

Investigating Plant Evolution Via Morphological And Molecular Phylogenetic Analysis

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Summary

This laboratory exercise explores plant diversity and evolution through phylogenetic analyses of 1) plant morphological and anatomical data collected by students and 2) molecular data provided to students to promote deeper understanding of plant structural features, plant diversity, trends in plant evolution, and methods of phylogenetic analysis. In this exercise, students use morphological and genetic data to construct structural and molecular phylogenies. By comparing the separate phylogenies and combining data sets to produce a single phylogeny, students can observe how structural and molecular data support one another and provide similar perspectives on plant evolution. Likewise, differences between the phylogenies provide an opportunity for students to critically think about the evolutionary meaning of the conflicting phylogenies and determine a logical resolution to those differences.

Learning Objectives

- Strengthen knowledge of plant anatomical and morphological features.
- Identify features that define different plant groups.
- Develop skills in conducting comparative structural analysis.
- Understand the evolutionary relationships among major plant groups
- Understand the evolutionary history of important plant features (*e.g.* vascular tissue, leaves, seeds, flowers, alternation of generations).
- Develop skills needed to conduct and interpret phylogenetic analyses.
- Evaluate phylogenetic information in structural and genetic data.

I. Overview

The study of plants and evolution are frequently unfamiliar topics for many undergraduates in introductory biology courses. Botany typically receives significantly less emphasis than other organismal topics (Uno 1994, 2009), and evolution is incorrectly taught or not taught in many high school biology courses (Rutledge and Mitchell 2002, Berkman *et al.* 2008, Moore 2008). Thus, students enter introductory biology courses with many misconceptions about, little experience with, and minimal knowledge of plants and evolution (Uno 1994, 2009; Wandersee and Schussler 1999; Rutledge and Mitchell 2002), which causes them to struggle with these topics when they are encountered in university courses (Moore and Cotner 2008, 2009). This is unfortunate because evolution and botany are fundamental biological topics. Failure to understand them can hinder students' success in upper-level coursework and inhibit exploration of prospective research and career opportunities (Alters and Nelson 2002, AAAS 2011, Losos *et al.* 2013).

To address these problems, we developed a guided-inquiry, laboratory exercise that combines comparative anatomy and morphology, molecular genetics, and phylogenetics to engage students in direct, hands-on, observation of plants to explore fundamental concepts in plant evolution and develop proficiency in phylogenetic analysis. In this exercise, students must observe plants directly to collect data they will need to construct their structural phylogeny. This activity requires them to engage directly with the plant specimens they are provided and utilize knowledge of plant anatomy and morphology they have developed from previous laboratory session. Thus, the laboratory exercise prompts students to use comparative anatomy, a highly valuable approach to investigate similarities and differences among organisms, to not only explore the structural variability among organisms but to better understand the significance of that variation.

The tree-thinking approach in this exercise emphasizes constructing, reading, and interpreting phylogenetic trees as a framework to improve students' understanding of fundamental evolutionary principles such as common ancestry and lineage diversification (Donovan 2005, Baum and Offner 2008, Gregory 2008). Tree-thinking also helps students appreciate how phylogenetic analyses are an important tool

biologists use to investigate questions about evolution and relationships among taxa (Losos *et al.* 2013). Baum and Offner (2008) and Gregory (2008) provide excellent reviews of tree-thinking and how to interpret phylogenetic diagrams.

II. Procedure

This exercise was developed for an introductory-level biology course for life science majors. Most of the exercise can be completed in a single, 3-hour laboratory session, with some work some outside of laboratory. It concludes with a discussion in the following laboratory meeting. Prior to conducting this exercise, students have completed exercises on phylogeny construction similar to those developed Duncan (1984), Gendron (2000), or Goldsmith (2003) and had a laboratory session to learn basic aspects of plant anatomy and morphology. To prepare for this laboratory session, students are expected to review algal and plant diversity sections in their laboratory manual, text, or other resource.

In this laboratory module, students directly observe and collect anatomical, morphological, and other data from slides and specimens at stations representing seven plant groups: green algae, non-vascular plants, seedless vascular plants, gymnosperms, monocots, and eudicots. These groups are not all monophyletic (e.g. gymnosperms, seedless vascular plants), but they do represent major groupings of terrestrial plants. Although the structural data can be collected from texts or online resources, the benefit of this activity is that the students must directly observe and collect data from the different specimens they are provided in laboratory. Data collected from observing specimens are used to construct a **structural phylogeny** based on features students choose and score. Next, students construct a **molecular phylogeny** using one of two DNA sequence data sets provided to them. The laboratory concludes with students comparing the two phylogenies and mapping structural traits onto the molecular phylogeny.

