EXPLORING FUNCTIONAL INFERENCE PATTERNS OF BIOSYNTHETIC GENE CLUSTERS VIA THEIR REGULATORY NETWORK

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ABSTRACT

In the search for novel antibiotic compounds, the exploration of predicted Biosynthetic Gene Clusters (BGCs) with genome mining has been a widely used and successful strategy. The family Streptomycetaceae, known to produce most of the current clinically used antibiotics, has shown potential for even more novel secondary metabolites. However, the functional annotation of predicted BGCs with precise functions remains difficult, as validation through laboratory experiments is often necessary. However, due to the frequently cryptic nature of the BGCs, where genes remain inactive or produce no detectable products, prioritizing novel BGCs with antimicrobial properties is difficult. Addressing this challenge, this study focused on the possibility to extend our knowledge about the functions of predicted BGCs, by exploring the complex regulatory system that governs them. Regulatory protein families, particularly those recognized as pathway-specific regulators, are known to directly regulate BGCs. Therefore, we sought to assess the predictive capacities of associations between the regulator families and their BGCs.

This study investigated all 784 experimentally characterized Streptomycetaceae BGCs from the MIBiG database, on which regulatory protein homologs were detected with 1375 regulatory protein families from the Pfam database and 36 regulatory protein families from antiSMASH's smCOGs library. Unfortunately, no clear associations between regulatory protein families and functions or types of the curated BGCs were exhibited. Further exploration of protein families' locations (within or outside of a BGC) in 625 Streptomycetaceae genomes revealed that none of the regulatory families, including SARPs (Streptomyces antibiotic regulatory proteins), exclusively function as pathway-specific regulators for BGCs. Subsequently, a phylogenetic examination of SARP family members revealed clades characterized by shared BGC functions, types, or compound production. For instance, well-known antibiotics like beta-lactam and prodigiosin were associated with SARP homologs that claded together.

In conclusion, this study underscores the complexity of Streptomycetaceae BGC regulation. Even though the regulatory LuxR and SARP protein families showed to be present in BGC regions more than any other regulatory protein family, no unique associations between BGCs and regulatory families were made. Nevertheless, the observed connections among specific clades within the extensive SARP regulator family still indicate the potential to establish associations between BGC types/functions/compounds and subsets of larger regulatory protein families.

1. INTRODUCTION

Over the last decades, the extensive use of antimicrobials within the clinical and agricultural field has led to an acceleration of antimicrobial multi- or even pan drug-resistance among bacterial pathogens. The fast pace at which this happens, combined with the scary slow pace at which new medications are being developed emphasizes the necessity to discover new antimicrobial drugs [1–3]. Most of the current clinically used natural antimicrobial compounds find their origin in bacteria from the Actinomycetota phylum. The most well-known and extensively studied *Streptomyces* are the biggest contributors of all [4]. These members are part of Streptomycetaceae family, a prominent group of gram-positive bacteria that inhabit a wide variety of environments. Streptomycetes are especially well-adapted to thrive in the nutrient-rich and complex organic ground. Their filamentous growth allows them to explore the soil, where they play crucial roles in the degradation of organic matter and recycling of nutrients[5]. Additionally, their ability to produce a wide range of secondary metabolites serves as a competitive advantage and increases their survivability.

The biosynthesis of a secondary metabolite is often encoded within groups of co-localized (clustered) genes, so called biosynthetic gene clusters (BGCs). These BGCs are responsible for the biosynthesis of various bioactive compounds, including antibiotics, antifungals, and antitumor compounds. Numerous BGCs have been identified and characterized and their data is stored in ***MIBiG*** [6,7]. MIBiG (Minimum Information about a Biosynthetic Gene cluster) serves as a repository for curated and standardized information about known BGCs from different microbial sources. Currently, 2502 secondary metabolite clusters are stored in there, from which 784 are from in members in the Streptomycetaceae family. Besides the sequences and genetic locations, it also holds information about the predicted compound and properties of the natural product. All the compounds are classified into the currently available categories, based on their conserved enzymes or pathway types; Polyketides (PK), Ribosomally synthesized and post-translationally modified peptides (RiPPs), Saccharides, Alkaloids, Non-ribosomal peptides, Terpenes and Other (Appendix A:C). Even though most of the BGCs have a type classification, a larger part's functional annotation is still lacking. From the 2502 BGCs, 1410 (56,35%) has no known function linked to it (Appendix A:B), which is emphasizing the need to find methods that could aid in extracting new information using the limited information (types/functions) that is known of the annotated BGCs.

Currently, the validation of the eventual secondary metabolite functions needs to be performed by wet-lab techniques. Unfortunately, this is often not possible, due to the BGC being silent or cryptic[3]. The production of secondary metabolites, thus the expression of the BGCs, is regulated by proteins, which cascades are initiated by external stimuli. However, if the required conditions are unknown, it is difficult to induce the expression in laboratory conditions[8]. Approaches, such as the One-Strain-Many-Compounds (OSMAC), address the latter challenge by submitting the natural compound producing bacteria to different conditions (e.g., co-culture with different bacteria and environmental stimuli or overexpression of regulators). Nonetheless, such techniques are cost and labour intensive and require the metabolite to be already functionally predicted or prioritized.

The prediction of novel BGCs and their core genes depends almost entirely on genome-mining tools like antiSMASH (antibiotics and secondary metabolite analysis shell)[9]. Since 2011, this powerful bioinformatics tool is, with over 1.6 million processed jobs, the most extensively used online bacterial BGC predictor and can detect 81 different BGC types [10]. A combination of rule-based algorithms and profile hidden Markov models (pHMMs) is used to identify BGC regions. Among these is a small-molecule HMM database, also known as the smCOGs (secondary metabolite – Clusters of Orthologue Genes), which represents protein family pHMMs with specific roles in secondary metabolite biosynthesis. These families were curated and classified based on their conserved domains and aid antiSMASH in the annotation of predicted BGC core genes, such as transporters and regulators. Despite huge amounts of genomic data and comprehensive genome mining tools, many predicted metabolites in these predicted BGCs in Streptomycetaceae are hard to functionally annotate. Over the past few years, there has been an increase in the development of algorithms using machine and deep learning models to aid in the prediction and characterization of BGCs. These methods mainly use the sequences of protein families which are localised in BGCs and combine these with the BGCs' currently available and curated annotations. Even though the latter methods show great potential to assist in the search for novel BGCs, they all have a limitation in common; the availability of known and experimentally validated BGC types and classes. Nevertheless, these

recent studies do show the potential to use pattern-based approaches on protein families as a method to extend the knowledge on BGCs and their properties [11–13].

This shown potential opens new methods to, ultimately, extend our knowledge on known and novel BGCs by using the protein families within them. Especially regulatory protein families are expected to aid here. Within the complex regulatory network, BGCs are often directly regulated by a combination of global and pathway-specific regulators. Global regulators oversee the expression of multiple BGCs across the genome. They act as master regulators that coordinate the expression of various secondary metabolite biosynthetic pathways in response to cellular and environmental signals[14–16]. Pathway-specific regulators, on the other hand, are associated with a particular BGC and directly control its activation or repression[16–18]. Therefore, a deeper understanding of the biological activities is expected to be gained from associations of pathway-specific regulator families with the corresponding BGCs. For example, if a BGC is associated with a pathway-specific regulator that is known to control the biosynthesis of a specific class of antibiotics, it is reasonable to hypothesize that the silent or cryptic BGC, regulated by the same family, could also be involved in the production of a related antibiotic compound. Similarly, the presence of global regulators associated with multiple BGCs in the same gene cluster might suggest that these BGCs are co-ordinately regulated and may produce complementary or similar metabolites. Even though evidential literature to support this concept is currently lacking, large regulatory families like the Streptomyces antibiotic regulatory protein (SARP) family have been shown to be directly involved in the regulation of BGCs and their products[19,20]. Not only as pathway-specific regulators, but also from a more pleiotropic position[21,22].

Here, we report on the exploration of regulatory protein families in Streptomycetaceae BGCs and the consequent hypotheses that arise from it. We assessed the possibility to make associations between regulatory protein families and (curated) BGCs in the 625 members of the Streptomycetaceae family. BGCs and complete genomes were analysed to see whether there might be regulator families, that could solely or mainly be associated with the regulation of (specific types of) BGCs. Subsequently, the association concept was assessed by a case study of the earlier mentioned SARP family.

2. MATERIALS & METHODS

During this study, different genomic perspectives were highlighted and used to create possibilities to associate regulatory families with biosynthetic functions in the biosynthetic compound producing Streptomycetaceae family. In the section below, the approaches and tools are being described. Tools were run with default parameters if none are specified. If no tool is mentioned, the task is performed with an in-house written python script (indicated by an asterisk (*), followed by the script number). An overview of these can be found in Appendix D. A visual representation of the scripts (data flow), during this thesis, is shown in supplementary data Appendix I Content of all the scripts, raw data locations (also described in Appendix C) and (intermediate) output files can be found on https://git.wur.nl/daan.vannassauw/thesis_BGC_functional_inference.

2.1. DATA COLLECTION

Multiple data sets were obtained from various data sources to be used as starting points. Firstly, 2502 GenBank and JSON files were retrieved from the MIBiG database to extract the most recent, available, and curated BGCs. (v3.1) [downloaded April 12th, 2023 (GenBank) & April 24th, 2023 (JSON)]. Coding sequences and identifiers of BGCs, with their origin in members of the *Streptomycetaceae* family, were extracted from the GenBank files and transformed to fasta files, containing a single BGC each (*1). Pseudo genes or incomplete translations were neglected to improve credibility of future hypotheses. Information (names, types, and functions) of the BGCs and their compounds were extracted from the JSON files (*2). Furthermore, 625 representative Streptomycetaceae genomes were collected from NCBI RefSeq [Downloaded April 21st, 2023 – search term: *txid2062[Organism:exp] AND (latest[filter] AND "representative genome"[filter] AND all[filter] NOT anomalous[filter]*]. These were selected to include a representative of each Streptomycetaceae member, while excluding assemblies with anomalies. Like the BGC extraction, solely available coding sequences of non-pseudo proteins were extracted (*3). For the eventual extraction of regulatory protein family profile Hidden Markov Models (pHMMs), the complete PFAM HMM library was downloaded as the most extensive and updated source

of protein families[23] [Downloaded March 14th, 2023], together with the smCOG BGC associated HMM library from antiSMASH[24] [Downloaded April 26th, 2023].

2.2. SELECTION OF REGULATORY PROTEIN FAMILIES

Protein families in the HMM libraries of antiSMASH' smCOGs and the PFAM database are involved in a wide variety of functions. For this project, solely protein families with regulatory properties were desired from both sources. The regulatory HMM subsets were created and consecutively indexed using HMMER's (v3.3.2) *hmmfetch* on the PFAM and smCOG HMM libraries by feeding it a list of regulatory-related keywords, which were expected to be found in the protein family descriptions in the Pfam and AntiSMASH's smCOG libraries (Appendix B | *4,5)[25]. There is a significant difference in key terms for both sources (see Appendix B) is explainable by the different aims of both extractions; the smCOG library only contained 36 regulatory protein families (annotated by the tool with 'R')[26], where the total number of regulatory protein families in the PFAM database is unknown. Therefore, these were extracted with the aim to capture as many as possible. Detection of unwantedly captured smCOG HMMs was done manually by comparing it to antiSMASH' annotations[26]. These were removed to solely capture regulatory protein family homologs. From now on, the subsets with regulatory proteins will be referred to as Pfam-R and smCOG-R.

2.3. REGULATORY PROTEIN DOMAIN DETECTION APPROACH

The presence of regulatory protein families was detected using HMMER's *hmmscan,* which takes a BGC or genome fasta file and Pfam-R or smCOG-R as input and returns all captured homologs of protein family pHMMs (*6,7). Trustworthiness of the domain homologs was increased by setting cut-offs. Pfam-R homology hits followed the in-house trusted cut-off from the Pfam database (often bit scores > 22), where the smCOG-R homologs with E-values < 1E-16 were neglected. The latter was done as it is in line with antiSMASH' internal annotation process[27]. For both the BGCs and complete genomes, all the locations, protein family IDs and bit scores of the homology hits were extracted and summarized into one overview (*8).

2.4. REGULATORY PROTEIN FAMILIES IN CURATED STREPTOMYCETACEAE BGCs

The overview of Pfam-R and smCOG-R matches occurring in BGCs from MIBiG was combined with the compound and type information of BGCs to create one large dataset, which is ideal for pattern recognition (*9). Visualization of this annotated list of homologs followed a network-structured approach using CytoScape (v3.10.0) [28]. SmCOG-R and Pfam-R IDs were set as source nodes, while the BGC IDs functioned as target nodes. Pattern detection and prioritizing further interesting events was done manually. Visualization of the nine most frequently occurring functions was done of families that had at least ten homologs in the entire MIBiG BGC dataset. This was performed using Python's Matplotlib (v3.7.2)[29] (*17).

