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1 Untangling the Contribution of Characters to Evolutionary Relationships: A Case Study Using
2 Fossils, Morphology, and Genes

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11 **Abstract**

12 Given the importance of phylogenetic trees to understanding common ancestry and evolution,
13 they are a necessary part of the undergraduate biology curriculum. However, a number of
14 common misconceptions, such as reading across branch tips and understanding homoplasy, can
15 pose difficulties in student understanding. Students also may take phylogenetic trees to be fact,
16 instead of hypotheses. Below I outline a case study that I have used in an upper-level
17 undergraduate evolution course that utilizes shark teeth (representing fossils), body characters,
18 and mitochondrial genes. Students construct their own trees using freely available software, and
19 are prompted to compare their trees with a series of questions. Finally, students explore
20 homoplasy, polytomies, and trees as hypotheses during a class discussion period. This case study
21 gives students practice with tree-thinking, as well as demonstrating that tree topology is reliant
22 on which characters and tree-building algorithms are used.

23 Keywords: Tree thinking; Phylogeny; Shark; Evolution

24 **Introduction**

25 Phylogenetic trees are now commonplace in introductory biology textbooks (e.g. Mason, Losos,
26 and Singer 2014; Morris et al. 2016; Urey et al. 2016). Not only are there chapters on how
27 phylogenetic trees are constructed and how to read them, but phylogenetic trees are also used
28 throughout these textbooks to illustrate biodiversity, speciation, and the mechanics and patterns
29 of evolution. Given the importance of phylogenetic trees to understanding common ancestry and
30 evolution, they are a necessary part of the undergraduate biology curriculum (Baum, Smith, and
31 Donovan 2005). However, the incorporation of “tree-thinking” (*sensu* Baum, Smith, and Donovan
32 2005) into a students’ mental toolbox has been challenging. A number of studies have addressed
33 common misconceptions students have with respect to reading, interpreting, and understanding
34 phylogenetic trees (e.g. Baum, Smith, and Donovan 2005; Halverson, Pires, and Abell 2011;
35 Gregory 2008 and references therein; Meir et al. 2007; Meisel 2010 and references therein;
36 Morabito, Catley, and Novick 2010; Novick, Catley, and Funk 2010), including reading across the
37 tips of branches, being confused by the ladderized structure, confusion around the meaning of
38 nodes, and not recognizing that dissimilar organisms can share a common ancestor. These
39 common misunderstandings can persist beyond the undergraduate level, including to the
40 graduate student (Sandvik 2008) and professional levels (Crisp and Cook 2005).

41 In addition, understanding the use and construction of phylogenetic trees in biology illustrates a
42 non-experimental method of hypothesis generation and/or testing (Cooper 2004). Despite
43 explicit language in textbooks stating otherwise, students may take phylogenetic trees to be the
44 definite answer with respect to the evolutionary relationships among the included taxa
45 (Whitenack, pers. obsv). However, phylogenetic trees are hypotheses, as the topology of a given

46 tree, and therefore the relationships illustrated by the tree, is dependent on the characters and
47 tree-building algorithm chosen (Haber 2005). For example, when studying evolutionary
48 relationships among extant hammerhead sharks, using morphological characters results in a tree
49 with the hammerhead with the least extreme head shape (*Sphyrna tiburo*) as a more basal
50 species (Compagno 1988), but using molecular characters from both mitochondrial and nuclear
51 genes results in the hammerhead with the most extreme head shape (*Eusphyra blochii*) as the
52 most basal species (Lim et al. 2010). Here, I describe a case study that can be used to reinforce
53 lessons on many of the common tree-thinking misconceptions, as well as to demonstrate how
54 character choice influences tree topography.

