Morphology should not be forgotten in the era of genomics—a phylogenetic perspective

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ABSTRACT

Morphology has traditionally played a pivotal role in animal phylogeny since the first evolutionary biologists began to decipher the animal tree of life. In recent times, however, morphological characters have lost prominence in inferring deep relationships among animals due to fundamental issues with homology statements and the choice of higher taxa as terminals, but most importantly, due to declining costs of generating molecular data. As genomes and transcriptomes become widely available, the burden of evidence for morphology in overcoming molecular tree topologies has become ever heavier. In addition, collection and coding of morphological characters must accelerate and become less subjective. These needs have led to developments in the field of ontology, the use of a controlled and formalized morphological vocabulary. Finally, the role of fossils for dating molecular phylogenies is discussed. While molecules seem to have displaced morphology for phylogenetic inference, the need to incorporate fossils as explicit terminals, including dating of molecular-driven trees, together with new methods for high-throughput morphological data acquisition and annotation, may rescue morphology to become again an important player in phylogenetics. The reasons for maintaining a research program in morphology are thus countless, as ultimately, a zoologist's interest is to understand form and function, ecology and evolution, as well as all other aspects that may explain how our favorite organisms live, behave and evolve.

Keywords: phylogenetics, phylogenomics, tip dating, node dating, total evidence dating

1. The historical role of morphology in systematics

Since the beginnings of systematics as the science for inferring organismal relationships, morphology has played a prominent role in elucidating the tree of life (e.g., Haeckel, 1866). With the advent of cladistics (phylogenetic systematics) (Hennig, 1950, 1966) and numerical methodologies (mostly parsimony-based methods), the prevalence of explicit morphological data sets exploded in the 1980s and 1990s with the aim of testing animal classification systems at various taxonomic levels. With respect to the interrelationships of the animal phyla, several morphological data matrices were assembled (e.g., Brusca and Brusca, 2003; Eernisse et al., 1992; Jenner, 2004; Jenner and Scholtz, 2005; Meglitsch and Schram, 1991; Nielsen et al., 1996; Schram, 1991; Sørensen et al., 2000; Zrzavý et al., 1998), some becoming the basis for subsequent combined analyses of morphological and molecular data (e.g., Giribet et al., 2000; Glenner et al., 2004; Peterson and Eernisse, 2001; Zrzavý et al., 1998).

The development of these matrices was however hampered by two methodological impediments and few advances have been made since the early to mid-2000s. First, these matrices often used higher taxa as terminals, a practice eloquently critiqued by Prendini (2001), among other things by not allowing such higher taxa to be non-monophyletic. For example, it is now broadly accepted that Acanthocephala nests within Rotifera (e.g., Garey et al., 1996; Sørensen and Giribet, 2006; Wey-Fabrizius et al., 2014), something that could never be discovered by coding Rotifera and Acanthocephala as terminals of their own—at most one could place them as sister taxa. Second, many characters coded as absent/present assumed homology of non-comparable conditions (this is independent from other issues with applying a/p coding; see for example Pleijel, 1995), as critiqued by Jenner (2002). For

example a character state such as presence of spiral cleavage bears a homology statement (Hejnol, 2010), but its absence coding assumes that all other types of cleavage are homologous—something that obviously is not the case. Many other issues with coding characters and recycling data sets have also been exposed (e.g., Jenner, 2001).

While the use of species as terminals is a logical, albeit time consuming, solution to the first problem—and may require coding multiple species with disparate morphologies per phylum—resolving issues of homology can be complicated in many cases, especially when dealing with complex characters such as egg cleavage patterns or segmentation (Scholtz, 2002). Large morphological data matrices based on the exemplar approach advocated by Prendini (2001) are occasionally assembled for many animal groups (e.g., Bieler et al., 2014; Lee et al., 2013). However, the exemplar approach has yet to be applied to broad metazoan relationships, and few efforts seem to be currently directed that way.

