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Phylogenomics: the beginning of incongruence?

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Until recently, molecular phylogenies based on a single or few orthologous genes often yielded contradictory results. Using multiple genes in a large concatenation was proposed to end these incongruences. Here we show that single-gene phylogenies often produce incongruences, albeit ones lacking statistically significant support. By contrast, the use of different tree reconstruction methods on different partitions of the concatenated supergene leads to well-resolved, but real (i.e. statistically significant) incongruences. Gathering a large amount of data is not sufficient to produce reliable trees, given the current limitation of tree reconstruction methods, especially when the quality of data is poor. We propose that selecting only data that contain minimal nonphylogenetic signals takes full advantage of phylogenomics and markedly reduces incongruence.

Introduction

Molecular characters, primarily DNA and derived protein sequences, provide a wealth of new information that sheds light on many parts of the 'Tree of Life'. However, molecular phylogenies based on single genes often lead to apparently conflicting results. To overcome this limitation, it is tempting to apply a genome-scale approach to phylogenetic inference (phylogenomics) by combining many genes. The number of published yeast genomes offers the opportunity to test this proposition. Indeed, using 106 genes from yeast genomes, a fully supported phylogeny has been obtained by the analysis of their concatenation [1,2]. Following from this, it has been anticipated that using large amounts of genomic data will mark the end of incongruence in phylogenetics [3].

The incongruence between two phylogenies can be the result of: (i) violations of the orthology assumption generated by mechanisms such as gene duplication, horizontal gene transfer or lineage sorting [4]; (ii) stochastic error related to the length of the genes; and (iii) systematic error leading to tree reconstruction artifacts generated by the presence of a nonphylogenetic signal in the data. Adopting a genome-scale approach theoretically overcomes incongruence because of the first two reasons: nonorthologous comparisons are gene-specific and will probably be buffered in a multigene analysis; and stochastic error naturally vanishes when more and more genes are considered. By contrast, systematic error is not expected to disappear with the addition of data [5]. Systematic error results from nonphylogenetic signals being present in the data, such as heterogeneity of nucleotide compositions among species (compositional signal), rate variation across lineages (rate signal) and also within-site rate variation (heterotachous signal) [6]. The bias causing systematic error creates a signal because, contrary to stochastic noise, it does not average out over several sites. If a bias is strong enough, it can dominate the true phylogenetic signal causing the tree reconstruction method to be inconsistent and lead to an incorrect, but highly supported tree [5,7]. Therefore, phylogenomics, instead of ending incongruence, might open an era of real, statistically significant incongruence resulting from the use of different methods, different taxon samplings, or different character partitions of the same data set.

To illustrate this paradox, we used the large data set of 106 genes (120 762 nucleotides) from 14 yeast species assembled by Rokas and Carroll [1]. Phylogenetic trees were inferred by maximum parsimony (MP) from nucleotide sequences, as in Ref. [1], and alternatively by probabilistic methods – Bayesian inference (BI) [8] or maximum likelihood (ML) [9–11] – because these methods are generally considered the most accurate [12,13]. In addition, because the diversification of these yeasts is ancient (>250 Mya [14]) and amino acid sequences evolve more slowly than nucleotide sequences, the translated protein sequences were also used to construct trees. Phylogenies were inferred from each of the 106 genes and from their concatenation, using two different methods (MP and BI) and two types of characters (nucleotides and amino acids), yielding a total of 428 trees. We estimated the level of incongruence as the number of bipartitions (or splits, i.e. groups of species defined by a branch of a phylogenetic tree), supported by more than a given bootstrap value, that are different between two trees. Our aim was to compare the level of among-gene incongruence for a given tree reconstruction method with the level of among-method incongruence for a given data set.

Congruence among phylogenetic markers

The trees inferred from each of the 106 genes are all different (data not shown), yielding an apparent high level of incongruence. However, there are 3×10^{11} possible binary trees connecting 14 taxa and it is possible that

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the different genes recover different, but very similar trees. Without taking statistical support into account, there are 25.9% and 24.6% different bipartitions when trees are inferred by either MP at the nucleotide level (MP_{nt}) or by BI at the amino acid level (BI_{aa}), respectively.

