INCOMPLETE DRAFT: Identification of *de novo* orthologous genes from comparative single-cell RNA-seq transcriptomics

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Abstract

We introduce `sencha`, a novel computational method for translating RNA-seq reads to putative protein sequence. As the direct assignment of protein-coding sequence skips both traditional alignment and gene orthology assignment it can a) be applied to transcriptomes from organisms with no or poorly annotated genomes, and b) identify putative functions of protein sequences contributing to shared cell types. Thus, we can identify orthologous cell types while discovering *de novo* orthologous genes across species. For widespread accessibility and usage, we implemented `sencha` into two distinct Nextflow pipelines following software best practices such as testing and continuous integration: (1) `nf-core/kmermaid` to compare translated transcriptomes across divergent species, and (2) `czbiohub/nf-predictorthologs` to infer functions of translated sequences. Using these pipelines, we were able to align transcriptomes and discover *de novo* homologous genes across species from a variety of divergence times: bulk RNA-seq transcriptomes across Amniotes (divergence ~312 millions of years ago), mouse single-cell RNA-seq with *Botryllus schlosseri* bulk RNA-seq, an early chordate (divergence ~684 millions of years ago), and Bilateria developmental atlases (divergence ~824 millions of years ago). By enabling analyses across divergent species' transcriptomes in an orthology-, genome- and gene annotation-agnostic manner, `sencha` illustrates the potential of non-model organisms in building the cell type evolutionary tree of life.

Identifying orthologous genes across species remains an open problem. We show how orthologous genes can be identified directly from RNA-seq reads of tissue and cell types that are shared across species.

Single-cell RNA-sequencing is a powerful technology for identifying cell types in a variety of species. However, the task of identifying even known cell types in species with poorly annotated genomes is nontrivial, as 99.999% of the predicted 8.7 million Eukaryotic species on Earth have no submitted genome assembly [1,2] and identifying orthologous genes, which remains an open problem [3,4]. Thus, there is an unmet need to quantitatively compare single-cell transcriptomes across species, without the need for orthologous gene mapping, gene annotations, or a reference genome. Short, k-long sequence substrings, or k-mers, have been proposed for clustering single cells [5] and here we implemented k-mers from putatitvely translated RNA-seq reads with reduced amino acid alphabets [**???**,6,6,7,8], to find shared cell types across species, and further identify *de novo* orthologous genes by querying the predicted protein sequences to a reference database. This method relies solely on divergence time between species, which we show can be estimated from RNA-seq nucleotide k-mers (Supplemental Figure [**???**]). We benchmark the genome-agnostic method on the Quest for Orthologs Opisthokonta dataset, showing that k-mers from reduced amino acid alphabets are sufficient to estimate orthology. Using human amino acid sequences, we show that one can extract putative protein-coding reads from 239 Opisthokonta species in ENSEMBL, and present the best k-mer size and alphabet for different divergence times. We first apply this method on a bulk comparative transcriptomic dataset consisting of nine amniote species and six tissues [11], showing that we achieve similar clustering results as using only reads mapping to 1:1 orthologs or Hierarchical Orthologous Groups (HOGs) [12,13,14] of protein-coding genes, but are able to resolve ... which can only be seen by using the k-mer method. We further demonstrate the utility of this method by comparing transcriptomes from organisms diverged by approximately 676 million years [15]: a single-cell atlas of a model organism, mouse from *Tabula Muris Senis* [16], and bulk RNA-seq from *Botryllus schlosseri* [17], a colonial tunicate which exhibits cell populations similar to the myeloid immune lineage. Across this evolutionary distance, only XX 1:1 orthologous genes exist as found by ... and XX HOGs via orthologous matrix (OMA) [18,19] We show that the myeloid-like cells from *B. schlosseri* not only cluster with the myeloid immune cells from *Tabula Muris Senis*, we also find *de novo* orthologous genes, such as ... We find that using k-mers has the advantage of resolving ... in comparison to using read counts from 1:1 gene orthologs. Using k-mers, we were able to resolve cell types ... , which was hidden using read counts alone. Thus, we have shown the reference-free method using the k-mers from single cells is a novel, annotation-agnostic method for comparing cells across species that is capable of identifying cell states unique to a particular organism, helping to build the cell type evolutionary tree of life.

To determine whether short segments of sequences could detect gene orthologues, we k-merized orthologous genes derived from the ENSEMBL version 97 [20] COMPARA database [21] (Figure [??]). We compared human protein sequences to orthologous chimpanzee, mouse, (orangutan, bonobo, gorilla, macaque, opossum, platypus, chicken) protein sequences, as these are species used in [11]. As a background, we randomly chose 10 non-orthologous genes relative to the human gene. In addition to k-merizing the protein-coding sequence, we also re-encoded the protein-coding sequence into a six-letter Dayhoff alphabet [22], a nine-letter encoding [9], and a two-letter hydrophobic-polar encodings [23,24], show in Table [2].

We found that, consistent with previous knowledge, that 1:1 orthologues had higher k-mer similarities as determined by the Jaccard Index. This approach is similar to SwiftOrtho [9], a k-mer based orthology relationship finder.

Additionally, more recently diverged genes had higher k-mer similarity as well.

Across tissues of the same time from the Brawand 2011 [11] dataset, we extracted protein-coding dayhoff signatures of k-mer size length 12, extracted hashes and thus k-mers

shared by samples from the same tissue, went back to the original protein sequence, and searched NCBI RefSeq NR for potential proteins. For each sample, we observed that shared k-mers appeared in 1:1 orthologous genes XX% of the time 1:many orthologs YY% of the time, many:many orthologs ZZ% of the time, in genes not known to be orthologs AA% of the time, in unannotated regions AA% of the time, in multimapped reads BB% of the time, and in unmapped reads CC% of the time. Overall, we observed XX de novo orthologs in each tissue. We removed genes that were already known to be orthol

Outline

- Kmers can approximate orthologies
  - Jaccard similarity of orthologues is higher than non-orthologues
  - Benchmarking using https://orthology.benchmarkservice.org/cgi-bin/gateway.pl
  - Finding orthologues
    - Gold standard
      - ENSEMBL COMPARA
      - Quest for Orthologs consortium, Altenhoff, A. M., Boeckmann, B., Capella-Gutierrez, S., Dalquen, D. A., DeLuca, T., et al. (2016). Standardized benchmarking in the quest for orthologs. Nature Methods, 13(5), 425–430. http://doi.org/10.1038/nmeth.3830 [25]
    - Orthologous groups/Conserved Domain Database [26]

Figure 1: Figure 2.

