

# BLAST Exercise: Detecting and Interpreting Genetic Homology

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## Recommended background tutorial

An Introduction to NCBI BLAST

## Resources

- [NCBI BLAST](#)
- [RepeatMasker web server](#)
- [The UniProt Protein Knowledgebase](#)

## Files for this exercise

The [package](#) containing the files for this exercise is available through the “[Detecting and Interpreting Genetic Homology](#)” page on the GEP website.

## Introduction

The Basic Local Alignment Search Tool (BLAST) is a program that reports regions of local similarity (at either the nucleotide or protein level) between a query sequence and sequences within a database. The ability to detect sequence homology allows us to determine if a gene or a protein is related to other known genes or proteins. Detecting sequence homology also facilitates the identification of conserved domains that are shared by multiple genes and the identification of members of a gene family.

BLAST is popular because it can efficiently identify regions of local similarity between two sequences. More importantly, BLAST is based on a robust statistical framework. This framework allows BLAST to determine if the alignment between two sequences is statistically significant (i.e., probability of obtaining an alignment with this score or higher by chance is low).

Before proceeding with annotation, it is important to understand the inferences that we are making when we use BLAST in our analysis. The theory of evolution proposes that all organisms descend by speciation from common ancestors. At the molecular level, an ancestral DNA sequence diverges over time (through accumulation of point mutations, duplications, deletions, transpositions, recombination events, etc.) to produce diverse sequences in the

genomes of subsequent organisms. Mutations to sequences with an important biological function, such as genes, have a higher probability of being deleterious to the organism, so they are less likely to become fixed in a population. We say that such sequences are under *negative selection*, which causes them to be *conserved* against change over time. We therefore expect that two homologous copies of a functional sequence, either in two species or within a single species, will exhibit a higher degree of conservation, and therefore of base-by-base similarity, than either two unrelated sequences or two sequences that are not under strong negative selection. This similarity is the “signal” detected by a BLAST search.

When we perform a BLAST search, we reverse the above line of reasoning to infer common function from sequence similarity. We first use the observed sequence similarity to infer that two sequences are in fact conserved homologs. Then we use this inferred conservation to infer that the sequences have a common function (e.g., that they encode the same protein). There are, of course, limitations to this line of reasoning. For example, two unrelated sequences might appear similar purely by chance. Alternatively, a pair of sequences may be conserved homologs, but they may have diverged only recently (as in human and chimpanzee), so that conservation implies nothing about whether they are under negative selection. Finally, a pair of sequences may indeed be conserved homologs because of strong negative selection, yet have different functions. For example, the *delta 1* and *delta 2 crystallin* genes in ducks have high sequence similarity and both serve structural functions in the eye lens. However, the *delta 2 crystallin* exhibits an additional enzymatic (arginosuccinate lyase) activity that is absent from the *delta 1 crystallin*. Similarly, the *ADH1* and *ADH2* genes in yeast are closely related **but have opposite enzymatic function!** While BLAST is a powerful tool for detecting similarity, we must also understand its inherent limitations in order to properly interpret its results.

## Overview of the annotation process

The main goal of this exercise is to identify interesting features (functional genes or non-functional pseudogenes) within a region of the *D. melanogaster* genome. We will use the programs BLAST and RepeatMasker to help us with the annotation. During the course of our investigation, we will also learn how to adjust some of the parameters in BLAST and in RepeatMasker to increase the sensitivity and specificity of our searches.

Much of this exercise consists of questions, which you should try to answer as you work through this exercise. You should also make note of the exact BLAST and RepeatMasker parameters and the databases that you use for your searches in order to ensure that your results are reproducible.

This exercise assumes that you are familiar with the basics of NCBI BLAST. You can find additional information on how to use BLAST at the [NCBI BLAST website](#).

## Finding interspersed repeats

The file *dmel\_seq1.fasta* contains a FASTA-formatted DNA sequence, which represents roughly 4,000 bases from the X chromosome of *D. melanogaster*.

Before we attempt to search for genes in this 4kb sequence, we should first annotate its repetitive elements using RepeatMasker. Repetitive DNA elements are sequence motifs repeated hundreds or thousands of times in the genome, constitutes the major proportion of all the nuclear DNA in most eukaryotic genomes, and its significance is not fully understood. RepeatMasker is a program that identifies transposable elements and low complexity repeats in DNA sequences. You can run the RepeatMasker program at the [RepeatMasker web server](#). Alternatively, the result of the RepeatMasker analysis of our sequence is available in the exercise package (files within the *DmelSeq1\_RpM* directory).

Open a new web browser window and navigate to the [RepeatMasker web service](#). Click on the “Browse” or “Choose File” button under the “Sequence” field, and then select the *dmel\_seq1.fasta* file from the exercise package.

We can use four different search engines with RepeatMasker: rmbblast, hmmer, cross\_match, and abblast. Because cross\_match is the most sensitive among these four search engines, we will change the “Search Engine” from “rmbblast” to “**cross\_match**”. Since RepeatMasker works by comparing the query sequence against a database of known repetitive elements, we should select the repeat database that best corresponds to the sequence we are annotating. After all, we would not want to waste time looking for Alu repeats in our fly sequence! Since our sequence is from a fruit fly, we will use the *Drosophila* repeat library. Click on the “DNA source” drop-down menu and change it to “**Fruit fly (*Drosophila melanogaster*)**”. Verify that the “Return Format” is set to “**html**” so that we can view the RepeatMasker results in the web browser (Figure 1).

### Basic Options

**Sequence:**  or

**Search Engine:**  rmbblast  hmmer  cross\_match  abblast

**Speed/Sensitivity:**  rush  quick  default  slow

**DNA source:**

**Return Format:**  html  tar file

Select a sequence file to process or paste the sequences(s) in FASTA format. Large sequences will be queued, and may take a while to process.

Select the search engine to use when searching the sequence. Cross\_match is slower but often more sensitive than the other engines. ABblast (formerly known as WUBlast) is very fast with a slight cost of sensitivity. RMBlast is a RepeatMasker compatible version of the NCBI Blast tool suite. HMMER uses the new nhmmer program to search sequences against the new Dfam database (human only).

Select the sensitivity of your search. The more sensitive the longer the processing time.

Select a species from the drop down box or select "Other." and enter a species name in the text box. Try the protein based repeatmasker if the repeat database for your species is small.

Select the format for the results of your search. The "tar" option will return the results as a compressed archive file, and "html" will present the results as a summary web page with links to the individual data files.