However, if the measure of incongruence is restricted only to those bipartitions that are supported above a predefined significance level, a statistically significant incongruence [bootstrap support (BS) >95%] among the 106 individual genes is almost nil. For MP_{nt} and BI_{aa}, only 0.4% or 0.6% of the significantly supported bipartitions are different, respectively. Yet, the nonparametric bootstrap test is often considered as conservative and the use of a *P*-value of 70% has been suggested [15]. Even with this reduced threshold, only 4.0% and 2.8% of the bipartitions are different, strongly arguing for the absence of statistically significant incongruence among the 106 genes when analyzed with the same method. These results are in line with a similar analysis based on the same 106 genes, although with only eight species [16]. In addition, single gene phylogenies are not significantly incongruent with concatenation-based trees. Only 1.8% (5.0%) and 3.1% (6.8%) of the bipartitions are different at 95% (70%) bootstrap confidence between the 106 gene trees and the concatenated tree for BI_{aa} and MP_{aa}, respectively.

In summary, all single-gene trees are different because of the predominant effect of stochastic error (except one paralogous comparison, Figure S1 in the supplementary material online), but there is no statistically significant incongruence among these 106 genes when the same tree reconstruction method is used (either MP_{nt} or BI_{aa}). In other words, there is no among-gene incongruence in this data set.

Strong incongruence when different tree reconstruction methods are used

By contrast, a non-negligible statistically significant incongruence exists because of the use of different tree reconstruction methods, and the use of nucleotide versus amino acid sequences. On average, 14.2% (23.2%) of the bipartitions are different at the 95% (70%) bootstrap confidence level between the MP_{nt} tree and the BI_{aa} tree, albeit inferred from the same genes.

Does the phylogenomic approach avoid this incongruence? The answer is no: when phylogenies are inferred from the concatenation of the 106 genes, 36.4% of bipartitions are different between the MP_{nt} and BI_{aa} trees. In fact, four out of 11 nodes, which are all highly supported, are different, indicating that incongruence has increased. Therefore, a large-scale genome approach only ends the statistically insignificant among-gene incongruence but opens the era of the real, statistically significant incongruence among methods and character sets.

Nucleotide compositional bias causes most of the incongruence

To understand better the source of this exceptionally high level of incongruence, trees inferred from the concatenation by MP_{nt}, BI_{nt}, MP_{aa} and BI_{aa} were compared (Figure 1a–d). This enables separation of the impact of the type of characters considered from the impact of the reconstruction method used. The topology within the clade containing the five *Saccharomyces* species, *Naumovia castellii* and *Candida glabrata* was identical in all four cases. In addition, *Debaromyces hansenii* invariantly appeared as the sister-group of *Candida albicans*. Incongruences are thus predominant for the most basal nodes. The MP_{nt} tree is the most different from the other three trees (four different bipartitions). The BI_{nt} tree differs from the BI_{aa} tree by three bipartitions, and the MP_{aa} tree from the BI_{aa} tree by only two bipartitions. This suggests that, in this case, the method (MP or BI) is less important than the type of characters used (nucleotides or amino acids).

Compositional bias is known to be more prominent in nucleotides than in amino acids [17], because the fast-evolving third codon positions accumulate mutational bias as a result of the degeneracy of the genetic code. The average G+C content at the third codon positions of the 106 genes for a given species is indeed highly heterogeneous, ranging from 27% in *C. albicans* to 68% in *Yarrowia lipolytica* (Figure S2 in the supplementary material online). Differences in nucleotide or amino acid composition can render tree reconstruction methods inconsistent if not properly accounted for [7,18]. Strikingly, groupings in the MP_{nt} tree (Figure 1a) seem to be strongly correlated with G+C content: the GC-rich *Y. lipolytica* (68%) is grouped with *Ashbya gossypii* (66%), then with *Kluyveromyces waltii* (51%) and finally with *Saccharomyces kluyveri* (45%), whereas the relatively GC-poor *Kluyveromyces lactis* (39%) is grouped with *D. hansenii* (34%) and *C. albicans* (27%). Therefore, nonphylogenetic compositional signal is probably dominating over genuine phylogenetic signal in the MP_{nt} tree.

