

Incongruence in the phylogenomics era

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Abstract

Genome-scale data and the development of novel statistical phylogenetic approaches have greatly aided the reconstruction of a broad sketch of the tree of life and resolved many of its branches. However, incongruence – the inference of conflicting evolutionary histories – remains pervasive in phylogenomic data, hampering our ability to reconstruct and interpret the tree of life. Biological factors, such as incomplete lineage sorting, horizontal gene transfer, hybridization, introgression, recombination and convergent molecular evolution, can lead to gene phylogenies that differ from the species tree. In addition, analytical factors, including stochastic, systematic and treatment errors, can drive incongruence. Here, we review these factors, discuss methodological advances to identify and handle incongruence, and highlight avenues for future research.

Sections

Introduction

Biological factors

Analytical factors

Detecting incongruence

Future directions

Conclusions

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Introduction

“The stream of heredity makes phylogeny; in a sense, it is phylogeny. Complete genetic analysis would provide the most priceless data for the mapping of this stream.”
George Gaylord Simpson¹

Phylogenetics aims to reconstruct the evolutionary histories of organisms, genes, traits or other biological features by examining the distribution of inherited characters in descendant lineages and tracing them back in time to identify ones that shared a common ancestor. ‘Trees’ inferred from phylogenetic analyses of biological features represent the best-supported hypotheses of their evolutionary histories, that is, the statistically most probable path rather than the ground truth. Phylogenetic approaches that use genome-scale data, or phylogenomics, have become the gold standard for understanding the evolution of lineages in the tree of life, a prerequisite for understanding the evolution of biological features^{2–5}. Defined initially as predicting gene function from phylogenies of homologous genes⁶, phylogenomics was later expanded to include phylogenetic inference using genome-scale data⁷. Phylogenomics has revolutionized systematic biology, resolving numerous branches of the tree of life that were previously contentious and increasing our confidence in many others^{8–14}.

Despite these successes, phylogenomic studies can sometimes support conflicting tree topologies^{15,16}, which suggests that certain branches of the tree of life are challenging to resolve, even with genome-scale data. Some of these branches concern relationships that are key to our understanding of the most exciting episodes in evolution (for one example, see Box 1), hindering our ability to resolve the tree of life. Incongruence is an umbrella term that describes the inference of conflicting tree topologies. This phenomenon can be observed at all time scales, from very ancient (hundreds of millions to billions of years old) to very recent (tens of thousands to millions of years old), and at all levels of genomic organization, from whole chromosomes to individual sites in a multiple sequence alignment (Fig. 1). The primary drivers of incongruence are biological processes that cause the genealogies of DNA sequences to differ from the genealogy of their species (for example, hybridization or horizontal gene transfer events)^{2,5} and analytical shortcomings that lead to errors in inference (for instance, erroneous orthologue detection or poor model fit)¹⁷. Dissecting the contribution of biological and analytical drivers of incongruence can improve phylogenetic inference and deepen our understanding of phylogenesis and the evolutionary process.

Now, more than two decades after the dawn of phylogenomics, our understanding of the factors contributing to incongruence has matured. Concomitant development of methods and software that aid in identifying and accounting for incongruence in phylogenomic analyses has improved accuracy in inference. This Review synthesizes the biological and analytical factors that drive incongruence, discusses methodological advances to identify and handle incongruence, and highlights avenues for future research.

Biological factors

Several processes influence the evolution of genomic regions; these biological factors can cause a gene tree (which shows the evolutionary relationships between sequences of a single gene) to differ from the species tree (which shows the evolutionary relationships between different species) and contribute to incongruence (Fig. 2). Note that the term ‘gene tree’ is often used as shorthand for any locus (for example,

a protein-coding or a non-coding region) in the genome; for simplicity, we follow this convention.

Incomplete lineage sorting

Incomplete lineage sorting can occur during the speciation process when alleles in a population fail to coalesce due to retention and random sorting of ancestral polymorphisms, causing, at times, alleles to first coalesce with alleles from more distantly related species (Fig. 2). It is common across sexually reproducing organisms because allelic polymorphisms often persist across multiple speciation events^{18–20}. Incomplete lineage sorting does not always result in gene trees that are incongruent with the species phylogeny but, when it does, it is referred to as hemiplasy²¹ (Table 1). Hemiplasy is particularly prevalent when populations are large and the time interval between speciation events is short²² and can affect a substantial fraction of the genome. Examination of the evolutionary history among 500-bp windows of the human, chimpanzee, bonobo, gorilla and orangutan genomes revealed that ~37% of the human genome exhibits hemiplasy, and the evolutionary histories of these loci conflict with the species tree topology¹⁸ (Fig. 2).

By modelling the underlying probability distribution of gene trees within a species tree, the multispecies coalescent model provides a framework that incorporates incomplete lineage sorting in phylogenomic inference²³. One approach for evaluating whether hemiplasy explains gene tree–species tree incongruence is by simulating trees under the multispecies coalescent model and comparing levels of observed and expected gene tree incongruence²⁴. If the observed incongruence is equal to the expected incongruence under the model, then hemiplasy is the major contributor; if not, other analytical or biological factors are likely (also) at play.

Other approaches, such as the one implemented by the BEAST software, use Bayesian statistics to co-estimate gene trees and species phylogenies in the presence of incomplete lineage sorting^{25,26} (Table 2). These fully coalescent methods are computationally expensive, hindering their use for large phylogenomic data matrices. To reduce computational costs, summary coalescent-based methods implemented in various software packages, including STAR, MP-EST, ASTRAL, ASTER and ASTEROID^{27–31} (Table 2), infer the species tree from pre-inferred single-gene trees in phylogenomic data matrices but at the cost of increased error rates in gene and species tree inference, especially for ancient divergences (see the section Analytical factors). Thus, although hemiplasy can contribute to the incongruence of both ancient and recent divergences, it is much more likely to be detectable in the latter.

Horizontal gene transfer

Genomic regions that experienced horizontal gene transfer also have histories that deviate from the species tree (Fig. 2 and Table 1). For example, eukaryotic acquisition of bacterial loci leads to gene phylogenies where eukaryotic sequences are nested within clades of bacterial sequences^{5,32}. The contribution of horizontal gene transfer to incongruence is asymmetric across the tree of life; horizontal gene transfer is very common in Bacteria and Archaea and is a notable driver of genome evolution in these lineages^{33,34}. Horizontal gene transfer in eukaryotes is less common, although evidence of its importance in eukaryotic genome evolution is increasing³⁵.

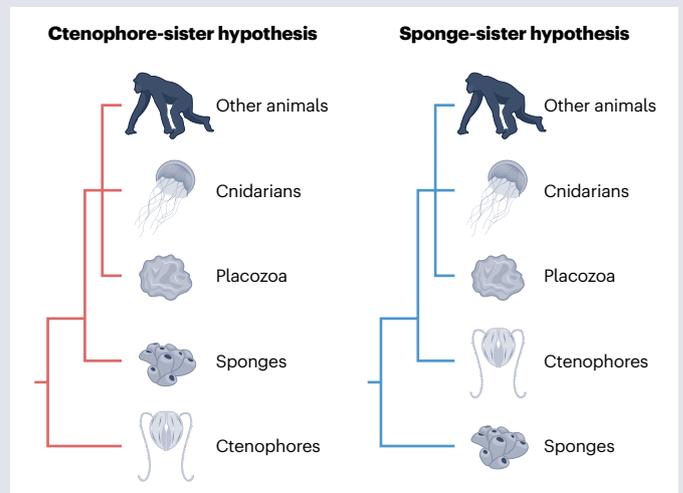
For lineages with low levels of horizontal gene transfer, incongruence stemming from horizontal gene transfer can be ameliorated by removing genes with signatures of transfer from the phylogenomic data matrix³⁶. Horizontally transferred genes can be identified using phylogeny-based methods such as topology tests (implemented

Box 1

Rooting the animal tree

Few branches in the tree of life are as intensely debated as the root of animal phylogeny. The two leading hypotheses debate whether sponges^{15,95,212–214} or comb jellies (ctenophores)^{12,16,67,198,215,216} are the sister group to a clade of all other animals. These two hypotheses have come to be known as the sponge-sister and ctenophore-sister hypotheses, respectively (see the figure). Resolution of the root of the animal tree has a bearing on our understanding of how animal cell types and tissues evolved²¹⁷. Sponges lack muscles and a nervous system and are sometimes thought of as morphologically ‘simpler’ animals compared to ctenophores, which have both^{218–220}. Which hypothesis is correct also has implications for whether ctenophore nervous systems are structurally and genetically homologous to those of bilaterian animals^{221,222}, with some arguing that the ctenophore nervous system evolved independently²²³.

