Investigating Gene Flow in Grasshopper Populations

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Based on data from:
Gerber, A. 1996. "The semiotics of subdivision: an empirical study of the population structure of *Trimerotropis saxatillus* (Acrididae)."
Division of Biomedical and Biological Sciences. Washington University, St. Louis.

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Investigating Gene Flow in Grasshopper Populations

Beginning of Instructor Pages

Purpose

This lab gives students the opportunity to participate in a population genetics experiment using grasshopper mitochondrial DNA. The lab is written to be inquiry-based and give students the opportunity to become involved in the research process. Students isolate mitochondrial DNA from grasshopper hind legs, set up sequencing reactions, analyze sequence data, and perform statistical analysis to draw a conclusion about the possible fragmentation of grasshopper populations. The topics of **gene flow** and **genetic drift** are the focus of this experiment.

Although this experiment is written using a grasshopper species native to the Missouri Ozarks, data are provided here, so that students may complete only the sequence and statistical analysis of the polymorphism frequency data. In addition, this lab may serve as a framework for creating a population genetics study on other insects.

Educational Context

This lab was created to accompany introductory curriculum in population genetics, specifically the topics of gene flow and genetic drift. This experiment gives students the opportunity to collect new sequence data then use these data to draw conclusions about population fragmentation. The students performing this lab have been first semester sophomores who are in their second semester of the introductory biology curriculum, having already completed a semester of cell and molecular biology. The experiment incorporates isolation of mitochondrial DNA from grasshopper hind muscle, PCR amplification, purification by gel electrophoresis, sequence analysis, identification of polymorphisms, and finally statistical analysis of the polymorphism frequency data.

Summary

This section contains a brief summary of the exercises contained in this lab. More thorough discussion of the materials follow in the General Materials section. The detailed protocol for each exercise is in the Student Section.

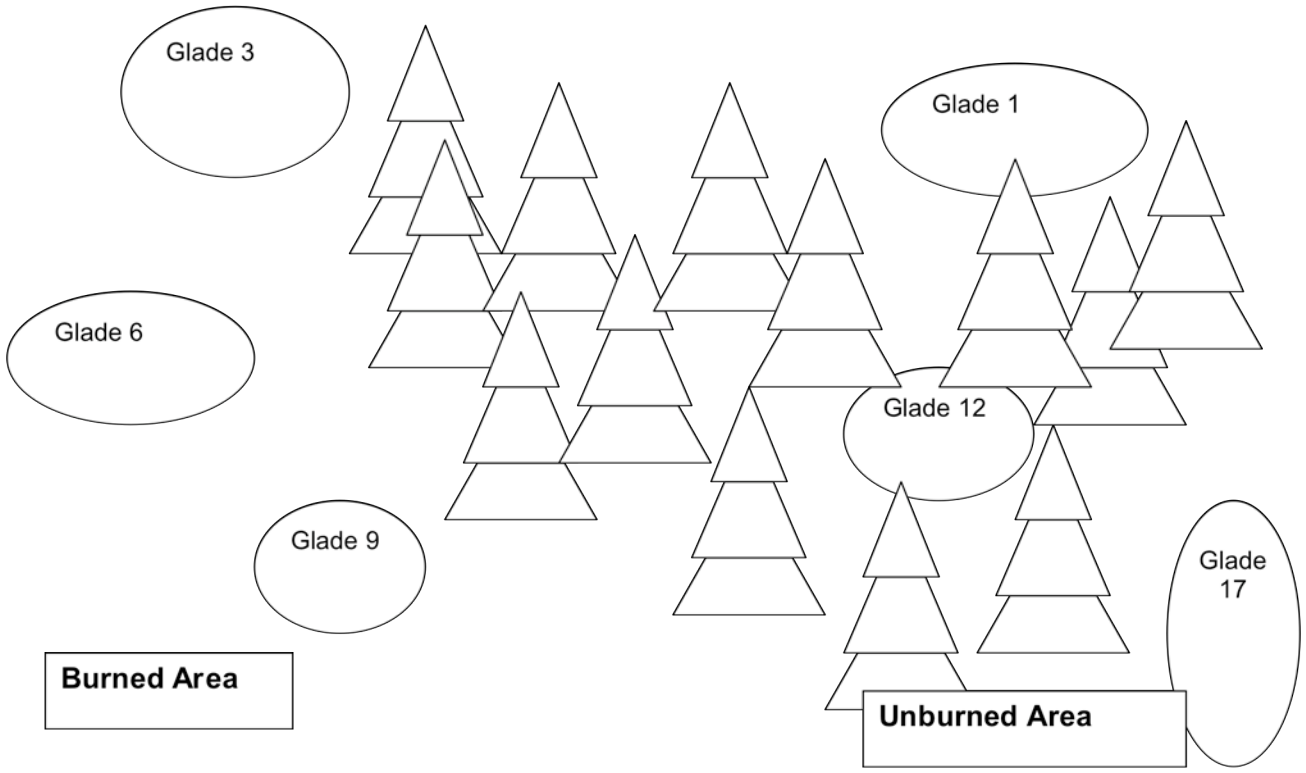
The lichen grasshopper *Trimerotropis saxatilis* lives in dry rocky areas, known as glades, ranging as far West as Oklahoma and East into Georgia. This medium sized grasshopper occurs exclusively on acidic glades where its cryptic coloration makes it nearly invisible against the lichen covered granite, rhyolite or sandstone dominating these habitats.

