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PHYLOGENOMICS

Hervé Philippe,[1] Frédéric Delsuc,[1] Henner Brinkmann,[1] and Nicolas Lartillot[2]

[1]Canadian Institute for Advanced Research, Département de Biochimie, Université de Montréal, Montréal, Québec H3C3J7, Canada; email: herve.philippe@umontreal.ca, frederic.delsuc@umontreal.ca, henner.brinkmann@umontreal.ca

[2]Laboratoire d'Informatique, de Robotique et de Mathématiques de Montpellier, Centre National de la Recherche Scientifique, Université de Montpellier, 34392 Montpellier Cedex 5, France; email: nicolas.lartillot@lirmm.fr

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■ **Abstract** The continuous flow of genomic data is creating unprecedented opportunities for the reconstruction of molecular phylogenies. Access to whole-genome data means that phylogenetic analysis can now be performed at different genomic levels, such as primary sequences and gene order, allowing for reciprocal corroboration of the results. We critically review the different kinds of phylogenomic methods currently available, paying particular attention to method reliability. Our emphasis is on methods for the analysis of primary sequences because these are the most advanced. We discuss the important issue of statistical inconsistency and show how failing to fully capture the process of sequence evolution in the underlying models leads to tree reconstruction artifacts. We suggest strategies for detecting and potentially overcoming these problems. These strategies involve the development of better models, the use of an improved taxon sampling, and the exclusion of phylogenetically misleading data.

INTRODUCTION

The newly arising discipline of phylogenomics owes its existence to the revolutionizing progress in DNA sequencing technology. The number of complete genome sequences is already high and increases at an ever-accelerating pace. The newly coined term "phylogenomics" (Eisen 1998, O'Brien & Stanyon 1999) comprises several areas of research at the interplay between molecular biology and evolution. The main issues are (*a*) using molecular data to infer species' relationships and (*b*) using information on species' evolutionary history to gain insights into the mechanisms of molecular evolution. The majority of publications on phylogenomics deal with the second aspect (see Sjolander 2004 for a review). However, our concern here is the use of data at the genomic scale to reconstruct the phylogeny of organisms.

A novel and interesting aspect of phylogenomics lies in the possibility of using molecular information above the primary sequence level. In particular, trees can be inferred from whole-genome features, such as gene content (Fitz-Gibbon & House 1999, Snel et al. 1999, Tekaia et al. 1999), gene order (Korbel et al. 2002, Sankoff et al.1992), intron positions (Roy & Gilbert 2005), or protein domain structure (Lin & Gerstein 2000, Yang et al. 2005). A potential advantage of these methods is that the complexity of some of these characters (e.g., gene order) renders the character-state space very large, reducing the risk of homoplasy by convergence and reversal, thus rendering the inferred phylogenies more reliable. However, these integrated approaches imply the use of a reduced number of characters relative to primary sequence-based approaches. There are about 300 times fewer genes than amino acid positions (assuming a mean protein length of about 300 amino acids), thus increasing the risk of stochastic error.

In fact, stochastic or sampling error constitutes one of the major limitations of standard phylogenetics based on single genes. Because the number of positions of a single gene is small, random noise influences the inference of numerous nodes, leading generally to poorly resolved phylogenetic trees. The idea of using large amounts of genomic data as a way to address this problem is not new. For example, to resolve the original tritomy between chimpanzee, human, and gorilla, about 10 kb were sequenced as early as the late 1980s (Miyamoto et al. 1988). However, technical and financial limitations have often confined molecular systematics to the use of a few markers (e.g., rRNA) for which a large diversity of organisms have been sequenced. Numerous important phylogenetic questions remained unsolved, and great hope was placed into the wealth of genomic data soon to be available.

Sampling (or stochastic) error should vanish as the number of genes added to the analysis gets large enough. In practice, this means that statistical support (e.g., bootstrap support) will eventually rise to 100% as more genes are considered. The use of tens of thousands, or millions, of aligned positions that provide a great deal of phylogenetic information should ultimately lead to fully resolved trees. Indeed, several empirical studies confirmed this premise (Bapteste et al. 2002, Madsen et al. 2001, Murphy et al. 2001, Qiu et al. 1999, Rokas et al. 2003, Soltis et al. 1999). This increased resolution leads to the optimistic view that phylogenomics would "end the incongruencies" observed in single-gene phylogenies (Gee 2003). However, whether the resulting, highly supported, phylogenetic trees are the true ones is not certain.

Systematic Error and Consistency

The most important challenge of phylogenomics is to verify that tree reconstruction methods are consistent, i.e., converge toward the correct answer as more and more characters are considered (Felsenstein 1978, 1988).[1] In principle, at least in a probabilistic framework, a lack of consistency can always be traced back to some violation of model assumptions by the data analyzed. Note that methods that are not explicitly model based, such as maximum parsimony, are equivalent to a statistical analysis under an implicit model (Steel & Penny 2000). The best understood causes of method inconsistency stem from models that do not properly account for (*a*) variable evolutionary rates across lineages, leading to the long-branch-attraction (LBA) artifact (Felsenstein 1978), (*b*) heterogeneous nucleotide/amino acid compositions, resulting in the artificial grouping of species that share the same bias (Lockhart et al. 1994), and (*c*) heterotachy, i.e., shift of position-specific evolutionary rates (Kolaczkowski & Thornton 2004, Lockhart et al. 1996, Philippe & Germot 2000). These systematic biases could be interpreted respectively as rate signal, compositional signal, and heterotachous signal, which we will collectively refer to as nonphylogenetic signals (Ho & Jermiin 2004). In other words, nonphylogenetic signals are due to substitutions that occurred along the true phylogeny but are misinterpreted by tree reconstruction methods as supporting an alternative topology.

