BITACORA:

A comprehensive tool for the identification and annotation of gene families in genome assemblies

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- Overview

Genome annotation is a critical bottleneck in genomic research, especially for the comprehensive study of gene families in the genomes of non-model organisms. Despite the recent progress in automatic annotation, the tools developed for this task often produce inaccurate annotations, such as fused, chimeric, partial or even completely absent gene models for many family copies, which require considerable extra efforts to be amended. Here we present BITACORA, a bioinformatics tool that integrates sequence similarity search algorithms and Perl scripts to facilitate both the curation of these inaccurate annotations and the identification of previously undetected gene family copies directly from DNA sequences. The pipeline generates general feature format (GFF) files with both curated and novel gene models, and FASTA files with the predicted proteins. The output of BITACORA is easily integrated in genomic annotation editors, greatly facilitating subsequent semi-automatic annotation and downstream evolutionary analyses.

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BITACORA Web Site

www.ub.edu/softevol/bitacora

Current version: 1.2

0 Workflow & Contents

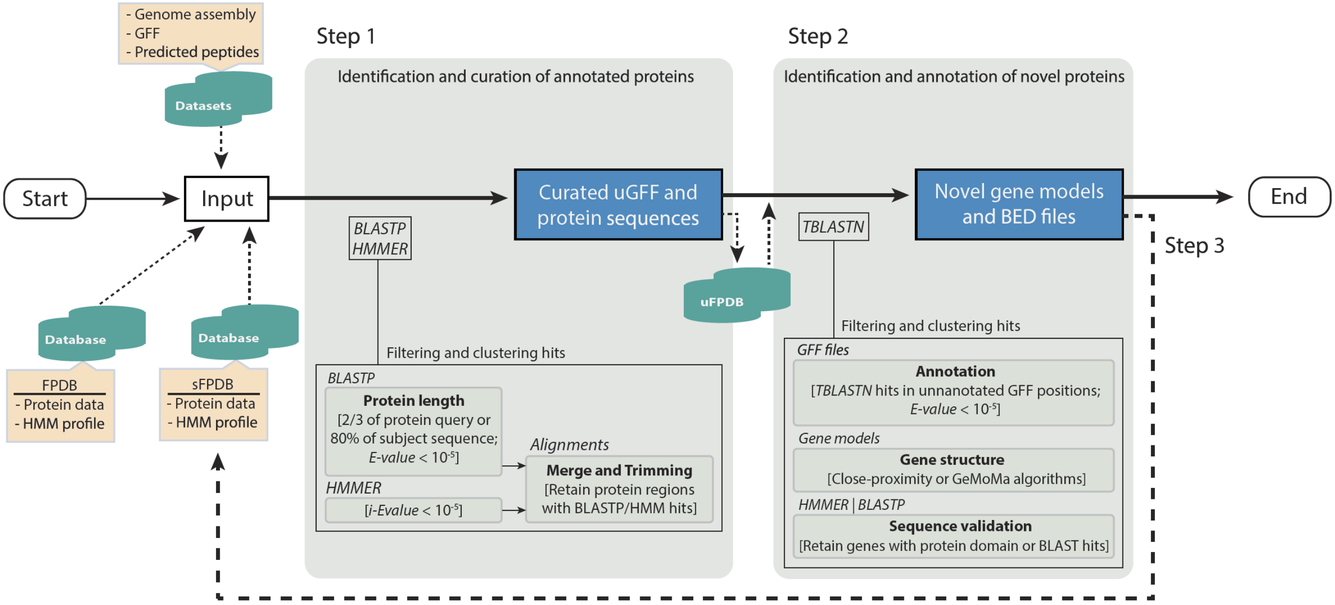
- Genome assembly
- GFF
- Predicted peptides
Datasets
Start
Input
Database
FPDB
- Protein data
- HMM profile
Database
sFPDB
- Protein data
- HMM profile
Step 1
Identification and curation of annotated proteins
Curated uGFF and protein sequences
BLASTP
HMMER
Filtering and clustering hits
BLASTP
Protein length
[2/3 of protein query or 80% of subject sequence; E-value < 10-5]
HMMER
[i-Evalue < 10-5]
Alignments
Merge and Trimming
[Retain protein regions with BLASTP/HMM hits]
uFPDB
Step 2
Identification and annotation of novel proteins
Novel gene models and BED files
TBLASTN
Filtering and clustering hits
GFF files
Annotation
[TBLASTN hits in unnanotated GFF positions; E-value < 10-5]
Gene models
Gene structure
[Close-proximity or GeMoMa algorithms]
HMMER | BLASTP
Sequence validation
[Retain genes with protein domain or BLAST hits]
Step 3
End