2.5. REGULATORY PROTEIN FAMILIES IN STREPTOMYCETACEAE GENOMES

BGC regions in the genomes were predicted by feeding the GenBank files to antiSMASH (v7.0) [10] and tabulated via Z. Reitz's existing workflow[30]. The locations of the Pfam-R and smCOG-R homologs were then compared to the predicted BGC region locations to determine whether the homolog was found in- or outside a predicted BGC region (*10). The same custom script also calculated the shortest distance from the homolog to the nearest predicted BGC edge. All information on the homolog locations, bit scores, predicted BGC locations, predicted products and distances to the nearest edges were combined into one single data frame (*11). Homologs occurring in draft genomes, indicated by a genome ID > 11 characters, were separated from complete genome homologs to improve the hit credibility. Simultaneously, homologs with close distances to the BGC edge (< 500 nucleotides) or with unknown values ("NA") were filtered out (*12). The fraction of in BGC-laying homologs was then calculated for the remaining homologs in complete genomes to identify possible BGC-associated protein families (*13).

2.6. SARP FAMILY OF REGULATORS - CASE STUDY

Matches of the SARP family in the complete, filtered genomes of the Streptomycetaceae family were located and its protein sequences were extracted (*14). Due to the large number of proteins, a dereplication approach was initiated. The collected sequences underwent a greedy incremental clustering with a 98% target coverage using MMseqs2 (v14.7.e284)[31]. It sorts the protein sequences from largest to smallest, aligns all sequences that

are covered for at least 98 percent & continues once there are none left. The representatives (from here on referred to as SARP-reps) of each cluster and their protein sequences were collected and visualized using CytoScape (v3.10.0)[28]. Subsequently, the SARP-reps' sequences were aligned against the original SARP profile HMM (SMCOG1041), which was caught from the smCOGs' HMM library using HMMER's, earlier mentioned, *hmmfetch*[25]. The alignment, executed by HMMER's *hmmalign,* simultaneously trimmed terminal tails of unaligned amino acids. After that, a transformation of the SARP-reps IDs took place (*15), while all insertions relative to the SARP HMM were removed from the alignment to compare the SARP homologs directly (*16). The resulted alignment of SARP-reps was used as input in IQ-TREE (v2.2.2.3)[32] for tree building by maximum likelihood (ML), which was performed with the ModelTest option and Ultrafast Bootstrap approximation to increase procedure speed[33]. Eventually, the tree was visualized and annotated with in/out BGC locations, BGC types and edge distances to the nearest BGC using the Interactive Tree of Life (iTOL) (v6.8)[34]. Pattern recognition and literature research was done manually.

3. RESULTS & DISCUSSION

3.1. EXTRACTION OF REGULATORY PROTEIN FAMILIES

Maximizing the amount of detected regulatory protein families started with the collection of them from the large PFAM and smCOG HMM libraries. To achieve this, regulatory related key terms that were expected to be present in regulatory family descriptions were created and used for the library subset creations, named Pfam-R and smCOG-R. The extraction of regulatory protein families led to library sizes of 1375 families in Pfam-R and 39 in smCOG-R. Since we were only interested in regulatory protein families, non-regulatory captured smCOGs ('SMCOG1132', 'SMCOG1210' and 'SMCOG1174') were manually removed from the list. These were unwantedly captured by HMMER's *hmmfetch* as a result. The Pfam-R could also contain non-regulatory or non-prokaryotic protein families; however, manual filtering 1375 families would take too much time and most of them were not expected to have a lot of hits anyways.

3.2. REGULATORY FAMILIES IN STREPTOMYCETACEAE BCGs

To find the regulatory protein families that are present in the current curated BGCs, 784 experimentally characterized BGC clusters with their origin in *Streptomycetaceae* were collected from MIBiG. Presence detection of these families was facilitated by the Pfam-R and smCOG-R pHMM subsets. Subsequently, the regulatory protein family homologs were assessed for possible links between families and BGC functions. Figure 1 shows the functional landscape of the BGCs in which the protein families occurred in.

As previously stated, more than 56% of both the currently identified and curated BGCs lacks definitive functional annotations. Ideally, BGCs with unknown functions could be annotated by the function of other BGCs, that share the same regulatory family. Almost all the regulatory families occur in one or more of the 784 BGCs that produce compounds annotated to have antibacterial, antifungal, cytotoxicity and inhibitory properties (often all of them).

The exception to occurring in multi-functional annotated BGCs was the Carboxypeptidase regulatory-like domain (CRL-D) (PF13620), which was found in BGCs with a single annotated function besides the non-annotated (NA) ones. Nine of the eleven BGCs harbouring this domain remain functionally uncharacterized, while the remaining two were annotated with an antibacterial function. Nevertheless, it is more likely that the CRL domain solely plays a role in the modification of EmrB/QacA drug resistance transporter proteins (smCOG1005), rather than fulfilling a regulatory position[35–42]. Therefore, following-up on the NA BGCs in which this CRL domain occurs to assess the potential association of the domain with the antibacterial function is unnecessary. At the same time, no consistent trends have been observed across all other protein families in relation to the functional annotations of available BGCs.

It was also noted that the Pfam-R library captured homologs in experimentally characterized BGCs, that were not captured by the smCOG-R library (see Appendix H). Among these were mainly helix-turn-helix domains that, after a literature search, have shown to have regulatory properties.

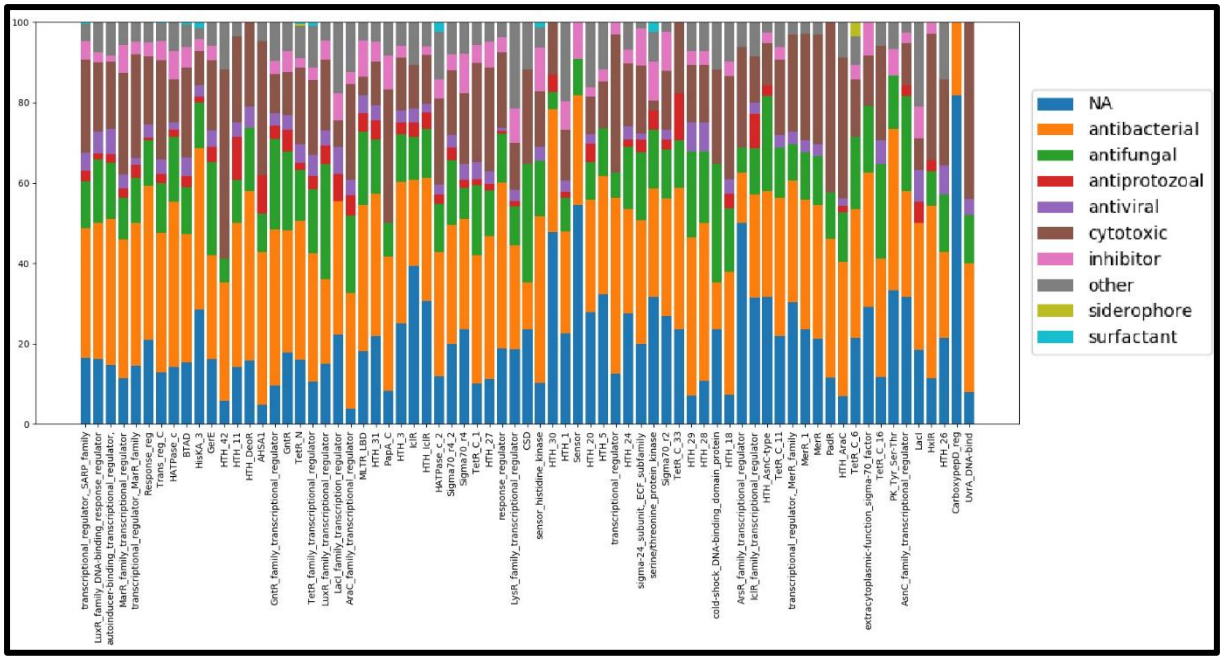


FIGURE 1 FRACTION OF BGC FUNCTIONS PER REGULATORY PROTEIN FAMILY (BOTH PFAM-R AND SMCOG-R) BASED ON THE 4444 DETECTED HOMOLOGS IN CURATED STREPTOMYCETACEAE BGCS. PROTEIN FAMILIES WITH TEN OR LESS DETECTED HOMOLOGS WERE EXCLUDED.

FRACTION OF REGULATORY FAMILIES WITHIN PREDICTED BGC AREAS

The identification of pathway-specific regulatory families that would exclusively be associated with the regulation of BGCs would lead to a significant increase in annotated BGCs. This detection method involved a comparison of occurrences of each regulatory protein family within the predicted BGC regions of antiSMASH against the total occurrences in Streptomycetaceae genomes. These values together yield the fraction of occurrences inside BGCs for each family.

Prior to this extraction, the detected homologs underwent filtering steps to improve the credibility of eventual associations and avoid missing broader patterns. Mainly, as the BGC regions, predicted by antiSMASH, consist of a core gene section and hard-coded extensions (between 5 and 20 kb) on both sizes. Therefore, only homologs that were found in complete genomes, without non-annotated distances to the nearest predicted BGC and distances larger than 500 bases were considered (see table 3).

TABLE 1 THE REMAINING NUMBER OF PROTEIN FAMILY MEMBERS PER TYPE OF STREPTOMYCETACEAE GENOME DATA TYPE BEFORE & AFTER APPLYING CONSECUTIVE FILTER CONDITIONS. THE NUMBER OF REMOVED HOMOLOGS ARE SHOWN BETWEEN BRACKETS

Filtering condition	All genomes	Complete genomes	Draft genomes
Unfiltered homologs	1,069,877	287,118	782,757
Homologs without an edge distance value (NA) excluded	723,698 (-346,179)	269,161 (-17,958)	454,538 (-328,219)
500 base pair distance to nearest BGC edge	710,161 (-13,539)	265,118 (-4,042)	445,042 (-9,496)
Remaining homologs	710,161 (-359,716; 33,63%)	265,118 (- 22,000; 7,66%)	445,042 (-337,715; 43,14%)

Starting with almost 1.07 million protein family homologs in the entire dataset turned into 265 thousand protein family homologs in the complete Streptomycetaceae genomes to continue with. The largest losses of the filtering steps are seen among the draft genomes (-43,1%; 337,715), where the complete genomes only lose a fraction of it (-7,2%; 22,000). The largest impact was delivered by the filtering on missing values, indicating the edge distances were not able to be calculated. Either no BGC regions were predicted in those genomes or, the data was too fragmented or lacked information, which is more likely to occur in draft genomes.

Figure 2A shows the total occurrences and the fraction of IN BGC locations for each protein family. Families containing high "IN BGC fraction" values often possess a relatively low overall count (<20), making them less reliable indicators of a family-BGC association. In contrary, protein families displaying a high number of matches still tend to fluctuate, but between a more constrained range (5% – 15%). The patterns demonstrate a 'baseline', which could be explained by the fact that 75% of the used Streptomyces genomes have a BGC content of 15% or less (see figure 2C). Therefore, IN BGC ratios around this baseline could be considered as random occurrences. Exceptions here are the families that have a significant number of occurrences and raise clearly above the 'baseline' (see figure 2B); the SARP family, LuxR family and the bacterial transcriptional activation domain (BTAD) family.

