Post-molecular systematics and the future of phylogenetics

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The time is past when a research program in systematics should be based on only a few genes, extant taxa, and ultrametric trees. Cheap genome sequencing, powerful statistical methods, and new fossil discoveries promise to reinvigorate research programs in evolutionary biology. Population genetics, phylogeography, and species delimitation all benefit from genomic data, not just tree building alone. Null-hypothesis testing and power analysis via simulation can increase the confidence and robustness of phylogenetic comparative methods. Merging morphological and molecular datasets for fossil and extant taxa gives a more complete view of the Tree of Life. Combined, these developments can foster a post-molecular systematics, integrating phylogenetic signal from the population up based on DNA and through time based on direct observation rather than inference.

Molecular systematics in the 21st century

For several years, molecular systematics has been the dominant phylogenetic paradigm [1]. By this, I mean an era in which the primary objective was obtaining DNA sequence data, primarily from protein-coding or ribosomal genes, to generate molecular phylogenies of extant taxa. This was facilitated by fast, cheap sequencing technologies, powerful statistical methods for inferring and analyzing phylogenies, and renewed interest in natural history collections for tissue samples. The result is that highly complete phylogenies are now available for groups such as plants [2], fishes [3], birds [4], amphibians [5], squamates [6], and mammals [7]. These phylogenies are increasingly supported by backbones of genomic data [8] and are being used to answer fundamental questions in ecology and evolution.

This tree-building effort has or will naturally plateau, since genomic information and species diversity are more or less finite. Of course, groups that are hyper-diverse and under-sampled (such as arthropods), as well as those whose evolutionary history cannot easily be represented by a dichotomous phylogeny (such as prokaryotes), have not reached this plateau. While much more work remains to be done in such groups, the developments discussed below remain relevant for such taxa. Once a clade is fully sampled for species and characters, the question becomes

what problems, limitations, and future directions face systematic efforts? In most cases, genomic data have not offered a full-scale revolution in phylogenetic understanding but have often simply reinforced results or increased support from smaller datasets.

Concurrently, these trees have been analyzed using increasingly sophisticated comparative methods designed for dated trees of extant species. A large proportion of recent molecular systematics literature follows a similar pattern. A tree is constructed based on DNA sequence data. This tree is then dated using clock-based methods, typically with internal node-age constraints derived from the *post hoc* association of fossils with extant clades. Then, comparative methods are applied to the dated tree, for aims such as estimating diversification rates, ancestral areas, community assembly processes, ancestral character states, and models of character evolution or some interaction between these, such as the effect of a trait on diversification. However, recent results suggest that many of these methods are underpowered, biased, and suffer from non-independence in ways not easily fixable, which may affect significant proportions of studies using them [9–12].

In general, as more groups approach phylogenetic completeness and stability, and comparative methods grow more complex, the era of molecular systematics as an end unto itself is ending. Now, I suggest we are entering an age of post-molecular systematics that will: (i) critically examine the utility and dimensionality of genomic data above and below the species level; (ii) reexamine the use of phylogenetic comparative methods in terms of scale and power; and (iii) place a renewed emphasis on total-evidence phylogenetics, including fossil species based on morphological data.

The strengths and weaknesses of genome-scale data

The genomics revolution seemed poised to offer massive benefits to molecular systematics. Hundreds or thousands of loci would hopefully provide unambiguous resolution and support for most, if not all, branches in the Tree of Life. The use of explicit species-tree methods would hopefully offer analytical stability and confidence in these estimates [13]. We might expect two scenarios to have arisen: (i) whole-genome data overturn long-held hypotheses about what were previously considered strongly supported branches in the Tree of Life; and (ii) genomic data unambiguously resolve and strongly support previously contentious rapid radiations in the Tree of Life. In general, these expectations seem not to have been met [14–16].

In the first instance, the early inception of molecular data did provoke a phylogenetic revolution, overturning numerous long-held phylogenetic hypotheses in groups such as basal animals [17], amniotes [18], birds [19], squamates [20], and amphibians [21]. The subsequent development of genomic datasets for these groups has not substantially altered these initial molecular estimates in many cases. For instance, over 1000 loci cement the placement of turtles with archosaurs but merely reaffirm early results from mitochondria or a few nuclear loci [22]. In squamates, sampling 44 nuclear genes for hundreds of taxa [23], or 15 mitochondrial and nuclear loci for thousands of species [6], yields essentially the same results as the initial tree from two nuclear loci [20].