Figure 1. Configure RepeatMasker to search the sequence in the *dmel\_seq1.fasta* file against a database of known transposons in *Drosophila melanogaster*.

By default, RepeatMasker also masks simple repeats and low complexity DNA. Low complexity DNA sequences may not have a highly repetitive structure, but these regions consist primarily of one or two out of the four possible nucleotides.

*Question 1: Why might it be a good idea to remove low-complexity DNA from a sequence before running blastn? Why might it be a bad idea to do so before running blastx? (Hint: consider proteins such as collagen that have highly regular sequences.)*

Because BLAST automatically filters low complexity regions when appropriate, we will tell RepeatMasker not to mask low complexity regions (Figure 2). Under “Advanced Options” change “Repeat Options” to “**Don’t mask simple repeats or low complexity DNA**”. Click “Submit Sequence”. Depending on how busy the server is, this analysis may take a few minutes to complete.

**Advanced Options**

<b>Alignment Options:</b>	No alignments returned	Select how you would like alignments displayed.
<b>Masking Options:</b>	Repetitive sequences replaced by strings of N	Select how you would like your sequence masked.
<b>Contamination Check:</b>	No contamination check	Check for contamination in your sequence.
<b>Repeat Options:</b>	Don't mask simple repeats or low complexity DNA	Select the types of repeats you would like to mask.

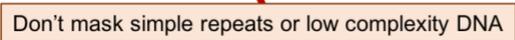


Figure 2. Turn off the filters for simple and low complexity repeats in RepeatMasker.

For class purposes, the RepeatMasker result files are also available inside the folder *DmelSeq1\_RpM* in the exercise package.

**Note:** RepeatMasker will cache the results of previous RepeatMasker analyses. If you see a message indicating that “Your request was previously run” or “Your request is still in the queue as id -1,” then click on the link to view the RepeatMasker results.

If the RepeatMasker search using the cross\_match search engine returns a blank page, navigate back to the [RepeatMasker web service](#) page, and then re-run the search using the “**rmbblast**” search engine. Alternatively, you can use the result files inside the *DmelSeq1\_RpM* folder in the exercise package.

The results page shows a table that summarizes the types and number of repeats identified by RepeatMasker (Figure 3). Scroll down to the “Results” section to view or download the Annotation Files, Masked File, and Alignment File produced by RepeatMasker (Figure 4).

**Summary:**

```

=====
file name: RM2_dmel_seq1.fasta_1667789542
sequences:      1
total length:  4000 bp (4000 bp excl N/X-runs)
GC level:      40.27 %
bases masked:  2453 bp ( 61.32 %)
=====

```

	number of elements*	length occupied	percentage of sequence
Retroelements	1	2453 bp	61.33 %
SINEs:	0	0 bp	0.00 %
Penelope	0	0 bp	0.00 %
LINEs:	0	0 bp	0.00 %
CRE/SLACS	0	0 bp	0.00 %
L2/CR1/Rex	0	0 bp	0.00 %
R1/L0A/Jockey	0	0 bp	0.00 %
R2/R4/NeSL	0	0 bp	0.00 %
RTE/Bov-B	0	0 bp	0.00 %
L1/CIN4	0	0 bp	0.00 %
LTR elements:	1	2453 bp	61.33 %
BEL/Pao	0	0 bp	0.00 %
Ty1/Copia	0	0 bp	0.00 %
Gypsy/DIRS1	1	2453 bp	61.33 %
Retroviral	0	0 bp	0.00 %

Figure 3. Summary table which shows the number and types of transposons identified by RepeatMasker.

**Results**

*Right-click and select "Save As" to save results to your computer or click on the link to view the file in the browser.*

Annotation File: [RM2\\_dmel\\_seq1.fasta\\_1667789542.out.html](#) ( **NEW** XHTML Format )

[RM2\\_dmel\\_seq1.fasta\\_1667789542.out.txt](#) ( Text File Format )

Masked File: [RM2\\_dmel\\_seq1.fasta\\_1667789542.masked](#)

Alignment File: [RM2\\_dmel\\_seq1.fasta\\_1667789542.align](#)

Figure 4. Click on the links under the “Results” section to view the RepeatMasker results.

If RepeatMasker found repeats in the query sequence, then it will produce the following files:

- A web page with a detailed list of repetitive elements found by RepeatMasker and their corresponding alignments, in a file with the extension “.out.html”
- A plain text version of the list of repetitive elements found by RepeatMasker, in a file with the extension “.out.txt”
- A copy of the original sequence with its repeats replaced by Ns, in a file with the extension “.masked”
- A list of alignments of the query sequence against each repetitive element identified by RepeatMasker, in a file with the extension “.align”

*Question 2: How many repetitive elements does our sequence contain, and what are their types? (Hint: examine the RepeatMasker Summary Table.)*

In the next section, we will be working with another sequence, *dmel\_seq2.fasta*. This 4.5kb sequence also comes from the X chromosome of *D. melanogaster*. Run RepeatMasker on this sequence using the same options as we have discussed above. Alternatively, the RepeatMasker analysis of our sequence is available in the exercise package (within the *DmelSeq2\_RpM* directory).

*Question 3: What is your result? Given the length of this sequence, would you expect the same result if it had come from a primate?*

## Translated query vs. protein database (*blastx*): the gene hunter

Following the initial annotation of repetitive elements found within our sequence (*dmel\_seq2.fasta*), we would like to see if any part of our sequence matches any known genes. We will use BLAST to help us detect these homologous regions.

There are a few decisions we must make before proceeding with the BLAST search. We could look for matches to our sequence at either the DNA or the protein level, using any one of several databases. In deciding which comparison tool to use, we should consider a few factors:

1. How sensitive will the comparison be? Is it likely to find genes or other meaningful features in our sequence?
2. How specific will the matches returned by our tool be? Will they cover the entire region or will they be confined to specific features of interest?
3. How good is the information associated with any matches we may find? Will we be able to interpret those matches?
4. How long will the tool take to run?

Considering all these factors, a reasonable first step to characterize anonymous DNA sequence is to compare the DNA sequence against the UniProtKB/Swiss-Prot protein database (a database of well characterized proteins) using *blastx*. In a *blastx* search, a nucleotide query sequence is translated into peptide sequences in all six reading frames (i.e., three reading frames on each strand) and compared against a protein database. This means *blastx* is good at specifically identifying parts of a DNA sequence that have the potential to code for proteins similar to those in the protein database. Hence it should provide us with a relatively good picture of potential genes (true positives) in our sequence without a lot of clutter (false positives).