In the Ozarks of Missouri the glades are habitat islands separated by patches of forest. Historically the glades were maintained by the frequent occurrence (around every four years) of forest fires. Recently, human suppression of forest fires has resulted in the loss, reduction, and fragmentation of glade habitats.

The fragmentation of the lichen grasshoppers' habitat could have a substantial impact on the way genetic variation is distributed throughout the population. As the habitat becomes patchier, gene flow may be reduced, causing genetic subdivision of the

population. Although this grasshopper can fly, it is doubtful that the grasshopper disperses across large tracts of forest to get from one glade to another. Recent work to preserve glade habitats has prompted controlled burns that enlarge and preserve the glades and may provide temporary corridors for lichen grasshoppers to disperse.

This lab studies the level of genetic subdivision (directly related to the level of gene flow) in this species. Each student isolates DNA from a *T. saxatilus* individual, PCR amplifies a 300 base-pair segment of its mitochondrial DNA, then sequences the resulting PCR fragment. (If grasshoppers are unavailable, please see note under **Timeline**.) Although most bases in this region are invariable, there are six bases that show variation in preliminary studies. The students look at each of these base positions and determine which haplotype (or allele) each grasshopper has. Each student then contributes his or her result to a class database. Population subdivision is examined using the F_{st} statistic. This statistic is compared between glades occurring in burned and unburned areas to examine whether controlled burns have an impact on population subdivision. It is hypothesized that grasshoppers will be able to disperse between the glades in the burned areas, but not in the unburned areas. The areas called "burned" have been subject to a controlled burn within the last 4 years. The areas that are called "unburned" have not had a forest fire in over 50 years. An example map is shown below.



For more information on PCR and DNA sequencing, please see the following Websites.

PCR:
http://www.dnalc.org/ddnalc/resources/pcr.html

Cycle sequencing:
http://www.dnalc.org/ddnalc/resources/cycseq.html
Sanger sequencing:
http://www.dnalc.org/ddnalc/resources/sangerseq.html
www.nslc.wustl.edu/elgin/genomics/

Select "Genome Sequencing Center Video Tour" in the first paragraph. This 30 min video provides a tour of the Washington University Genome Sequencing Center with explanations and animations of each step of the sequencing process, which includes PCR and cycle sequencing.

Timeline

The experiment can be completed in 3, 2-hour lab meetings. Additional instructor time is needed between lab meeting 1 and 2 in order to run the PCR reactions and isolate the product through gel electrophoresis. In addition, access to a sequencing core is required between lab meeting 2 and 3.

NOTE: Data is presented here for students to complete only the sequence analysis portion of the lab (if grasshoppers are unavailable). This can be completed in one, 2-hour lab meeting.

Activity	Instructor Preparation	Student Lab Time
Isolation of mitochondrial DNA and PCR amplification	Collecting grasshoppers Preparing PCR mix	~1 ½ hours
Agarose gel isolation of PCR product and Setting up sequencing reaction	Run PCR reactions Run PCR samples on gel and cut fragment	~1 ½ hours
Analyzing Sequencing Results	Obtain sequence information from a sequencing core facility	~1 ½ hours

General Materials and Equipment:

Collecting and Storing Grasshoppers

Trimerotropis saxatilus grasshoppers are collected from the Ozarks region of Missouri during the late summer to early fall when the grasshoppers have already mated. They are collected by walking slowly across the rocky glade and sweeping an insect net on the ground in front of each collector. The grasshoppers are caught in the net as they jump. Once they are caught, they are kept alive, but cold, in coolers on ice and brought back to the lab. In the lab, each grasshopper is put in a 2.0 mL vial, labeled with the glade number that it was collected in, then placed in a $^{-}80°C$ freezer to prevent decomposition. A rough map should be made that shows which glades are from the "unburned" area (therefore fragmented by trees and brush) and which glades are from the "burned" area.