- Overview of kmermaid pipeline
  - Comparison of tissue across species
    - Partition reads to coding/noncoding bins
    - MinHash the Dayhoff-encoded coding sequences
    - Jaccard similarity on the MinHashes
- Which reads are found to have coding features but didn't map to the genome?
- Do these features map to novel genes or gene fusions?
- Kmers can find correct reading from of RNA-seq reads
  - Human peptides → human, chimp, bonobo, orangutan, gorilla, macaque, mouse, opossum, playtpus, chicken RNAseq from Brawand2011 data
- Comparison to other methods: RNASamba [27]

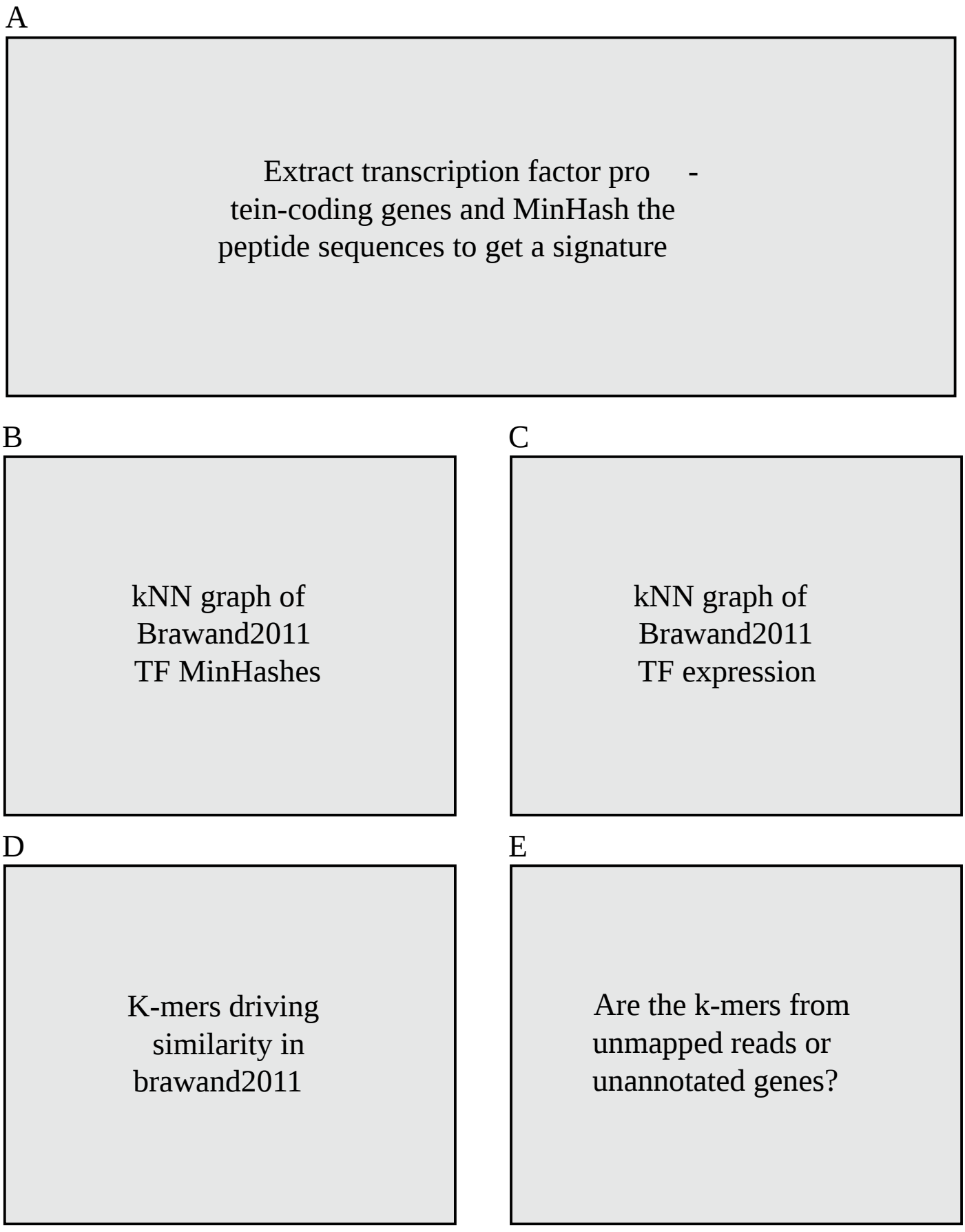


Figure 2: Figure 3.

- Kmers can find only transcription factor reads of TFs from RNA-seq reads
  - Human peptides → human, chimp, bonobo, orangutan, gorilla, macaque, mouse, opossum, playtpus, chicken RNAseq from Brawand2011 data

`kmermaid` implements the concept of lightweight orthology assignment using k-mers to the problem of cross-species RNA-seq analyses and achieves unprecedented speed of analysis. By removing the orthology inference step, `kmermaid` opens up the possibilty of finding shared and divergent tissue and cell types across a broad range of species, paving the way for evolutionary analyses of cell types across species. `kmermaid` can be used in *de novo* setting for non-model organisms, finding similar cell types within an organism, or finding similar cell types relative to a reference organism, without the need for a reference genome or transcriptome. The memory usage of `kmermaid` is quite low, using only 50MB for extracting coding sequences and 50MB for assigning protein k-mer signatures. As the number of RNA-seq datasets, especially single-cell RNA-seq datasets continues to grow, we expect `kmermaid` to be widely used for identifying cell types in non-model organisms.

`kmermaid` is free and open-source software and is available as Supplementary Data and at http://github.com/czbiohub/kmermaid and as a scalable Nextflow workflow at http://github.com/nf-core/nf-kmermaid.

Some potential references

Gene expression evolution through duplications

- Farre, D., & Alba, M. M. (2010). Heterogeneous Patterns of Gene-Expression Diversification in Mammalian Gene Duplicates. Molecular Biology and Evolution, 27(2), 325–335. http://doi.org/10.1093/molbev/msp242 [28]
- Thornton, J. W., & DeSalle, R. (2000). Gene family evolution and homology: genomics meets phylogenetics. Annual Review of Genomics and Human Genetics, 1(1), 41–73. http://doi.org/10.1146/annurev.genom.1.1.41 [29]
- Farre, D., & Alba, M. M. (2010). Heterogeneous Patterns of Gene-Expression Diversification in Mammalian Gene Duplicates. Molecular Biology and Evolution, 27(2), 325–335. http://doi.org/10.1093/molbev/msp242 [28]

Taxa-restricted genes

- Human-specific genes in fetal neocortex Florio, M., Heide, M., Pinson, A., Brandl, H., Albert, M., Winkler, S., et al. (2018). Evolution and cell-type specificity of human-specific genes preferentially expressed in progenitors of fetal neocortex. eLife, 7, D635. http://doi.org/10.7554/eLife.32332 [30]
- Insects – Santos, M. E., Le Bouquin, A., Crumière, A. J. J., & Khila, A. (2017). Taxon-restricted genes at the origin of a novel trait allowing access to a new environment. Science, 358(6361), 386–390. http://doi.org/10.1126/science.aan2748 [31]

Correlated evolution of celltypes?

- Liang, C., Musser, J. M., Cloutier, A., Prum, R. O., & Wagner, G. P. (2018). Pervasive Correlated Evolution in Gene Expression Shapes Cell and Tissue Type Transcriptomes. Genome Biology and Evolution, 10(2), 538–552. http://doi.org/10.1093/gbe/evy016 [32]

Cell type homology

- Thornton, J. W., & DeSalle, R. (2000). Gene family evolution and homology: genomics meets phylogenetics. Annual Review of Genomics and Human Genetics, 1(1), 41–73. http://doi.org/10.1146/annurev.genom.1.1.41 [29]
- Tschopp, P., & Tabin, C. J. (2017). Deep homology in the age of next-generation sequencing. Philosophical Transactions of the Royal Society B: Biological Sciences, 372(1713), 20150475–8. http://doi.org/10.1098/rstb.2015.0475 [33]

- Hejnol, A., & Lowe, C. J. (2015). Embracing the comparative approach: how robust phylogenies and broader developmental sampling impacts the understanding of nervous system evolution. Philosophical Transactions of the Royal Society B: Biological Sciences, 370(1684), 20150045–16. http://doi.org/10.1098/rstb.2015.0045 [34]
- Santos, M. E., Le Bouquin, A., Crumière, A. J. J., & Khila, A. (2017). Taxon-restricted genes at the origin of a novel trait allowing access to a new environment. Science, 358(6361), 386–390. http://doi.org/10.1126/science.aan2748 [31]
- Mammalian decidual cell