This result is borne out by numerous empirical and simulation studies using species-tree methods, which show that topologies usually stabilize with a relatively small (<20) number of loci in most instances [24]. Although the number of potential resolutions is large but finite, the number of realistic or likely resolutions is usually small, and phylogenetic signal is usually strong and broadly distributed in sampled loci. Thus, such a result is not really unexpected. It is difficult to imagine a case where 1000 loci provide strong support for a novel rearrangement of a branch that was not found in previous datasets of 500, 50, or even five loci [14].

Similarly, many contentious nodes in rapid radiations have not been fully resolved by genome-scale data [14,15,25–29]. In these studies, some previously uncertain relationships were strongly resolved by phylogenomic data, but others were not [27,30]. This issue has been noted for some time: if speciation events occurred too rapidly and branching events are tightly clustered, too few substitutions may become fixed to resolve relationships, regardless of the volume of data used to address the problem [31–33]. A possible avenue of investigation to resolve such nodes is phylogenetic inference based on gene order and synteny mapping, although these models and analyses remain in their infancy [34].

Thus, genomic data may be useful for providing a strongly supported backbone, even when that backbone has been contentious, provided phylogenetic signal is present [29,30]. However, this is not guaranteed for more recalcitrant radiations [15,35]. Species-tree methods can help to resolve some ambiguous branches [36], but poorly supported nodes may persist across the Tree of Life due to various evolutionary mechanisms [14,32]. Even then, taxon sampling is likely to have as strong an impact at the phylogenomic scale as it does for smaller concatenated datasets. Thus, the effort already expended to build current phylogenies would need to be equaled or exceeded to leverage fully the power of phylogenomics to resolve nodes with both extensive character and taxon sampling.

By contrast, genome-scale data seem to be somewhat underappreciated in the context of species delimitation, phylogeography, and population genetics [37]. For these applications, increases in the amount of data seem to increase accuracy and precision more linearly as more variable sites from more regions of the genome are included. Investigating species boundaries of morphologically similar groups [38], phylogeographic structure [39], and

parameters such as gene flow and effective population size [40] is boosted substantially by phylogenomic datasets.

Thus, genome-scale data have at least three major applications for post-molecular systematics that have previously been underappreciated:

Studies below the species level. Coalescent species delimitation, multilocus phylogeography, and population genetics are all significantly improved by genome-scale datasets, particularly with new models for analyzing large-scale genomic variation [37,41–46].

Adaptive molecular evolution. Relatively few studies have leveraged the power of genome-scale data to investigate molecular evolution across the genome itself, and within and among lineages through time, to highlight processes such as adaptation and convergence [47,48]. This may be particularly relevant when adaptive convergence in phenotypic traits is driven by adaptive convergence in the genome [49,50]. While a few genomes have been analyzed thus, this awaits a larger number of well-annotated genomes for large-scale comparison.

Genomes as traits. A final interesting and under-studied potential of genomics is treating the genome itself as a phenotypic character expressed by the organism. While the genome sequences of closely related species might be similar, the functional expression patterns of those genomes can vary widely among tissues, developmental stages, and environmental conditions. Comparative transcriptomics can use this information to analyze adaptation in functional traits, patterns of selection, and processes of genome evolution [51].

The necessities and failures of phylogenetic comparative methods

The ascendance of phylogenetics as a dominant paradigm in biology stemmed from the realization that species are not epistemologically or statistically independent; all comparative observations in biology are affected by shared ancestry. Even a simple bivariate correlation needs a phylogenetic term when it involves multiple species [52]. Such methods have become increasingly complex, including models of trait evolution [9], character correlation [53], estimation of speciation and extinction rates [54], and the association of traits and diversification rates [54]. These models permit sophisticated statistical analyses based on traditional model-fitting techniques such as analysis of variance (ANOVA) as well as situations (such as diversification rate estimation) specific to a phylogenetic context [55].