Numerous biological and analytical factors contribute to this challenging phylogenetic problem. Much of the controversy has centred around whether site-homogeneous (with gene partitioning) or site-heterogeneous models of sequence evolution are most appropriate for reconstructing the animal phylogeny^{198,224}. These models are largely employed to combat long-branch attraction, an artefact central to the debate because ctenophores have a long branch leading up to the lineage²²⁵. Site-heterogeneous models with many categories tend to support the sponge-sister hypothesis^{15,198}, whereas site-heterogeneous models with fewer categories and site-homogeneous models tend to support the ctenophore-sister hypothesis¹⁹⁸. Some simulation analyses suggest that site-heterogeneous models underperform site-homogeneous models with gene partitioning²²⁶ and others suggest the opposite²²⁵. Aimed at reducing saturation and compositional biases, data matrix recoding analyses supported the sponge-sister hypothesis^{153,212}; however, some of these analyses²¹² failed to recover well-established monophyletic clades, such as Chordata, suggesting that analyses of non-recoded data were more accurate²²⁷. Poor taxon sampling has also long impacted this phylogenetic question, but new genomes and transcriptomes have recently been made available for key lineages — sponges, ctenophores, cnidarians and placozoans^{15,16,153}. Outgroup choice has also been important to the debate: the sponge-sister hypothesis is most frequently supported when choanoflagellates are chosen as the outgroup, whereas



the ctenophore-sister hypothesis is supported when a broader sampling of single-celled relatives of animals (Holozoa) and fungi (Opisthokonta) is used¹⁹⁸.

Several other factors, such as orthologue inference errors and multiple sequence alignment errors, are likely at play. The possibility that additional biological factors, such as hybridization or incomplete lineage sorting, also contribute cannot be excluded; however, detecting the effect of multiple analytical and biological factors in such an ancient divergence is challenging. Resolving the root of the animal tree may require extensive amounts of new (high-quality) data such as expanded taxon sampling of sponge, ctenophore and choanoflagellate genomes²¹⁷. Similarly, other lines of evidence, such as investigations of synteny conservation using chromosome-level genome assemblies²²⁸ — an independent line of evidence that does not have the same pitfalls as sequence data analyses — may shed light on the root of the animal tree. Interestingly, a very recent study identified seven genomic regions displaying synteny conservation across animals except for ctenophores but no regions with conserved synteny across animals except for sponges, providing support for the ctenophore-sister hypothesis²²⁹.

in major programmes such as RAXML and IQ-TREE 2) that evaluate whether the gene tree topology indicative of horizontal gene transfer is significantly better than topologies that do not invoke transfer³⁷. Horizontally transferred loci can also be detected by sequence composition-based methods, where notable changes in the GC content or codon usage bias of one or more loci relative to the rest of the genome are used to identify signatures of horizontal transfer³⁸, or using sequence similarity-based methods to detect foreign sequences such as alien index³⁹. Sequence composition-based and similarity-based methods are faster, can be implemented across entire genomes, and are primarily suitable for recent events where the acquired sequence has not substantially diverged from the donor sequence; by contrast,

phylogeny-based methods are generally more accurate, especially for ancient episodes of transfer, but slower and typically used to test horizontal transfer for one or a few loci.

An alternative approach is to infer the species phylogeny through a probabilistic model of sequence evolution that explicitly models horizontal gene transfer as one of the processes that lead to gene tree–species tree incongruence^{40,41}, using programmes such as SpeciesRax⁴². Horizontal transfer can occur between both closely related species as well as between distantly related ones. However, irrespective of the method used, inference of gene transfer — and amelioration of its effects on incongruence — among distantly related species is much easier than among close relatives. This is because horizontal

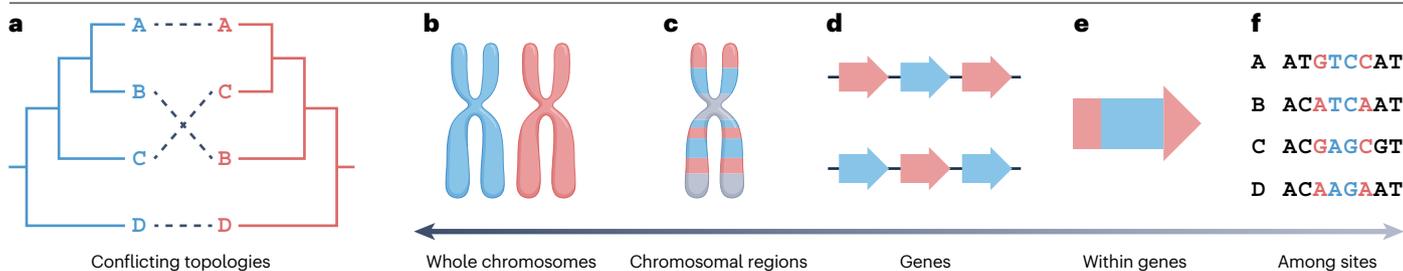


Fig. 1 | Incongruence at different levels of genomic organization. The topology shown in blue supports a sister group relationship of taxa A and B, whereas the red topology supports a sister group relationship of taxa A and C (part a). The inference of such conflicting topologies defines incongruence. Incongruence can occur at different levels in the genome, such as among whole chromosomes (for example, analyses of one chromosome support the blue

topology but analyses of another support the red topology) (part b), regions of a chromosome (grey regions represent lack of homology) (part c), genes (or loci) (part d), within a gene or locus (for example, different domains support different topologies) (part e) and among sites in a multiple sequence alignment (part f). Note that incongruence is also prevalent in other types of data (for example, behavioural or morphological traits) and can occur at all evolutionary depths.

gene transfer between closely related species is much more difficult to distinguish from other evolutionary processes such as differential gene duplication and loss.

Hybridization, introgression and recombination

The exchange of genetic material between species during hybridization or introgression introduces alleles with evolutionary histories that deviate from the history of species, leading to gene tree–species tree incongruence^{43,44} (Fig. 2). When the hybrid species has the same ploidy as the parental species, hybridization can be detected through phylogeny-based and sequence read-mapping methods. In phylogeny-based methods, phylogenomic data matrices containing loci from the hybrid and both parental species are expected to show nearly equal support (using measures such as internode certainty, gene support frequency and concordance factors; see the section Detecting incongruence) for two distinct topologies because roughly one-half of the hybrid genome derives from each parent⁴⁵. Similarly, in sequence read-mapping methods, such as the one implemented in *sppIDer*⁴⁶ (Table 2), half of the sequence reads of the hybrid are expected to map to one parental species and the other half to the other parental species. Hybrid species that differ in their ploidy from the parental species (for example, allodiploid hybrids) can also be detected using the above methods but their gene number is also expected to be the sum of the genes in the parental species⁴⁷. Approaches that ameliorate the contribution of hybridization to incongruence include first separating the hybrid genome into parental subgenomes prior to phylogenomic inference⁴⁷ and using probabilistic models that explicitly incorporate hybridization as one of the processes contributing to incongruence⁴⁸.

