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Implications of ancient DNA for phylogenetic studies

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Abstract. The utility of DNA sequence characters from fossil specimens is examined from a phylogenetic perspective. Four ways that fossil characters can alter phylogenetic hypotheses are discussed. Two empirical examples and a third hypothetical example concerning amber-preserved insects are presented to illustrate these phenomena. Fossil DNA sequences as characters will be affected by the problem of missing data and missing taxa. In general, cladogram accuracy will be more greatly affected by missing taxa and cladogram resolution will be affected more acutely by missing data. Due to these points, an examination of the importance of the phylogenetic question being addressed, the utility of the fossil DNA sequences and the rarity of the fossil should be considered before damage of a fossil is undertaken.
Key words. Ancient DNA; insect systematics; fossils; cladistics; DNA sequences.

Introduction

The utility of fossils in systematic studies has been the subject of intense debate. Hennig[21] recognized that phylogenetic systematics could be applied to living organisms and extinct organisms, both individually and simultaneously[10] and that fossils could aid in the detection of convergence. Concomitant with these functional aspects of fossils, Hennig[21] also pointed to several shortcomings in fossil data, the most critical being that fossils are often more morphologically fragmented than living specimens and thus might have less of an impact on determining phylogenetic relationships. Yet a great deal of his research was spent examining phylogenetic relationships of Baltic amber flies. Patterson[30] made the strong statement that, operationally, fossils will have little if any impact on the establishment of relationships among extant taxa.

More recently, Goodman[16] claimed that since fossil remains were not amenable to genetic analysis, fossils should take a backseat in phylogenetic reconstruction. In this view, fossils are to be used as 'embellishments' of genetically and morphologically based extant taxon phylogenies[29]. Several strong systematic arguments against this view have appeared[11,14,29] and, in fact, it is now possible to compare the DNA sequences of long extinct organisms through the Polymerase Chain Reaction (PCR) and DNA sequencing techniques[2–4,8,15]. Novacek[29] suggests that we should be beyond the debate as to whether fossils are useful. We should rather, be more interested in those analytical problems that accompany phylogenetic reconstruction.

In this paper, certain predictions made from an understanding of phylogenetic analysis are examined that are pertinent to incorporating ancient DNA (and indeed any fossil characters) into phylogenetic analysis. The strengths and limitations of phylogenetic analysis using ancient DNA are examined. The effects of missing fossil DNA data and the length of DNA sequences obtained from fossils are also discussed. My preference is for cladistic methodology and hence only this approach will be discussed with respect to the predictions, questions, strengths and limitations involved. The use of amber-preserved fossils (since these fossils will be the best preserved and most likely to yield useful molecular information) is the focal point of this discussion. Amber appears to preserve DNA more consistently than any other kind of fossilization. To date, several examples of DNA isolated from amber-preserved organisms have been published[2–4,7,8,32]. The consistent preservation of DNA in amber must be attributable to the same processes that preserve entire organs, tissues and cellular ultrastructure with a high degree of fidelity[19,22–24]. Rapid fixation by low molecular weight aldehydes and isoprenes, dehydration and bactericidal activity in amber all contribute to the remarkable preservation of tissue[26,27].

Topological changes after addition of taxa

The first and perhaps most pressing factor involved with the use of fossils in systematic studies is the effect that the addition of taxa (either extinct or extant) will have on topology. As stated earlier, Patterson[30] suggested that fossils would have little if any impact on cladogram topology. This statement was made because of the predominant and perhaps false impression that most fossils are so incomplete that they could not be accurately placed in a phylogeny. Since Patterson's statement[30], several studies have shown that fossil character state information can alter cladogram topology. Table 1 summarizes four major effects that fossil character state information will have on phylogenetic analysis[10,12,14,29]. In the following discussion I

Table 1. Topological changes in cladograms produced by the addition of fossils.