If our *blastx* search against the UniProtKB/Swiss-Prot database fails to produce any significant matches, we could search our sequence against all proteins in the GenBank non-redundant (nr) protein database. The nr protein database contains most of the real and hypothetical peptide sequences that have been submitted to GenBank. While this would increase our chances of seeing a match, the quality of the supplementary information associated with each protein record in the nr database is much lower than those in the UniProtKB/Swiss-Prot database.

Note that there are also species-specific databases [e.g., FlyBase for fruit flies (Figure 5), WormBase for *C. elegans*] available on the web. Using these species-specific databases can substantially reduce the computational time needed to perform the BLAST searches. These species-specific databases might also contain additional metadata and references for each gene. However, we will use the generic databases in this exercise.

Figure 5. The [FlyBase website](https://flybase.org).

When performing BLAST searches, we will typically use the repeat-masked version of the sequence to reduce the search time and the number of spurious matches. However, because our sequence did not contain any repetitious elements, we will use the original sequence for our BLAST searches.

Open a new web browser window, navigate to the [NCBI BLAST web server](https://blast.ncbi.nlm.nih.gov/). Click on the *blastx* image under the “Web BLAST” section. Click on the “Browse” or “Choose File” button under the “Enter Query Sequence” section, and then select the *dmel\_seq2.fasta* file from the exercise package. Under the “Database” drop-down menu, select “**UniProtKB/Swiss-Prot (swissprot)**” (Figure 6).

**Note:** NCBI is constantly updating the databases and will [occasionally change the default BLAST parameters](#). Hence you might not get exactly the same results if you were to run the searches yourself. You should run these searches to practice using these web pages. However, you may wish to use the results saved in the exercise package to answer the questions below. The results of this BLAST search are in the file “*blx\_swissprot\_dmel\_seq2.txt*” inside the “*blast\_results*” folder.

Translated BLAST: blastx

blastn blastp **blastx** tblastn tblastx

BLASTX search protein databases using a translated nucleotide query. more...

**Enter Query Sequence**

Enter accession number(s), gi(s), or FASTA sequence(s) [Clear](#) Query subrange [?](#)

From  To

Or, upload file  dmel\_seq2.fasta [?](#)

Genetic code

Job Title

Enter a descriptive title for your BLAST search [?](#)

Align two or more sequences [?](#)

**Choose Search Set**

Databases  Standard databases (nr etc.): New  Experimental databases [Try experimental clustered nr database](#) [For more info see What is clustered nr?](#)

Compare  Select to compare standard and experimental database [?](#)

**Standard**

Database  [?](#)

Organism Optional   exclude

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown [?](#)

Exclude Optional  Models (XM/XP)  Non-redundant RefSeq proteins (WP)  Uncultured/environmental sample sequences

Search database swissprot using Blastx (search protein databases using a translated nucleotide query)  Show results in a new window

Figure 6. Configure a *blastx* search against the UniProtKB/Swiss-Prot database

By default, *blastx* uses a Word size of 5 (i.e., a sliding window of 5 amino acids) to identify the positions within the query and subject sequences where *blastx* will initiate the local alignments. In order to increase the sensitivity of the *blastx* search, we will **change the Word size to 3**. Click on the “+” icon next to the “Algorithm parameters” header under the “BLAST” button to expand the section. Change the “Word size” parameter to **3** under the “General Parameters” section (Figure 7). Click on the “**BLAST**” button to run the *blastx* search.

Note: Parameter values that differ from the default are highlighted in yellow and marked with **+** sign

**Algorithm parameters**

**General Parameters**

Max target sequences  [?](#)  
Select the maximum number of aligned sequences to display [?](#)

Expect threshold  [?](#)

Word size  [?](#) **Word size = 3**

Max matches in a query range  [?](#)

Figure 7. Change the Word size parameter to 3 in order to increase the sensitivity of the *blastx* search

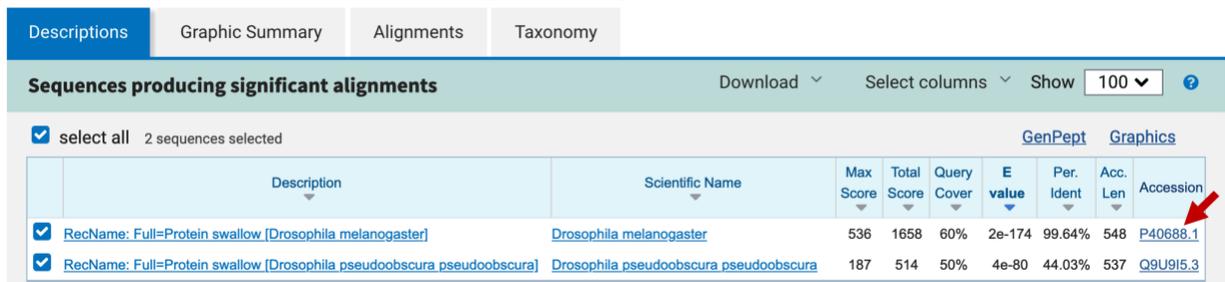
*Question 4: How many blastx hits to distinct sequences were returned? What are the best and worst E-values reported?*

When searching a large database, it is good practice to ignore matches with poor (high) E-values. In principle, a match with an E-value less than 1 is a significant hit (because we expect to find an alignment this good or better by chance less than once when searching the database). In practice, you should allow a large margin of safety when interpreting E-values because of the simplified model used by BLAST to calculate the alignments and E-values. As a rule of thumb, unless you are working with very short sequences, you should be suspicious of matches with E-values greater than  $1e-10$  and extremely skeptical of hits with E-values above  $1e-5$ .

*Question 5: Based on the search result, what does BLAST say about the content of this sequence? What caveats might you consider in interpreting these results?*

## Interpreting the *blastx* output

If you emulated our analysis thus far, the hit table under the “Descriptions” tab should show two strong hits to the swallow protein (Figure 8). However, remember that sequence similarity does not necessarily imply that our sequence contains the *D. melanogaster* version of the swallow protein. We need to gather more evidence before deciding how to annotate our sequence.



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
RecName: Full=Protein swallow [Drosophila melanogaster]	<a href="#">Drosophila melanogaster</a>	536	1658	60%	2e-174	99.64%	548	<a href="#">P40688.1</a>
RecName: Full=Protein swallow [Drosophila pseudoobscura pseudoobscura]	<a href="#">Drosophila pseudoobscura pseudoobscura</a>	187	514	50%	4e-80	44.03%	537	<a href="#">Q9U915.3</a>

Figure 8. The “Descriptions” tab shows the two matches to our query sequence that were detected by the *blastx* search. Click on the accession number to retrieve the GenBank record for the BLAST hit.