Cell type evolution

- Erkenbrack, E. M., Maziarz, J. D., Griffith, O. W., Liang, C., Chavan, A. R., Nnamani, M. C., & Wagner, G. P. (2018). The mammalian decidual cell evolved from a cellular stress response. PLOS Biology, 16(8), e2005594–27. http://doi.org/10.1371/journal.pbio.2005594 [35]

In summary, we developed a method to identify both known cell types in a non-model organism using a reference atlas from another organism, without the need for a genome or gene annotation from the non-model organism. This method can be used to combine single-cell cell atlases from well-annotated, model organisms, with sequencing data from poorly annotated non-model organisms, to directly find homologous cell types and orthologous genes. By eliminating read alignment and orthologous gene mapping, `kmermaid` enables comparison of transcriptomes of the remaining 99.999% Eukaryotic species on Earth without submitted genome assemblies, with the cell atlases of a handful of model organisms to identify shared and novel cell types, and *de novo* identify orthologous genes. By identifying homologous cell types across a broad variety of species, we come closer to an understanding of the evolution of genes, cells, and thus life itself.

Online Methods

Implementation

Reduced alphabets

At the core of `kmerslay` is the ability to cheaply compare sequences using k-mers. As k-mers are very brittle to substitutions and thus to compare across species, one must allow for minor base substitutions that still maintain similar chemical or structural properties. A reduced alphabet can encode useful information into a smaller alphabet space, and enable sequence comparisons across a broader variety of species than the original alphabet alone.

Reduced amino acid alphabets

Reduced amino acid alphabets have been useful for over 50 years [22] in finding related protein sequences [6,7,8,10,36]. Recently, a reduced amino acid alphabet (specifically, `aa9` below) combined with k-mers were used to find homologous protein-coding sequences [9]. We build on this concept by enabling prediction of protein-coding sequences from RNA-seq reads, and by enabling users to perform a parameter sweep in an all-by-all comparison to identify putative homologs using a variety of alphabet metrics.

Dayhoff and `HP` alphabets

Table 1: Dayhoff and hydrophobic-polar encodings are a reduced amino acid alphabet allowing for permissive cross-species sequence comparisons. For example, the amino acid sequence `SASHAFIERCE` would be Dayhoff-encoded to `bbbdbfecdac`, and HP-encoded to `phpphhhpppp`, as below.

Amino acid	Property	Dayhoff	Hydrophobic-polar (HP)
C	Sulfur polymerization	a	p
A, G, P, S, T	Small	b	AGP: h ST: p
D, E, N, Q	Acid and amide	c	p
H, K, R	Basic	d	p
I, L, M, V	Hydrophobic	e	h
F, W, Y	Aromatic	f	h

```
protein20: SASHAFIERCE
dayhoff6:  bbbdbfecdac
hp2:       phpphhhpppp
```

All implemented alphabets (with citations, not as nicely organized)

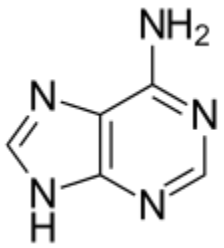
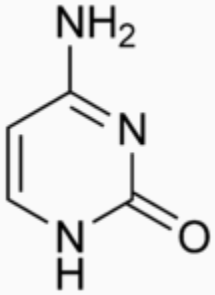
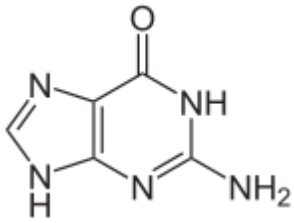
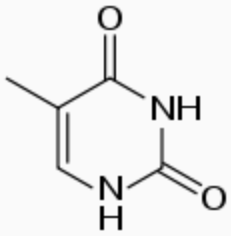
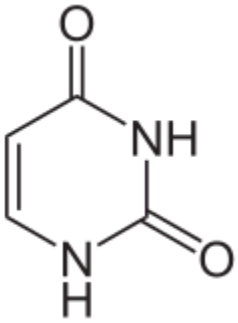
[NOTE: maybe this should go into the supplementary? The main alphabets that have been successful for me are dayhoff and HP]

Citation	Alphabet	Amino acid groups
Phillips, R., *et al.* (2012). [23]	hp2	AFGILMPVWY CDEHKNQRST
Peterson, E. L., *et al.* (2009) [6]	gbmr4	G ADKERNTSQ YFLIVMCWH P
Dayhoff, M. O., & Eck, R. V. (1968). [22]	dayhoff6	AGPST HRK DENQ FWY ILMV C
This paper	botvinnik8	AG DE RK NQ ST FY LIV CMWHP
Hu, X., & Friedberg, I. (2019). [9]	aa9	G AST KR EQ DN CFILMVY W H P
Peterson, E. L., *et al.* (2009) [6]	sdm12	G A D KER N TSQ YF LIVM C W H P
Peterson, E. L., *et al.* (2009) [6]	hsdm17	G A D KE R N T S Q Y F LIV M C W H P
Dayhoff, M. O., & Eck, R. V. (1968). [22]	protein20	G A D E K R N T S Q Y F L I V M C W H P

Reduced nucleotide alphabets

The IUPAC degenerate nucleotide code [37] includes several two-letter codes for the original 4-letter nucleobase alphabet. The first, Weak/Strong, indicates the strength of the hydrogen bond across the double strand. The bond of adenine to thymine has two hydrogen bonds, making it weak; and the bond of guanine to cytosine has three hydrogen bonds, making it 50% stronger. The second, Purine/Pyrimidine, encodes the ring size of the nucleobase, where Adenine and Guanine both have larger Purine double rings, while Cytosine and Thymine/Uracil have smaller Pyrimidine rings. The third, Amino/Keto, designates the main functional group of the ring, where Adenine and Cytosine both have an Amino group, while Guanine and Thymine/Uracil both have a Keto group.

Nucleotide	Hydrogen Bonding	Ring type	Ring functional group	Nucleobase chemical structure

