

RATE VARIATION CAUSED BY DISCORDANCE

Gene tree discordance causes apparent substitution rate variation

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ABSTRACT

25 Substitution rates are known to be variable among genes, chromosomes, species, and
lineages due to multifarious biological processes. Here we consider another source of
substitution rate variation due to a technical bias associated with gene tree discordance, which
has been found to be rampant in genome-wide datasets, often due to incomplete lineage sorting
(ILS). This apparent substitution rate variation is caused when substitutions that occur on
30 discordant gene trees are analyzed in the context of a single, fixed species tree. Such
substitutions have to be resolved by proposing multiple substitutions on the species tree, and we
therefore refer to this phenomenon as “SPILS” (Substitutions Produced by Incomplete Lineage
Sorting). We use simulations to demonstrate that SPILS has a larger effect with increasing levels
of ILS, and on trees with larger numbers of taxa. Specific branches of the species trees are
35 consistently, but erroneously, inferred to be longer or shorter, and we show that these branches
can be predicted based on discordant tree topologies. Moreover, we observe that fixing a species
tree topology when performing tests of positive selection increases the false positive rate,
particularly for genes whose discordant topologies are most affected by SPILS. Finally, we use
data from multiple *Drosophila* species to show that SPILS can be detected in nature. While the
40 effects of SPILS are modest per gene, it has the potential to affect substitution rate variation
whenever high levels of ILS are present, particularly in rapid radiations. The problems outlined
here have implications for character mapping of any type of trait, and for any biological process
that causes discordance. We discuss possible solutions to these problems, and areas in which
they are likely to have caused faulty inferences of convergence and accelerated evolution.

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(Keywords: substitution rates, incomplete lineage sorting, hybridization, gene tree discordance)

Characterizing the rate of molecular evolution and the forces driving variation in this rate is a major goal of evolutionary biology. Studying how and why substitution rates vary allows us to make inferences about the biology and evolutionary past of extant species and genes (Bromham 2009). Examples of such inferences include: learning about past episodes of adaptive evolution (e.g., Larracuenta et al. 2008; Studer et al. 2008), whether species' life-history traits influence how fast they evolve (e.g., Martin and Palumbi 1993; Bromham et al. 1996; Smith and Donoghue 2008), and whether specific environments make species prone to higher mutation rates (e.g., Wright et al. 2006). A better understanding of the tempo of molecular evolution can also be used to improve the dating of evolutionary events (Lanfear et al. 2010).

Substitution rates vary at several different scales, including among genes, regions with different recombination rates, chromosomes, species, and clades. Variation among genes can be driven by natural selection. Adaptive evolution increases the rate of substitution, while negative selection decreases it (Li 1997). Even in the absence of differences in selective effects there can be variation in the substitution rate due to variation in the underlying mutation rate. For instance, regions of low recombination have been found to have lower substitution rates (Hellmann et al. 2003). This association has been explained by the mutagenic effects of recombination (Hellmann et al. 2003) or by linked selection in recent ancestral populations (Begun et al. 2007).

Substitution rates also vary among chromosomes, with sex chromosomes such as the X chromosome in mammals exhibiting lower substitution rates (e.g., Shimmin et al. 1993; Chang et al. 1994; Ebersberger et al. 2002; Malcom et al. 2003; The Chimpanzee Sequencing and Analysis Consortium 2005). Male mammals undergo more germline cell divisions than females, and therefore because the X spends less time in males it accumulates fewer mutations (Miyata et al. 1987; Makova and Li 2002). Species also vary in their rate of substitution, for which several

70 underlying forces have been put forward. Possible causes include variation in the metabolic rate
(Martin and Palumbi 1993), generation time (Laird et al. 1969; Wu and Li 1985), population size
(Lynch 2010), and body size (Bromham et al. 1996). Finally, the number of speciation events in
a clade is correlated with its substitution rate, leading to the assertion that speciation is an active
force promoting bouts of accelerated evolution (Webster et al. 2003; Pagel et al. 2006; Venditti
75 and Pagel 2009; Zhang et al. 2014).

Here we consider another source of apparent substitution rate variation due to a technical
bias associated with gene tree discordance. Gene trees can differ in topology from the species
tree because of diverse phenomena such as incomplete lineage sorting (ILS), hybridization, and
lateral gene transfer (Maddison 1997). Gene tree discordance – differences between gene trees
80 and the species tree – caused by ILS is common in nature (Pollard et al. 2006; Scally et al. 2012;
Brawand et al. 2014; Jarvis et al. 2014; Zhang et al. 2014; Suh et al. 2015), with ILS levels being
proportional to ancestral population sizes and inversely proportional to time between speciation
events (Pamilo and Nei 1988; Maddison 1997). ILS can occur among species at any point in the
past, and therefore can affect phylogenetic inference across all timescales (Oliver 2013).

85 How does gene tree discordance lead to apparent substitution rate variation? If a single
species tree is used for analysis, nucleotide substitutions that occur on internal branches of
discordant gene trees that are absent from the species tree must then be placed on existing
species tree branches (Fig. 1). All bifurcating discordant gene trees contain one or more internal
branches that are not present in the bifurcating species tree (see e.g. Fig. 1; Robinson and Foulds
90 1981). Substitutions occurring on these internal branches of discordant gene trees (filled symbols
in Fig. 1) must be accounted for by multiple substitutions on the species tree (hollow symbols in
Fig. 1). Consider the filled cross (representing a substitution from 0→1) on discordant tree 1

(Fig. 1). Because taxa A and C now both have state 1, on the species tree this substitution must be explained by multiple substitutions, in this case an initial 0→1 change on the branch subtending A, B, and C (hollow cross in Fig. 1) and a back substitution on branch B (also represented by a hollow cross in Fig. 1). The same reasoning applies to substitutions occurring on internal branches of discordant gene tree 2 that do not exist in the species tree (circles in Fig. 1). No matter how many taxa are involved, when substitutions on discordant gene trees are analyzed in the context of a single species tree, spurious substitutions will be inferred. Therefore, with more discordant gene trees included in an analysis (i.e. the more ILS is present), we expect a higher inferred substitution rate.

The technical bias described above has been previously considered in a wider context, irrespective of the character's nature, and dubbed "hemiplasy" (Avice and Robinson 2008). The rationale behind this term is that a homoplasy-like effect is observed (i.e., character transitions are mapped to two or more branches mimicking convergent evolution or character-state reversal), yet the event is not actually homoplasious (Avice and Robinson 2008). In the particular case of analyses of sequence data in the presence of ILS, we hypothesized that there would be consistent biases and predictable patterns of substitution rate variation. To distinguish this phenomenon from the general pattern of hemiplasy we henceforth refer to it as SPILS (Substitutions Produced by Incomplete Lineage Sorting), returning later to discuss the relationship between the two. In the present work we use simulated data and data from multiple *Drosophila* species to demonstrate the magnitude and ubiquity of SPILS. We indeed observe predictable biases, including consistent patterns of apparent substitution rate acceleration and deceleration on individual branches, and an effect of SPILS on increasing the false positive rate in tests for positive selection.