First, we need to find out more about the swallow protein. A good place to start is the UniProtKB/Swiss-Prot database, which is manually curated and has links to many other databases. To access the UniProtKB/Swiss-Prot record for the *D. melanogaster* swallow protein, click on the accession number “**P40688.1**” in the *blastx* hit table (red arrow, Figure 8). This will bring you to the GenBank record for the swallow protein (Figure 9). According to the GenBank record, the locus name (i.e., the UniProtKB accession string) for the swallow protein is SWA\_DROME (blue arrow, Figure 9). A UniProtKB accession string consists of an abbreviated gene name followed by an abbreviated species name. For example, the name SWA\_DROME corresponds to the swallow protein from DROsophila MElanogaster.

**RecName: Full=Protein swallow**

UniProtKB/Swiss-Prot: P40688.1

[Identical Proteins](#) [FASTA](#) [Graphics](#)

Go to:

LOCUS SWA\_DROME 548 aa Linear INV 08-NOV-2023

DEFINITION RecName: Full=Protein swallow.

ACCESSION P40688

VERSION P40688.1

DBSOURCE UniProtKB: locus SWA\_DROME, accession [P40688](#);

class: standard.  
extra accessions:Q9W400  
created: Feb 1, 1995.  
sequence updated: Feb 1, 1995.  
annotation updated: Nov 8, 2023.

xrefs: X56023.1, CAA39500.1, AE014298.5, AAF46160.3, AY069487.1, AAL39632.1, S20806, NP\_511060.2, 3BRL\_C, 3E2B\_C, 6X0R\_A, 6X0R\_B  
xrefs (non-sequence databases): PDBsum:3BRL, PDBsum:3E2B, PDBsum:6X0R, AlphaFoldDB:P40688, BMRB:P40688, SMR:P40688, BioGRID:58067, DIP:DIP-17669N, ELM:P40688, IntAct:P40688, STRING:7227.FBpp0070884, iPTMnet:P40688, PaxDb:7227-FBpp0070884, ABCD:P40688, DNASU:31580, EnsemblMetazoa:FBtr0070922, EnsemblMetazoa:FBpp0070884, EnsemblMetazoa:FBgn0003655,

**Customize view**

**Analyze this sequence**

Run BLAST

Identify Conserved Domains

Highlight Sequence Features

Find in this Sequence

**Protein 3D Structure**

Crystal Structure of LC8 S88E / Swa  
PDB: 3BRL  
Source: Drosophila melanogaster, synthetic construct  
Method: X-ray Diffraction  
Resolution: 1.9 Å

Figure 9. The GenBank record shows the UniProtKB accession string for the *D. melanogaster* swallow protein is SWA\_DROME (blue arrow). Click on the “P40688” link under the DBSOURCE field (red arrow) to navigate to the UniProtKB record.

We can navigate to the UniProtKB record for the *D. melanogaster* swallow protein from the GenBank record directly by clicking on the “P40688” link under the “DBSOURCE” field (red arrow, Figure 9). Alternatively, we can search for the protein record using the accession string “SWA\_DROME” at the [UniProt website](#). The UniProtKB record for SWA\_DROME is also available in the exercise package (file named *UniProtEntry.pdf*).

*Question 6: Based on the UniProtKB entry (Figure 10), what does swallow do? Does your blastx output match the swallow genes from more than one species, and if so, which species? If you want to talk about the D. melanogaster and D. pseudoobscura swallow genes in your own work, whom should you cite as having discovered it? (Hint: check the Publications section of the UniProtKB record.)*

UniProtKB · P40688 · SWA\_DROME

Function | Protein | Protein swallow | Amino acids | 548 (go to sequence)

Names & Taxonomy | Gene | swa | Protein existence | Evidence at protein level

Subcellular Location | Status | UniProtKB reviewed (Swiss-Prot) | Annotation score | 5/5

Phenotypes & Variants | Organism | Drosophila melanogaster (Fruit fly)

PTM/Processing

Expression | Entry | Variant viewer | Feature viewer | Genomic coordinates | Publications | External links | History

Interaction | BLAST | Download | Add | Add a publication | Entry feedback

Structure

Family & Domains

Sequence

Similar Proteins

**Function<sup>1</sup>**

Has a role in localizing bicoid mRNA at the anterior margin of the oocyte during oogenesis, and a poorly characterized role in nuclear divisions in early embryogenesis. [1 Publication](#)

**GO annotations<sup>1</sup>**

Access the complete set of GO annotations on QuickGO [↗](#)

Slimming set:

generic

Figure 10. UniProtKB/Swiss-Prot entry for the swallow protein in *D. melanogaster*.

Now that we know a bit more about the candidate matches to our gene, let's take a closer look at the *blastx* output. To produce an annotation, we need to verify that the query sequence really does contain the *D. melanogaster swallow* gene. In particular, the match should be full-length, including all the coding exons of the gene. If there are exons missing, then this may indicate that the feature is a pseudogene.

From the “**Graphic Summary**” tab of the *blastx* output, we see that there are many alignment blocks distributed across our entire query sequence (Figure 11). To determine if there are parts of the protein that are missing or if there are multiple hits to the same part of the protein, we need to examine the alignment more closely. Go back to the hit table in the “Descriptions” tab and click on the description for the match with the lowest E-value (i.e., “**RecName: Full=Protein swallow [Drosophila melanogaster]**” with E-value 2e-174).

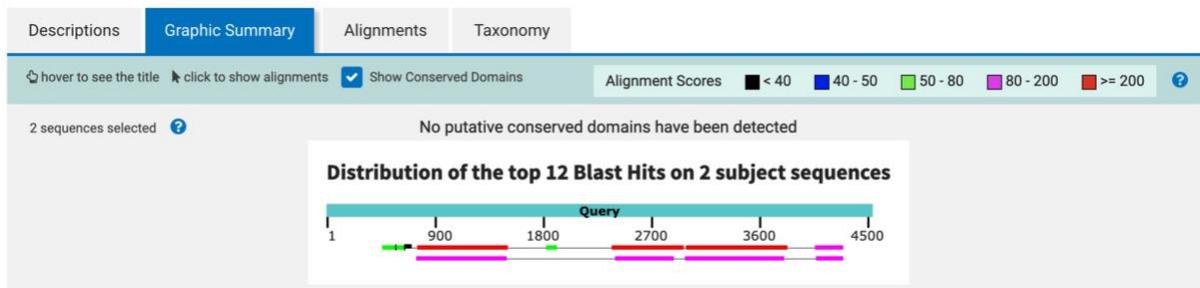


Figure 11. Graphical representation of the alignments to our unknown sequence.