Figure 1. Workflow showing the basic steps used in BITACORA

1. Installation

2. Prerequisites

3. Computational Requirements

4. Usage modes

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1 Installation

BITACORA is distributed as a multiplatform shell script (`runBITACORA.sh`) that calls several other perl scripts, which include all functions responsible of performing all pipeline tasks. Hence, it does not require any installation or compilation step.

You can download all package contents from GitHub: https://github.com/molevol-ub/bitacora

To run the pipeline, edit the master script `runBITACORA.sh` variables described in Prerequisites, Data, and Parameters.

2 Prerequisites

- **Perl**: Perl is installed by default in most operating systems. See https://learn.perl.org/installing/ for installation instructions.

- **BLAST**: Download blast executables from: ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/

- **HMMER**: The easiest way to install HMMER in your system is to type one of the following commands in your terminal:

```
% brew install hmmer                 # OS/X, HomeBrew
% port install hmmer                 # OS/X, MacPorts
% apt install hmmer                  # Linux (Ubuntu, Debian...)
% dnf install hmmer                  # Linux (Fedora)
% yum install hmmer                  # Linux (older Fedora)
% conda install -c bioconda hmmer    # Anaconda
```

Or compile HMMER binaries from the source code: http://hmmer.org/

HMMER and BLAST binaries require to be added to the PATH environment variable. Specify the correct path to bin folders in the master script `runBITACORA.sh`, if necessary.

```
$ export PATH=$PATH:/path/to/blast/bin
$ export PATH=$PATH:/path/to/hmmer/bin
```

- **GeMoMa (optional)**: By default, BITACORA reconstructs new gene models using the "close-proximity" algorithm. To use GeMoMa algorithm (Keilwagen et al., 2016; Keilwagen et al., 2018), the GeMoMa jar file (i.e. `GeMoMa-1.6.2.jar`) must be specified in `GEMOMAP` variable in `runBITACORA.sh`. GeMoMa is implemented in Java using Jstacs and can be downloaded from: http://www.jstacs.de/index.php/GeMoMa.

`GEMOMAP=/path/to/GeMoMa.jar` (within `runBITACORA.sh` script)

3 Computational requirements

BITACORA have been tested in UNIX-based platforms (both in Mac OS and Linux operating systems). Multiple threading can be set in blast searches, which is the most time-consuming step, by editing the option THREADS in `runBITACORA.sh`

For a typical good quality genome (~2Gb in size and ~10,000 scaffolds) and a standard modern PC (16Gb RAM), a full run of BITACORA is completed in less than 24h. This running time, however, will depend on the size of the gene family or the group of genes surveyed in a particular analysis. For gene families of 10 to 100 members, BITACORA spends from minutes to a couple of hours.

In case of larger or very fragmented genomes, BITACORA should be used in a computer cluster or workstation given the increase of RAM memory and time required.