While they conduct this exercise, students should focus their investigations on addressing three questions:

1. What relationships among taxa and patterns in plant evolution do the structural and molecular data show?

2. What are the similarities and differences between structural and molecular phylogenies?
3. What insights on plant evolution and diversification do combined structural and molecular data provide?

II.A. Materials

This exercise requires a collection of fresh and preserved plants, anatomical slides, microscopes, and computers. A list of suggested specimens is provided in Table 1. It can be expanded and adjusted based upon specimens available commercially, in greenhouses, or growing naturally. For data analyses, we provide computers with Mesquite (Maddison and Maddison, 2011), a free, multi-platform program for generating phylogenetic trees based on morphological or molecular data, already loaded. However, because this is free software, students can conduct this part of the module on their own computers. Our Mesquite User's Guide for Students is included with this exercise. The student users guide and a more detailed user's guide for instructors are available at http://www.ou.edu/gibsonlab/Tree_Thinking/Tree-Thinking_Modules.html.

II.B. Set-up

Seven stations are prepared on separate tables (Figure 1). Each table has fresh specimens and microscope slides representing a single "group" (Table 1). The algae table is identified as the outgroup, and the remaining stations are randomly labeled as Unknowns A-F.

Table 1. Materials for each plant station. Slides are whole mount (wm) longitudinal (ls), cross (cs) or tangential (ts) sections and are commercially available.

Station	Fresh or Preserved Specimens	Microscope Slides
Green Algae	<ul style="list-style-type: none"> • <i>Chlamydomonas</i> • <i>Gonium</i> • <i>Volvox</i> • <i>Chlorella</i> • <i>Spirogyra</i> • <i>Coleochaete</i> 	<ul style="list-style-type: none"> • <i>Chlamydomonas</i> (wm) • <i>Oedogonium</i> (wm) • <i>Spirogyra</i> vegetative and zygote stages (wm,) • <i>Ulva</i> (wm) • <i>Volvox</i> (wm)
Non-Vascular Plants	<ul style="list-style-type: none"> • <i>Marchantia</i> (liverwort) gametophyte • <i>Polytrichum</i> (moss) gametophyte with sporophyte • <i>Sphagnum</i> (peat moss) gametophyte 	<ul style="list-style-type: none"> • <i>Polytrichum</i> capsule (ls) • <i>Mnium</i> (moss) antheridium (ls) and archegonium (ls) • <i>Marchantia</i> sporophyte (ls)
Seedless Vascular Plants	<ul style="list-style-type: none"> • Fern sporophytes with sori • <i>Psilotum</i> (whisk fern) sporophyte • <i>Azolla</i> (water sprite) or <i>Salvinia</i> (water fern) sporophyte • <i>Equisetum</i> (horsetail) sporophyte with strobili • <i>Lycopodium</i> (club moss) sporophyte with strobili 	<ul style="list-style-type: none"> • <i>Equisetum</i> stem (cs), rhizome (cs), and strobilus (ls) • <i>Psilotum</i> stem (cs) and rhizome (cs) • <i>Lycopodium</i> stem (cs), root (cs), and strobilus (ls) • <i>Dryopteris</i> (fern) sori (cs) • Fern prothallium (wm)
Gymnosperms	<ul style="list-style-type: none"> • <i>Pinus</i> (pine) branches with needles, male and female cones • <i>Ginkgo</i> (maidenhair tree) branch with leaves • <i>Juniperus</i> branch and cones • Cycads with cones 	<ul style="list-style-type: none"> • <i>Pinus</i> macerated wood, wood sections (cs, ls, ts), male cone (cs or ls), female cone (ls) • <i>Ephedra</i> (ephedra) male cone (ls) and female cone (ls)
Monocots	<ul style="list-style-type: none"> • Orchids, iris, lilies and other species with showy flowers • <i>Zea</i> (corn) seedlings and seeds • <i>Cyperus papyrus</i> (papyrus) plant • <i>Billbergia</i>, <i>Tillandsia</i>, or other bromeliads • <i>Musa</i> (banana) fruit • Assorted grains/caryopses 	<ul style="list-style-type: none"> • <i>Lilium</i> (lily) anthers (cs) and ovary (cs), • <i>Zea</i> leaf (cs), stem (cs) and root (cs); • <i>Triticum</i> or <i>Zea</i> grain (ls)
Eudicots	<ul style="list-style-type: none"> • <i>Helianthus</i> (sunflower) flowers • <i>Solenostemon</i> (coleus) • <i>Quercus</i> (oak) branches • <i>Rosa</i> (rose) flowers • Assorted eudicots showing leaf and floral variation • Assorted dry and fleshy fruits. 	<ul style="list-style-type: none"> • <i>Helianthus</i> (sunflower) stem (cs) and root (cs) • <i>Ligustrum</i> (privet) leaf (cs) • <i>Salix</i> (willow) root (cs) • <i>Quercus</i> (oak) wood (cs, ls, ts) • <i>Pelargonium</i> (geranium) young and older stem (cs)

