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A Complete Guide to Tardigrade Isolation and Phylogenetic Characterization for Undergraduate Students

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ABSTRACT

Here we present a complete guide for the isolation and phylogenetic characterizations of tardigrades suitable for the undergraduate level. The protocol provides guidelines for isolating tardigrades from moss and lichens along with simple steps for isolating their DNA and performing PCR amplification of ribosomal genes. Sequenced DNA is first processed with the free online DNA Subway platform. DNA sequences are then used to collect orthologs from the NCBI database for phylogenetic analysis using the free bioinformatics software MEGA. Following this simple protocol will allow students to explore the diversity of tardigrade, explore their various morphological features using microscopy and to characterize tardigrades using DNA sequencing and phylogenetic analysis.

IMAGE ATTRIBUTION

Martin Alberg, IRSC

SAFETY WARNINGS

Use caution when using cutting utensils during lichen and moss collection.
Use gloves when handling DNA gels stained with SYBR safe or other DNA staining reagents.
Use proper eye protection when viewing DNA gels under UV light.

Keywords: Tardigrades, Eutardigrade, Tardigrada, DNA, DNA Subway, Phylogenetics, BLAST, MEGA, Undergraduate research, DNA extraction, Lichen, Moss, Multiple Sequence Alignment, Undergraduate

Collecting Lichens and Moss for Tardigrade Isolation

1 Gather materials required to collect Tardigrades from environmental samples.

- Lab notebook
- Petri dish or shallow clear container
- Razor blade or pocket knife
- Pen or marker
- GPS enabled device

2 Find a lichen or moss growing on any substrate, such as trees, mulch beds, decaying wood, shaded rocks or brick walls. Lichen and moss can grow in a variety of different habitats, but in general, mosses like areas that are damp and lichens like porous surfaces. Take pictures of the sample site and record relevant observations like date, temperature, rain/moisture levels and height of the sample.

3 Holding a petri dish under the moss or lichen you have found, begin scraping the sample off into

the dish. Collect approximately enough sample to cover a quarter to half of a petri dish. Close the petri dish for storage. Try to leave some moss or lichen behind for it to regrow.

4 Record sample data on your petri dish with permanent marker. Write down your name, create a sample number (Moss 1/Lichen 1, etc.), and the date you collected the sample. In a lab notebook, record a description of the location, the type of substrate from which the sample was collected, GPS coordinates of the sample location (drop a pin at your location using your phone's "maps" app, click on the pin and it should give you GPS information), local weather, and description of the moss or lichen sample (color, texture, general appearance). Write down any additional notes that may be relevant. Record all this information in a lab notebook to keep your labeling consistent and to have a backup when the paper bag eventually gets thrown out.

5 Store each sample in a closed petri dish in a refrigerator (4°C) until ready to saturate and isolate Tardigrades.

Finding Tardigrades in Lichens and Moss

6 Soak your sample in the petri dish with deionized (DI) water. Use enough water to allow the sample to be submerged, but you do not need to cover the entire plate with water. We have found that the longer it sits in water, the more Tardigrades start waking up. So, you may want to soak your sample in the morning to look through in the afternoon. One strategy is to soak the sample in water, gently stir then incubate for 5-10 min at 37°C. Observe the petri dish for Tardigrades as described below, then re-incubate for another 10 min at 37°C and look for more Tardigrades. Repeat this process as more Tardigrades become visible. This may cause a selection bias in which Tardigrades are isolated. However, it seems to expedite the rehydration process. Another approach is to simply soak the samples at room temperature and check periodically hourly, or in 24-hour internals.

7 When you are ready to look for Tardigrades, use the 4X objective lens (40X total magnification) on a light microscope to look around the petri dish. Move the dish slowly as you scan the dish in all directions. If you move too fast, the water will slosh around making it very difficult. Pay close attention to the areas near the moss or lichen sample, these are the areas you are most likely to find the Tardigrades. Look for movement and for motion under fragments of moss or lichens. Switch to the 10X (100X total magnification) objective and observe closely. Record the types of organisms observed. Many rotifers, nematodes, moss mites, amoebas, paramecium and other microorganisms will be visible with patient observation.

In your notebook, write down the sample number for the moss or lichen sample you are analyzing, all the information you gathered about that sample and how many Tardigrades you found as you find them.

8 As you find Tardigrades, you will want to give them a number (e.g., Moss 1, Tardigrade 1; Lichen 4, Tardigrade 18). Again, keeping track of information and being able to relate it back to the sample it came from is extremely important, so try to organize this all in a way that is consistent.

When you find a Tardigrade, take several pictures so that it can be identified using a Tardigrade identification key. Even if the Tardigrade cannot be identified by morphological features, simply recording the total number of Tardigrades per sample is valuable data.

9 You now need to collect the single Tardigrade using a Pasture pipette. Collect the Tardigrades one at a time, taking care to label the microcentrifuge tube they go in to match your records. Collecting multiple Tardigrades per tube will provide difficulties with the DNA sequencing steps. To collect the individual Tardigrades, while looking through the microscope at 40X, bring the pipette into the field of view. Then, slowly move the tip of the pipette close to the Tardigrade. Slowly pull up water, bring up as little as possible - less than 100 µl if possible. Be careful not to pull water all the way up into the bulb of the pipette, which will make it hard to transfer the Tardigrade to the collection microcentrifuge tube.

10 Squirt the water and the Tardigrade out of the pipette and into a 1.5 mL microcentrifuge tube. Label the top of the microcentrifuge tube with whatever number you gave that individual Tardigrade. The best way to label it is something referring to what number moss/lichen it came from and which number Tardigrade it is, and the date. Good example labels would be: M2T4 (moss sample 2, Tardigrade 4) or L3T2 (lichen sample 3, Tardigrade 2).

11 The isolated Tardigrades can be safety stored in the refrigerator at 4 °C for later identification and to extract their DNA. Store tubes in a labeled rack at 4 °C until ready to proceed. The samples should be stable for an extended period, weeks to months, or longer.

Isolation of Tardigrade DNA

12 Guidelines: Make sure all materials are sterilized ahead of time.

Materials:
1.5 mL microcentrifuge tubes

50% Ethanol

Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10-977-015**

Invitrogen UltraPure DNase/RNase-Free Distilled Water (ThermoFisher)

Equipment	
Fisherbrand™ Pellet Pestles™	NAME
Pestles with microcentrifuge tubes	TYPE
Thermo Fisher Scientific	BRAND
12-141-367	SKU
https://www.fishersci.com/shop/products	LINK
Resuspend protein and DNA pellets or grind soft tissue in microcentrifuge tubes	SPECIFICATIONS

Pestles

GeneJET Genomic DNA Purification Kit **ThermoFisher Scientific Catalog #K0721**
GeneJET Genomic DNA Purification Kit (ThermoFisher)

13 Isolate one Tardigrade in a sterile 1.5 mL microcentrifuge tube and grind it up with a sterile pestle. Make sure there is not too much water in the collection tube, less than 100 µl is best. If excess water needs to be removed, take care to not accidently remove the Tardigrade with the excess water.

14 Add 180 µL of Digestion Solution and 20 µL of Proteinase K Solution. Mix thoroughly by vortexing or pipetting the volume up and down to obtain a homogeneous suspension. The sample can be ground in the tube using a microcentrifuge pestle. If a pestle is used, carefully spin the pestle in the solution to make sure all residual Tardigrade tissue is removed from the pestle and remains in the solution.

15 Incubate the sample at 56 °C until the Tardigrade is completely lysed. Since the sample is small (< 5 mg) it is recommended to incubate for at least one hour. During incubation, vortex the centrifuge tube occasionally.