2. Molecular systematics in the era of genomics

While morphological data sets played a pioneering role in elucidating the animal phylogenetic tree, molecules entered the game relatively early (Field et al., 1988), and included data from nearly all the animal phyla just a decade later (e.g., Giribet et al., 2000; Zrzavý et al., 1998). But these phylogenies relied mostly on sequences from one or a few genes obtained through Sanger sequencing of PCR (polymerase chain reaction)-amplified fragments, even after the first invertebrate genomes were rapidly being sequenced and assembled (Adams et al., 2000; Consortium, 1998). Sanger-based sequencing approaches were however limited in the number of genes that could be amplified and they often lacked signal in many deep nodes. Soon, with the availability of the first animal genomes, several

80 data sets used information from 100 (Blair et al., 2002) to 3,000 gene sequences (Dopazo et
81 al., 2004). These data sets, severely limited in taxonomic scope, questioned what had
82 become recent "dogmas" of molecular phylogenetics, such as the clade Ecdysozoa (Philip et
83 al., 2005; Wolf et al., 2004). These studies however omitted most of the diversity of
84 invertebrates, save for a few model organisms for which genomes were available.
85 While these few genomes were available for a handful of model organisms,
86 generating whole genome data remained unreachable to the average systematist. The first
87 attempts to analyze large data sets using broad animal sampling efforts thus combined the
88 previous genomic data with a rapidly evolving set of multigene data sets derived from
89 expressed sequence tags (ESTs) (Delsuc et al., 2006; Delsuc et al., 2005; Lartillot and
90 Philippe, 2008; Philippe et al., 2007; Philippe et al., 2005; Philippe and Telford, 2006). Still,
91 these studies mostly relied on publicly available data until Dunn et al. (2008) published the
92 first comprehensive analysis of animal relationships using transcriptomics, soon expanded
93 to include a few additional missing species and key lineages like acoelomorphs and
94 placozoans (Hejnol et al., 2009). Many other analyses followed, especially re-evaluating the
95 base of the animal tree (e.g., Lartillot and Philippe, 2008; Moroz et al., 2014; Philippe et al.,
96 2009; Pick et al., 2010; Ryan et al., 2013), or some of its largest clades (e.g., Andrade et al.,
97 2014; Dordel et al., 2010; González et al., 2015; Kocot et al., 2011; Lemer et al., 2015;
98 Meusemann et al., 2010; Nesnidal et al., 2013; Philippe et al., 2011; Rehm et al., 2011; Rehm
99 et al., 2014; Sharma et al., 2014; Smith et al., 2011; Struck et al., 2014; von Reumont et al.,
100 2012; Weigert et al., 2014; Zapata et al., 2014). The newer analyses transitioned from small
101 and sparsely populated EST data matrices, generated by Sanger sequencing, to more
102 complete ones using genomes or transcriptomes obtained by 454 pyrosequencing, or
103 Illumina sequencing, among other techniques. These high-throughput sequencing
104 techniques have exploded, with an associated decrease in sequencing costs, shifting the

105 paradigm of nucleic acid sequencing, and resulting in a low cost per base and a throughput
106 on the gigabase order of magnitude. Some phylogenetic studies therefore now include
107 thousands of genes—mostly protein-coding genes are used in phylogenomic studies—and
108 dozens of taxa, and larger data sets will soon be available (see several global initiatives in
109 Kumar et al., 2012; Lopez et al., 2014; Robinson et al., 2011). One such data set recently
110 explored the phylogeny of insects with an unprecedented amount of data (Misof et al.,
111 2014). The first multi-genome phylogeny of an animal group appeared more than half a
112 decade ago (Clark et al., 2007), and dozens of genomes can now be obtained for non-model
113 invertebrate species, as recently shown by the 101 genomes of the monarch butterfly
114 *Danaus plexippus* (Zhan et al., 2014). It is thus unavoidable that rapidly decreasing costs of
115 sequencing genomes coupled with analytical developments will result in sufficient data to
116 thoroughly examine many key nodes in the animal tree.
117 Whereas the available amounts of molecular data scale up faster than requisite
118 technological developments (i.e., computation power, algorithmic efficiency), the gradual
119 expansion of morphological data is far from paralleling the growth of molecular data
120 acquisition, in spite of more efficient data collection. This has led some to question whether
121 one should collect morphological data at all for the purpose of inferring phylogenies
122 (Scotland et al., 2003).
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125 **3. Why is it important to maintain a research program in morphology?**
126
127 The reasons for maintaining a research program in morphology are countless, as
128 ultimately, a zoologist's interest is to understand form and function, ecology and evolution,
129 as well as all other aspects that may explain how our favorite organisms live, behave and

