A Snakemake-Based Pipeline for Comprehensive Functional Annotation of Protein Sequences

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Abstract. The functional annotation of protein sequences remains a challenge in bioinformatics, particularly with the growing influx of uncharacterized sequences from genomic and metagenomic studies. While traditional homology-based methods are effective for well-characterized proteins, they have limitations when applied to novel or divergent sequences. Emerging approaches based on protein structure and machine learning (ML) offer complementary advantages, enabling broader and more accurate functional inference. This project aims to contribute to this evolving field by identifying the most effective open-source tools across homology-based, structure-based, and ML-based strategies, rigorously evaluating their performance, and integrating them into a unified, automated, and reproducible workflow using Snakemake. The resulting pipeline enables scalable, modular annotation of protein datasets and generates comprehensive reports that consolidate predictions across methods. This approach enhances reproducibility and supports the functional characterization of proteins with limited existing annotations.

Keywords: Functional annotation, protein sequences, workflow, Snakemake, homology-based methods, structure-based inference, machine learning, bioinformatics pipeline, reproducibility, automated annotation

1 Motivation and objectives

The exponential increase in genomic and metagenomic sequencing has led to a growing repository of protein sequences with unknown functions, posing a significant challenge for functional annotation. Traditional sequence homology-based methods are widely used and effective for annotating well-characterized proteins. However, they may be less successful when applied to proteins whose homologs are either uncharacterized or only distantly related, limiting their applicability in some cases. This limitation has driven the need for different approaches, such as leveraging structural analysis and machine learning (ML) techniques, which can infer function based on protein folds or learned patterns beyond sequence similarity. Despite the growing availability of powerful open source tools for protein annotation, whether based on sequence homology, structural analysis, or machine learning (ML), these methodologies are relatively recent and rapidly evolving. Many of these tools show great promise for uncovering functions of previously

uncharacterized proteins. Given the complexity of protein annotation, relying on a single method is often insufficient. Instead, combining complementary approaches increases the likelihood of obtaining accurate predictions, since some methods may outperform others depending on the protein, and concordant results across methods can serve as a form of validation. However, such tools are rarely integrated into unified, automated, and reproducible workflows. A standardized and modular pipeline that brings together homology-based, structure-based, and ML-based annotation strategies would thus provide a more comprehensive and scalable solution for protein function prediction. This project aims to contribute to that goal by identifying the most effective open-source tools in each of these three categories, rigorously testing them, and integrating them into a cohesive workflow using a workflow manager such as Snakemake.

2 State of the art

Proteins play a vast array of biological roles, and functional annotation is the process of assigning information to proteins regarding their biochemical activity, cellular localization, and involvement in biological processes [39]. Functional annotation can rely on various sources of evidence, such as sequence similarity, structural characteristics, or patterns learned by computational models [41-43]. In this section, we provide an overview of the key concepts and current methodologies used in protein functional annotation.

2.1 Gene Ontology (GO) Terms and Enzyme Commission (EC) Numbers and Clusters of Orthologous Groups (COGs).

Accurate functional annotation of proteins requires standardized systems that clearly describe and categorize their biological roles. Three widely used systems in bioinformatics and protein annotation are Gene Ontology (GO) terms, Enzyme Commission (EC) numbers, and Clusters of Orthologous Groups (COGs). The Gene Ontology (GO) system provides a structured vocabulary for describing protein functions consistently and precisely. GO categorizes protein functions into three distinct areas: Molecular Function, which describes specific biochemical activities at the molecular level; Biological Process, capturing broader biological objectives or pathways in which a protein participates; and Cellular Component, indicating the specific cellular locations where a protein performs its function. GO terms are structured hierarchically, ranging from general terms to highly specific annotations, facilitating automated and consistent annotations across databases and studies [17]. In contrast, the Enzyme Commission (EC) classification specifically categorizes enzymes based on the chemical reactions they catalyze. EC numbers consist of four digits separated by periods (for example, EC 2.7.11.1), with each digit representing increasingly specific information about the enzyme reaction type. EC numbers provide clarity and precision in biochemical communication, significantly improving enzyme-related annotations, especially in metabolic pathway analyses and bioinformatics applications [18,19].

Another important resource is the Clusters of Orthologous Groups (COGs), which classify proteins into families of orthologous genes based on evolutionary relationships. Orthologous genes are genes found in different species that originated from a common ancestral gene and typically retain similar functions. Each COG represents a group of such genes inferred to be functionally equivalent across multiple organisms [38].