Compared to single-gene studies, inconsistency is more pronounced in phylogenomic analyses. For example, a reanalysis of the large dataset of Rokas et al. (2003) demonstrates that, depending on the method used, mutually incongruent, yet 100% supported, trees could be obtained (Phillips et al. 2004). It is well accepted that the analysis of phylogenomic datasets will necessarily increase the resolution of the trees through the "increase of the signal-to-noise ratio" (Rokas et al. 2003). Indeed, the signal-to-random-noise ratio increases, but the phylogenetic-to-nonphylogenetic signal ratio remains constant whatever the number of genes considered (assuming that the gene sampling is not biased). In this review, we focus on best practices for enhancing the phylogenetic signal in genomic data, while reducing the impact of erroneous signals, in order to obtain accurate and robust trees.

ASSEMBLY OF PHYLOGENOMIC DATASETS

The reliability of a phylogenetic tree depends on the quality of the data and the accuracy of the reconstruction method. In 1988, Felsenstein noted that "molecular evolutionists who use methods for inferring phylogenies do not engage in much

[1] The concept of consistency is formally defined as a property of a statistical estimator. Bayesian statistics is not so much concerned with statistical estimators but with the posterior distribution of a parameter, leading some to question the relevancy of consistency to Bayesian analysis (Felsenstein 2004). However, this view is not shared by many Bayesian statisticians. Indeed, if the underlying model is misspecified, this may result in an asymptotically vanishing posterior probability for the true tree, which is exactly the problem that the concept of consistency tries to capture. This suggests a Bayesian analogue of consistency: A model is consistent if the posterior distribution tends to a point mass concentrated on the true value of the unknown parameter, as the number of observations tends to infinity (Diaconis 1986).

discussion of the properties of the methods they use since they focus on the difficult task of collecting the data" (Felsenstein 1988, p. 523). Almost 20 years later, molecular systematists still spend much of their time assembling larger and larger datasets, and the crucial discussion about inference methods remains neglected. In phylogenomics, the reliability of the inference is often simply justified by the large number of characters used. Nevertheless, the problem of data acquisition deserves further discussion, as it can heavily compromise the subsequent analysis.

Importance of a Rich Taxon Sampling

A long-standing debate in phylogenetics concerns the relative importance of improving taxon versus gene sampling (Graybeal 1998, Hillis et al. 2003, Rosenberg & Kumar 2003). In the genomic age, gene sampling would seem not to be an issue. However the limited resources devoted to systematics often prevent sequencing the genomes of all relevant species. Depending on the importance accorded to taxon sampling, two strategies can be used: (*a*) gathering complete genome sequences from a few key organisms or (*2*) gathering incomplete, yet large, genome sequences from a great diversity of organisms.

The first approach is supported by some computer simulation studies (Rosenberg & Kumar 2003) and is the most frequently used in phylogenomic analyzes (Blair et al. 2002, Goremykin et al. 2004, Misawa & Janke 2003, Philip et al. 2005, Rokas et al. 2003, Wolf et al. 2004). However, the design of computer simulations and the interpretation of their results make it difficult to draw firm conclusions from this approach (Hillis et al. 2003, Rosenberg & Kumar 2003). Empirical evidence seems nevertheless to argue against the taxon-poor approach, as illustrated by the phylogeny of metazoans. Two new clades (Ecdysozoa, the moulting animals, including among others arthropods and nematodes, and Lophotrochozoa, including among others annelids, molluscs and Platyhelminthes) were proposed from rRNA analyses (Aguinaldo et al. 1997). However, several phylogenomic studies strongly supported the paraphyly of Ecdysozoa when considering a few model organisms and using a distant out-group (Blair et al. 2002, Dopazo et al. 2004, Philip et al. 2005, Wolf et al. 2004). In contrast, when 49 species and 71 genes (20,705 positions) are used (Philippe et al. 2005), the monophyly of Ecdysozoa and Lophotrochozoa is recovered with strong support (Figure 1*a*). However, the removal of close out-groups leads to drastic changes (Figure 1*b*): the fast-evolving lineages emerge paraphyletically at the base of the tree. Such an asymmetrical tree shape is expected to result from an LBA artifact when the out-group is distantly related (Philippe & Laurent 1998), as fungi are. Contrary to the situation with a few species discussed above, the statistical support for these incorrect placements is weak (bootstrap values between 32 and 63), demonstrating that an increased taxon sampling (from 4–10 to 45) has reduced, but not eliminated, the impact of LBA. In fact, the low bootstrap support (Figure 1*b*) demonstrates that the nonphylogenetic signal becomes equivalent to a phylogenetic signal when species sampling is impoverished.

A rich-taxon sampling is not, however, the panacea. First, computation time increases rapidly with the number of species, rendering exhaustive searches impossible with more than 20 species and most heuristic searches with probabilistic methods intractable with more than ~200 species. Second, adding taxa can sometimes degrade the phylogenetic inference (Kim 1996). Third, the number of extant species can be naturally sparse, forever preventing the assembly of a rich and balanced taxon sample. For example, *Amborella* is proposed to constitute the first, or one of the first, emerging angiosperm lineages (Qiu et al. 1999), but it is the only extant representative of an ancient group. Even if the heated debate about its placement (Goremykin et al. 2004, Soltis et al. 2004) could be solved by improved taxon sampling, the assumed basal position of *Amborella* might prove difficult to attest in the absence of closely related extant taxa (Stefanovic et al. 2004). In conclusion, an adequate taxon sampling, as balanced as possible, is important to increase the accuracy of phylogenomic trees.