By contrast, in the BI_{aa} tree (Figure 1d), the species do not seem to be grouped according to their G+C content: *K. lactis* (39%) with *A. gossypii* (66%), *S. kluyveri* (45%) with *K. waltii* (51%), and *Y. lipolytica* (68%) together with *C. albicans* (27%) and *D. hansenii* (34%). Similarly, in the MP_{aa} tree (Figure 1c), the groups seem to be no longer determined by nucleotide composition, even if they are slightly different from the ones observed in the BI_{aa} tree – paraphyly of *K. lactis* (39%) and *A. gossypii* (66%). This indicates that the BI_{aa} tree (Figure 1d) is not, or less, biased by the compositional signal, and is likely to be closer to the correct phylogeny than the probably erroneous MP_{nt} tree. As we will show in the following, this conclusion is supported by an approach known to increase the impact of G+C bias and converges towards the MP_{nt} tree (case 1), and two approaches known to decrease its impact converge towards the BI_{aa} tree (cases 2 and 3).

Case 1: When only the most biased third codon positions are analyzed by BI, the inferred phylogeny is identical to the MP_{nt} tree (Figure S3 in the supplementary material online), indicating that for these fast-evolving characters BI is unable to extract the phylogenetic signal correctly, which is overwhelmed by the compositional signal.

Case 2: When the least biased first two codon positions are analyzed by MP, the inferred phylogeny (Figure S4 in the supplementary material online) is identical to the BI_{nt}