Week 1 Materials and Instructor Preparation

Assigning and Distibuting Grasshoppers:
If working with multiple lab sections of students, assign one glade per lab section and distribute only grasshoppers from that glade to the students. Each student should remove the hind leg from a different grasshopper collected from that glade. It works well if each student is given a "code" to identify their grasshopper to use when labelling tubes throughout the experiment. The example data provided here were obtained from 6 lab sections of approximately 20 students each. Two lab sections were given grasshoppers from glades in burned areas and three lab sections were given grasshoppers from glades in unburned areas. Data were obtained from approximately 30 grasshoppers from the burned glades and 79 grasshoppers from the unburned glades.

Isolating mitochondrial DNA:
10% Chelex (10 g/100mL), aliquoted into 1.5 mL tubes, one tube with 250 µL per student
Proteinase K (20 mg/mL), need 50 µL per student

PCR reaction:
0.2 mL tubes with 45 µL PCR amplification mix per student
In PCR amplification mix:

Volume (per reaction)	Reagent	Stock Concentration
1.1 µL	dNTP	10 mM
0.2 µL	Taq polymerase	5 U/µL
5.5 µL	Taq buffer	10X
1.65 µL	$MgCl_2$	50 mM
2.75 µL	Primer 1	20 pmol/µL
2.75 µL	Primer 2	20 pmol/µL
31.05 µL	dH_2O	----

Students will add 10 µL of their mitochondrial DNA for a final volume of 55 µL.

Other materials:
Containers of dry ice (for storing grasshoppers during lab)
Pointed forceps (for handling the grasshoppers and removing hind leg)
P200 and P20 micropipetters and appropriate sterile tips
Sterile wooden sticks or toothpicks (for grinding hind leg)
Eppendorf tabletop centrifuge
Kim wipes
0.2 mL tubes
Air incubator set at 56 °C with shaker (for Proteinase K digestion)
Boiling water bath
Ice buckets (with wet ice)

Thermocycler Program for PCR Reaction:
95°C – 3 min, then 45 cycles of:

95 °C – 1 min - denature
45 °C – 1 min - anneal
72 °C – 1 min elongation

Polyacrylamide Gel Electrophoresis:
Run each student's PCR reaction out on a 1% agarose gel containing ethydium bromide (1 µL of 10 mg/mL added to a 50 mL gel). Also load a 100 bp DNA ladder for 45 min at 200 mV. Cut out the band at 300 bp with a razor blade and place in a 1.5 mL Eppendorf tube. Label with students' grasshopper sample numbers. Store fragments at 4 °C. If time permits, allow students to cut out the gel band themselves. For more information on agarose gel electrophoresis, please see:

http://www.methodbook.net/dna/agarogel.html

Week 2 Materials and Instructor Preparation

Gel Extraction:
QIAQuick Gel Extraction Kits

Each student will need 450 µL of the Gel Extraction Buffer, one QIAquick column, 150 µL isopropyl alcohol, and 750 µL QIAquick wash solution. For more information on this procedure, the manual is available online at:

http://www1.qiagen.com

Sequencing Reaction:
Each reaction:
8 µL - BigDye mix
0.32 µL - Primer 1 (20 pmol/µL)
1.68 µL H_2O
10 µL total

Students will each add 10 µL of their purified PCR fragment for a final volume of 20 µL.

Thermocylcer Program for Sequencing Reaction:
Same program as used for the PCR amplification.

Submit samples to a sequencing core. Follow the directions from the sequencing core for any further sample preparation. Obtain an electropherogram, if possible, so students can analyze the quality of their sequence data.

Other materials:
P10, P20, and P200 micropipetters with appropriate sterile tips

Clean, sterile 1.5 mL Eppendorf tubes (1 per student) and sterile 0.2 mL Eppendorf tubes (1 per student)
Water bath set at 50 °C

Ordering Information

General chemicals not listed here can be purchased from Sigma-Aldrich (www.sigmaaldrich.com)
General materials not listed here can be purchased from Fisher Scientific (www.fishersci.com)