55 **Curricular context**

56 This case study is used in two courses. The first is an upper-level undergraduate course in
57 evolution, which is typically taken by biology majors and minors. Students have been briefly
58 exposed to reading phylogenetic trees in their introductory biology sequence. Because of the
59 importance of tree-thinking to understanding evolution, phylogenetic trees are the first topic
60 covered in the upper-level evolution course. Before completing this case study, students read
61 Baum et al.'s 2005 paper on tree thinking (and complete the associated quiz), have two to three
62 class periods of lecture introducing them to phylogenetic trees and characters, and complete an
63 in-class activity in small groups that allows them to make their own phylogenetic trees of candy
64 (Whitenack 2014). The case study outlined below is given as a homework assignment, as there
65 is no laboratory period, and is used to spur discussion in the last class period that we spend on
66 phylogenetic trees exclusively. The second course is an upper-level ichthyology course that is a
67 mixture of graduate and undergraduate students.

68

69 **Organismal background**

70 This case study uses sharks (Chondrichthyes, Elasmobranchii) as the organism of interest. In
71 addition to being a charismatic group of animals that many students are generally familiar with
72 from popular culture and aquaria, sharks are one of the most common vertebrates in the fossil
73 record. However, their fossil record is overwhelmingly composed of teeth, as chondrichthyans
74 have a cartilaginous skeleton, which fossilizes poorly. On the other hand, teeth are highly
75 mineralized and individual sharks produce hundreds to thousands of them in their lifetimes
76 (Correia 1998). Because of the paucity of informative non-tooth material, evolutionary
77 relationships of fossil sharks are typically determined by tooth morphology.

78 Specifically, the elasmobranch order Lamniformes is used in this case study. Lamniformes
79 includes seven families and a morphologically diverse array of fourteen extant species, including
80 makos (*Isurus*), white sharks (*Carcharodon*), thresher sharks (*Alopias*), basking sharks

81 (*Cetorhinus*), and megamouth sharks (*Megachasma*). The sharks in this order not only vary in
82 body form, but also in tooth morphology. For example, both *Cetorhinus* and *Megachasma* are
83 filter feeders with very small teeth; *Alopias* have extremely long caudal fins and “typical” shark
84 teeth. Additionally, the Lamniformes are particularly well studied in terms of their phylogenetic
85 relationships (e.g. Bowden et al. 2015; Martin & Naylor 1997; Naylor et al. 1997; Shimada 2005;
86 Vélez-Zuazo & Agnarsson 2011).

87 This case study includes all fourteen extant species of Lamniformes, plus two outgroups:
88 *Carcharhinus plumbeus* (the sandbar shark) and *Heterodontus francisci* (the horn shark).
89 *Carcharhinus plumbeus* is in the order Carcharhiniformes, which is a sister taxon to Lamniformes,
90 while *H. francisci* is a member of the Heterodontiformes, a sister taxon to Lamniformes +
91 Carcharhiniformes. (Carvalho 1996; Maisey, Naylor, and Ward 2004; Naylor et al. 2012; Shirai
92 1996) While we do not directly use fossil species, such as *Carcharocles/Carcharodon megalodon*,
93 we simulate the use of fossils by having students also examine tooth characters (see below).

94 **The case study**

95 The following is a summary of the case study, which can be found in its full form in the appendix.
96 Students are asked to examine images of the sixteen included taxa and come up with an informal
97 phylogenetic tree and list of characters. Suggested resources for these images include FAO
98 Species Guides (Compagno 1984, 2001) which are freely available online, but students are
99 welcome to also use images from the Internet. Students usually hand-draw their trees for this
100 portion of the assignment, and use morphological characters seen from photos and drawings.