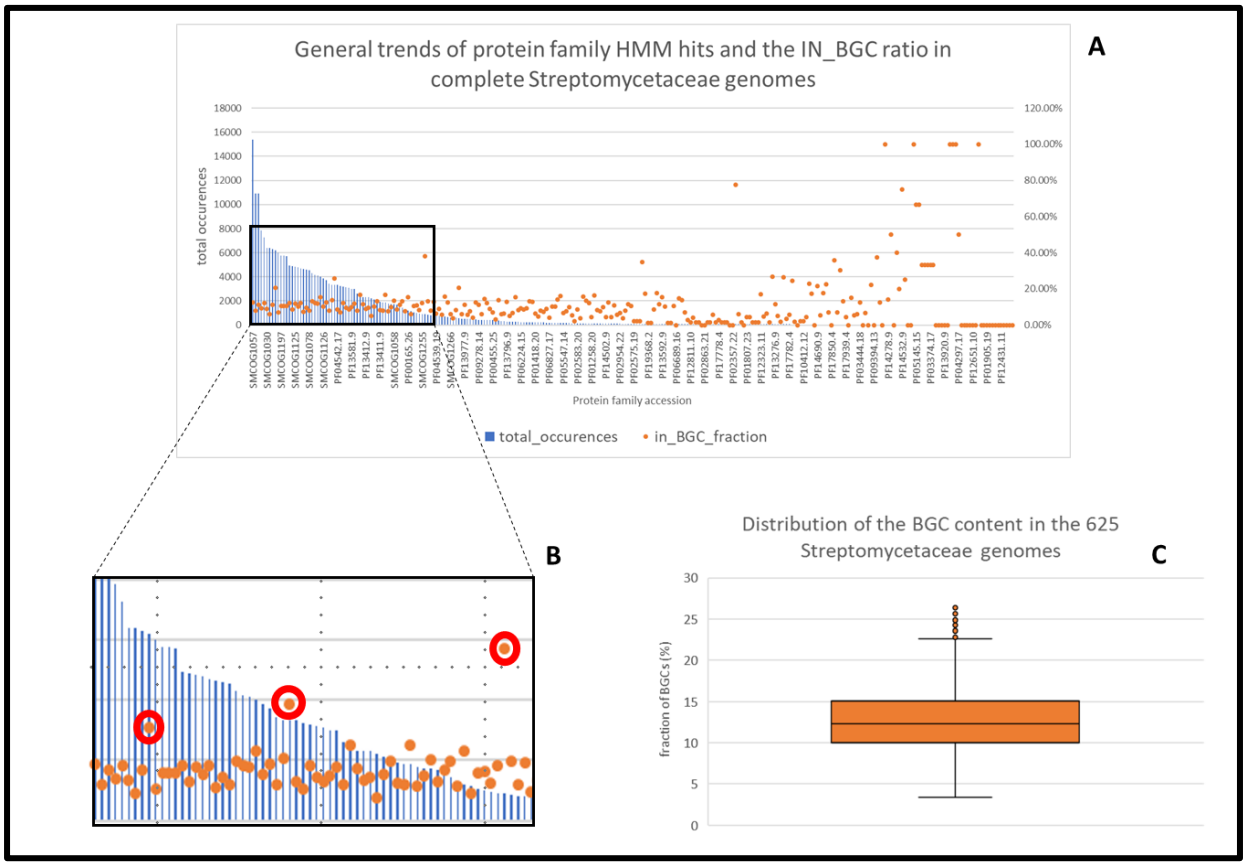


FIGURE 2 A) THE TOTAL AMOUNT OF OCCURRENCES PER PROTEIN FAMILY AND THE FRACTION THAT IS DETECTED INSIDE ANTISMASH'S PREDICTED BGC REGIONS. THE FAMILIES ARE SORTED FROM HIGHEST TO LOWEST NUMBER OF TOTAL HOMOLOGS IN STREPTOMYCETACEAE. **B)** REGULATORY PROTEIN FAMILIES WITH >900 TOTAL OCCURRENCES. RED CIRCLE 1 = SARP FAMILY (6191 HOMOLOGS - 20.7% IN BGC RATIO), RED CIRCLE 2 = LUXR FAMILY (3333 HOMOLOGS – 25.7% IN BGC RATIO), RED CIRCLE 3 = BTAD (906 HOMOLOGS – 38.0 % IN BGC RATIO), **C)** DISTRIBUTION OF BGC CONTENT FRACTIONS ACROSS ALL 625 STREPTOMYCETACEAE SPECIES (ALL VALUES CAN BE FOUND IN APPENDIX J).

In the dataset, the members of the LuxR regulatory family appeared 3333 times, with an IN BGC ratio of 25.7%. This observed elevated IN BGC ratio is in line with expectations, given the major role of the LuxR family in quorum sensing[43,44]. Furthermore, research onto the LuxR family in gram-positive bacteria has revealed an evolutionary history that contributed to a large diversity among its members. It led to LuxR regulators functioning within one-

or two-component signalling systems, with numbers up to 69,000 solo LuxR instances across 800 gram-positive bacterial genomes [45,46]. This indicates that finding high occurrences for this family is not entirely unexpected.

The BTAD is a domain located after the N-terminal DNA-binding domain of in Mycobacterium's EmbR regulator and in SARP family members[47–49]. This means that this domain is not entirely exclusive to the SARP family[50]. However, given the inclusion of solely Streptomycetaceae genomes, we expect that these 906 found homologs are found in members from the SARP family. Unfortunately, this has not been assessed in this study. Moreover, we still noticed a difference of 5285 detected homologs, that were detected with AntiSMASH's smCOG1041, but not with Pfam-R's BTAD. Differences between the HMM profile sizes (811AA for SARP vs. 146AA for BTAD) could aid as it makes the smCOG1041 more inclusive, capturing a broader range sequences. To better understand these differences, further analysis and comparison of the two HMMs and the detected homologs would be valuable by direct profile-profile comparison tools as HHsuite[51].

Furthermore, given the association of the SARP family with the regulation of BGCs and its usual localization inside those regions, raises questions why we observed such a low IN BGC ratio for this family. Multiple factors might contribute to this latter finding. Firstly, antiSMASH might not yet be able to identify a broader spectrum of novel BGC types, if SARP regulators exclusively regulate BGCs. However, given the extensive presence of SARP homologs in Streptomycetaceae, this scenario appears improbable. A second hypothesis was based on a study by *Krause et al*[52]., who performed a similar approach to detect SARPs using SMCOG1041 in Actinobacteria, revealing hits in Proteobacteria even without the BTAD or HTH motif [52]. This suggests the possibility that SMCOG1041's detection scope might be marginally wider than intended. Lastly, the most plausible explanation is based on the entire scientific perspective on SARP members. While their characterization as pathway specific BGC regulators is widely accepted, it is also known that some members, like AfsR, regulate multiple pathways from a more global perspective[22]. Therefore, it is not unlikely to think that there might be more SARP family members regulating from a more pleiotropic perspective[53].

VARIATION AMONG THE SARP FAMILY

To explore the diversity within the SARP family members and find the underlying reasons for the observed low IN BGC ratio, all 3093 homologs in the Streptomycetaceae genomes were collected. They were subjected to clustering and representatives for each cluster (SARP-reps) were subsequently aligned against the SARP HMM (smCOG1041). Alignment showed SARPs with minimal alignment lengths of 620 AA to the 811 AA long SARP HMM. A phylogenetic tree was reconstructed of the alignment and annotated with the IN_BGC Boolean, distances to the nearest BGC edge and compound annotations. The resultant tree contains 601 SARP-reps and 42 distinct BGC types, as shown in Figure 4.

Across the circular tree, elevated areas of SARP members found in predicted BGC regions are seen and indicated by the red circle ("SARP hit location"). These segments exhibit homologs that are evidently linked to BGCs. However, these segments frequently contain SARP-reps that are not localized in predicted BGC regions. When considering the distances to the nearest predicted BGC boundaries, some of these homologs display distances exceeding 150,000 nucleotides. Such distances could potentially signal the presence of novel, undetected BGCs. Moreover, during the clustering process of all SARPs, instances emerged where an entire cluster was situated within a BGC region, except for the cluster's representative member (Appendix E – red circles). Notably, these SARP-reps were primarily observed in the segments with BGC-associated SARP homologs (figure 4 – red labels).

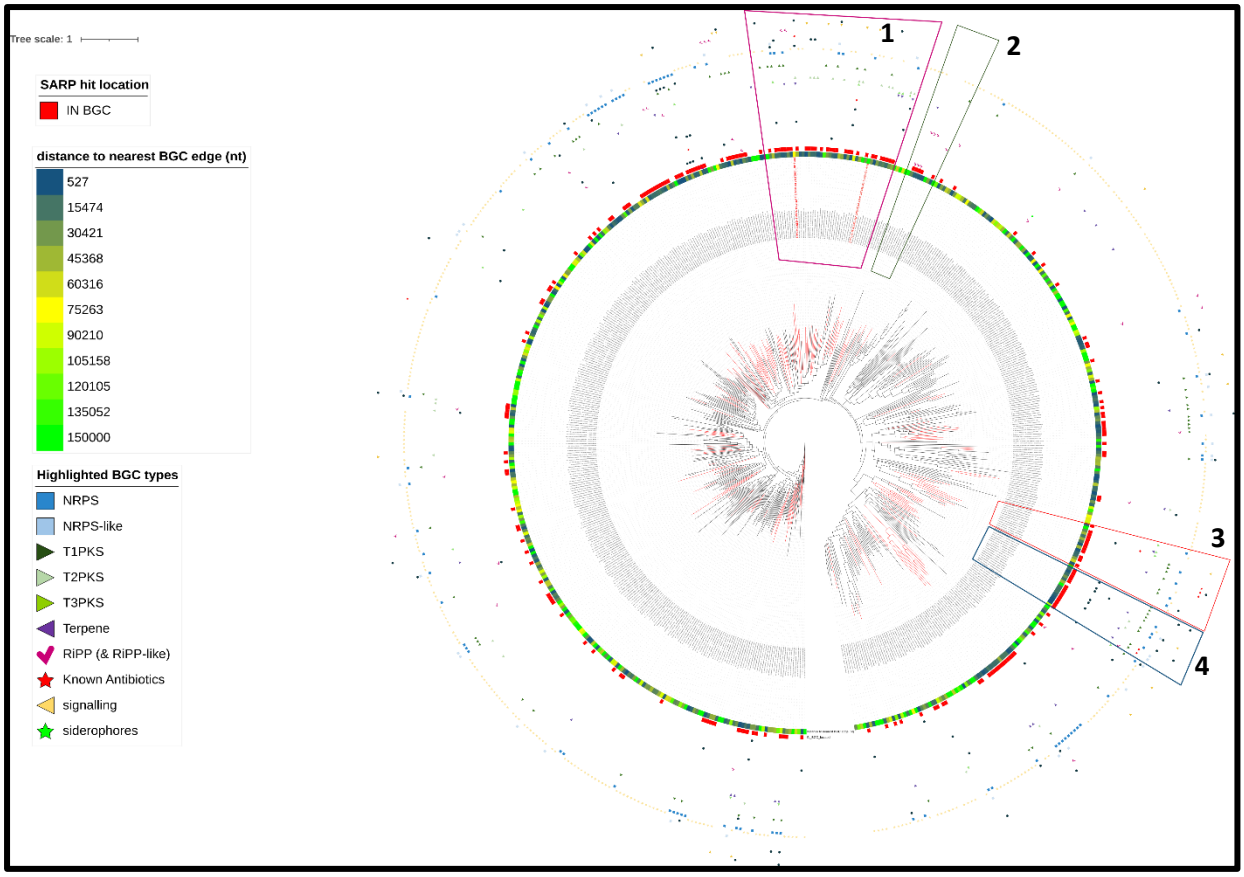


FIGURE 3 601 REPRESENTATIVE SARP HOMOLOGS WITH ANNOTATED LOCATION (IN OR OUT BGC), DISTANCE TO NEAREST BGC EDGE AND EVENTUAL BGC PROPERTIES. THE CIRCULAR TREE, BASED ON MAXIMUM LIKELIHOOD, WAS MIDPOINT-ROOTED AND BUILT WITH MODELTEST AND ULTRAFAST BOOTSTRAP APPROXIMATION. SUPPLEMENTARY VISUALIZATIONS OF COLOURED BOXES 1, 2, 3 AND 4 ARE AVAILABLE IN APPENDIX F.

Manual checking led to some noteworthy patterns (figure 4 - coloured boxes). Firstly, the purple area (#1) shows SARP homologs in a clade with visually elevated levels (30/44) of Type-II polyketide synthase (T2PKS) BGCs of which two are known antibiotics (2dos[54,55], phenazine[56,57]). Often, these T2PKS type BGCs are double annotated with either the T1PKS or T3PKS in the MIBiG database. In general, polyketide types cover a long list of clinically used antimicrobial compounds as tetracycline, anthracycline, amphotericin and avermectin[58–61]. Therefore, there might be potential to associate that clade of SARP-reps with T2PKS type BGCs, which could lead to the discovery of novel BGCs with compounds that have antimicrobial properties. Secondly, the dark-green area (#2) contains a clade with visually elevated levels (5/9) of SARPs in ribosomally synthesized and post-translationally modified peptide classes (RiPPs). This type embodies an enormous family of small molecule natural products with diverse functions, which caused the huge interest in assessing their potential in antibiotic compound discovery[62,63]. Lastly, the red box (#3) and blue box (#4) contain visually elevated (12/15 & 14/15) SARP-reps that are found in BGCs of the T1PKS type. Among the same segments, multiple known antibiotic compound producing BGCs are present. Multiple SARP-reps, that are found in BGCs for beta-lactam were clustered immediately next to each other. The same was shown for SARP-reps that are in prodigiosin producing BGCs. This indicates that there could be an association between those clades of SARP-reps and antibiotic compound synthesizing BGCs. Obviously, statistical substantiation is necessary to prove these predictions as there is phylogenetic non-independence among the Streptomycetaceae and their BGCs. This means that their characteristics are not statistically independent, due to their evolutionary history. If these are ignored, conclusions could be incorrect or overestimate the relationships between traits like a SARP homolog and the traits of the BGC they are found in. Phylogenetic regression models could aid in this situation, an example is the Caper package[64,65]. Unfortunately, this was outside of this project's scope due to the lack of time.