Figure 1. A typical set-up for a gymnosperm station.



Activity 1: Morphological & Anatomical Data Collection & Phylogenetic Analysis

1. The laboratory session begins with a brief review of the alternation of generations and plant structure.
2. During the next 60-90 minutes, students move from station to station observing specimens, recording plant traits, and comparing characteristics among specimens at each station. To fully experience the learning benefits of comparative analyses and promote inquiry and consideration of plant traits, we suggest that students initially visit stations without their laboratory manual. This encourages them to look at specimens closely to self-identify traits that may be useful in their analysis and facilitates a more active engagement with the specimens; thereby making this a stronger inquiry experience. Students should try to identify approximately 10 traits with simple, binary character states (e.g., trait present/absent, sporophyte/gametophyte dominant) that are indicative of plants at each station (Table 2). More than two character states can be used,

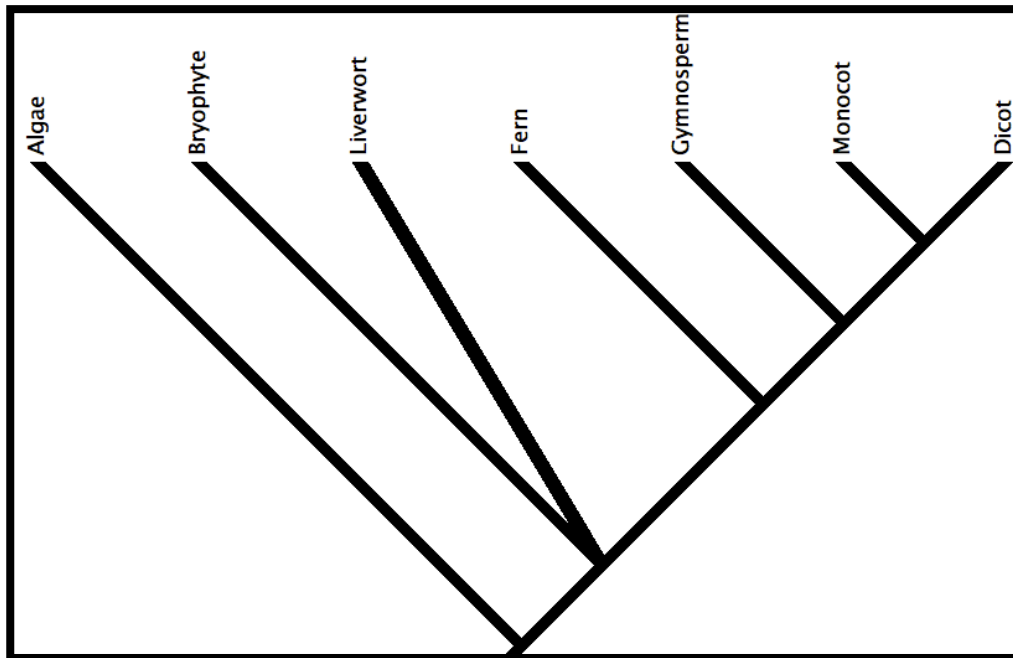
but the analysis becomes more challenging. Instructors should be aware that how students choose and code characters can produce difficulties in tree construction, but these problems are often immediately identifiable in the analysis and can be used as instantaneous indicators that students need to reconsider traits they chose.

