Project Goal:

The main goal of this project is to use *rbcL* gene sequences to study phylogeny of green plants. We are going to gather DNA sequences of *rbcL* gene from Genbank, and compare them to build a phylogenetic tree.

Learning Objectives:

By the end of this project, SWAT:

- Recognize the availability of vast pool of molecular data at the <u>NCBI Genbank website</u> (<u>http://www.ncbi.nlm.nih.gov/genbank/</u>).
- 2. Learn mutations among DNA sequences.
- **3.** Reconstruct tree using freely available software such as ClustalX and MEGA.
- **4.** Understand the meaning of ancestral vs. recent species, clade, and interpret evolutionary relationships among species.

Requirements:

A. Internet access for data mining.

- B. Installation of free software
 - ClustalX (download from http://www.clustal.org/download/current/ by choosing clustalx-2.1-win.msi) for windows and clustalx-2.1-win.msi) for MAC. This software allows you to align DNA or protein sequences.
 - 2. MEGA5 (download from http://www.megasoftware.net/mega.php for windows and http://www.megasoftware.net/mega.php for MAC)

Getting Started:

SECTION 1

- 1. Google "Genbank and click on the link (<u>GenBank Home National Center for Biotechnology Information</u>: http://www.ncbi.nlm.nih.gov/genbank/)
- 2. Use the dropdown next to the word "GenBank" to change from defaulted "Nucleotide" and select "Gene"
 - <u>Type</u> "rbcl" in the entry box to the right of dropdown and <u>search</u>
- 3. Select the link: **rbcL** RuBisCO large subunit [Chlamydomonas reinhardtii] (<u>http://www.ncbi.nlm.nih.gov/gene/2717040</u>)
 - Click on "Genomic regions, transcripts, and products" in the table on contents.
 - Select "FASTA".

Your new screen should look like this.

Chlamydomonas reinhardtii chloroplast, complete genome
NCBI Reference Sequence: NC_005353.1
GenBank Graphics
GenBank Graphics >gil 41179002:cl24130-122275 Chlamydomonas reinhardtii chloroplast, complete genome AAGTAACTGCGTAAGACGACCGACATATACCTAAGGCCCTTTCTATGCTCGACTGATAGACCAGTAC ATAAATTGCTAGTTACATTATTTTTATATCTAAATATATAT
ATTCGAATTTGATACTATTGACAAACTTTAATTTTTATTTTCATGATGTTTATGTGAATAGCATAAACA
AAAGTTATCTTTGTTTAAATTTGCCTGTGCTTTATAAATACCTCATCGATGTGCCAGAAAAATAAAATTAAGTTCT
TTTTTATTAGAATTTATCTTTATGTATTATATTT

- 4. <u>Copy</u> the sequence of the Chlamydomonas.
 - <u>Paste</u> into a NotePad file(PC) or Texteditor(Mac)
 - To find Notepad go to Start menu, All programs then click Accessories you should see Notepad
- 5. After pasting into *NotePad*, leave the prompt sign">" and <u>delete</u> text before the DNA sequence, <u>Replace</u> deleted text with "Algae"

Your NotePad should appear as shown below.