*Question 7: What is the orientation of the swallow gene relative to our query sequence? (Hint: check the frame in your alignment.)*

There is considerable confusion in the collection of *blastx* alignments to the SWA\_DROME protein. As an annotator, your job is to produce order from this chaos.

*Question 8: Look at all the matches to SWA\_DROME in your blastx output. Is the entire protein matched? If not, which residues are missing? Are there any regions of the protein that are aligned to multiple places in our query sequence?*

**Hint:** You may find it helpful to draw a figure of the BLAST results in order to check the coordinates of all the alignment blocks relative to the subject (i.e., the swallow protein) sequence. For example, the first alignment block maps to 94-372 of the swallow protein, so on a map we would draw a block that corresponds to this region of the swallow protein (Figure 12). Use the same strategy to draw the rest of the alignment blocks onto the map.

# BLAST result:

RecName: Full=Protein swallow [Drosophila melanogaster]  
 Sequence ID: [P40688.1](#) Length: 548 Number of Matches: 8

Range 1: 94 to 372 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps	Frame
536 bits(1380)	2e-174	Compositional matrix adjust.	278/279(99%)	279/279(100%)	0/279(0%)	-1
Query 3799	HKLGCEKAPLGSGRSSKAVSYQDIHSAYTKRRFQHVTSKVGQYIAEIQADQKRRNVKFA					3620
Sbjct 94	HKLGCEKAPLGSGRSSKAVSYQDIHSAYTKRRFQHVTSKVGQYIAEIQADQKRRNVKFA					153
Query 3619	GFQRVNSMPESLTPTLQQVYVHDGDFKVDKNCQTHSnsdsnynsnsnnssSFDRLLAEN					3440
Sbjct 154	GFQRVNSMPESLTPTLQQVYVHDGDFKVDKNCQTHSnsdsnynsnsnnssSFDRLLAEN					213
Query 3439	ESLQKINSLRVEAKRLQGFNEYVQERLDRKTDDFVKMKCNFETLRTELSECQQLRRQQ					3260
Sbjct 214	ESLQKINSLRVEAKRLQGFNEYVQERLDRKTDDFVKMKCNFETLRTELSECQQLRRQQ					273
Query 3259	DNSQHFMYHIRSATSAKATQDFLVDTPASGNVLVTPHPLGDLTYNSSKGSIELALLS					3080
Sbjct 274	DNSQHFMYHIRSATSAKATQDFLVDTPASGNVLVTPHPLGDLTYNSSKGSIELALLS					333
Query 3079	VAPSARVAQNPVQVQRAIHPQSLDFSSVSTEADGSGSGK			2963		
Sbjct 334	VAPSARVAQNPVQVQRAIHPQSLDFSSVSTEADGSGSGE			372		

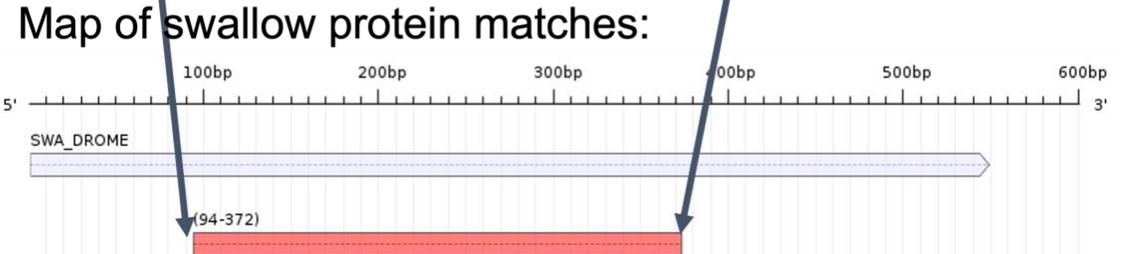


Figure 12. Since the BLAST alignment block spans from residue 94 to residue 372, indicate this region on the map by drawing a line from coordinate about 94 to about 372 (as shown by the red box). Do this for all the alignment blocks relative to the swallow protein to help you answer question 8.

We will continue our investigation with a more detail analysis of the missing residues. Go back to the UniProtKB entry for SWA\_DROME and find the part of the protein that is not represented by any of the BLAST alignments between SWA\_DROME and our sequence, as shown by your analysis in Question 8.

*Question 9: Which amino acids predominate in the missing region? Given that blastx will, by default, mask low-complexity sequence in the query before a search, do you have a reasonable explanation for why this part of the protein is missing? What evidence would support your hypothesis?*

Around amino acid 190 of the protein (subject) sequence, you will see a series of gray letters representing masked residues (Figure 13) in the query. BLAST apparently decided that the protein in the region, rich in serines and asparagines, should be marked as low-complexity. Aligning a residue to a masked base yields a negative score.

RecName: Full=Protein swallow [Drosophila melanogaster]

Sequence ID: [P40688.1](#) Length: 548 Number of Matches: 8

Range 1: 94 to 372 [GenPept](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps	Frame
536 bits(1380)	2e-174	Compositional matrix adjust.	278/279(99%)	279/279(100%)	0/279(0%)	-1
Query 3799		HKLGCEKAPLGSGRSSKAVSYQDIHSAYTKRRFQHVTSKVGQYIAEIQADQKRRNVKFA				3620
Sbjct 94		HKLGCEKAPLGSGRSSKAVSYQDIHSAYTKRRFQHVTSKVGQYIAEIQADQKRRNVKFA				153
Query 3619		GFQRVNSMPESLTPTLQQVYVHDGDFKVDKNCQTHsnssdsnynsnsnnsssfDRLLAEN				3440
Sbjct 154		GFQRVNSMPESLTPTLQQVYVHDGDFKVDKNCQTHSNSDSNYNSNSNNSSSSFDRLLAEN				213
Query 3439		ESLQQKINSLRVEAKRLQGFNEYVQERLDRKTDDFVKMKCNFETLRTTELSECQQKLRQQ				3260
Sbjct 214		ESLQQKINSLRVEAKRLQGFNEYVQERLDRKTDDFVKMKCNFETLRTTELSECQQKLRQQ				273
Query 3259		DNSQHHFMYHIRSATSAKATQTDFLVDTIPASGNVLVTPHPLGDLTYNSSKGSIELALLS				3080
Sbjct 274		DNSQHHFMYHIRSATSAKATQTDFLVDTIPASGNVLVTPHPLGDLTYNSSKGSIELALLS				333
Query 3079		VAPSARVAQNVPVQVQRAIHPQSLDFSSVSTEADGSGSGK		2963		
Sbjct 334		VAPSARVAQNVPVQVQRAIHPQSLDFSSVSTEADGSGSGE		372		

Figure 13. Lowercase gray letters in the *blastx* alignment denote masked low complexity sequences.