Taq polymerase and buffer
Invitrogen (www.invitrogen.com)
Catalog number 18038-018
PCR-grade dH_2O
Invitrogen (www.invitrogen.com)
Catalog number 10977-015
QIAQuick Gel Extraction Kit (50)
Qiagen
Catalog number 28704
Big Dye® Terminator v1.1 Cycle Sequencing Kit
Applied Biosystems (www.appliedbiosystems.com)
Product number 4336774
dNTP mix (10 mM)
Invitrogen (www.invitrogen.com)
Catalog number 18427-013
Primers:
Primer 1: 5' – GTTCGAGTTCTAACTAGAGCATCAG – 3'
Primer 2: 5' – CAAGGTGTACTAATATTTAGTTATT – 3'
IDT (Integrated DNA Technologies) at www.idtdna.com
Each primer was shipped dry and was 0.5 mg, or 69 nmoles. We diluted each primer in 3.45 mL of dH_20 to a final concentration of 20 pmol/µL.
Proteinase K (30 units/mg)
Sigma-Aldrich
Catalog number P2308
Chelex 100 Resin
Bio-Rad
Catalog number 142-1253

Sample Data and Results

Sample Sequence Data:

Allele AATAT:

```
5'-GTTCGAGTTC  TAACTAGAGC  ATCAGATGTT  TTACATTCAT  GAGCCGTACC
     ❶            ❷                      ❸
CGCTCTAGGA  ATTAAAATAG  ATGCAACTTC  TGGACGTTTA  AATCAAGGAA
               ❹
CATTTTTAAT  TAAACGTCCA  GGATTATTTT  TTGGACAATG  TTCAGAAATT
                                ❺
TGTGGAGCAA  TTCATAGATT  TATACCAATT  GTAATTGAAA  GAACATCAGT
AAAATTATTT  ATTAAATGAT  TATCAAGTAT  AATATAAGGA  GTTAGTTAAA
ATATAACATT  AGAATGTCAA  TCTAAAATAA  CTAAATATTA  GTACACCTTG -3'
```

Allele AGTAT:

```
5'-GTTCGAGTTC  TAACTAGAGC  ATCAGATGTT  TTACATTCAT  GAGCCGTACC
     ❶            ❷                      ❸
CGCTCTAGGA  ATTAAAATGG  ATGCAACTTC  TGGACGTTTA  AATCAAGGAA
               ❹
CATTTTTAAT  TAAACGTCCA  GGATTATTTT  TTGGACAATG  TTCAGAAATT
                                ❺
TGTGGAGCAA  TTCATAGATT  TATACCAATT  GTAATTGAAA  GAACATCAGT
AAAATTATTT  ATTAAATGAT  TATCAAGTAT  AATATAAGGA  GTTAGTTAAA
ATATAACATT  AGAATGTCAA  TCTAAAATAA  CTAAATATTA  GTACACCTTG -3'
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Allele AGTTT:

```
5'-GTTCGAGTTC  TAACTAGAGC  ATCAGATGTT  TTACATTCAT  GAGCCGTACC
     ❶            ❷                      ❸
CGCTCTAGGA  ATTAAAATGG  ATGCAACTTC  TGGACGTTTA  AATCAAGGAA
               ❹
CATTTTTAAT  TAATCGTCCA  GGATTATTTT  TTGGACAATG  TTCAGAAATT
                                ❺
TGTGGAGCAA  TTCATAGATT  TATACCAATT  GTAATTGAAA  GAACATCAGT
AAAATTATTT  ATTAAATGAT  TATCAAGTAT  AATATAAGGA  GTTAGTTAAA
ATATAACATT  AGAATGTCAA  TCTAAAATAA  CTAAATATTA  GTACACCTTG -3'
```

Sample Polymorphism Data

Allele	Glade 3	Glade 6	Glade 9	Glade 1	Glade 12	Glade 17
AATAT	2	4	6	14	9	17
AGTAT	5	7	4	18	15	6
AGTTT	1	0	1	0	0	0

Glades 3, 6, and 9 are burned glades.
Glades 1, 12, and 17 are unburned glades.

Lab Report Results Based on Sample Data

BURNED GLADES							
	Glade 3		Glade 6		Glade 9		Overall frequency (p_i)
Allele (*i*)	number	frequency (p_{ij})	number	frequency (p_{ik})	number	frequency (p_{im})	
AATAT	2	0.250	4	0.364	6	0.545	0.400
AGTAT	5	0.625	7	0.636	4	0.364	0.533
AGTTT	1	0.125	0	0.000	1	0.091	0.067
Total number	**8**		**11**		**11**		
H_{sj}	**0.531**		**0.464**		**0.563**		
H_S	**0.518**						
H_T							**0.554**
F_{ST}	**0.064**						

UNBURNED GLADES							
	Glade 1		Glade 12		Glade 17		Overall frequency (p_i)
Allele (*i*)	number	frequency (p_{ij})	number	frequency (p_{ik})	number	frequency (p_{im})	
AATAT	14	0.437	9	0.375	17	0.739	0.506
AGTAT	18	0.563	15	0.625	6	0.261	0.494
AGTTT	0	0.000	0	0.000	0	0.000	0.000
Total number	**32**		**24**		**23**		
H_{sj}	**0.491**		**0.468**		**0.386**		
H_S	**0.453**						
H_T							**0.499**
F_{ST}	**0.092**						

A. Which F_{ST} is larger, burned or unburned glades?
The F_{ST} for the unburned glades is larger (0.092 vs. 0.064).

B. What does the answer to the previous question imply about the impact of forest fires on the dispersal of *T. saxatilis*?

When F_{ST} is 0, gene flow dominates. When F_{ST} is 1, genetic drift dominates. Forest fires promote slightly more gene flow (emigrating/immigrating) among the *T. saxatilis* population when compared to unburned glades. However, since both F_{ST} values are closer to 0 than 1, gene flow is dominating for both types of glades.

Blank Tables (for photocopying)

BURNED GLADES							
	Glade		Glade		Glade		Overall frequency (p_i)
Allele (*i*)	number	frequency (p_{ij})	number	frequency (p_{ik})	number	frequency (p_{im})	
AATAT							
AGTAT							
AGTTT							
Total number							
H_{sj}							
H_S							
H_T							
F_{ST}							

UNBURNED GLADES							
	Glade		Glade		Glade		Overall frequency (p_i)
Allele (*i*)	number	frequency (p_{ij})	number	frequency (p_{ik})	number	frequency (p_{im})	
AATAT							
AGTAT							
AGTTT							
Total number							
H_{sj}							
H_S							
H_T							
F_{ST}							

References

Gerber, A,1994, “The semiotics of subdivision: an empirical study of the population structure of *Trimerotropis saxatilis* (Acrididae).” PhD Thesis, Division of Biomedical and Biological Sciences. Washington University, St. Louis.

Gerber, AS and Templeton, AR,1996, “Population sizes and within-deme movement of *Trimerotropis saxatilis*, a grasshopper with a fragmented distribution,” *Oecologia*, Vol 105 (3), p. 343-350.

Templeton, AR, 1998, “Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history,” *Molecular Ecology*, Vol 7 (4), p. 381 - 397.

Brisson, JA, Strasburg, JL, and Templeton, AR, 2003, “Impact of fire management on the ecology of collared lizard (*Crotaphytus collaris*) populations living on the Ozark Plateau,” *Animal Conservation*, Vol 6, p. 247 – 254.

Investigating Gene Flow in Grasshopper Populations

Beginning of Student Pages

Gene Flow in *Trimerotropis saxatilis*

Background:

The lichen grasshopper *Trimerotropis saxatilis* lives in dry rocky areas, known as glades, ranging as far west as Oklahoma and east into Georgia. This medium sized grasshopper occurs exclusively on acidic glades where its cryptic coloration makes it nearly invisible against the lichen covered granite, rhyolite or sandstone dominating these habitats.

In the Ozarks of Missouri the glades are habitat islands separated by patches of forest. Historically the glades were maintained by the frequent occurrence (around every four years) of forest fires. Recently, human suppression of forest fires has resulted in the loss, reduction, and fragmentation of glade habitats.

The fragmentation of the lichen grasshoppers' habitat could have a substantial impact on the way genetic variation is distributed throughout the population. As the habitat becomes patchier, gene flow may be reduced, causing genetic subdivision of the population. Although this grasshopper can fly, it is doubtful that the grasshopper disperses across large tracts of forest to get from one rocky area to another. Recent work to preserve glade habitats has prompted controlled burns that enlarge and preserve the glades and may provide temporary corridors for lichen grasshoppers to disperse.