Extant taxon scenario	Effect of fossil information	Reference
1. Limited character evidence for particular nodes	1. Overturn the weakly supported nodes	10, 29
2. Long branches with numerous apomorphies	2. Partition clades along the long branch and alter topology	10, 14, 29
3. Well established sister group relationships among taxa where distinct alternative hypotheses are evident as discrete dichotomous alternatives	3. Fossil plesiomorphic sister taxa will alter or switch the sister-group relationships	10, 29
4. Highly resolved dichotomies in basal branches of extant taxon phylogeny	4. Addition of fossil will alter topology in parts of the phylogeny	29

Details and examples are discussed in text

examine some of these effects using three amber fossil examples, the first with a completed study, the second with a work in progress and the final with a planned study.

Termites

The first and perhaps most common effect in table 1 will occur in situations where a cladogram for a set of extant taxa results in a phylogeny with certain relationships that are only marginally supported. Often phylogenetic hypotheses obtained for extant taxa can be controversial, supported by only weak character evidence. Addition of fossil information can sway the weight of the evidence to support a hypothesis that is at odds with the current understanding of a phylogeny, or alter a phylogeny that is not congruent with accepted morphological understanding.

In such cases, fossil evidence is most likely to alter relationships of clades that have the weakest support. This is exactly the case discussed in DeSalle et al.[8] where addition of DNA sequence information of an extinct taxon alters the topology of a cladogram. (fig. 1). The classical understanding of insect phylogeny is that termites evolved as a grade from cockroaches. In particular, certain termite families such as the Mastotermitidae were thought to be more primitive and perhaps to have phylogenetic links to the cockroaches (fig. 1a), based on a suite of retained morphological features. A molecular systematics approach was used to examine these phylogenetic hypotheses using several isopteran families (Mastotermitidae, Kalotermitidae and Termopsidae), cockroaches (*Blaberus*), mantids (*Mantis*) and several outgroup taxa (*Drosophila*, *Warramaba*, *Pteronarcys*). DNA sequence data from the 18S ribosomal RNA gene were used to generate phylogenetic hypotheses concerning the extant taxa (fig. 1b). The placement of the extant Mastotermitidae was ambiguous from this preliminary analysis of the molecular data. The extant *M. darwiniensis* was hypothesized as either the sister taxon of the other two isopteran taxa or sister to the other Isoptera and the mantid. Ambiguity as to the placement of *M. darwiniensis* is most assuredly due to weak character support for either hypothesis (fig. 1b). Addition of the fossil specimen, *M. electrodominicus* adds support for the isopteran clade and produces the parsimony cladogram in figure 1c. In this case the hypothesis of mantids being embedded in the Isoptera is overturned by the addition of the fossil DNA sequence.

Another interesting aspect of the 18S phylogeny is the exclusion of the hypothesis that cockroaches graded into termites and that Mastotermitidae are ancestral taxa or missing links. In addition, Thorne and Carpenter[35] have also overturned these classical scenarios on morphological grounds. I have added a short segment of sequence information for *Cryptocercus* to the data base and present here a total evidence phylogeny including this taxon (fig. 2). This cladogram is highly concordant with the scheme in Thorne and Carpenter[35]. The morphological data set is the stronger of the two character sets. This high degree of concordance stems from the fact that the DNA sequences do not conflict with the morphological data, but do not add a great deal of resolution to certain parts of the cladogram.

Wood gnats

In some situations, strong statements on the monophyly of certain extant groups can be made whereas higher level relationships between such groups are either poorly resolved or misleading. Such a situation results in character evolution where the groups are defined by large numbers of autapomorphies that are distributed on the branch leading to the strongly supported monophyletic group (the second effect in table 1). Numerous autapomorphies, in fact, can seriously impede the ability to accurately place a taxon in a phylogeny. Addition of fossil character state information results in the reassignment of these autapomorphic characters as