2.2 Workflow Management and Pipeline Development in Bioinformatics

Modern bioinformatics analyses often require the combination of multiple tools and processing steps, applied systematically to large datasets. Constructing such pipelines manually can be time-consuming, error-prone, and difficult to reproduce [26]. To address these challenges, workflow management systems have been developed to streamline the creation, execution, and maintenance of bioinformatics pipelines. These systems allow users to define each analysis step, manage data dependencies, and ensure that workflows are both reproducible and scalable across computing environments.

Among the most widely used workflow systems Snakemake, enables researchers to specify computational steps as modular rules, clearly defining inputs, outputs, and commands [16]. Its flexibility and strong adoption in genomics and proteomics make it particularly suitable for building comprehensive annotation workflows [16]. For this project, Snakemake was selected to integrate the chosen annotation tools into a unified and reproducible pipeline.

2.3 Homology-Based Functional Annotation Approaches

One of the most common and reliable ways to predict the function of a protein is by comparing its sequence to other proteins whose functions are already known [1]. Sequence similarity methods such as BLAST and PSI-BLAST are widely used to identify homologous proteins by comparing input sequences to annotated entries in reference databases. PSI-BLAST enhances sensitivity through iterative refinement of position-specific scoring matrices (PSSMs), improving detection of distant homologs [8].

However, this approach also has certain limitations. While it performs well for proteins that are closely related to well annotated sequences [1,8], it may not always provide reliable annotations for novel or highly divergent proteins lacking characterized homologs in current databases [8]. Nonetheless, homology-based methods remain a valuable first step in many annotation pipelines due to their robustness, interpretability, and widespread support across bioinformatics tools [1-4].

Another approach is domain-based methods that focus on conserved functional regions within proteins rather than full sequences. These regions are identified using profile models from databases like Pfam and CDD, which capture shared patterns across domain families. Tools such as reCOGnizer map query sequences to orthologous groups and curated domain models, enhancing functional

annotation with evolutionary information [5–7]. Several existing tools follow this approach and are candidates for integration into the annotation pipeline. These include BLAST and PSI-BLAST (NCBI), which identify homologous sequences through pairwise and iterative alignments [1]; InterProScan, which detects functional motifs and domains using curated databases [27]; eggNOG-mapper, which assigns orthologs and annotations based on precomputed orthologous groups [4]; UPIMAPI, which performs fast similarity searches using DIAMOND and UniProt [7]; reCOGnizer, which refines domain-based annotations using HMMER and Reverse PSI-BLAST [7]; and GO-Figure, which groups and visualizes GO terms based on semantic similarity [20].

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2.4 Structure-Based Functional Annotation Strategies

A powerful way to predict a protein's function is by analyzing its three-dimensional (3D) structure, which is often more conserved than its sequence [9,14]. Structural configurations frequently indicate shared functions, even when sequence similarity is low [13–15].

Traditionally, protein structures were determined using experimental methods like X-ray crystallography or NMR, which are time-consuming and costly [42]. Recent advances, such as AlphaFold2, allow accurate structure prediction from amino acid sequences. This open-source tool has enabled structural modeling for millions of proteins, including many without prior characterization [10,42].

Once a structure is available, it can be compared to known proteins in databases like the Protein Data Bank (PDB). Tools like FoldSeek perform fast alignments to identify proteins with similar structural features, even in the absence of sequence similarity [12]. This is especially useful for annotating proteins with no close homologs.

Structure-based annotation tools increasingly integrate machine learning. For example, DeepFRI represents structures as graphs and applies graph convolutional networks to predict Gene Ontology (GO) terms [15]. COFACTOR combines structure alignment and interaction data to infer GO terms, EC numbers, and ligand-binding sites [28].

Because structural similarity does not always imply identical function, results are often interpreted alongside other evidence, such as gene context or biochemical data [14].

A number of tools support structure-based functional annotation and are being considered for integration into the pipeline. These include DeepFRI, which

uses graph-based deep learning on protein structures to predict GO terms [15]; COFACTOR, which combines structure alignment and interaction data to predict GO terms, EC numbers, and ligand-binding sites [28]; and FoldSeek, which performs fast structural alignments to identify proteins with similar 3D folds [12].

