessary genes in the FunOrder analysis; \(b = \) number of gap genes, that clustered with the genes needed for the SM biosynthesis in the FunOrder analysis; \(d = \) number of analysed genes in the BGC.

The negative control value (NCV) is a relative value that expresses the robustness of the FunOrder tool. The NCV was calculated using Equation 3, resulting in values between 0 and 1, with 1 representing no detected co-evolved genes in the tested negative control BGC.
Equation 3. NCV = negative control value; d = number of genes in the BGC; g = number of strict distances < 0.7 and combined distances < (0.6 * max value) in all matrices.

Next, the obtained values for FCGM and ERM were classified as true positives (TP) or false negatives (FN), and the values for NCV were classified as true negative (TN) or false positives (FP). As the FCGM, the ERM, and the NCV are linked, a shared threshold was used for the classification. The threshold value was determined as the first quartile of all NCVs. This ensured an unbiased evaluation of the FunOrder tool. All values above the threshold were classified as true positives (TP) for the FCGM and the ERM and as true negatives (TN) for the NCV. All values below the threshold were classified as false negatives (FN) for the FCGM and the ERM and as false positives (FP) for the NCV. Finally, the results of the FunOrder analysis of the positive control BGCs (TP vs. FN) were assayed in two confusion matrices.

RESULTS AND DISCUSSION

Exemplary analysis of the Lovastatin BGC (lov)

FunOrder compares the genes in a BGC regarding their evolutionary background and calculates two distance matrices, the strict distance, and the evolutionary distance. These distance matrices are then combined, analysed, and visualized in different ways (Fig. 3, Supplementary data Fig. S1 – S7). To evaluate if and which genes are necessary for the biosynthesis of a SM, we searched for genes that were clustering together with the backbone producing genes (e.g., PKS or NRPS) in the visualisations. Genes clustering together can be considered as co-evolutionary linked genes, it is thus likely that the respective enzymes are part of the same biosynthetic pathway. We exemplary describe the analysis for the positive control Lovastatin BGC of Aspergillus terreus (lov, Fig. 3). This analysis strategy was used for all control BGC in this study and can be used for the analysis of yet uncharacterized BGCs.

For the evaluation and interpretation of the FunOrder results for the Lovastatin BGC (Fig. 3, Supplementary data Fig. S1 – S7), we first had a look at the numerical distance matrices (Supplementary data, Tables S5 – S7). Not all of the calculated values for the genes necessary for the biosynthesis of lovastatin (lovA-D, F,G; Fig. 1, red arrows) fell within the previously defined limit of < 0.7 for strict and evolutionary distance and ≤ (0.6 * max value) for the combined distance. In our experience, evaluating only the numerical values is not enough for a thorough analysis of a BGC and it is necessary to consider all provided visualisations for a thorough data interpretation (Fig. 3, Supplementary data Fig. S1 – S7). All genes necessary to produce lovastatin (lovA-D, F,G) formed distinct clusters in the heatmap, in the dendrograms and in the PCA of the strict distance (Supplementary data Fig. S1, S4; Fig. 3A). In the visualizations of the evolutionary distance, on the other hand, the necessary genes did not cluster together and were indistinguishable from the gap genes (Supplementary data Fig. S2, S5, S7). This results are expected, as the strict distance has been suggested to be more significant in the detection of
Co-evolution between protein families (34). Consequently, we recommend using the strict distance values as the basis for data interpretation. In our experience, it was often helpful to additionally take the combined distance values into consideration to get a more comprehensive picture of the BGC. As mentioned before, the combined distance also considers speciation history. In the case of the Lovastatin BGC, orf10c clustered together with lovA, B, D, F, G in the PCA of the combined distance, whereas lovC was not clustering with the other necessary genes when considering the speciation history (Fig. 3B). The gene orf10c encodes for an MFS (major facilitator superfamily) transporter that is necessary for export of lovastatin (16), it is thus not directly necessary for the biosynthesis of lovastatin, but important for a lovastatin-producing organism. Analogously, lovE did not cluster with the other genes in the PCA of the combined distance matrix, nor in any other distance measure used for the detection of co-evolution (Fig. 3 and Supplementary data Fig. S1-S7). Notably, LovE is a transcription factor and the main regulator of the lovastatin cluster (16) and thus not directly part of the biosynthetic pathway of lovastatin. The obtained results show how varying speciation history of different genes influences the FunOrder output and that both distances should be considered for evaluation.

The Lovastatin BGC is a clear example of the ability of FunOrder to discover the correct genes for SM production and to differentiate them from gap genes. In this case differentiating orf1, orf2, orf10c, orf13, orf14, orf15, orf16, orf17, orf18, lovE and orf80c from the needed lovA, lovB, lovC, lovD, lovF and lovG. This exemplary analysis further points out how we needed to adopt a wider view of the data to make a more precise decision on which genes are co-evolutionary linked. When considering only one output, one might get a distorted view of the analysed BGC. Notably, the more visualisations point towards a co-evolution of two or more genes, the more reliable the prediction of FunOrder.