*Question 10: Given blastx seems happy enough to include masked residues in its alignments (as shown in the figure above), why didn't it include residues 78-93 of the protein? [Hint: look at the reading frames (specified in the header) of the matches ending at 77 and beginning at 94. What happens if you add negative-scoring residue pairs to the end of an alignment?]*

With careful inspection of the results, you should see that most of the residues of the swallow protein align to two places in the query. We will need to investigate this further in order to fully annotate the query. To get a more detailed graphical representation of all the alignment blocks between the swallow protein and the query sequences, we will go back and perform another *blastx* search. Start by clicking on the “**Edit Search**” button at the top of the *blastx* output to go back to the BLAST input page. Because we are interested specifically in the swallow protein, we will use BLAST to directly align this protein sequence with our query.

Click on the “**Align two or more sequences**” checkbox on the BLAST input page. This will change the BLAST input page so that the “Choose Search Set” section is replaced by the “Enter Subject Sequence” section. As before, click on the “Browse” or “Choose File” button under the “Enter Query Sequence” section and then select the *dmel\_seq2.fasta* file from the exercise package. In the bottom text box, we want to enter the amino acid sequence of the swallow protein. We could retrieve the sequence from the GenBank record, copy the sequence, and then paste it into the text box. However, BLAST will automatically retrieve the protein sequence if we enter the accession number of the swallow protein into the text box. Looking back at the GenBank record for our first result (Figure 8), we see that the accession number for the swallow protein is P40688. Enter “**P40688**” into the “Enter Subject Sequence” text box to indicate that you want to use the swallow protein as the subject sequence in your *blastx* search (Figure 14).

The screenshot shows the NCBI BLASTX search interface. At the top, there are tabs for different BLAST programs: blastn, blastp, **blastx**, tblastn, and tblastx. The main heading is "Align Sequences Translated BLAST: blastx". Below this, there are two main sections: "Enter Query Sequence" and "Enter Subject Sequence".

In the "Enter Query Sequence" section, the text box contains "dmel\_seq2.fasta" and is highlighted with a red box. A red arrow points to the "Browse..." button next to it, which also shows "dmel\_seq2.fasta". Below this, there are fields for "Genetic code" (set to "Standard (1)") and "Job Title". A red arrow points to the "Align two or more sequences" checkbox, which is checked.

In the "Enter Subject Sequence" section, the text box contains "P40688" and is highlighted with a red box. A red arrow points to the text box. Below this, there is a "Browse..." button with "No file selected." next to it.

At the bottom, there is a blue "BLAST" button and a checkbox for "Show results in a new window" which is checked. The text "Search protein sequence using Blastx (search protein subjects using a translated nucleotide query)" is displayed above the checkbox.

Figure 14. Compare the *dmel\_seq2.fasta* sequence (query) against the *D. melanogaster* swallow protein (which has the accession number P40688; subject).

To determine if the low complexity filter is the cause of the missing residues in our original *blastx* alignment, we will turn off the low complexity filter when we run this BLAST search. Click on the “**Algorithm parameters**” header to expand this section and then uncheck the option “**Low complexity regions**” under the “Filters and Masking” section. In addition, we will also change the “Compositional adjustments” field to “**No adjustment**”. The use of a compositionally adjusted scoring matrix will generally produce BLAST results with more accurate E-values and reduce the number of spurious matches when searching a large database. However, this adjustment could remove conserved residues from the alignment when we are comparing only two sequences against each other.

Because our previous BLAST results show the relevant alignments all have E-values below  $1e-5$ , we will also set the “Expect threshold” to “**1e-5**” (Figure 15). In addition, verify that the “Word size” parameter is set to **3**. Click on the “**BLAST**” button after you have changed these settings.

For teaching purposes, the *blastx* comparison of these two sequences is available in the exercise package (*blx\_swallow\_nolow\_dmel\_seq2.txt*).

The screenshot shows the "Algorithm parameters" section of the BLAST interface. A note at the top states: "Note: Parameter values that differ from the default are highlighted in yellow and marked with a diamond sign." The interface is divided into three sections: "General Parameters", "Scoring Parameters", and "Filters and Masking".

- General Parameters:**
  - Max target sequences: 100
  - Expect threshold: 1e-5 (highlighted in yellow, with a callout box: "Expect threshold = 1e-5")
  - Word size: 3 (highlighted in yellow, with a callout box: "Verify word size = 3")
  - Max matches in a query range: 0
- Scoring Parameters:**
  - Matrix: BLOSUM62
  - Gap Costs: Existence: 11 Extension: 1
  - Compositional adjustments: No adjustment (highlighted in yellow, with a callout box: "No compositional adjustment")
- Filters and Masking:**
  - Filter: Low complexity regions (unchecked, highlighted in yellow, with a callout box: "Turn off filter for low complexity regions")
  - Mask: Mask for lookup table only (unchecked), Mask lower case letters (unchecked)

Figure 15. Under the “Algorithm parameters” section of the BLAST interface, turn off the low complexity filter and compositional adjustments, set the Expect threshold to  $1e-5$ , and verify that the Word size is set to 3.

Click on the “**Alignments**” tab to examine the *blastx* alignment. We found that the original alignment block that spans from 1-77 of the swallow protein has been extended to 1-91 in the new *blastx* result (Figure 16).