4 Usage modes

4.1. Full mode

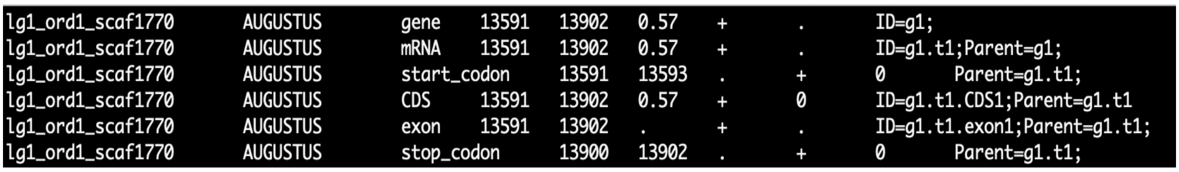
BITACORA has been designed to work with genome sequences and protein annotations (full mode). However, the pipeline can also be used either with only protein or only genomic sequences (protein and genome modes, respectively). These last modes are explained in next subsections.

Preparing the data: The input files (in plain text) required by BITACORA to run a full analysis are (update the complete path to these files in the master script `runBITACORA.sh`):

I. File with genomic sequences in FASTA format

II. File with structural annotations in GFF3 format. [*NOTE: mRNA* or *transcript*, and *CDS* are mandatory fields].

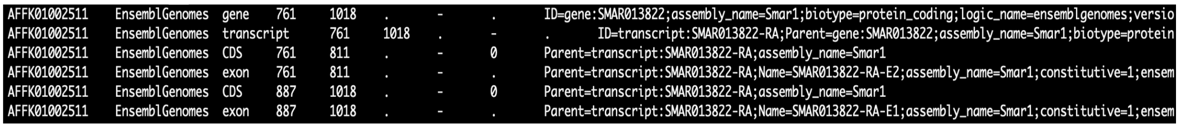
-------------------- GFF3 example



```
lg1_ord1_scaf1770       AUGUSTUS        gene    13591   13902   0.57    +       .       ID=g1;
lg1_ord1_scaf1770       AUGUSTUS        mRNA    13591   13902   0.57    +       .       ID=g1.t1;Parent=g1;
lg1_ord1_scaf1770       AUGUSTUS        start_codon     13591   13593   .       +       0       Parent=g1.t1;
lg1_ord1_scaf1770       AUGUSTUS        CDS     13591   13902   0.57    +       0       ID=g1.t1.CDS1;Parent=g1.t1
lg1_ord1_scaf1770       AUGUSTUS        exon    13591   13902   .       +       .       ID=g1.t1.exon1;Parent=g1.t1;
lg1_ord1_scaf1770       AUGUSTUS        stop_codon      13900   13902   .       +       0       Parent=g1.t1;
```

BITACORA also accepts other GFF formats, such as Ensembl GFF3 or GTF. [NOTE: GFF formatted files from NCBI can cause errors when processing the data, use the supplied script "`reformat_ncbi_gff.pl`" (located in the folder `/Scripts/Tools`) to make the file parsable by BITACORA]. See Troubleshooting in case of getting errors while parsing your GFF.

-------------------- Ensembl GFF3 example



```
AFFK01002511    EnsemblGenomes  gene    761     1018    .       -       .       ID=gene:SMAR013822;assembly_name=Smar1;biotype=protein_coding;logic_name=ensemblgenomes;versio
AFFK01002511    EnsemblGenomes  transcript      761     1018    .       -       .       ID=transcript:SMAR013822-RA;Parent=gene:SMAR013822;assembly_name=Smar1;biotype=protein
AFFK01002511    EnsemblGenomes  CDS     761     811     .       -       0       Parent=transcript:SMAR013822-RA;assembly_name=Smar1
AFFK01002511    EnsemblGenomes  exon    761     811     .       -       .       Parent=transcript:SMAR013822-RA;Name=SMAR013822-RA-E2;assembly_name=Smar1;constitutive=1;ensem
AFFK01002511    EnsemblGenomes  CDS     887     1018    .       -       0       Parent=transcript:SMAR013822-RA;assembly_name=Smar1
AFFK01002511    EnsemblGenomes  exon    887     1018    .       -       .       Parent=transcript:SMAR013822-RA;Name=SMAR013822-RA-E1;assembly_name=Smar1;constitutive=1;ensem
```