evolve (see Richter and Wirkner, 2014). Put plainly, one's interest in animal phylogeny is not the phylogeny per se, but to understand how the organisms evolve and adapt to their environment, and how this reflects upon their morphology, ecology and behavior. And of course, morphology will always be required for recognizing new taxa representing novel body plans (e.g., Funch and Kristensen, 1995; Just et al., 2014; Kristensen, 1983; Kristensen and Funch, 2000), "testing" molecular hypotheses, and to understand the evolution of body plans (*evo-devo*). Therefore, rephrasing the title of this section, we may ask, "*Why is it important to maintain a research program in morphology for estimating phylogenies?*", especially when entire genomes can now be collected for hundreds of species. It is thus unquestionable that large molecular data sets derived from next-generation sequencing techniques are becoming available and the preferred way to infer the relationships of many animal groups, including most phyla. It is also evident that cladistic matrices to infer deep metazoan relationships are comparatively stagnant, given the amount of effort and expertise required to code properly a matrix using an exemplar approach and produce sound hypotheses of homology for animals that share few macroscopic features, such as between for example the two "*C. elegans*", *Crella*, the sponge, and *Caenorhabditis*, the nematode. There are obviously some sound alternatives to the exemplar approach advocated here, such as combining actual observations from different exemplars for analytical purposes, especially to minimize amounts of missing data, but still this strategy does not seem to scale up with molecular data. Nevertheless, molecular hypotheses face the insurmountable limitation of necessarily omitting vast amounts of biodiversity—those that are extinct but still provide sound explanations of a group's phylogeny (Edgecombe, 2010).

Fossils constitute a key element for understanding the phylogenies of many animal groups (Donoghue et al., 1989; Edgecombe, 2010; Wiens, 2004), from placental mammals (O'Leary et al., 2013) to early panarthropods (Legg et al., 2013). In some cases, fossils in fact

155 increase congruence between morphological and molecular phylogenies. The role of
156 morphology in coding fossil taxa for phylogenetic inference is thus undeniable and such
157 practice becomes a strong advocate (one of many) for maintaining a healthy program in
158 morphology. However, the proportion of phylogenies produced using fossils as terminals is
159 just a tiny fraction of the total number of phylogenies generated every year, as they require
160 expertise in more complex data than molecular phylogenies, and access to material often
161 not broadly available for study.

162 Another aspect worth exploring, but seldom touched upon in the literature, is the
163 information content of morphology and molecules, or even in some cases, that of the
164 different character systems. Some early studies compared measures such as the consistency
165 index of molecular and morphological data (Sanderson and Donoghue, 1989), but to my
166 knowledge, few invertebrate studies have focused on discerning the information content of
167 different systems. A seminal study by Harper et al. (2000) analyzed 43 anatomical and shell
168 characters in anomalodesmatan bivalves, and then re-analyzed 18 characters which could
169 be scored from shell characters alone—as many of these could also be scored for fossils.
170 They then showed conflict between the shell-based tree and the cladistic hypothesis based
171 on all the evidence (Harper et al., 2000). More recently, Bieler et al. (2014) analyzed
172 broader bivalve relationships using a combined approach with multiple molecular markers
173 and a specimen-based morphological approach of 210 characters (for a total of 22,680 cells
174 with 16.6% of missing data), and then assessed the phylogenetic signal of multiple
175 character systems. From the 210 characters, 99 had significant phylogenetic signal (when
176 compared to the molecular and total evidence results), but a few character systems,
177 including sperm ultrastructure and shell gross morphology and microstructure contained
178 above average signal, while other character systems, such as alimentary and gill characters,
179 are less informative than the average. Studies such as these can direct morphological