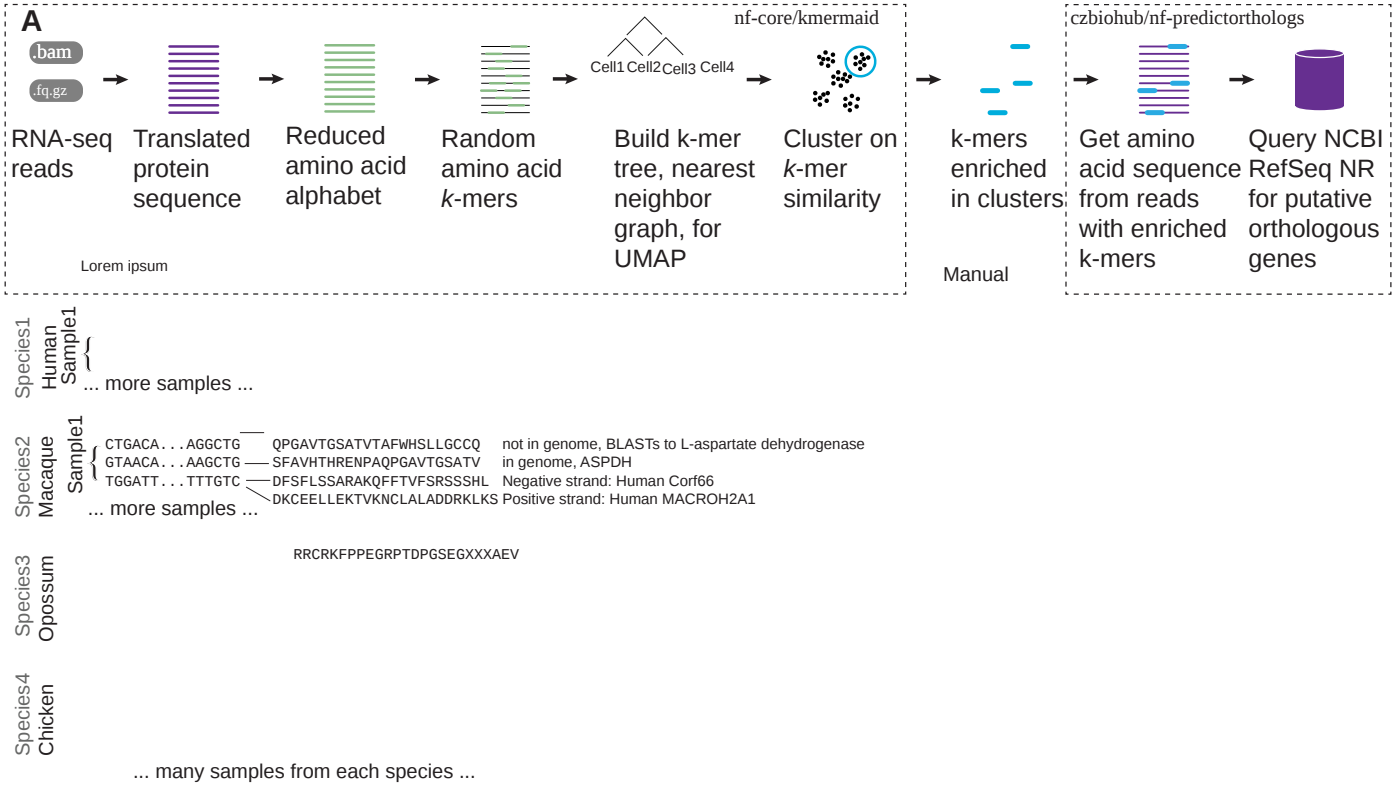
Nucleotide	Hydrogen Bonding	Ring type	Ring functional group	Nucleobase chemical structure
A	Weak (W)	Purine (R)	Amino (M)	
C	Strong (S)	Pyrimidine (Y)	Amino (M)	
G	Strong (S)	Purine (R)	Keto (K)	
T	Weak (W)	Pyrimidine (Y)	Keto (K)	
U	Weak (W)	Pyrimidine (Y)	Keto (K)	

Thus, the nucleotide string `GATTACA` would be re-encoded into the following:

```
Nucleotide:         GATTACA
Weak/Strong:        SWWWWSW
Purine/Pyrimidine: RRYYRYR
Functional group:   KMKKMMM
```

`kmerslay extract-coding`

Figure 1



Overview of `kmerslay extract-coding` **A.** First, each read is translated into all six possible protein-coding translation frames. Next, reading frames with stop codons are eliminated. Each protein-coding frame is k -merized, then the fraction of k-mers which appear in the known protein-coding database is computed. Frames which contain a fraction of coding frames exceeding the threshold are inferred to be putatively protein-coding. **B.** Worked example of an RNA-seq read with a single putatitive reading frame. **C.** Worked example of an RNA-seq read with multiple reading frames, and a `UCSC` genome browser shot of the read showing that both reading frames are present in the annotation.

Set Jaccard threshold of `extract-coding` by controlling false positive rate of protein-coding prediction

To set a threshold of the minimum Jaccard overlap between a translated read's frame and the reference proteome, the most statistically principled way is to control the false positive rate of predicing a protein-coding read.

Probability of random k-mers from a read

If k-mers from reads were independent, identically distributed (i.i.d.) variables, then a translated read of length $L_{\text{translated}}$ drawing letters from the alphabet Σ, whose size is $|\Sigma|$, would contain

$$\left(\frac{1}{|\Sigma|^k} \right)^{L_{\text{translated}} - k + 1} \tag{1}$$

However, k-mers drawn from reads are not i.i.d. Let's take a simple example. If we have a two-letter alphabet,
$\Sigma =$
a, b

, thus $|\Sigma| = 2$. Let us use an example sequence $S = abbabba$. If $k = 4$, then the first k-mer is $abba$. The second k-mer is thus either $bbaa$ or $bbab$, with equal probability. We can generalize this: Given the

first k-mer, the first $k-1$ letters from the second k-mer are known, and thus the probability of guessing the next k-mer is $\frac{1}{|\Sigma|}$.

Probability of future k -mers is influenced by the existence of previous k-mers.

Thus, the probability of a random k-mer from a sequencing read is completely dependent on the alphabet size $|\Sigma|$ and its translated sequence length, $L_{\text{translated}}$:

$$\begin{aligned}\Pr(\text{FPR}) &= \left(\frac{1}{|\Sigma|}\right)^{k} \times \left(\frac{1}{|\Sigma|}\right)^{L_{\text{translated}}-k} \\ &= \frac{1}{|\Sigma|^{k} \times |\Sigma|^{L_{\text{translated}}-k}} \\ &= \frac{1}{|\Sigma|^{L_{\text{translated}}}}\end{aligned} \tag{2}$$

Bloom filter collision probability

The probability of error of the `khmer` bloom filter implementation [38] used in `kmerslay`, given N distinct k-mers counted, a hash table size of H, and Z total number of hash tables, is

$$\Pr(\text{FPR}_{\text{bloom}}) = \left(1 - \exp^{N/H}\right)^{Z}. \tag{3}$$

Theoretically, the total number of k-mers is limited by the alphabet size and choice of k. Empirically, the number of possible k-mers is limited by the k-mers which are compatible with life, and by $k = 5$, the number of theoretical protein k-mers exceeds the number of observed protein k-mers. Additionally, the mass of all possible k-mers of a certain size, exceeds the mass of the planet earth by $k = X$ [get the data for this]. The UniProtKB Opisthokonta manually reviewed dataset contains 4.8×10^7 7-mers in the protein alphabet. Thus, we can give an upper bound to the number of theoretical k-mers to be 10^8. Therefore, the total number of k-mers in the bloom filter is,

$$N = \min\left(10^8, |\Sigma|^{k}\right). \tag{4}$$

Number of theoretical k -mers given alphabet size, compared to observed k-mers in ENSEMBL human translated proteome. The number of observed k-mers is distinct from the number of theoretical k-mers, as the total number of observed k-mers is limted by k-mers compatible with life. Rerun this with uniprot uniref data.

False positive rate of protein-coding prediction

Combining Equations 2, 4, and 3, for an RNA-seq read of length L where its translated length $L_{\text{translated}} = \lfloor \frac{L}{3} \rfloor$, containing a possible six frames of translation, then the false-positive rate (FPR) protein-coding read is,

$$\Pr\left(\mathrm{FPR}\right) = 6 \times \left(1 - \exp^{\min\left(10^8, |\Sigma|^k\right)}\right)^Z \times \frac{1}{|\Sigma|^{L_{\text{translated}}}}. \quad (5)$$

Similarity thresholds for percentage of matching k-mers

A single SNP in a read affects k k-mers.

