Advances using molecular data in insect systematics

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Abstract

The size of molecular datasets has been growing exponentially since the mid 1980s, and new technologies have now dramatically increased the slope of this increase. New datasets include genomes, transcriptomes, and hybrid capture data, producing hundreds or thousands of loci. With these datasets, we are approaching a consensus on the higher level insect phylogeny. Huge datasets can produce new challenges in interpreting branch support, and new opportunities in developing better models, and more sophisticated partitioning schemes. Dating analyses are improving as we recognize the importance of careful fossil calibration selection. With thousands of genes now available, coalescent methods have come of age. Barcode libraries continue to expand, and new methods are being developed for incorporating them into phylogenies with tens of thousands of individuals.

Introduction

While molecular data has revolutionized the higher level phylogeny of many taxa, the backbone phylogeny of insects was recovered quite accurately in 1904 [1] . Subsequent morphological treatments refined insect phylogeny, and corroborated many nodes [2] but differences among hypotheses had been difficult to resolve. The period from 1995-2010 was dominated by molecular studies from Sanger sequencing. Datasets typically ranged from 1,000 to 10,000 nucleotides and usually included nuclear rRNA, one or two mitochondrial genes, and sometimes one or two nuclear single copy genes.

The seemingly revolutionary discoveries from this period have not been confirmed by much larger phylogenomic datasets [3]. Conflict was amplified by philosophical and analytical differences, recently reviewed [4]. At the dawn of the phylogenomic age, much controversy over higher-level insect phylogeny remained, and we awaited the age of “big data” to mediate our differences.

Datasets of extraordinary size are now common in phylogenetics, involving hundreds or thousands of genes. The size of datasets has been growing exponentially since the earliest studies in the 1980s (Fig. 1) and the recent works [3][5][6][7][8][9] are converging on consensus in higher level insect phylogeny (Fig. 2).

Branch support and confidence

Branch support measures such as bootstrap values and posterior probabilities provide confidence when a range of values are needed to distinguish signal from stochasticity. However, with very large datasets, stochasticity is effectively eliminated, and support values are often 100%. Of course, this is not a bad thing. However, even small biases amplified by millions of nucleotides can result in strong support for erroneous results. Recognizing this, Misof et al. [3] examined model mis-specification [10] and used quartet mapping [11], which led them to question certain strongly supported relationships, most notably the monophyly of Palaeoptera and the placement of Psocodea as sister to Holometabola. Congruence among data sources can also be used for building confidence for a particular hypothesis. Although it is often stated that morphological data should simply be mapped onto molecular trees, we find this opinion to be too limiting, especially for insects, whose morphological characters are abundant, and still accumulating with advanced techniques such as micro computed tomography (μ CT)[12]. Morphological data can provide corroboration and focus attention on problematic nodes. Corroboration can also come from embryological data [13] or from comparative studies of spermatozoa [14]. Despite its utility, congruence does not give us a quantitative or statistically meaningful value, and conflict from quartet mapping or the discovery of model mis-specification simply provides reason for skepticism. We are still looking for a more meaningful method of branch support for very large datasets.

Data partitioning and model selection

As phylogenetic datasets increase in size, the variation in the pattern of evolution among those data also increases. Two methods are often used for accounting for this increased variation: mixture models and partitioning. For large datasets, partitioning is the arguably the more popular method, though some studies have used mixture model methods on large datasets [15][16]. Partitioned models account for evolutionary variation by estimating independent model parameters for different subsets of sites within a concatenated alignment. In early model based molecular studies, the decision of how to partition the dataset was generally made *a priori* by the researcher based on some known biological feature of the data, e.g. gene boundaries, codon positions within a gene, or stems and loops of rRNA. The process of determining these boundaries is sometimes referred to as “more of an art than a science” [17]. More recently, algorithms have been proposed for the selection of partitioning schemes from pre-

defined data blocks [18][19]. These heuristic algorithms join the pre-defined data blocks and accept joins based on whether they improve the score of an information theoretic metric such as AICc or BIC. For algorithms like these, the more data blocks pre-defined, *a priori*, the better (since subsequent improvements are made by joining data blocks). For example, Misof et al. [3] identified protein domains as their initial data blocks with the argument that the domain, not the gene, is the unit of selection, and showed that partitioning by domain outperformed partitioning by gene. Other methods have explored estimating partitioning schemes without *a priori* knowledge of partitioning boundaries and, instead, partition by clustering sites with similar site patterns or rates [20][21].