Worryingly, recent results suggest that many of these methods have low overall power [56]. Methods for estimating speciation regimes may be relatively robust [57] but the outlook for extinction is typically weak even under optimal conditions [54,58]. Summary statistics that were popular in the past, such as γ, are now known to be relatively inaccurate and underpowered, while diversification scenarios are often so complex that analytical likelihoods cannot be calculated [59]. Other methods for estimating rate shifts such as modeling evolutionary diversity using stepwise AIC (MEDUSA) appear to have excessive Type I error rates and biased parameter estimates that essentially preclude their use [60]. Other authors have pointed out the shaky epistemological and methodological

footings on which many analyses have been based, as the models used are often limited in their ability to answer the desired questions and the questions themselves are often poorly defined [61].

The limitations of ancestral-state reconstruction have long been known [62]. For instance, the power to reconstruct a character at the root of placental mammals using a tree of 4507 species modeled using Brownian motion (BM) is equivalent to a measurement from five data points [9]. For more complex models such as Ornstein–Uhlenbeck (OU), current approaches are non-unique or unidentifiable, often inaccurate, and pathologically biased toward excessively complex scenarios [9]. In general, available models such as BM and OU appear to be relatively inadequate and poor absolute fits to real clades when examined from an empirical perspective [10,63]. Methods linking ancestral-state estimation to quantitative trait variation using BM and OU models have also been proposed [53,64] but it is unclear whether the methods are robust to the fundamental shortcomings that seem to plague parameter estimation using the models [10,63]

In a particularly spectacular case, the use of state-dependent speciation and extinction (SSE-based) methods [specifically binary (BiSSE)] to estimate the relationship between a binary trait and speciation and extinction rates yields Type I error rates of ~100% [11]. This is not, apparently, due to any conceptual or mathematical flaws in the method or algorithm, but simply extreme sensitivity to assumptions of rate constancy across lineages, which are nearly always violated in empirical cases. The effect is so severe that even characters such as the length of a species' Latin name are found by the method to influence speciation and extinction significantly. All of these methods have been used in dozens or hundreds of studies, potentially calling into question the conclusions from a large proportion of molecular systematics literature from the past 15 years.

The relevant question for post-molecular systematics is twofold: (i) what can we reasonably expect to learn from phylogenies; and (ii) how confident can we be in these inferences? The information contained in a dated phylogeny generally reduces to $n-1$ node ages and $2(n-1)$ branch lengths, which are subject to numerous upstream biases of phylogenetic and temporal inference that can strongly affect the results of comparative methods [65–67]. Then, we demand from this graph information on speciation, extinction, ancestral states, ancestral areas, links between traits or areas and diversification process, community assembly processes, phylogenetically informed regressions and ANOVAs, etc. Perhaps our favorite clade has only 50–100 species; is all of this information contained in those branches? Or when analyzing large-scale phylogenies, do our methods adequately capture among-lineage variation and the diversity of processes such as biotic interactions like competition, predation, and facilitation?

A way forward has been suggested by several recent papers testing comparative methods, implemented by a few studies introducing new methods, but rarely employed by any empirical studies. This is the evaluation of model adequacy and absolute fit via *a posteriori* simulations and the use of summary statistics [9,10,60]. For any

model-based scenario, ranging from estimating speciation, fitting a model of trait evolution, or linking traits to speciation, it will be possible to generate simulated phylogenies within the estimated parameter space of the best-fit model. These can be compared with the observed data via summary statistics to evaluate how well the model captures the empirical conditions [60] and to evaluate the power of the test to reject null or alternative models [10,11].

Even for tests as simple as comparing BM and OU for a trait, this approach should be used to estimate power and model adequacy. Nearly all comparative-methods packages include the facility for simulations, yet few empirical studies employ this approach. While tedious, it seems absolutely necessary for future investigations of complex evolutionary dynamics, given the frequent and substantial failings that many commonly used comparative methods exhibit. Furthermore, the potential for exhausting the available degrees of freedom in a phylogeny through numerous tests of different rates, characters, and scenarios should not be overlooked [9,12] and, if possible, should be simulated and assessed jointly. Clearly [11], it is often possible to retrieve strong significance from comparative analysis of large-scale phylogenies. However, recent results suggest that it is often difficult to determine the biological importance of this significance or whether it is real or artifactual in empirical datasets.

