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

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Abstract

We introduce `kmermaid`, a novel computational method for identifying orthologous cell types discovering *de novo* orthologous genes across species. As `kmermaid` skips both traditional alignment and gene orthology assignment it can a) be applied to transcriptomes from organisms with no or poorly annotated genomes, b) predicts protein-coding sequences from raw RNA-seq reads, and c) identify putative functions of protein sequences contributing to shared cell types. By enabling analyses across divergent species' transcriptomes in an orthology-, genome- and gene annotation-agnostic manner, `kmermaid` 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].

Typesetting math: 100% et 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.

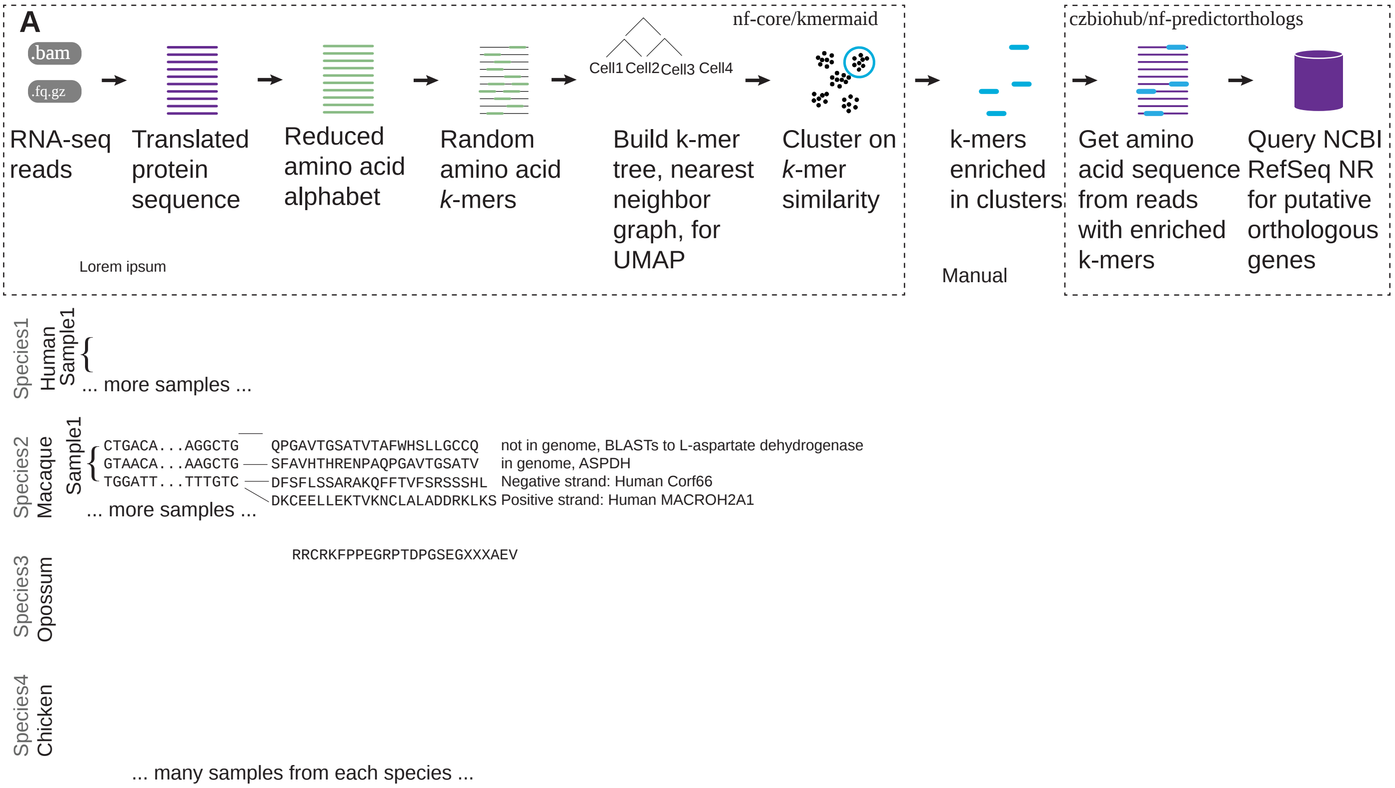
A
nf-core/kmermaid
czbiohub/nf-predictorthologs
.bam
.fq.gz
RNA-seq reads
Translated protein sequence
Reduced amino acid alphabet
Random amino acid k-mers
Cell1 Cell2 Cell3 Cell4
Build k-mer tree, nearest neighbor graph, for UMAP
Cluster on k-mer similarity
k-mers enriched in clusters
Get amino acid sequence from reads with enriched k-mers
Query NCBI RefSeq NR for putative orthologous genes
Lorem ipsum
Manual
Species1
Human
Sample1
... more samples ...
Species2
Macaque
Sample1
CTGACA...AGGCTG
GTAACA...AAGCTG
TGGATT...TTTGTC
QPGAVTGSATVTAFWHSLLGCCQ
SFAVHTHRENPAQPGAVTGSATV
DFSFLSSARAKQFFTVFSRSSSHL
DKCEELLEKTVKNCLALADDRKLKS
not in genome, BLASTs to L-aspartate dehydrogenase
in genome, ASPDH
Negative strand: Human Corf66
Positive strand: Human MACROH2A1
... more samples ...
RRCRKFPPEGRPTDPGSEGXXXAEV
Species3
Opossum
Species4
Chicken
... many samples from each species ...