Missing Data

Although genome sequencing has become ever easier, it seems unlikely that complete genomes will be soon available for a rich diversity of organisms. In addition, a bias in favor of sequencing small genomes leads to potential problems. Because small genomes are generally derived from larger genomes, whole-genome features, such as gene content or gene order, will evolve much faster, rendering tree reconstruction susceptible to artifacts such as LBA and compositional bias (Copley et al. 2004, House & Fitz-Gibbon 2002, Korbel et al. 2002, Lake & Rivera 2004, Wolf et al. 2001). Moreover, genome reduction is often associated with an accelerated rate of protein evolution (Brinkmann et al. 2005, Dufresne et al. 2005) or extremely biased nucleotide compositions (Herbeck et al. 2005). The sampling of a fraction of the genome from species with huge genomes is therefore a necessity to represent some key taxa and/or to include less biased representatives.

Two low-cost approaches can be used: (*a*) the selection of a limited set of genes potentially useful for the phylogenetic question of interest, followed by their targeted PCR amplification and sequencing (Murphy et al. 2001) and (*b*) the sequencing of thousands of expressed sequence tags (ESTs), which generally provides hundreds of relevant genes (Bapteste et al. 2002). The first method is more adapted to phylogeny at small evolutionary scales and has the advantage that genes can be a priori selected to obtain an optimal phylogenetic signal. The second might be preferable at larger evolutionary scales (e.g., among protists) and allows the discovery of many other genes, which can shed light on the evolution of important features such as metabolic pathways.

Phylogenomic reconstruction methods based on gene content/order cannot be applied to incomplete genomic sampling, but those based on DNA strings and on primary sequences can. In the latter case, missing data will occur even when complete genomes are used, especially at a large evolutionary scale because most, if not all, genes can be lost, duplicated, or horizontally transferred in some organisms.

To our knowledge, no theoretical reasons suggest that sequence-based approaches can not be used on incomplete alignments, i.e., containing cells coded as missing data. Nevertheless, "the problem of missing data is widely considered to be the most significant obstacle. . .in combining datasets. . . that do not include identical taxa," as suggested by empirical studies and computer simulations (Wiens 2003). Two problems need to be distinguished: (*a*) the potential lack of resolution induced by the presence of taxa with too many missing cells and (*b*) the possible interaction between missing entries and artifact-inducing model violations.

Recent computer simulations using a large number of characters (Philippe et al. 2004, Wiens 2003) suggest that the inaccurate placement of incomplete taxa is not the result of missing data but rather the result of an insufficient number of informative characters. As an extreme example, the tree reconstruction method remains accurate when positions have an average of 4 known and 32 unknown character states because each species is nevertheless represented by about 3000 amino acids (Philippe et al. 2004). However, the presence of missing cells unevenly distributed across the data matrix potentially affects estimates of model parameters. It is not yet clear how the induced model misspecifications in turn influence phylogenetic inference. Interestingly, it seems that the advantage of adding an incomplete taxon that breaks a long branch is greater than the disadvantage of the induced model misspecification (Wiens 2005).

Few attempts at assessing the effect of missing data have been made with empirical data (Bapteste et al. 2002, House & Fitz-Gibbon 2002, Philippe et al. 2004). For instance, a bipartition of the supermatrix (25% of missing data) into the most complete genes and the less complete genes appears to be indistinguishable from random bipartitions of the same size (Bapteste et al. 2002, Philippe et al. 2004). However, when the level of missing data is extreme (92%), the quality of the inference appears to be affected (e.g., strong support for the paraphyly of Glires and of Ecdysozoa) (Driskell et al. 2004), despite the large size of the dataset (70 taxa and 1131 genes). In summary, even if the problem generated by missing data has been overrated, additional work is needed to characterize its impact more precisely.

INFERENCE OF PHYLOGENOMIC TREES

The methods used in phylogenomic inference are either (*a*) primary sequence-based methods, which are very similar to the classical tree reconstruction, and for which several excellent reviews and textbooks are available (Felsenstein 2004, Holder & Lewis 2003, Swofford et al. 1996) or (*b*) methods above the sequence level (Wolf et al. 2002). The two approaches are fundamentally similar; the main difference is the characters used. In both cases, we believe that probabilistic methods are more powerful and more reliable. First, they have a more robust theoretical justification in that they rely on an explicit account of their assumptions by using stochastic models describing the pattern of molecular evolution (Felsenstein 2004). Second, not only do they allow estimates of the phylogeny and a confidence level, but they also provide general methods to evaluate the fit of the model used (Goldman 1993).

Approaches Based on Whole-Genome Features

Because genomes are the results of evolution, virtually any features comparable between organisms can be used to infer phylogenies, as evidenced by the plethora of new approaches recently published (Fitz-Gibbon & House 1999, Henz et al. 2005, House & Fitz-Gibbon 2002, House et al. 2003, Korbel et al. 2002, Lin & Gerstein 2000, Pride et al. 2003, Qi et al. 2004, Snel et al. 1999, Tekaia et al. 1999, Wolf et al. 2001, Yang et al. 2005). The justifications of whole-genome tree approaches are generally that the phylogeny of organisms can not be equated to the phylogeny of single genes (such as rRNA) and that this classical phylogeny is sensitive to hidden paralogy, horizontal gene transfer (HGT) or tree reconstruction artifacts (Fitz-Gibbon & House 1999, Lin & Gerstein 2000, Snel et al. 1999, Tekaia et al. 1999). Because tree reconstruction artifacts can affect any approach and are difficult to detect, we rather believe that these new approaches based on various character types are of paramount importance to corroborate phylogenetic inference (Miyamoto & Fitch 1995, Swofford 1991, Wolf et al. 2002).