MATERIALS AND METHODS

Quantifying the Effects of SPILS on Branch Lengths

In order to test whether ILS leads to inferences of spurious substitutions, we simulated 1-
120 kb sequences using a population size of $N_e=10^6$ and a mutation rate of $\mu=10^{-9}$. There was no recombination within loci. Ten thousand (10,000) sequences were simulated along species tree $((A,B),C),D$ and $((((A,B),C),D),E)$ under 4 different ILS conditions, named ILS0, ILS1, ILS2 and ILS3, in order of increasing gene tree discordance starting from no ILS (ILS0). The amount of ILS was increased by changing the N_e of the internal branches of the two species trees
125 (Supplementary Fig. 1): in the first, three taxa are involved in the discordance $[((A,B),C)]$, and three different topologies are possible (Supplementary Fig. 2a). In the second, four species are involved in the discordance $[(((A,B),C),D)]$, and fifteen different topologies are possible (numbered according to their probabilities; Supplementary Fig. 2b). Simulations were done using the *egglib* package (Mita and Siol 2012).

130 For each simulated alignment, branch lengths were inferred using two kinds of trees, fixed (F) and unfixed (U), using PhyML (Guindon and Gascuel 2003) with the GTR nucleotide sequence model. “F” trees were constrained to be topologically equivalent to the species tree, that is, they were forced to be either $((A,B),C),D$ or $((((A,B),C),D),E)$. “U” trees were free to have the topology that maximized their likelihood given the alignment.

135 We quantified the effects of SPILS by comparing the lengths of F and U trees, as SPILS should make the former spuriously longer. Comparisons are made between these two conditions rather than with the known, simulated topology because with increasingly more ILS, terminal branches tend to become longer and internal branches shorter as a result of deeper coalescence in

the ancestral populations (Gillespie and Langley 1979). These length differences are not caused
140 by SPILS, and are not the subject of this study (see Angelis and dos Reis 2015 for a discussion of
this problem).

In order to detect an increase in total tree length due to SPILS, we calculated total tree
length differences ($F - U$) for each of the 10,000 simulated alignments. We then proceeded to
determine which branches were affected using two approaches. First, for each F tree branch, we
145 compared its average length across all 10,000 sequences in the ILS3 condition for the ((A,B),C)
and (((A,B),C),D) scenarios. Second, again for each F tree branch, we looked for any increase in
length by calculating ($F - U$) branch length differences for all 10,000 sequences, and then
grouped these differences by topology.

Both these approaches must deal with the complication that discordant trees differ from
150 the species tree by one or more internal branches that do not exist in the species tree, making
these branches hard to compare. Given species tree ((A,B),C), for instance, an internal branch
subtends (A,B); but in the discordant topologies resulting from ILS the internal branch subtends
either (A,C) or (B,C). It is precisely these internal branches lacking a counterpart in the species
tree that give rise to SPILS – substitutions on these branches cannot be directly mapped to the
155 species tree's internal branch, which necessitates mapping to multiple other branches. Therefore,
we employed the same procedure used for terminal branches (calculation of $F - U$ differences),
but with a key difference: when allowing genes to take their own trees (U trees), we used the
lengths of the internal branches present in those trees (and absent in the species tree).

160 *Quantifying the Effect of SPILS on Inferences of Positive Selection*

The three and fifteen different topologies resulting from ILS among ((A,B),C) and (((A,B),C),D), respectively, were used to simulate 999-bp coding sequences with *evolver*, a program from the PAML suite (Yang 2007). One thousand coding sequences were simulated for each topology, with 60% and 40% of the sites being constrained ($\omega = 0.1$) and unconstrained ($\omega = 1$), respectively. No sites were simulated with positive selection.

Branch lengths used in the *evolver* simulations were the average lengths obtained from the ILS3 scenario when either ((A,B),C) or (((A,B),C),D) were the clades experiencing ILS. Positive selection was inferred with M1a-M2a site tests (as implemented in PAML v4.7a, [Yang 2007]), specifying species tree (((A,B),C),D) or (((((A,B),C),D),E) as the topology to use regardless of the topology employed in the simulations. We counted a gene as under positive selection using a likelihood ratio test between model M1a and M2a, with 2 degrees of freedom and $P < 0.05$ assuming a χ^2 distribution.

Quantifying the Effects of SPILS on Drosophila

8,565 gene sequence alignments from the four *Drosophila* species, *D. yakuba*, *D. erecta*, *D. melanogaster*, and *D. ananassae* (henceforth abbreviated Y, E, M, and A) used in Larracunte et al. (2008) were obtained from ftp://ftp.flybase.net/genomes/12_species_analysis/clark_eisen/alignments/melangaster_group.gui_de_tree.longest.cds.masked.tar.gz. Each alignment was used to produce two gene trees each, similar to the simulations described above: F trees had their topology fixed to the species tree topology [(((Y,E),M),A)], and U trees were free to take the topology that maximized the gene tree likelihood in PhyML.

In order to investigate how the presence of intragenic recombination in the *Drosophila* data could influence the effect of SPILS, we concatenated segments of alignments simulated
185 with *evolver* under the three *Drosophila* alternative topologies into a total of 3,000 999-bp “mixed” alignments. Branch lengths used in these simulations were the averages across all *Drosophila* genes trees of each specific topology. These mixed alignments had 70% of their sites from one of the three alternative topologies, and 15% from each of the other two topologies.

190 RESULTS

Total and Individual Branch Lengths

We generated 10,000 simulated alignments for each of the eight ILS scenarios: ILS0, ILS1, ILS2, and ILS3 with either ((A,B),C) or (((A,B),C),D) under ILS. To quantify the effects of SPILS, each simulated alignment was used to infer a pair of trees, either requiring the
195 topology to be fixed (F) to the species tree, or allowing it to be freely inferred (unfixed, or U). Each scenario differed with respect to the level of gene tree discordance exhibited. ILS0, ILS1, ILS2 and ILS3 produced increasingly larger numbers of discordant gene trees, with approximately 0%, 10%, 30%, and 45% of discordant gene trees in each condition when ((A,B),C) was allowed to vary, and 0%, 20%, 50% and 70% when (((A,B),C),D) was allowed to
200 vary (Supplementary Fig. 2). As increasing the number of species allowed for more possible discordant topologies, we predicted it would also lead to more SPILS.

The mean change in total tree length due to SPILS is shown in Figure 2. If SPILS occurs, F trees should be longer than U trees for the same alignment, and this is exactly what we see. The mean change in total tree length (F - U) was positive, and became increasingly larger with
205 increasing levels of discordance. The effect of SPILS was greater when more taxa were involved

in the discordance, as trees were inferred to be disproportionately longer when four taxa were involved in the ILS compared to cases where only three taxa were involved (Fig. 2).

Specific branches are expected to become longer or shorter due to SPILS. SPILS is largely caused because some internal branches exist on discordant trees that do not exist on the species tree, whereas some internal branches on the species tree do not exist on the discordant trees. Substitutions that occur on discordant-only branches are mapped twice onto the species tree, causing certain branches to lengthen. While the details of this mapping are surely dependent on the models of sequence evolution used and on the length of species tree branches, we have found that certain branches consistently get longer: PhyML favored a substitution on the ancestral branch subtending the clade undergoing ILS, followed by a reversion on an external branch in all cases (similar results were found with RAxML, [Stamatakis 2014]; results not shown). For example, assuming the species tree ((A,B),C), analyzing a gene that has the discordant gene tree ((A,C),B) should cause branch B and the branch leading to ((A,B),C) to become longer due to SPILS (Fig. 1). If the discordant gene tree ((B,C),A) is used, then branch A and the same branch subtending ((A,B),C) should get longer (Fig. 1). Conversely, because the internal branch subtending (A,B) in the species tree does not exist in either of the two discordant topologies for a rooted three-taxon tree, no substitutions can occur on this branch in these trees and we expect it to therefore become shorter (Fig. 1).