**Speed and scalability**

As the manually curated proteome database contained only 134 fungal proteomes, we were able to use the blastp algorithm for sequence similarity search. The analysis of the Lovastatin BGC of *Aspergillus terreus* (lov) with 17 genes, took 1 h 19 m 48 sec real time using 22 threads on an Ubuntu Linux system with 128 GB DDR4 RAM, demonstrating that the analysis of such a large cluster as the Lovastatin cluster is fast and feasible. The number of threads can be defined, to increase the scalability and the overall performance of FunOrder.

**Performance evaluation**

*Negative control BGCs.* FunOrder did not find any sequence similarities between the 42 synthetic BGCs and the manually curated fungal proteome database. This demonstrates the robustness of FunOrder towards non-biological random amino acid sequences. Consequently, the synthetic BGCs were not considered when calculating the confusion matrix to evaluate the performance of FunOrder.

The random BGCs, on the other hand, consist of randomly sampled genes from the fungal proteome database itself and represent a suitable negative control set where each gene has a biological
background. For each of the random BGC the NCV was calculated as described in equation 3. A low NCV value indicates that FunOrder detected co-evolutionary linked genes in the random BGC, a high value indicates that FunOrder could not detect linked genes. The NCV minimum value was 0.5 and the maximum 1, the first quartile was 0.66, the third quartile 0.83 and the median 0.75. Based on a Shapiro-Wilk normality test (alpha significance value 0.05) the NCVs can be considered normally distributed (Fig. 4A), demonstrating that the used set of BGCs was indeed random and suitable for the usage as negative controls. Based on the first quartile of the NCVs an unbiased threshold was set to 0.66 for the classification into true negatives (TN) or false positives (FP). 49 of the 60 negative control BGCs were above the set threshold and were considered as TN. 11 of the negative control BGCs were below the threshold and considered FP (Supplementary data, Table S4).

Positive control BGCs. We used 30 empirically verified BGCs (Table 2) as positive controls for benchmarking the FunOrder tool. These BGCs were all previously characterized by in vivo molecular biology techniques, such as gene deletions or heterologous expression of the genes in different hosts. To evaluate how well the FunOrder tool performs in finding co-evolutionary linked genes and distinguish them from gap genes in positive control BGCs, we compared the results of the FunOrder analysis to the previously published experimental data. To this end, we first analysed each BGC with the FunOrder tool and looked for genes that are co-evolutionary linked with the core biosynthetic gene. These gene sets were considered as “detected” and compared to the empirically proven set of necessary genes. Based on these numbers, we calculated the FCGM and ERM (Equations 1 and 2) for each positive control BGC. A high FCGM value indicates that FunOrder was able identify a large proportion of the necessary genes in the analysed BGC. The FCGM minimum value was 0.33 and the maximum 1, the first quartile was 0.55, the third quartile 0.82 and the median 0.73. Based on a Shapiro-Wilk normality test (alpha significance value 0.05) the FCGMs can be considered normally distributed (Fig. 4B). 20 of the 30 positive control BGCs were above the set threshold of 0.66, these were considered TP. 10 of the positive control BGCs were below the threshold and considered FN (Table 3). A high ERM indicated a low error rate (undetected necessary genes or detected gap genes). The ERM minimum value was 0 and the maximum 1, the first quartile was 0.53, the third quartile 0.8 and the median 0.64. Based on a Shapiro-Wilk normality test (alpha significance value 0.05) the ERMs can be considered normally distributed (Fig. 4C). 15 of the 30 positive control BGCs were above the set threshold 0.66 and were considered as TP, whereas 15 of the positive control BGCs were below the threshold and considered FN (Table 3). As mentioned above a single threshold was chosen as the 1st quartile of the NCV, to avoid a biased choice and a transparent evaluation. The previously exemplarily characterised Lovastatin BGC of Aspergillus terreus (lov) had a FCGM of 0.83 and an ERM of 0.88.

The numbers of TP and FN (Table 3) were used to create two confusion matrices (Fig. 5A and 5B), which were the basis for a thorough statistical testing of the applicability of the FunOrder tool (Table 4). We calculated the normalized Matthews correlation coefficient (normMCC) and other classical metrics and global metrics as indicated by Chicco and Jurman (184). To determine the degree of balance
between positive and negative controls we calculated the no-information error rate $n_i$, which is best for balanced test sets with the value 0.5. The evaluation showed that the $n_i$ value was at 0.67. This still allowed for the usage and confirmed the validity of the classical metrics such as F1 score and Accuracy. Based on the FCGM and the stringent classification threshold (0.66), FunOrder displays overall high metrics in identifying necessary genes in a BGC. Based on the ERM and the stringent classification threshold (0.66) the evaluation metrics can still be considered high regarding the ability of FunOrder to identify necessary genes and distinguish them from gap genes (Table 4).