`kmerslay compare-kmer-content` performs all-by-all or pairwise k-mer similarity of protein or nucleotide sequences using reduced alphabets

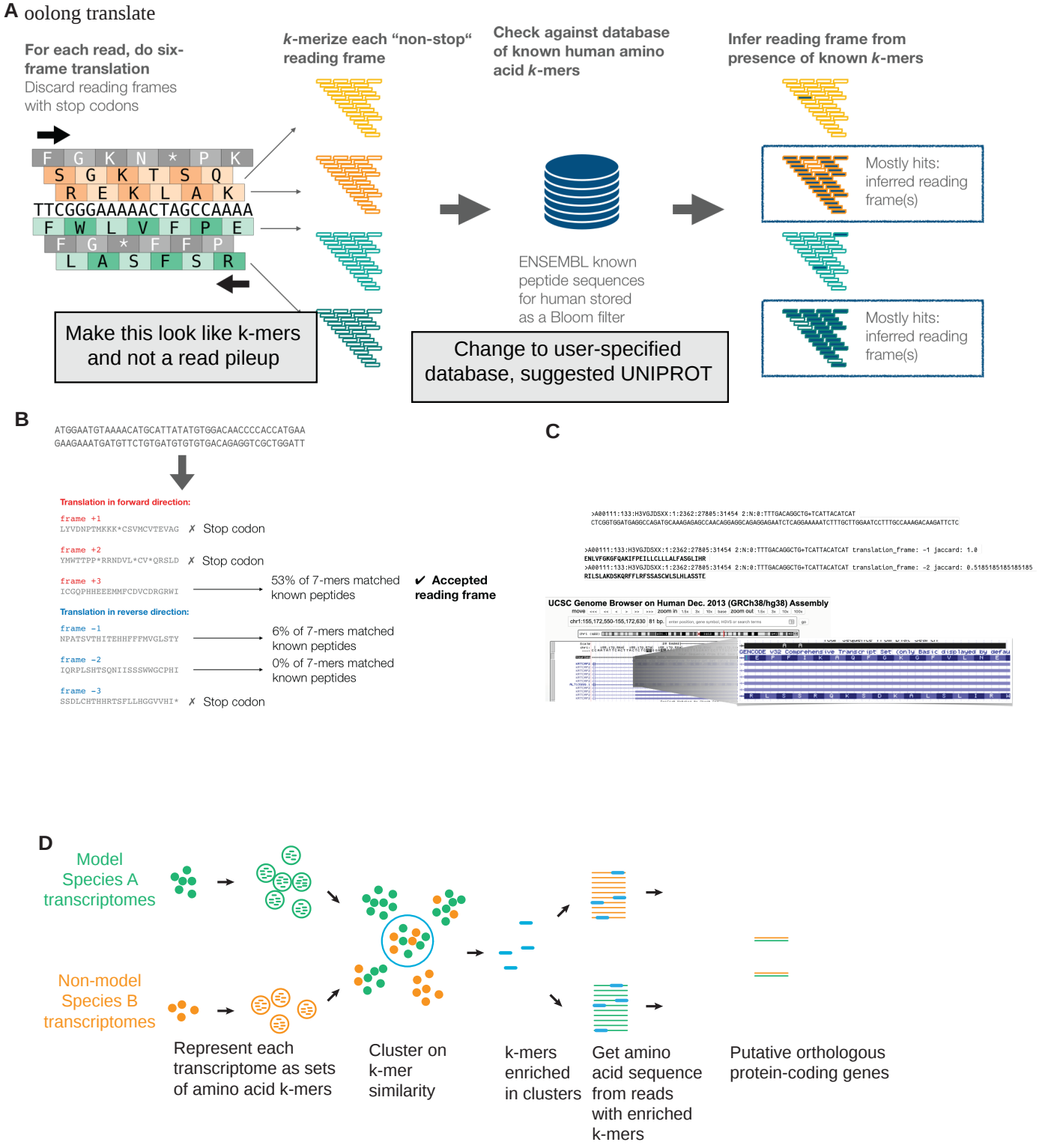
Overview of `kmerslay compare-kmer-content` **A.** Protein sequences are k -merized by converting into a bag of words using a sliding window of size k, potentially re-encoded to a lossy alphabet, and then their fraction of overlapping k-mers is computed into a Jaccard similarity. **B.** One option for `kmerslay compare-kmer-content` is to specify a pair of sequence files, and compute a background of k-mer similarty using randomly shuffled pairs. **C.** Another option for `kmerslay compare-kmer-content` is to do an all-by-all k-mer similarity comparison.

Benchmarking

Methods go here.

Computational

Supplemental Figure 1



A. Overview of `nf-core/kmermaid` pipeline. 1. If input is bam, extract per-cell sequences. 2. Predict amino acid sequence of each RNA-seq read using `khtools extract-coding`. 3. Randomly subsample amino acid k-mers via MinHash using `sourmash sketch`. 4. Compare all k-mer sketches to one another using `sourmash compare` to compute cell-cell Jaccard similarities. 5. Build sequence bloom tree using `sourmash index`. 6. Build k-nearest neighbor graph using sequence bloom tree. 7. Build UMAP off of KNN. **B.** Overview of `czbiohub/nf-predictorthologs` pipeline for prediction of homologous genes from sequences. 1. If input is bam, must also have a convert bam reads to `amtools fastq` subcommand (samtools version 1.9). If input is fastqs, go directly to second

step. 2. Trim adapters, poly-A, polyG using the `fastp` tool. 3. Predict protein-coding sequence using khtools extract_coding, using conservative UniProt/SwissProt manually curated database as examples of known protein-coding sequences, for most stringent definition of protein-coding. 4. Query predicted protein in permissive NCBI RefSeq non-redundant protein database for most complete search query. **C.** Example of predicting protein-coding sequence using Brawand2011 RNA-seq data, and human proteome as the reference. x-axis, percentage of reads falling into that category, y-axis, the species which the reads are from.

k-mer comparison of orthologous genes

We used ENSEMBL version 97. We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [25]. Biorxiv example: [39]. Multiple citations per line example: [25,39].

Extraction of putative coding reads from RNA-seq

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [25]. Biorxiv example: [39]. Multiple citations per line example: [25,39].

Supplemental Methods

Table 2: Dayhoff and hydrophobic-polar encodings are a reduced amino acid alphabet allowing for permissive cross-species sequence comparisons. For example, the amino acid sequence `SASHAFIERCE` would be Dayhoff-encoded to `bbbdbfecdac`, and HP-encoded to `phpphhhpppp`.

Amino acid	Property	Dayhoff	Hydrophobic-polar (HP)
C	Sulfur polymerization	a	p
A, G, P, S, T	Small	b	A, G, P: h
			S,T: p
D, E, N, Q	Acid and amide	c	p
H, K, R	Basic	d	p
I, L, M, V	Hydrophobic	e	h
F, W, Y	Aromatic	f	h

Applications

Installation

`kmerslay` can be installed with the Anaconda package manager, `conda` (preferred),

```
# Note: not actually on bioconda yet ... this is aspirational
conda install --channel bioconda kmerslay
```

or from the Python Package Index (PyPI) with the Python package manager, `pip`,

```
# Note: not actually on PyPI yet ... this is aspirational
pip install kmerslay
```

Prediction of protein-coding sequences across a variety of species

We used `kmerslay extract-coding` to obtain putative protein-coding sequences from a comaparative transcriptomic dataset spanning nine species and six tissues [11].

Read preprocessing

As the protein-coding score is assessed on the entire read, we recommend RNA-seq reads be removed of library artifacts to the best of the user's ability. This means, the adapters should be trimmed, and if there was a negative insert size such that the R1 and R2 reads overlap, then the read pairs should be merged.