During this study, it was attempted to demonstrate associations between regulatory protein families and BGC properties (types, functions, etc.) in Streptomycetaceae with the idea to, ultimately, use these for functional predictions of yet unannotated BGCs. To the extent of our knowledge, such a concept has not been attempted and proofed before. An important necessity that allows making associations in the first place, is a high completeness of the dataset you are working with. Unfortunately, with over 56 percent of available, curated BGCs from MIBiG being unannotated, this has not come to our advance. Luckly, detection of regulatory protein families was not affected by the lack of annotative information, as you solely require the presence of complete genomes and the pHMMs of protein families. Both sources of the pHMM libraries (Pfam and antiSMASH) aided in the detection of protein families and followed the same principle, however, they displayed some fundamental differences along the way. The Pfam database is suggested to contain more specific pHMMs compared to the library of smCOGs. Not only does the Pfam database contain more pHMMs per regulatory protein family (example TetR: 41 vs. 4), but they also showed lower numbers of detected homologs per family in the complete Streptomycetaceae genomes (examples in Appendix G). Similarly, the Pfam database lacks a pHMM that covers the entire SARP as a protein family, but it does have pHMMs for SARP characterizing domains; BTAD, N-terminal winged HTH DNA-binding domain (Trans_reg_C) and TPR_12 (for larger SARPs)[52,66]. The latter was not included in this study. With the detected differences in specificity between the databases in mind, we would like to suggest an update of the smCOG library of AntiSMASH. Not only by adding novel regulatory domains, that capture regulators in experimentally validated BGCs (examples in Appendix H), but also as more specific pHMMs (e.g., of family subdivisions) might lead to more specific associations between regulator families and BGC annotations.

To gain more perspective in the locations of the regulatory homologs, the shortest distances from the homologs to the nearest predicted BGC edge was calculated and used as a label in the manual pattern finding in the SARP case. Initially, it was intended to predict potential novel BGC types, that could not be detected by antiSMASH yet (SARP homologs with huge differences to the nearest predicted BGC). Simultaneously, it could help manually assessing cases, where the regulator would be very close to the predicted BGC edge. Especially, knowing that the edges of the predicted BGC types are hardcoded by antiSMASH as extensions between 5 kb and 20 kb. Therefore, we would argue that an alternative approach i.e., calculating the distance to the nearest core genes of predicted BGCs would be more effective in the concept of making meaningful associations. Only not to establish associations between regulator families and complete BGCs, but to assess the possibility to predict associations between regulators and certain core genes.

No direct associations between a single regulatory protein family and one of the curated BGC types or known functions were displayed, which was not entirely unexpected. The BTAD, LuxR and SARP families of regulators showed a higher occupation in predicted BGC areas than any other regulatory protein family. This study displayed 1280 SARP homologs and 857 LuxR homologs within (predicted) BGC regions among the complete Streptomycetaceae genomes. SARPs have been described before to have multiple occurrences within a single BGC, which means that the 1280 homologs are probably not directly present in 1280 BGCs (detected and undetected)[67–69]. Estimation of the SARP distribution within this study has not been evaluated but could give additional insight into the complex regulatory network. Similarly, indications that subdivisions of the SARP family could be enriched in clades of certain BGC traits based on phylogenetic analysis were there, but they require statistical comparative analysis (phylogenetic regression models) to make the predictions meaningful. Nevertheless, phylogenetic analysis of large regulator families, involved in BGC regulation, is suggested to be helpful in prioritizing interesting cases of pathway-specific regulators in clades with specific BGC traits.

4. Conclusion

While the library of novel predicted BGCs in Streptomycetaceae keeps expanding, the need for functionally inferring them those does too. While the eventual functions of novel metabolites need to be validated experimentally, computational predictions could aid in prioritizing and save time and money that way. This study assessed the possibility to extend our knowledge on BGC functions by attempting to predict associations of enriched regulatory protein families in known with specific BGC types and functions. The initial linkage on protein regulator families to known BGCs and their types/functions did not show any cases with a full association

between a regulatory protein family and a BGC property (function or type). The locational assessment only showed the two large regulator families, LuxR and SARP, to have affinity with BGCs. It did become clear that, within the SARP family, there is potential to find associations between enriched sub-groups of the regulator family in specific BGCs with specific properties. Nevertheless, this requires further investigation and statistical substantiation. A follow-up on SARP homologs that are in the earlier mentioned boxes with large distances (>150k nucleotides) to the nearest BGC would be necessary to proof the concept of associating regulatory members to functionally infer unannotated BGCs. The functional diversity of the analysed SARP family does highlight the complex regulatory networks that the Streptomycetaceae uses to adapt to their environment and produce a wide range of bioactive compounds.

5. Recommendations

The following section presents key recommendations derived from the findings and analyses conducted in this study. Firstly, the assessment of SARP homologs that were found in clades with enriched BGC-associated SARPs and showing high distances to the nearest BGCs. Such cases could be an indication of novel BGC types in that area. One option would be the use of different BGC prediction tools, that use machine- or deep learning principles (e.g., GECCO or SanntiS) to evaluate the potential of those regions with, by antiSMASH, (yet) undetected BGCs[70,71]. Secondly, as there are large quantitative detection differences between the smCOG-R and Pfam-R libraries (examples in Appendix G), a direct comparison of Pfam-R and smCOG-R HMMs through tools like *HHsuite* might be worth exploring. This could aid in creating subdivisions among regulatory families and creation of the respective pHMMs. The main example being here the SARP smCOG and the SARP characterizing domains from the Pfam database. Lastly, it would be worth to propose an update of the smCOG library, as it showed not capturing all regulatory domains in experimentally validated BGCs (see Appendix H). Besides the addition of novel regulatory protein families, the update could focus on the creation of more specific pHMMs of already available families. This fine-tuning could potentially lead to more targeted connections in the future between regulator families and BGC annotations.

6. REFERENCES

1. Cooper, M. A. & Shlaes, D. Fix the antibiotics pipeline. *Nature 2011 472:7341* **472**, 32–32 (2011).
2. Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M. & Hanage, W. P. Antibiotics in agriculture and the risk to human health: how worried should we be? *Evol Appl* **8**, 240–247 (2015).
3. Miethke, M. *et al.* Towards the sustainable discovery and development of new antibiotics. *Nature Reviews Chemistry 2021 5:10* **5**, 726–749 (2021).
4. Donald, L. *et al.* Streptomyces: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective. *Microbiol Res (Pavia)* **13**, 418–465 (2022).
5. Anandan, R. *et al.* An Introduction to Actinobacteria. *Actinobacteria - Basics and Biotechnological Applications* (2016) doi:10.5772/62329.
6. Medema, M. H. *et al.* Minimum Information about a Biosynthetic Gene cluster. *Nat Chem Biol* **11**, 625–631 (2015).
7. Terlouw, B. R. *et al.* MIBiG 3.0: a community-driven effort to annotate experimentally validated biosynthetic gene clusters. *Nucleic Acids Res* **51**, D603–D610 (2023).
8. Hoskisson, P. A. & Seipke, R. F. Cryptic or Silent? The Known Unknowns, Unknown Knowns, and Unknown Unknowns of Secondary Metabolism. *mBio* **11**, 1–5 (2020).
9. Medema, M. H. *et al.* antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res* **39**, (2011).

10. Blin, K. *et al.* antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. *Nucleic Acids Res* **51**, W46–W50 (2023).

11. Walker, A. S. & Clardy, J. A Machine Learning Bioinformatics Method to Predict Biological Activity from Biosynthetic Gene Clusters. *J Chem Inf Model* **61**, 2560–2571 (2021).

12. Rios-Martinez, C., Bhattacharya, N., Amini, A. P., Crawford, L. & Yang, K. K. Deep self-supervised learning for biosynthetic gene cluster detection and product classification. *PLoS Comput Biol* **19**, e1011162 (2023).

13. Liu, M., Li, Y. & Li, H. Deep Learning to Predict the Biosynthetic Gene Clusters in Bacterial Genomes. *J Mol Biol* **434**, 167597 (2022).

14. Wei, J., He, L. & Niu, G. Regulation of antibiotic biosynthesis in actinomycetes: Perspectives and challenges. *Synth Syst Biotechnol* **3**, 229–235 (2018).

15. Novakova, R. *et al.* A New Family of Transcriptional Regulators Activating Biosynthetic Gene Clusters for Secondary Metabolites. *Int J Mol Sci* **23**, 2455 (2022).

16. Van Der Heul, H. U., Bilyk, B. L., McDowall, K. J., Seipke, R. F. & Van Wezel, G. P. Regulation of antibiotic production in Actinobacteria: New perspectives from the post-genomic era. *Nat Prod Rep* **35**, 575–604 (2018).

17. Kormanec, J., Novakova, R., Mingyar, E. & Feckova, L. Intriguing properties of the angucycline antibiotic auricin and complex regulation of its biosynthesis. *Appl Microbiol Biotechnol* **98**, 45–60 (2014).

18. Bibb, M. J. Regulation of secondary metabolism in streptomycetes. *Curr Opin Microbiol* **8**, 208–215 (2005).

19. Ramos, J. L. *et al.* The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev* **69**, 326–356 (2005).

20. Chen, J. & Xie, J. Role and regulation of bacterial LuxR-like regulators. *J Cell Biochem* **112**, 2694–2702 (2011).

21. Tanaka, A., Takano, Y., Ohnishi, Y. & Horinouchi, S. AfsR Recruits RNA Polymerase to the afsS Promoter: A Model for Transcriptional Activation by SARPs. *J Mol Biol* **369**, 322–333 (2007).

22. Floriano, B. & Bibb, M. afsR is a pleiotropic but conditionally required regulatory gene for antibiotic production in Streptomyces coelicolor A3(2). *Mol Microbiol* **21**, 385–396 (1996).

23. Blum, M. *et al.* The InterPro protein families and domains database: 20 years on. *Nucleic Acids Res* **49**, D344–D354 (2021).

24. antismash/antismash/detection/genefunctions/data/smcogs.hmm at master · antismash/antismash · GitHub. https://github.com/antismash/antismash/blob/master/antismash/detection/genefunctions/data/smcogs.hmm.

25. HMMER. http://hmmer.org/.

26. antismash/antismash/detection/genefunctions/data/cog_annotations.txt at master · antismash/antismash · GitHub. https://github.com/antismash/antismash/blob/master/antismash/detection/genefunctions/data/cog_annotations.txt.

27. antismash/antismash/detection/genefunctions/smcogs.py at master · antismash/antismash · GitHub. https://github.com/antismash/antismash/blob/master/antismash/detection/genefunctions/smcogs.py.

28. Shannon, P. *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* **13**, 2498–2504 (2003).

29. Hunter, J. D. Matplotlib: A 2D graphics environment. *Comput Sci Eng* **9**, 90–95 (2007).

30. GitHub - zreitz/multismash. https://github.com/zreitz/multismash/.

31. Steinegger, M. & Söding, J. MMseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. *Nature Biotechnology 2017 35:11* **35**, 1026–1028 (2017).

32. Minh, B. Q. *et al.* IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol Biol Evol* **37**, 1530–1534 (2020).

33. Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q. & Vinh, L. S. UFBoot2: Improving the Ultrafast Bootstrap Approximation. *Mol Biol Evol* **35**, 518–522 (2018).

34. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res* **49**, W293–W296 (2021).

35. BGC0000705. https://mibig.secondarymetabolites.org/repository/BGC0000705/index.html#r1c1.

36. BGC0000704. https://mibig.secondarymetabolites.org/repository/BGC0000704/index.html#r1c1.

37. BGC0000703. https://mibig.secondarymetabolites.org/repository/BGC0000703/index.html#r1c1.

38. BGC0001003. https://mibig.secondarymetabolites.org/repository/BGC0001003/index.html#r1c1.

39. Yanai, K., Murakami, T. & Bibb, M. Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of Streptomyces kanamyceticus. *Proc Natl Acad Sci U S A* **103**, 9661–9666 (2006).

40. Piepersberg, W., Aboshanab, K. M., Schmidt-Beißner, H. & Wehmeier, U. F. The Biochemistry and Genetics of Aminoglycoside Producers. *Aminoglycoside Antibiotics: From Chemical Biology to Drug Discovery* 15–118 (2007) doi:10.1002/9780470149676.CH2.

41. Kharel, M. K. *et al.* A gene cluster for biosynthesis of kanamycin from Streptomyces kanamyceticus: Comparison with gentamicin biosynthetic gene cluster. *Arch Biochem Biophys* **429**, 204–214 (2004).