16 Add 20 µL of RNase A Solution. Mix by vortexing then incubate for 00:10:00 at room temperature. 10m

17 Add 200 µL of Lysis Solution. Mix thoroughly by vortexing for 00:00:15 until a homogeneous mixture is obtained. 15s

18 Add 400 µL of 50% ethanol and mix by vortexing or pipetting up and down.

19 Transfer the prepared lysate to a GeneJET Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 00:01:00 at 6000 x g. Discard the collection tube containing the flow-through solution. Place the GeneJET Genomic DNA Purification Column into a new 2 mL collection tube. 1m

20 Add 500 µL of Wash Buffer I (with ethanol added). Centrifuge for 00:01:00 at 8000 x g. Discard the flow-through and place the purification column back into the collection tube. 1m

21 Add 500 µL of Wash Buffer II (with ethanol added) to the GeneJET Genomic DNA Purification Column. Centrifuge for 00:03:00 at maximum speed ≥ 12000 x g. (Optional: If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 00:01:00 at maximum speed. Discard the collection tube containing the flow-through solution and transfer the GeneJET Genomic DNA Purification Column to a sterile 1.5 mL microcentrifuge tube.) 4m

22 Add 30 µL of sterile Invitrogen UltraPure DNase/RNase-Free Distilled Water to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 00:02:00 at room temperature and centrifuge for 00:01:00 at 8000 x g. 3m

23 Discard the purification column and use the purified DNA immediately in downstream applications. Otherwise, store the DNA at -20 °C.

PCR Amplification and Gel Electrophoresis 45m

24 PCR amplification of the isolated Tardigrade DNA can be completed using a variety of primers.

Primers for the 18S and 28S rRNA genes, ITS2 and COI are available from this reference (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0192210)
For our analysis we primarily used the 18S rRNA gene primers.

25 PCR Master Mix: prepare the following master mix for PCR amplification. Multiply the volume needed of each reagent by the total number of samples to be amplified, plus 2 for pipetting error.

PCR Master Mix:

- Promega Green GoTaq: 12.5 µL
- Forward primer ([M] 10 micromolar (µM)): 0.5 µL
- Reverse primer ([M] 10 micromolar (µM)): 0.5 µL
- Sterile water: 12.5 µL

26 Setting up PCR reactions 12m

- After preparing enough master mix for all of your samples, dispense 24 µL of the master mix into 0.2 µL PCR tubes.
- Then, add 1 µL of one sample of Tardigrade DNA to only one tube.
- Place tubes in thermocycle and run following these parameters

PCR Program Steps:

1. 95 °C for 00:01:00
2. 95 °C for 00:01:00
3. 60 °C for 00:01:30
4. 72 °C for 00:01:30
5. Repeat steps 2-4; 35 times
6. 72 °C for 00:07:00

27 Gel electrophoreses and DNA Sequencing preparation 45m

- In a 250 mL Erlenmeyer flask, prepare a 1% agarose gel using 1X TAE ([M] 40 millimolar (mM) Tris-acetate and [M] 1 millimolar (mM) EDTA at pH 8.3). Example: 0.4 g agarose and 40 mL of 1X TAE. Include a SYBR Safe dye at the appropriate volume (4 µL SYBR safe for 40 mL gel)
- Microwave to completely dissolve agarose. Stop periodically to carefully stir to prevent solution from bubbling over.
- Load 10-15 µl of PCR product directly from PCR tube (no loading dye required).
- Use appropriate ladder.
- Run gel for 00:45:00 at 100 volts
- View gels under UV light, take images and record notes in lab book. Document the band intensity and any non-specific amplification (second bands). Also, record if there is any notable smearing or other indications of degradation. A single bright band should be visible around 1300 bp. Light or weak amplified products may still be sequenced.
- For DNA sequencing, prepare DNA following guidelines for your DNA sequencing center. From our work using the 18S rRNA gene primers, PCR products that showed strong bands on gels were diluted 1:1 with sterile water and PCR products that showed faint bands were not diluted prior to sending for sequencing. Sequence each sample with a forward and reverse primer, separately.

Using DNA Subway for DNA Sequence Clean-up

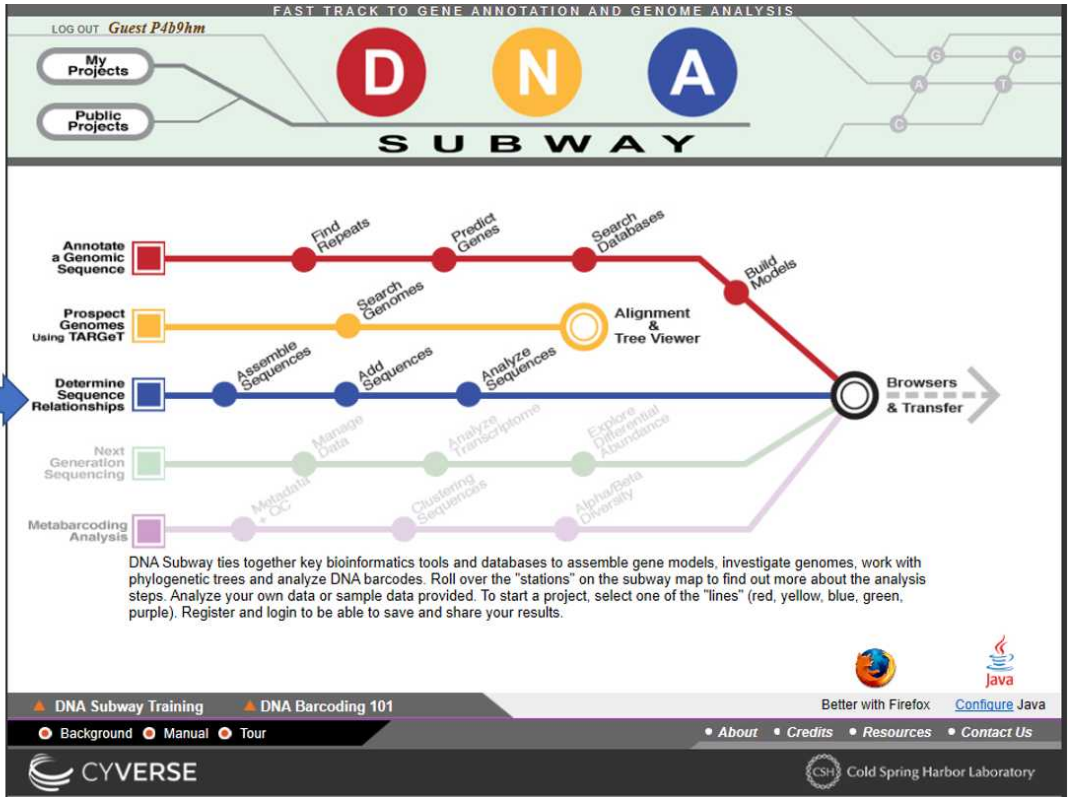
28 Analysis of the sequencing quality and merging forward and reverse sequences can be completed using the Blue Station: Determine Sequence Relationships.

Overall, DNA subway allows you to:

- Trim sequence files: remove any poorly sequenced regions that show up as an "N" or otherwise ambiguous.
- Align the forward and reverse sequences to make a consensus sequence
- BLASTn to search for related sequences at GenBank within DNA Subway, or generate a FASTA file for BLASTn searches on your own at NCBI.

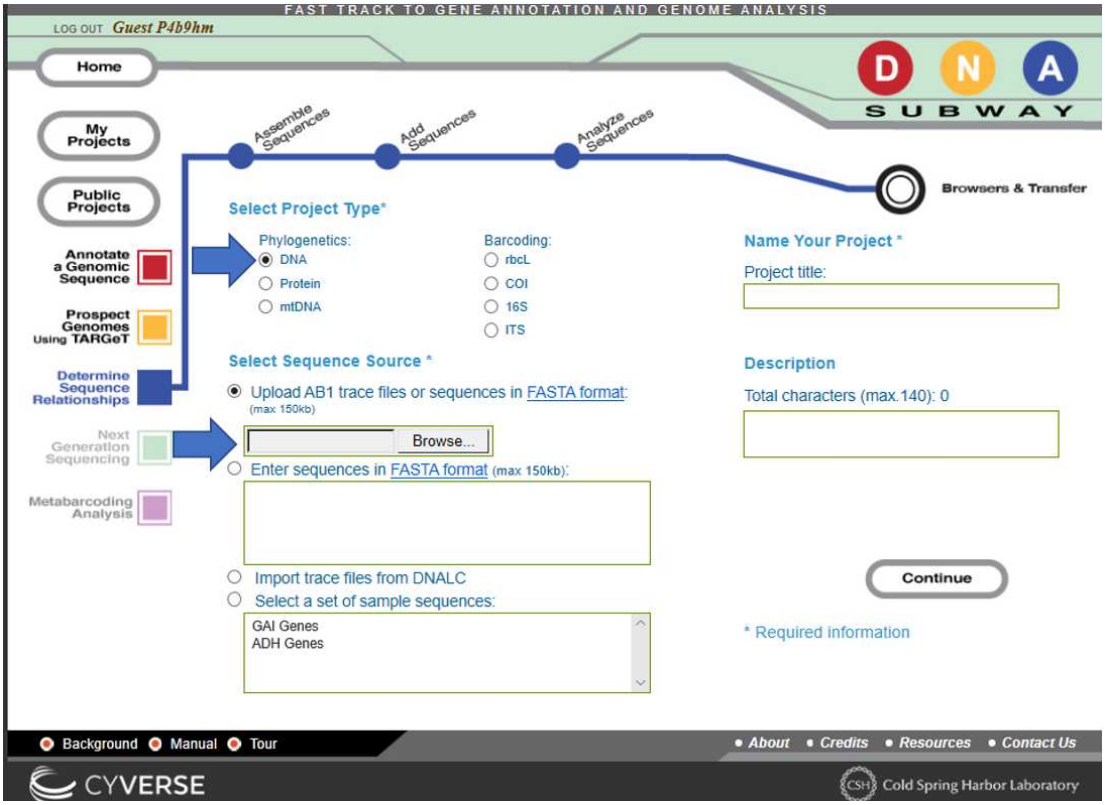
29 Importing DNA sequences into DNA Subway