Introgression can also affect large genomic regions that can be several megabases in size or greater and lead to incongruence but it is potentially more challenging to detect because the percentage and distribution of introgressed regions can vary. Methods for introgression detection typically aim to identify allele patterns across species that significantly deviate from a null model in which these patterns are governed only by incomplete lineage sorting (and no introgression). These include the D-statistic (also known as the ABBA-BABA test), which is designed to detect gene flow between two taxa in a four-taxon phylogeny⁴⁹; D_{FOIL} , which expands the D-statistic for the five-taxon case⁵⁰; and D_3 and the branch-length test, which use the signal of pairwise divergence⁵¹ – wherein gene trees that support introgression have shorter branch lengths⁵² – for introgression detection (Table 2). Removing loci

with signatures of introgression or directly modelling the process can ameliorate incongruence stemming from introgression⁴⁴. For example, inclusion of introgressed regions (detected using the D-statistic) in a phylogenomic data set of passerine birds led to an incorrect species phylogeny; more accurate inference of the species phylogeny required careful examination not only of the topologies of individual loci but also of some of their properties such as recombination frequency and nucleotide diversity⁴³.

Recombination, a frequent phenomenon in diverse lineages, including prokaryotes and viruses, can also give rise to mosaic sequences and incongruence. In these instances, incongruence depends on the fraction of recombinant sites and how closely related the taxa are⁵³. Sequences with evidence of recombination can be detected using *PhyPack* or *RDP*^{54,55} and removed from the data matrix before inference. Accurate inference of all three processes is inversely proportional to the ages of the events; therefore, evaluating their contribution to incongruence in ancient divergences is challenging.

Natural selection

Natural selection generally leads to the divergence of sequences; however, selection for the same or similar traits in distantly related taxa can result in convergent molecular evolution⁵⁶ (Table 1). Thus, trees that contain sequences that experienced convergent evolution may erroneously suggest that these sequences are closely related, reflecting the shared influence of selection rather than common ancestry (Fig. 2). For example, phylogenetic analysis of the gene *prestin*, which encodes a transport protein present on the membrane of cochlear outer hair cells, groups sequences from echolocating organisms, such as bats and whales, together. This grouping occurs because the bat and whale sequences of *prestin* have experienced convergent molecular evolution as bats and whales are not sister lineages⁵⁷. One method for detecting convergent sequence evolution is reconstructing ancestral sequences and identifying convergent amino acid substitutions in independent branches of the species phylogeny, if known⁵⁸. Ancestral sequence reconstruction can be done with diverse software, including *IQ-TREE*⁵⁹, *FireProt*^{ASR60} and *PhyloBot*⁶¹ (Table 2). Cases of convergent molecular evolution that affect one or a few genes are best handled by removing such genes, if they are known, from the data matrix before tree inference but their contribution to incongruence is generally expected to be small.

Convergent molecular evolution can also be observed in phylogenomic analyses of entire genomes or proteomes. For example,

convergent amino acid usage, such as the convergence observed in high-salt adapted Methanotroph archaea and Haloarchaea towards similarly acidified amino acid compositions in their proteomes, can obfuscate phylogenomic inference⁶². In such cases, incongruence can be reduced through exclusion of affected sites, character recoding (see the section Character recoding) and the use of models that explicitly account for compositional heterogeneity. For example, recent analyses on the evolutionary origins of mitochondrial genomes, a case of incongruence where compositional biases are at play⁶³, using a model that accommodates both across-site and across-branch compositional heterogeneity supported mitochondria as the sister lineage to Alphaproteobacteria⁶⁴.

Analytical factors

The content of phylogenomic data sets and choices in how these data sets are constructed and analysed can also contribute to incongruence (Fig. 3). Incongruence due to stochastic errors stems from statistical uncertainty when too few molecular markers or taxa are analysed. Incongruence from systematic errors stems from incorrect or inadequate assumptions in analysis such as substitution model misspecifications, a lack of realistic models or erroneous orthologue detection. Finally, choices in experimental design or treatment of phylogenomic data are an emerging category of error that can also lead to incongruence, sometimes exacerbating or leading to additional stochastic and/or systematic errors; we term these treatment errors.

Stochastic errors

Taxon sampling. Taxon sampling has a critical role in species tree inference and incongruence (Fig. 3a) because the number and taxonomic distribution of the sampled taxa influence numerous downstream analyses such as predicting orthologous groups of genes and the estimation of substitution model parameters (Table 1). Generally, including more taxa improves tree inference but can lead to speed versus accuracy trade-offs (see the section Treatment errors). In some cases, incongruence can guide the sampling of additional taxa. For example, the placement of the family *Ascoideaceae*, represented by a single taxon, was unstable in early phylogenomic studies of Saccharomycotina yeasts^{65–67}, but the inclusion of three additional taxa from *Ascoideaceae* stabilized its placement⁶⁸. Similarly, the inclusion of additional taxa that diverged near the base of the land plant phylogeny increased the stability of phylogenetic inference^{69–71}. However, taxon pruning, such as removing rogue taxa whose placement is unstable across a set of trees (for example, across a set of gene trees), may also improve congruence and accuracy in some cases^{72,73}. Comprehensive taxon sampling may not always be possible, for example, for ancient lineages that contain one or a few closely related extant species such as coelacanths and lungfish⁷⁴. However, studies of ancient DNA can shed light on phylogenetic relationships in cases where extant taxon sampling is difficult or impossible^{75,76}.

Locus sampling. How much sampling of sequence data is required depends on the specific evolutionary history of the lineage examined and how ancient or recent it is, on the information content of the loci used to reconstruct it, and on the evolutionary history of the loci^{9,77,78} (see the section Biological factors). Thus, incongruence stemming from limited sampling of sequence data can affect the resolution of ancient and recent divergences^{79,80} but can generally be improved with additional sampling of molecular markers (Table 1). Additional molecular markers can be obtained using programmes that identify

single-copy orthologues from multi-copy gene families, for example, OrthoSNAP or DISCO^{81,82} (Table 2). However, there is a limit imposed by the sequence divergence of the genomes examined, such that the resolution of relationships of genome sequences that contain relatively few informative sites and/or many taxa, such as the SARS-CoV-2 whole-genome alignments⁸⁰, will be challenging from sequence data alone. Additionally, data sets that contain short sequences (for example, gene fragments or short genes) often contain insufficient numbers of sites for robust gene tree inference when using summary-based coalescence methods and can contribute to incongruence⁸³ (Fig. 3a) but, at times, this limitation can be overcome by collapsing poorly supported branches before species tree inference²⁹.

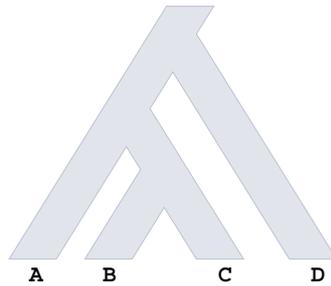
Molecular markers included in phylogenomic data matrices typically exhibit partial or incomplete taxon coverage. This can increase statistical uncertainty, leading to identical support for multiple topologies, referred to as tree terraces^{84,85}. For example, in a 3-locus, 298-taxon data matrix from grasses (Poaceae), with taxon coverage of 66%, the optimal tree is on a terrace with 61.2 million other equally supported topologies⁸⁴. Tree terraces can be addressed through increased taxon coverage across molecular markers and locus sampling. For example, analysis of a 129-locus, 117-taxon data matrix of arthropods, with a coverage density similar to that of the data set of grasses (65%), yielded a single optimal tree^{84,86}. The Gentrus function in IQ-TREE can help identify and characterize phylogenetic terraces⁵⁹ (Table 2).

Partial taxon coverage can stem from genuine differences in the gene content of organisms or from missing data (for example, from incomplete genome assemblies or errors in gene annotation). To reduce the negative effects of partial taxon coverage on inference, phylogenomic studies typically implement a taxon occupancy threshold of 50% or higher per locus^{68,87}. However, different taxon occupancy thresholds may be optimal for different clades. For example, among Lori and Lorikeet birds, a taxon occupancy threshold of 70% was necessary to ameliorate the impact of missing data⁸⁸.