42. Bihlmaier, C. *et al.* Biosynthetic gene cluster for the polyenoyltetramic acid α-lipomycin. *Antimicrob Agents Chemother* **50**, 2113–2121 (2006).

43. Fuqua, W. C., Winans, S. C. & Greenberg, E. P. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269–275 (1994).

44. Brotherton, C. A., Medema, M. H. & Greenberg, E. P. luxR Homolog-Linked Biosynthetic Gene Clusters in Proteobacteria. *mSystems* **3**, (2018).

45. Rajput, A. & Kumar, M. In silico analyses of conservational, functional and phylogenetic distribution of the LuxI and LuxR homologs in Gram-positive bacteria. *Scientific Reports 2017 7:1* **7**, 1–13 (2017).

46. Santos, C. L., Correia-Neves, M., Moradas-Ferreira, P. & Mendes, M. V. A Walk into the LuxR Regulators of Actinobacteria: Phylogenomic Distribution and Functional Diversity. *PLoS One* **7**, 46758 (2012).

47. Rehakova, A., Novakova, R., Feckova, L., Mingyar, E. & Kormanec, J. A gene determining a new member of the SARP family contributes to transcription of genes for the synthesis of the angucycline polyketide auricin in Streptomyces aureofaciens CCM 3239. *FEMS Microbiol Lett* **346**, 45–55 (2013).

48. Novakova, R., Rehakova, A., Kutas, P., Feckova, L. & Kormanec, J. The role of two SARP family transcriptional regulators in regulation of the auricin gene cluster in Streptomyces aureofaciens CCM 3239. *Microbiology (Reading)* **157**, 1629–1639 (2011).

49. Kurniawan, Y. N., Kitani, S., Maeda, A. & Nihira, T. Differential contributions of two SARP family regulatory genes to indigoidine biosynthesis in Streptomyces lavendulae FRI-5. *Appl Microbiol Biotechnol* **98**, 9713–9721 (2014).

50. Alderwick, L. J. *et al.* Molecular structure of EmbR, a response element of Ser/Thr kinase signaling in Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* **103**, 2558–2563 (2006).

51. Steinegger, M. *et al.* HH-suite3 for fast remote homology detection and deep protein annotation. *BMC Bioinformatics* **20**, 1–15 (2019).

52. Krause, J., Handayani, I., Blin, K., Kulik, A. & Mast, Y. Disclosing the Potential of the SARP-Type Regulator PapR2 for the Activation of Antibiotic Gene Clusters in Streptomycetes. *Front Microbiol* **11**, (2020).

53. Huang, J. *et al.* Cross-regulation among disparate antibiotic biosynthetic pathways of Streptomyces coelicolor. *Mol Microbiol* **58**, 1276–1287 (2005).

54. Kudo, F. & Eguchi, T. Biosynthetic genes for aminoglycoside antibiotics. *J Antibiot (Tokyo)* **62**, 471–481 (2009).

55. Llewellyn, N. M. & Spencer, J. B. Biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics. *Nat Prod Rep* **23**, 864–874 (2006).

56. Wang, Y., Kern, S. E. & Newman, D. K. Endogenous phenazine antibiotics promote anaerobic survival of Pseudomonas aeruginosa via extracellular electron transfer. *J Bacteriol* **192**, 365–369 (2010).

57. Kudo, F. & Eguchi, T. Biosynthetic genes for aminoglycoside antibiotics. *The Journal of Antibiotics 2009 62:9* **62**, 471–481 (2009).

58. Barajas, J. F., Blake-Hedges, J. M., Bailey, C. B., Curran, S. & Keasling, J. D. Engineered polyketides: synergy between protein and host level engineering. *Synth Syst Biotechnol* **2**, 147–166 (2017).

59. Klaus, M. & Grininger, M. Engineering strategies for rational polyketide synthase design. *Nat Prod Rep* **35**, 1070–1081 (2018).

60. Katz, L. & Baltz, R. H. Natural product discovery: past, present, and future. *J Ind Microbiol Biotechnol* **43**, 155–176 (2016).

61. Fang, L., Guell, M., Church, G. M. & Pfeifer, B. A. Heterologous erythromycin production across strain and plasmid construction. *Biotechnol Prog* **34**, 271–276 (2018).

62. Arnison, P. G. *et al.* Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat Prod Rep* **30**, 108–160 (2013).

63. Montalbán-López, M. *et al.* New developments in RiPP discovery, enzymology and engineering. *Nat Prod Rep* **38**, 130–239 (2021).

64. Orme, D. The caper package: comparative analysis of phylogenetics and evolution in R. (2023).

65. Ives, A. R. & Garland, T. Phylogenetic Logistic Regression for Binary Dependent Variables. *Syst Biol* **59**, 9–26 (2010).

66. Liu, G., Chater, K. F., Chandra, G., Niu, G. & Tan, H. Molecular Regulation of Antibiotic Biosynthesis in Streptomyces. *Microbiol Mol Biol Rev* **77**, 112 (2013).

67. Karray, F., Darbon, E., Nguyen, H. C., Gagnat, J. & Pernodet, J. L. Regulation of the biosynthesis of the macrolide antibiotic spiramycin in Streptomyces ambofaciens. *J Bacteriol* **192**, 5813–5821 (2010).

68. Bunet, R. *et al.* Characterization and manipulation of the pathway-specific late regulator AlpW reveals Streptomyces ambofaciens as a new producer of kinamycins. *J Bacteriol* **193**, 1142–1153 (2011).

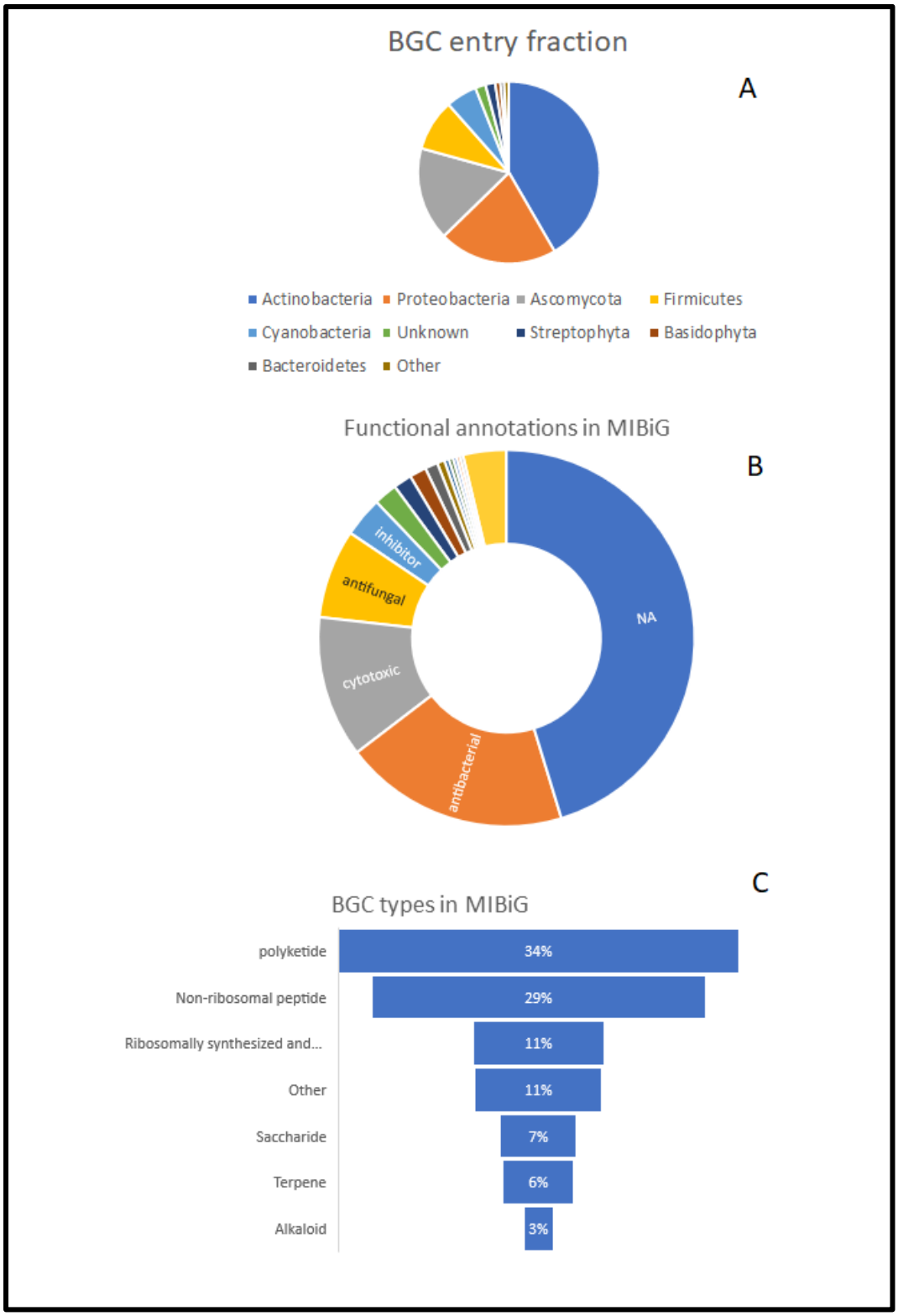
69. Bate, N., Butler, A. R., Gandecha, A. R. & Cundliffe, E. Multiple regulatory genes in the tylosin biosynthetic cluster of Streptomyces fradiae. *Chem Biol* **6**, 617–624 (1999).

70. Carroll, L. M. *et al.* Accurate de novo identification of biosynthetic gene clusters with GECCO. *bioRxiv* 2021.05.03.442509 (2021) doi:10.1101/2021.05.03.442509.

71. Sanchez, S. *et al.* Expansion of novel biosynthetic gene clusters from diverse environments using SanntiS. *bioRxiv* 2023.05.23.540769 (2023) doi:10.1101/2023.05.23.540769.

7. APPENDICES

APPENDIX A: CURRENT CURATED BGCS AND THEIR ANNOTATIONS IN THE MIBIG DATABASE. A) the fraction of present phyla, B) division of functions and C) fraction of BGC types



APPENDIX B: KEY TERMS USED TO CAPTURE REGULATORY PROTEIN FAMILIES FROM THE HMM LIBRARIES

Key terms	
PFAM	**antiSMASH's smCOG**
Regulator	Regulator
Transcription	Transcriptional
Repressor	Factor
Activator	Kinase
Histidine kinase	DNA-binding
DNA-binding	Repressor
DNA binding	ECF
Inducer	
Helix-turn-helix	
HTH	
Helix-loop-helix	
HLH	
Winged	
Sigma	
Serine/threonine	
Fork	
Ribbon-helix-helix	
RHH	
Sensor	
Inhibitor	
Quorum-sensing	
Leucine zipper	
sRNA regular	
Zinc-finger	
Zinc finger	
Co-activator	
Response	

APPENDIX C: LOCATIONS OF THE THESIS'S INITIAL DATASETS

Stored data name	Location on the server
MIBiG Genbank files	/lustre/BIF/nobackup/nassa006/MIBiG_regulatory_Pfams/mibig_gbk_3.1
MIBiG JSON files	/lustre/BIF/nobackup/nassa006/MIBiG_regulatory_Pfams/mibig_json_3.1
Extracted BGCs from MIBiG	/lustre/BIF/nobackup/nassa006/MIBiG_regulatory_Pfams/BGC_seq_files
Streptomycetaceae Genbank files	/lustre/BIF/nobackup/reitz001/seq_data/streptomycetaceae/genbanks/

APPENDIX D: CUSTOM SCRIPT NAMES

All can be found in: https://git.wur.nl/daan.vannassauw/thesis_BGC_functional_inference/-/tree/main/scripts

#	Script name
1	extract_MIBiG_clusters.py
2	parse_json_info.py
3	extract_streptomycetacaea_cds.py
4	filter_pfams.py
5	filter_smcogs.py
6	regs_from_mibig.py
7	regs_from_streptomycetaceae.py
8	Hmmer_parser.py
9	attribute_table_maker.py
10	location_analysis.py
11	regions_overview.py
12	homolog_split_and_filtering.py
13	location_stats.py
14	extract_prot_fam.py
15	fasta_ID_converter.py
16	alignment_trimming.py
17	functions_rep_view.py
18	Prediction_stats.py
19	
20	