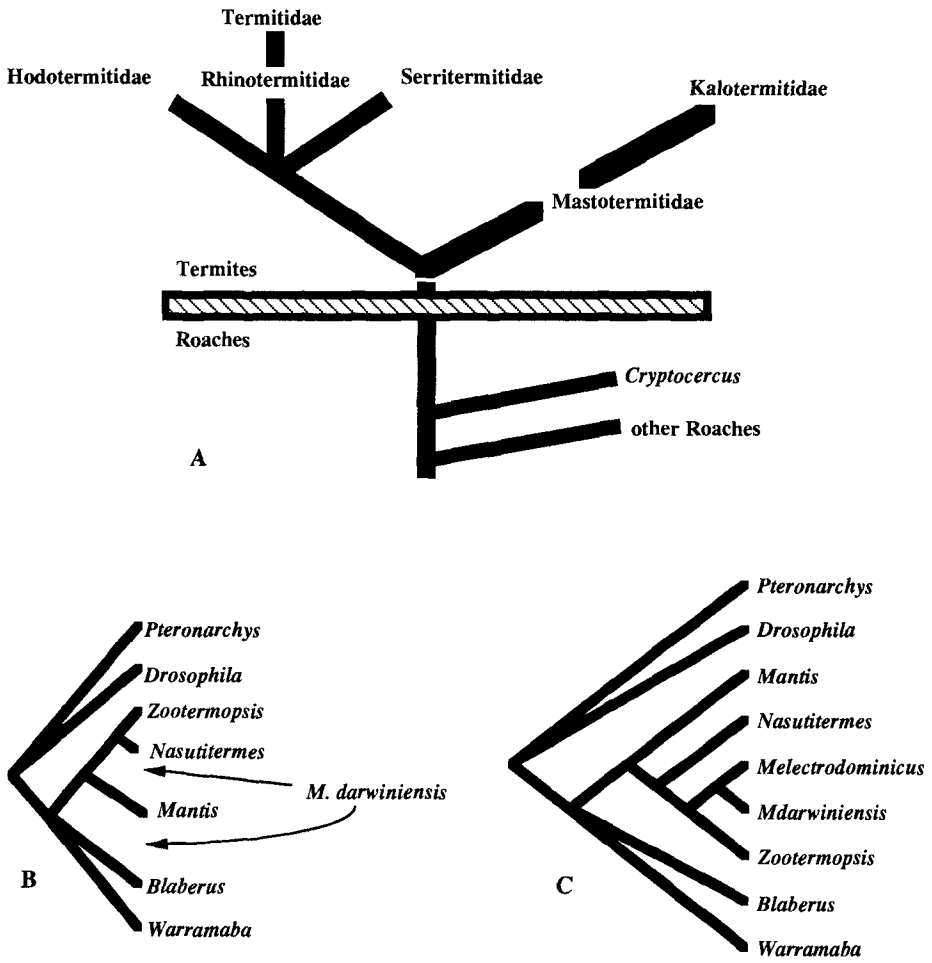

Figure 1. Phylogenetic analysis of Dictyopteran relationships using fossil DNA. *A* Classical interpretation of termite and cockroach relationships. Redrawn from DeSalle et al.[8]. *B* Analysis of 400 bases of 18S rDNA sequence without the fossil taxon *Mastotermes electrodominicus*. Note that the extant *M. darwiniensis* can be placed in two positions in the cladogram hypothesizing rather different relationships. *C* Analysis of the molecular data including the fossil DNA sequences. Note that the position of the genus *Mastotermes* now becomes resolved as the sister to other termites and that the genus is monophyletic.

synapomorphies at a higher taxonomic level. Gauthier et al.[14] describe the case of amniote relationships (birds, mammals, lepidosaurs, crocodilians and turtles) where several morphological characters support the monophyly of extant taxa in each of the five groups. The relationships of the five groups to each other using extant taxa, however, is controversial. Addition of fossil amniotes breaks the long autapomorphic branches of the phylogeny into shorter more intermediate steps by addition of taxa that are closer to the most basal part of the amniote cladogram and results in several of the autapomorphies being distributed as synapomorphies. In addition, by adding fossil morphological evidence several new characters are added that are important to resolving basal branches in the phylogeny. Gauthier et al.[14] point out that this is due to certain fossil characters having been either lost or so extremely transformed in extant groups that the extant group relationships are unaffected by these fossil characters due to a lack of information.

An example of the second effect from table 1 using fossil DNA sequences concerns a wood gnat, family Anisopodidae (*Valeseguya disjuncta*). Only one extant species exists in the genus from Australia and one extinct species in Dominican amber[18a]. Moreover, the genus is probably basal to other living genera of wood gnats in the family Anisiopodidae so it is an example of a long branch. Morphologically these insects are distinct and very autapomorphic. Oddly, the extinct specimens of this genus are numerous with over 50 males and females versus only two males known for the living species. Due to the rarity of fossils, most groups will have more extant exemplars than extinct exemplars. Some mammals follow this pattern of having more fossil taxa, such as horses and elephants[28], but in general most groups have more extant examples than extinct.