Comparing the average F and U branch lengths from the three and fifteen alternative topologies allowed us to confirm our predictions for branch length changes in the species tree due to SPILS. In the case where only three topologies are possible, branches A, B, and that leading to ((A,B),C) became slightly longer as expected, and the internal branch subtending (A,B) became slightly shorter (Fig. 3a, left panel). Length changes are more pronounced when

230 fifteen alternative topologies are possible (Fig. 3a, right panel), possibly because a greater proportion of the total tree length can be involved in the discordance (see below).

Breaking down the effect of SPILS by topology allows us to further explore which branches are affected given alternative gene trees. As predicted, when the discordant gene trees were ((A,C),B) or ((B,C),A), branches A and B became longer due to SPILS, respectively (Fig. 3b). Moreover both topologies showed the ancestor of A, B, and C becoming longer due to SPILS (Fig. 3b). Also as predicted, the number of substitutions by which the internal branch subtending (A,B) gets shorter matches the length increase of the branch subtending ((A,B),C) and either A or B (Fig. 3b). Supplementary Figure 3 shows the predictions for the case of fifteen alternative topologies and Supplementary Figure 4 their confirmation.

240 Finally, the overall effect of SPILS varied among trees with different discordant topologies, though this was only apparent for the case where ILS involved four taxa. Discordant topologies with a greater number of internal branches absent from the species tree, and with a greater total length of internal branches absent from the species tree, were more affected by SPILS (Fig. 4). Both these gene tree characteristics underlie the effect of SPILS because in fixed-tree analyses longer internal branches absent in the species tree will cause a larger number of substitutions to be misplaced on other branches, causing them to lengthen (Supplementary Fig. 5).

Inferences of Positive Selection

250 The results presented above demonstrate that using a fixed species tree when analyzing a collection of possibly discordant gene trees leads to incorrect inferences about the number of substitutions that have occurred. We reasoned that such a phenomenon might also affect

inferences of positive selection when studying protein-coding genes. Though nonsynonymous substitutions (as measured by d_N) and synonymous substitutions (d_S) are expected to be affected equally by SPILS, the increased variance in number of substitutions of each type may result in
255 some genes accumulating multiple excess nonsynonymous substitutions. This could in turn lead to spurious inferences of positive selection on individual genes.

To test this idea we simulated protein-coding sequences along topologies that arise from ILS within the species tree ((A,B),C) and (((A,B),C),D) (Methods). To detect positive selection we used the “site” models implemented in PAML (Yang 2007), fixing the tree to be analyzed as
260 the species tree in each case. The genes experienced only negative selection in the simulations, and we therefore expect no positive selection to be detected (or rather, we should only find as much as expected under our null hypothesis of $\alpha=0.05$).

Overall, we found an excess of genes inferred to be under positive selection when discordant trees were analyzed using the species tree. When there is no discordance the fraction
265 of genes under positive selection was 1.2% for both the three-taxon and four-taxon rooted trees. However, when all topologies are included, the fractions of genes under positive selection were 1.7% and 3% for the same two species trees. Topologies more affected by SPILS (for the reasons discussed above) were particularly more prone to be erroneously classified as being under positive selection: we found a close association between the total increase in tree length due to
270 SPILS and the fraction of trees incorrectly inferred to be under positive selection (Fig. 4b). The specific discordant topologies most affected by SPILS (topologies 6 – 9) showed false positive rates $>5\%$ (Fig. 4b). The concordant topology also showed a very slightly lower value of estimated d_N/d_S than the discordant topologies (0.491 ± 0.005 vs. 0.496 ± 0.001).

Interestingly, the topologies that are most affected by SPILS – and also most responsible
275 for the higher false positive rate – are not necessarily the most (or least) frequent of all the
possible topologies in the presence of ILS. Topologies numbered 10 to 15 are the five least
frequent topologies among the 15 possible ones when $((A,B),C),D$ undergoes ILS
(Supplementary Fig. 2b), but the number of false positives from these topologies was similar to
that from the most frequent discordant topologies (numbered 2 and 3 in Fig. 4b). These results
280 indicate that although the overall influence of SPILS on inferences of positive selection is
modest, for certain topologies it may have a much larger effect.

Drosophila Data

We estimated branch lengths for 8,565 genes from three *Drosophila* species (and an
285 outgroup) known to exhibit considerable levels of ILS (Pollard et al. 2006). We followed the
same procedure used for the simulated data, either fixing the tree topology to $((Y,E),M),A$, or
allowing each gene to take on its own topology. Note, however, that in this case we do not know
whether the inferred trees for each gene are the correct topologies; this fact becomes important
below.

290 Our specific predictions are: that total tree lengths from trees fixed for the species tree
topology will be longer for genes with discordant trees, that terminal branches Y and E, and the
branch subtending $((Y,E),M)$, will get longer, and that the internal branch leading to (Y,E) will
get shorter. Our predictions were confirmed in the *Drosophila* data (Fig. 5a-b). When analyzed
under a fixed species tree topology, total tree lengths of genes with discordant gene trees
295 $((Y,M),E)$ and $((E,M),Y)$ are inferred to be 2.1% and 1% longer than when each gene is allowed
to take on its most likely topology. We also see that the terminal branches leading to Y and E,

and the internal branch leading to the common ancestor of ((Y,E),M), are all longer in the fixed-tree analysis, while the internal branch leading to the (Y,E) ancestor is shorter (Fig. 5b).

300 We noticed two differences in these data compared to the simulated data above. First, it often appeared that the external branch leading to species M was shorter in the fixed-tree condition (Fig. 5a), and that this was due to discordant gene trees (Fig. 5b). Second, the increase in length of the branch leading to the ((Y,E),M) common ancestor was smaller than length increases in the Y and E branches (Fig. 5a, b), though these were of similar magnitude in our simulated datasets (Fig. 3b).

305 We believe that intragenic recombination is the reason for the difference between the results from simulated data and from the *Drosophila* data. SPILS results from assuming one tree when analyzing genes whose sites have for the most part evolved along different topologies as a result of ILS. Recombination within genes, however, means that most genes will have a mix of sites from different topologies; regardless of the major topology inferred for each gene, it is likely that a minority of sites have evolved along another topology. Therefore, even in our “unfixed” trees, enforcing a single (possibly discordant) topology for each gene means that some fraction of sites will show a SPILS-like pattern. The fact that we are now enforcing a different topology means that a different set of branches may be inferred to be longer or shorter. In fact, we can recapitulate the patterns seen in the *Drosophila* data by generating simulated data with intragenic recombination. We generated “recombinant” sequences where 70%, 15%, and 15% of 315 the sites within loci simulated along the three possible topologies using branch length averages obtained from *Drosophila* gene trees. Performing the same comparisons as before recovers the same qualitative and quantitative patterns of branch length differences as in the *Drosophila* genes (Fig. 5c).

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DISCUSSION

The increasing availability of genomic data from multiple species has revealed substantial variation in nucleotide substitution rates (Bromham 2009; Lanfear et al. 2010). It is now recognized that there are a multitude of factors potentially contribution to variation in substitution rates, including variation in functional importance among genes, sex-related differences in mutation rates among chromosomes, variation in life-history traits among species, and even variation in the number of speciation events among clades.