research or even assess whether fossils would contribute with significant signal—as in this case, two of the best character systems are available in the fossils. Analyses of phylogenetic signal in morphological characters are however scarce, or in some cases, limited to just a few characters to assess their possible value for inferring phylogenies (e.g., Gainett et al., 2014). Further and rigorous assessment of the phylogenetic signal in morphological data is certainly needed (see Wiens, 2004).

Another aspect to consider when relying on morphological data for inferring phylogenetic relationships is the time of data collection and interpretation, an aspect that has been properly outlined by just a few authors (e.g., Ramírez et al., 2007). Discussion has emerged in the community about "high throughput morphology", and novel terms such as "next-generation histology", or "phenomics", have appeared, often trailing on the newest tendencies in molecular data generation. The use of automated methods for generating the raw data that will be transformed into the comparative morphological data is becoming more widespread, at least for technologies such as micro-computed tomography and magnetic resonance imaging (e.g., Ziegler et al., 2011), leading to accelerated acquisition of anatomical data (Ziegler, 2012; Ziegler et al., 2008; Ziegler and Menze, 2013). A major impediment to the phylogenetic analysis of such data is the so-called linguistic problem of morphology, or the translation of the anatomical raw data into anatomical descriptions, and these into morphological data matrices (Vogt et al., 2010). A solution to this problem can be provided by the development of ontologies—the use of a controlled and formalized morphological vocabulary (e.g., Ramírez et al., 2007; Richter et al., 2010; Vogt et al., 2010; Vogt et al., 2012; Vogt et al., 2013), but the practice and application of ontologies to distantly related groups is difficult. Modernizing data collection, storage, display and annotation are, together with the training of morphological experts who can interpret the characters, key aspects for returning morphological data to a more prominent position in

inferring phylogenetic patterns. However, there will always be a limiting step, character conceptualization, that will require expert assessment and that will limit automated procedures.

4. Morphology, fossils, dating, and phylogenomics

Dating trees has become common in systematics (Donoghue and Benton, 2007), as a time-tree allows not only discussing patterns, but also tempo, of evolution, and thus allows for testing specific hypotheses. The hypotheses often pertain to biogeography, and increasingly to comparative methods evaluating diversification rates and alternative models for evolutionary dynamics through time. Comparative methods are thus on the rise (Harmon et al., 2008; Rabosky and Alfaro, 2010) and require both precise dating for testing key innovations (Slater et al., 2012), where morphological or behavioral innovations cause differential diversification rates (e.g., Dimitrov et al., 2012) and allow testing for adaptive radiations, where niches are defined on the basis of morphology (e.g., Gillespie, 2004; Losos and Ricklefs, 2009). It is thus ironic that fossils have become formidable allies of molecules wherever inference of divergence times is required, while molecules have relegated morphological characters of living organisms to an almost marginal role in phylogenetics.