Systematic errors

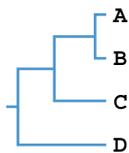
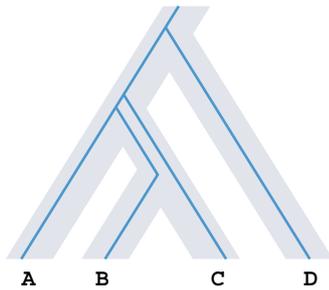
Orthologue inference. Phylogenomic analyses often rely on single-copy orthologous genes, but errors in orthology inference, such as hidden orthology, can lead to incongruence. The over-splitting of orthologous groups of genes can stem from sequence length biases among orthologues because both BLAST bit scores and expectation values have a length dependency: longer sequences that contain many hundreds or thousands of base pairs have higher maximum bit scores and lower expectation values. Thus, variation in sequence length within an orthologous group of genes can lead to the exclusion of shorter sequences⁸⁹ (Fig. 3a and Table 1). Hidden orthology can also stem from a failure to detect rapidly evolving orthologues, an issue exacerbated across large evolutionary distances⁹⁰, resulting in artefactual inferences of lineage-specific genes. Hidden orthologues can be detected using 'bridging' methods such as Leapfrog, an algorithm for the identification of instances of reciprocal best BLAST hits in two different orthologous groups of genes⁹¹ (Table 1). Probabilistic modelling approaches, such as profile Hidden Markov Models implemented in HMMER that leverage site-specific parameterization of conservation (or lack thereof) from multiple sequence alignments, are more sensitive in detecting rapidly evolving orthologues⁹² and reduce the risk of hidden orthology (Table 2). Improved taxon sampling (for example, inclusion of under-represented lineages) in multiple sequence alignments used to construct profile Hidden Markov Models, such as those

Species tree

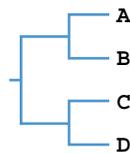
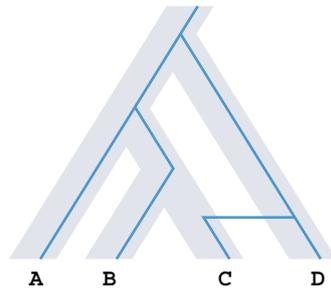


Incongruent locus trees

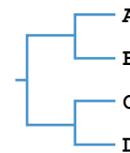
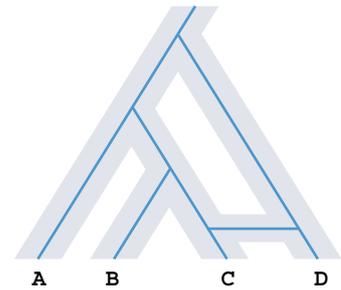
Incomplete lineage sorting



Horizontal gene transfer

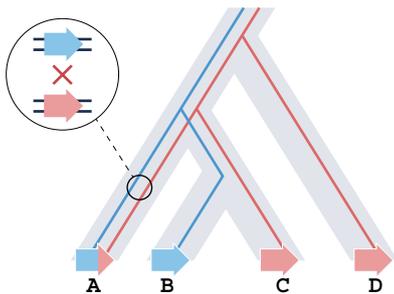


Hybridization or introgression



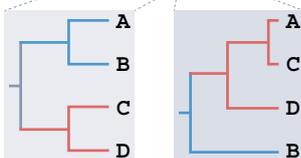
Time

Recombination

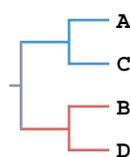
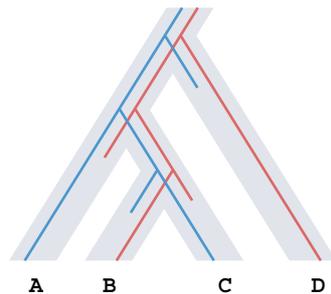


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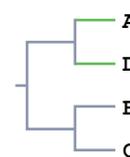
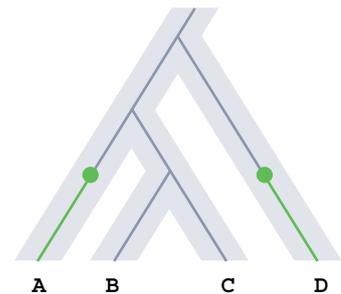
>A
ATG---GATCC
>B
ATG---GATTG
>C
ATGATCGATCG
>D
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Duplication and loss



Convergent evolution



Time

Fig. 2 | Major biological factors that contribute to incongruence. The true species phylogeny is depicted in grey (top). Six exemplary processes that contribute to incongruence are depicted (bottom). For each example, the true species history is depicted in grey and the evolutionary history of a locus is depicted as lines within the species history. Incomplete lineage sorting can lead to gene trees that differ from the species phylogeny due to variation in the sorting of ancestral polymorphisms. Horizontal gene transfer, hybridization and introgression can also lead to gene phylogenies that differ from the species tree due to non-vertical evolution. Recombination can result in loci with chimeric evolutionary histories. Here, two distinct loci depicted in red and blue recombine on the branch leading to species A, resulting in one part of the gene that tracks

with the evolutionary history of the red locus and another that tracks with the evolutionary history of the blue locus. Ancestral gene duplication followed by asymmetric patterns of paralogue loss can lead to hidden paralogy. Here, gene duplication occurred in the last common ancestor of all four taxa, giving rise to blue and red paralogues. Loss of the red paralogue in taxa A and C and loss of the blue paralogue in taxa B and D results in a one-to-one paralogous group of genes whose evolutionary history differs from that of the species. Independently evolved traits in different phylogenetic lineages can be associated with convergent molecular evolution (green), meaning that loci independently underwent similar nucleotide or amino acid substitutions in their sequences. One or a combination of these factors can contribute to loci incongruent with species histories.

implemented in TIAMMAT, can further improve the sensitivity of sequence similarity searches⁹³ (Table 2).

Another systematic error source is the asymmetry in rates of gene duplication and loss between species, which can result in hidden paralogy. At shallow evolutionary depths (that is, when comparing species that diverged less than a hundred million years ago), hidden paralogy can be detected by examining synteny, for example, examining the synteny of six yeast species that underwent differential patterns of gene loss since a shared whole-genome duplication event revealed that ~10% of inferred single-copy orthologues were hidden paralogues⁹⁴. Detecting hidden paralogy instances deeper in time (that is, when comparing species that diverged hundreds of millions or billions of years ago) is more challenging because synteny is likely not conserved. In such cases, hidden paralogues can potentially be detected by searching for gene trees where well-known clades are not monophyletic^{95,96}. Alternatively, because hidden paralogues can be quite divergent from the rest of the sequences in an orthogroup, they can also be identified by examining gene trees for taxa that have unexpectedly long terminal branches using software such as TreeShrink⁹⁷, PhyloFisher⁹⁸ and PhyKIT⁹⁶ (Table 2). In paralogues, especially species-specific ones, can easily be handled by retaining one of the two sequences as implemented in PhyloTreePruner⁹⁹ and OrthoSNAP⁸¹.

Errors in orthologue inference can also stem from contaminated sequences in genome assemblies, a key concern in metagenome-assembled genomes. The degree of contamination (and completeness) of a given genome can be evaluated with the CheckM¹⁰⁰ and miComplete¹⁰¹ programmes, and contaminant sequences can be removed prior to inference.