3. After collecting data for all groups, students prepare a character matrix of traits they observed. Students enter their data into the phylogenetic analysis program and run an initial analysis of their data. This step typically generates several, equally parsimonious trees. When multiple trees are produced, students will need to create a single consensus tree that combines the results from equally parsimonious trees into a single phylogeny (Figure 2). Using this tree, students can evaluate how each of the characters they selected influences branching patterns in the tree and investigate patterns of trait evolution that define different plant groups.
4. After preparing their initial trees, the instructor identifies the “unknown” groups of plants at each station and discusses with the class what traits students identified as characteristic for each group. Afterwards, students revisit stations using their notes and laboratory manual to review specific features of groups, clarify or correct their data, and include additional traits for their analysis as needed.

Table 2. Potential traits for structural phylogeny data set construction.

Character	Character States
Dominant stage of life cycle	Sporophyte – Gametophyte
Embryo produced in archegonium or ovary	Yes – No
Flowers	Present – Absent
Flowers with parts in multiples of 3	Present – Absent
Flowers with parts in multiples of 4 or 5	Present – Absent
Free-living gametophyte	Present – Absent
Free-living sporophyte	Present – Absent
Fruits	Present – Absent
Leaves with single vein (microphyll)	Present – Absent
Leaves with multiple, branched veins (megaphyll)	Present – Absent
Ovary	Present – Absent
Ovule	Present – Absent
Pollen	Present – Absent
Roots	Present – Absent
Secondary (woody) growth	Yes – No
Seeds	Present – Absent
Strobili or cones	Present – Absent
Stamens	Present – Absent
True leaves	Present – Absent
Vascular tissue	Present – Absent
Vessel elements	Present – Absent

Figure 2. Sample consensus tree based on structural and life cycle characteristics.



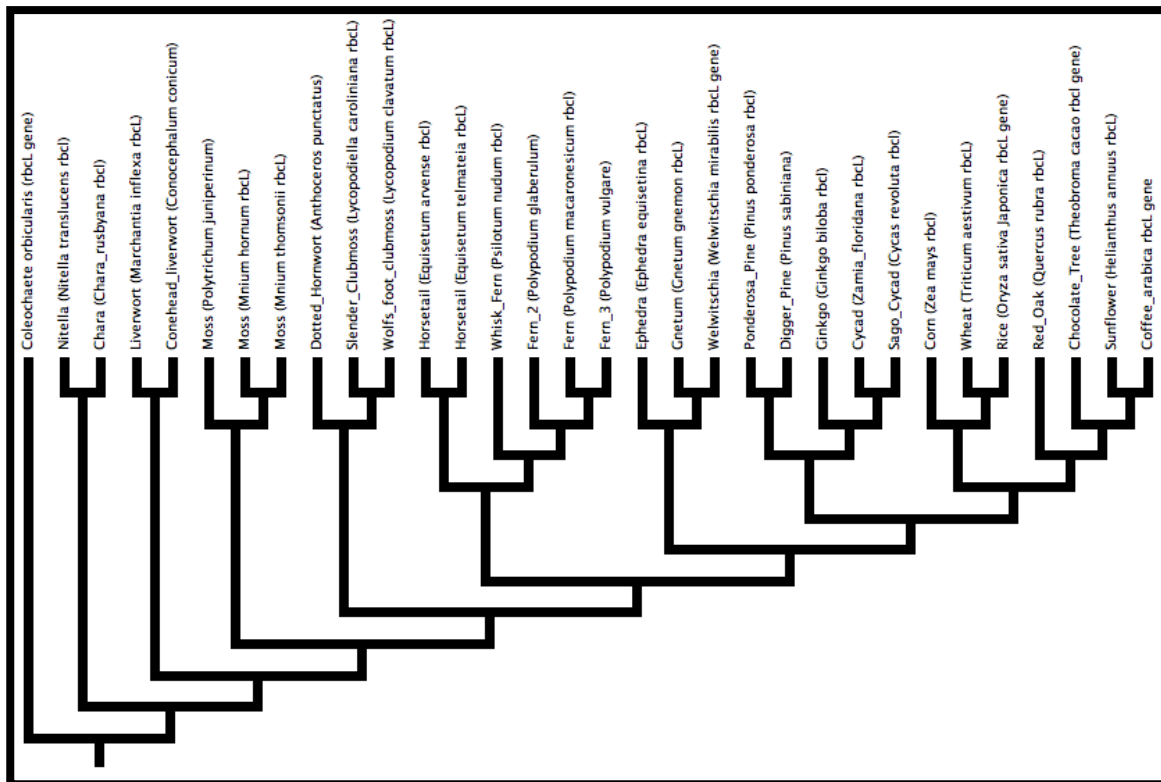
Activity 2: Phylogenetic Analysis of Molecular Data