DISTRIBUTION OF SEQUENCE STRINGS The frequencies of small oligonucleotides (up to eight) or oligopeptides (up to six) observed in the genome or the proteome can be transformed into distances then used to construct phylogenies (Blaisdell 1986). Numerous well-accepted clades, including deep ones, were recovered using this approach, confirming that a phylogenetic signal is present in these characters (Edwards et al. 2002, Pride et al. 2003, Qi et al. 2004). However, several undisputed clades were significantly rejected, and the comparison of the branch lengths of the genome tree obtained using tetranucleotide frequencies with those of the tree obtained from the standard analysis of rRNA sequences (Pride et al. 2003) suggests that the phylogenetic signal contained in sequence strings saturates rapidly. However, the methods proposed so far are extremely crude. Oligonucleotide or oligopeptide frequencies are transformed into distances without any underlying model of evolution. It is nevertheless remarkable that something considered as a bias in standard sequence-based methods (Lockhart et al. 1994) contains a phylogenetic signal, but it is not yet clear whether accurate methods can be developed to extract it.

HOMOLOGY AND ORTHOLOGY ASSESSMENT All other approaches require the establishment of homology, or more often, orthology of genes. By definition, the phylogenetic history displayed by orthologous genes is the organismal phylogeny (Fitch 1970), whereas paralogous or xenologous genes display a combination of organismal and gene-specific history. In practice, the identification of orthologous genes involves a certain amount of circularity because it requires an a priori knowledge of the organismal phylogeny. Indeed, finding orthologous genes is difficult because the organismal phylogeny is generally unknown (or at best partially known), and its reconstruction represents the goal. This problem is much akin to the alignment/phylogeny problem, in which alignment and phylogeny should be estimated simultaneously (Sankoff et al. 1973, Wheeler 2003), but the practical difficulties are such that the two steps are generally separated. Moreover, a careful phylogenetic reconstruction of all gene families is a Herculean labor most researchers want to avoid (but see Storm & Sonnhammer 2002). Therefore, an operational, yet approximate, definition of orthology is used. Schematically, all genomes are compared to each other at the amino acid level; only the pairs of sequences that are the best reciprocal hits are considered further. The clusters of orthologous groups are then constructed by a single-linkage analysis of all orthologous pairs (Tatusov et al. 1997) or by Markov cluster algorithms (Remm et al. 2001). These approaches, albeit reasonably effective in practice, do not guarantee the identification of orthologous genes because, if a gene has been transferred from a distant organism and replaced the original copy, it will fulfill all the requirements while being xenologous. Information from synteny will probably improve the accuracy of orthology assignment (Zheng et al. 2004), but much more work is needed before obtaining perfect assignment of orthologous genes.

GENE-ODER METHODS The character space of gene-order data is huge, so it probably constitutes the most promising genome feature-based method. However, it is also technically and computationally the most difficult (see Moret et al. 2005 for a review). Briefly, distances can be computed by minimizing the number of inversions, transpositions, insertions, and deletions necessary to transform one unichromosomal genome into another (Sankoff et al. 1992), a method which is most often further simplified by considering only inversions (Bourque & Pevzner 2002). Alternatively, the "break-point distance" between two genomes, defined as the minimum number of pairs of genes next to each other in one of the two genomes, but not in the other (Nadeau & Taylor 1984), can be used to infer phylogeny (Blanchette et al. 1997). However, the evolution of gene order involves additional rearrangement mechanisms that are not easily accounted for using these methods, such as translocation (i.e., transposition between chromosomes) and fusion or fission events of chromosomes.

More recently, methods based on maximum-likelihood (ML) distances (Wang & Warnow 2005), or on Bayesian inference (Larget et al. 2005, York et al. 2002), have been proposed that take multiple changes in gene order into account. They revealed an important level of saturation (York et al. 2002) and a limited tree-resolving power (Larget et al. 2005). The small size of mitochondrial genomes might explain the predominance of stochastic noise in the latter case. Prokaryotic genomes contain much more information, but are too large for analysis by current software. The drastic simplifying assumption that gene order can be reduced to the presence/absence of gene pairs allows the inference of prokaryotic phylogenies that are similar to the ones based on gene content (Korbel et al. 2002, Wolf et al.

2001). Further methodological and computational developments are needed to realize the full potential of gene-order methods.

GENE-CONTENT METHODS Phylogenomic analyses based on gene content generally use orthologs, but a few variants exist that use homologous instead of orthologous genes (Fitz-Gibbon & House 1999), protein domain content (Yang et al. 2005), or fold occurrence (Lin & Gerstein 2000, Yang et al. 2005). Interestingly, an orthologous gene present in all organisms (the Holy Grail of the sequence-based approach) has the same character state and is not informative for gene-content methods. This is an important source of corroboration because patterns informative for sequence-based approaches are not informative for gene-content approaches. Otherwise, the distribution of orthologs will be informative in the cases of (*a*) gene loss, which have a more than negligible probability of being convergent, (*b*) gene genesis, which is potentially the most informative, and (*c*) horizontal gene transfers (i.e., the acquisition of a new gene from a distantly related organism at the base of a clade will create a synapomorphy for this clade and a homoplasy for locating this clade). Gene content, albeit more integrated than primary sequences, is thus far from providing an unambiguous phylogenetic signal.

The binary matrices of presence/absence of homologous or orthologous genes can be analyzed by distance methods (Lin & Gerstein 2000, Snel et al. 1999), parsimony (Fitz-Gibbon & House 1999), or Dollo parsimony (Wolf et al. 2001). However, big/small genome attraction (Lake & Rivera 2004) appears to affect all these methods, as demonstrated by the artificial grouping of unrelated species with small genomes (e.g., *Mycoplasma*, *Buchnera*, *Chlamydia*, or *Rickettsia*). For instance, Copley et al. (2004) demonstrated that phylogenies based on gene content and protein domain combinations support the paraphyly of Ecdysozoa, but these results are biased by a systematic high rate of character loss in nematodes. When this bias is accounted for by computing the number of losses expected randomly, a slight support for the monophyly of Ecdysozoa is recovered. Interestingly, this artifact yields the same inconsistency phenotype as the one displayed by sequence-based analyses (Philippe et al. 2005); in both cases, arthropods are the sister group of vertebrates to the exclusion of nematodes. Such a convergence between the two methods could be explained by the fact that the fast-evolving species are also those that have undergone the most extreme genome reduction. This observation weakens the strength of the corroboration between gene-content and primary sequence approaches.