III. File with predicted proteins in FASTA format. BITACORA requires identical IDs for proteins and their corresponding mRNAs or transcripts IDs in the GFF3. [NOTE: we recommend using genes but not isoforms in BITACORA; isoforms can be removed or properly annotated after BITACORA analysis]

IV. Specific folder with files containing the query protein databases (`YOURFPDB_db.fasta`) and HMM profiles (`YOURFPDB_db.hmm`) in FASTA and hmm format, respectively, where the "YOURFPDB" label is your specific data file name. The

addition of "_db" to the database name with its proper extension, `fasta` or `hmm`, is mandatory.
BITACORA requires one protein database and profile per surveyed gene family (or gene group). See `Example/DB` files for an example of searching for two different gene families in BITACORA: OR, Odorant Receptors; and CD36-SNMP.

[NOTE: profiles covering only partially the proteins of interest are not recommended]

Notes on HMM profiles:

HMM profiles are found in InterPro or PFAM databases associated to known protein domains. If you don't know if your protein contains any described domain, you can search in InterPro (http://www.ebi.ac.uk/interpro/) using the protein sequence of one of your queries to identify domains.

For example, for the chemosensory proteins (CSPs) in insects, you can download the HMM profile from pfam (Curation & model PFAM submenu):

http://pfam.xfam.org/family/PF03392#tabview=tab6

In the case of searching for proteins with not described protein domains, or with domains not covering most of the protein sequence, it should be performed an alignment of the query proteins to create a specific HMM profile.

Example of building a protein profile (it requires an aligner, here we use mafft as example):

```
$ mafft --auto FPDB_db.fasta > FPDB_db.aln

$ hmmbuild FPDB_db.hmm FPDB_db.aln
```

Notes on the importance of selecting a confident curated database:

The proteins included in the database to be used as query (FPDB) in the protein search is really important; indeed, the inclusion of unrelated or bad annotated proteins could lead to the identification and annotation of proteins unrelated to the focal gene family and can inflate the number of sequences identified.

On the other hand, if possible, we recommend to include proteins from phylogenetically-close species to increase the power of identifying proteins, particularly in fast-evolving and divergent gene families. If your organism of interest does not have an annotated genome of a close related species, we suggest to perform a second BITACORA round (step 3 described in the manuscript; Figure 1), including in the query database (sFPDB) the sequences identified in the first round, along with a new HMM profile build with these sequences. This step may facilitate the identification of previously undetected related divergent sequences.

4.2. Protein mode

BITACORA can also run with a set of proteins (i.e. predicted proteins from transcriptomic data; script `runBITACORA_protein_mode.sh`) by using the input files described in points **III** and **IV** of the section **4.1**.

Under this mode, BITACORA identifies, curates when necessary, and report all members of the surveyed family among the predicted proteins. The original protein sequences (not being curated) are also reported (located in `Intermediate_Files` if cleaning output is active).

4.3. Genome mode

BITACORA can also run with raw genome sequences (i.e., not annotated genomes; script `runBITACORA_genome_mode.sh`), by using the input files described in points **I** and **IV** of the section **4.1**.

Under this mode, BITACORA identifies *de novo* all members of the surveyed family and returns a BED file with gene coordinates of the detected exons, a FASTA file with predicted proteins from these exons and a GFF3 file with the corresponding structural annotations.

[NOTE: By default, BITACORA applies the "close-proximity" algorithm to generate gene models; these models are only semi-automatic predictions and require further manual annotation (i.e. using genomic annotation editors, such as Apollo). More accurate gene models can be predicted using the GeMoMa algorithm. In addition, we highly recommend running an iterative search round, at least, to properly identify all putative gene family copies.]

5 Parameters

- The option CLEAN can be used to create the `Intermediate_files` directory where all intermediate files will be stored (see output section).

```
CLEAN=T  #T=true, F=false
```

- BLAST and HMMER hits are filtered with a default cut-off E-value of 10^{-5} (in addition to an internal parameter for filtering the length covered by the alignment).