APPENDIX E: SARP CLUSTERS

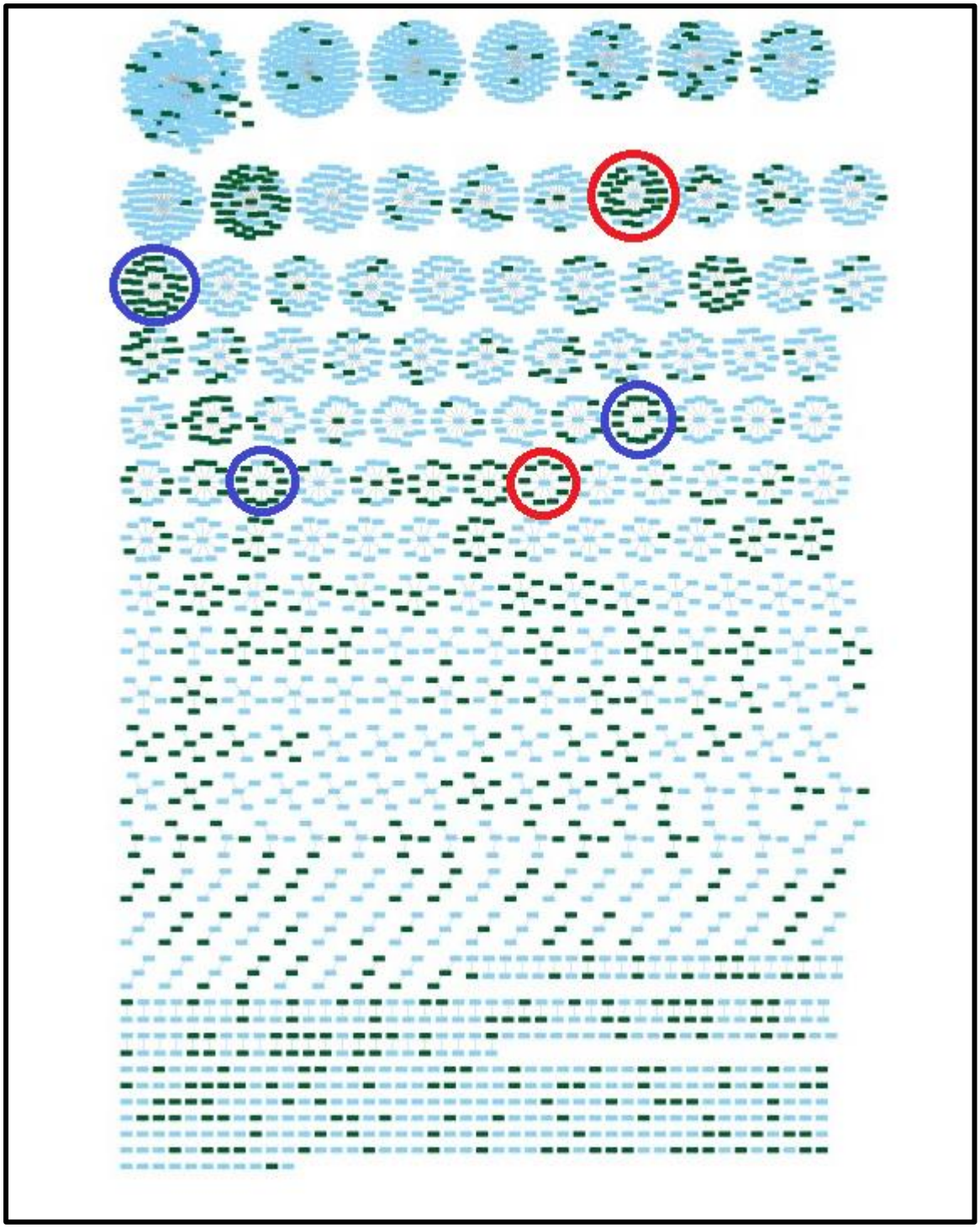
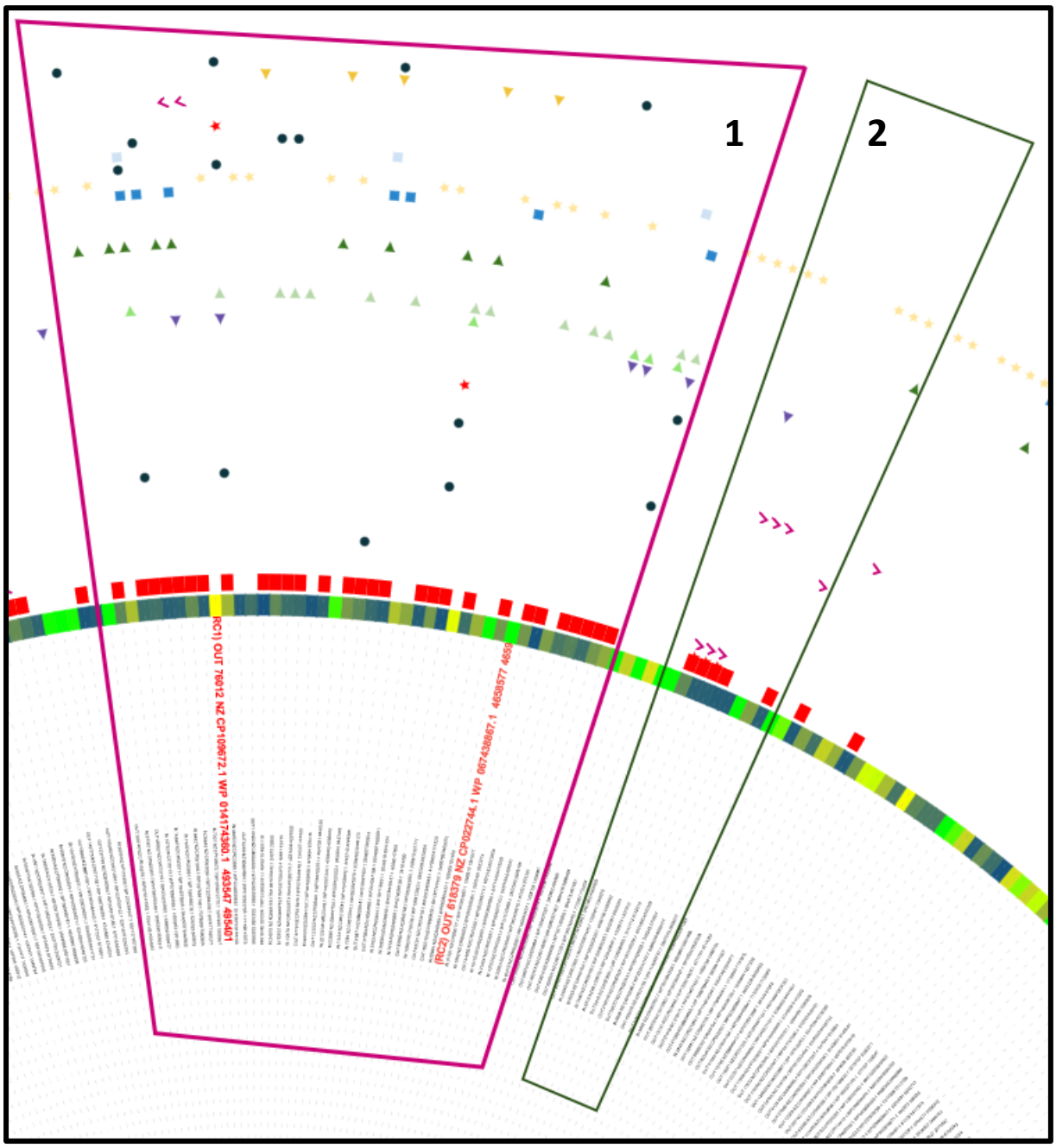
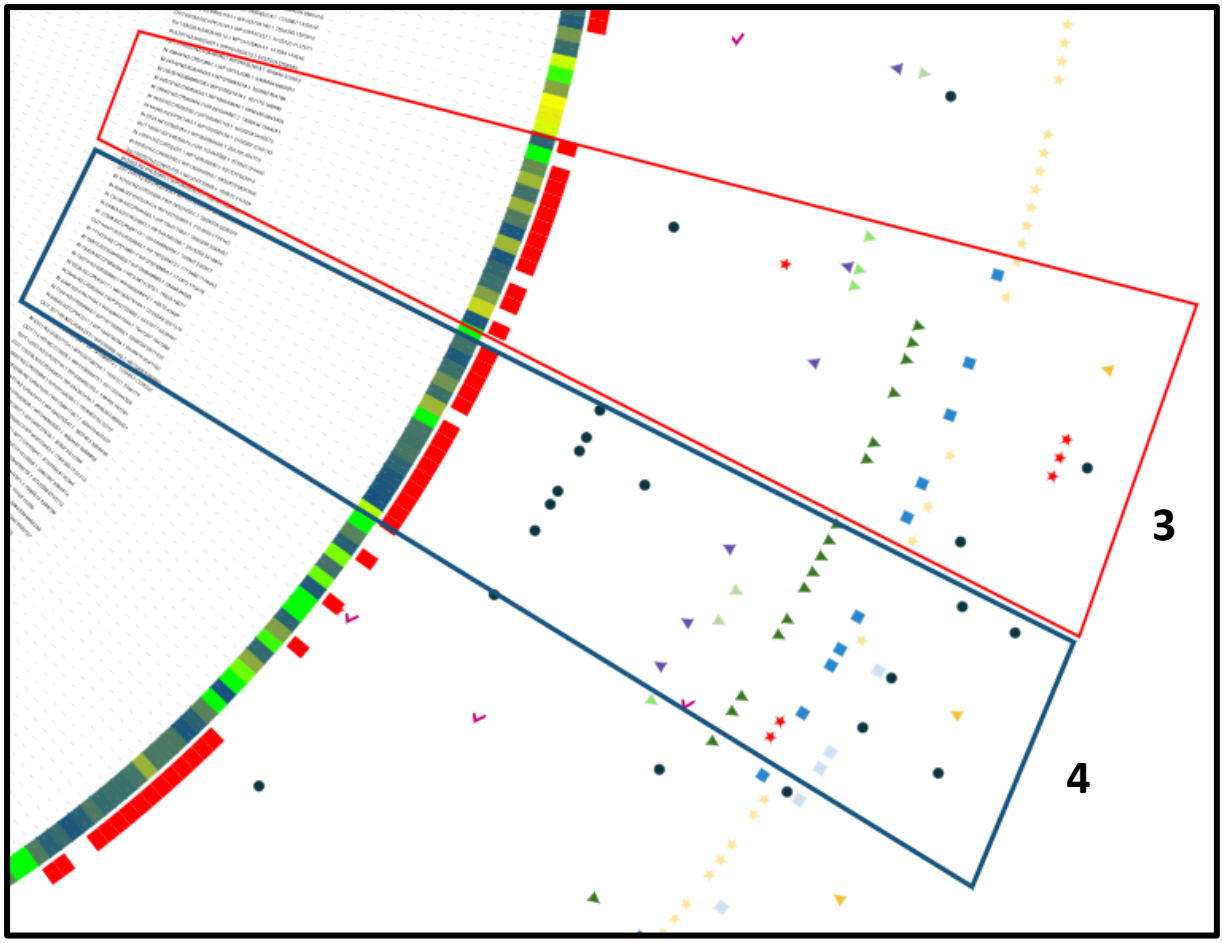


FIGURE 4 THE SARP HOMOLOGS BASED ON INCREMENTAL CLUSTERING WITH 98 TARGET COVERAGE. DARK GREEN SARPS ARE LOCATED IN PREDICTED BGC REGIONS. A LIST OF NAMES OF EVERY HOMOLOGUE CAN BE FOUND ON THE GIT. RED CIRCLE INDICATES CLUSTERS WHOSE REPRESENTATIVE HAS A DIFFERENT LOCATION THAN IT MEMBERS. BLUE CIRCLES INDICATE CLUSTERS WITH INDIVIDUAL OUT BGC-LAYING SARPS AS INTERESTING FOLLOW-UP CASES

APPENDIX F: ZOOMED-IN IMAGES OF THE SARP REPRESENTATIVE TREE





APPENDIX G: TOTAL AMOUNT OF DETECTED HOMOLOGS PER DATABASE (FEW EXAMPLES)

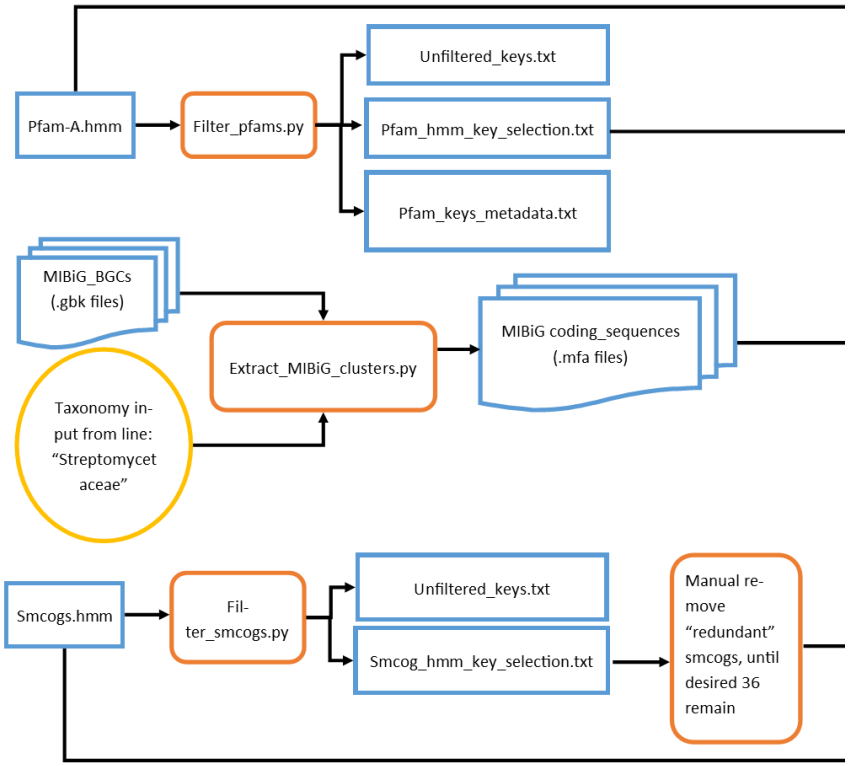
The full list of occurrences per accession can be found on the git (filename; Genomes_filter_and_count_comparison.txt). The values are the sum of all the HMMs dedicated to a single family. Thus, overlapping hits or HMMs were not analysed.