Log in to DNA Subway as a Guest: https://dnasubway.cyverse.org/
Click on the Blue Route (the blue box)



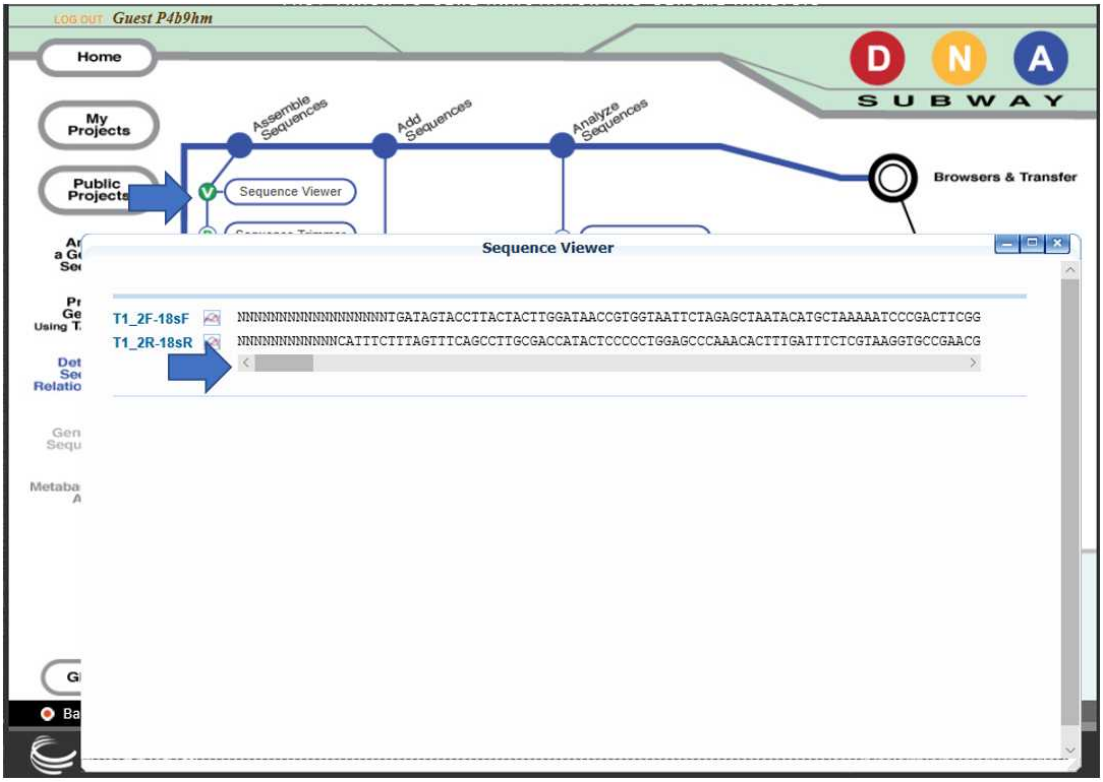
30 Select the Phylogenetics DNA option

Click on browse to find your two matching AB1 files from your folder. Select both of the files to be uploaded. These should be the forward and reverse DNA sequences for a sample.



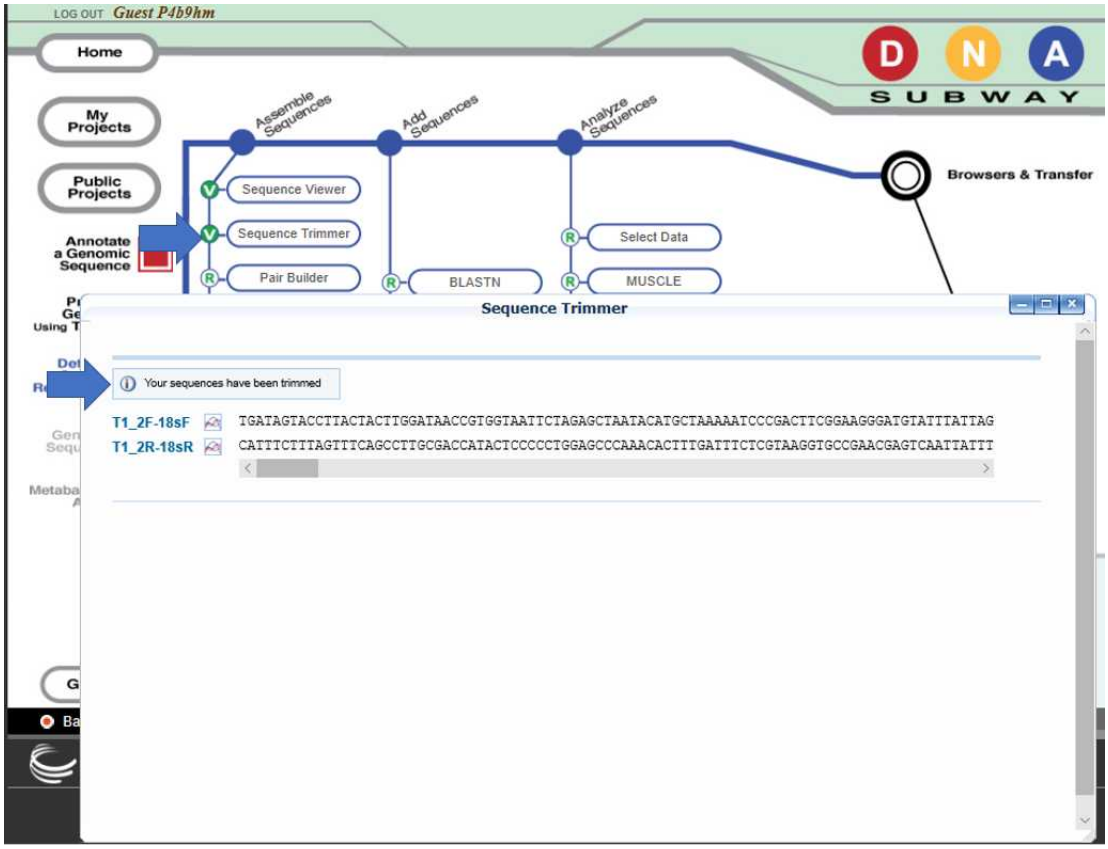
31 Click Sequence Viewer

- Scroll left to right along your sequences
- Notice the “Ns” these are bases that were not sequenced well. We will remove them in the next step.