Estimation of Divergence dates

Historically, the timing of insect divergences was estimated using the fossil record, with palaeo-entomologists using stratigraphic and radiometric information. With the advent of molecular phylogenies, branch lengths could be used to date the age of nodes in a phylogeny. However, a “molecular clock” is more of a hope than a reality. Thus fossil are used calibrate substitution rates in one part of the tree, which may then be extrapolated across the phylogeny to estimate divergence dates of taxa across the tree. Unfortunately, the “clock” may tick at wildly different rates across lineages, and among genes which suggests the need for multiple calibration points, and some method to accommodate rate heterogeneity. In addition, fossil calibrations can be problematic, due to misapplication of stem and crown taxa, and ambiguously identified fossils [22][23]. Other factors such as gene choice, and modeling parameters can negatively influence molecular age estimates. Indeed, the dates produced by many of the earliest dating studies were considerably older than the palaeontological data suggested [24][25](Ware and Barden, this issue). The most recent insect node molecular ages were recovered by Misof et al. [3] and Tong et al. [26] differed in their fossil calibration choice, and the types of priors they used to model fossil calibrations, which resulted in significant age differences for some nodes [27]. Future divergence studies will benefit from a cooperative effort between paleontologists and entomologists, to ensure that fossil interpretation, calibration priors, and phylogenetic methods are rigorously implemented.

Coalescence methods

In the last 30 years, the fields of population genetics and systematics have become more fully synthesized. This is because sampling extant genetic variation has become the major source of evidence for understanding evolutionary processes, their effects on nucleotide evolution, and nucleotide-based estimation of phylogeny. Coalescent models incorporate population-level parameters and tree inference into a model of the estimated time to common ancestry (coalescence) for alleles in a population [28]. The method is particularly valuable for understanding the unique phylogenetic patterns revealed by different genes sampled among close relatives at the species level and below [29]. Because rates and the amount of available variation differ among genes, the

pattern of population or species level relationships can differ for each gene. For this reason, it has been widely acknowledged that simple concatenation of genes into large datasets can obscure patterns of species diversification that are confounded by incomplete lineage sorting – a pattern of genetic variation with ancestral polymorphism retained through branching events [30][31][32]. Computationally powerful new methods have been developed that reconstruct phylogeny for each sampled gene individually using coalescent-based models, and these multispecies gene trees are summarized by various statistical consensus methods to evaluate support for the best supported topologies that spread the effects of lineage specific genetic biases more equitably in the analysis [33] [34] [35][36]. These methods are computationally challenging, but provide a powerful way to incorporate more aspects of the complex history that influenced extant genetic variation into the study. Multispecies coalescent-based methods have been applied in groundbreaking phylogeographic analyses various insect groups that have undergone rapid and relative recent species divergences [37][38][39][40][41]. These methods are also being extended to address deep divergences where rapid radiations may have been particularly prone to incomplete lineage sorting and the resulting phylogenetic pattern is difficult to resolve by more traditional methods [42][43][44].

SNPs and population genetics

Success in phylogenetics has always been tied to targeting the data to the question, which is linked to the range and depth of the question through time. SNPs, Single Nucleotide Polymorphisms, are variable sites that can be used to track relationships from alleles, to individual organisms, through populations and above the species level. SNPs are especially useful for revealing population structure and phylogeography [45]. However, the increasing availability of genomes, and the ease of sequencing and assembling partial genomes make it possible to map SNPs back to specific loci, establish homology through alignment, and to infer phylogeny. SNP datasets gathered for extensive genetic surveys of closely related species have been shown to have great power for resolving phylogenetic relationships [46][47][48].

Barcodes

Using “barcodes” to identify species has advanced over the past decade, and the Barcode Of Life Database (BOLD) now includes records from over 200,000 insect species. Compared with high-throughput sequencing, Sanger sequencing seems to be outdated and relatively expensive, when calculated at cost per nucleotide. However, when considering the combined cost of library preparation, tagging, and sequencing, if only a single locus is required, such as the barcode fragment, Sanger sequencing may still be a wise choice. Coissac *et al.* [49] discussed a strategy of augmenting standard barcode approaches with genomics. A reasonable phylogeny for a genus of Trichoptera was constructed by adding a single fragment of 28S to the existing barcode data [48]. Zhou *et al.* [50] extended this idea by constructing backbone constraints from transcriptomes and then adding 16,000 unique barcode haplotypes from 40,000 individuals to fill out the leaves of the Trichoptera tree of life. Both examples explored

the utility of maximizing the number of taxa in a phylogeny with a minimum amount of data, made possible by leveraging the enormous amount of available barcode data in combination with data from other sources. Recent efforts in barcoding have expanded to include high throughput sequences, and metabarcoding now routinely collects hundreds or potentially thousands of entire mitochondrial genomes from hundreds or potentially thousands of species. Initially, these studies [51][52][53][54] required PCR amplification, which can result in taxonomic biases based on differential amplification[54][55]. However, Zhou *et al.* [56] successfully applied a shotgun sequencing approach without PCR, and Liu *et al.* [57] dramatically enriched the relative yield of mitochondrial genomes with PCR-free approaches.

Transcriptomes

Initially, it was feared that transcriptomes would result in a haphazard matrix, filled with mostly missing data, as pioneering studies seemed to indicate. However, improvements in illumina sequencing technology, followed by matrix reduction with programs such as Aliscore [58], and MARE [59] resulted in data matrices that are largely complete. According to the OrthoDB database [60], there were a total of 1478 genes in common among all the arthropods in the Misof *et al.* [3] sample. All 1478 genes were recovered from most taxa, which means that genomes would not have provided more genes. Transcriptomes recover easy to model protein coding genes without introns, and do not require the processing, annotation, or data storage of genomes, making them ideal for phylogenetics.