1. Students are next given a data set with either a ribulose-1,5-bisphosphate carboxylase oxygenase large subunit (*rbcL*) gene sequences or ATPase subunitB (*atpB*) gene sequences. Both are chloroplast genes. The *rbcL* gene is highly conserved due to its critical function in the photosynthetic enzyme rubisco. Likewise, *atpB* is highly conserved in plants due to its involvement in ATP synthesis. Both have been demonstrated to be useful in plant phylogenetic analysis using molecular data. Gene sequence data sets were obtained from the National Center for Biotechnology Information (2013) Nucleotide search engine and are available with this exercise and at http://www.ou.edu/gibsonlab/Tree_Thinking/Tree-Thinking_Modules.html. It contains two or three genera from each plant group and generally corresponds to specimens from Activity 1.
2. Students import molecular data into Mesquite, align the sequence data using MUSCLE alignment software (Edgar 2004) available for free download at <http://www.drive5.com/muscle/>. The instructor should explain that alignment is

necessary so that the same positions in the gene sequence can be compared across taxa to construct a phylogeny.

3. After alignment, a phylogeny can be constructed as described for the structural data analysis. Again, several equally parsimonious trees will be generated and need to be combined into a single consensus tree (Figure 3).
4. Students evaluate the branching and topology of the consensus tree to evaluate relationships among taxa in the molecular analysis. This activity can be conducted in laboratory or completed outside of laboratory if students have access to Mesquite on laboratory or personal computers.

Figure 3. Sample *rbcL* molecular sequence data consensus tree.



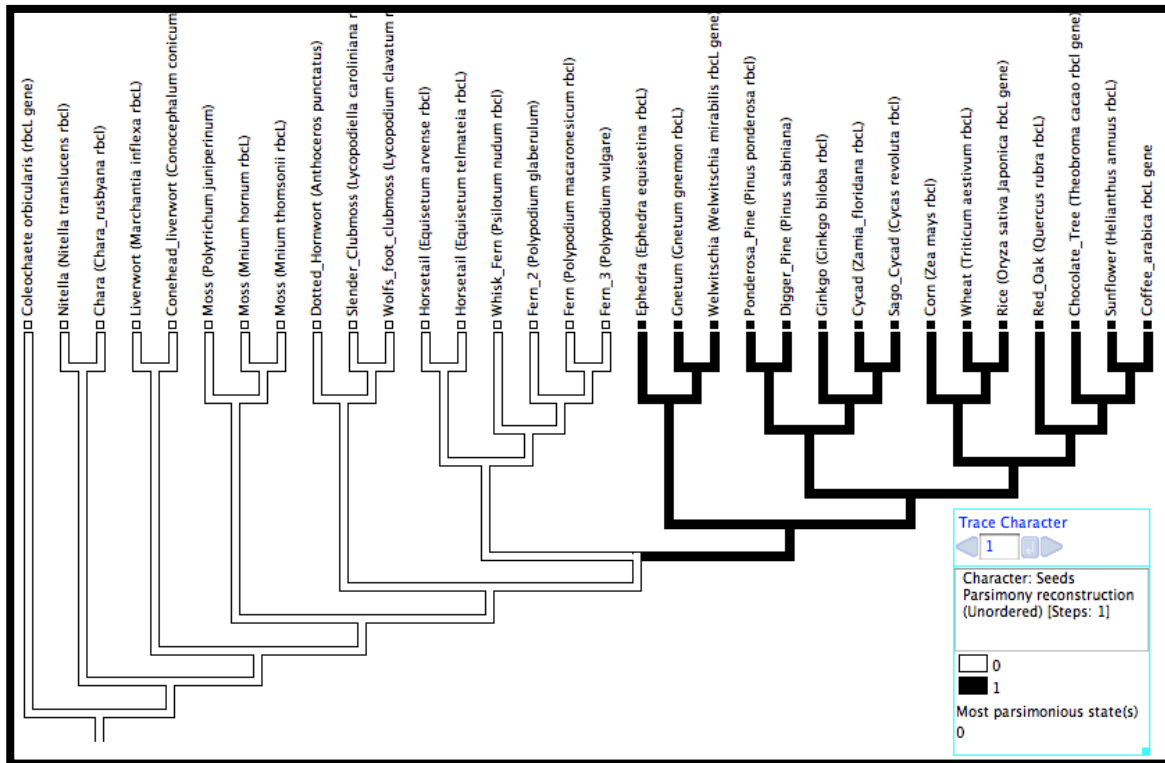
Activity 3: Comparing Trees & Combining Data