For the most part, researchers use fossils to calibrate molecular trees, providing information to constrain certain nodes (Donoghue and Benton, 2007). Some even explore issues of uncertainty of calibrations and multiple models (e.g., Mao et al., 2012; Warnock et al., 2012), but often assume that the taxonomy reflects the correct phylogenetic position of the fossils, something difficult to justify without proper phylogenetic assessment. It is for the latter reason that a minority of workers has opted to treat fossils as actual terminals

230 (the so-called "tip dating" or "total evidence dating" approach), using morphological data to
231 infer fossil placement—as commonly done by paleontologists—while the molecular data
232 guides the dating (Murienne et al., 2010; Pyron, 2011; Sharma and Giribet, 2014; Wood et
233 al., 2013). Tip dating addresses on the one side the aspect of phylogenetic uncertainty of
234 fossils (see also Parham et al., 2012), but also the concern that the date estimates are more
235 accurate when fossils appear in a specific phylogenetic position, and not just as
236 approximate constraints to nodes (Pyron, 2011), especially when taxon sampling is limited.
237 This has been illustrated in a large arachnid data set with an exemplar fossil record (Sharma
238 and Giribet, 2014), and has now been tested with ancient DNA from fossil humans (Rieux et
239 al., 2014). The latter study, using a comprehensive data set of 350 ancient and modern
240 human complete mitochondrial DNA genomes, shows that for the same data set, estimates
241 based on individual dated tips are far more consistent with each other than those based on
242 node calibration, and should thus be considered as more reliable. This result thus provides
243 the scientific justification for preferring tip dating (using fossils as terminals) over node
244 dating (using fossils to constrain nodes), encouraging the use of tip dating whenever
245 possible—which may not always be possible.
246 There may also be other reasons for preferring tip versus node dating, these having
247 to do with the idiosyncrasies of the fossil record. Some groups (e.g., molluscs, arthropods,
248 brachiopods) fossilize well, while others (e.g., platyhelminths, nemerteans, rotifers) do not.
249 In addition, some geological periods have more rock available to us or more favorable
250 conditions for fossilization, so in some cases, a group that has missed a fossilization window
251 (e.g., in the Carboniferous) may not appear again until many millions of years later. To
252 provide an empirical example, the fossil record of Opiliones (harvestman arachnids) is
253 exceptional in many respects, with the first Devonian fossils (Dunlop et al., 2003) probably
254 close to the first appearance of this ancient arachnid order, with multiple lineages

255 preserved as three-dimensional fossils in the Carboniferous (Garwood et al., 2011; Garwood
256 et al., 2014), and several other lineages known from the Mesozoic (see Dunlop, 2010 for a
257 recent review). The phylogeny of Opiliones is well understood (e.g., Giribet et al., 2002;
258 Giribet et al., 2010; Hedin et al., 2012a; Sharma et al., 2014; Shultz, 1998; Shultz and Regier,
259 2001), and while stem-group Eupnoi and Dyspnoi are known from the Paleozoic (Garwood
260 et al., 2011), the first fossil Cyphophthalmi is only from Cretaceous amber (Poinar, 2008),
261 between 45 and more than 300 million years after the diversification of the group
262 (following Sharma and Giribet, 2014), and its exact phylogenetic position is unclear. In the
263 case of Laniatores—the largest Opiliones suborder, which separated from its sister group
264 around the Devonian—the oldest known fossil is from Eocene Baltic amber (Ubick and
265 Dunlop, 2005). Therefore, while for some clades the first fossil occurs near their time of
266 origin and thus may provide good estimates of the age of a clade, in other cases the first
267 fossil occurrences may be closer to the tip than to the base of a clade. If the latter are used
268 for dating a clade, the excessively young fossil may produce a "push towards the present"
269 effect for the whole clade, and thus grossly underestimate the age of the clade in question
270 (Fig. 1).
271 It is clear that this may oversimplify the way node dating is done, as this fossil would
272 generally be treated as some sort of minimum age, but still, in the absence of much older
273 fossils in nearby clades, this effect should be noticeable. However, if the fossil were included
274 as a morphological terminal in a phylogeny instead of used for constraining a clade, its
275 position within the phylogeny should reflect its more derived position, and thus engender a
276 more precise dating of the whole clade. Of course one could argue that a more precise node
277 than "Cyphophthalmi" or "Laniatores" should be chosen when using those groups, but the
278 Burmese cyphophthalmid fossil was described in the wrong family and the phylogenetic
279 position of the Baltic fossils is difficult to ascertain without a comprehensive phylogenetic

280 analysis of Laniatores, which was entirely lacking at the time of its description. One could
281 also argue that the taxonomy should be fixed first, but in the real world, molecular
282 systematists tend to follow the taxonomy from the taxonomists describing the fossils and
283 such fossils may often not be precisely positioned.