We will study the level of genetic subdivision (directly related to the level of gene flow) in this species. Each student will isolate DNA from a *T. saxatilis* individual, amplify a 300 base-pair segment of its mitochondrial DNA, and sequence the resulting PCR fragment. Although most bases in this region are invariable, there are six bases that show variation in preliminary studies. We will look at each of these base positions and determine which haplotype (or allele) each grasshopper has. Each student will then contribute his or her result to a class database. Population subdivision will be examined using a statistic, called F_{st}. We will compare this statistic between glades occurring in burned and unburned areas to examine whether controlled burns have an impact on population subdivision. The experiment will be broken down between three lab meetings as described on the following page.

Week 1
-Isolate mitochondrial DNA from hind limb of a grasshopper
-Initiate a PCR reaction using this DNA

Week 2
-Visualize PCR products by gel electrophoresis
-If PCR product is observed, clean-up PCR product to remove residual nucleotides and primers
-Prepare sequencing reaction from each cleaned PCR product and run sequencing reaction in a thermocycler.
-Sequencing reactions will be further prepared (as required) by the instructors for automated sequencing and submitted to a sequencing core. The sequencing core will provide the electropherogram along with the sequence for the PCR product.

Week 3
-Computer analysis of the electropherogram and sequence
-Determine the haplotype of each individual
-Accumulate class data and analyze

Week 1

DNA isolation: (Work individually)
Note: It is crucial that the grasshoppers do not thaw during this procedure. Once you remove your grasshopper from the dry ice, work as quickly as possible.

1. Record the grasshopper number that you have been assigned: _____

2. Find one tube containing 10% chelex solution and label the top of the tube with your grasshopper number.

3. Add 50 µl proteinase K (20 mg/ml) to the chelex solution.

4. Find a Kim wipe tissue and place it on the work area of your bench. Find a sterile wooden stick and place it on the Kim wipe.

5. Obtain a 2 ml tube containing one lichen grasshopper from the dry ice (avoid contact with the dry ice, since it can burn your skin).

6. Use forceps to remove the grasshopper from the tube and place it on the Kim wipe.

7. Use the forceps to break off one of the hind limbs (the large back leg) of the insect. Immediately place the leg in your tube of chelex.

8. Place the grasshopper back in its original 2 ml tube place the tube back in the dry ice bucket labeled "USED GRASSHOPPERS" to prevent other students from using the same grasshopper. Make sure the tube is down in the ice, not just sitting on top of it.

9. Use the sterile stick to crush the leg in the chelex solution. Break up the leg material as much as possible. The tube should become murky with pinkish/orange muscle tissue.

10. Close the tube and place the tube in the appropriate tube rack at the front of the room. When all tubes have been collected, the samples will be shaken at 56°C for 30 minutes.

11. After the 56°C incubation is complete, incubate your sample at 100°C for 10 minutes to inactivate the Proteinase K.

12. Cool your sample on ice for 1 minute.

13. Centrifuge the tube at full speed for 30 seconds to pellet the chelex and undigested tissue.

14. Place your sample on ice.

PCR reaction:

1. Obtain a 0.2 ml PCR tube and label the top of the tube with your grasshopper number.

2. Add 45 µl of amplification mixture (amp. mix) to the tube. The amp. mix contains:
   A) 2 mtDNA primers (Forward and Reverse)
   B) $MgCl_2$
   C) dNTPs (dATP, dCTP, dGTP, dTTP)
   D) *Taq* polymerase buffer
   E) *Taq* polymerase

3 Add 10 µl of your sample (i.e., grasshopper mtDNA template) to the tube (be careful to pipet only supernatant from your DNA tube). Pipet the DNA directly into amp. mix that you deposited in the reaction tube. Do not transfer beads or undigested tissue.

4. Hand your PCR reaction to your instructor or TA to place on ice. Subsequently, when all of your sections samples have been collected, the samples will be placed in the thermal cycling machine for amplification.

Week 2

A 1 % agarose gel was run to visualize PCR products and to separate it from spurious products, primers, nucleotides and the polymerase. If an appropriately sized product was amplified, a band containing the product was cut out of the gel and the gel slice was stored in the refrigerator. The DNA must be purified away from the agarose for the next step of our experiment, the sequencing reaction.