Altogether, gene-content phylogenies are not in excellent agreement with previous knowledge, even if we ignore the problem caused by small genome attraction. The monophyly of the three domains (Archaea, Bacteria, and Eukaryota) is always recovered, but the phylogeny within domains is much more problematic. For instance, *Halobacterium* never clusters with *Methanosarcina*, probably because of many HGTs from Bacteria that attract it toward the base of Archaea (Korbel et al. 2002); except with threshold parsimony (House et al. 2003), the monophyly of Proteobacteria is never recovered (Dutilh et al. 2004, Gu & Zhang 2004, Henz et al. 2005, Wolf et al. 2001).

Several technical improvements have recently been proposed. Because big/small genome attraction is akin to the problem of compositional bias in sequence-based approaches, this nonphylogenetic signal can be reduced by the use of the LogDet/paralinear transformation (Lake & Rivera 2004). A simple model of gene genesis and gene loss allows ML estimates of evolutionary distances (Gu & Zhang 2004, Huson & Steel 2004), but simulations showed that their performance appears to be slightly poorer than the performance of Dollo parsimony (Huson & Steel 2004).

In summary, the methods for inferring trees based on whole-genome features are at an early stage of their development, which might be comparable to that of sequence-based methods in the early 1970s. In particular, they generally lack a global probabilistic modeling. Numerous works are ongoing, and it will be important to extensively evaluate the accuracy of present and future methods.

Approaches Based on Primary Sequences

SUPERMATRIX VERSUS SUPERTREE The question of how to analyze multiple datasets has been the subject of intense debate (for reviews see Bull et al. 1993, de Queiroz et al. 1995). In brief, three approaches are mainly used: (*a*) total evidence (Kluge 1989), in which all datasets are combined together, called hereafter the supermatrix approach; (*b*) separate analysis (Miyamoto & Fitch 1995), in which the datasets are analyzed individually and resulting topologies are combined using consensus or supertree methods, called hereafter the supertree approach; and (*c*) conditional combination (Bull et al. 1993, Lecointre & Deleporte 2005), in which only the datasets considered as congruent are combined, called hereafter the conditional supermatrix approach. Generally, their respective advantages are minimizing stochastic error, increasing the significance of corroboration, and minimizing conflicting signals. In the practice of phylogenomics, the supermatrix is by far preferred (Murphy et al. 2001, Philippe et al. 2005, Qiu et al. 1999, Rokas et al. 2003), followed by a few cases of the supertree method (Daubin et al. 2002, Philip et al. 2005).

Although Bull et al. (1993) state that "no rational systematist would suggest combining genes with different histories to produce a single reconstruction," the reliance in the power of the supermatrix approach is quite strong, and this methodology is generally applied. Against Bull et al., one may argue that discordant genes will each display a different discordant history, which will be averaged away through a combined analysis (but see Matte-Tailliez et al. 2002). In most of the large multigene studies, the possibility of incongruencies is voluntarily minimized by selecting genes having a priori the same evolutionary history [e.g., single-copy genes (Lerat et al. 2003, Murphy et al. 2001, Philip et al. 2005), organellar genes (Qiu et al. 1999, Soltis et al. 1999), or orthology assessment based on synteny (Rokas et al. 2003)]. But generally, the homogeneity of the datasets was not tested, indicating a strict application of the total evidence principle.

The problem of homogeneity is important because several recurrent processes (gene duplication, HGT, or lineage sorting) can lead to incongruent gene trees.

In particular, the very notion of a "tree of life" has been questioned because of rampant HGTs (Doolittle 1999). Several studies have nevertheless argued that a strong phylogenetic signal is present in the prokaryotic genomes (for reviews see Brown 2003, Philippe & Douady 2003) and therefore that HGTs do not wipe out the notion of organismal phylogeny. It is clear, however, that few, and probably none, of the genes have followed exactly the organismal phylogeny during the entire history of life on Earth. Thus, HGTs constitute a source of nuisance that should be addressed to improve the accuracy of phylogenetic reconstructions.

The infrequent use of the conditional supermatrix approach is likely not due to a philosophical rejection of its principle but rather to the difficulty of detecting homogeneous datasets in practice. The incongruence length difference (ILD) test (Farris et al. 1995) was initially designed for parsimony and has been recently adapted to distance methods (Zelwer & Daubin 2004). However, the interpretation of this test is complicated by the fact that stochastic noise can generate by itself significant results (Dolphin et al. 2000). Its efficiency in detecting incongruence and determining data combinability has been repeatedly questioned (Darlu & Lecointre 2002, Dowton & Austin 2002, Yoder et al. 2001). Parametric incongruence tests have also been proposed for ML methods (Huelsenbeck & Bull 1996), or in a Bayesian framework (Nylander et al. 2004), although they are not yet in widespread use. A promising model has been proposed in which each gene can, with a certain prior probability, choose between either conforming to the common global topology or relying on its own topology (Suchard et al. 2003).

Nevertheless, the conditional supermatrix approach is used, despite the fact that the criteria used to discard gene/sequence are not well validated (Brochier et al. 2002, Brown et al. 2001, Lecointre & Deleporte 2005, Matte-Tailliez et al. 2002). However, the nature of the test used to decide whether a gene significantly supports a different topology yields divergent interpretations of the same data with regards to the importance of HGTs (Bapteste et al. 2004, Lerat et al. 2003, Zhaxybayeva et al. 2004). An improvement of these incongruence tests (Goldman et al. 2000) therefore constitutes an important avenue of future research.