In the present study we systematically characterized how a phenomenon we refer to as “SPILS” affects substitution rates. This phenomenon is expected to occur in any analysis where a fixed species tree topology is used to analyze rates of substitution, but where gene tree discordance is present among the genes being analyzed. SPILS occurs because substitutions on internal branches of discordant gene trees are mapped to multiple species tree branches when the topology is fixed, artificially inflating the length of these branches but shortening the length of species tree branches that are not present in discordant gene trees. Although the effects of SPILS are modest per gene, with more genes, more species, and more discordance present in any dataset, the larger the effects are expected to be. Additionally, we demonstrate that the branches that become spuriously longer (and shorter) due to SPILS can be predicted depending on the discordant gene tree topologies.

Interestingly, the two factors that most affect the degree of discordance due to ILS – the time between successive speciation events and population size – are also often observed to covary with substitution rates. SPILS may therefore contribute to variation in substitution rates at many levels. For example, genes located in genomic regions with lower recombination rates may

have lower rates of substitution (Hellmann et al. 2003; Begun et al. 2007). Due to an increased effect of linked selection in these regions there is also a lower effective population size and less discordance due to ILS (Pease and Hahn 2013), and therefore less of an effect of SPILS. 345

Similarly, the X chromosome has a lower effective population than the autosomes, and at least in mammals also has a lower substitution rate (Makova and Li 2002; Wilson Sayres et al. 2011). Many species differ in effective population size, and again larger sizes are associated with higher rates of nucleotide substitution (Lanfear et al. 2014). Though there are multiple life-history traits 350 that are correlated with population size (Bromham 2009), we hypothesize that population size itself may drive a positive correlation with substitution rates via SPILS. Finally, clades containing a larger number of species will by necessity have more, shorter internal branches between speciation events, which can result in more ILS and gene tree discordance. It follows that, in addition to any intrinsic effect speciation may have on rates of molecular evolution 355 (Pagel et al. 2006), SPILS is expected to increase the substitution rate of clades with more species.

Although SPILS is expected to occur and to affect substitution rates in all of the aforementioned cases, it does not explain all the variation in any of them. We know this because it is possible to study substitution rates without any effects of SPILS. When only analyzing pairs 360 of species (with or without an outgroup) gene tree discordance is not possible, making SPILS impossible. This analysis has also been suggested in order to control for the node density effect when measuring substitution rates (Witt and Brumfield 2004; Hugall and Lee 2007). Pairwise comparisons have been used to calculate substitution rates among regions differing in recombination rate (e.g., Hellmann et al. 2003), among sex chromosomes and autosomes (e.g., 365 The Chimpanzee Sequencing and Analysis Consortium 2005), among species with different

population sizes (e.g., Wu and Li 1985), and among clades with different numbers of species (Barraclough and Savolainen 2001; Lanfear et al. 2010). In each case the expected effect was observed; therefore SPILS cannot explain the entire effect, though it may change the size of the effect observed. However, in cases in which SPILS could affect inferred substitution rates or
370 branch lengths, using only pairs of species – or other possible species trees where no discordance is possible – will ensure no effect of SPILS. In fact, we were able to recover the expected branch lengths from our simulations when removing all but two species and the outgroup (results not shown).

Another strategy for eliminating or minimizing the effect of SPILS is to make an
375 individual tree for each gene to be analyzed. Such an approach is especially relevant when testing each individual gene for positive selection, as we have demonstrated that SPILS causes an increase in false positives. While to our knowledge this problem had not been explicitly discussed before, it is clear that the problem was implicitly recognized in multiple studies. For instance, Larracuente et al. (2008) and Good et al. (2013) inferred trees for each individual gene
380 before testing for positive selection, whereas Scally et al. (2012) simply masked discordant sites consistent with ILS. We note, however, that the former strategy is not optimal because it mitigates the effect of SPILS but does not completely remove them. Within-gene recombination means that any gene likely contains sites that have evolved across multiple different topologies. We observed exactly this pattern in our analysis of the *Drosophila* data, though we have not
385 quantified its effects on inferences of positive selection.

There are additional problems with the strategy of making a tree for every gene. First, in some cases where SPILS is of great magnitude, there may not be enough informative substitutions to accurately reconstruct the history of individual genes. One important instance in

which this likely occurs is during species radiations. Radiations involve multiple, closely spaced
390 speciation events, and as a consequence discordance may involve many taxa (e.g., Brawand et al.
2014; Lamichhaney et al. 2015; Suh et al. 2015). There will likely be very few genes that contain
substitutions that are informative about all speciation events during a radiation. Furthermore,
radiations are expected to be especially hard hit by SPILS because of high levels of discordance
among a large number of species, with an outsized accumulation of substitutions on the branch
395 subtending the radiation. In fact, just such a pattern was observed in the radiation of African
cichlids (Brawand et al. 2014), with an apparent acceleration of both nucleotide substitutions and
gene duplications on this branch. Our results suggest that this pattern may be in part an artifact
caused by SPILS.

The strategy of generating individual gene trees may not even be applicable to all
400 situations that are affected by SPILS. Any method attempting to assess the rate of substitution
across a tree, for any kind of molecular change, should be affected by SPILS. For example,
studies of the rate of gene gain and loss (e.g., Han et al. 2013) or the rate of karyotype evolution
(e.g., Jónsson et al. 2014) could both be affected by SPILS, but it is unclear what individual trees
one could make to account for discordance. There is no clear single locus or region that all such
405 changes should track, and it may be difficult or impossible to find a region associated with each
individual change to make a tree from. In these cases – and in the case of radiations – we suggest
that reducing the number of taxa being studied in order to eliminate discordance would lead to
more accurate inferences, even though some data is being excluded. Certainly such an approach
would make it possible to test for an accelerated rate of evolution on branches leading to
410 radiations, with little loss of power.

As mentioned in the Introduction, SPILS is just one manifestation of the larger problem of hemiplasy (Avisé and Robinson 2008). Character-mapping in the presence of ILS will result in incorrect inferences for any kind of character, molecular or not (Hahn and Nakhleh in prep.). These inferences often involve the appearance of convergent changes when none has actually
415 occurred, and we predict that there will be many individual cases of molecular convergence that are ascribable to ILS (Parker et al. 2013). While the problem of mapping character changes onto species trees with underlying discordance has generally gone unaddressed, interestingly it has been addressed in multiple papers concerned with gene-tree/species-tree reconciliation algorithms (e.g., Vernot et al. 2008; Rasmussen and Kellis 2012; Stolzer et al. 2012). In the
420 presence of ILS, naive reconciliation algorithms place gene duplications on the branch subtending the discordance, with multiple convergent gene losses on descendent branches (Hahn 2007). Newer reconciliation algorithms deal with this bias either by treating the species tree as a polytomy (Vernot et al. 2008; Stolzer et al. 2012) or by assuming there is no hemiplasy (Rasmussen and Kellis 2012). Regardless of the details of these algorithms, it is clear that they
425 offer a way forward for dealing with the mapping of any types of characters onto a “fixed” species trees undergoing ILS (cf. Pollard et al. 2006). Note also that the problem we describe here is not limited to ILS as a cause of discordance: hybridization and introgression between species will also cause discordance, as will errors in phylogenetic reconstruction (Duchêne and Lanfear 2015). All of these will also lead to SPILS-like patterns. However, unlike in the case of
430 ILS – where the proportion of each discordant topology can be predicted (Degnan and Salter 2005) – in the case of introgression one specific discordant topology may be overrepresented. In these situations the set of branches whose lengths are affected is much less predictable.