101 Next, students move on to creating phylogenetic trees from morphological data using the
102 freeware PAST (version 2.17, Hammer, Harper, and Ryan 2001) (Figure 1). Students first begin
103 with a data set that only uses tooth characters, as this is meant to simulate the fossil record
104 (Supplement). This data set is provided to the students, in Nexus format, in the interest of time
105 for this particular course (Supplement). The instructions provided to the students guide them
106 through creating a phylogenetic tree using parsimony analysis, and they are prompted to use
107 heuristic nearest neighbor interchange search with Wagner character criterion. PAST will
108 generate a number of most parsimonious trees (MPTs) and has an option to show a consensus
109 tree (either strict or majority rules). Students are asked to save the majority rules consensus tree
110 and record the number of MPTs. After their simulated-fossil tree is completed, students then
111 create a second majority rules consensus tree using a data set of non-tooth morphological data
112 from the literature, and also record the number of MPTs. Students are also asked to do the same
113 for a data set that combines the tooth and non-tooth characters that they used previously.
114 Finally, students are provided with a consensus tree based on cytochrome-b and NADH mtDNA
115 (Naylor et al. 1997). Later in the course, students gain experience making trees from molecular
116 data as we discuss molecular clocks and neutral theory, so I did not include it in this particular

117 assignment. However, we do come back to this case study during class discussion at that point in
118 the semester.

119 Every time a student completes a new phylogenetic tree, they are asked to compare and contrast
120 all of the generated and provided trees. Students work through the different evolutionary
121 relationships illustrated in each tree, which requires them to read trees correctly in general
122 (understanding nodes, not reading across the tips of branches). This also requires them to
123 examine differences in polytomy location and anatomy. This allows the students to understand
124 not only do different characters yield different results, but also each tree also contains unknown
125 relationships.

126 Last, students are asked to consider the following questions for our class discussion, which are
127 not part of the graded assignment:

128 (1) Which one do you think is the “right tree” (molecular, morphological, fossil, or
129 morphological + fossil) and why? How would you argue your point to a classmate who
130 disagrees?

131 (2) Notice the position of *C. maximus* and *M. pelagios* on each tree, and look at the FAO
132 Guide’s information on these sharks. Why do we see different relationships on the
133 morphological trees than we do on the molecular tree?

134 (3) On the fossil tree, look at the position of *C. plumbeus*. Even though it’s an outgroup and
135 not part of Lamniformes, it seems to be included in Lamniformes on this tree and not the
136 others. Why might this be?

137 **Potential modifications**

138 This case study would be easily adaptable into a lab activity. Students could compile their own
139 data in a more formal way using the literature and Internet sources, instead of using provided
140 data. In this case, it would be helpful to provide a template of the Nexus file format. Students
141 could also download the genetic sequences from GenBank and create their own molecular trees;
142 all of these taxa have mitochondrial DNA sequences available.

143 **Educational implications**

144 On the day that this assignment is due in class, we dedicate the class period to discussing the
145 three discussion questions. The first question requires them to convince a classmate that their
146 opinion is the “correct one,” and asks the student to consider the strengths and weaknesses of
147 different types of characters. Students discuss this question in small groups, and often do not
148 come to a conclusion with regards to one “right” tree. This question also is effective at reinforcing
149 the idea that trees are hypotheses and thus there is no “right” tree, when we come back to
150 discussing this question as a class.

151 The last two discussion questions touch on homoplasy and how that can influence tree topology.
152 When examining tooth shape of the sharks in question, it becomes apparent that there is a link
153 between tooth shape and function. Filter feeding sharks (*C. maximus* and *M. pelagios*) have small
154 non-functional teeth, whereas the other sharks in the case study all use their teeth for processing
155 prey (Compagno 1984, 2001). *Heterodontus francisci*, one of the outgroups, has molariform teeth
156 for crushing hard prey, which no lamniform has (Compagno 2001). On the other hand, *C.*
157 *plumbeus* has blade-like teeth and tends to eat softer prey, much like the other non-filter-feeding
158 lamniforms (Compagno 1984, 2001). These two questions together lead to discussion about
159 adaptation, functional morphology, and what paleontologists need to consider when using teeth
160 as a taxonomic character. This also serves as a lead-in for the next part of the evolution course,
161 which focuses on adaptation.