The supertree approach is most often used to combine trees from the literature that were obtained from diverse sources of data (Sanderson et al. 1998), the most popular method being matrix representation with parsimony (MRP) (Baum 1992, Ragan 1992). The comparative efficiency of supermatrix and supertree approaches is poorly studied, especially in a phylogenomic context (Gatesy et al. 2004, Philip et al. 2005). We have therefore analyzed the dataset of 71 genes (Figure 1*a*) using a supertree MRP approach. Interestingly, almost all nodes inferred from the supermatrix or using the supertree are identical, except the position of urochordates within deuterostomes (not shown) and the relationships among protostomes, indicating an excellent congruence of the supermatrix and supertree (only 3 differences for 46 bipartitions). When the supertree is reconstructed with maximum parsimony (Figure 1*c*), Platyhelminthes are grouped with tardigrads + nematods instead of with other lophotrochozoans (annelids and molluscs), disrupting the monophyly of both Ecdysozoa and Lophotrochozoa. When the supertree is reconstructed using

Bayesian inference (Figure 1*d*), the results were slightly more consistent because the monophyly of Ecdysozoa, but not Lophotrochozoa, was recovered. The MRP supertree approach appears to have difficulty in placing the fast-evolving lineages (e.g., Platyhelminthes). The synergy among all positions in the supermatrix might explain the ability of the method to better deal with LBA artifacts. However, refined studies are urgently required to evaluate the relative accuracy and efficiency of the supermatrix and supertree methods.

SUPERMATRIX: SCALING UP CURRENT METHODS In contrast to the genome-feature approaches mentioned above, the main advantage of sequence-based methods is that their properties have been intensively explored, tested, and validated, so that many of their strengths and weaknesses are known. Indeed, in most sequence-based phylogenomic analyses published to date, almost the same protocols as for single-gene studies have been applied. The congruence among results obtained by different methods is high, with some notable exceptions (Canback et al. 2004, Goremykin et al. 2004, Soltis et al. 2002, Stefanovic et al. 2004). These incongruencies confirm the presence of a nonphylogenetic signal and suggest that the increase in resolution obtained by analyzing larger datasets is not in itself a guarantee of accuracy. Conversely, the agreement between the methods does not mean that the obtained tree is correct (see Brinkmann et al. 2005).

The dramatic change of scale of the data matrices implies the need for a corresponding increase in computational power, in particular for probabilistic methods. Two factors have to be considered. First, there is a simple scaling up of both the memory requirement and computational load. Second, the reliability of the heuristic search procedures underlying ML programs, or the Monte Carlo devices of the Bayesian samplers, is anything but guaranteed. A particular concern is that conflicting signals result in the presence of many secondary maxima separated by high potential barriers in the space of tree topologies. Standard procedures are likely to get trapped in these local optima (Salter 2001) and thus do not yield reliable phylogenetic estimates.

A number of algorithmic innovations have led to better heuristic searches in the space of topologies; these innovations include the following: genetic algorithms (Brauer et al. 2002, Lemmon & Milinkovitch 2002), disk-covering methods (Huson et al. 1999), and parallelized computing (Keane et al. 2005). In the case of the Bayesian methods, an interesting approach has been proposed consisting of using coupled "heated" Monte Carlo Markov Chains, which can be easily parallelized (Altekar et al. 2004, Feng et al. 2003). Thanks to these advances, ML and Bayesian phylogenetic reconstructions will soon be able to handle phylogenomic datasets. However, their overall reliability has been evaluated mainly on simulated data, which are probably much more "funnel shaped" toward the true phylogeny than are real sequences (Brinkmann et al. 2005, Stamatakis et al. 2005).

Another possible stance toward efficient tree space searches is to restrict the analysis by constraining nodes that have been found with high support when each gene of the concatenation was analyzed separately (Philippe et al. 2005). The number of trees compatible with these constraints is still large, but accessible to an exhaustive analysis. Such approaches may not be considered as a definitive method but could provide a good proxy.

TOWARD MORE COMPLEX MODELS If the availability of large data matrices poses new computational challenges to probabilistic methods, it allows the development of more realistic, parameter-rich models. As long as the number of parameters increases more slowly than the number of sites, a model does not fall into the infinitely many parameter trap (Felsenstein 2004) and thus has good consistency properties. Of course, no probabilistic model will ever capture evolutionary patterns in their full complexity, but their most important aspects, at least the ones that cause inconsistency of the current methods, can be accounted for (Steel 2005). The main idea is an improved flexibility that accounts for the diverse kinds of heterogeneities and disparities of the substitution processes.

One possible research direction is to account for disparities in the evolutionary process across the genes that make up the concatenation. A simple solution is to constrain the model to have a global topology, but gene-specific branch lengths (Yang 1996). More generally, any parameter other than the topology can be considered as gene-specific in such "separate" (or partitioned) models. Another possible avenue of research is to account for site-specific patterns of substitution, using mixture models (Kolaczkowski & Thornton 2004, Lartillot & Philippe 2004, Pagel & Meade 2004). A mixture model combines several different classes to describe the substitution process, each of which is characterized by its own set of parameters (e.g., equilibrium frequencies or exchangeability probabilities).

Thus far, few studies have tried to address the relative performances of alternative probabilistic models on phylogenomic datasets. A recent study (Brinkmann et al. 2005) has confirmed that accounting for site-specific rates, or having a good empirical substitution rate matrix, is an important factor, resulting in a higher phylogenetic accuracy. In contrast, separate models, in spite of their overall better statistical fit, do not seem to fundamentally improve phylogenetic inference. Because separate models handle a substantial part of heterotachy, this suggests that heterotachy, despite recent interest, may not constitute a major source of systematic bias. In any case, a much wider analysis of the impact of model choice on the prevalence of artifacts in phylogenomic inference has to be performed.