Figure 1: 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

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e 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.

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 [1]). 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 [1].

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 sequences, generated 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 2: 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]

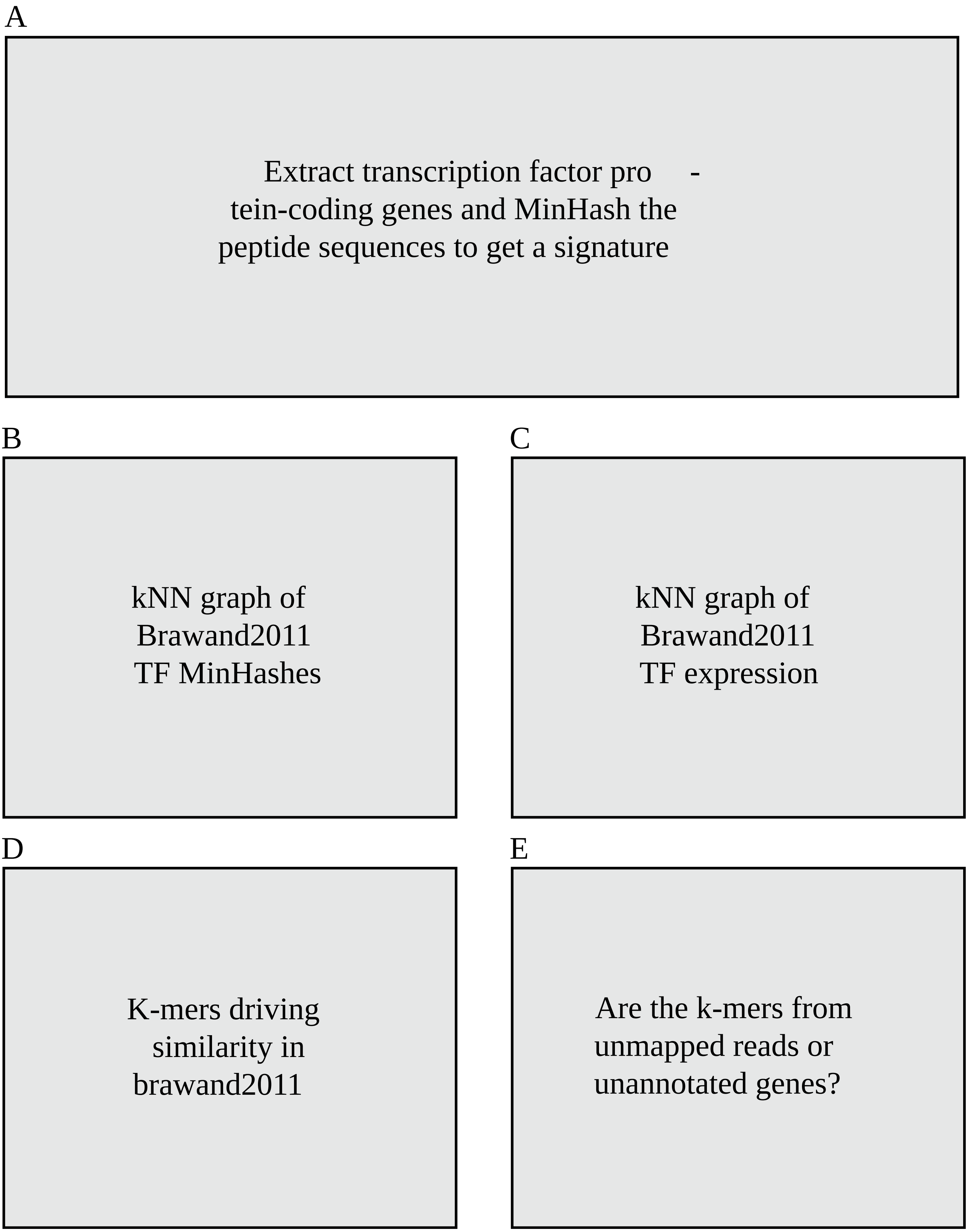
A
Extract transcription factor protein-coding genes and MinHash the peptide sequences to get a signature
B
kNN graph of Brawand2011 TF MinHashes
C
kNN graph of Brawand2011 TF expression
D
K-mers driving similarity in brawand2011
E
Are the k-mers from unmapped reads or unannotated genes?