162 This case study also requires students to exercise their tree-reading and interpreting skills. In
163 order to answer questions about relatedness and homoplasy, students must be able to
164 successfully find common ancestors and sister groups. This addresses the common
165 misconceptions of reading along the tips and understanding the meaning of nodes (e.g. Baum,
166 Smith, and Donovan 2005; Gregory 2008; Morabito, Catley, and Novick 2010). Having students
167 examine two possible cases of homoplasy also addresses the misconception of similarity versus
168 relatedness (Gregory 2008).

169 **Preliminary Learning Outcome Evaluation:**

170 This case study has been used in five semesters at Allegheny College, from 2010-2015 (total
171 students N=147). Students average (+SD) 86.2+16.3% on the assignment, and 83.8+12.2% on the
172 subsequent exam that includes the material.

173 In addition, during the Fall semester of 2017, students in the Ichthyology (course W4112) course
174 at Columbia University (N=14) were given identical pre- and post-case study assessments to
175 evaluate the impact of the case study and associated lectures. The assessment consisted of six
176 questions, modified from Baum et al. 2005 and a review question from Bergstrom & Dugatkin's
177 evolution textbook (2012). We calculated the change in the average student performance on the
178 assessment using an unpaired T-test. Changes in individual student performance on the six
179 assessment questions were evaluated using McNemar's test in QuickCalcs (GraphPad Software,
180 La Jolla California USA). This test is similar to a Chi Square test and is appropriate for changes in
181 paired binary (e.g. correct/incorrect answers) data. (Hu et al. 1998)

182 On average the students overall performance improved from a mean 48.8% correct on the pre-
183 test (0.17% SD) to a mean post-test performance of 59.5% (SD=0.26%), an increase of 10.7%
184 points or 21% over the initial scores (Table 1). These means were nonsignificant at $\alpha=0.05$ ($t=$
185 1.727, $df=13$, $P=0.107$), although we did have a small sample size and therefore low power. The

186 questions that dealt with character states, reading incongruent trees from different data sets,
187 and homoplasy showed the greatest increase in student comprehension (an increase in average
188 of 21.4%, 28.6%, and 14.3%; $P = 0.24$, 0.24 , and 0.63 respectively) (Table 1). We emphasize that
189 these preliminary results are from a small sample size that influences the P-values and power of
190 the test. Qualitatively, we found that our students had a richer understanding of phylogenies and
191 tree thinking after doing this exercise; students' grades have increased and their subsequent in-
192 class discussions about phylogenies are more mature and are able to incorporate nuances of
193 phylogenetic thought. Replications over multiple semesters will deliver a richer data set more
194 appropriate for robust statistical analyses.

195 **Conclusions**

196 Tree thinking is a critical step in developing a thorough understanding of evolutionary biology.
197 Moreover as phylogenies underscore much of ecology and conservation, it is important that our
198 graduates recognize the multitude of ways that understanding phylogenetics provides lenses for
199 understanding biodiversity. Here we outline a simple, low-cost, yet theoretically well-developed
200 case study that helps students meet core learning objectives centered on phylogenies. Our
201 preliminary results indicate that students are not only gaining a quantifiable increase in
202 competency in the material, but more importantly, qualitatively, that they are incorporating
203 these new tools into critical thinking and reasoning skills.

204

205 **Acknowledgments**

206 Thank you to Tricia Humphreys and our anonymous reviewers for their valuable comments. Use
207 of student grade data was approved by the Allegheny College Institutional Research Board (IRB).
208 Assessment procedures and analysis were approved by the Columbia University IRB.

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291

292 **Figure captions**

293 Figure 1. Majority consensus trees generated in PAST. (A) Tooth data, representing 7 most
294 parsimonious trees (MPTs). (B) Non-tooth morphological data, representing 96 MPTs. (C)
295 Combination of tooth & non-tooth morphological data, representing 5 MPTs.

296 **Table captions**

297 Table 1. Preliminary pre- and post-test results (N = 14 students), including the results of
298 McNemar's test (df = 1 for all tests).