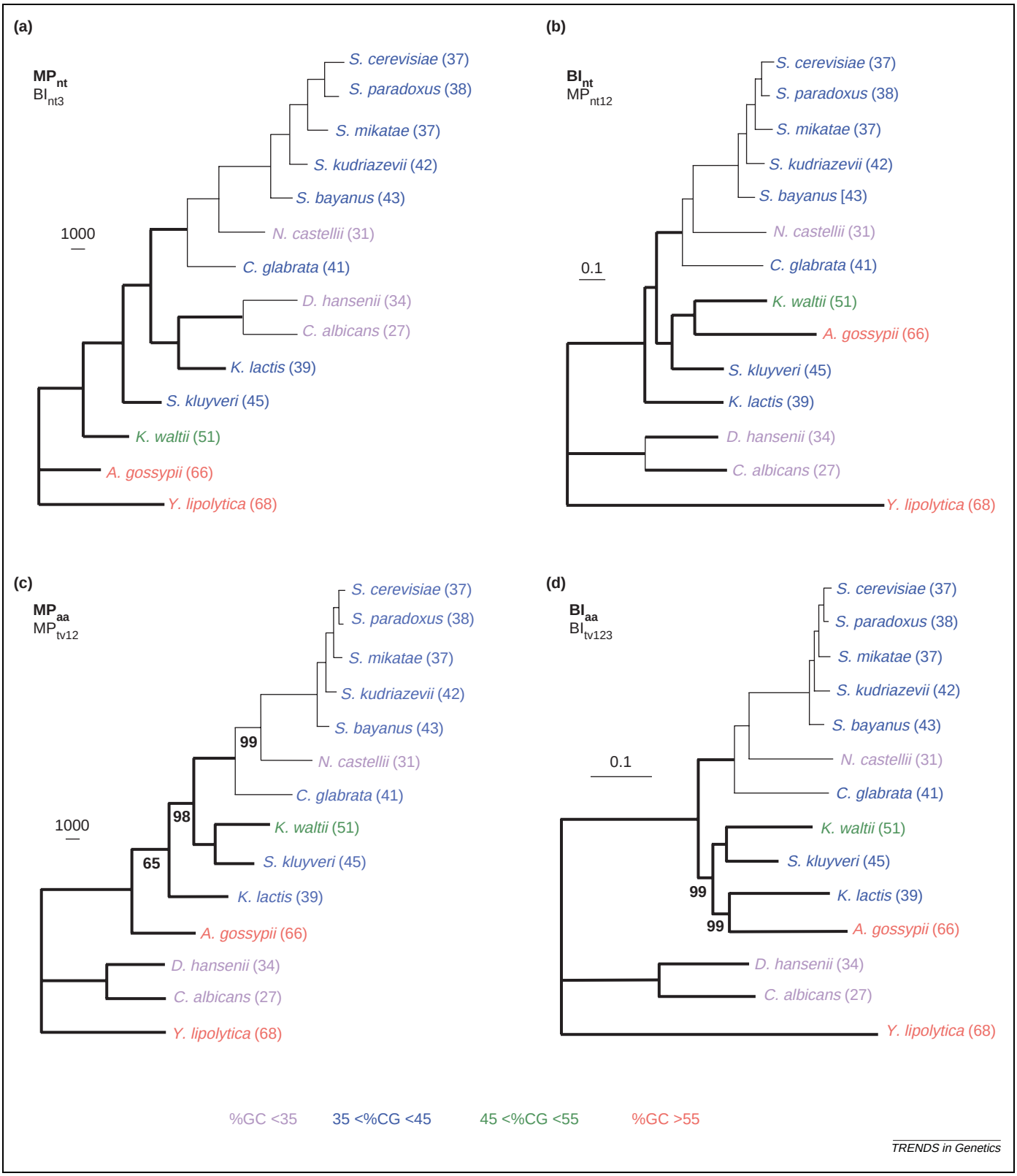

Figure 1. Phylogenies based on the concatenation of 106 genes. Trees were inferred using MP **(a,c)** and BI **(b,d)** with both nucleotide (a,b; 120 762 positions) and deduced amino acid (c,d; 40 254 positions) sequences. They are highly supported and only bootstrap values $<100\%$ are indicated to the left of the corresponding node; 1000 replicates for MP using PAUP* (Phylogenetic Analysis Using Parsimony and Other Methods) [9] [ten random species were added with tree bisection reconnection (TBR) branch swapping] and 100 replicates for BI using MrBayes [8] (GTR+Γ model for nucleotides and WAG+Γ model for amino acids) following Douady *et al.* [34] were performed. The ML trees and bootstrap supports inferred using PAUP*, PHYML [10] and Treefinder [11] are nearly identical. The G+C content at the third codon position is indicated in brackets and the color varies from purple to red with increasing G+C content. *Y. lipolytica* is used as the outgroup in all tree representations. Parts of phylogenies that are not identical among the four trees are shown in bold. The scale bar indicates the number of substitutions (MP) and the number of substitutions per position (BI). When a different approach gives the same topology as the one indicated in bold, its name is indicated using normal typeface (e.g. BI_{nt3}). The complete species names are *Saccharomyces cerevisiae, Saccharomyces paradoxus, Saccharomyces mikatae, Saccharomyces kudriazevii, Saccharomyces bayanus, Naumovia castellii, Candida glabrata, Debaromyces hansenii, Candida albicans, Kluyveromyces lactis, Saccharomyces kluyveri, Kluyveromyces waltii, Ashbya gossypii* and *Yarrowia lipolytica*. The phylogeny shown in part (d) is most likely to be the correct tree.

tree, indicating that the removal of the third codon positions renders MP less sensitive to the compositional bias.

Case 3: The use of the slowly evolving transversions is known to avoid artifacts caused by the compositional signal [19], because the purine (and pyrimidine) content is generally homogeneous even when the G+C content is not (Figure S2 in the supplementary material online). As expected, when the transversions at the first two codon positions are analyzed by MP, the same tree as that obtained with amino acid sequences is recovered (Figure S5 in the supplementary material online). Finally, the same result as with BI on amino acids is obtained with BI on transversions from all three codon positions (Figure S6 in the supplementary material online).

In conclusion, all analyses indicate that the strong statistical incongruence between MP_{nt} and BI_{aa} trees is caused by a greater sensitivity of MP to a systematic error related to the compositional bias at the nucleotide level whose effects are attenuated on translation. In addition, the difference in the type of characters used (nucleotides versus amino acids) explains a greater part of the huge differences observed between the MP_{nt} (Figure 1a) and the BI_{aa} trees (Figure 1d) than the use of two different tree reconstruction methods (MP versus BI).

Saturation as an indicator of incongruence

Tree reconstruction artifacts are the result of the accumulation of multiple substitutions at the same position over time: convergences and reversions erase the genuine phylogenetic signal. When multiple substitutions are dominating, the data set is said to be mutationally saturated. Without any bias, a highly saturated data set will produce an unresolved starlike phylogeny. However, when sequences have been generated by a heterogeneous evolutionary process, saturation will ultimately lead to the accumulation of an erroneous nonphylogenetic signal in the alignments.