Morphologically the extinct forms are very similar to the extant form and so little morphological character evidence for breaking the long branch into shorter branches with these fossils exists. However, it could be

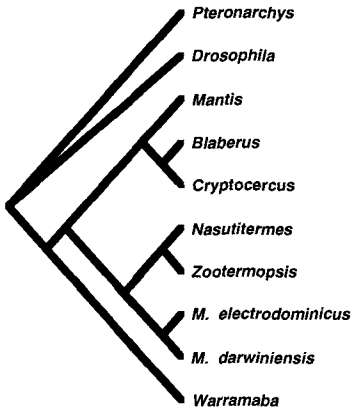

Figure 2. Morphological and 18S rDNA B fragment[8] data were combined and the basal cockroach *Cryptocercus* was added to the combined data set. One parsimony tree was obtained using the 74 morphological characters and 115 molecular characters. The tree is 131 steps long and has a CI = 0.85 and RI = 0.88. Note that the total evidence cladogram is very similar to the hypothesis of Thorne and Carpenter[35]. The molecular characters when analyzed alone give three parsimony trees that are, in general, congruent with the morphological data set, except that the mantid is seen as a sister to the Isoptera (not shown).

possible to break the long branch to the extant form using DNA sequences. A first step in determining the feasibility of this approach is the demonstration of the isolation and characterization of fossil wood gnat DNA. Procedures similar to those for the isolation of the amber termite DNA were used, except that the fossil DNA was isolated using CHELEX. We isolated DNA from three individual amber-preserved wood gnats and used the 18S rDNA primers to generate DNA sequence information. Figure 3 gives the details of the isolation, amplification and sequencing of these specimens and lists the sequences pertinent to the discussion of the implications of these sequences. Final assessment of the congruence of the placement of wood gnats awaits using the sequences shown in figure 3 and the isolation and characterization of DNA sequences from extant anisopodids. Due to the fact that we prefer to manipulate the fossil taxa well before any extant forms are introduced into the lab we have not yet attempted this. Figure 3, however, demonstrates that the DNA sequences we obtained from the amber wood gnat specimen is dipteran and in fact basal in the phylogeny. The placement of this dipteran as most basal disagrees with classical dipteran systematics but the general placement of the fossil with other 'Nematocera' is correct. No doubt, additional taxa, whether extinct or extant, will have major effects on the topology of this cladogram. We have demonstrated that it is possible to isolate and characterize DNA sequences from these extinct wood gnats and that the DNA sequences will possibly break the long branches to the extant form. All of these extinct specimens are from Dominican amber spanning a time period of from 20 to 30 million years ago (a critique of the oft cited 25 to 40 million year age of Dominican amber is in preparation; Grimaldi, pers. commun.) so a 10 million year range of character state changes is possible if enough amber preserved wood gnats are sampled from appropriate mines.

Fruit flies

The third example in table 1 concerns plesiomorphic sister taxa and their effects on phylogenetic analysis. A

A

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Drosophila gcctgcggcttaatttgactcaac acgggaaaacttaccaggt-cgaacataagtgtgtaagacagattgatagctctttctcg aatctatgggtggtggtgcat
anisoamb   gcctgcggcttaatttgactcaac acgggaaaacttaccaggtccgaacatnnnnnnntaagacagattgatagctctttctcg aatctgtgggtggtggtgcat
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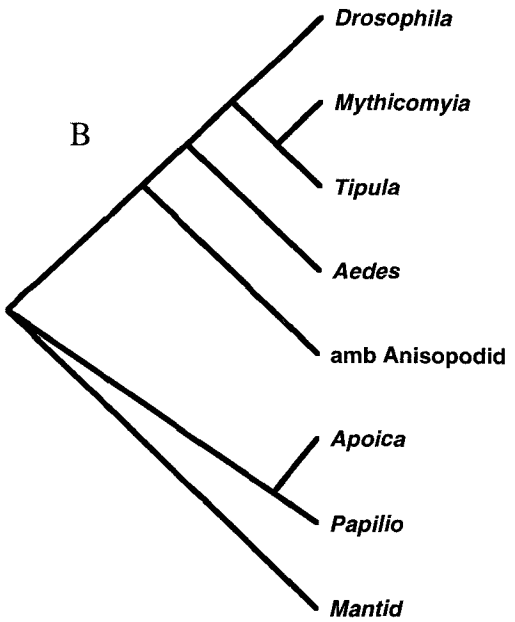