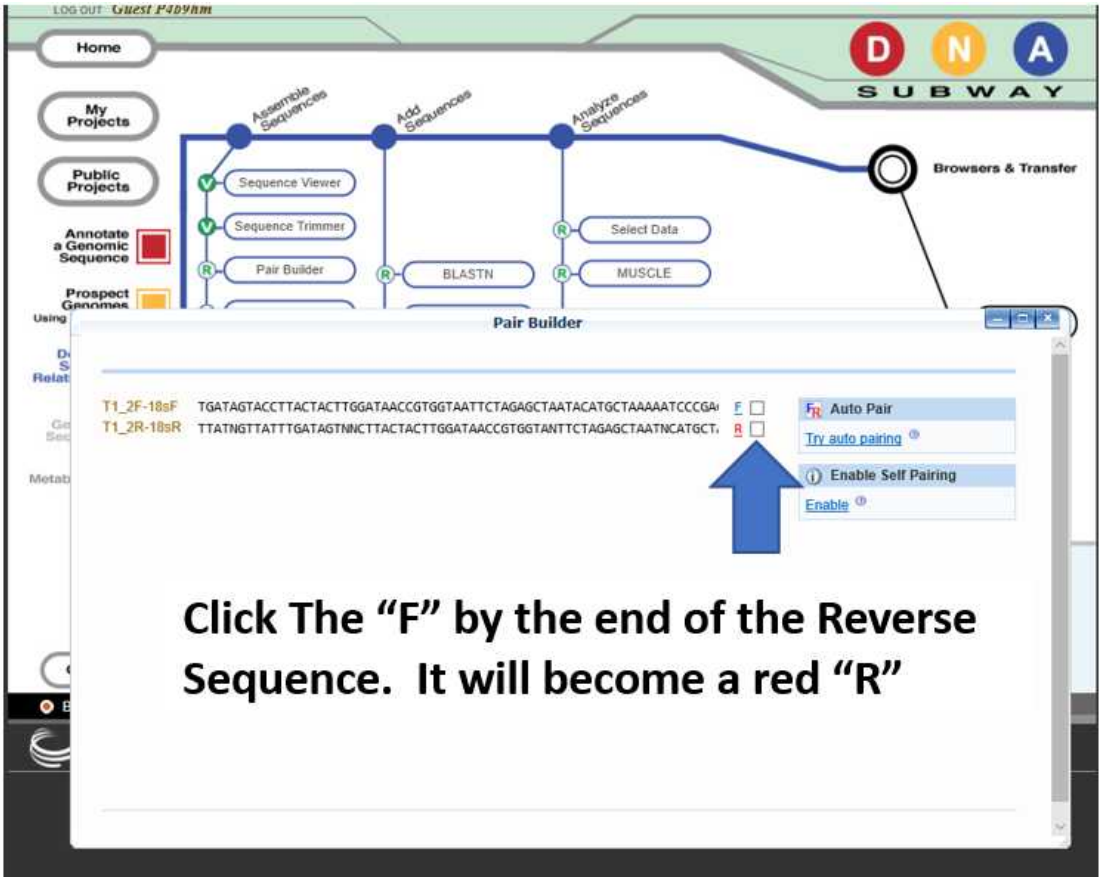
32 Click Sequence Trimmer

- Scroll left to right along your sequences
- Notice the “Ns” have been removed

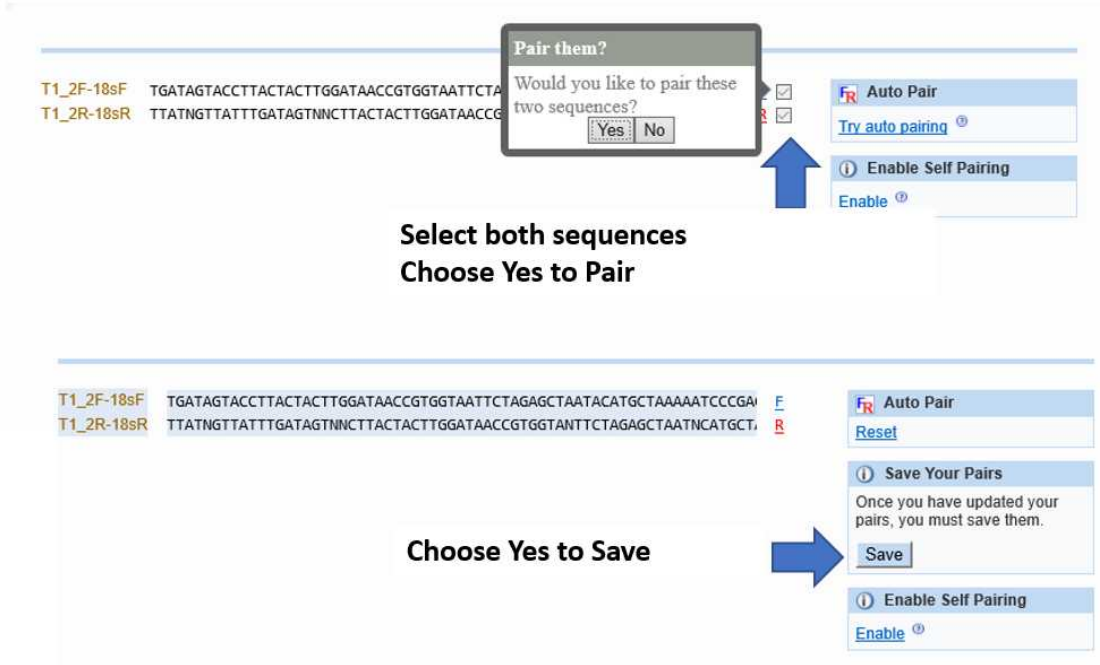


33 Click Pair Builder

- We need to pair the forward and reverse.
- But, we need to reverse compliment the reverse sequence so it aligns.

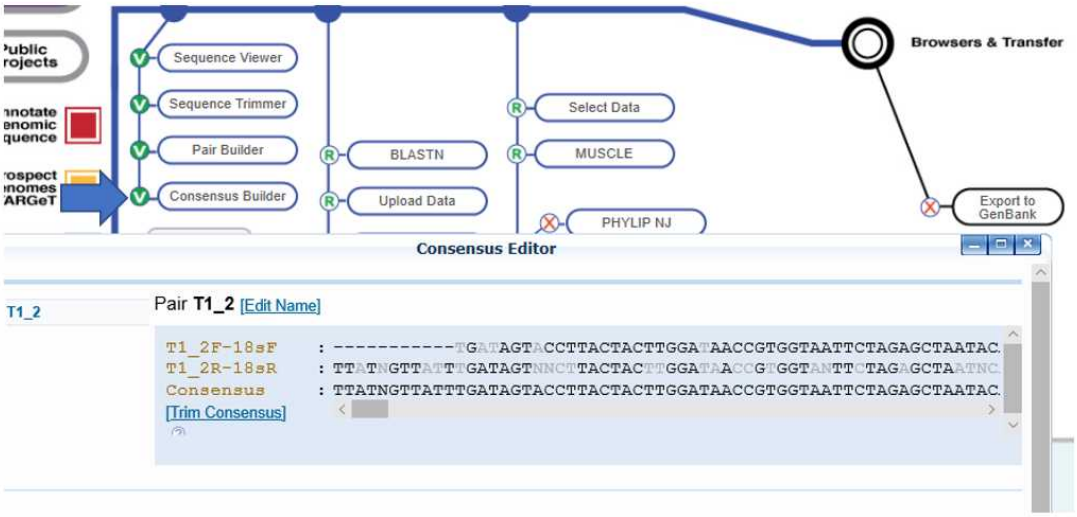


34 Pair and Save your sequences. First, mark each box at the end of the forward and reverse sequences. Choose "Yes" to pair them. Then, click on "Save" to save your pairs.



35 Click Consensus Builder

- Scroll across and see your Forward and Reverse sequences aligned, and the consensus sequence on the bottom.
- Notice how the consensus uses the two sequences to generate one sequence that is more complete.