2.5 Machine Learning Approaches for Functional Protein Annotation

Machine learning has become a cornerstone in protein annotation, enabling the prediction of protein functions, structures, and interactions directly from amino acid sequences. By training on extensive datasets, machine learning models identify intricate patterns within protein sequences, facilitating accurate annotations [21]. A critical aspect of this process is feature extraction, where models discern relevant characteristics from sequences, such as physicochemical properties, motifs, domains, and evolutionary information. Advanced models, including protein language models, can autonomously learn these features from raw sequences, capturing complex patterns essential for precise predictions [22]. Machine learning applications in protein annotation are diverse. For instance, models can predict the biological roles of proteins by analyzing sequence patterns and comparing them to known functional motifs, assigning Gene Ontology (GO) terms. Tools like DeepGO-SE exemplify this application by leveraging pre-trained language models to predict GO functions from protein sequences [24]. In addition to function prediction, machine learning is also widely applied to other aspects of protein annotation, such as subcellular localization [21]. Predicting a protein's subcellular localization is vital for understanding its function, as many biological activities are compartment specific. Machine learning models analyze signal peptides and localization signals within sequences to predict subcellular compartments, aiding in elucidating protein roles and interactions [25].

Various machine learning-based tools are also being evaluated for inclusion in the pipeline. These include CLEAN, which predicts EC numbers using neural networks trained on enzyme data [29]; DeepLoc 2.0, which predicts subcellular localization using deep learning [25]; DeepGO-SE, which uses sequence embeddings and structural features to predict GO terms [24]; ProteInfer, which uses convolutional neural networks to classify protein functions [31]; and ProtENN, which predicts EC numbers using an ensemble of neural networks [32].

3 Methodology

To construct the functional annotation pipeline, a comprehensive survey and evaluation of bioinformatics tools was conducted, focusing on three main annotation strategies: sequence homology, protein structure, and machine learning. The selection process was guided by criteria such as open-source availability, ease of automation within a Snakemake-based pipeline, demonstrated performance in

functional annotation tasks, interpretability of the results, and relevance in the bioinformatics community.

For the sequence homology-based annotation module, three tools were chosen. reCOGnizer performs domain-based annotations using multiple domain databases and provides reliable GO and EC annotations by leveraging sequence profiles and PSI-BLAST results [7]. UPIMAPI offers a fast and flexible way to retrieve functional annotations from UniProtKB, including GO terms and EC numbers based on DIAMOND similarity searches[7]. eggNOG-mapper provides scalable annotations through precomputed orthologous groups and delivers high-quality outputs for GO, EC, and KEGG terms, making it ideal for large-scale protein datasets[33]. In the structure-based annotation module, DeepFRI was selected for its use of graph convolutional neural networks to predict Gene Ontology (GO) terms from protein 3D structures, offering residue-level resolution and direct applicability to AlphaFold-predicted models [15]. FoldSeek complements DeepFRI by enabling ultra-fast 3D structure alignment, supporting the identification of structural analogs even in the absence of sequence similarity [12]. For the machine learning-based annotation module, the tools selected include DeepGO-SE, which combines protein sequence embeddings with semantic modeling of the Gene Ontology to predict GO terms accurately and consistently[35]. CLEAN was chosen for its use of contrastive learning with deep transformer models trained on curated enzyme data to predict EC numbers, offering high interpretability and classification performance [36]. Finally, ProteInfer was incorporated due to its use of convolutional neural networks trained on large, curated protein datasets to predict functional classes and EC numbers directly from amino acid sequences [37].

All selected tools are open source and can be integrated into Python-based pipelines, ensuring reproducibility and ease of automation within the Snakemake workflow. The pipeline will be implemented using Snakemake, which provides a robust and flexible framework for defining and managing computational workflows. Each tool will be integrated as an independent module, with Snakemake rules clearly specifying inputs, outputs, and commands. The pipeline will begin with a FASTA file provided by the user, containing protein sequences to be annotated. These sequences will be preprocessed as needed and passed through each of the three modules. Outputs will be collected in organized directories by module and tool, allowing for straightforward downstream integration.

Upon completion of all modules, the pipeline will automatically generate a comprehensive report summarizing the annotations obtained for each protein. This report will consolidate the results from all tools, indicate overlapping or divergent predictions, and include relevant visualizations when applicable. To evaluate the performance of the pipeline, it will be tested on protein sequences that are currently unannotated or poorly characterized in public databases.

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