We evaluated the saturation level of the yeast phylogenomic data set by comparing the number of substitutions inferred by ML with the number of observed differences for each pair of species [20], for the complete alignment (Figure 2). The lesser the slope of the linear regression, the greater the level of saturation, and therefore the greater the probability of tree reconstruction artifacts (and hence of incongruence). As expected, nucleotides (slope = 0.31, Figure 2d) are more saturated than amino acids (slope = 0.51, Figure 2a). However, the saturation of nucleotides is highly concentrated in third codon positions (slope = 0.16, Figure 2b), and much less pronounced for the first two codon positions (slope = 0.47, Figure 2c).

Multiple substitutions are so frequent at the third codon positions (up to ten inferred substitutions for only one observed difference) that the BI method, albeit efficient in detecting multiple substitutions [12,13], is seriously misled by the compositional signal (Figure S2 in the supplementary material online). The high level of saturation suggests that third-codon positions should not be used for inferring ancient phylogenies. However, the sparse taxon sampling considered here (only 14 species) probably aggravates the case, and this conclusion might have to be re-evaluated with a denser sampling of species.

Interestingly, Figure 2f shows a negative correlation between the level of saturation of a given data set and the number of differences to the least biased tree (BI_{aa} or Bayesian interference of transversion, BI_{tv123}, Figure 1d). In other words, when fast-evolving positions are removed from the analysis, the inferred phylogeny is less biased by nonphylogenetic (in particular compositional) signals, even if the tree reconstruction method is not accurate. As a result, a more reliable phylogeny is obtained with a poorly performing method (MP) and a relatively unsaturated data set (amino acids) than with a more accurate method (BI or ML with a complex model) and a highly saturated data set (third codon positions only). The importance of the quality of the data set therefore equals or exceeds that of the accuracy of the tree reconstruction method.

A greater sensitivity of MP to mutational saturation compared with BI could explain why the MP_{aa} and BI_{aa} trees are slightly different. In fact, *K. lactis* and *A. gossypii* evolve faster than *K. waltii* and *S. kluyveri* (Figure 1d) and are likely to be attracted by the long unbroken branch of the outgroup in the MP tree (Figure 1c) because of a long branch attraction (LBA) artifact [5]. To obtain a less saturated data set than the complete amino acid alignment, we removed the 18 075 positions that display at least two different amino acids in the outgroup species (*D. hansenii*, *C. albicans* and *Y. lipolytica*). This approach has the additional advantage of efficiently shortening the branch length of the outgroup, thus reducing the impact of the LBA. The MP tree inferred from the remaining 22 179 slowly evolving positions (Figure 3) is identical to the BI_{aa} tree, albeit with a reduced bootstrap support. When the saturation is much reduced (slope = 0.58 for these 22 179 amino acid positions, Figure 2e), MP recovers exactly the same tree as BI, strongly arguing that this tree is the best current working hypothesis for the relationships of these 14 yeast species, in light of which yeast genomic evolution should be interpreted.

Conclusions and recommendations

We do not dispute the use of numerous genes for phylogenetic inference [1,2,21], because it is generally required to solve difficult phylogenetic questions [22,23]. However, contrary to some current opinions [1,2], obtaining a highly supported tree from the analysis of a concatenation of multiple genes does not guarantee that 'it accurately represents the historical relationships' [2]. Highly supported groupings can prove to be incorrect because of the inconsistency of the tree reconstruction method (Figure 1a). Because these errors are the result of systematic biases that generally become apparent when using large data sets, phylogenomic trees should be regarded with greater caution than single-gene trees for possible tree reconstruction artifacts [6,7,23–27].

We stress that phylogenomics should emphasize not only the quantity of data under study but also their quality (i.e. their degree of saturation). Because current tree reconstruction methods are not always able to handle the presence of multiple substitutions correctly, efforts should be made to