**Table 3.** Analysis of the positive control BGCs and benchmarking of the FunOrder tool. The experimentally characterized BGCs were analysed with the FunOrder tool and the find correct genes measure (FCGM) and the error rate measure (ERM) were determined by manual comparison to the literature. Based on the thresholds of 0.66, the ERM and FCGM were classified as false negative (FN) or true positive (TP).

| Positive control BGC                  | Species           | Genes | ERM  | FCGM | (FN / TP) ERM | (FN / TP) FCGM |
|---------------------------------------|-------------------|-------|------|------|---------------|---------------|
| 2-Pyridon-Desmethylbassianin (dmb)   | *B. bassiana*     | 4     | 0.50 | 0.50 | FN            | FN            |
| Aflatoxin (afl)                       | *A. flavus*       | 23    | 0.35 | 0.43 | FN            | FN            |
| Botrydial (bot)                       | *B. cinera*       | 7     | 0.36 | 0.67 | TP            | TP            |
| Cephalosporin (cef)                   | *A. chrysogenum*  | 7     | 0    | 0.33 | FN            | FN            |
| Compactin (mlc)                       | *P. citrinum*     | 9     | 0.55 | 0.67 | FN            | TP            |
| Cyclosporin (cy2)                     | *B. felina*       | 13    | 0.31 | 0.38 | FN            | FN            |
| Destruxin (dtxs)                      | *M. robertsi*     | 21    | 0.62 |      | FN            | FN            |
| Fumagillin (fma)                      | *A. fumigatus*    | 15    | 0.87 | 0.75 | TP            | TP            |
| Fumitremorgin (ftm)                   | *A. fumigatus*    | 9     | 0.55 | 0.62 | FN            | FN            |
| Fumonisin (fum1)                      | *F. oxysporum*    | 16    | 0.59 | 0.82 | FN            | TP            |
| Fumonisin (fum2)                      | *F. verticilloides* | 23   | 0.52 | 0.82 | FN            | TP            |
| Fusaric acid (FUB)                    | *F. fujikuroi*    | 15    | 0.87 | 0.92 | TP            | TP            |
| Ilicolin H (ili)                      | *N. sp. DH2*      | 6     | 0.67 | 0.75 | TP            | TP            |
| Leporin (lep)                         | *A. flavus*       | 10    | 0.80 | 0.67 | TP            | TP            |
| Lovastatin (lov)                      | *A. terreus*      | 17    | 0.88 | 0.83 | TP            | TP            |
| Mycophenolic acid (mpa1)              | *P. brevicompactum* | 8   | 0.50 | 0.50 | FN            | FN            |
| Mycophenolic acid (mpa2)              | *P. roqueforti*   | 7     | 0.71 | 0.71 | TP            | TP            |
| Mycophenolic acid (mpa3)              | *P. roqueforti*   | 7     | 0.43 | 0.43 | FN            | FN            |
| Paxillin (pax)                        | *P. paxilli*      | 8     | 1    | 1    | TP            | TP            |
| Penicillin (pen1)                     | *P. chrysogenum*  | 15    | 0.87 | 1    | TP            | TP            |
| Compound                  | Producing Organism | Count | Sensitivity | Specificity | PPV | NPV | FNR | FPR | FDR | FOR | Accuracy | F1 Score | MCC     | normMCC |
|--------------------------|--------------------|-------|-------------|-------------|-----|-----|-----|-----|-----|-----|----------|----------|---------|---------|
| Penicillin (pen2)        | *P. chrysogenum*   | 15    | 0.93        | 1           |     |     |     |     |     |     |         |          |         |         |
| Pestheic acid (pta)      | *P. fici*          | 18    | 0.55        | 0.78        | FN  |     |     |     |     |     |         |          |         |         |
| Pneumocandin (GL)        | *G. lozoyensis*    | 25    | 0.68        | 0.80        |     |     |     |     |     |     |         |          |         |         |
| Sorbicillinol (sor1)     | *P. rubens*        | 7     | 0.57        | 0.57        | FN  |     |     |     |     |     |         |          |         |         |
| Sorbicillinol (sor2)     | *T. reesei*        | 15    | 0.80        | 0.80        |     |     |     |     |     |     |         |          |         |         |
| Tenellin (ten)           | *B. bassiana*      | 5     | 0.80        | 0.75        |     |     |     |     |     |     |         |          |         |         |
| Terrein (ter)            | *A. terreus*       | 10    | 0.57        | 0.86        | FN  |     |     |     |     |     |         |          |         |         |
| Tetramic acid (tas)      | *H. irregularis*   | 8     | 0.50        | 0.50        | FN  |     |     |     |     |     |         |          |         |         |
| Ustiloxin B (ust)        | *A. flavus*        | 19    | 0.68        | 0.54        |     |     |     |     |     |     |         |          |         |         |
| Xanthocillin (xan)       | *A. fumigatus*     | 7     | 0.71        | 0.67        |     |     |     |     |     |     |         |          |         |         |