Modelling substitutions. Traditional substitution models are site-homogeneous models, which use one reversible substitution matrix and the same nucleotide or amino acid frequencies for all sites in a data matrix. Early nucleotide models assumed equal substitution rates and base frequencies¹⁰². Later models incorporated biologically informed parameters such as accounting for differences in the rates of transitions and transversions or base frequencies^{103,104}. The most parameter-rich model among reversible models for nucleotide sequences is the generalized time-reversible model, which uses unequal substitution rates and base frequencies¹⁰⁵. Nucleotide substitution models that relax the assumptions of reversibility (that is, the rate at which a particular nucleotide, say A, changes to another one, say G, is not the same as the rate of a G changing to an A), stationarity (nucleotide frequencies do not change over time) and independence (changes at each site in the alignment are independent of changes at other sites) also exist, but they are computationally expensive and not typically used in phylogenomic studies¹⁰⁶.

In contrast to these mechanistic substitution models for nucleotide sequences, substitution models for amino acid sequences are often inferred from empirical multiple sequence alignments. For example, the amino acid exchange probabilities in the mtMAM substitution model were estimated empirically by examining the rates of amino acid substitutions across the mitochondrial proteomes of 20 mammals¹⁰⁷; other substitution models, such as WAG¹⁰⁸ and LG¹⁰⁹, are derived by estimating substitution rates from larger, more diverse data bases of amino acid sequence alignments like Pfam.

Determining the best-fitting nucleotide and amino acid substitution models is often done using likelihood ratio tests and Akaike or Bayesian information criteria¹¹⁰. The latter outperform likelihood ratio tests but also have their shortcomings, which can result in the wrong model being favoured¹¹¹. Of note, model fit does not always predict phylogenetic tree accuracy, and models of variable fit can sometimes result in consistent phylogenetic trees¹¹². For example, the generalized time-reversible model is often the best-fitting nucleotide reversible model; however, the large number of estimated parameters in this model may need to be revised for specific analyses¹¹³. In general, the modelling of substitutions is more challenging in ancient divergences than in more recent ones because the variation of mutational processes and evolutionary rates is typically greater in analyses of distantly related taxa. Another avenue of modelling sequence evolution is through direct experimental

Table 1 | Drivers of incongruence

Driver of incongruence	Factor	Refs.
Incomplete lineage sorting	Biological	20,24,194
Horizontal gene transfer	Biological	36,40,195,196
Hybridization or introgression and recombination	Biological	43,44
Natural selection	Biological	57,58
Sampling (taxon and locus)	Analytical, stochastic error	83,197,198
Insufficient number of genes or divergent sites	Analytical, stochastic error	2,9,11,80
Erroneous orthologue detection	Analytical, systematic error	87,89,95,97,199
Model misspecification	Analytical, systematic error	8,125,126,200
Multiple sequence alignment errors	Analytical, treatment error	143,144
Excessive trimming	Analytical, treatment error	148,149
Inappropriate character recoding	Analytical, treatment error	154,155

Table 2 | Tools to investigate incongruence in large genomic data sets

Software or method	Utility category	Utility details	Refs.
Bag of little bootstraps	Bipartition support metric	Median bagging of bootstrap support assessed using few little samples and a small subset of sites is a rapid method to infer bootstrap trees and provides similar patterns of support compared to traditional bootstrapping procedures	201
Gene and site concordance factors	Bipartition support metric	Bipartition support that details how many 'decisive' genes or sites support a given bipartition in a reference tree	168
Internode or tree certainty	Bipartition support metric	Identifies bipartitions in a reference phylogeny that also have a well-supported alternative topology	172–175
UFBoot2	Bipartition support metric	Ultrafast bootstrap approximations that are robust to model violation	202
IQ-TREE 2, FireProt ^{ASR} , PhyloBot	Convergent sequence evolution	Software for inferring ancestral sequences across nodes of a phylogeny; these pieces of software can be used to detect convergent sequence evolution	59–61
RERconverge	Convergent sequence evolution	Identifies genes in phylogenomic data matrices with signatures of convergent relative evolutionary rates in lineages with similar phenotypes	203
ClipKIT	Data processing and analysis	Multiple sequence alignment trimming wherein informative sites are retained rather than removing highly divergent sites	149
Concaterpillar	Data processing and analysis	Identifies congruent loci in a phylogenomic data matrix	204
ConJak	Data processing and analysis	Identifies sequence outliers compared to the central mean of a phylogenomic data matrix	205
ConWin	Data processing and analysis	Tests for within-protein incongruence using a sliding window approach	205
PhyKIT	Data processing and analysis	Broadly applicable phylogenomic toolkit for data processing and analysis such as examining information content biases, gene–gene coevolution and polytomy testing	96
PhyloFisher	Data processing and analysis	Collection of scripts for data set building and trimming phylogenomic data sets; also features a data base of eukaryotic orthologues	98
RogueNaRok	Data processing and analysis	Identification of rogue taxa in a phylogenomic data set	72
Root Digger	Data processing and analysis	Uses a non-reversible Markov model to calculate the likelihood of the root position in a tree	140
TreeShrink, PhyloFisher and PhyKIT	Data processing and analysis	Identifies spurious orthologues from unexpectedly long terminal branches	96–98
abSENSE	Homology and/or orthology detection	Calculates the probability that homologue detection may fail	90
BLAST	Homology and/or orthology detection	Searches for similar sequences by using measures of local similarity	206
Leapfrog	Homology and/or orthology detection	Combines over split orthologues using reciprocal best BLAST hits	91
OrthoFinder	Homology and/or orthology detection	Infers groups of orthologous genes	89
OrthoSNAP and DISCO	Homology and/or orthology detection	Decompose multi-copy gene families into subgroups of single-copy orthologous genes	81,82
Profile hidden Markov models	Homology and/or orthology detection	Probabilistic inference method that accounts for position-specific variation in sequences	92
TIAMMAT	Homology and/or orthology detection	Increases sensitivity of sequence similarity searches by incorporating under-represented lineages in profile Hidden Markov Models	93
ASTRAL and PhyKIT	Hypothesis testing	Both pieces of software enable researchers to conduct polytomy testing at a specific bipartition in a phylogeny	29,96
Gene-wise and site-wise log-likelihood scores; gene-wise quartet scores	Hypothesis testing	Allows researchers to examine gene-wise and site-wise support between two topologies using maximum likelihood; gene-wise support can also be examined using quartet scores	67,158
D-statistic (also known as the ABBA-BABA test), D_{FOIL} , D_3 and the branch-length test	Introgression detection	Diverse methods that detect introgression events using sequence or phylogenetic information	44, 49–51
NetRAX	Phylogenetic network inference	Maximum likelihood inference of phylogenetic networks when incomplete lineage sorting is not a factor	180
PhyloNet	Tree inference	Maximum parsimony, maximum likelihood and Bayesian inference of phylogenetic networks from locus tree estimates	179

Table 2 (continued) | Tools to investigate incongruence in large genomic data sets

Software or method	Utility category	Utility details	Refs.
SplitsTree	Phylogenetic network inference	Splits graph inference using multiple sequence alignments, distance matrices or sets of trees	177
GHOST	Substitution models	Edge-unlinked mixture model consisting of several site classes with separate sets of model parameters and edge lengths on the same tree topology	8
QMaker	Substitution models	Estimates general time-reversible protein matrices, which describe rates of substitutions between amino acids, from multiple sequence alignments	200
Asteroid	Tree inference	Supertree method for species tree inference that is robust to missing data	31
ASTRAL, ASTRAL-PRO and ASTER	Tree inference	Quartet-based supertree method that accounts for partial gene trees, paralogs and gene tree uncertainty	29,30, 207
BEAST	Tree inference	Bayesian approach for phylogenetic tree inference and divergence time estimation	26
BPP	Tree inference	Full-likelihood implementation of the multispecies coalescent	25
IQ-TREE 2	Tree inference	Maximum likelihood tree inference method that uses hill-climbing and stochastic perturbation to search tree space; moreover, the Gentrus function can help identify and characterize phylogenetic terraces	59
MP-EST	Tree inference	Maximum pseudo-likelihood approach for species tree inference	28
PhyloBayes MPI	Tree inference	Bayesian tree inference method that incorporates finite and infinite mixture models to account for site variation	208
RAxML-NG	Tree inference	Maximum likelihood tree inference method that uses a greedy tree search algorithm to explore tree space	209
STAR	Tree inference	Inference of species trees using average ranks of coalescences	210
SpeciesRax	Tree inference	Maximum likelihood species tree inference method that explicitly accounts for incomplete lineage sorting, gene duplication, gene loss and horizontal gene transfer	42
SVDQuartets	Tree inference	Inference of relationships using quartets and the coalescent model	211

measurement – mutagenesis, functional selection and deep sequencing. These experimentally derived models have substantially improved fit compared to those with few or hundreds of parameters¹¹⁴.