Figure 3: Figure 3

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- 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]
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Correlated evolution of celltypes?

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Cell type homology

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- Mammalian decidual cell

Cell type evolution

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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.

Methods

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Experimental

Primate brain organoid protocols

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Single-cell capture of primate brain organoids

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Long read library prep

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [25]. Biorxiv example: [36]. Multiple citations per line example: [25,36].

Short read library prep

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Sequencing

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Computational

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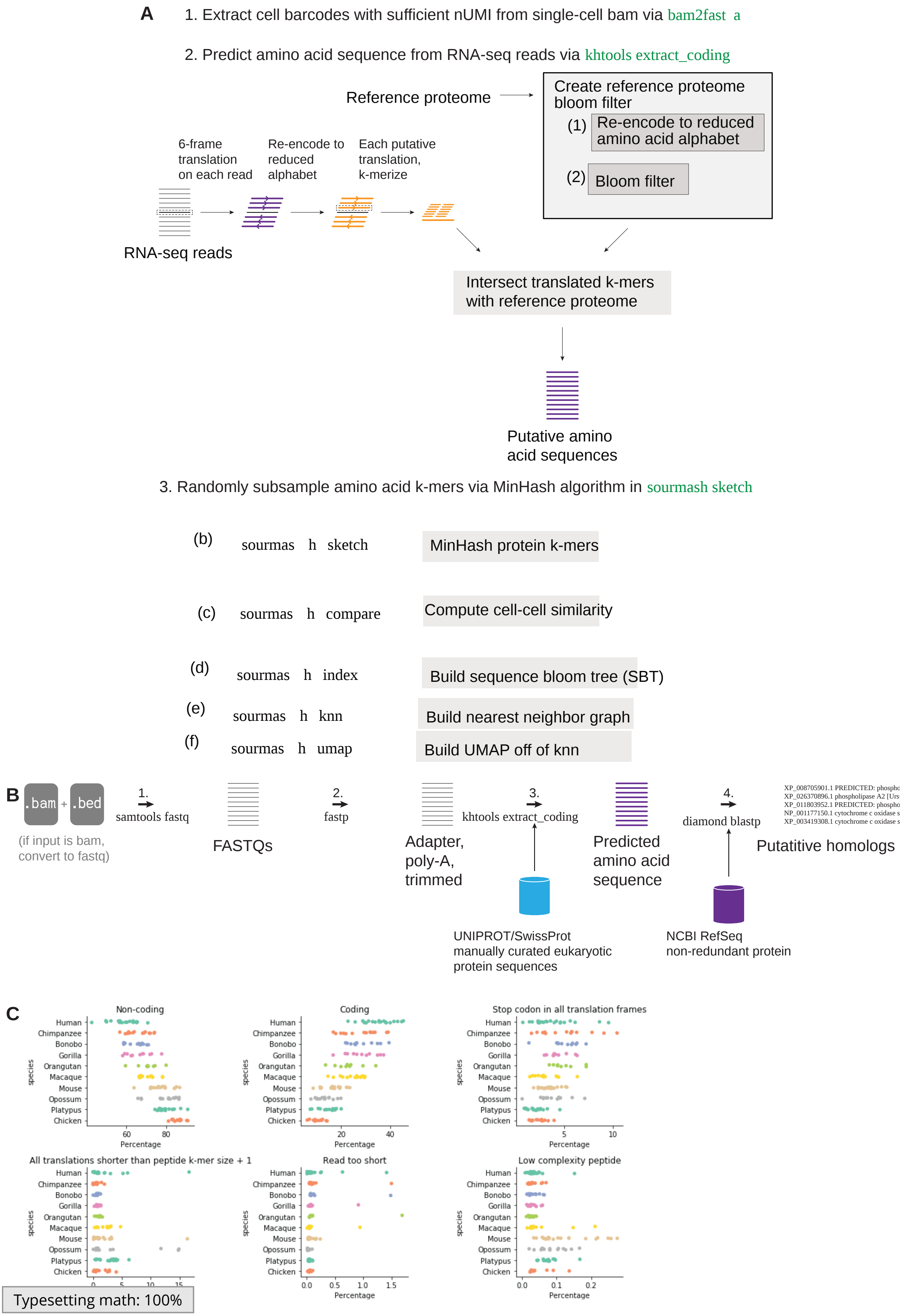
A
1. Extract cell barcodes with sufficient nUMI from single-cell bam via bam2fast a
2. Predict amino acid sequence from RNA-seq reads via khtools extract_coding
Reference proteome
Create reference proteome bloom filter
(1) Re-encode to reduced amino acid alphabet
(2) Bloom filter
6-frame translation on each read
Re-encode to reduced alphabet
Each putative translation, k-merize
RNA-seq reads
Intersect translated k-mers with reference proteome
Putative amino acid sequences