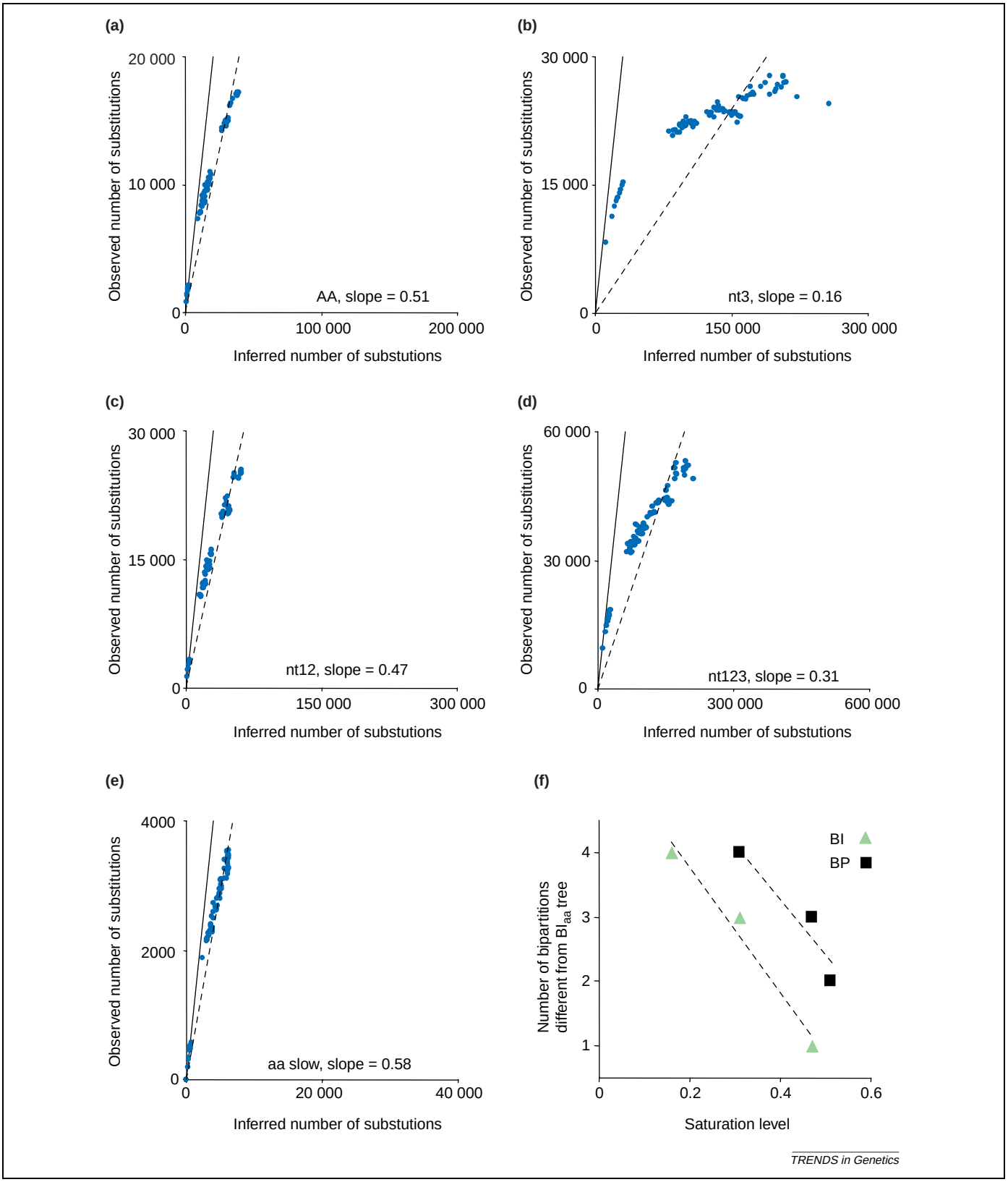

Figure 2. Mutational saturation of the concatenation of 106 genes. The level of saturation was estimated using the method described in Philippe *et al.* [20], as implemented in MUST (management utilities for sequences and trees) [35]. The *x*-axis corresponds to the number of substitutions inferred from the ML tree, whereas the *y*-axis corresponds to the number of differences observed in a pair-wise comparison, for the same pair of species. A linear regression is performed and the slope of the dotted line starting from the origin is used as an indicator of the saturation level (e.g. multiple substitutions at the same position), the steeper the slope the less saturated the data set. Analyses were performed for **(a)** amino acids (40 254 positions); **(b)** third codon positions (40 254 positions); **(c)** first two codon positions (80 508 positions); **(d)** all three codon positions (120 762 positions); and **(e)** a data set where variable positions in the outgroup species (*Debaromyces hansenii*, *Candida albicans* and *Yarrowia lipolytica*) have been removed (22 179 positions). The diagonal that corresponds to the case where no multiple substitutions occurred is indicated by a bold line. Finally, **(f)** shows the relation of the number of bipartitions different to the least biased tree (BI_{aa} or BI_{tv123}; see Figure 1d) as a function of the saturation level expressed as the slope of the regression line. The three data sets used are nt_3, nt_{123} and nt_{12}.