**Table 4.** Metrics based on the two confusion matrices for FCGM (find correct genes measure) and ERM (error rate measure) (Fig.5). True positive rate (TPR), true negative rate (TNR), positive predictive value (PPV), negative predictive value (NPV), false negative rate (FNR), false positive rate (FPR), false discovery rate (FDR), false omission rate (FOR), Accuracy, F1 score, Matthews correlation coefficient (MCC) and normalized Matthews correlation coefficient (normMCC).
Concluding remarks

In this study, we demonstrated that FunOrder can reliably find co-evolutionary linked genes in BGCs from ascomycetes. The basis but also limitation for FunOrder is the manually curated database. Here we used a set of ascomycete proteomes and were thus able to detect co-evolved genes in ascomycetes. The underlying strategy and workflow of FunOrder can be adapted to analysing genomic regions in other phyla, orders, or even kingdoms by using different databases. In case a larger database is integrated into FunOrder, alternative search algorithms, such as DIAMOND (185) or HMMER (186) (similarity search using hidden Markov models) might be used instead of blastp to enhance the performance.

Here, we further demonstrated that FunOrder can be applied to identify the genes of a BGC that are necessary for the biosynthesis of a SM. Based on a stringent threshold (Fig. 4), we calculated a normalized MCC of 0.7397 (Table 4) for the identification of the necessary genes. Further, with a normMCC of 0.6647 (Table 4) we were able to differentiate between necessary and gap genes in the analysed BGCs, this is also reflected in a high Accuracy of 71.1% (Table 4). The FunOrder tool is delivering data on co-evolution, that needs to be critically evaluated and interpreted keeping the biological background in mind. We introduced the previously discussed measures FCGM and ERM to enable a stringent and robust performance determination to prevent an overestimation of the evaluation metrics. In this study, we looked on genes that share the same or a similar evolutionary background with the core gene(s) of a BGC (e.g., PKS or NRPS-encoding genes). The optional remote search of the non-redundant NCBI protein database delivers further information on the analysed BGC, which can support the data interpretation. FunOrder is a fast and powerful tool that can support scientists to decide which genes of a BGC are promising study objects. The application of FunOrder is not limited to fungal BGC. It can be used for any applications, where information of a shared co-evolution can contribute to a better understanding, such as a genome wide analysis of co-evolving transcription factors or detection of functionally connected protein-protein interactions (29). As a future perspective, FunOrder might be even used for the analysis of total proteomes to detect evolutionary linked genes.

DATA AVAILABILITY

The FunOrder tool, the relevant database, and the sequences and the FunOrder output of the negative and positive control BGCs are available in the GitHub repository (https://github.com/gvignolle/FunOrder).

SUPPLEMENTARY DATA

Supplementary data file 1: MEM_values.xlsx
Supplementary data file 2: supplementary_tables_figures.pdf
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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Figure 1. Schematic representation of the Lovastatin BGC from *Aspergillus terreus* (lov). In red the necessary genes for SM production and in blue the genes not involved in the biosynthetic pathway.
Figure 2. Schematic representation of the workflow of FunOrder.
Figure 3. A selection of the standard output of the FunOrder analysis of the Lovastatin BGC (lov, Fig. 1A). (A) Score plot of the first two principal components from a PCA performed on the combined distance matrix. (B) Score plot of the first two principal components from a PCA performed on the strict distance matrix. Gene necessary for the biosynthesis of lovastatin are highlighted in red.
Figure 4. Kernel density plots of the (A) Negative control values (NCV) calculated based on equation 3, (B) the Find the correct genes measure (FCGM) calculated based on equation 1, and (C) the error rate measure (ERM) calculated based on equation 2. The vertical orange line indicates the 1st quartile of the NCV and the chosen threshold.
Figure 5. Confusion matrices calculated with (A) the Negative control values (NCV) and find the correct genes measure (FCGM) as basis for true negatives (TN), false positives (FP), true positives (TP) and false negatives (FN), (B) the NCV and error rate measure (ERM) as basis for TN, FP, TP, and FN.

| FCGM     | Predicted |       |
|----------|-----------|-------|
|          | positive  | negative |
| positive | 20        | 10    |
| negative | 11        | 49    |

| ERM      | Predicted |       |
|----------|-----------|-------|
|          | positive  | negative |
| positive | 15        | 15    |
| negative | 11        | 49    |