The sequencing technology we will use in this experiment is similar to the Sanger sequencing method, but with significant improvements. First, *Taq* polymerase is used instead of the Klenow fragment. This enables the use of much less template than is required in the traditional Sanger method. Second, each dideoxynucleotide is coupled with a different dye that fluoresces with a unique color. This allows the reaction to be performed in a single tube rather than in four separate tubes; the reaction products can then be separated in a single lane of a polyacrylamide gel. As the gel runs, the terminating base of each sequencing product is revealed by the color emitted under a laser. Visit the following website for an on-line animation of cycle-sequencing:

http://vector.cshl.org/resources/BiologyAnimationLibrary.htm

Isolation of DNA from Agarose:

1. Using your grasshopper number, identify and obtain the 1.5 ml tube that contains an agarose gel block that contains your PCR product from the PCR reaction that you assembled last week.

2. Add 450 µl of agarose Gel Extraction Buffer to the 1.5 ml tube containing your PCR product. Use a blue (P-1000) pipet tip to *carefully* push the agarose gel block down until it is completely immersed in the Gel Extraction Buffer.

3. Incubate the tube at 50^{o}C and, occasionally, invert it several times once during this incubation. Incubate until no agarose is visible (*depending upon the size of the agarose block, this can take a while; 30 minutes or so*).

4. After the gel slice has completely dissolved, check the color of the mixture to confirm that it is still yellow. If not, consult your laboratory instructor.

5. Add 150 µl of isopropyl alcohol to the tube. Your may DNA may precipitate out of solution.

6. Obtain a purple spin column and label the top of the spin column with your grasshopper number.

7. Transfer 750 µl of the dissolved gel slice/buffer/isopropanol mixture onto the column.

8. To bind the DNA to the column, centrifuge for 1 minute in the HIGH SPEED MICROCENTRIFUGE. *Make sure the centrifuge is properly BALANCED and the rotor lid is placed on the rotor and tightened*.

9. Discard the liquid that flows through the column and place the column back into the same collection tube.

10. If you have any remaining gel slice/buffer/isopropanol mixture in the original 1.5 ml tube, repeat steps 7–9.

11. Wash the DNA bound to the column by adding 750 µl of wash solution to the column and centrifuge for 1 minute on the HIGH SPEED MICROCENTRIFUGE.

12. Discard the liquid that flows through the column and centrifuge the empty column at top speed in the bench-top microcentrifuge on the your bench for an additional 1 minute.

13. Place the purple spin column into a new 1.5 ml microcentrifuge tube.

14. Add 40 µl of dH_2O to the column.

15. Allow the column to stand for 1 minute.

16. Centrifuge in the HIGH SPEED MICROCENTRIFUGE for 1 minute.

17. Your purified PCR product is now in the 1.5 ml tube and is ready for use in a sequencing reaction.

Sequencing reaction:

1. Obtain a 0.2 ml PCR reaction tube from your instructor or TA that contains the following:
   A) BigDye Terminator sequencing mixture (8 µl)
      This sequencing mixture contains *Taq* polymerase, dye-coupled dideoxynucleotides (ddNTPs), normal dNTPs, $MgCl_2$, and buffers.
   B) One mtDNA primer (2 µl)
2. Label the top of the PCR reaction tube with your grasshopper number.
3. Pipet 10 µl of your purified PCR reaction from step 17 above into the same tube.
4. Return your sequencing reaction to your lab instructor or TA.

Your instructor will purify the products of your sequencing reactions and will send them to a sequencing facility where they will be run and read on an automated sequencing unit for analysis next week.

Week 3

Sequence determination, data collection, and analysis: (Work individually)
We will view the sequencing results on computers. There are five nucleotides within this sequence that have shown variation in preliminary experiments (the data from the class may reveal more) and we will focus on those sites. The following table shows the rough location of the bases in which we are interested and the sequence surrounding the bases of interest. Find these sites within your DNA sequence and record the base that occurs at that location in the table below.

5'-**GTTCGAGTTC TAACTAGAGC ATCAG**ATGTT TTACATTCAT GAGCCGTACC

❶ ❷ ❸

CGCTCT□GGA ATTAAAAT□G ATGCAACTTC TGGACG□TTA AATCAAGGAA

❹

CATTTTTAAT TAA□CGTCCA GGATTATTTT TTGGACAATG TTCAGAAATT

❺

TGTGGAGCAA TTCATAGATT TATACCAAT□ GTAATTGAAA GAACATCAGT

AAAATTATTT ATTAAATGAT TATCAAGTAT AATATAAGGA GTTAGTTAAA

ATATAACATT AGAATGTCAA TCTAAAATAA CTAAATATTA GTACACCTTG -3'

Key:
underline…PCR primer sites

bold & underline…
sequencing primer site

❶ - ❺ sites of variation

Results Table:

Site	Base number from start (⇨):	Sequence	Base Observed
❶	7	ACCCGCTCT□GGAATT	
❷	19	AATTAAAAT□GATGCA	
❸	37	TTCTGGACG□TTAAAT	
❹	64	TTTAATTAA□CGTCCA	
❺	130	TATACCAAT□GTAATT	

Your allele _____ (Read bases for sites ❶ - ❺)
Your glade _____

We will accumulate the sequence data for sites ❶ - ❺ for all the lab sections. The data will be distributed at the end of the week for you to use in your calculations. An example of the table that will be distributed is shown in Figure 2 below.

UNBURNED GLADES							