🔄 Untitled - Notepad
File Edit Format View Help
>Algae
AAGTAAACTGCGTAAGACGACCGACATATACCTAAAGGCCCTTTCTATGCTCGACTGATAAGACAAGTAC
ATAAATTTGCTAGTTTACATTATTTTTATTTCTAAATATATAT
CAACAATTTTTAAATTATATTTCCGGACAGATTATTTTAGGATCGTCAAAAGAAGTTACATTTATTATA
TAAATGGTTCCACAAACAGAAACTAAAGCAGGTGCTGGATTCAAAGCCGGTGTAAAAGACTACCGTTTAA
CATACTACACCTGATTACGTAGTAAGAGATACTGATATTTTAGCTGCATTCCGTATGACTCCACAACT
AGGTGTTCCACCTGAAGAATGTGGTGCTGCTGTAGCTGCTGAATCTTCAACAGGTACATGGACTACAGTA
TGGACTGACGGTTTAACAAGTCTTGACCGTTACAAAGGTCGTTGTTACGATATCGAACCAGTTCCGGGTG
AAGACAACCAATACATTGCTTACGTAGCTTACCCAATCGACTTATTCGAAGAAGGTTCAGTAACTAAC
GTTCACTTCTATTGTAGGTAACGTATTCGGTTTCAAAGCTTTACGTGCTCTACGTCTTGAAGACCTTCGT
ATTCCACCTGCTTACGTTAAAACATTCGTAGGTCCTCCACACGGTATTCAGGTAGAACGTGACAAATTAA
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TTAGCTATCTACTGTCGTGACAACGGTCTTCTTCTACACATCCACCGTGCTATGCACGCGGTTATTGACC
GTCAACGTAACCACGGTATTCACTTCCGTGTTCTTGCTAAAGCTCTTCGTATGTCTGGTGGTGACCACCT
TCACTCTGGTACTGTTGTAGGTAAACTAGAAGGTGAACGTGAAGTTACTCTAGGTTTCGTAGACTTAATG
CGTGATGACTACGTTGAAAAAGACCGTAGCCGTGGTATTTACTTCACTCAAGACTGGTGTTCAATGCCAG
GTGTTATGCCAGTTGCTTCAGGCGGTATTCACGTATGGCACATGCCAGCTTTAGTTGAAATCTTCGGTGA
TGACGCATGTCTTCAGTTCGGTGGTGGTACTCTAGGTCACCCTTGGGGTAACGCTCCAGGTGCTGCAGCT
AACCGTGTAGCTCTTGAAGCTTGTACTCAAGCTCGTAACGAAGGTCGTGACCTTGCTCGTGAAGGTGGCG
ACGTAATTCGTTCAGCTTGTAAATGGTCTCCAGAACTTGCTGCTGCATGTGAAGTTTGGAAAGAAA
ATTCGAATTTGATACTATTGACAAACTTTAATTTTTATTTTTCATGATGTTTATGTGAATAGCATAAACA
TCGTTTTTATTTTTATGGTGTTTAGGTTAAATACCTAAACATCATTTTACATTTTTAAAATTAAGTTCT
AAAGTTATCTTTIGTTTAAATTTGCCTGTGCTTTATAAATTACGATGTGCCAGAAAAATAAAATCTTAGC
TTTTTATTATAGAATTTATCTTTATGTATTATATTT

Now save this file as "tree.fasta". In the drop down choose "All files". We Now have the *rbcL* gene sequence for one species. Now we will get similar sequences from other plant species.

SECTION 2

- 1. Go to NCBI BLAST website http://blast.ncbi.nlm.nih.gov/
 - a. Select "nucleotide blast" under the Basic Blast section.
 - b. It will bring you to this window.



<u>Copy</u> the DNA sequence from *NotePad*

- b. 1st arrow. "Enter accession numbers......FASTA sequences"- <u>Paste</u> your algae sequence in the area indicated by the
- c. 2nd arrow. Type moss in the organism box. In the dropdown choose "Mosses(taxid:3208)"
- d. 3rd arrow. Choose somewhat similar sequences(blastn)
- e. 4th arrow. Click "Show results in a new windown". Click BLAST

*Wait until program is finished, a new Tab should appear like the one below.

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Eunaria hygrometrica ribulose-1,5-bisphosphate carboxylase large subunit-like (rboL) gene, partial sequence; of	roplast gene for chloroplast product		1310 1310 72% 0.0 82% AF22081
Physicomitrella patens chloroplast rbol, gene for ribulose-1,5-bisphosphate carboxylase/oxygenase			1305 1305 75% 0.0 81% <u>X74158.</u>
Eunaria hygrometrica ribulose-bisphosphate carboxylase large subunit (rboL) gene, chloroplast gene encoding o	oroplast protein, partial ods	<i></i>	1297 1297 71% 0.0 82% AF00551

*The first red line on your page represents your reference sequence "Algae". Below that line the other lines contain DNA sequences similar to your initial reference sequence.