The resurgence and importance of total-evidence phylogenetics

Morphological systematics commonly included both extinct and extant taxa, as there was little epistemological distinction between them in terms of the available data [68]. The ascendance of molecular systematics saw a decline in total-evidence phylogenetics incorporating morphological data, primarily due to the ease of capturing large amounts of DNA sequence data quickly and the difficulty of generating large morphological matrices [69]. However, recent methods allow the integration of morphological and molecular data for fossil and living taxa in a unified, dated phylogenetic framework [70,71]. It is time for a reunion of paleontology and neontology and a resurrection of total-evidence phylogenetics. The Tree of Life contains both extinct and extant branches across time and including fossil taxa and morphological data can improve topological and branch-length estimation [68,72–74].

Importantly, many of the limitations of phylogenetic comparative methods described above are lessened or alleviated when fossil lineages are included in analyses [9,75]. Ancient DNA can also provide an avenue for merging datasets of extinct and extant species [76]. Fossil lineages give direct observations of speciation and extinction rates and character states through time. For a tree with fossil lineages, speciation and extinction can be estimated with much higher precision and accuracy [77]. When trait data are available, ancestral states and areas can be observed much closer to nodes of interest [78] and character models such as OU and BM can be assessed far more powerfully [9].

Of course, this is not a problem-free directive. Many traits, particularly ecological variables, cannot be measured easily for extinct species. The true diversity of extinct

lineages is vastly under-sampled and stochastically limited by the availability of sediments in appropriate areas and the time spent describing fossil material. Finally, the preparation of morphological matrices is tedious and time consuming and requires substantial expertise possessed by relatively few. There are substantial ontological arguments regarding character states and homology statements across large phylogenetic scales [79]. This is in addition to empirical problems arising from taphonomic artifacts that may mislead phylogenetic inference [80].

There is no easy way forward here, other than to continue to bolster paleontological and morphological expertise and to begin constructing larger joint matrices of fossil and living taxa. Issues such as data incongruence and homoplasy are well known [81]. The impact of these issues on total-evidence phylogenetics has been assessed in only a few groups [72] but suggests that incongruence may be resolved through combined analyses [82]. Epistemological issues of homology, character-state definition, and treatment of multistate characters all have a long history in the literature, with which many younger systematists may be unfamiliar, and these issues will need to be revisited and revised [83]. Finally, little methodological work has been done since 2001 on the models used for phylogenetic inference of discrete morphological data [84]. Improving on these might substantially enhance efforts to infer total-evidence phylogenies as matrices grow larger and more complex [85].

Concluding remarks

For more than three decades, molecular systematists have followed a fruitful, productive research plan for phylogenetic inference, including more taxa and more characters, inferring trees using more sophisticated methods, and analyzing evolutionary dynamics using more complex statistical tools. The benefits and advances from this approach have been incalculable. Many parts of the Tree of Life are now saturating in terms of taxa and characters. Undoubtedly, many groups remain under-sampled, and new species are constantly discovered, but even then saturation is still on the horizon. Concurrently, many comparative methods have become mathematically unwieldy and are poorly understood and menacingly underpowered or prone to invisible, pathological biases. Furthermore, a decline or omission of substantial amounts of historical data from fossils has led to a deep divide between paleontology and neontology and extinct lineages are rarely considered in this molecular context.

Thus, it is now time for a post-molecular systematics where at least three major research programs should be reinvigorated, all of which have a strong historical literature but reduced prominence in the molecular systematics paradigm. The first is what to do with genomes beyond simply building trees with more characters. Genomic data may be particularly helpful below the species level, genomic architecture may provide a richly independent source of phylogenetic signal, and studying genome expression developmentally and physiologically may open new frontiers in evolutionary biology. Contrastingly, a return to basics is needed in phylogenetic comparative methods, to ensure that the evolutionary scenarios being modeled

are not so complex that they are unidentifiable and that the methods are not being misled by invisible violations of model assumptions. This can primarily be accomplished via posterior simulations and assessment of model adequacy, absolute fit, and relative power. Finally, a reunion is needed between paleontology and neontology, with a resurrection of total-evidence phylogenetics. Combining fossil and living taxa on a large phylogenetic scale holds the promise to advance numerous questions in evolutionary biology in a significant and powerful way.

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