1. To complete the exercise, students are instructed to compare the structural and molecular phylogenies for similarities and differences in branching patterns and relationships among groups.
2. To evaluate what the molecular tree indicates about evolution of structural traits, students add structural data to their molecular data matrix and produce a

combined structural-molecular phylogeny. Once this is done, tools in Mesquite and other software can trace individual, structural characters to analyze their evolution (Figure 4).

- There are often minor differences between phylogenies, but they are generally similar in most instances. Likewise, polytomies with three or more taxa will occur. These should indicate to students the characters they have chosen have low phylogenetic information and that they should refine their character states or consider not using that character.

Figure 4. Sample *rbcL* molecular tree showing the trace character function.



Assessment

To complete the exercise, students prepare a written report for the next laboratory session that compares their trees, explains patterns of relationships among plant groups, and describes patterns of structural trait evolution in the different phylogenetic trees they have produced. More advanced students may want to discuss how specific mutations in the molecular data are associated with different clades.

At the beginning of the next laboratory meeting, students discuss as a class what their trees show about relationships and patterns of evolutionary relationships in plants and discuss how their analyses answer the three questions posed at the beginning of the exercise. The group discussion also provides an opportunity to explore the relative strengths and limitations of molecular and structural data, how the data conflict or corroborate in the different phylogenies, how DNA is a record of successful reproductive events passed from ancestors to descendants, and other insights students may have from the exercise.

When grading reports, the focus is on a student's ability to describe and interpret their phylogenetic trees using appropriate terminology. We evaluate accuracy of their phylogenies, ability to map structural traits onto a phylogeny, and descriptions of informative patterns and relationships in plant evolution such as changes in the alternation of generations, evolution of vascular tissue, and evolution of reproductive features.

Conclusion

The primary objective for this laboratory is to use plants as a study system to improve students' understanding of taxa as lineages whose patterns of evolutionary relationships can be shown in phylogenetic trees. The exercise gives them first hand experience with phylogenetic data collection, data analysis, and interpretation of results. It has an additional benefit of providing a platform to apply their knowledge of plant anatomy and morphology in a problem-solving context to explore plant diversity. Upon completion of the exercise, students should have an improved understanding of how phylogenetic trees are produced, the ways different types of data can be used in phylogenetic analyses, and that trees are evolutionary hypotheses of relationships among taxa.

The tree-thinking approach used in this exercise combines Dobzhansky's (1973) oft-cited observation that, "Nothing in biology makes sense except in the light of evolution," with its logical corollaries that "Nothing in evolution makes sense except in the light of phylogeny," (Society of Systematic Biologists 2001) and, "Nothing in evolution makes sense except in the light of DNA," (Kalinowski et al 2010). This

laboratory activity reinforces student understanding of evolution as a process of diversification from shared ancestry, a topic students often struggle with that is fundamental to their understanding of evolution. By using a tree-thinking approach that requires students to construct and interpret phylogenetic trees, they can examine how different lineages share traits that indicate their common ancestry and how unique traits define lineages. The exercise provides hands-on experience with phylogenetic study of plants that exposes students to different plant groups and strengthens their understanding of fundamental evolutionary concepts. Kalinowski *et al.* (2010) correctly articulate that student understanding of evolution can be strengthened and misconceptions corrected by combining the study of evolution in general and natural selection in particular with data about genes, alleles, and phenotypes to demonstrate the essential links between DNA and evolution that are basic evidence of how evolution operates. The phylogenetic approach described here expands this idea to demonstrate how molecular data support evolution on a macroevolutionary scale and give insights on how phylogenetic approaches can be used to study trait evolution among organisms.

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