284 Another common problem of using fossils to date nodes is that sometimes such
285 nodes are misrepresented, because most authors "recycling" fossil papers for their analyses
286 without critical phylogenetic assessment tend to take conservative approaches. For
287 example, Hedin et al. (2012b) used the age estimate of two members of closely related
288 families of Eupnoi as the age for the entire suborder (see Sharma and Giribet, 2014),
289 resulting again in a *push towards the present* effect, but in this case for a different reason.
290 Had they coded the fossils as terminals, the two species represented molecularly would
291 have not been placed with the fossil they used for dating the node. A clear demonstration of
292 these was recently provided in two analyses of fossil Opiliones (Garwood et al., 2014;
293 Sharma and Giribet, 2014).

294 Tip dating however requires on the one hand morphological expertise, and also
295 algorithmic developments, as currently no software allows analyzing simultaneously
296 complex genomic-scale data (i.e., thousands of loci) and morphological data in a
297 probabilistic framework. To my knowledge, no software additionally enables efficient
298 dating using morphological and phylogenomic data matrices (see Sharma and Giribet,
299 2014). Due to the complexity of nucleotide and/or amino acid transition matrices, assigning
300 accurate models to simultaneous analyses of morphological and molecular sequence data is
301 inherently difficult, and recapitulates an older problem of how different data classes should
302 be weighted in total evidence analyses (Bull et al., 1993; Giribet, 2010; Kluge, 1989).
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305 **5. Final Remarks**

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307 Morphological data have traditionally played a key role in inferring animal
308 phylogeny but recently have been replaced by molecular data due to multiple factors,
309 perhaps most prominently due to the efficiency and low cost of sequence data acquisition
310 and the development of analytical methods to deal with a vast amount of characters with a
311 small number of states. This is unfortunately leading to a large asymmetry in the amount of
312 studies using either type of data, while morphological data can still provide an important
313 framework to assess molecular trees—congruence between the two data sources generate
314 confidence in the results, while incongruence often leads to interesting aspects of body plan
315 evolution, including convergence. Several developments, including high throughput
316 morphological data collection and the establishment of clear ontologies are now required to
317 restore the place of morphology as a main player in elucidating phylogenetic relationships.
318 These will also aid in ameliorating previous problems with higher taxa as terminals and
319 poorly formulated homology statements. But most important is the need of using fossils to
320 improve phylogenetic inference and to help generate time-trees. In the latter arena, it is
321 beginning to become evident that tip dating (total evidence dating) may be philosophically
322 preferable to other forms of dating using fossils to calibrate accepted nodes. As refined
323 bioinformatic tools become available, it should be possible to analyze genomic and
324 morphological data in conjunction and to use these analyses for providing the most
325 accurate estimate of the timed phylogenies of a group. Molecular data may easily provide
326 the skeleton of the animal tree of life; morphology will still be required to test such skeleton,
327 to place key fossils, and to provide a sound temporal framework for such animal tree.

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341 **References**

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795 **Fig. 1.** Effect of a young fossil on the phylogeny of an old lineage, represented in this case by
796 an amber fossil for a clade with an old history (A). Supposing that the fossil could be
797 assigned to the red clade, based on morphology, it could be used for tip dating (B) or for
798 constraining a node (C). In tip dating, the clade could accommodate for extensive
799 cladogenesis occurring prior to the fossil, if the fossil were positioned in a derived position
800 in the phylogeny of the group. In node dating, the fossil may cause a "push towards the
801 present" effect. See text for discussion.