Partitioning concatenated data matrices – that is, applying different site-homogeneous substitution models to distinct molecular markers or portions of an alignment – can account for heterogeneity in substitutions among sites and lead to more accurate estimates of phylogeny¹¹⁵. Supermatrices can be partitioned by biological features (for example, genes or codon positions) or be algorithmically defined¹¹⁶. An alternative to partitioning is site-heterogeneous models, wherein nucleotide or amino acid equilibrium frequencies differ across sites of a multiple sequence alignment. Site-heterogeneous models fit data better than site-homogeneous models and are thought to be superior at ameliorating long-branch attraction artefacts^{117,118}. Consequently, site-heterogeneous models have risen in popularity and helped resolve the placement of several anciently diverged lineages^{119,120} but are also the focal point of controversies such as the rooting of the animal tree (Box 1). In other cases, using site-heterogeneous models has shed light on the evolutionary relationships among the three domains of life, supporting the hypothesis that eukaryotes originated from within Archaea (the two-domain hypothesis)¹²¹.

Substitution model misspecification can bias topology estimation, contributing to incongruence^{17,122–124} (Fig. 3c and Table 1). One well-known source of incongruence that stems from model misspecification is long-branch attraction^{125,126}. Long-branch attraction is common in phylogenomic data matrices containing taxa that greatly vary in their evolutionary rates or lineages undergoing accelerated evolutionary

rates as observed in bacterial endosymbionts¹²⁷ and parasitic fungi¹²⁸. Outgroup taxa may also introduce long branches, increasing the potential for long-branch attraction artefacts (see the section Rooting strategy). In addition to using site-heterogeneous models¹²⁵, long-branch attraction artefacts can sometimes be ameliorated by including taxa whose placements break long branches^{129,130} (see the section Taxon sampling). Notably, long-branch attraction can also occur when models are correctly specified and be exacerbated when partitioning phylogenomic data sets¹²⁶. Other approaches attempt to better approximate true processes of sequence evolution. For example, heterotachy, which is not accounted for by either site-homogeneous or site-heterogeneous models¹³¹, can decrease phylogenetic accuracy due to long-branch attraction artefacts^{126,132}. The GHOST (general heterogeneous evolution on a single topology) model of sequence evolution can partly account for heterotachy by incorporating features of mixed substitution and mixed branch-length models. The GHOST model has helped resolve some phylogenetic controversies such as the placement of turtles⁸.

Rooting strategy. Rooting strategies have been debated for a long time, especially in the context of outgroup taxa driving long-branch attraction artefacts¹³³. The recent controversy surrounding the root of animal phylogeny has highlighted the relevance of these debates (Box 1). Although there is no consensus on selecting outgroup taxa¹³⁴, it is broadly accepted that thorough sampling of representatives of diverse lineages improves phylogenetic inference¹³⁵.

Other methods aim to infer the root of a phylogenetic tree without using outgroup taxa. These include the use of paralogues as

Review article

a Taxon selection



Contributor of incongruence

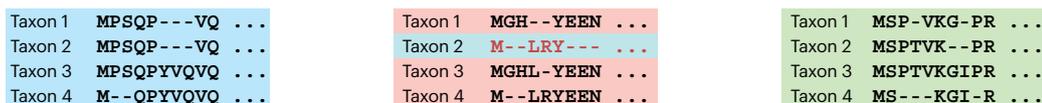
- Insufficient taxon sampling
- Insufficient locus sampling
- Fast-evolving lineages
- Rogue taxa
- Outgroup choice

b Orthology inference



- Sequence length biases
- Erroneous orthologue inference (hidden paralogy and orthology)

c Alignment and site trimming



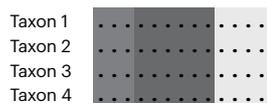
- Misalignment
- Excessive trimming
- Inappropriate recoding

d Selection of substitution model

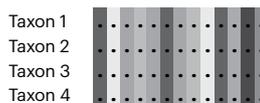
Site-homogeneous model



Site-homogeneous with partitioning



Site-heterogeneous model



- Long-branch attraction
- Model misspecification
- Inadequate model complexity

e Method of tree inference

Concatenation

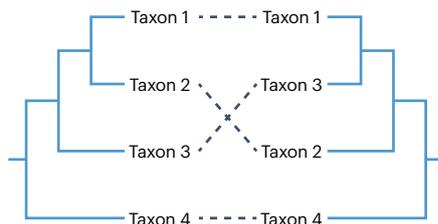


Coalescence



- Irreproducibility
- Single-locus accuracy

f Incongruent gene or species trees



- Biological factors

implemented in the software STRIDE¹³⁶⁻¹³⁸; non-reversible Markov models as the one implemented in the software Root Digger^{139,140}; relaxed molecular clock models as implemented in BEAST¹⁴¹; the minimal ancestor deviation method, which is also based on molecular clocks¹⁴²; and modelling dynamics of gene family evolution⁴¹.

For example, modelling genome duplication, horizontal gene transfer and gene loss helped root the archaeal tree of life, placing it between Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, Nanohaloarchaea (known as DPANN) and other Archaea⁴¹.

Fig. 3 | Analytical factors can contribute to incongruence at every step in a phylogenomic workflow. **a**, Taxon sampling, including sampling of taxa from fast-evolving lineages or of rogue taxa, can affect all downstream analyses in phylogenomic studies. **b**, During orthology inference, biases (for example, sequence length biases) and analytical errors (for example, erroneous orthology inferences) can contribute to incongruence. Each colour corresponds to a unique orthologue present in each of the four taxa. **c**, Misalignment and excessive trimming of individual groups of orthologous genes can further decrease the accuracy of phylogenetic inferences and contribute to incongruence. An example of erroneous orthologue inclusion is depicted using red font. **d**, Selection of substitution model can influence phylogenetic inferences.

Some common approaches include site-homogeneous models, site-homogeneous models with partitioning or site-heterogeneous models. Sources of error include model misspecification, inadequate model complexity and long-branch attraction artefacts. **e**, The method of tree inference, for example, concatenation (left) or coalescence (right), can be susceptible to multiple additional sources of error, including irreproducibility and poor accuracy of trees inferred from single genes (single-locus accuracy). **f**, Even if all analytical factors have been adequately addressed and there is no incongruence due to them, the resulting locus or species trees may still be exhibiting incongruence due to the action of diverse biological factors (Fig. 2).

Treatment errors

Multiple sequence alignment. Errors in multiple sequence alignment can result in inaccurate phylogenetic inferences and incongruence^{143,144}. Alignment errors can stem from errors in orthologue inference (from either hidden paralogy or hidden orthology) but can also occur when truly orthologous sequences are aligned. Such errors are particularly common when sequences in the alignment exhibit high levels of divergence¹⁴⁵ (Fig. 3b). Approaches to remedy errors in multiple sequence alignments include alignment trimming (see the section Alignment trimming), probabilistic modelling to identify clusters of homologous characters and dividing the alignment accordingly (as implemented in Divvier)¹⁴⁶, or masking putative errors in multiple sequence alignments using two-dimensional outlier detection methods (as implemented in TAPER)¹⁴⁷.

