Genetic Analysis of Lady Slippers

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Abstract

Showy lady's slippers orchids (*Cypripedium reginae*) are critically endangered in New Hampshire, existing in less than 5 places in the wild. The intent of this experiment was to investigate the genetic diversity of the flowers in the wild, as a lack of genetic diversity can be a contributing factor for a population's especially when facing environmental a global climate change. We performed PCR on DNA samples extracted from plants in an artificial fen located in Lyme, NH. These plants were grown in axenic seed culture from seeds collected from the Eshqua Bog in Vermont. We utilized PCR primers that had been previously used to target variable microsatellite DNA regions on another species of *Cypripedium*. We targeted these specific microsatellite regions because they are likely to change between two distinct *Cypripedium* populations of the same species. Our experiments identified primers that could be used for our *Cyp. reginae* samples and yielded PCR products for one microsatellite region from 16 plants that were submitted for DNA sequencing. This produced sequencing results for the very short microsatellite segments of DNA. The obtained sequences were analyzed for quality and aligned to determine the amount of difference between individuals. There were very few differences, with the sequences aligning almost identically. This suggests our analytic approach works for *Cyp. reginae* and can be used to analyze the genetic diversity of this orchid species. Based on these results, the plants within the Lyme fen were not substantially genetically different from one another, but comparisons to populations in the wild are needed to fully confirm this suspicion. We would like to analyze the genetic diversity of plants from wild fens to determine if they have robust genetic diversity within and among different fens.

Introduction

Lady's Slippers

- Terrestrial orchids in upper mid-west and eastern parts of US
- Critically endangered in NH
- Can reproduce by 2 methods:
  1) Asexually via underground stems (faster)
  2) Sexually via seeds (~250,000 seeds per pod, <1% germinate first year, <1% of germinated make it to flowering stage – in 8-10yrs)

Showy Lady's Slipper

- Are Showy Lady's Slippers populations expected to grow and survive?
  - What is the genetic variation within the surviving population?

Genetic Variation

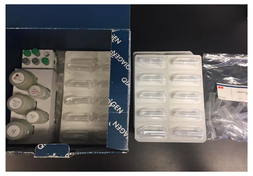
- High genetic variation means higher potential for some part of population to be preferred and survive
- Genetic variation can be assessed by looking at sequences of different parts of the genome

Our Research

- Purpose: To start to measure genetic variation in artificial fen in Lyme, NH (originally populated by 15 axenically grown seedlings from Eshqua Bog, VT)
- Hypothesis: High genetic variation in fen, most likely sexual reproduction
- Initially focused on one variable microsatellite region of genome
- Studying variable regions gives idea of genetic diversity in a population

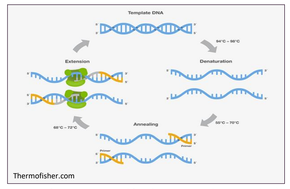
Methods and Materials

1. Collect Samples



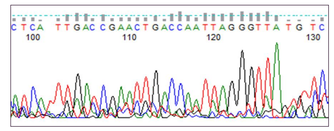
2. Extract DNA

3. Nanodrop



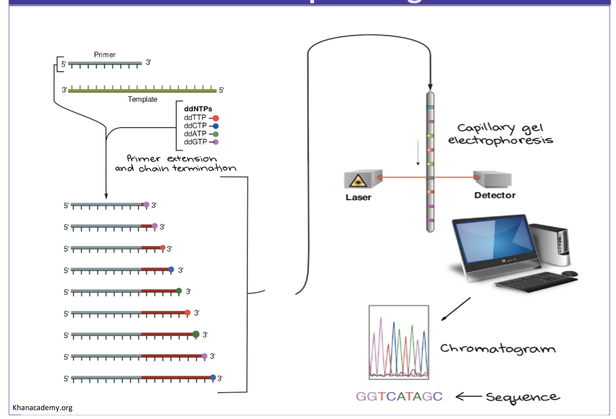
4. Polymerase Chain Reaction (PCR)

5. Gel Electrophoresis



6. DNA Sequencing

DNA Sequencing



Results

Original PCR

- On Root Tip 10, Root Tip 9, and Leaf Tip 8 – no bands
- Troubleshooted PCR with 3 different buffer + enhancer combinations possible with the OneTaq we used and also increased DNA amounts
- Original PCR with GC Buffer did not work during troubleshooting. Standard buffer with no enhancer worked best.

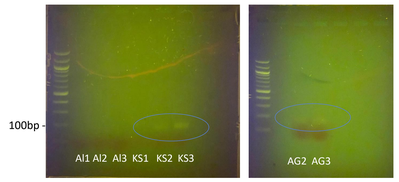


Figure 1. Gel electrophoresis of troubleshooting PCR. First lane in each gel is a 100bp Ladder (New England Biolabs). Lanes are: Al1, KS1 – GC Buffer + GC Enhancer; Al2, KS2, AG2 – GC Buffer + Water; Al3, KS3, AG3 – Standard Buffer + Water. Bands for the microsatellite primers m209 can be seen in KS2 and KS3, faint on AG2 and AG3. Dark spots in lanes are loading dye shadows.