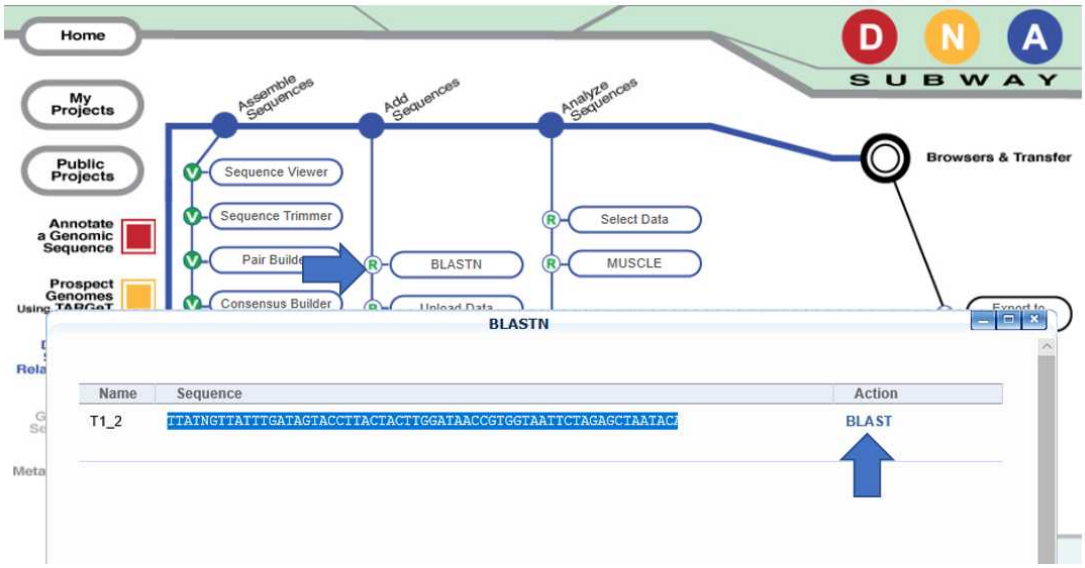


36 Click BLASTN

You can then select the consensus sequence in the pop up box and copy and paste it to use in BLASTN on your own (recommended), or run BLASTN in DNA Subway by clicking BLASTN. Save your sequence to your electronic records for BLAST searching and phylogenetic analysis. Save sequence in FASTA format by giving your sequence a name that relates to the sample the DNA sequence came from.

Example of FASTA format is:

>Tardiagrade_Sample_1
TTATNGTTATTTGATAGTACCTTACTACTTGGATAAC...

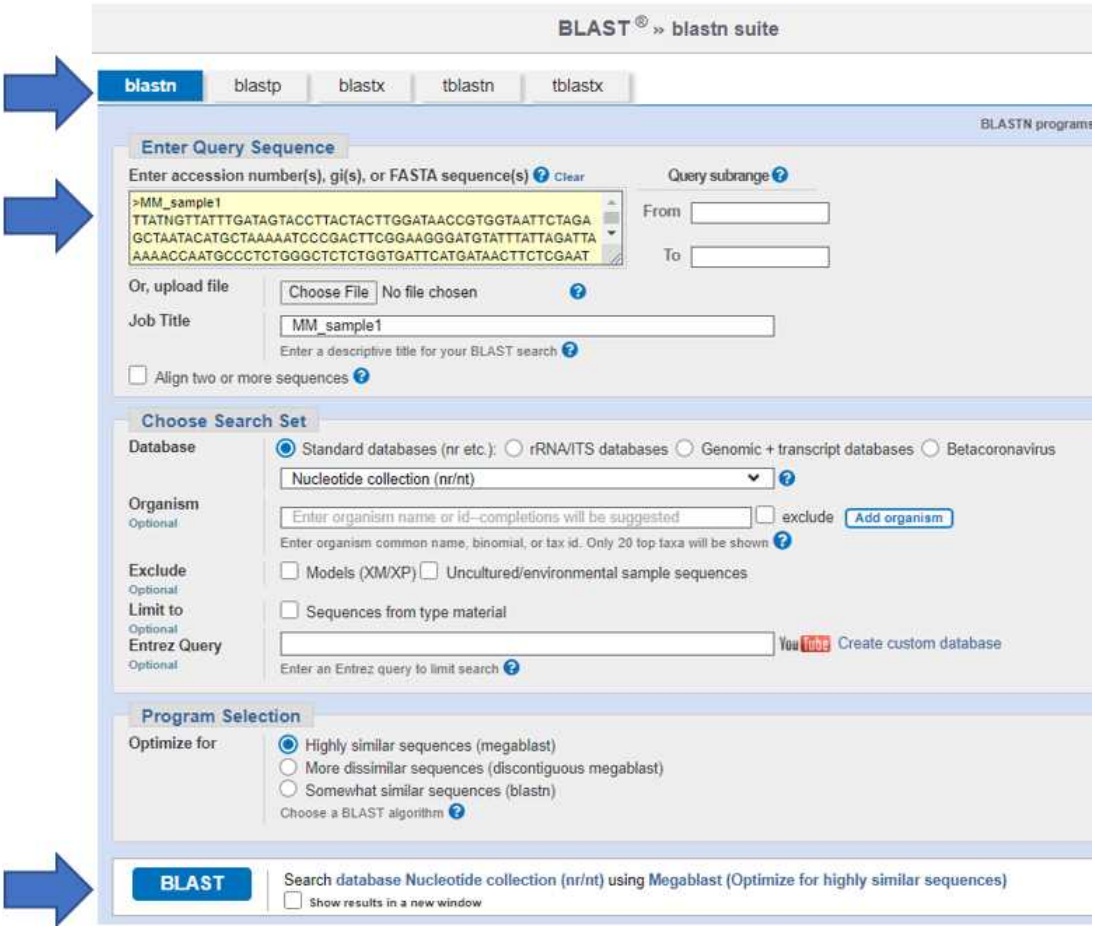


Using NCBI BLASTN to collect sequences from closely relat...

37 NCBI BLASTN tool is used to search for close matches to your Tardigrade DNA sequences. Following the BLASTN search, matches, or "hits" will be displayed. You will want to collect matches that have high query coverage (>85%). When you collect these sequences from NCBI, you will want to record the sequence accession number, the Tardigrade's scientific name or taxonomic description, the location and year the sample was collected, if there is any associated journal article and any other information of interest. Save the sequence for the top matches in your electronic lab book. Make sure to keep the accession number with each corresponding sequence.

38 Running a BLASTN search.

Go to BLASTN at NCBI. Paste a single DNA sequence into the "Enter Query Sequence Box". Scroll down and click "BLAST."

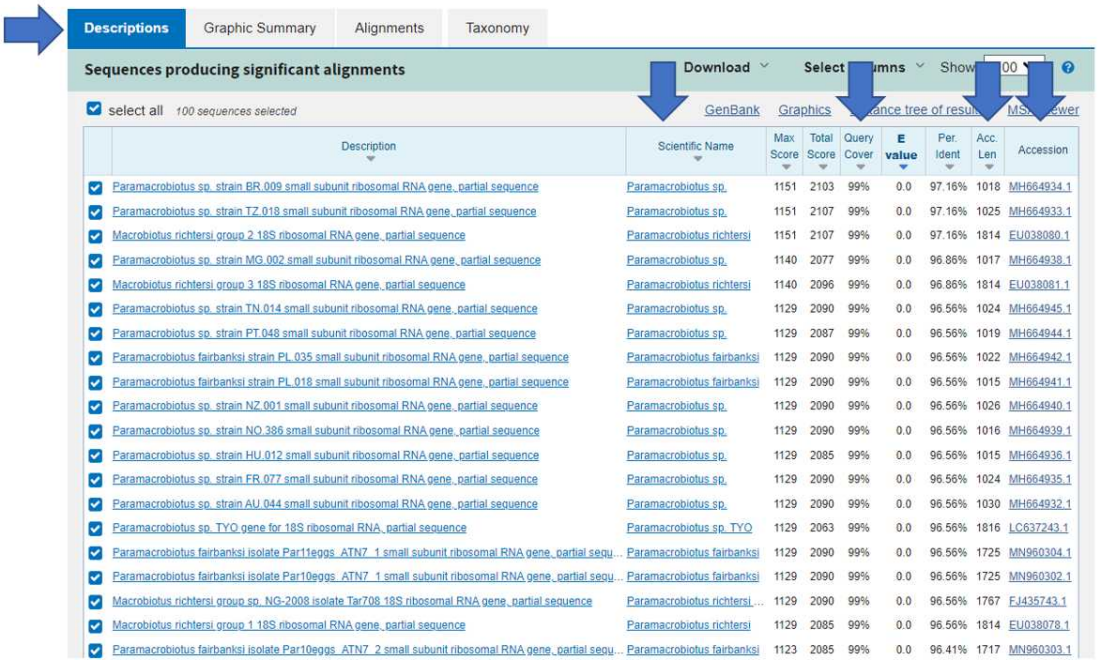


39 Once the results load, scroll down and view the results listed under the descriptions tab. You will want to choose 3-5 sequences to collect for phylogenetic analysis.