3. Randomly subsample amino acid k-mers via MinHash algorithm in sourmash sketch
(b) sourmas h sketch
MinHash protein k-mers
(c) sourmas h compare
Compute cell-cell similarity
(d) sourmas h index
Build sequence bloom tree (SBT)
(e) sourmas h knn
Build nearest neighbor graph
(f) sourmas h umap
Build UMAP off of knn
B
.bam + .bed
(if input is bam, convert to fastq)
1. samtools fastq
FASTQs
2. fastp
Adapter, poly-A, trimmed
3. khtools extract_coding
UNIPROT/SwissProt manually curated eukaryotic protein sequences
Predicted amino acid sequence
4. diamond blastp
NCBI RefSeq non-redundant protein
XP_008705901.1 PREDICTED: phospho
XP_026370896.1 phospholipase A2 [Urs
XP_011803952.1 PREDICTED: phospho
NP_001177150.1 cytochrome c oxidase s
XP_003419308.1 cytochrome c oxidase s
Putatitive homologs
C
Non-coding
Coding
Stop codon in all translation frames
All translations shorter than peptide k-mer size + 1
Read too short
Low complexity peptide
species
Human
Chimpanzee
Bonobo
Gorilla
Orangutan
Macaque
Mouse
Opossum
Platypus
Chicken
Percentage
60
80
20
40
5
10
0
15
0.0
0.5
1.0
1.5
0.1
0.2

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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 raw fastq files using the `samtools 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: [36]. Multiple citations per line example: [25,36].

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: [36]. Multiple citations per line example: [25,36].

Supplemental Methods

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`.

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

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