7.2- Find the description "Pohlia drummondii chloroplast rbcL gene for ribulose 1.5-bisphosphate carboxylase/oxygenase large subunit, complete cds, specimen_voucher: personal:H. Akiyama:21292"

- a. Put a check mark to the left of the text and double click the description.
- b. It will appear as shown below.

S Chia	mydomonas	reinhard × 😣 Nucleotide BLAST: Search × 😣 NCBE Blast:Algae 🛛 × 🛄
$ \leq \rightarrow $	C Db	last.ncbi.nlm.nih.gov/Blast.cgi#269784565
	Download	* GenBank Graphica
-	Pohila drum Sequence ID	mondii chloroplast rbcL gene for ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, complete cds, specimen_vouc :: dbi/AB405053.11 Length: 1428 Number of Matches: 1
	Range 1: 7 t	e 1418 gentano graphos Vicot Hatch 🛦 Previous Hatch
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	Query 299 Sbjcb 186	AACAGETACLIGEACTACAGETGAGGETGAGGETTACAAGETGIGGGGTACAAGE 455
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- c. Click download and press Continue
- 2. A new sequence is downloaded. <u>DoubleClick</u> to open the sequence.

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Query	699	AAACAAATAD96705969CTTTT366776TRCAATCAAACTAAATTA667CTT7CA6C	758
Brjen	456	AAACAAATA5605005CCRTTRITA66856CSCTRTTRA6CCAAAAT6601TTA5CT6C	848
Query	759	TAAAAACTACOOTCOTOCASITITATSAATOTITACOTOOTOOTCITSACTITACTAAASA	818
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Query	1478	TASCECTERASCETERACEAASCECETAACEAASECCEDEACETERCECEDEAASEDE	1517
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Query.	1998	GGRAAGRAATTAAATTCGRAFTTGREACTATTGR 1691	
seqdump (1	4).txt		

3. When sequence is open delete text after ">" but before the beginning of the gene sequence *example below*



- 4. Name this new sequence "moss". Hit enter if the sequence and the name came together.
 - a. Your window should look like this.
- 5. Copy and paste this new sequence "moss" into your tree.fasta file
 - a. Your tree.fasta file should look like this.



SECTION 3

You now have an *Algae* and *Moss* sequence in your tree.fasta file.

- 1. You take your Algae sequence and compare to ferns.
 - a. Open BLAST window(<u>http://blast.ncvi.nlm.nih.gov/</u>)
 - b. Copy your Algae sequence into blast box.
 - c. <u>Type fern</u> in organisms place use the dropdown to select "ferns (taxid:241806)", and *optimize* for "somewhat similar sequences".
 - d. <u>BLAST</u>
- 2. In new result window check the first result in the "Description" section. You can locate this section after the "red lines".
 - a. After checking your selected sequence click Download
 - b. Choose "FASTA (aligned sequences).
 - c. Click Continue.
 - d. Open downloaded sequence and copy it to your "tree.fasta" file give it the name *fern*.

*Your tree.fasta file should look like this-



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Cardiomanes reniforme chlorop	last rbcL ar

SECTION 4

Using the same steps as above you will now BLAST pine, soybean and wheat:

- 1. Organism: pine family (taxid:3318), optimize for somewhat similar sequences.
 - a. After checking your selected sequence (1st option) click Download
 - b. Choose "FASTA (aligned sequences).
 - c. Click Continue.
 - d. Open downloaded sequence and copy it to your "tree.fasta" file, give it the name *pine*