Here are some guidelines to help you choose which sequences to collect. Look for matches that:

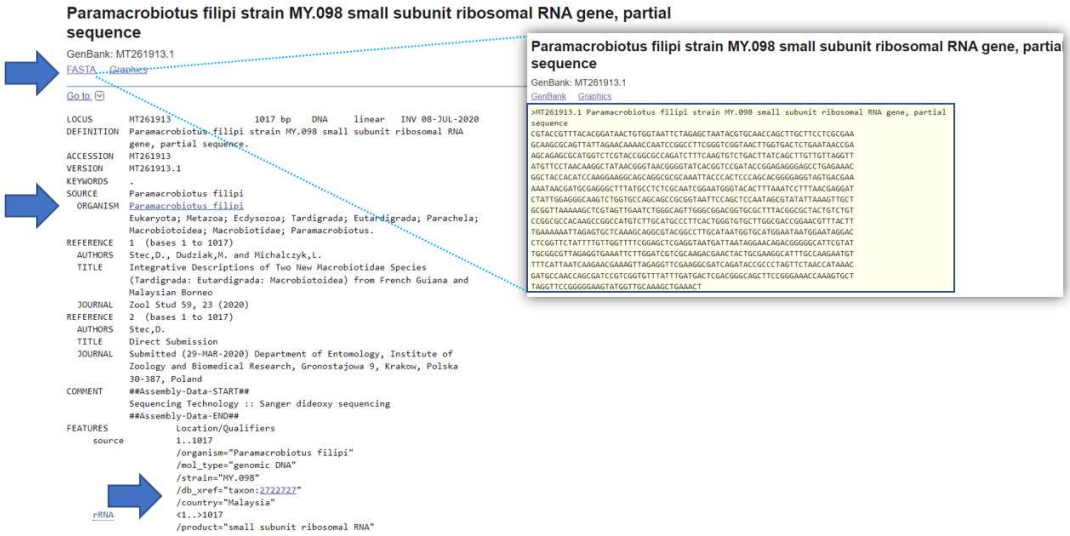
- Are nearly as long as your sequence. See column labeled Acc. Len (accession length).
- Have high query coverage
- Have distinct accession numbers. For example sequences with accession numbers MH66434.1 and MH66433.1 are likely very similar, have same predicted scientific name and will not likely add to our phylogenetic analysis.
- Have different predicted scientific names. From the image below, it would be good to choose a representative sample from the *Paramacrobiotus* sp, *Paramacrobiotus richtersi* and *Paramacrobiotus fairbanksi*.

See next step for directions for collecting the sequence.



40 Collect sequences in FASTA format. Once you know which sequences from the blast results you would like to collect, click on the accession number to access the GenBank Nucleotide page for that sequence. Record important metadata about the sequence, like the taxonomic classification, any associated journal articles and information about the sample (like data and geographic location the sample was collected).

To collect the sequence, click on "FASTA" in the top right of the page. Then, copy and paste the entire sequence, including the ">sample name" line, as shown highlighted in yellow below.



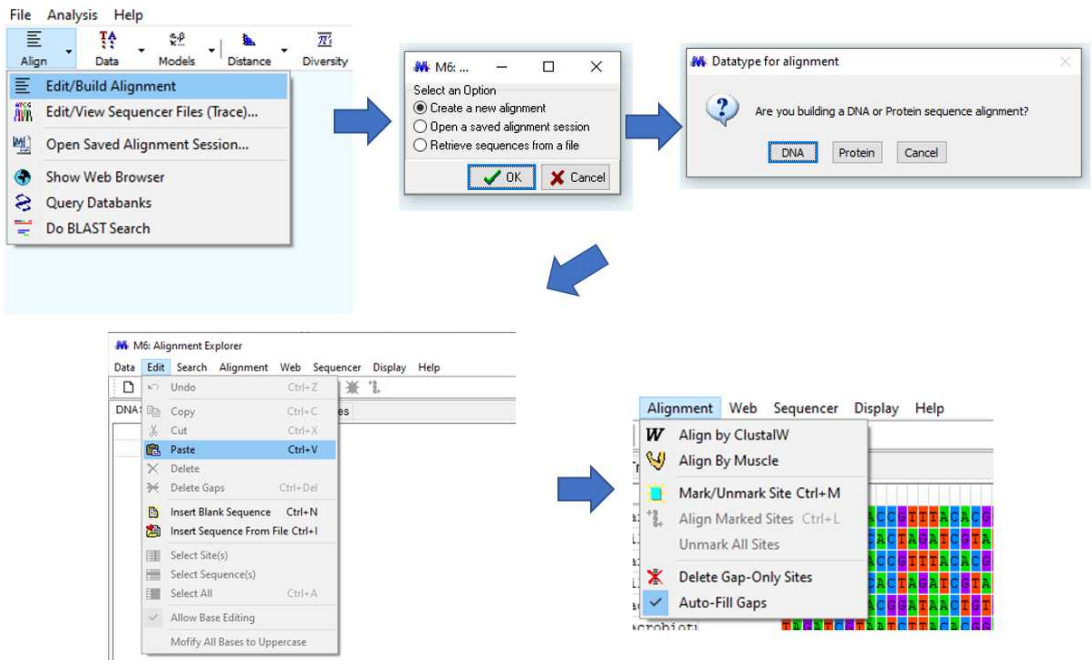
Phylogenetic Analysis using MEGA

41 The free bioinformatics software MEGA can be used to complete the phylogenetic analysis of the DNA sequences prepared in DNA Subway and the sequences collected from the BLAST results. We recommend MEGA version 7 to allow easy editing of the tree in PowerPoint.

The general workflow is to generate a multiple sequence alignment of all of the DNA sequences and construct a phylogenetic tree.

42 Perform a **multiple sequence alignment** in MEGA:

1. Before you start, prepare all of your DNA sequences by copy and pasting each sequence on a text file in notepad. Make sure each sequence is in FASTA format.
2. Start the MEGA software.
3. From the home page, click on "**Align**" found in upper right hand corner.
4. Then, select "**Edit/Build**" from the drop down menu.
5. Choose "**Create a new alignment**" from the dialogue box that opens.
6. On the next pop-up box, select "**DNA**"
7. An Alignment Explorer window will open.
8. At the top menu, click on "**Edit**" then "**Paste**"
9. All of your sequences should now be available.
10. At the top menu, click "**Alignment**" then choose either ClustalW or Muscle to align the DNA sequences. Use default settings.
11. Once alignment is completed, scroll left to right and make any observations of a sequence that appears significantly shorter. If there is one, remove that sequence and replace with a longer one from that same species if possible.



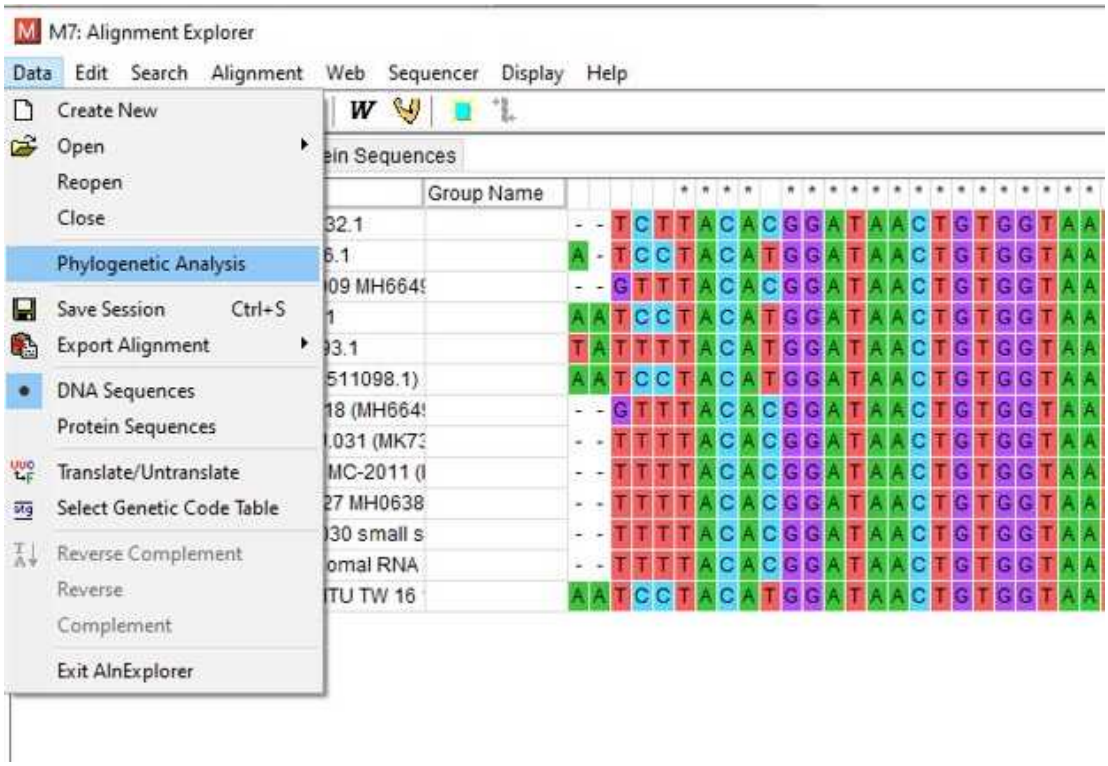
Steps for completing multiple sequence alignment of DNA sequences in MEGA