Finally, it is important to recognize the effect of SPILS on inferences of branch lengths when constructing species trees. Often these branch lengths are used as a first indicator of substitution rate variation, especially if questions concerning the molecular clock are relevant; subsequently they are also important in dating events on a tree. Though the practice of concatenation in species tree inference has received a lot of attention (Edwards et al. 2007; Gatesy and Springer 2014), most of the concern about this approach has involved the accuracy of the tree topology in the “anomaly zone” (Degnan and Rosenberg 2006; Kubatko and Degnan 2007). But concatenation will also force discordant substitutions to be resolved on the most common topology, which will result in SPILS. This problem will be amplified as one concatenates a greater number of loci and a greater number of taxa. Multiple coalescent methods have been developed to overcome the topology-based problems with concatenation (e.g., Drummond and Rambaut 2007; Liu et al. 2009, 2010; Larget et al. 2010; Bryant et al. 2012; Mirarab and Warnow 2015; Yang 2015), but some of these do not estimate branch lengths (e.g., Mirarab and Warnow 2015), and even those that do may report lengths in coalescent units (e.g., Larget et al. 2010; Liu et al. 2010). Moving forward, it is clear that more methods that can simultaneously account for the topological discordance and branch-length discrepancies caused by ILS will be needed, and that they will need to be scalable to genome data. Only with such methods in hand will we be able to get an accurate picture of true substitution rate heterogeneity across large clades.

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670

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FIGURES

FIGURE 1

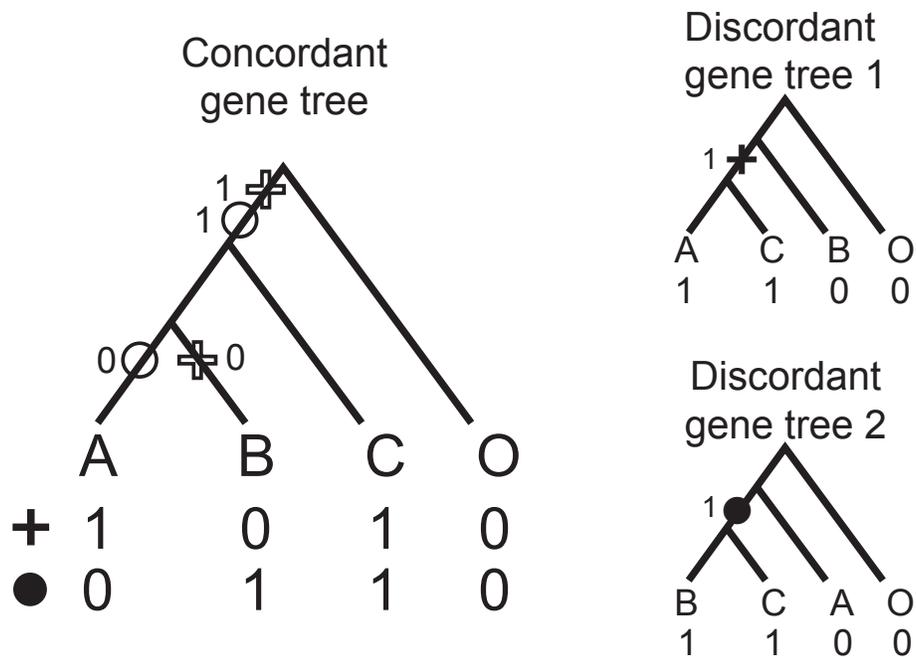


FIGURE 2

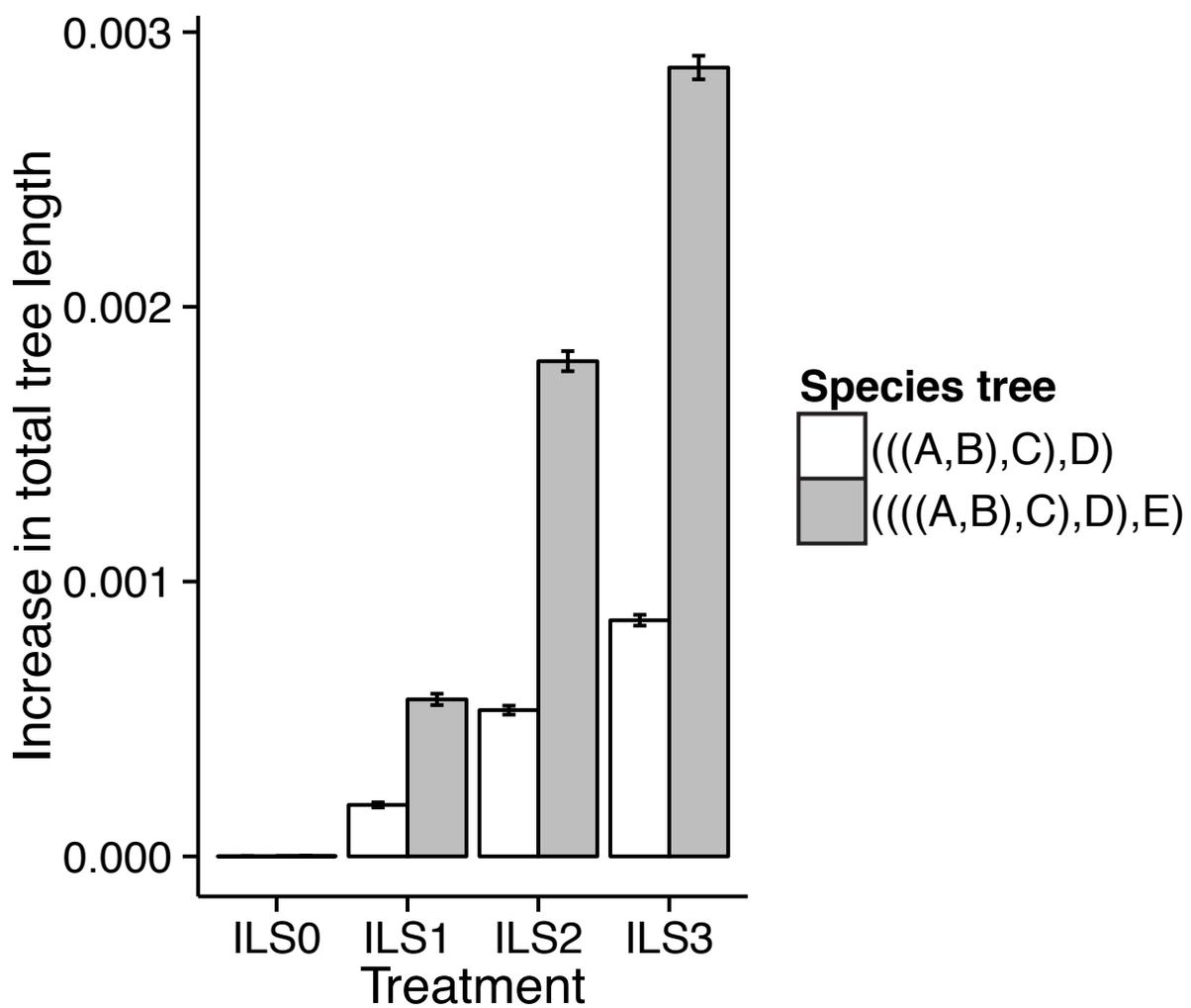
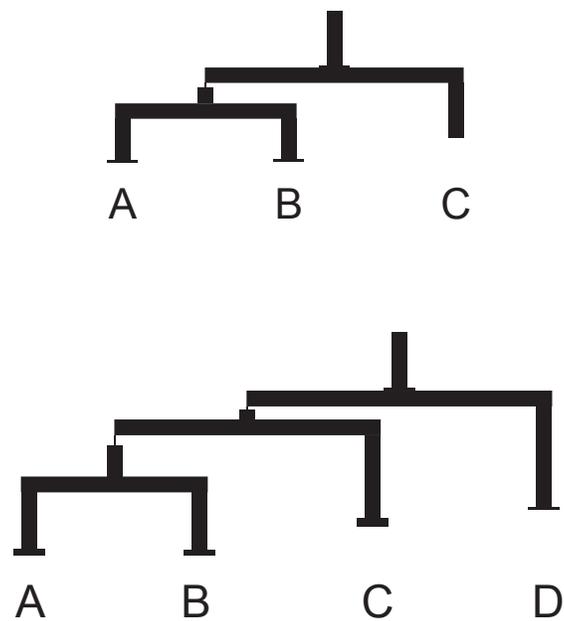