E-value can be modified in the master script runBITACORA.sh:

```
EVALUE=10e-5    #Default
```

- Number of threads to be used in blast searches, default is 1.

```
THREADS=1    #Default
```

- BITACORA can generate new gene models (for those putative genes not included in the input GFF) using two different methods. Set `GEMOMA=T` (with upper case) if you want to use the GeMoMa software to predict novel genes from TBLASTN alignments (the user should specify the PATH to jar file in `GEMOMAP` variable described in prerequisites). Otherwise (`GEMOMA=F`), BITACORA will predict new genes by exon proximity (close-proximity method described in the manuscript).

```
GEMOMA=F    #Default
```

- For the close-proximity method (`GEMOMA=F` option), BITACORA uses by default an upper limit value of 15 kb to join putative exons from separate but contiguous (and in the same scaffold) genome hits to build a gene model

This value can be modified in the master script runBITACORA.sh:

```
MAXINTRON=15000    #Default
```

- New generated gene models are subsequently assessed for the presence of the specific protein family domain (with HMMER) or BLASTP hits against the FPDB, using homology-based alignments. Set `GENOMICBLASTP=T` to perform both BLASTP and HMMER searches to curate putative errors in these novel annotated genes. BITACORA will retain putative new genes that exhibit the protein domain OR a BLASTP hit. BITACORA can also perform this filtering step assessing only if novel annotated genes have the specific protein domain (`GENOMICBLASTP=F` option). The first option (`GENOMICBLASTP=T`) is the most sensitive but at the expense of the specificity, especially for poor quality annotations (or repetitive regions) in the FPDB that might generate wrong gene models (e.g., including non-homologous fragments). The second option (`GENOMICBLASTP=F`) has a higher specificity, but it could not retain some members (e.g. divergent members) that could not match the protein profile.

```
GENOMICBLASTP=F    #Default
```

Notes about using GeMoMa:

We highly recommend the use of GeMoMa to construct more accurate gene models. This method uses intron position conservation for homology-based gene prediction. Nevertheless, it is more sensitive to the presence of putative errors in genome assemblies (i.e. punctual errors or fragmented scaffolds) and, therefore, could fail to identify some gene models; opposite to the close-proximity method which could report putative pseudogenes or fragment copies. Therefore, we encourage the user to check both methods in order to ensure the identification of all putative gene family copies in the surveyed genome.

Notes on the parameter MAXINTRON:

Estimating the intron length distribution in your genome:

MAXINTRON is a critical parameter affecting the quality of the gene models built after joining *de novo* identified exons in the close-proximity method after BLASTN search (see BITACORA article). BITACORA is distributed with a script (`get_intron_size_fromgff.pl`) to compute some summary statistics, such as the mean, median, and the 95% and 99% upper limits of the intron length distribution, of an input GFF, which can contain all genes from genome or only the genes identified for a particular gene family (i.e. GFF generated in BITACORA output).

Note that a very high value could join exons from different genes, generating a putative chimeric gene. On the other hand, a very low value could not join exons from the same gene. Therefore, it is very important to set a MAXINTRON biological realistic value, which could vary across species or assemblies. As default, BITACORA uses a conservative high value, as a compromise between ensuring the joining of all exons from a same gene, and avoiding the generation of erroneous gene fusions. In any case, a large value of MAXINTRON parameter prevents the annotation of fragmented genes but can generate gene models with multiple gene fusions. Putative gene fusions (proteins with two or more domains predicted by BITACORA) are tagged with the label "`Ndom`" at the end of the protein name in the output file, being N the number of putative genes (detected domains).