Alignment trimming. Although trimming of sites during multiple sequence alignment is a widespread practice to reduce alignment errors, it can also reduce the accuracy of phylogenetic inference, increase statistical uncertainty and lead to incongruence (Fig. 3b and Table 1). Generally, more aggressive alignment trimming that removes larger numbers of sites increases errors in single-gene tree inferences¹⁴⁸. For example, entropy-based trimming, which removes divergent sites, or multiple rounds of trimming, which often remove more than 20% of sites in an alignment, can significantly worsen phylogenetic inferences of tree topology, support and branch-length estimation^{148,149}. Recently developed approaches that focus on retaining phylogenetically informative sites, such as ClipKIT (Table 2), can be equally accurate and are more time-saving than no-trimming approaches¹⁴⁹.

Character recoding. Saturation by multiple substitutions and compositional biases can lead to inaccurate phylogenetic inferences and contribute to incongruence. Recoding nucleotides or amino acids into fewer character states can combat these issues^{150–153} (Fig. 3b). However, the benefit of combating compositional heterogeneity and substitutional saturation can be outweighed by the loss of information from reducing the number of character states during recoding and increase statistical uncertainty, especially among shorter alignments^{154,155}. Thus, recoding can also increase, rather than ameliorate, error. Appropriate ways forward include adequately assessing how recoding affects compositional heterogeneity or implementing alternative recoding schemes; for example, in amino acid sequence alignments, a greater number of recoding states outperformed the most frequently implemented six-state recoding strategies¹⁵⁴. Notably, errors in multiple sequence alignment, excessive trimming and inappropriate character recoding all contribute to erosion of the phylogenetic signal.

Concatenation versus coalescence. Phylogenomic data matrices can be analysed as a single supermatrix (an approach known as concatenation) or each gene alignment can be analysed separately under the multispecies coalescent framework (an approach known as coalescence). The two approaches sometimes yield different tree topologies, contributing to incongruence^{68,87}. Determining which approach is more appropriate for a phylogenomic data set is difficult. For example, using simulated multi-locus data, concatenation slightly outperformed a fully coalescent-based approach (wherein gene trees and species trees are coestimated), whereas using coalescent independent sites, both approaches performed comparably¹⁵⁶. However, an extensive evaluation of coalescent-based and concatenation-based approaches when different biological and analytical factors are at play is lacking, hindering our knowledge of best practices. Moreover, there can be differences in the performance of fully and summary coalescent-based methods (wherein gene trees are first estimated and then the species tree is estimated by summarizing the collection of gene trees). Summary coalescent-based methods are more vulnerable to errors in gene tree inference than fully coalescent-based methods but newer implementations of summary coalescent-based methods take gene tree uncertainty into account³⁰. Analyses with both fully and summary coalescent-based methods can be improved through targeted data filtering such as removing loci with low phylogenetic informativeness¹⁵⁷. Loci that are inconsistent between concatenation-based and coalescence-based methods can also be pruned from data matrices¹⁵⁸.

Irreproducibility. A tenet of scientific inquiry is reproducibility. Phylogenetic irreproducibility contributes to incongruence and can be caused by increasing the number of threads (because threads can be initialized in different orders between runs); errors in floating point arithmetic, such as rounding errors, and numerical overflow and underflow (the storing of a value greater than or smaller than the maximum and minimum supported value, respectively); and differences in software compilers that result in binaries with slightly different orders of operations^{159,160}. Genes with a low phylogenetic signal (few parsimony-informative sites) are particularly susceptible to irreproducibility; this means that summary coalescent-based methods, which typically rely on accurately inferred gene tree topologies, can be particularly susceptible¹⁶⁰. Some problems of irreproducibility and issues plaguing bioinformatic software can be remedied through rigorous software development practices such as extensive testing and continuous integration pipelines^{149,159}. Studies that further our understanding of the accuracy and information content of multiple sequence alignments may facilitate predicting genes with a greater phylogenetic signal^{77,161–163}.

Detecting incongruence

Because multiple biological and analytical factors, often initially unknown, can contribute to incongruence, several methods examine the presence and magnitude of incongruence per se in phylogenomic data sets without assuming the presence of specific underlying biological or analytical factors.

Measures of branch support

Traditional approaches, such as non-parametric bootstrapping¹⁶⁴ and Bayesian posterior probabilities, are frequently used to examine bipartition support in a phylogeny; low branch support values can be indicative of incongruence. Other branch support methods include approximate likelihood ratio tests and the Shimodaira–Hasegawa approximate likelihood ratio test¹⁶⁵. The transfer bootstrap expectation method – an approach based on traditional bootstrapping but that measures the presence of branches among bootstrap trees as a gradual ‘transfer’ distance rather than as a binary presence or absence – is more accurate for assessing support among deep branches in data sets with large numbers of taxa¹⁶⁶. The usefulness of many of these measures in concatenation analyses of phylogenomic data sets is rather low because they almost invariably yield absolute support values, even if there is substantial incongruence between sites or loci⁷⁹. However, these measures are highly informative when using summary coalescent-based methods to remove loci with low amounts of phylogenetic signal¹⁶⁷.

Gene support frequencies and concordance factors

Gene support frequencies measure the frequency of recovering an individual branch in a set of gene trees from a phylogenomic data matrix^{96,168}. Branches with low gene support frequencies are likely to be incongruent. Concordance factors were initially defined as the proportion of the genome that supports a given branch in the species tree^{169,170} and can be measured using BUCKy, a Bayesian approach that estimates the joint probability distribution of genes and their phylogenies (or a gene-to-tree map) genome-wide^{169,171}. Recently, concordance factors were redefined as equivalent to gene support frequencies¹⁶⁸, which can be calculated using IQ-TREE^{59,96} (Table 2).

Internode certainty

Internode certainty is an information theory-based approach that considers the relative prevalence of a branch and the second most common conflicting branch in a set of trees; internode certainty-all considers the relative prevalence of a branch relative to all alternative conflicting branches in a set of trees^{172–175}. Internode certainty measures can help identify branches with substantial conflict, which can then be examined further for underlying causes contributing to incongruence. Internode certainty measures are distinct in that the prevalence of conflicting alternative branches is accounted for, thereby providing a measure of the degree of conflict for every branch in a phylogenomic tree. Internode certainty can be calculated using the software QuartetScores¹⁷⁴ (Table 2).

Phylogenetic networks

Evolutionary relationships among organisms are often depicted as bifurcating trees, which may not always be appropriate. As discussed earlier, many genomes bear the hallmarks of biological factors that make the histories of genes and genomes deviate from strict vertical inheritance. By relaxing the assumption of a strictly bifurcating topology, reconstruction of the histories of loci from such lineages as phylogenetic networks enables the description and visualization of

incongruence. The underlying data and theory used to infer a phylogenetic network can differ¹⁷⁶; for example, split networks depict all possible splits in a set of phylogenies¹⁷⁷, whereas reticulate networks depict putative evolutionary events such as hybridization¹⁷⁸. Software for inferring phylogenetic networks includes SplitsTree¹⁷⁷, PhyloNet¹⁷⁹ and NetRAX¹⁸⁰ (Table 2).

Incongruence search protocols

In addition to the above methods, several protocols have been used to search for incongruence in phylogenomic data sets. These include repeated subsampling of smaller subsets of loci with a robust phylogenetic signal and re-inference of the species phylogeny¹⁶², gene genealogy interrogation¹⁸¹, examination of phylogenetic signal¹⁶⁷ and quartet sampling¹⁸².

Polytomies

Several clades in the tree of life, such as cichlids and finches, have experienced elevated rates of speciation, giving rise to evolutionary radiation. Such clades have often been influenced by multiple biological (for example, introgression or lineage sorting) and analytical (for example, long-branch attraction for ancient radiations) factors, making phylogenomic inference particularly challenging. They often present as polytomies, a node where more than two descendant lineages stem from an ancestral one. Polytomies can be detected by identifying cases of equal support for multiple distinct topologies in sets of single-gene trees^{96,183}. Support can be measured using gene trees or the quartets of taxa present in these gene trees using ASTRAL²⁹, PhyKIT⁹⁶ and IQ-TREE⁵⁹ (Table 2).