FIGURE 3

a)



b)

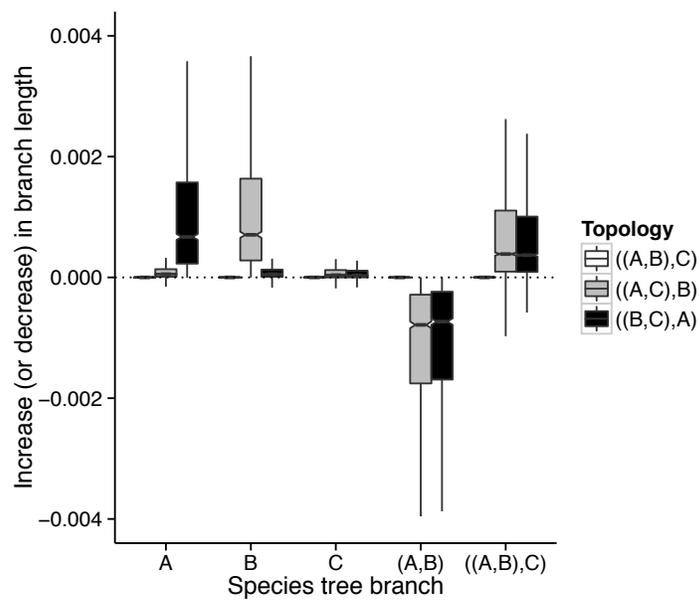
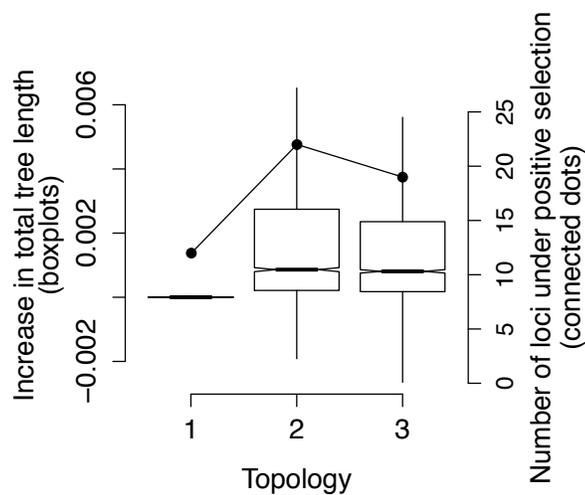


FIGURE 4

a)



b)

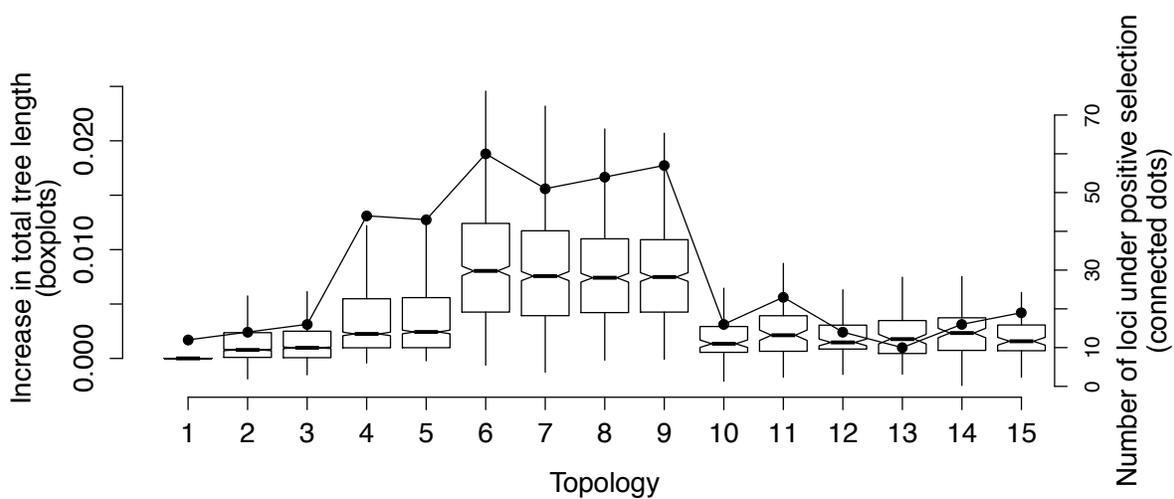
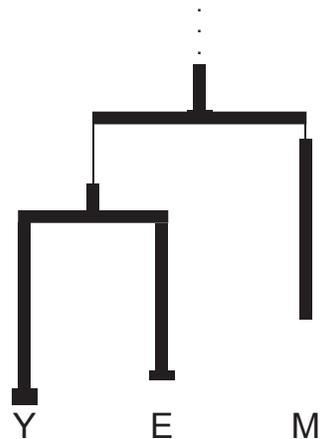
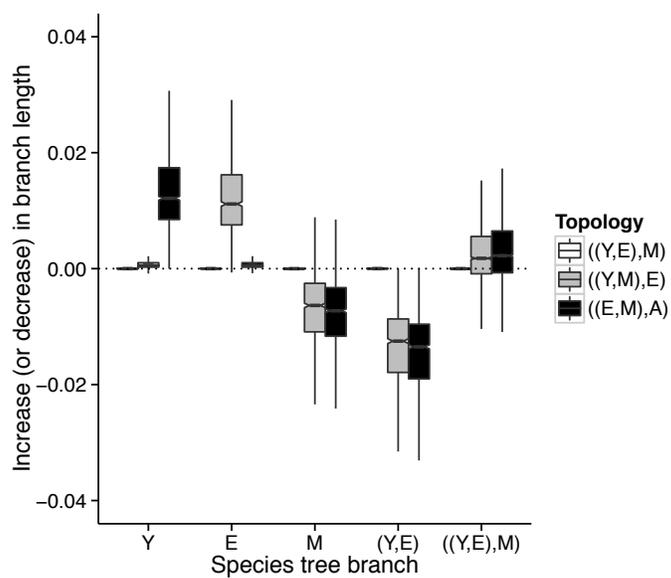