Creation of amino acid k-mer database with `kmerslay bloom-filter`

Before predicting protein-coding sequences, `kmerslay` must create a database of known amino acid k-mers, which is stored in the form of a probabilistic set membership data structure known as a bloom filter. `kmerslay` uses the bloom filter implementation in `khmer` / `oxli` [38,40], called a NodeGraph. We created a dataset of known amino acid k-mers from the manually annotated UniProtKB/Swiss-Prot databases [41,42]. We used only protein sequences observed in *Opisthokont* species [43], previously known as a "Fungi/Metazoa" group that encompasseses "Fungus-like" *Holomycota* and "Animal-like" *Holozoa*. [NOTE: Does this need a figure/phylogenetic timetree?]

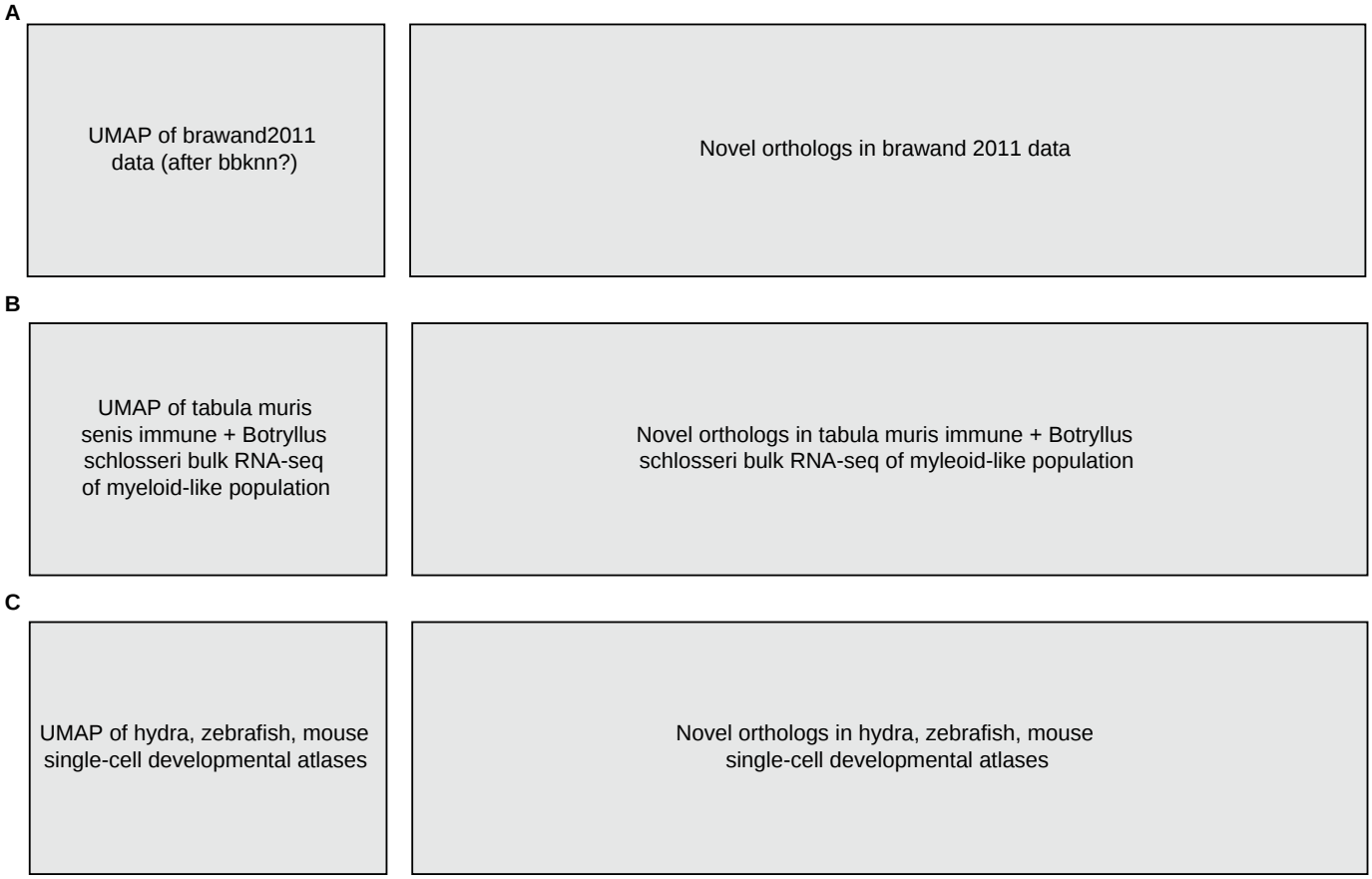
```
kmerslay bloom-filter \
  --tablesize 100000000 \
  --molecule protein \
  --peptide-ksize 7 \
  --save-as uniprot-reviewed_yes+taxonomy_2759__molecule-protein_ksize-
7.bloomfilter \
  uniprot-reviewed_yes+taxonomy_2759.fasta.gz
```

Prediction of protein-coding sequences with `kmerslay extract-coding`

We then predicted protein coding reads using the created bloom filter using `kmerslay extract-coding`.

```
kmerslay extract-coding \
  --molecule protein \
  --coding-nucleotide-fasta
SRR306800_GSM752653_ggo_br_F_1__coding_reads_nucleotides.fasta \
  --csv SRR306800_GSM752653_ggo_br_F_1__coding_scores.csv \
  --json-summary SRR306800_GSM752653_ggo_br_F_1__coding_summary.json \
  --jaccard-threshold 0.8 \
  --peptides-are-bloom-filter \
  uniprot-reviewed_yes+taxonomy_2759__molecule-protein_ksize-7.bloomfilter \
  SRR306800_GSM752653_ggo_br_F_1_trimmed.fq.gz >
SRR306800_GSM752653_ggo_br_F_1__coding_reads_peptides.fasta
```

Figure 2



Applications of `kmerslay extract-coding`. **A.** We simulated RNA-seq data using Opisthokonta species from the Quest for Orthologs dataset for true positive protein-coding RNAs, reads completely contained within intergenic, intronic, and UTR sequences as true positive noncoding RNAs, and reads partially overlapping a coding and noncoding region as an adversarial test set. We then predicted protein-coding sequences and computed false positive and false negative rates. False Positive coding reads were found to be ... False negative noncoding reads were found to be ... **B.** Number of putative protein-coding sequences per read. **C.** This method could also be used to extract only reads whose putative protein-coding sequences are transcription factors. **D.** We ran `kmerslay extract-coding` on the five tissues and nine species from the Brawand 2011 dataset.

`kmerslay compare-kmer-content` is a simple method to identify homologs

Applications of `kmerslay compare-kmer-content`. **A.** We used `kmerslay compare-kmer-content` on pairs of orthologous protein sequences between humans and the remaining Opisthokonta species in the Quest for Orthologs dataset. x-axis, k -mer size, y-axis, mean difference. **B.** False positive calls by `kmerslay compare-kmer-content` are either paralogs or read-through protein products. **C.** We applied `kmerslay compare-kmer-content` to ... to find putative orthologs. We found ... the accuracy was ...