Figure 3. *A* 18S rDNA sequence of a 20–30 million-year-old anisopodid aligned to the corresponding sequence from *D. melanogaster*. *B* Cladogram generated from the 18S rDNA data in *A* in combination with other insect species (see below). All isolations were done in a part of the AMNH lab complex where dipteran DNA had not been previously manipulated. Sterile positive displacement pipette tips were used in all manipulations. DNA was isolated from an amber preserved wood gnat using CHELEX. The DNA was then amplified using the 18S primers described in DeSalle et al.[8] A reagent control and a large DNA control[8] were run simultaneously with the fossil DNA amplification. Only when both controls showed absolutely no amplification were the fossil PCR products analyzed. PCR products were purified using GENECLEAN and double strand sequenced using SEQUENASE. Sequences were read and placed into a data matrix with several dipteran sequences and examples of other insects kindly provided by M. Whiting (pers. commun.). n's indicate inability to assign a base unambiguously for this position. Phylogenetic analysis was done with PAUP[34]. This strict consensus cladogram was constructed using 200 molecular characters. The parsimony trees used to construct this tree are 32 steps long and have an RI = 0.94 and a CI = 0.92.

plesiomorphic sister taxon has at least one derived character that is shared with a terminal taxon but possesses the ancestral condition for all other informative traits. In this case, sister-group relationships can be altered by the addition of this plesiomorphic sister taxon. Donoghue et al.[10] discuss this possibility and Novacek[29] elaborates on this subject with a hypothetical example. This prediction will be most important where distinct alternative hypotheses are evident as in the discrete dichotomous alternatives in figure 4. In most real situations, however, certain parts of phylogenies are not well resolved due to character conflict among the taxa involved. Figure 4 gives a hypothetical example of three extant taxa X, Y and Z with character states for six characters as in table 2. The most parsimonious solution for these data hypothesizes a YZ sister group relationship. A fossil taxon (F) that has the derived morphological characters of one of the extant taxa and plesiomorphic DNA characters is added to the data set. In this case the parsimony solution overturns the YZ sister-group relationship supported in the extant data set and supports, rather, a ZX sister grouping.

Donoghue et al.[10] state that the most profound topological changes in cladistic analysis will occur in phylogenies where taxa have been added that necessarily introduce character conflict. In particular, when a fossil is added that has the derived state of a character of one of the terminal taxa, but has ancestral character states for every other character in the data set then the effect on the final tree topology will be the most pronounced. This is exactly the situation that could be attained for addition of fossil DNA sequences in studies of Drosophilidae systematics (fig. 5). The molecular and morphological data base for these insects is quite substantial[6, 9, 18, 25, 31, 33]. Figure 5 shows a phylogeny of the Drosophilidae based on morphological and molecular information with the position of several fossils[17] mapped onto the tree.

In the case of the Drosophilidae, the fossil taxa mapped on the tree in figure 5 all have derived morphological characters, that allowed Grimaldi[17] to reconstruct a phylogeny including these fossils and several extant taxa. So fossil Drosophilidae will fulfil the first requirement for a plesiomorphic sister taxon – that at least one derived character be present in the taxon. The critical data then become the molecular data. If the DNA sequences have plesiomorphic character states then the extant taxa plylogenies will most likely be overturned.