FIGURE 5

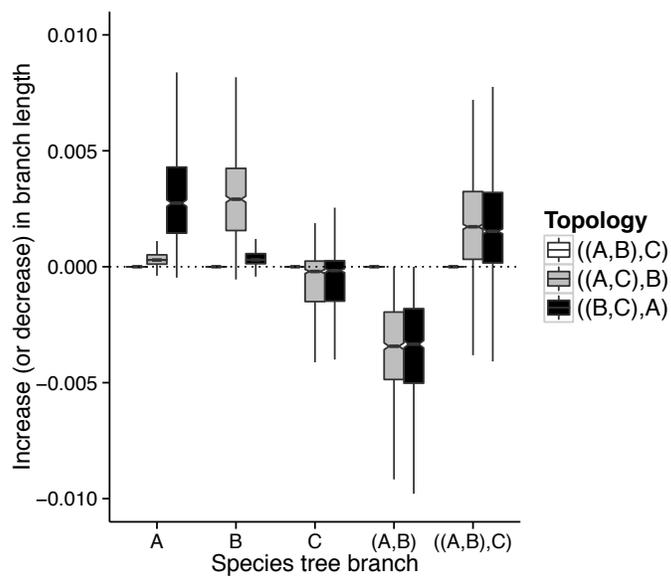
a)



b)

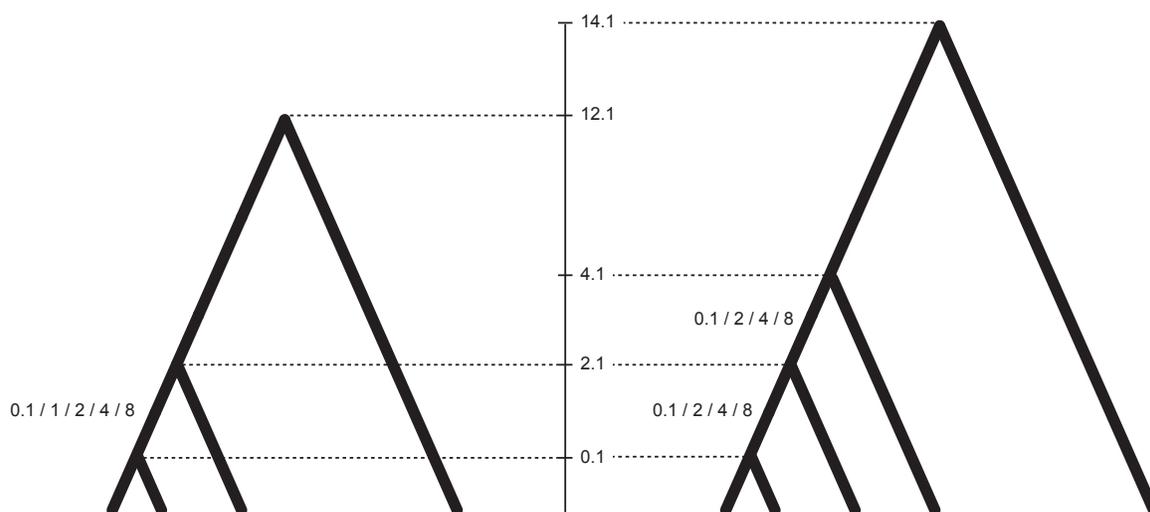


c)