The number of putative fussed genes identified as new proteins in not annotated regions of the genome can be obtained using the following command in the terminal:

```
$ grep '>.*dom' DB/DB_genomic_and_annotated_proteins_trimmed.fasta
```

6 Running BITACORA

After preparing the data as indicated in sections 4 (Usage) and 5 (Parameters), you can execute BITACORA with the following command:

```
$ bash runBITACORA.sh
```

7 Output

BITACORA creates an output folder for each query database, and three files with the number of proteins identified in each step, including a summary table. For the genome and protein modes, only one summary table will be reported with the number of identified genes.

In each folder, there are the following **main files** (considering you chose to clean output directory. If not, all files will be found in the same output folder):

- `YOURFPDB_genomic_and_annotated_genes_trimmed.gff3`: GFF3 file with information of all identified protein curated models both in already annotated proteins and unannotated genomic sequences.

- `YOURFPDB_genomic_and_annotated_proteins_trimmed.fasta`: A fasta file containing the protein sequences from the above gene models.

Non-redundant data: Relevant information excluding identical proteins, or those considered as artefactual false positives (i.e. duplicated scaffolds, isoforms…).

- `YOURFPDB_genomic_and_annotated_genes_trimmed_nr.gff3`: GFF3 file containing all identified non-redundant protein curated models both in already annotated proteins and unannotated genomic sequences.

- `YOURFPDB_genomic_and_annotated_proteins_trimmed.fasta`: A fasta file containing the non-redundant protein sequences from the above gene models.

BED files with non-redundant merged blast hits in genome sequence:

- `YOURFPDBtblastn_parsed_list_genomic_positions.bed`: BED file with only merged blast alignments in non-annotated regions.

- `YOURFPDBtblastn_parsed_list_genomic_positions_nogff_filtered.bed`: BED file with merged blast alignment in all genomic regions.

Intermediate files: BITACORA generates the following Intermediate files (located into `Intermediate_files` folder if `CLEAN=T`). These files contain information of some intermediate steps of the analysis, such as the **original or untrimmed gene models**:

- `YOURFPDB_annot_genes.gff3` and `YOURFPDB_proteins.fasta`: GFF3 and fasta file containing the original untrimmed models for the identified proteins.

- `YOURFPDB_annot_genes_trimmed.gff3` and `YOURFPDB_proteins_trimmed.fasta`: GFF3 and fasta containing only the curated model for the identified annotated proteins (trimming exons if not aligned to query FPDB sequences or split putative fused genes).

- `YOURFPDB_genomic_genes.gff3`: GFF3 containing novel identified and untrimmed proteins in genomic sequences.

- YOURFPDB_genomic_genes_trimmed.gff3: GFF3 containing novel identified proteins in genomic sequences curated by the positions identified in the HMM profile.

- YOURFPDBgfftrimmed.cds.fasta and YOURFPDBgfftrimmed.pepfasta: Files containing CDS and protein sequences translated directly from YOURFPDB_annot_genes_trimmed.gff3

- YOURFPDBgffgenomictrimmed.cds.fasta and YOURFPDBgffgenomictrimmed.pep.fasta: Files containing CDS and protein sequences translated directly from YOURFPDB_genomic_genes_trimmed.gff3

- hmmer folder containing the output of HMMER searches against the annotated proteins and novel proteins identified in the genome.

- YOURFPDB_blastp.outfmt6: BLASTP output of the search of the query FPDB against the annotated proteins.

- YOURFPDB_tblastn.outfmt6: TBLASTN output of the search of the query FPDB against the genomic sequence.

- YOURFPDB_blastp_parsed_list.txt; YOURFPDB_hmmer_parsed_list.txt; YOURFPDB_allsearches_list.txt; YOURFPDB_combinedsearches_list.txt: Parsed files combining all hits and extending the hit positions from BLASTP and HMMER outputs.

- YOURFPDB_tblastn_parsed_list_genomic_positions.txt (and _notgff_filtered) : File containing the positions identified after parsing the TBLASTN search.