43

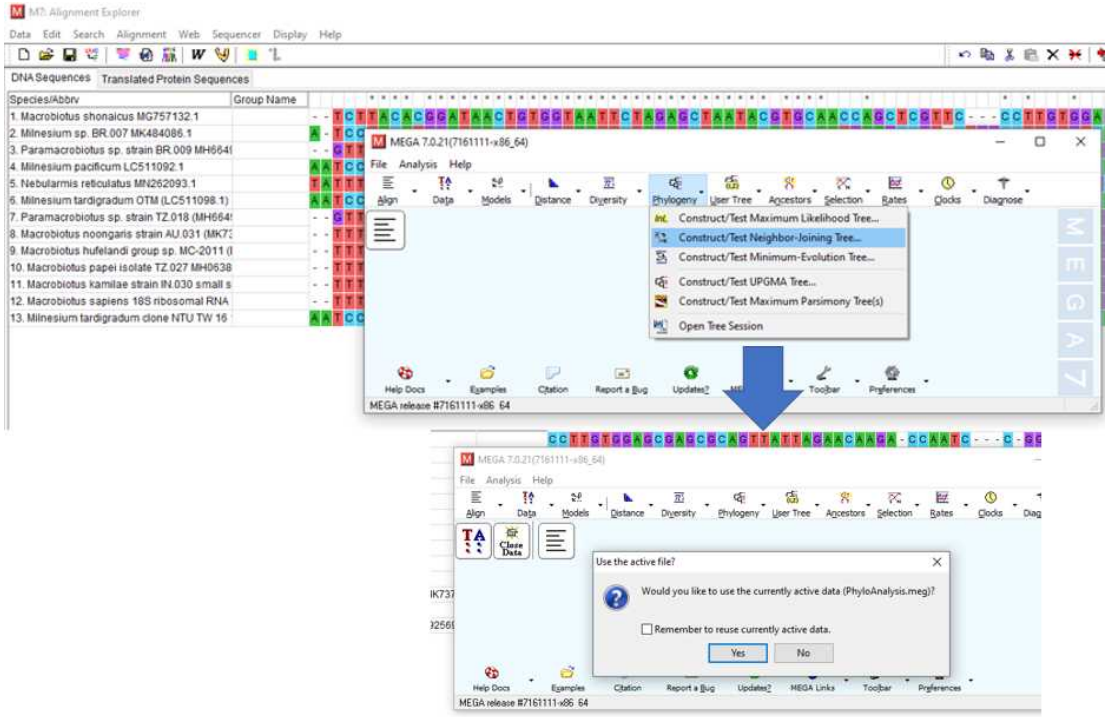
Perform **phylogenetic analysis** in MEGA:

Construct the Phylogenetic Tree

- After the multiple sequence alignment is completed, click on "**Data**" then "**Phylogenetic Analysis**". On the pop up box, click "**No**" when asked if the data is for protein-coding nucleotides (when using 18S rRNA gene or ITS primers).

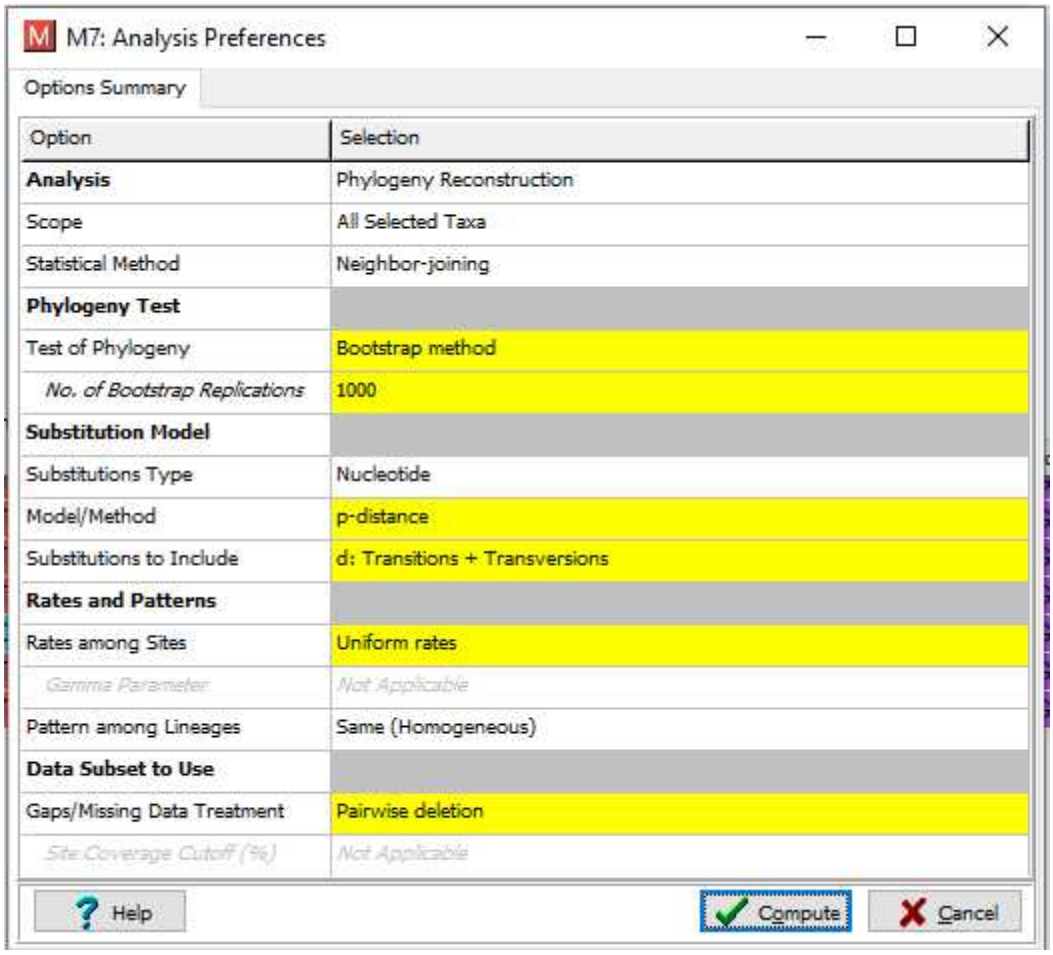


- Next, navigate back to the main MEGA window and select "**Phylogeny**" then "**Construct/Test Neighbor-Joining Tree...**".
- A pop up box will ask if you want to use the currently active data. Choose "Yes"