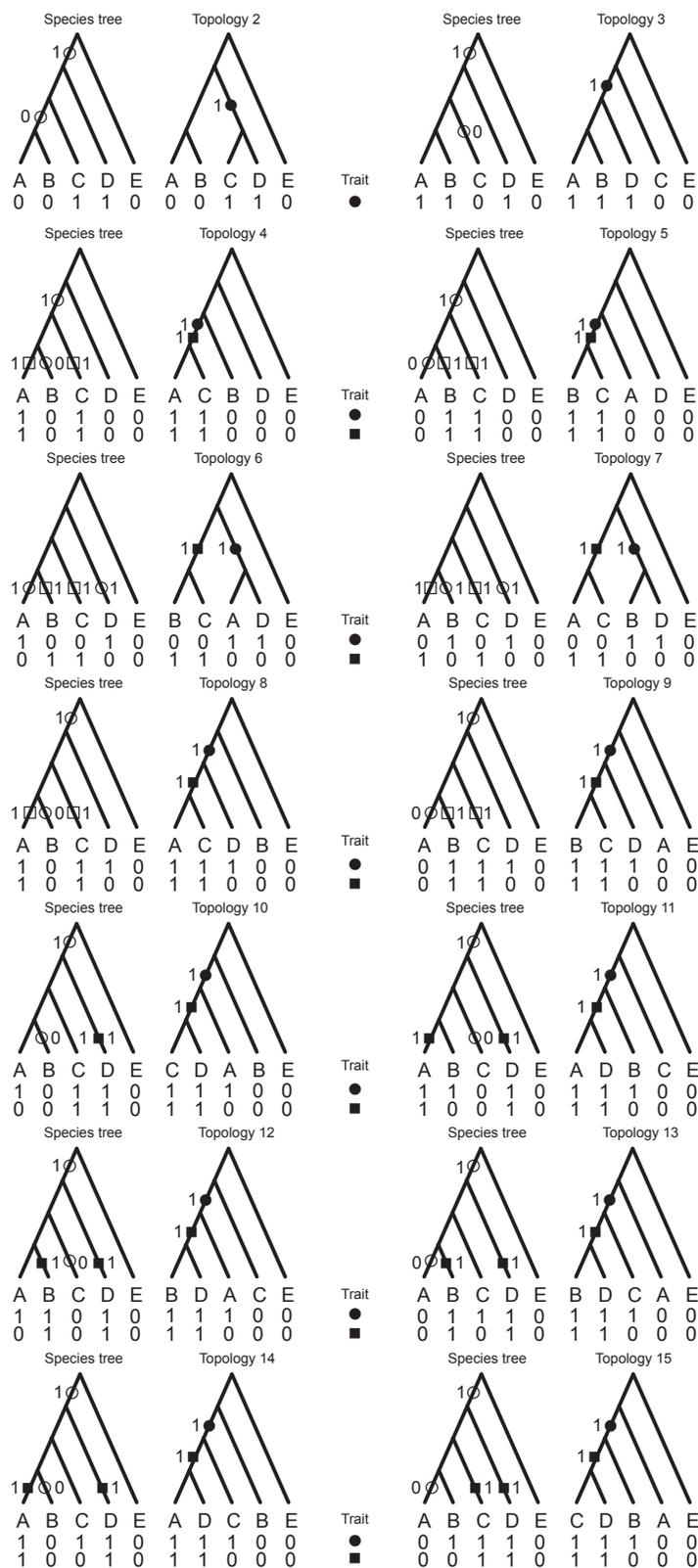


SUPPLEMENTARY FIGURES

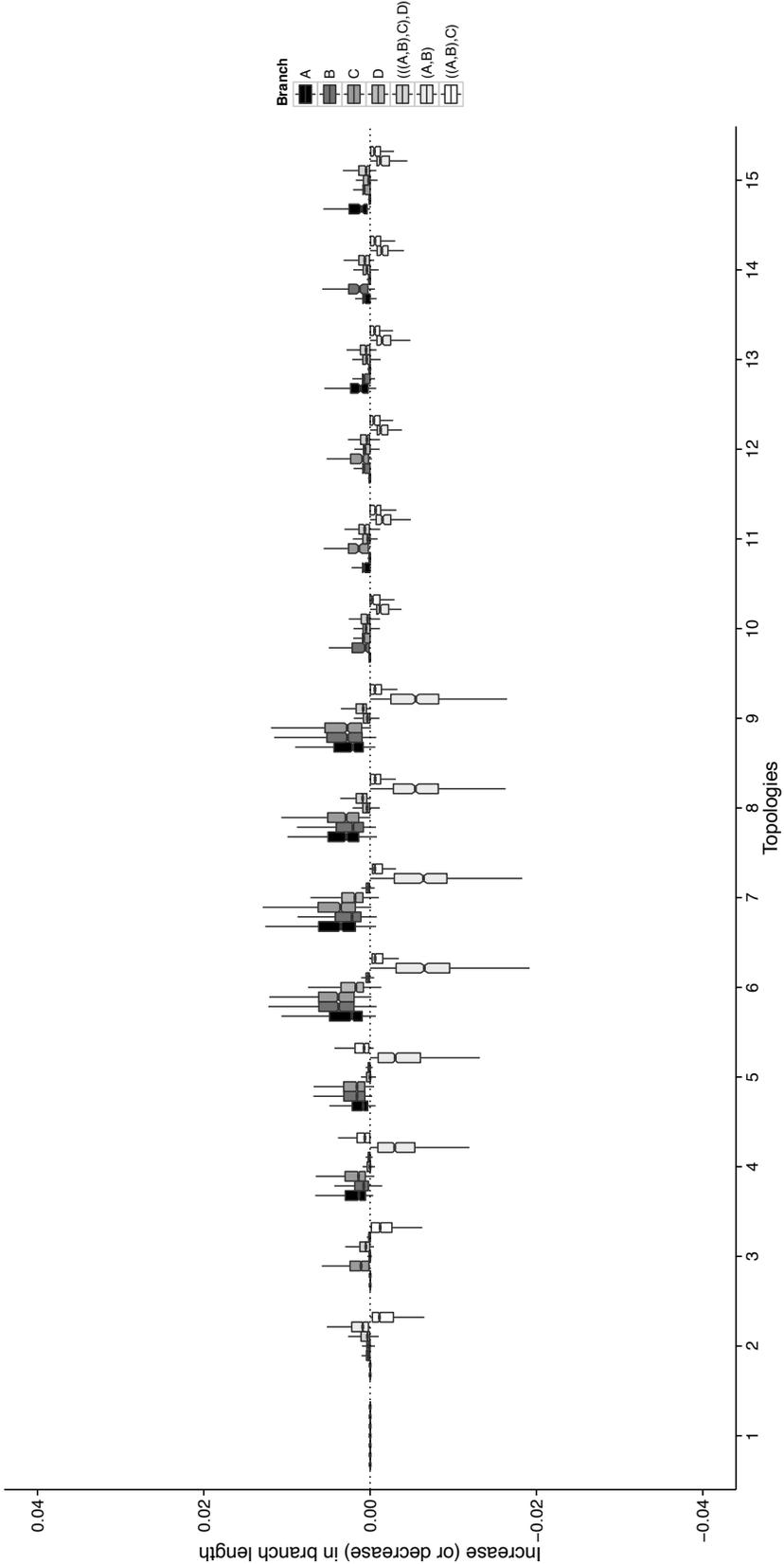
SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4



SUPPLEMENTARY FIGURE 5

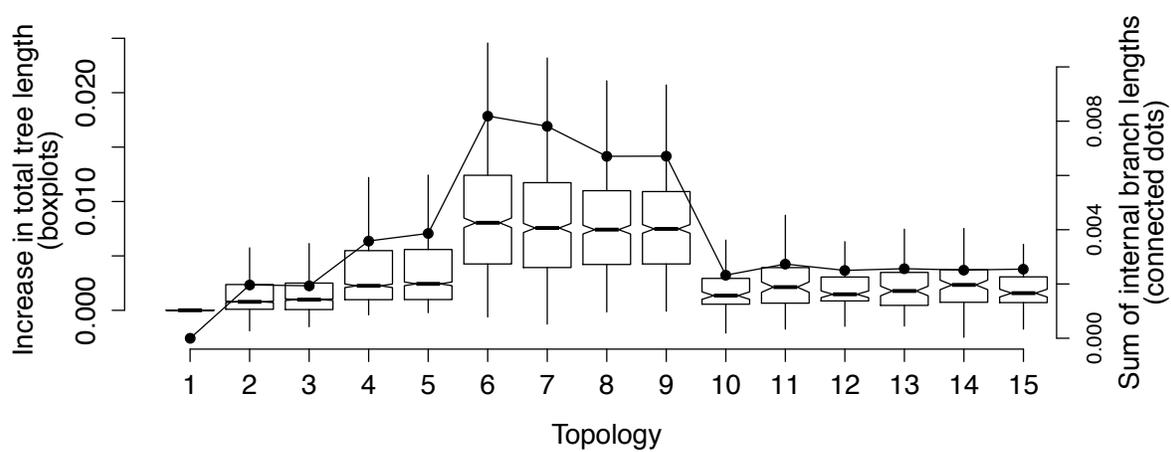


FIGURE CAPTIONS

FIGURE 1. Species tree and two discordant gene trees. The filled cross and filled circle on gene tree
680 1 and 2, respectively, correspond to $0 \rightarrow 1$ substitutions that occurred in two different hypothetical
loci. When both genes are analyzed using the species tree, these substitutions are incorrectly
mapped (hollow symbols) on the branch leading to ((A,B),C) and as reversals on either branch A
or B. As a result, branches A, B, and the branch leading to ((A,B),C) get spuriously longer.

685 FIGURE 2. Mean total tree length increase (calculated as the difference in length of “Fixed”
topologies and “Unfixed” topologies) by ILS treatment, for ILS among ((A,B),C) and
(((A,B),C),D). Based on 10,000 simulated topologies for each treatment.

FIGURE 3. Branch-specific effects of SPILS using simulated data. a) Average species tree branch
690 lengths for ILS among ((A,B),C) and (((A,B),C),D). Wider segments represent the increase in
length due to SPILS, while thinner lines represent the decrease in length due to SPILS. b)
Increase or decrease in branch length by topology for ILS among ((A,B),C).

FIGURE 4. Total tree length increase or decrease ($F - U$) by tree topology (left axis). Number of
695 genes inferred to be under positive selection using codon site models and a likelihood ratio test
(right axis). a) ILS among ((A,B),C). b) ILS among (((A,B),C),D).

FIGURE 5. Branch-specific effects of SPILS using *Drosophila* data. a) Average species tree branch
lengths for ILS among ((Y,E),M). b) Increase or decrease in branch length by topology for
700 *Drosophila* sequences. c) Increase or decrease in branch length by topology for “recombinant”
simulated sequences.

SUPPLEMENTARY FIGURE CAPTIONS

705 SUPPLEMENTARY FIGURE 1. Simulation parameters. The vertical scale shows divergence times in coalescent units. Numbers beside internal branches are factors by which their N_e 's were multiplied in order to produce varying levels of ILS.

SUPPLEMENTARY FIGURE 2. Gene tree topology counts for ILS among a) ((A,B),C), and b) (((A,B),C),D). Topologies are numbered from most to least frequent.

710 SUPPLEMENTARY FIGURE 3. Substitution placement on branches of species tree (left) for each of the 14 possible discordant tree topologies in the presence of ILS (right). Filled symbols correspond to $0 \rightarrow 1$ substitutions that occurred on the discordant genealogy of a locus, hollow symbols represent substitutions incorrectly mapped to the species tree.

715 SUPPLEMENTARY FIGURE 4. Increase or decrease in branch lengths by topology when ILS is present for species tree (((A,B),C),D),E). Branch length differences ($F - U$) come from 10,000 simulated loci.

SUPPLEMENTARY FIGURE 5. Total tree length increase or decrease ($F - U$) by tree topology (left axis).
720 Sum of internal branches on each tree topology not present in species tree (((A,B),C),D),E).