- YOURFPDB_prots_VsGFF_badannot_list.txt and YOURFPDB_goodannot_list.txt: Debugging files: These files are for checking that the identified proteins and the protein models in the GFF3 codify the same protein. If the file badannot_list.txt contains some identifier, it means that the GFF3 annotation is incorrect pointing to a bad annotation in the original GFF3. Please, try to translate the CDS for that protein into the 3 reading frames and check if the 2nd or 3rd frame codify for the protein in question stored in "YOURFPDB_genomic_and_annotated_proteins_trimmed_nr.fasta". If correct, modify the GFF3 by adding 1 or 2 nucleotide position in the start of the GFF3 (take into account if it is transcribed from forward or reverse strand). If negative, please report the error via GitHub.

- YOURFPDB_genomic_genes_proteins.fasta: It contains all merged exons from putative novel proteins identified in the genome before filtering those without the protein domain identified with HMMER. This file could be useful in case of using an HMM profile not trained with your sequences which cannot detect divergent sequences, such as remote homologs.

- YOURFPDB_genomic_exon_proteins.fasta: contains the exon sequences joined into genes in the aforementioned file.

- Additional generated files are stored for pipeline debugging and controls.

Notes on BITACORA output:

The identified proteins could be used for further prospective analyses, or to facilitate a more curated annotation using genome annotation editors (such as Apollo). In particular, it is recommended to perform a first validation of the new identified proteins when using the close-proximity method. Among other aspects, it is important to check the parameter used to join putative exons, or to determine if some models need to be split or joined (erroneous joined genes, or absence of joined exons from the same gene). It is important to note that the identified proteins (from the genomic regions) are illustrative, and other members could be encoded in the genome but were not identified (true negatives, i.e. divergent genes that do not match the protein profile). We recommend using the parameter `BLASTPHMMER=T` in order to retain new divergent copies also based on BLASTP alignments.

In addition, if there are BITACORA identified sequences containing stop codons, codified as "X", it could be artefactual from TBLASTN hits if they are in the beginning or end of an exon or, otherwise, those genes are probably pseudogenes.

A validation to identify putative erroneously assigned proteins (mainly caused by the inclusion of contaminant sequences in the query database) could consist in aligning all proteins and checking the MSA, constructing the phylogeny of the gene family including related species; doing a reduced blast with NCBI-nr database or obtaining structural particularities of the proteins (i.e. characterizing protein domains as transmembrane domains, signal peptides, etc.). See Vizueta *et al.* (2018) for an example of such analyses.

In particular, BITACORA full and genome mode is also designed to facilitate the gene annotation in editors as Apollo (Figure 2). For that, the use of the following files would be useful (see an example in Documentation/example_Apollo.png):

- Original GFF3
- Final GFF3 with curated models for the annotated proteins
- BED files from TBLASTN search