- 2. Organism: wheat (taxid:4565), optimize for somewhat similar sequences.
 - a. After checking your selected sequence (1st option) click Download
 - b. Choose "FASTA (aligned sequences).
 - c. Click Continue.
 - d. Open downloaded sequence and copy it to your "tree.fasta" file, give it the name wheat
- 3. Organism: Soybean (taxid:3847), optimize for somewhat similar sequences.
 - a. After checking your selected sequence (1st option) click Download
 - b. Choose "FASTA (aligned sequences).
 - c. Click Continue.
 - *d.* Open downloaded sequence and copy it to your "tree.fasta" file, give it the name *soybean*

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***You now should have rcbL sequences for algae, moss, fern, pine, wheat and soybean in your "tree.fasta" file. **Double check** your work to make sure every organism has the respective label.

SECTION 5

- 1. Open program clustalX2 on your computer.
 - a. When clustalX2 is open, go to "file", "load sequences"
 - b. Find your tree.fasta file.
 - c. A portion of your clustalX2 file should look like this:

ClustalX 2.0.11		
<u>File Edit Alignment Trees</u>	<u>C</u> olors <u>Q</u> uality <u>H</u> elp	
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	*	*
Algae	AAGTAAACTCCGTAACACCACCACATA	T <mark>acctaaa</mark> ggcc
moss	ATGTCACCACAAACGGAGACTAAAGCAG	G <mark>TGTTGGA</mark> TTTA
Fern	TAACATATTACACACCAGATTACCAAAC'	TAAAGACACTGA
pine	CCACAAACAGAAACTAAAGCTAGTGTCG	G <mark>attc</mark> aaag <mark>ct</mark> g
wheat	CCACAAACAGAAACTAAAGCAGGTGTTG	G <mark>atttaaaget</mark> g
soybean	CCACAAACAGAGAGGAAAGCAAGTGTTG	GG <mark>TTC</mark> AAAGCTG

- d. Click the "alignment" tab, and click the option "do complete alignment"- a small window will come up, click "OK".
- e. After alignment is complete, you can scroll over in the screen and see the mutations among sequences.
- f. Go to "file", select "save sequence as", a new window will show up, <u>uncheck</u> the "clustalXformat" and <u>check</u> "FASTA format" and click "OK". *remember where you saved that file too.
 - a. We have now created the input file to run in the program MEGA

SECTION 6

Constructing a tree using MEGA5

- 1. Open MEGA by double-clicking on the program.
- 2. Click on the "data" tab and "open a file/session"
- 3. Open the "tree.fasta" file.
 - Hit "analyze".
- 4. Choose "nucleotide sequence", "Ok" and then "Yes" in the next steps, then hit "OK" *Your Mega window should look like this:



- a. Click on the "phylogeny" tab, choose the first option in the dropdown. Click "okay" and finally "compute"
- b. After your data has been analyzed a tree will pop up and will look similar to this:



c. Now click algae on your tree and click on the second option on your left sidebar (one with the green arrow). This will root the tree to show evolution of organisms. Your tree will now look like this:



Now you have a beautiful evolutionary tree as shown below.

<u>Reading tree (Example)</u>



You can play with various options about the cosmetic of the tree. You can save the tree in different format by clicking the second tab "image" on the menu bar or directly print as pdf from the file menu.

How to read the above tree (five of many messages are included)

- 1. All green plants had ancestor similar to the Alga
- 2. Fern and Moss form a group (=clade) that is supported by a bootstrap support of 67. This means out of 100 replications, 67% of the time they are together. They are more closely relagted each other than to other plants sampled in the present data set.
- 3. Pine is ancestral to both wheat and soybean (BS = 99).
- 4. Soybean and wheat are the most recent plants among the six sampled. They are more closely related to each other (BS=96) than any other plants sampled here.
- 5. Soybean, wheat and pine form a clade (a group of ancestror and all of its descendants) and fern and moss form another clade. All five together form a bigger clade. The inner lines represent ancestor for respective members, and those outer lines on the right are the descendants.