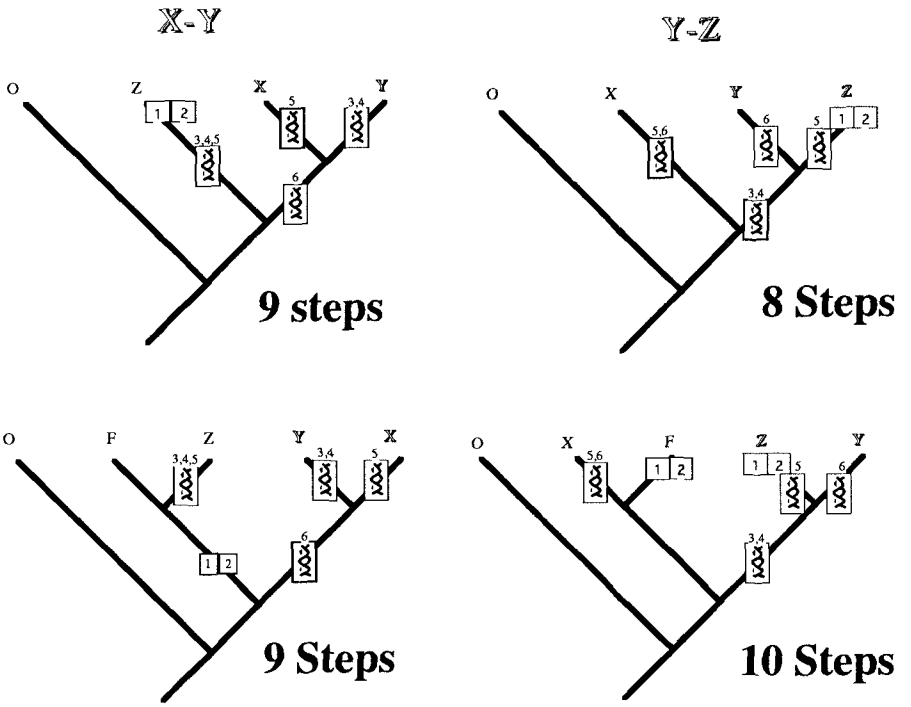

Figure 4. Hypothetical example showing the effect of adding a plesiomorphic fossil sister taxon to an analysis. Characters are numbered as in table 2 with DNA sequence characters represented by a helix and morphological characters in boxes. The top panel shows the most parsimonious character distribution for the extant taxa (X, Y and Z) in table 2 under different hypotheses of sister group relationships. The tree on the left hypothesizes an XY sister group relationship and is nine steps long. The tree on the right hypothesizes a YZ sister group relationship and is eight steps long. The third possible hypothesis (an XZ sister grouping) is ten steps long and is not shown. The most parsimonious sister group hypothesis, therefore, is an XY sister grouping. The bottom panel shows the effect of adding a plesiomorphic fossil sister taxon (F) with the character states shown in table 2. The tree on the left hypothesizes an XY sister group relationship and is nine steps long while the tree on the right hypothesizes a YZ sister group relationship and is ten steps long. Note that the most parsimonious hypothesis after addition of the fossil (F) is now a YZ sister group relationship.

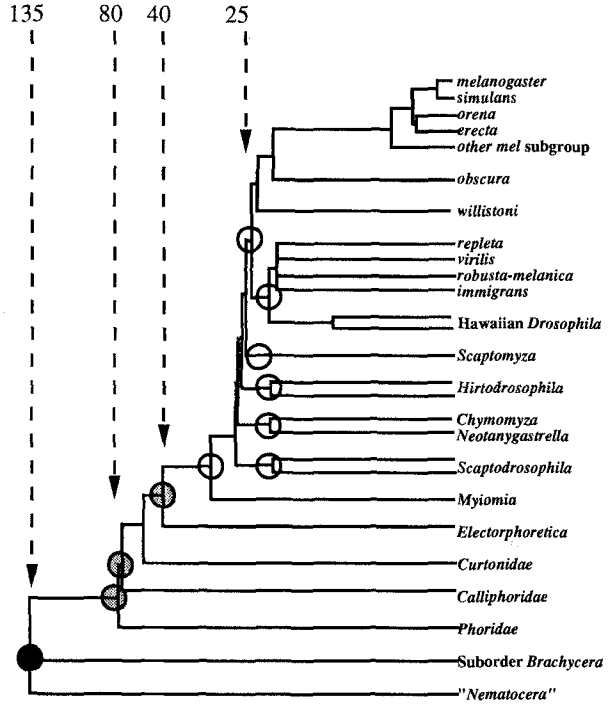

Figure 5. Phylogenetic relationships of several genera, subgenera and species groups in the Drosophilidae with fossils mapped onto the tree. Arrows indicate the approximate latest ages of the fossils.