Pipelines

Figure 3: **A.** Overview of the `kmermaid` pipeline. (**a**, **b**, **c**) `kmermaid` consists of a protein-coding prediction phase (**a**) that is invoked by the command `khtools extract_coding`, a k-mer sketch computation phase (**b**) invoked by the command `sourmash sketch`, a signature similarity comparison phase (**c**) invoked by the command `sourmash compare`, and an optional database-creation phase (**d**) invoked by the command `sourmash index`. The coding prediction phase has three components: (1) six-frame translation, removal of stop-codon frames, and subsequent k-merization of RNA-sequencing reads; (2) a degenerate protein alphabet which allows for protein-coding detection from a wide variety of species; (3) a bloom filter containing known protein-coding sequences from a well annotated organism; and (4) computation of the Jaccard index of translated RNA-seq reading frames. The sketch computation phase involves randomly subsetting the degenerate peptide k-mers using a MinHash algorithm. The sketch comparison phase consists of computing the Jaccard intersection of MinHashed degenerate peptide k-mers between all pairs of samples.

References

1. **How Many Species Are There on Earth and in the Ocean?**
   Camilo Mora, Derek P. Tittensor, Sina Adl, Alastair G. B. Simpson, Boris Worm
   PLoS Biology (2011-08-23) https://doi.org/fpr4z8
   DOI: 10.1371/journal.pbio.1001127 · PMID: 21886479 · PMCID: PMC3160336

2. **Genome List - Genome - NCBI** https://www.ncbi.nlm.nih.gov/genome/browse

3. **The origin and evolution of cell types**
   Detlev Arendt, Jacob M. Musser, Clare V. H. Baker, Aviv Bergman, Connie Cepko, Douglas H. Erwin, Mihaela Pavlicev, Gerhard Schlosser, Stefanie Widder, Manfred D. Laubichler, Günter P. Wagner
   Nature Reviews Genetics (2016-11-07) https://doi.org/f9b62x
   DOI: 10.1038/nrg.2016.127 · PMID: 27818507

4. **How Single-Cell Genomics Is Changing Evolutionary and Developmental Biology**
   John C. Marioni, Detlev Arendt
   Annual Review of Cell and Developmental Biology (2017-10-06) https://doi.org/ggb632
   DOI: 10.1146/annurev-cellbio-100616-060818 · PMID: 28813177

5. **K-mer counting with low memory consumption enables fast clustering of single-cell sequencing data without read alignment**
   Christina Huan Shi, Kevin Y. Yip
   bioRxiv (2019-08-02) https://www.biorxiv.org/content/10.1101/723833v1
   DOI: 10.1101/723833

6. **Reduced amino acid alphabets exhibit an improved sensitivity and selectivity in fold assignment**
   Eric L. Peterson, Jané Kondev, Julie A. Theriot, Rob Phillips
   Bioinformatics (2009-04-07) https://doi.org/btqmnp
   DOI: 10.1093/bioinformatics/btp164 · PMID: 19351620 · PMCID: PMC2732308

7. **Simplified amino acid alphabets for protein fold recognition and implications for folding**
   Lynne Reed Murphy, Anders Wallqvist, Ronald M. Levy
   Protein Engineering, Design and Selection (2000-03) https://doi.org/bdtngh
   DOI: 10.1093/protein/13.3.149 · PMID: 10775656

8. **Local homology recognition and distance measures in linear time using compressed amino acid alphabets**
   R. C. Edgar
   Nucleic Acids Research (2004-01-02) https://doi.org/ckg5d4
   DOI: 10.1093/nar/gkh180 · PMID: 14729922 · PMCID: PMC373290

9. **SwiftOrtho: A fast, memory-efficient, multiple genome orthology classifier**
   Xiao Hu, Iddo Friedberg
   GigaScience (2019-10-01) https://doi.org/ggcr5x
   DOI: 10.1093/gigascience/giz118 · PMID: 31648300 · PMCID: PMC6812468

10. **Fast databank searching with a reduced amino-acid alphabet**
    Claudine Landès, Jean-Loup Risler
    Bioinformatics (1994) https://doi.org/cvrjmw
    DOI: 10.1093/bioinformatics/10.4.453 · PMID: 7804879

11. **The evolution of gene expression levels in mammalian organs**
David Brawand, Magali Soumillon, Anamaria Necsulea, Philippe Julien, Gábor Csárdi, Patrick Harrigan, Manuela Weier, Angélica Liechti, Ayinuer Aximu-Petri, Martin Kircher, … Henrik Kaessmann
Nature (2011-10) https://doi.org/fcvk54
DOI: 10.1038/nature10532 · PMID: 22012392

12. **Inferring Hierarchical Orthologous Groups from Orthologous Gene Pairs**
Adrian M. Altenhoff, Manuel Gil, Gaston H. Gonnet, Christophe Dessimoz
PLoS ONE (2013-01-14) https://doi.org/ggkv2j
DOI: 10.1371/journal.pone.0053786 · PMID: 23342000 · PMCID: PMC3544860

13. **Conceptual framework and pilot study to benchmark phylogenomic databases based on reference gene trees**
B. Boeckmann, M. Robinson-Rechavi, I. Xenarios, C. Dessimoz
Briefings in Bioinformatics (2011-07-07) https://doi.org/c78rwm
DOI: 10.1093/bib/bbr034 · PMID: 21737420 · PMCID: PMC3178055

14. **Big data and other challenges in the quest for orthologs**
E. L. L. Sonnhammer, T. Gabaldon, A. W. Sousa da Silva, M. Martin, M. Robinson-Rechavi, B. Boeckmann, P. D. Thomas, C. Dessimoz,
Bioinformatics (2014-07-26) https://doi.org/f6ntvb
DOI: 10.1093/bioinformatics/btu492 · PMID: 25064571 · PMCID: PMC4201156

15. **TimeTree :: The Timescale of Life** http://timetree.org/

16. **A Single Cell Transcriptomic Atlas Characterizes Aging Tissues in the Mouse**
The Tabula Muris Consortium, Angela Oliveira Pisco, Aaron McGeever, Nicholas Schaum, Jim Karkanias, Norma F. Neff, Spyros Darmanis, Tony Wyss-Coray, Stephen R. Quake
bioRxiv (2019-11-18) https://www.biorxiv.org/content/10.1101/661728v2
DOI: 10.1101/661728

17. **Complex mammalian-like haematopoietic system found in a colonial chordate**
Benyamin Rosental, Mark Kowarsky, Jun Seita, Daniel M. Corey, Katherine J. Ishizuka, Karla J. Palmeri, Shih-Yu Chen, Rahul Sinha, Jennifer Okamoto, Gary Mantalas, … Ayelet Voskoboynik
Nature (2018-12) https://doi.org/gfkzvm
DOI: 10.1038/s41586-018-0783-x · PMID: 30518860 · PMCID: PMC6347970

18. **Assigning confidence scores to homoeologs using fuzzy logic**
Natasha M. Glover, Adrian Altenhoff, Christophe Dessimoz
PeerJ (2019-01-11) https://doi.org/ggkv2k
DOI: 10.7717/peerj.6231 · PMID: 30648004 · PMCID: PMC6330999

19. **Orthologous Matrix (OMA) algorithm 2.0: more robust to asymmetric evolutionary rates and more scalable hierarchical orthologous group inference**
Clément-Marie Train, Natasha M Glover, Gaston H Gonnet, Adrian M Altenhoff, Christophe Dessimoz
Bioinformatics (2017-07-12) https://doi.org/ggkv2h
DOI: 10.1093/bioinformatics/btx229 · PMID: 28881964 · PMCID: PMC5870696

20. **Ensembl 2018**
Daniel R Zerbino, Premanand Achuthan, Wasiu Akanni, M Ridwan Amode, Daniel Barrell, Jyothish Bhai, Konstantinos Billis, Carla Cummins, Astrid Gall, Carlos García Girón, … Paul Flicek