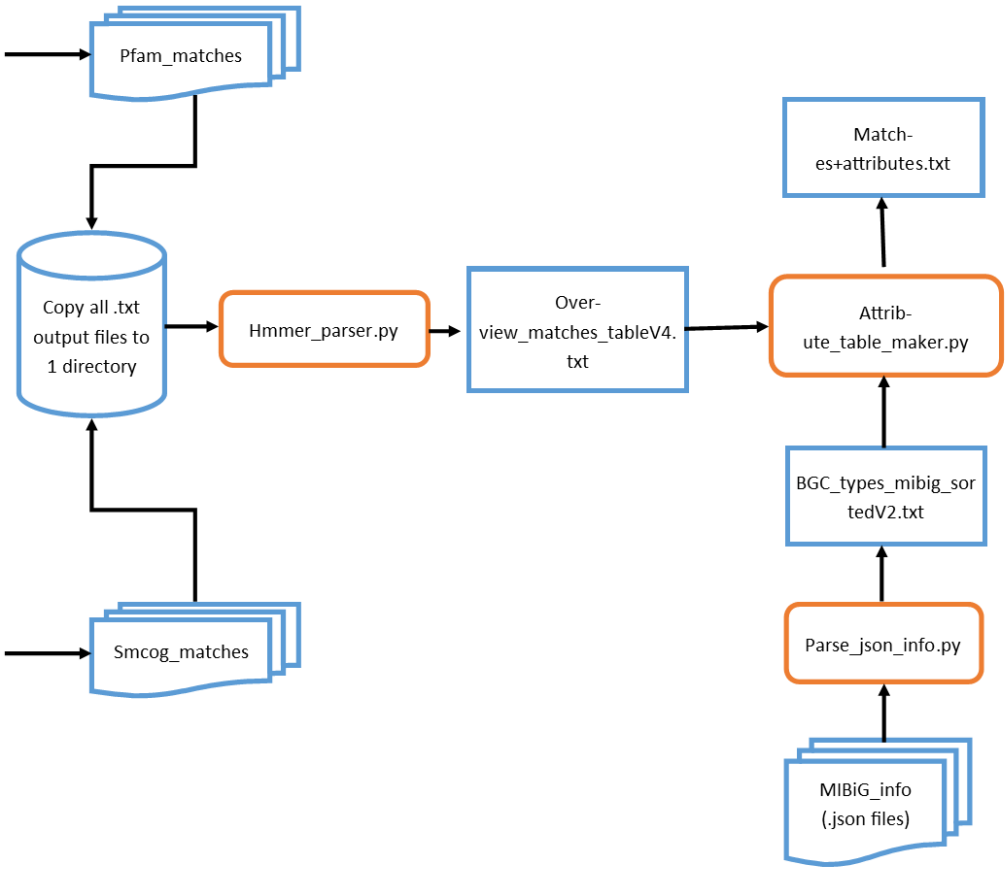
FAMILY NAME	**smCOG-R**	**Pfam-R**
LuxR	10929	3997
LysR	14473	2104
Sigma-70 factor	3845	4617
GntR	10646	3224
SARP (BTAD in Pfam-R)	6191	906

APPENDIX H: (REGULATORY) PROTEIN FAMILY OCCURRENCES IN BGCS, NOT CAPTURED BY ANTISMASH' SMCOGS

HMM_accession	HMM_match_protein_family_description	domain_description	Comments	literature	GO-terms
PF17755.4	UvrA DNA-binding domain	UvrA hypothesized to locate DNA damage as a part of a UvrABC complex		https://doi.org/10.1038%2Fnsmb.1973	
PF08220.15	HTH_DeoR	HTH DNA-binding TF (negative) regulator family (involved in sugar catabolism)		PMID: 10714997	GO:0006355, GO:000
PF03551.17	PadR; TF regulator family	winged helix-like DNA-binding TF family, possibly involved in nitrone-oligomycin A resistance & produ		https://doi.org/10.1186/s13036-018-0103-x , https://d	
PF01381.25	HTH_3	the full protein fold incorporates a helix-turn-helix motif, but the function of this member is unlikely to		https://doi.org/10.1002/prot.22698	
PF13693.9	HTH_35	winged HTH DNA-binding	has 1 hit --> BGC0001386_BAU98050.1_[23801:24392](+)		
PF13560.9	HTH_31	showed homology hits with lambda repressor-like & Cro/XRE domains (xenobiotic response element)		https://www.uniprot.org/uniprotkb/Q53895/entry	
PF13443.9	HTH_26	Cro/C1-type = Cro/XRE domains (xenobiotic response element)		https://www.ebi.ac.uk/interpro/entry/pfam/PF1344	
PF13413.9	HTH_25	'probably" binds to DNA	has 1 hit --> BGC0002350_QTT77481.1_[89142:89760](-)		
PF12844.10	HTH_19	This family contains many example antitoxins from bacterial toxin-antitoxin systems. In other domain databases, these are refered to as lambda repressor-li			
PF03444.18	HrcA_DNA-binding TF_repressor (also HTH domain)	This domain is always found with a pair of CBS domains. CBS doma	has 1 hit --> BGC0001200_ctg1_orf262	PMID:14722619	
PF06224.15	HTH_42	tend to include recently found DNA glycosylases, that play essenti	This family contains two copies of a v	PMID: 28396405 , PMID: 35311535 , PMID: 26400161	
PF02082.23	Rrf2; Iron-dependent Transcriptional regulator	HTH, Several proteins in this family form iron-sulfur clusters enabling iron dependent DNA transcriptior		PMID:23644595	
PF13589.9	HATPase_c_3 (histidine kinases)	ATPase domains of histidine kinase, DNA gyrase B and HSP90			
PF13581.9	HATPase_c_2 (histidine kinases)	ATPase domains of Sensor histidine kinases			
PF13551.9	HTH_29	This helix-turn-helix domain is often found in transferases and is l			
PF13518.9	HTH_28	This helix-turn-helix domain is often found in transposases and is	sharing hits		
PF13592.9	HTH_33	This helix-turn-helix domain is often found in transferases and is l			
PF08327.14	AHSA1	It is probably a general upregulator of Hsp90 function, particularly contributing to its efficiency in condi		PMID:12504007	
PF13556.9	HTH_30	often found at the C-terminus of PucR-like transcriptional regulat	activator for purine metabolic proces	https://www.uniprot.org/uniprotkb/O32138/publicat	
PF17765.4	MLTR_LBD	MmyB-like transcription regulator ligand binding domain, found in a family of actinobacterial transcript		PMID:22844465	
		> high chance of being a transcriptional regulator			
		> indication to be a TF, but lack of evidental literature			
		> low chance of being a transcriptional regulator			

APPENDIX I: DATA FLOW VISUALISATION WITH SCRIPT NAMES

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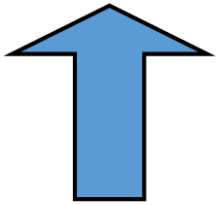
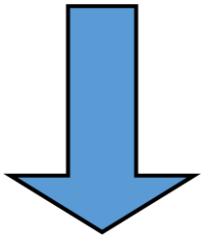
Copy all .txt output files to 1 directory
Hmmer_parser.py
Over-view_matches_tableV4.txt
Match-es+attributes.txt
Attrib-ute_table_maker.py
BGC_types_mibig_sortedV2.txt
Parse_json_info.py
MIBiG_info (.json files)
Smcog_matches