Future directions

Our knowledge of the tree of life and of the evolution of traits and genomes has been transformed by phylogenomics, but incongruence continues to cloud our understanding of some of its branches. We discussed biological and analytical factors contributing to incongruence, methods for its detection, and approaches that have helped improve the accuracy of phylogenomic inference. In this final section, we identify avenues ripe for research and discovery.

Which factors matter and when?

Although the effects of multiple factors on specific instances of incongruence have been investigated^{32,157,160}, a unified framework to assess the contribution of multiple biological and analytical factors to a given case of incongruence is lacking. The evolutionary depth of each case of incongruence further complicates assessment of the relative importance of any factor because our ability to detect their effects varies across time scales. For example, incomplete lineage sorting and hybridization likely contribute to incongruence of ancient and recent relationships but are typically detectable only in studies of recently diverged lineages. By contrast, it is typically much easier to detect horizontal gene transfer between distantly related taxa than between closely related ones. We also know that errors in orthologue inference or multiple sequence alignment are greater contributors to incongruence when studying ancient divergences than recent ones^{90,184}. However, for a given case of incongruence in deep time, simultaneously evaluating the relative contribution of incongruence stemming from multiple biological and analytical factors is challenging (Box 1). A related issue is identifiability, that is, ascribing an observed conflict to certain factors and ruling out others. For example, ancient horizontal gene transfer is often difficult to distinguish from gene duplication followed by extensive gene loss; attributing incongruence

to one specific factor is challenging and often depends on a priori knowledge regarding which process is more likely. Developing methods and computational pipelines that enable simultaneous evaluation of potential contributing factors will be key to fully understanding the drivers of incongruence.

Data and data sets of ever higher quality

Data quality is paramount to phylogenomic inference. As sequencing technologies and other downstream processes, such as methods for genome assembly and gene annotation, improve, so does the field of phylogenomics. Higher quality and more complete genomes, coupled with increased sampling of organisms from taxa under-represented in genomic data bases, will help to reduce the impact of hidden paralogy and orthology in phylogenomic data sets. Denser data sets will also help increase confidence in inferences of the underlying analytical or biological drivers of incongruence; for example, confidence in inferring hybridization as a potential driver of incongruence may be weak in a data set of 100 molecular markers but strong in a 5,000-marker data set.

Mitigating errors in data set construction

Errors that contribute to incongruence can be introduced at all stages of phylogenomic analyses, including data matrix construction. Some errors may stem from certain strategies employed in a phylogenomic pipeline, such as multiple sequence alignment and trimming, being suitable for some, but not all, genes. Some features that may influence the efficacy of alignment and trimming strategies may be the taxa sampled and their evolutionary breadth, although numerous other technical contributors of incongruence may be at play. The development of pipelines to reproducibly handle phylogenomic data matrix construction will greatly facilitate comparative analyses of analytical drivers of incongruence across studies.

The forest grows: how can tree space be efficiently examined?

As genomic data increase, phylogenomic studies sampling several hundreds to thousands of organisms are becoming commonplace. One challenge with inferring phylogenies from such taxon-rich data sets is that tree space is vast, making computation challenging. For example, the numbers of possible unrooted trees for 3, 5, 7 and 9 taxa are 1, 15, 945 and 135,135, respectively. As tree space grows, the likelihood of finding the non-optimal tree increases, leading to speed-accuracy trade-offs and incongruence. However, efficiently searching tree space is key to finding an optimal tree; phylogenetic inference programmes that yield the highest likelihood scores on phylogenomic data matrices are the ones that perform the most extensive explorations of tree space and require the longest runtimes¹⁸⁵. Moreover, gene-rich data sets present their own challenges such as optimizing tree parameters. It is possible that the phylogenetic signal in whole genomes will prove insufficient for resolving phylogenies of all known species in each major lineage. Developing algorithms, including those that leverage the power of machine learning^{163,186–188}, that can heuristically explore tree space in a reasonable amount of time or evaluate the degree of difficulty in the inference task will be critical for resolving the tree of life.

Phylogenomics and green computing

End-to-end phylogenomic analysis requires substantial computational resources and large amounts of energy. As the planet grapples with the consequences of global climate change, we must work to minimize the environmental toll of phylogenomic analyses¹⁸⁹. We can reduce the carbon footprint of phylogenomics through judicious use of computing infrastructure, careful experimental design and software choice. For example, evaluating substitution model fit using fast and robust software such as ModelTest-NG¹⁹⁰ and jModelTest¹⁹¹ can result in a 90% reduction in energy use, resulting in 10% less greenhouse gas

Glossary

Convergent molecular evolution

Independent evolution of similar or identical molecular changes (for example, gene deletions, nucleotide substitutions, gene order rearrangements) in organisms from different lineages that exhibit similar adaptations.

Evolutionary radiation

The occurrence of an elevated rate of speciation events in a narrow window of evolutionary time.

Heterotachy

The phenomenon of changes in the evolutionary rate of a nucleotide or amino acid sequence through time.

Hidden orthology

Undetected orthologous relationships of genes.

Hidden paralogy

Orthologous groups of genes that contain orthologues and paralogues (inparalogues and outparalogues) stemming from asymmetric patterns of duplication and loss.

Horizontal gene transfer

Also known as lateral gene transfer. The transfer of genetic material between organisms of the same or different species through non-reproductive means.

Hybridization

The interbreeding of two distinct species or lineages.

Inparalogues

Lineage-specific or species-specific paralogues wherein the duplication event occurred after divergence from a reference common ancestor.

Introgression

The interbreeding of two distinct species or lineages, followed by backcrossing with one of the parental species.

Long-branch attraction

The inaccurate inference of taxa with high evolutionary rates (giving rise to long branches in their phylogenetic trees) as closely related.

Model of sequence evolution

Also known as the substitution model. Markov models that describe rates of nucleotide or amino acid substitutions in a locus during evolution.

Partial or incomplete taxon coverage

The lack of sequences (either because they are genuinely absent or because

they were not collected) from particular taxa in a group of orthologous genes.

Phylogenetic irreproducibility

Lack of reproducibility of a tree topology between two replicate tree inferences using the same software parameters (for example, same model of sequence evolution or starting seed).

Phylogenetic networks

Graphs of evolutionary relationships that, in addition to depicting the splitting of lineages, also depict the merging of lineages (due to events such as hybridization and convergent molecular evolution or due to different gene tree topologies).

Taxon sampling

Which and how many taxa are selected for a phylogenetic analysis.

emissions¹⁹². Similarly, choosing faster programmes in quantifiably difficult-to-analyse data sets does not alter the quality of inference but can save energy, according to a recent preprint¹⁹³.

Conclusions

Phylogenomics has revolutionized evolutionary biology by providing a clearer picture of the tree of life and a more accurate reconstruction of the evolution of biological features. The study of genome-scale data from diverse organisms has deepened our understanding of the various biological factors that cause the histories of genomic regions to differ from those of their species, spurring the development of models that consider them in inference. At the same time, it has improved knowledge of the analytical factors that contribute to errors in inference and the development of models and protocols for their amelioration. These advances, together with ongoing work that tackles some of the greatest challenges in the field, will continue to improve our mapping and understanding of, as Simpson eloquently put it, “*The stream of heredity [that] makes phylogeny*”¹.

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All authors researched the literature, contributed substantially to discussions of the content, and reviewed and/or edited the manuscript before submission. J.L.S. and A.R. wrote the article.

Competing interests

J.L.S. is a scientific adviser for LifeGen Biotechnologies and an adviser for ForensisGroup. A.R. is a scientific consultant for LifeMine Therapeutics. The other authors declare no competing interests.

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