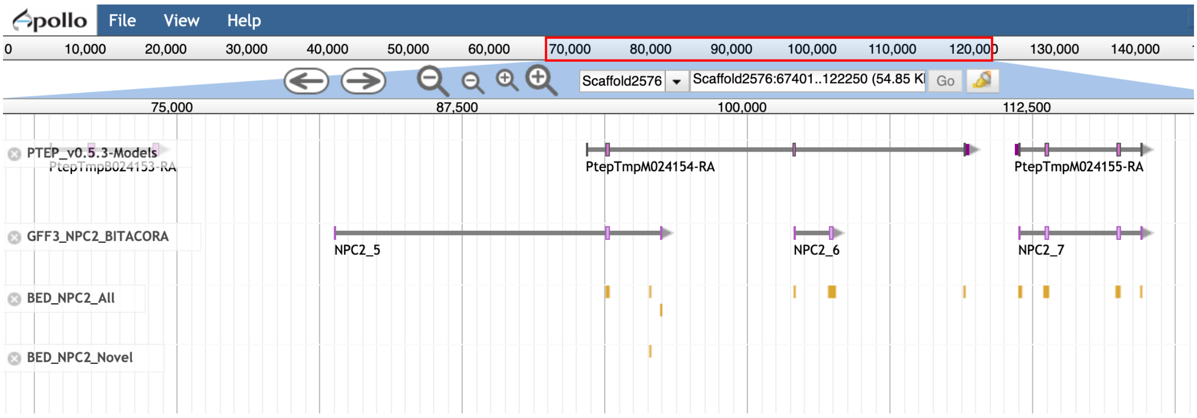


Figure 2. Visualization of BITACORA output in Apollo genome editor. The figure includes the annotation features of three NPC2 genes arranged in cluster in the genome of *P. tepidariorum*. The automatic annotation of this region using MAKER2 (track PTEP_v0.5.3-Models), generated a chimeric gene model (PtepTmpM024154-RA; a gene fusion of two different genes), which could be curated using BITACORA (NPC2_5 and NPC2_6 gene models). The next three tracks, generated by BITACORA, show the final BITACORA output (GFF3_NPC2_BITACORA track) with information of the identified and curated gene models, and the BED_NPC2_All and BED_NPC2_Novel tracks that show information of all identified putative exons, and a putative novel exon not annotated in the available annotation, respectively. For clarity, the name of these tracks has been renamed.

GFF3_NPC2_BITACORA

(file named as: `NPC2_genomic_and_annotated_genes_trimmed_nr.gff3`)

BED_NPC2_All

(named as: `NPC2tblastn_parsed_list_genomic_positions_nogff_filtered.bed`)

BED_NPC2_Novel

(named as: `NPC2tblastn_parsed_list_genomic_positions.bed`)

8 Example

An example to run BITACORA can be found in `Example` folder. First, unzip the `Example_files.zip` file to obtain the necessary files for BITACORA. In this example, two chemosensory-related gene families in insects: Odorant receptors (ORs), and the CD36-SNMP gene family; will be searched in the chromosome 2R of *Drosophila melanogaster*. The GFF3 and protein files are modified from original annotations, deleting some gene models, to allow that BITACORA can identify novel not-annotated genes.

To run the example, edit the master script `runBITACORA.sh` to add the path to BLAST and HMMER binaries and run the script. It will take around 1 minute with 2 threads.

```
$ bash runBITACORA.sh
```

9 Citation

Joel Vizueta, Alejandro Sánchez-Gracia, and Julio Rozas. 2019. BITACORA: A comprehensive tool for the identification and annotation of gene families in genome assemblies. *bioRxiv*. https://doi.org/10.1101/593889

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Moreover, if you use GeMoMa, please cite:

J. Keilwagen, M. Wenk, J. L. Erickson, M. H. Schattat, J. Grau, and F. Hartung. Using intron position conservation for homology-based gene prediction. *Nucleic Acids Research*, 2016. doi: 10.1093/nar/gkw092

J. Keilwagen, F. Hartung, M. Paulini, S. O. Twardziok, and J. Grau Combining RNA-seq data and homology-based gene prediction for plants, animals and fungi. *BMC Bioinformatics*, 2018. doi: 10.1186/s12859-018-2203-5

10 Troubleshooting

When BITACORA detects any error related to input data, it stops and prints the description of the error. Please check the error and your data.

If you are getting errors related to parsing the GFF file, take into account that BITACORA expects proteins ID to be as ID in mRNA rows from GFF3.

In case of protein ID and mRNA ID causing error as they are not the named equally, first, you can use the script located in `Scripts/Tools/get_proteins_notfound_ingff.pl` to check which proteins are not found in the GFF3 file, as detailed in the Error message. You could use only those proteins found in the GFF3 in BITACORA.

If all proteins are named differently in the GFF3, you can obtain a protein file from the GFF3 using the script `Scripts/gff2fasta_v3.pl` and use that protein file as input to BITACORA.

You could also modify the perl module `Readgff.pm` to allow BITACORA to read your data. Otherwise, modify the GFF, preferably, as GFF3 format.

If you cannot solve the error, create an issue in Github specifying the error and all details as possible.