Reducing Systematic Errors Through Data Exclusion

Phylogenomic datasets contain a large amount of genuine phylogenetic signal, but they also contain nonphylogenetic signals that current methods of tree reconstruction are not able to perfectly handle. To avoid the perils of inconsistency, one can take advantage of the fact that the quantity of phylogenetic signal is no longer a serious limiting factor. More precisely, the part of the datasets that contains mainly nonphylogenetic signals can be excluded, allowing the concentration of phylogenetic signal in the remaining dataset. This increase of the phylogenetic to nonphylogenetic signal ratio reduces the probability of inconsistency even without the use of improved tree reconstruction methods.

The rationale of most methods of data exclusion is straightforward: Tree reconstruction artifacts are due to multiple substitutions that are not correctly identified as convergences or reversions by inference methods (Olsen 1987). The simplest possibility consists in removing the fast-evolving species, which by definition accumulate multiple substitutions. This approach efficiently reduces the misleading effect of the rate signal (Philippe et al. 2005, Stefanovic et al. 2004). In many cases, the exclusion of odd taxa (Sanderson & Shaffer 2002) is implicit because investigators never envision using them in their analyses (e.g., microsporidia as a fungal representative).

When all of the available species representing a clade of interest are fast evolving, the specific removal of the fastest-evolving sequences of this clade from the supermatrix appears to be efficient. For example, when a complete supermatrix of 133 genes is used, all tree reconstruction methods strongly, albeit artifactually, locate microsporidia at the base of eukaryotes, but probabilistic methods with a complex model (WAG + F + Γ) avoid this LBA artifact when >70% of the microsporidial sequences are coded as missing data (Brinkmann et al. 2005). It is interesting to note that a highly incomplete taxon (70% of the positions are unknown) is more accurately located than a complete one. A similar approach, in which genes in their totality are discarded, was successful at avoiding the attraction between the fast-evolving nematodes and Platyhelminthes (Philippe et al. 2005) or between nematodes and the out-group (Dopazo & Dopazo 2005). Interestingly, in the first case, the statistical support for the monophyly of Ecdysozoa and Lophotrochozoa increases when more and more genes are discarded (as long as more than 40 genes are considered).

These approaches are rather crude because sequences from genes and/or species are discarded in their totality. Nevertheless, even if these sequences contain much nonphylogenetic signal, they likely also contain some phylogenetic signal. Refined methods have been proposed to selectively eliminate fast-evolving characters in part (Lopez et al. 1999) or completely (Brinkmann & Philippe 1999, Burleigh & Mathews 2004, Dutilh et al. 2004, Pisani 2004, Ruiz-Trillo et al. 1999). In several cases, taxa that emerged at the base of the tree when all the characters are used are relocated later in the tree when fast-evolving positions are removed, strongly suggesting that their observed basal position is due to an LBA artifact (Brochier & Philippe 2002, Philippe et al. 2000, Pisani 2004). However, the number of remaining slowly evolving positions is often too small to yield high statistical support for most of the clades in single-gene analyses but not in phylogenomic analyses (Burleigh & Mathews 2004, Delsuc et al. 2005).

The RY-coding strategy (Woese et al. 1991) discards all fast-evolving transitions and improves inference without drastically compromising the resolution (Phillips et al. 2004). Importantly, this coding not only addresses the problem of rate signal but also of compositional signal. Indeed, the G + C content can be extremely variable among homologous sequences from various organisms, whereas the frequency of purines is remarkably homogeneous (Woese et al. 1991). This constitutes a method of choice to avoid inconsistency resulting from compositional bias.

Finally, it is possible to remove characters whose evolutionary history violates most the assumptions of the underlying model of sequence evolution instead of the fastest-evolving characters/species. In fact, the RY coding eliminates transitions that are mainly responsible for the nonstationarity of the nucleotide composition (see Hrdy et al. 2004 for a similar approach in the case of proteins). Similarly, the constant sites violate the assumptions about the distribution of site rates (Lockhart et al. 1996), and their elimination constitutes an efficient way of improving inference (Hirt et al. 1999, Phillips et al. 2004). Heterotachous sites violate the assumption that the evolutionary rate of a position is constant through time, made by all current models except the covarion model (Fitch & Markowitz 1970). As expected, the elimination of these sites reduced LBA artifacts in the case of the eukaryotic phylogeny (Inagaki et al. 2004, Philippe & Germot 2000).

We believe that these data removal approaches are complementary to the improvement of tree reconstruction methods through the implementation of more realistic models of sequence evolution. So far, they are also more readily accessible in practice because they are less demanding in terms of the complexity of bioinformatic methods and computational time.

CONCLUDING REMARKS

In this review, we have emphasized that inconsistency of tree reconstruction methods constitutes the major limitation of phylogenomics. We have explored ways to reduce its impact, whether at the level of data assembly or at the level of tree building per se. Adequate taxon sampling, probabilistic methods, and the exclusion of phylogenetically misleading data constitute the three most important criteria required to obtain reliable phylogenomic trees. However, this might not be sufficient to ensure that the inferred tree is the correct one. We believe that an important test of the reliability of tree reconstruction methods is their robustness with respect to species sampling. In particular, a reliable method should be able to recover exactly the same topology with a taxon-poor and a taxon-rich sampling, which is far from being the case at present (Figure 1). This is particularly important to be confident in the phylogenetic location of poorly diversified clades (e.g., *Amborella*, or monotremes). The stability of phylogenies in face of variation of species sampling will constitute one of the best guarantees that the nonphylogenetic signal has been correctly handled.

The correctness of inferences should also be verified via corroboration from independent sources (Miyamoto & Fitch 1995, Swofford 1991). When complete genomes are used, this may appear hopeless (even if internal verifications of homogeneity are possible). However, genomes can be conveniently subdivided into various character types that can be considered as more or less independent, e.g., oligonucleotide composition, sequences of orthologous gene, gene content, and gene order. If inferences based on these "independent" sets of characters converge to the same results, an increased confidence can be placed in the corresponding phylogeny, although the same bias can theoretically affect several approaches. We therefore encourage the development of sophisticated probabilistic methods for all types of data and not just for primary sequences.