Strength of inference, missing data and length of fossil sequence

A current controversy in systematic studies concerns the problem of strength or robustness of inference. Donoghue et al.[10] and Novacek[29] implicitly discuss the effect of adding taxa to analyses where character support for particular nodes is weak. In fact, the greatest effects on tree topology will occur in trees where character support is limited. The robustness of a phylogenetic hypothesis reflects the characters supporting the nodes in a tree. This appears to be the approach that is the most consistent with phylogenetic systematics as formulated by Hennig[21]. Other methods exist such as bootstrapping[13] and decay indices[11]. Both techniques attempt to impart a metric or statistic to nodes that should indicate the relative strength of that node. A summary of the bootstrap and decay index information for three ancient DNA studies is presented in table 3 to indicate the ranges of values that will be obtained for particular molecules, lengths of the sequence information and systematic question. This table demonstrates that some of the nodes in these fossil DNA studies are well supported using bootstrap values. The decay indices are actually a bit more informative in that sister-group relationships of the fossil taxon to its nearest sister group is only weakly supported in both the nemonychid study[2] and anisopodid study (still in progress), while in the termite study it takes trees four steps greater than parsimony to break the sister-group relationships. Conversely, the nodes more basal to the fossil and its sister group are more robust in the nemonychid and anisopodid study and relatively weak in the termite study.

Missing data has also been a major problem in fossil studies. Due to the supposed fragmentary nature of fossils, morphological data are often coded as missing for certain characters. This is particularly true of mineralized fossils where a great deal of dissociation of body parts occurs during fossilization. For amber fossils, nearly complete external morphologies can be obtained, but internal morphology is difficult to observe. Fossil DNA may not be different in that, due to the highly

Table 2. Hypothetical character state matrix for plesiomorphic sister group example.

Taxon	Morphological characters	DNA characters
	1 2	3 4 5 6
X	0 0	g g a a
Y	0 0	a a g a
Z	1 1	a a a g
O	0 0	g g g g
F	1 1	g g g g

Characters 1 and 2 are morphological and 3 thru 6 are DNA sequence characters. X, Y and Z are extant taxa with O as the outgroup. F is the plesiomorphic sister fossil taxon.

Table 3. Bootstrap and decay indices for insect fossil DNA studies.

Study	Reference	Number of bases	Parsimony trees	Bootstrap		DI	
				A	B	A	B
Nemonychid	2	315	1	76	84	1	3
Anisopodid	this study	200	1	67	100	1	10
Mastotermes	8	215	3	85	29	4	1

All studies used the 18S nuclear rDNA gene. DI refers to the decay index. Bootstrap values were determined by performing 1000 replicates. The bootstrap values and the DI's are given for A) the node including the fossil and its sister taxon and B) for the node just basal to A. These values are indicative of values that appear at other nodes in the trees. The Nemonychid parsimony tree is identical to the distance tree in Cano et al.[2] The Anisopodid tree is shown in figure 5. The consensus tree for the *Mastotermes* study is given in DeSalle et al.[8]

Table 4. Missing data due to alignment ambiguity in some selected genes.

Study	Bases missing	Bases	System (N)	Gene
Van Le et al.[36]	532	143	Gnathostomes (38)	18S
Hedges and Bezy[20]	397	50	Xantusids (8)	16S
Baldwin[1]	659	26	Compositae (14)	ITS
deSa and Hillis[5]	1486	240	Amphibians (4)	18S and 28S
DeSalle[6]	905	0	Drosophilidae (15)	16S
Wheeler et al.[38]	699	38	Arthropods (24)	18S

Three structural rRNA genes were chosen and publications reporting the use of these genes in systematics were examined. Alignment ambiguity was the major source of missing data in most of these studies. In the Wheeler et al.[38] study gaps were coded as missing data and hence the value for that study reflects the coding of gaps as missing and not caused by removing data due to ambiguity. N indicates number of taxa in the study.

degraded nature of most fossil DNA preps, only small fragments can be amplified and analyzed, greatly limiting the amounts of sequence that can be obtained relative to extant taxa. This dilemma suggests that the problems encountered with missing data in fossil morphological character sets will be similar to those for fossil DNA sequences.