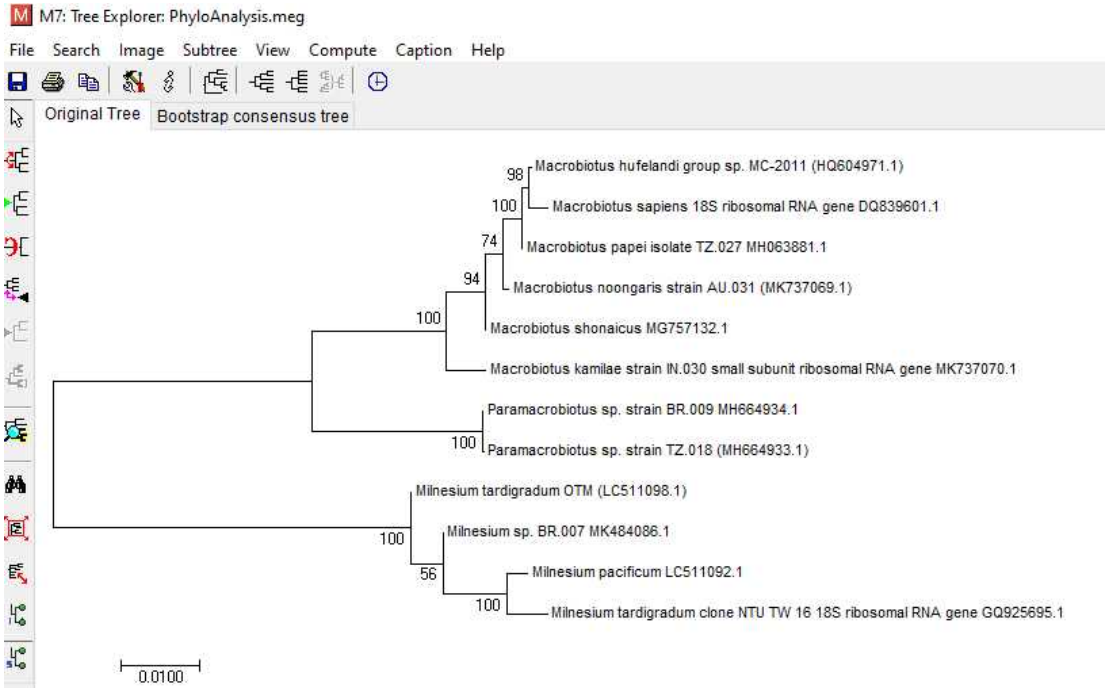


- An, "Options Summary" box will appear. Under Test of Phylogeny, choose Bootstrap method. For "**No. of Boostrap Replications**" input 1000.

- Leave all other default settings.
- Click on "Compute". The tree will be constructed.



The tree will be generated and displayed:

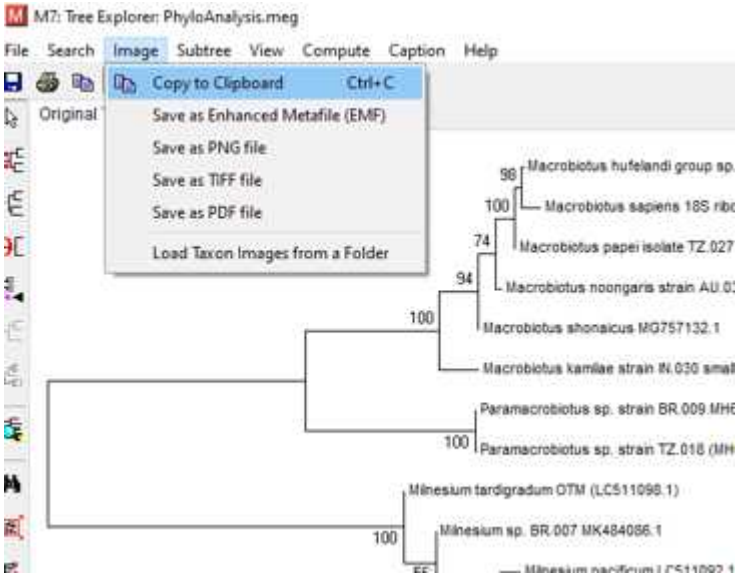


Review the Tree. Consider these questions to help you evaluate the quality of the tree constructed.

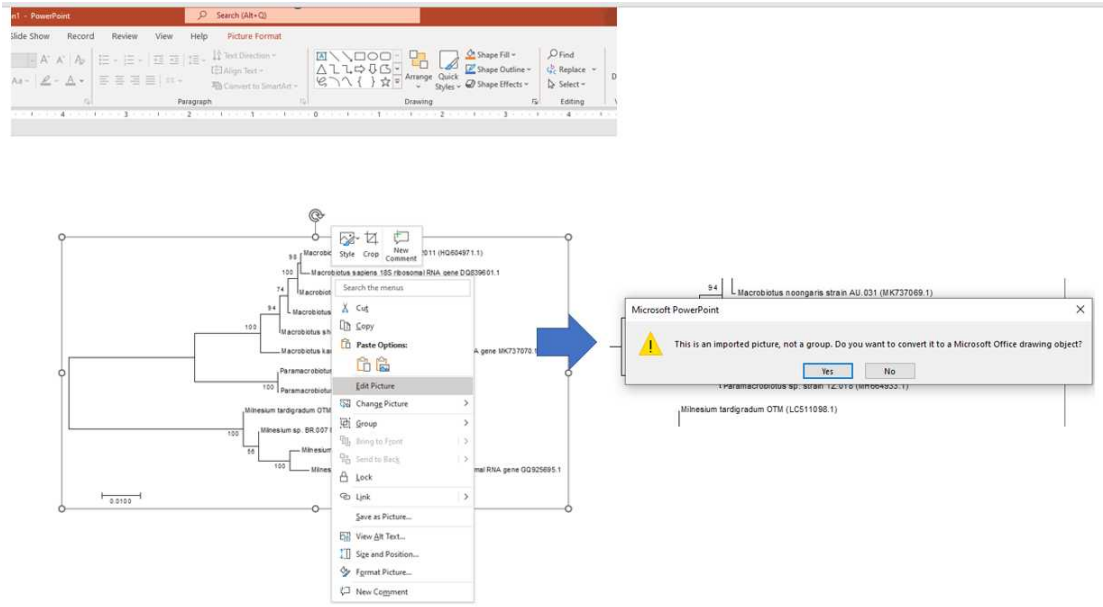
- Do your samples group with species you would have predicted based on your BLASTN results of each sample?
- Are there any of your samples that are not in a clade (group) with sequences collected from NCBI? If so, take your sample and re-blast to collect more sequences to add. You will have to re-run the multiple sequence alignment and make a new tree.
- Are several of the NCBI sequences grouped closely together, without any horizontal lines (connected by vertical line that is basically straight up and down? If so, some of these sequences are redundant and could be removed to clean up the tree.

Basic Editing of the Phylogenetic Tree

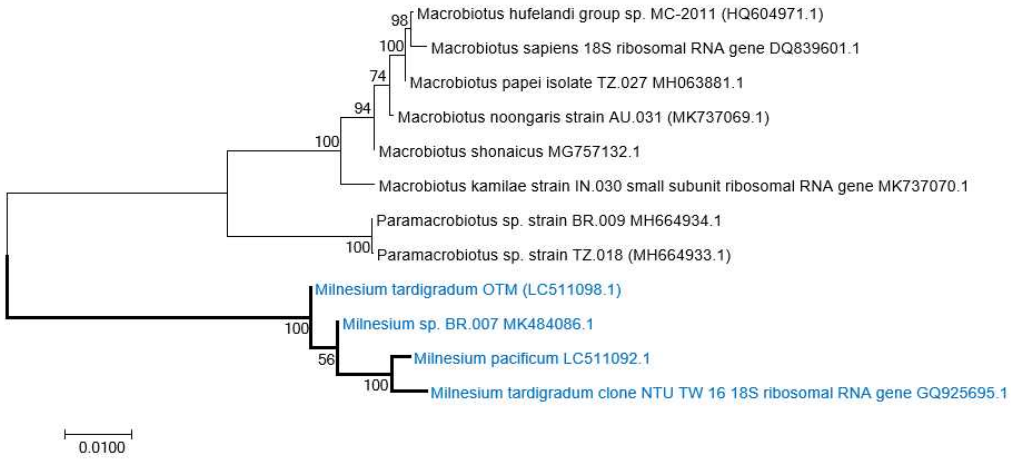
- The tree can be edited to change the names and colors in MEGA.
- It can be easier to export the image to PowerPoint and convert it to a line drawing to complete the final edits of the tree to prepare a clean and easy to read version for presentation.
- With the final tree open in MEGA, click on "Image" then "Copy to Clipboard."



- Open PowerPoint and paste the tree onto a blank slide.
- Right click on the tree and choose "**Edit Picture**." A dialogue box will open confirming the change to convert the image to a Microsoft Office drawing object. Select "Yes."



- The entire tree can now be edited using the PowerPoint edit features. For example, the names of the taxa on the tree can be changed, the font colors, and the weight of the lines can be easily edited.



Tardigrade phylogenetic tree that has been edited. Notice the bottom half of the tree has had the weight of the lines increased and the font changed to blue. Further editing to the remainder of the tree could still be completed, left undone for comparison of edited versus non-edited.