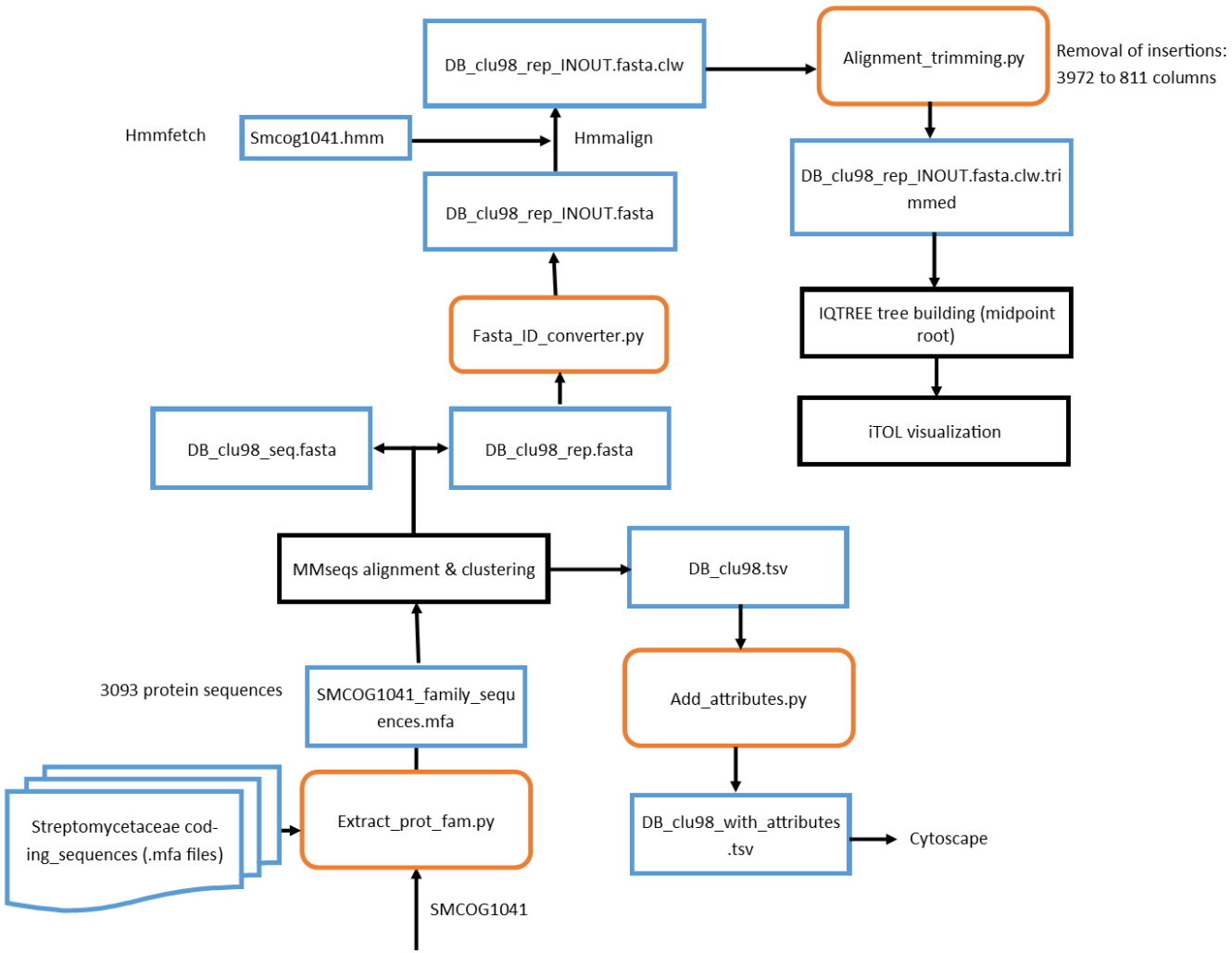


BGCs only

Streptomycetaceae

Whole-genomes

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Alignment_trimming.py
Removal of insertions:
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Smcog1041.hmm
Hmmalign
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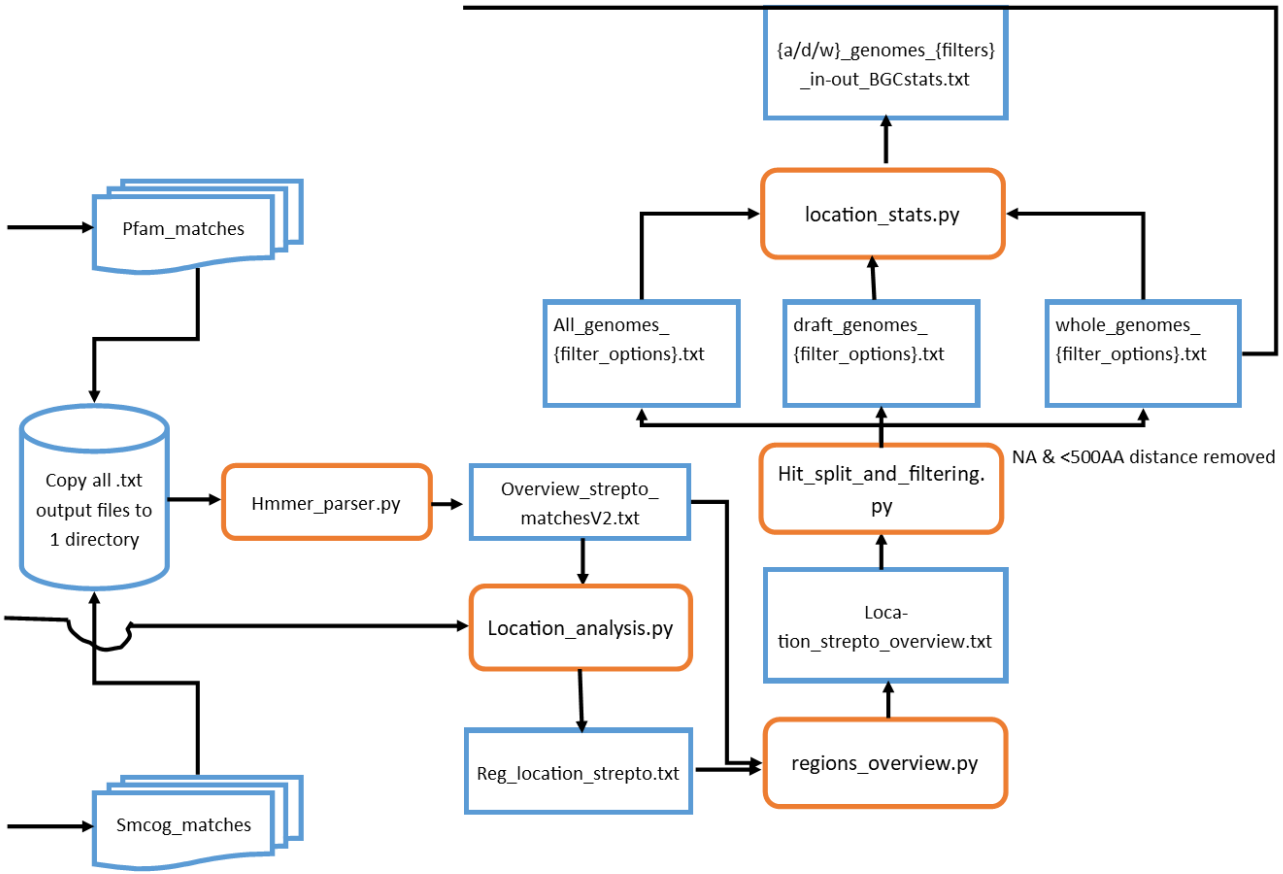
DB_clu98_rep_INOUT.fasta.clw.trimmed
Fasta_ID_converter.py
IQTREE tree building (midpoint root)
DB_clu98_seq.fasta
DB_clu98_rep.fasta
iTOL visualization
MMseqs alignment & clustering
DB_clu98.tsv
3093 protein sequences
SMCOG1041_family_sequences.mfa
Add_attributes.py
Streptomycetaceae coding_sequences (.mfa files)
Extract_prot_fam.py
DB_clu98_with_attributes.tsv
Cytoscape
SMCOG1041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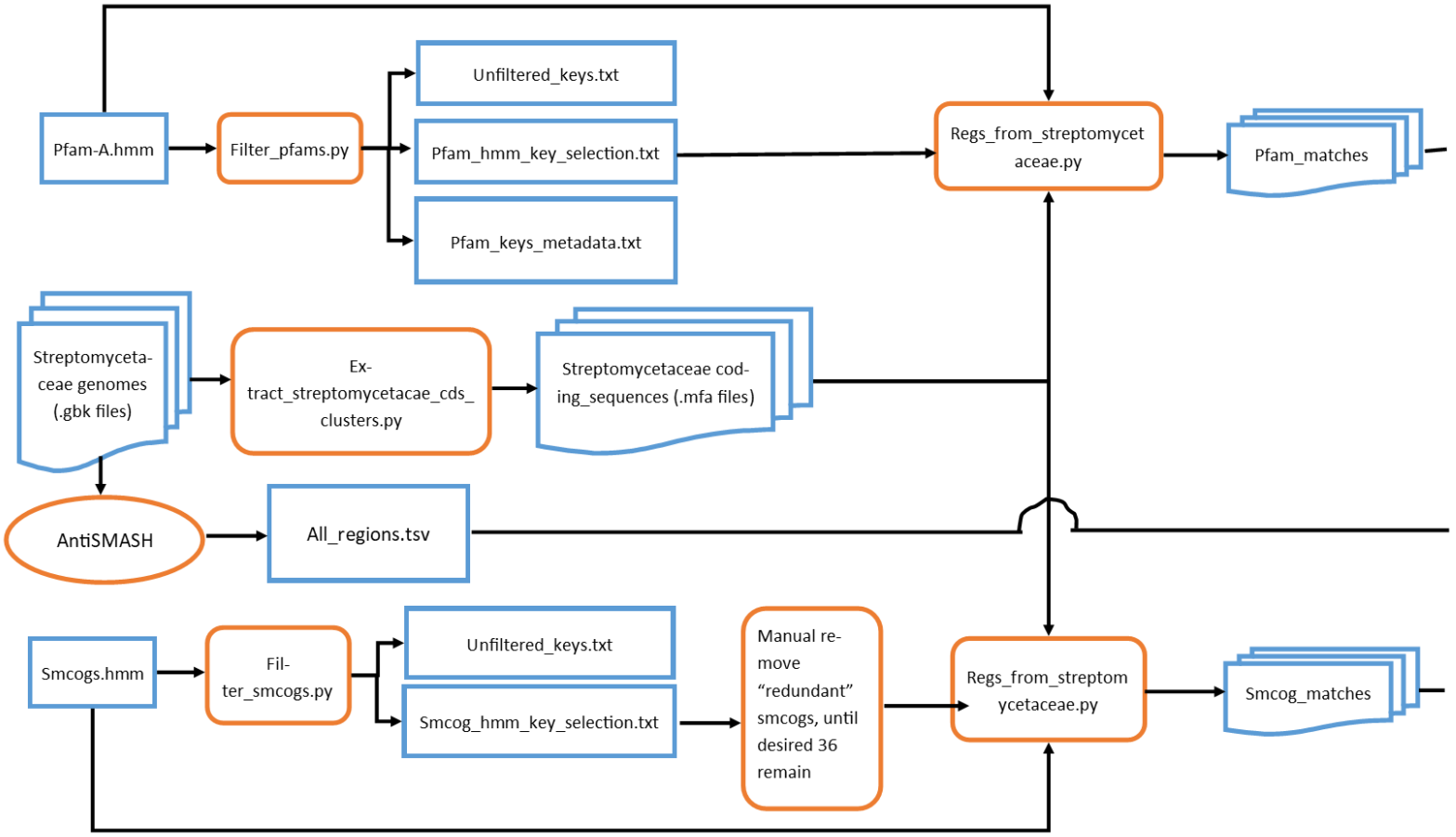
_in-out_BGCstats.txt
location_stats.py
Pfam_matches
All_genomes_
{filter_options}.txt
draft_genomes_
{filter_options}.txt
whole_genomes_
{filter_options}.txt
Copy all .txt
output files to
1 directory
Hmmer_parser.py
Overview_strepto_
matchesV2.txt
Hit_split_and_filtering.
py
NA & <500AA distance removed
Location_analysis.py
Loca-
tion_strepto_overview.txt
Reg_location_strepto.txt
regions_overview.py
Smcog_matches


Pfam-A.hmm
Filter_pfams.py
Unfiltered_keys.txt
Pfam_hmm_key_selection.txt
Pfam_keys_metadata.txt
Regs_from_streptomycetaceae.py
Pfam_matches
Streptomyceta-ceae genomes (.gbk files)
Ex-tract_streptomycetacae_cds_clusters.py
Streptomycetaceae cod-ing_sequences (.mfa files)
AntiSMASH
All_regions.tsv
Smcogs.hmm
Fil-ter_smcogs.py
Unfiltered_keys.txt
Smcog_hmm_key_selection.txt
Manual re-move "redundant" smcogs, until desired 36 remain
Regs_from_streptomycetaceae.py
Smcog_matches

APPENDIX J: FRACTION OF BGCS IN RELATION IN ALL STREPTOMYCETACEAE GENOMES (extracted with *18)

mean fraction: 12.852339167026784
median fraction: 12.252232381438137

genome	amount_of_BGCs	fraction_of_genome (%)
GCF_000717595.1	35	10.428897599280038
GCF_001008345.1	27	10.284363331050178
GCF_026339895.1	18	9.863593357909451
GCF_006334995.2	23	9.989899061464302
GCF_003751605.1	27	11.695062325544015
GCF_014649895.1	30	8.17498659473775
GCF_000444875.1	36	16.983340427914243
GCF_000009765.2	36	15.752209866451313
GCF_001514065.1	32	9.317470222169831
GCF_000242715.1	47	15.863426539020503
GCF_014648775.1	37	19.623643703507536
GCF_000158915.1	41	21.259099023302745
GCF_019933235.1	32	15.56169978199646
GCF_023155275.1	26	10.542432797355024
GCF_014650095.1	33	10.68187120016651
GCF_016741875.1	78	23.573752286815264
GCF_016745505.1	29	7.776394305373099
GCF_000725565.1	46	21.771716808750362
GCF_019890725.1	27	6.15916886482877
GCF_016598475.1	24	7.456380323158306
GCF_009811555.1	39	14.859561429487075
GCF_000966975.1	52	6.229302866888363
GCF_001418575.1	29	3.6239014056646943
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GCF_000225525.1	34	10.194857653422762
GCF_003097515.1	29	16.4612226224176
GCF_003696235.1	30	8.459893413147013
GCF_014649015.1	32	10.659401176627501
GCF_000158955.1	31	12.504556085323662
GCF_001294335.1	49	15.115260477341428
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GCF_001514205.1	39	11.967561956840553
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GCF_003391135.1	45	25.718504396187285
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GCF_023923245.1	32	15.181258387530244
GCF_005768555.2	46	7.537369132779865
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GCF_002804165.1	30	15.539436679944346
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GCF_014650155.1	41	12.742662610649738	
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GCF_009811575.1	34	13.850837499083502	
GCF_009377205.1	48	7.696598407558013	
GCF_017874715.1	68	23.623312243189215	
GCF_014649035.1	37	12.584793184871284	
GCF_020010925.1	33	12.373328935277902	
GCF_017676385.1	23	9.435551546247211	
GCF_020521255.1	21	8.160842970065158	
GCF_000429085.1	24	11.892584386243165	
GCF_028657195.1	51	15.433186346169256	
GCF_008369065.1	42	9.634496141500202	
GCF_000696185.1	32	12.950278630317172	
GCF_000716445.1	36	9.788047742851145	
GCF_001879105.1	10	3.3305538915350175	

GCF_001270025.1	27	6.848975649600892	
GCF_002891435.1	35	4.981317417306841	
GCF_002843305.1	34	12.175102557375826	
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GCF_001905345.1	36	15.938621918823184	
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GCF_002335465.1	39	10.015815779885308	
GCF_900112355.1	24	12.971634701040994	
GCF_000787775.1	33	8.96242054182905	
GCF_014656295.1	31	11.510072792834976	
GCF_007829875.1	34	17.817194666292252	
GCF_000802245.2	29	13.904459183986006	
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GCF_015767775.1	35	17.06594630896187	
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GCF_014648915.1 41	12.7483945266841
GCF_005048155.1 33	14.783608769185788
GCF_014203705.1 27	11.340539197116337
GCF_016107395.1 32	10.6655112979884
GCF_017352335.1 21	11.807799969584368

GCF_027270315.1	32	14.875831465077818
GCF_012273655.1	38	22.56655051060125
GCF_000787855.1	36	12.324305999977202
GCF_014649115.1	51	19.03276821910648
GCF_003626645.1	42	23.864782581543615
GCF_000349325.1	35	13.973304590333772