Donoghue et al.[10] examine the amounts of missing data in morphological fossil studies and point out that there are two kinds of missing data relevant to fossils. The first concerns missing data as a result of the non-preservation of fossils, and the second is missing data produced by 'divergence'. In their analysis of the Gauthier et al.[14] study, incompleteness due to non-preservation is completely restricted to fossils. On the other hand, the missing characters due to divergence occur in both fossils and extant taxa. More surprisingly is the fact that, of the 411 cases of missing data on hard body parts in the study of Gauthier et al.[14] only 79 are cases due to non-preservation. In addition, extant taxa are missing on average about twice as many characters as fossils. In some extreme cases (turtles and mammals) extant taxa are missing information for up to 43 characters.

The main cause of missing data in DNA sequencing studies stems from sequence alignment[37]. DNA sequence alignment problems are produced by divergence of sequence regions that accrue insertions and deletions. This phenomenon in DNA sequence alignment is exactly analogous to the insertion of missing data in morphological studies produced by divergence. Some studies treat gaps as missing data[38] and in fact PAUP by default scores a gap as missing[34]. This problem will be most acute in structural RNA sequences such as mitochondrial 12S and 16S genes and nuclear 18S and 28S genes. Some authors have used alignment ambiguity to eliminate characters from analyses and these positions could also be considered a class of missing data. DNA studies may actually be no different than morphological studies as concerns the amount of missing data due to gaps placed into sequence alignment. Table 4, while by no means an exhaustive summary of studies using ribosomal RNAs as a source of characters, demonstrates that a considerable percentage of characters in sequence data sets could potentially be coded as missing. This observation implies that the effects of the fragmentary nature of ancient DNA data may be analogous to the morphological studies.

Wheeler[37] has examined the effect of missing data on phylogenetic analysis of molecular sequences. Using simulations he showed that for molecular studies, although missing taxa (produced by extinction or poorly sampled representative taxa) and missing characters (produced by alignment) both have the overall effect of reducing phylogenetic information, each has a specific effect on phylogenetic reconstruction. In particular, missing taxa are most important to cladogram accuracy and missing data are most important to cladogram resolution. Cladograms produced from data sets with missing data or small data bases will produce unresolved but congruent phylogenies.

The accuracy of cladogram reconstruction is affected more sharply by missing taxa for the reasons outlined in Donoghue et al.[10]. They argue forcefully that poorly represented fossils may reveal combinations of characters that are absolutely necessary for establishing relationships. Since fossils presumably preserve relatively unmodified conditions of major lines, they may have more relevant character information on the branching orders of those lineages.

Conclusions

The primary use of fossils in modern biology is in systematics, and so should the primary use of fossil DNA sequences. Fossil DNA information should be treated no differently than the morphological information from the same fossils. This calls into question whether fossils should be used as primary sources of character information or whether they should be used as 'embellishments'[16]. It is obvious from the examples given in Gauthier et al.[14], Doyle and Donoghue[12] and Donoghue et al.[10], that fossils can play a critical role in understanding the relationships of extant taxa. Novacek[29] points out that addition of fossil taxa often destabilizes a cladogram, but that this undesirable attribute of adding fossils coincides with two more impor-

tant aspects of phylogenetic analysis. The first is that a more complete taxonomic array is obtained, and the second is that a more complete picture of character transformation is obtained by adding fossil information. An examination of the strength of inference of fossil DNA studies (table 3) suggests that character support in these studies is not overwhelming. However, the results of each study indicate that the fossil DNA data can resolve or overturn relationships that extant taxon-based analyses support. The effects of missing data in DNA studies using fossils are most likely of the same magnitude and certainly have the same impact as fossil morphology studies. It is of even greater importance that the effects and approaches to using ancient DNA in systematics be carefully considered because many fossil DNA studies require that the fossil be destroyed or at least partially damaged in order to isolate the DNA for PCR and sequencing. Assessment of the importance of the systematic question, the utility of the potential DNA sequence characters and the rarity of the fossil should dictate whether or not a certain fossil should be damaged.

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