After Troubleshooting

- Tested m172 and m209 primer sets
- Only m209 primer set produced results
- From 24 samples amplified with m209 primer set, only 10 worked
- When all but one sequence aligns, like base 93, check if the sequences agree at all positions - Must check for double peaks of both bases in **all** chromatograms – Double peaks in Figure 2, circled.
- Double peaks = a position that is heterozygous in the gene, meaning different copies of the gene in that individual have different bases at that position; if two DNA samples have same heterozygous mutation, very likely that are closely related.
- If all match up – sequences from different plants identical at position
- If they do not all match up – important difference for determining relationships between individuals using alignment

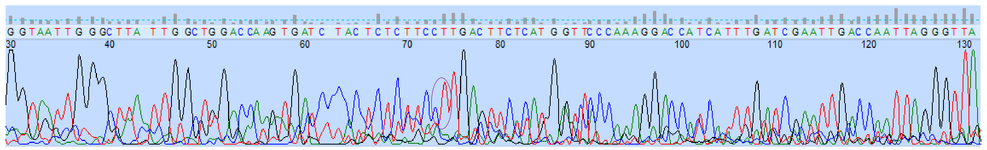


Figure 2 Chromatogram from DNA Sequencing of CA4 with m209 forward primer. X-axis shows sequence identified. Y-axis shows graph of bases' fluorescence intensity as labeled base passed by detector where each color represents a different base. The beginning of the chromatogram omitted, as it is generally messy. An example of a double peak is circled.

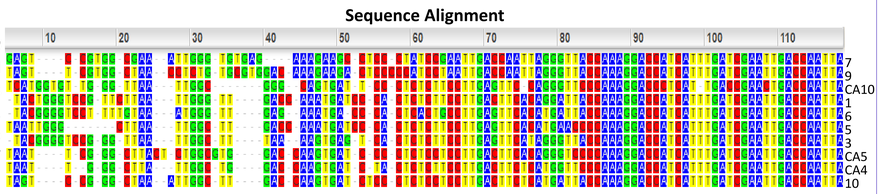


Figure 3. Alignment of sequences received from all 10 samples. Alignment performed by webPRANK and viewed in Wasabi browser (www.ebi.ac.uk). Different colors indicate different bases with initial of base in colored box. Dashes show alignment software skipped base to continue alignment. Order of samples marked on right of the figure.

Conclusions & Discussion

Process

- Trying to find usable primers for genetic analysis, starting with primers previously used on *Cyp. tibeticum*.
- Tested 10 new *C. tibeticum* primers, none worked.
- Used *C. tibeticum* primer set that worked for previous student
- Note for below:
  - CA from Crossroads Academy fen – Experiment 1
  - 1 through 10 from Crossroads Academy fen – Experiment 2

Results

- 10 DNA samples with bands meaning amplification worked
- The ten samples (CA4, CA5, CA10, 1, 3, 5, 6, 7, 9, 10) had similar sequences
- Suggest genetically similar, maybe even closely related?
- No sequences matched exactly
- 1 and 6 appear to be more similar to one another than to 10; sequences were more identical to one another's than to 10

Results vs. Hypothesis

- Results do not confirm our hypothesis
- Plants closer genetically than expected
- Since only one small region sequenced, cannot draw concrete conclusions about genetic similarity

Further Work

- Repeat the experiment, in a more organized manner
- Repeat sequencing to confirm that double peaks represent actual heterozygosity, not an unclear chromatogram
- Analyze more regions in the genome for better sense of genetic difference between plants

References

National Geographic Society. "Endangered Species." National Geographic Society, 9 Oct. 2012, www.nationalgeographic.org/encyclopedia/endangered-species/.

Li, Jing, et al. "Development of Microsatellite Markers for Cypripedium Tibeticum (Orchidaceae) and Their Applicability to Two Related Species." Applications in Plant Sciences, Botanical Society of America, 21 Dec. 2017, www.ncbi.nlm.nih.gov/pmc/articles/PMC5749819/#lpr50.0000.

Owen, Wayne. "Showy Lady's Slipper (Cypripedium Reginae)." U.S. Forest Service, www.fs.fed.us/wildflowers/plant-of-the-week/cypripedium_reginae.shtml.

Showy Lady's Slipper picture - https://render.fineartamerica.com/images/rendered/default/poster/8/10/break/images-medium-5/showy-ladys-slipper-bill-morgenstern.jpg

Yellow Lady's Slipper picture - https://images.fineartamerica.com/images-medium-large/large-yellow-lady-slipper-orchid-dspf251-gerry-gantt.jpg

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