Nucleic Acids Research (2017-11-16) https://doi.org/gcwg6r
DOI: 10.1093/nar/gkx1098 · PMID: 29155950 · PMCID: PMC5753206

21. **Ensembl comparative genomics resources**
Javier Herrero, Matthieu Muffato, Kathryn Beal, Stephen Fitzgerald, Leo Gordon, Miguel Pignatelli, Albert J. Vilella, Stephen M. J. Searle, Ridwan Amode, Simon Brent, … Paul Flicek
Database (2016) https://doi.org/ggb9tv
DOI: 10.1093/database/bav096 · PMID: 26896847 · PMCID: PMC4761110

22. **Atlas of protein sequence and structure**
Margaret O Dayhoff
National Biomedical Research Foundation. (1969)

23. **Physical biology of the cell**
Rob Phillips, Julie Theriot, Jane Kondev, Hernan Garcia
Garland Science (2012)

24. **Theory for the folding and stability of globular proteins**
Ken A. Dill
Biochemistry (1985-03-12) https://doi.org/fnj5k7
DOI: 10.1021/bi00327a032 · PMID: 3986190

25. **Standardized benchmarking in the quest for orthologs**
Adrian M AltenhoffBrigitte Boeckmann, Salvador Capella-Gutierrez, Daniel A Dalquen, Todd DeLuca, Kristoffer Forslund, Jaime Huerta-Cepas, Benjamin Linard, Cécile Pereira, … Christophe Dessimoz
Nature Methods (2016-04-04) https://doi.org/f3rpzx
DOI: 10.1038/nmeth.3830 · PMID: 27043882 · PMCID: PMC4827703

26. https://www.ebi.ac.uk/miriam/main/collections/MIR:00000119

27. **RNAsamba: coding potential assessment using ORF and whole transcript sequence information**
Antonio P. Camargo, Vsevolod Sourkov, Marcelo F. Carazzolle
Cold Spring Harbor Laboratory (2019-04-28) https://doi.org/ggdtxk
DOI: 10.1101/620880

28. **Heterogeneous Patterns of Gene-Expression Diversification in Mammalian Gene Duplicates**
D. Farre, M. M. Alba
Molecular Biology and Evolution (2009-10-12) https://doi.org/dxrtmd
DOI: 10.1093/molbev/msp242 · PMID: 19822635

29. **GENEFAMILYEVOLUTION ANDHOMOLOGY: Genomics Meets Phylogenetics**
Joseph W. Thornton, Rob DeSalle
Annual Review of Genomics and Human Genetics (2000-09) https://doi.org/bjp5pm
DOI: 10.1146/annurev.genom.1.1.41 · PMID: 11701624

30. **Evolution and cell-type specificity of human-specific genes preferentially expressed in progenitors of fetal neocortex**
Marta Florio, Michael Heide, Anneline Pinson, Holger Brandl, Mareike Albert, Sylke Winkler, Pauline Wimberger, Wieland B Huttner, Michael Hiller
eLife (2018-03-21) https://doi.org/gc678k
DOI: 10.7554/elife.32332 · PMID: 29561261 · PMCID: PMC5898914

31. **Taxon-restricted genes at the origin of a novel trait allowing access to a new environment**
M. Emília Santos, Augustin Le Bouquin, Antonin J. J. Crumière, Abderrahman Khila
Science (2017-10-19) https://doi.org/gcgjbs
DOI: 10.1126/science.aan2748 · PMID: 29051384

32. **Pervasive Correlated Evolution in Gene Expression Shapes Cell and Tissue Type Transcriptomes**
Cong Liang, Jacob M Musser, Alison Cloutier, Richard O Prum, Günter P Wagner
Genome Biology and Evolution (2018-01-23) https://doi.org/gc69v9
DOI: 10.1093/gbe/evy016 · PMID: 29373668 · PMCID: PMC5800078

33. **Deep homology in the age of next-generation sequencing**
Patrick Tschopp, Clifford J. Tabin
Philosophical Transactions of the Royal Society B: Biological Sciences (2017-02-05) https://doi.org/gfzpbg
DOI: 10.1098/rstb.2015.0475 · PMID: 27994118 · PMCID: PMC5182409

34. **Embracing the comparative approach: how robust phylogenies and broader developmental sampling impacts the understanding of nervous system evolution**
Andreas Hejnol, Christopher J. Lowe
Philosophical Transactions of the Royal Society B: Biological Sciences (2015-12-19) https://doi.org/ggcd2m
DOI: 10.1098/rstb.2015.0045 · PMID: 26554039 · PMCID: PMC4650123

35. **The mammalian decidual cell evolved from a cellular stress response**
Eric M. Erkenbrack, Jamie D. Maziarz, Oliver W. Griffith, Cong Liang, Arun R. Chavan, Mauris C. Nnamani, Günter P. Wagner
PLOS Biology (2018-08-24) https://doi.org/gd5b9s
DOI: 10.1371/journal.pbio.2005594 · PMID: 30142145 · PMCID: PMC6108454

36. **RAPSearch: a fast protein similarity search tool for short reads**
Yuzhen Ye, Jeong-Hyeon Choi, Haixu Tang
BMC Bioinformatics (2011-05-15) https://doi.org/dgt5rt
DOI: 10.1186/1471-2105-12-159 · PMID: 21575167 · PMCID: PMC3113943

37. **Incomplete nucleic acid sequences** https://www.qmul.ac.uk/sbcs/iubmb/misc/naseq.html

38. **These Are Not the K-mers You Are Looking For: Efficient Online K-mer Counting Using a Probabilistic Data Structure**
Qingpeng Zhang, Jason Pell, Rosangela Canino-Koning, Adina Chuang Howe, C. Titus Brown
PLoS ONE (2014-07-25) https://doi.org/f6kb9b
DOI: 10.1371/journal.pone.0101271 · PMID: 25062443 · PMCID: PMC4111482

39. **OrthoFinder: phylogenetic orthology inference for comparative genomics**
David M. Emms, Steven Kelly
bioRxiv (2019-04-24) https://www.biorxiv.org/content/10.1101/466201v2
DOI: 10.1101/466201

40. **The khmer software package: enabling efficient nucleotide sequence analysis**
Michael R. Crusoe, Hussien F. Alameldin, Sherine Awad, Elmar Boucher, Adam Caldwell, Reed Cartwright, Amanda Charbonneau, Bede Constantinides, Greg Edvenson, Scott Fay, … C. Titus Brown

F1000Research (2015-09-25) https://doi.org/9qp
DOI: 10.12688/f1000research.6924.1 · PMID: 26535114 · PMCID: PMC4608353

41. **UniProt: a worldwide hub of protein knowledge** *Nucleic Acids Research* (2018-11-05) https://doi.org/gfwqck
DOI: 10.1093/nar/gky1049 · PMID: 30395287 · PMCID: PMC6323992

42. **UniProtKB/Swiss-Prot, the Manually Annotated Section of the UniProt KnowledgeBase: How to Use the Entry View**
Emmanuel Boutet, Damien Lieberherr, Michael Tognolli, Michel Schneider, Parit Bansal, Alan J. Bridge, Sylvain Poux, Lydie Bougueleret, Ioannis Xenarios
Plant Bioinformatics (2016) https://doi.org/f79kbb
DOI: 10.1007/978-1-4939-3167-5_2 · PMID: 26519399

43. **Opisthokont**
Wikipedia
(2020-03-26) https://en.wikipedia.org/w/index.php?title=Opisthokont&oldid=947511780