**Original *blastx* result:**

Range 7: 1 to 77 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#) [First Match](#)

Score	Expect	Method	Identities	Positives	Gaps	Frame
168 bits(425)	2e-42	Compositional matrix adjust.	77/77(100%)	77/77(100%)	0/77(0%)	-3
Query 4259	MSLQDESFPDDELFDQLNNLSSSGARNTWFAEHHKPAVFERDTAPFLEICYADPDFDADG					4080
Sbjct 1	MSLQDESFPDDELFDQLNNLSSSGARNTWFAEHHKPAVFERDTAPFLEICYADPDFDADG					60
Query 4079	DVANKSAKTCVSDPVGR 4029					
Sbjct 61	DVANKSAKTCVSDPVGR 77					

***blastx* result with no compositional adjustments or low complexity filter:**

Range 4: 1 to 91 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#) [First Match](#)

Score	Expect	Identities	Positives	Gaps	Frame	
194 bits(494)	6e-57	91/91(100%)	91/91(100%)	0/91(0%)	-3	
Query 4259	MSLQDESFPDDELFDQLNNLSSSGARNTWFAEHHKPAVFERDTAPFLEICYADPDFDADG					4080
Sbjct 1	MSLQDESFPDDELFDQLNNLSSSGARNTWFAEHHKPAVFERDTAPFLEICYADPDFDADG					60
Query 4079	DVANKSAKTCVSDPVGRDQEDEDYDEDVDG 3987					
Sbjct 61	DVANKSAKTCVSDPVGRDQEDEDYDEDVDG 91					

Figure 16. Turning off the BLAST filters extends the alignment block. (Top) The alignment from the original *blastx* search result covers residues 1-77 of the swallow protein. (Bottom) After turning off the BLAST filters, the *blastx* alignment covers residues 1-91 of the swallow protein.

Similarly, the first alignment block in the original *blastx* search result covers residues 94-372 of the swallow protein. After turning off the filters, the beginning of the *blastx* alignment block has been extended to cover residues 91-372 of the swallow protein (Figure 17).

Range 1: 91 to 372 [GenPept](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Positives	Gaps	Frame	
561 bits(1445)	0.0	281/282(99%)	282/282(100%)	0/282(0%)	-1	
Query 3808	GDDHKLGC EKAPLGSGRSSKAVSYQDIHSAYTKRRFQHVTSKVGQYIAEIQADQKRRNV					3629
Sbjct 91	GDDHKLGC EKAPLGSGRSSKAVSYQDIHSAYTKRRFQHVTSKVGQYIAEIQADQKRRNV					150
Query 3628	KFAGFQRVNSMPESLTPTLQQVYVHDGDFKVDKNCQTHSNSDSNYSNSNNSSSSFDRLL					3449
Sbjct 151	KFAGFQRVNSMPESLTPTLQQVYVHDGDFKVDKNCQTHSNSDSNYSNSNNSSSSFDRLL					210
Query 3448	AENESLQQKINSLRVEAKRLQGFNEYVQERLDRKTDDFVKMKCNFETLRTELSECQKLR					3269
Sbjct 211	AENESLQQKINSLRVEAKRLQGFNEYVQERLDRKTDDFVKMKCNFETLRTELSECQKLR					270
Query 3268	RQQDNSQHFFMYHIRSATS AKATQTDFLVDTIPASGNVLVTPHPLGDLTYNSSKGSIELA					3089
Sbjct 271	RQQDNSQHFFMYHIRSATS AKATQTDFLVDTIPASGNVLVTPHPLGDLTYNSSKGSIELA					330
Query 3088	LLSVAPSARVAQNPVQVQRAIHPQSLDFSSVSTEADGSGSGK 2963					
Sbjct 331	LLSVAPSARVAQNPVQVQRAIHPQSLDFSSVSTEADGSGSGE 372					

Figure 17. Turning off the BLAST filters extend the beginning of the first alignment block by three residues. (The alignment begins at residue 91 of the swallow protein instead of residue 94.)

Now that we have accounted for all the missing amino acids in the swallow protein, we should get an overall view of how all the alignment blocks are organized. Click on the “**Dot Plot**” tab in the *blastx* output. This will open a section that contains a dot matrix comparison of the query and subject sequences. The *x*-axis of the dot matrix corresponds to the query sequence and the *y*-axis corresponds to the swallow protein sequence. The lines within the dot matrix correspond to the positions of the alignment blocks.

*Question 11: Looking at these results, how many “swallow” like features are found in the query, which (if any) seems most likely to be the true swallow gene? What might the other matches be, and what biological mechanisms might have produced them?*

## Further exploration Using *blastn*

To make further progress in determining the correct annotation for our sequence, we will try to obtain additional evidence at the nucleotide level, specifically looking at *Drosophila* mRNAs.

In order to detect homology at the nucleotide level, we will use nucleotide BLAST (*blastn*) instead of *blastx*. As was the case for the *blastx* search, we must make some decisions before we can perform the search. For example, we could compare our query sequence against the GenBank non-redundant nucleotide database (also known as nr/nt) or one of the EST databases. Because ESTs are pretty noisy and do not come with easily accessible annotations, we will use the nucleotide collection (nr/nt) database in this exercise.

To do the *blastn* search, go back to the [BLAST page](#) and click on the “**Nucleotide BLAST**” image under the “Web BLAST” section. As before, click on the “Browse” or “Choose File” button under the “Enter Query Sequence” section, and then select the *dmel\_seq2.fasta* file from the exercise package. Select “**Nucleotide collection (nr/nt)**” in the “Database” field. To limit the scope of the search to mRNA sequences, add the search term “**biomol\_mrna[properties]**” to the “Entrez Query” field. Lastly, because *blastn* is more sensitive than *megablast*, we will select “**Somewhat similar sequences (blastn)**” under the “Program Selection” field (Figure 18). Click on the “**BLAST**” button and then wait for your results. Alternatively, the *blastn* output is available in the exercise package (*bln\_nt\_dmel\_seq2.txt*).

*Question 12: Had your query contained a repetitive element such as a transposon, what would have happened had you forgotten to repeat-mask the query sequence before running the BLAST search?*

**Standard Nucleotide BLAST**

blastn   blastp   blastx   tblastn   tblastx

BLASTN programs search nucleotide databases using a nucleotide query. [more...](#)

### Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) [?](#) [Clear](#)

**dmel\_seq2.fasta**

Query subrange [?](#)

From

To

Or, upload file  dmel\_seq2.fasta [?](#)

Job Title

Enter a descriptive title for your BLAST search [?](#)

Align two or more sequences [?](#)

### Choose Search Set

Database  Standard databases (nr etc.):  rRNA/ITS databases  Genomic + transcript databases  Betacoronavirus

**New**  Experimental databases [< Try experimental taxonomic nt databases](#) [Download](#)

For more info see [What are taxonomic nt databases?](#)

Nucleotide collection (nr/nt) **nr/nt database** [?](#)

Organism Optional   exclude [Add organism](#)

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown [?](#)

Exclude Optional  Models (XM/XP)  Uncultured/environmental sample sequences

Limit to Optional  Sequences from type material **biomol\_mrna[properties]**

Entrez Query Optional  [YouTube](#) [Create custom database](#)

Enter an Entrez query to limit search [?](#)

### Program Selection

Optimize for

Highly similar sequences (megablast)

More dissimilar sequences (discontiguous megablast)

**blastn**  Somewhat similar sequences (blastn)

Choose a BLAST algorithm [?](#)

[BLAST](#) Search database nt using **Blastn** (Optimize for somewhat similar sequences)

Show results in a new window

Figure 18. Configure the *blastn* search of *dmel\_seq2.fasta* (query) against the nr/nt database (subject).