	Glade *j*		Glade *k*		Glade *m*		Overall frequency (p_i)
Allele (*i*)	number	frequency (p_{ij})	number	frequency (p_{ik})	number	frequency (p_{im})	
ATACT	9	0.563	26	0.650	22	0.489	0.564
ATATT	7	0.438	13	0.325	23	0.511	0.426
ATACG	0	0.000	1	0.025	0	0.000	0.010
Total number	**16**		**40**		**45**		
H_{sj}	**0.492**		**0.471**		**0.500**		
H_S	**0.487**						
H_T							**0.500**
F_{ST}	**0.026**						

Figure 2 Example of data table from *Trimerotropis saxitillus* experiment. The graph you obtain from your instructor will only have the "number" columns filled in. Follow the directions under "calculations" to fill in the rest of the table.

Calculations

F_{st} Analysis: (Work individually)

The F_{st} statistic was designed by Sewall Wright to measure the amount of genetic variation found among **s**ubpopulations relative to the **t**otal population (hence, the subscript "**st**"). Wright used a model of the balance between gene flow (genetic interchange among the glade populations in this case) versus genetic drift (due to the small populations sizes on each glade, between 20-100 individuals in this case). If gene flow were very common among the glade populations, each glade population should have the same alleles at the same frequency.

To calculate F_{st}, we start by calculating the probability that two genes drawn at random from glade population *j* are different alleles, H_{sj}. It is actually easier to calculate first the probability that two randomly drawn genes are the same allele, which is 1-H_{sj}. Let p_i be the frequency of allele *i* in the total collection of glade populations being analyzed. If

gene flow dominates, then $p_{ij} = p_i$ for every glade *j*. As gene flow becomes weaker relative to genetic drift, we expect glade populations to show different allele frequencies.

Let ***i*** designate an allele or haplotype and $\boldsymbol{p_{ij}}$ be the frequency of allele *i* in glade *j*. Therefore:

Equation 1:

$$1 - H_{sj} = \sum_{i=1}^{a} p_{ij}^2$$

where ***a*** is the number of different alleles or haplotypes and

Equation 2:

$$H_{sj} = 1 - \sum_{i=1}^{a} p_{ij}^2$$

Subsequently, average the H_{sj} over all glade populations of a particular type of interest:

Equation 3:

$$H_s = \frac{\sum_{j=1}^{g} n_j H_{sj}}{n}$$

where ***g*** is the number of glade populations being averaged, $\mathbf{n}_j$ is the number of individuals sampled in glade *j*. **n** is the total number of individuals sampled in all the glades of interest and thus,

Equation 4:

$$n = \sum_{j=1}^{g} n_j$$

The next step is to calculate the probability that two genes drawn at random from the total pooled sample of glade populations being considered are different alleles:

Equation 5:

$$H_T = 1 - \sum_{i=1}^{a} p_i^2$$

Now, F_{st} can be calculated as:

Equation 6:

$$F_{st} = \frac{(H_T - H_s)}{H_T}$$

Note that F_{st} can take on any value between 0 (which occurs only when all allele frequencies are identical in all glades; that is, gene flow dominates) and 1 (which occurs only when each glade population has only one allele; that is, genetic drift dominates).

Lab Report Instructions:

Once your instructor has provided the pooled class data in two tables like the table shown in Figure 2, you can use the instructions below to finish filling in the tables and calculate F_{st} values for burned and unburned glades. Attach sheets as necessary to **SHOW YOUR WORK** and report the final F_{st} calculations for comparison. Perform the calculations separately for each type of glade (burned or unburned).

1. For each glade population, determine the frequency (*p*) of each allele (*i*) as:

Equation 7:

$$p_{ij} = \frac{\text{number of times allele } i \text{ is observed in glade } j}{n_j}$$

Where *j* is the particular glade being examined. For **Total number** of alleles in a particular glade, n_j , do not use $2n_j$ because mtDNA is haploid in its inheritance.

2. Calculate H_{sj} for each glade using equation 2:

Equation 2:

$$H_{sj} = 1 - \sum_{i=1}^{a} p_{ij}^2$$

3. Average them over for the type of glades (burned or unburned) using equation 3 to obtain H_s where n is the **Total number** of alleles from one type of glade.

Equation 3:

$$H_s = \frac{\sum_{j=1}^{g} n_j H_{sj}}{n}$$

4. For the set of glades from the burned area, determine the frequency of each allele in the total population:

Equation 8:

$$p_i = \frac{\sum_{j=1}^{g} n_j p_{ij}}{n}$$

5. For the set of glades from the burned area, calculate the overall H_T using equation 5.

Equation 5:

$$H_T = 1 - \sum_{i=1}^{a} p_i^2$$

6. Now you can calculate F_{st} for the burned area using equation 6.

Equation 6:

$$F_{st} = \frac{(H_T - H_s)}{H_T}$$

7. Repeat steps 2, 3, 4 and 5 for the set of glades from the unburned area.

8. Contrast the values of F_{st} obtained in the burned versus unburned areas.
    A. Which F_{st} is larger?

    B. What does this imply about the impact of forest fires on dispersal in this grasshopper?