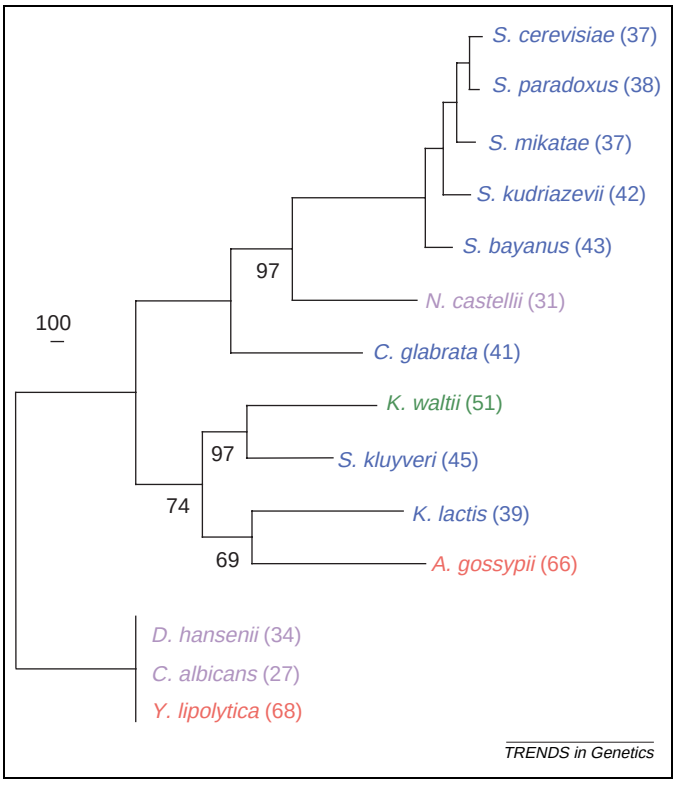

Figure 3. Removal of fast-evolving positions. The 18 075 amino acid positions that are variable in the outgroup species (*Debaromyces hansenii, Candida albicans* and *Yarrowia lipolytica*) were eliminated and a MP analysis (as in Figure 1c) was performed on the remaining 22 179 slowly evolving positions. This removal reduces the impact of the long-branch attraction artifact and the resulting MP tree is identical to the one obtained using BI based on the complete amino acid concatenation (Figure 1d). Color code and species names are the same as in Figure 1.

reduce their potentially misleading effects. First, because using several taxa enables a better detection of multiple substitutions, increasing the taxon sampling is particularly important. All recent empirical phylogenomic studies [23,25,28–31] except one [1] support this conclusion. This latter study [1] should be treated with caution because the tree used as reference (the MP_{nt} tree of Figure 1a) is almost certainly incorrect. Second, probabilistic methods should be used with models of sequence evolution that handle the most flagrant aspects of real substitution patterns to reduce the inconsistency of current methods caused by model misspecification [32]. Nonstationary models for dealing with heterogeneous G+C content and mixture models certainly represent steps in the right direction.

Finally, we believe that an efficient way to take advantage of the wealth of genomic data currently produced is to voluntarily discard a part of the data from phylogenetic analyses. This is already a common practice, as demonstrated by the removal of ambiguously aligned regions or of odd species (e.g. the fast-evolving microsporidia are never used to represent fungi) or by the use of amino acids instead of nucleotides for ancient divergences. Extensive data removal is often impractical in single-gene analyses because too few positions remain available, producing a poorly resolved tree [33]. This limitation becomes negligible in phylogenomics, and highly supported trees cleared of tree reconstruction artifacts can be recovered when more than half of the data have been discarded [23,28,30]. We therefore suggest putting the emphasis on the development and refinement of objective methods aimed at detecting and removing the part of the data containing a high level of nonphylogenetic signal [6]. As we showed in yeasts, the application of these guidelines will hopefully avoid domination by incongruence in the phylogenomic era.

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Supplementary data

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