Target enrichment methods

Targeted capture is an increasingly popular method that involves hybridization of custom-made molecular probes to loci of interest followed by sequencing of the captured sequences on a next-generation platform [61]. Two approaches utilizing this technique have become popular among phylogeneticists: Anchored Hybrid Enrichment (AHE; [62] and the so-called Ultraconserved Elements (UCEs; [63]). The main difference between AHE and UCEs is the nature of loci targeted, the former focusing on protein-coding genes and the latter utilizing any highly conserved regions that are flanked by more variable sequences. A large number of additional AHE projects with dense taxon sampling for Diptera, Hymenoptera, Lepidoptera, and Trichoptera are in progress, but yet unpublished. The first AHE analysis in flies resolved higher-level phylogeny of the flower flies, family Syrphidae [64]. UCEs have been applied to elucidate relationships among aculeate Hymenoptera [65] and within several groups of ants [66][67][68]. Low cost (as low as \$40 per sample), high data quality, and time-efficient sample preparation mean that target enrichment datasets are likely to range into thousands of taxa in the near future.

Genomes: rare events

As fully sequenced genomes become available, additional synapomorphic evidence can be inferred from rare genomic events, such as chromosomal duplications or rearrangements [69][70][71][72], changes in chromosome number or genome size [73]

[74], and rearrangement or conservation in gene order or synteny relationships between organisms [75][76][77]. Large scale changes in genome architecture are increasingly considered to be important drivers of speciation itself [78] and are also leave signatures of hybridization and introgression [79]. Empirical data are only beginning to be available for larger phylogenetic comparisons, but genomic patterns are likely to be informative due to conservation and rarity once sampling is thorough enough to reveal the limits of their taxonomic distribution. A recent dramatic example comes from the analysis of 16 *Anopheles* mosquito genomes which revealed high rates of gene gain and loss and convincing evidence for significant interspecific hybridization leading to autosomal gene introgression among closely related malaria vectors [80]. Reconstruction of species phylogeny is obscured by hybridization, thus complicating the use of small numbers of genes to do so, but phylogenomics reveals the complexity of genetic, ecological, and behavioral factors that shape the history of species diversification and their impact on the genome.

Phylogeography, diversification rate analysis, predicting future distributions

Phylogenetic analyses of molecular data play a central role in testing evolutionary hypotheses, both at the population/species interface and at more ancient macroevolutionary timescales. Phylogeographic studies that track the history of closely related species in time and space are increasingly sophisticated in their use of multiple data sources and analytical approaches. A rich conceptual and analytical framework is flourishing that combines genetic, population, and species level data to connect evolutionary patterns through estimates of gene flow, migration, and historical changes in geographic range [30]. These powerful tools are used in tracking invasive species [81][82][83], delimiting hybrid zones and speciation events [84], revealing cryptic species [85], and documenting the influence of selective sweeps and habitat degradation [86][87] --to name just a few of many applications. Macroevolutionary methods that capitalize on computing power and the availability of phylogenetic trees have also made it possible to evaluate changes in diversification rate through time [88][89][90]. These methods look for evidence of significant changes in the number of new lineages originating through time, or track the possible slow downs in lineage accumulation due to periods of greater than expected rates of extinction. Diversification rate tests have been applied at deep levels among insect orders [91] [92] and below [93] [94], but are most powerful in their application to fully sampled studies evaluating the diversification of rapidly radiating species in diverse clades [95][91][96]. Diversification rate studies provide insight 'key adaptation' hypotheses such as powered flight, complete metamorphosis, or phytophagy as causes for the rapid increases in species diversity that are a repeated and compelling feature of the history of insects [97][92][98]. Phylogenetic trees provide important temporal context connecting organisms and their changing environments. Phylogenies are a key parameter in studies that evaluate current biogeographic distributions, link taxa to their historic distribution and areas of endemism, and organize the specificity of their ecological requirements [99][100]. Phylogenetic assessment thus plays a key role in evaluating the affects of climate change and habitat loss for insects [101][102]. Ultimately, molecular systematic

data and their broad application are relevant not only as a historical record of phylogeny, but provide the framework for understanding ecological and evolutionary patterns, contextualizing changes in biotic interactions and community-level processes, and for predicting and evaluating the impact of these changes on insect biodiversity.

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Figure Captions:

Fig. 1. Size of phylogenetic datasets through time. X axis; Dates of publication of selected phylogenetic works. Y axis; number of sites multiplied by the number of taxa. For transcriptome datasets this number was then multiplied by (1 minus the percent missing data). “Ribosomal RNA” points frequently included other data. W09=; M10=[6]; N12=[77]; J13=[42]; S13=[7]; L13=[8]; P14=[9] M14=[3]; B15=[66]; B16=[68]; Z16=[50]. Others are as in Kjer et al. [4], Fig 5B.

Fig. 2. Consensus phylogeny from Misof et al. [3]. Collapsed nodes at Palaeoptera and Acercaria are discussed in the supplementary materials of that paper.

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Figure1

Figure 2