In the long term, one might envision that the tree of life, or at least its global scaffold, will be established within the next 10 years. Then, to numerous molecular systematists the important question will be: What next?

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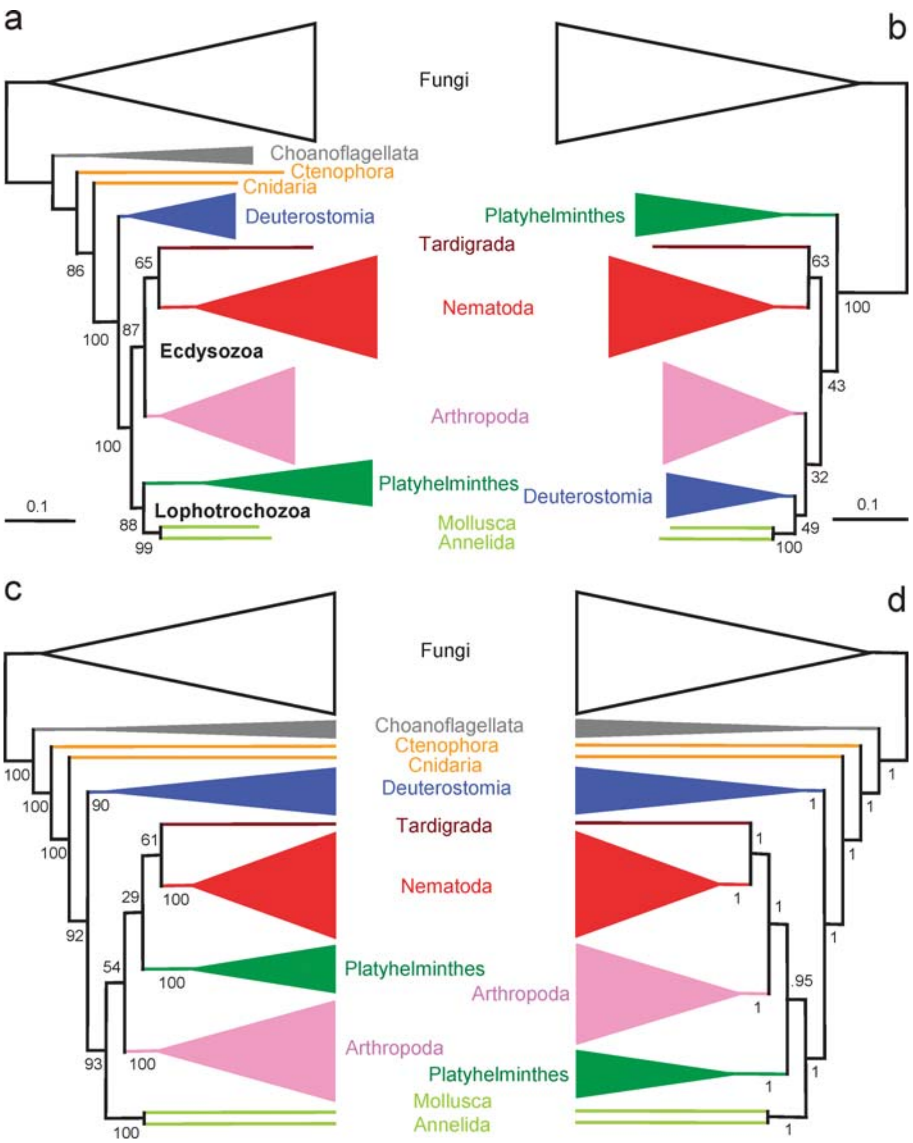

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Figure 1 Supermatrix, taxon sampling and supertree in animal phylogenomics. A dataset constituted of 71 slowly evolving nuclear proteins corresponding to 20,705 amino acid positions (Philippe et al. 2005) was used for phylogenetic analyses based on maximum likelihood (ML) with a separate WAG + F + Γ model. Panel (*a*) presents the ML tree obtained with 49 species (redrawn from Philippe et al. 2005). This tree strongly supports the new animal phylogeny (Aguinaldo et al. 1997), dividing Bilateria into Deuterostomia and Protostomia, which comprises Lophotrochozoa and Ecdysozoa. Note that the major division between Deuterostomia and Protostomia is supported by a 100% bootstrap value. In panel (*b*), the exclusion of only four close out-group sequences (two Choanoflagellata, Cnidaria, and Ctenophora) creates an LBA artifact via the distant fungal out-group leading to the successive early emergence of the fast-evolving Platyhelmintes and then nematoda plus Tardigrada. Note that the overall bootstrap support substantially decreases, requiring caution when the existence of a radiation is extrapolated from low statistical supports because limited resolution can also be due to poor taxon sampling or to unreliable tree reconstruction methods (not shown). Branch lengths are drawn proportionally to evolutionary rates, and the height of triangles represents the taxonomic diversity of the different groups. Panel (*c*) presents the supertree obtained by maximum parsimony analysis conducted with PAUP* (Swofford 2000) of the matrix representation of 71 source trees obtained from Bayesian analyses of individual genes with MrBayes (Ronquist & Huelsenbeck 2003) using a WAG + F + Γ model. This supertree recovers both the monophyly of Deuterostomia and Protostomia with strong bootstrap support, but fails to find Ecdysozoa and Lophotrochozoa. Panel (*d*) shows the supertree obtained on the same matrix from a Bayesian analysis using MrBayes and a simple two-state model. This supertree strongly supports the respective monophyly of Deuterostomia, Protostomia and Ecdysozoa, but not of Lophotrochozoa. Numbers on branches are bootstrap values (*a*–*c*) or posterior probabilities (*d*).

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CONTENTS

INDEXES

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