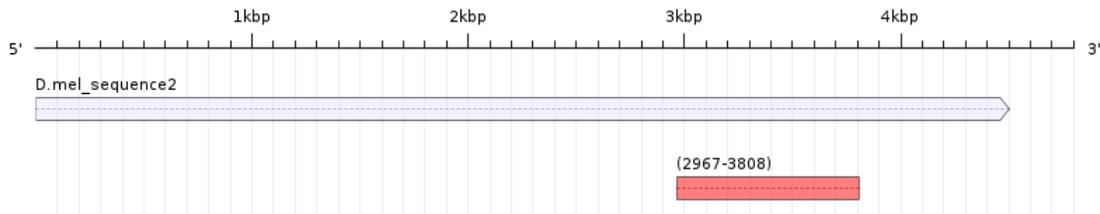
The sequences in the GenBank nr/nt database come from many sources, including genomic contigs from whole-genome sequencing projects and mRNAs/cDNAs. A particular useful class of mRNA entries is the NCBI RefSeqs, these sequences come from a curated database of full-length mRNAs from various genes. You can find out more information about the RefSeq database through the “Using RefSeq” section of the [RefSeq home page](#). You can easily recognize RefSeq matches in the BLAST output because their accession numbers always begin with two letters (NM or XM for mRNA records and NP or XP for protein records), followed by an underscore and a unique ID (Figure 19). See Table 1 in [Chapter 18 of the NCBI Handbook](#) for the list of accession prefixes used by the RefSeq database.

Descriptions		Graphic Summary	Alignments	Taxonomy				
<b>Sequences producing significant alignments</b>								
Download <span>▼</span> Select columns <span>▼</span> Show 100 <span>▼</span> <span>?</span>								
<input checked="" type="checkbox"/> select all 89 sequences selected <span style="float: right;"> <a href="#">GenBank</a> <a href="#">Graphics</a> <a href="#">Distance tree of results</a> <a href="#">MSA Viewer</a> </span>								
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/> <a href="#">Drosophila melanogaster AT14971 full insert cDNA</a>	<a href="#">Drosophila me...</a>	2149	3912	62%	0.0	94.48%	1388	<a href="#">BT032879.1</a>
<input checked="" type="checkbox"/> <a href="#">Drosophila melanogaster swallow (swa). mRNA</a>	<a href="#">Drosophila me...</a>	1519	5439	76%	0.0	100.00%	2022	<a href="#">NM_078505.4</a>
<input checked="" type="checkbox"/> <a href="#">Drosophila melanogaster LD21771 full length cDNA</a>	<a href="#">Drosophila me...</a>	1519	5441	76%	0.0	100.00%	2095	<a href="#">AY069487.1</a>
<input checked="" type="checkbox"/> <a href="#">PREDICTED: Drosophila simulans protein swallow (LOC6725279). mRNA</a>	<a href="#">Drosophila si...</a>	1291	4983	78%	0.0	94.18%	2094	<a href="#">XM_016172909.3</a>
<input checked="" type="checkbox"/> <a href="#">PREDICTED: Drosophila mauritiana protein swallow (LOC117147092). mRNA</a>	<a href="#">Drosophila ma...</a>	1281	4834	74%	0.0	93.71%	1993	<a href="#">XM_033313856.1</a>
<input checked="" type="checkbox"/> <a href="#">PREDICTED: Drosophila sechellia protein swallow (LOC6612274). mRNA</a>	<a href="#">Drosophila se...</a>	1277	4140	65%	0.0	93.59%	1777	<a href="#">XM_002036748.2</a>
<input checked="" type="checkbox"/> <a href="#">PREDICTED: Drosophila santomea protein swallow (LOC120456458). mRNA</a>	<a href="#">Drosophila sa...</a>	885	3135	73%	0.0	83.37%	1999	<a href="#">XM_039643305.2</a>
<input checked="" type="checkbox"/> <a href="#">PREDICTED: Drosophila teissieri protein swallow (LOC122624856). mRNA</a>	<a href="#">Drosophila tej...</a>	876	3271	76%	0.0	83.14%	2034	<a href="#">XM_043804592.1</a>
<input checked="" type="checkbox"/> <a href="#">PREDICTED: Drosophila yakuba protein swallow (LOC6524028). mRNA</a>	<a href="#">Drosophila ya...</a>	863	3077	74%	0.0	82.78%	2027	<a href="#">XM_002099864.4</a>

Figure 19. The accession numbers for the RefSeq mRNA records begin with either NM or XM, followed by an underscore and a unique ID number (e.g., NM\_078505.4).

Click on the description for the *D. melanogaster* swallow RefSeq mRNA (NM\_078505.4) in order to navigate to the corresponding *blastn* alignment.

*Question 13: What is the best RefSeq match to the query? How good is the match to what you think is the true swallow gene? (Hint: Use the figure below to create a map showing all the blastn hits to the query sequence as you did for Question 8.) Based on the blastn alignment, how many exons does the gene have, and roughly where do the introns occur?*



*Question 14: How well does the RefSeq match the other part of the query? Can you see regions that were not aligned at the protein level? Why might this be?*

The main question at this point is what is the other set of matches outside the region that we believe to be the *D. melanogaster swallow* gene? Do these matches reveal a real gene or pseudogene? Pseudogenes are rare in *Drosophila* compared to mammals, but they are not unknown.

There are two signals that strongly suggest a putative match to a gene might be a pseudogene: internal stop codons that produce a truncated protein and gaps in the alignment that causes frame shift mutations. The *blastx* alignment uses an asterisk (\*) to represent a stop codon in the alignment. Gaps in the coding region of a *blastn* alignment would result in a frame-shift if the size of the gap were indivisible by three. Frame shift mutations may introduce internal stop codons that prematurely terminate the translation of the protein.

*Question 15: Keeping in mind the exon boundaries you inferred above, could you find evidence of premature stop codons and/or frame shift-inducing gaps that would cause you to diagnose a pseudogene adjacent to the swallow gene? Describe any evidence you find.*

## Summary

*Question 16: Based on all the evidence gathered in this exercise, how would you annotate the query sequence? What uncertainties remain? Compose a short (a few sentences) paragraph that you could add to an annotation database summarizing your findings.*