The Impact of Species Tree Estimation Error on Cophylogenetic Reconstruction

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ABSTRACT

Just as a phylogeny encodes the evolutionary relationships among a group of organisms, a cophylogeny represents the coevolutionary relationships among symbiotic partners. Both are primarily reconstructed using computational analysis of biomolecular sequence data. The most widely used cophylogenetic reconstruction methods utilize an important simplifying assumption: species phylogenies for each set of coevolved taxa are required as input and assumed to be correct. Many studies have shown that this assumption is rarely – if ever – satisfied, and the consequences for cophylogenetic studies are poorly understood.

To address this gap, we conduct a comprehensive performance study that quantifies the relationship between species tree estimation error and downstream cophylogenetic estimation accuracy. We study the performance of state-of-the-art methods for cophylogenetic reconstruction using *in silico* model-based simulations. Our investigation also includes assessments of cophylogenetic reproducibility using genomic sequence datasets sampled from two important models of symbiosis: soil-associated fungi and their endosymbiotic bacteria, and bobtail squid and their bioluminescent bacterial symbionts.

Our findings conclusively demonstrate the major impact that upstream phylogenetic estimation error has on downstream cophylogenetic reconstruction quality. Relative to other experimental factors such as cophylogenetic estimation method choice and coevolutionary event costs, phylogenetic estimation error ranked highest in importance based on a random forest-based variable importance assessment. We conclude with practical guidance and future research directions. In particular, among the many considerations needed for accurate cophylogenetic reconstruction – choice of cophylogenetic reconstruction method and method settings, sampling design, and others – just as much attention must be paid to careful species phylogeny estimation using modern best practices.

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CCS CONCEPTS

• **Applied computing** → *Computational genomics*; *Computational biology*; *Molecular sequence analysis*; *Molecular evolution*; *Computational genomics*; *Bioinformatics*; *Population genetics.*

KEYWORDS

cophylogeny, cophylogenetic reconciliation, species tree, simulation study, *Mortierella*, bobtail squid, symbiont, symbiosis

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1 INTRODUCTION

A cophylogeny represents the evolutionary and coevolutionary relationships among multiple sets of coevolved taxa, and cophylogenies are widely used to study fundamental and applied topics throughout biology and the life sciences [4, 26]. For example, untangling coevolutionary histories is essential to reconstructing the web of life [49], as symbiosis and coevolution have played an important role in evolution at different scales – from genes to proteins, biomolecular pathways, organisms, populations, and beyond [22].

As is the case in phylogenetic estimation, cophylogenies are principally reconstructed using computational analyses of biomolecular sequences – increasingly abundant thanks to next-generation biomolecular sequencing technologies [43] – as well as other types of biological data [12]. The most widely used computational approach for cophylogenetic estimation consists of a multi-stage pipeline where: (1) a species tree is independently estimated for each coevolved set of taxa using the same approaches as in a traditional phylogenetic study, and (2) cophylogenetic analysis proceeds using the estimated species trees as input, alongside the known host and symbiont associations (Figure 1).

Many cophylogenetic methods have been developed and they fall into two broad categories. (1) Global-fit methods [4] evaluate overall congruence between host and symbiont tree topologies, and examples include PARAFIT [21], PACo [1], and MRCAlink [42]. (2) Event-based methods perform phylogenetic reconciliation using either parsimony-based optimization or, less commonly, model-based statistical optimization. These optimization problems are known

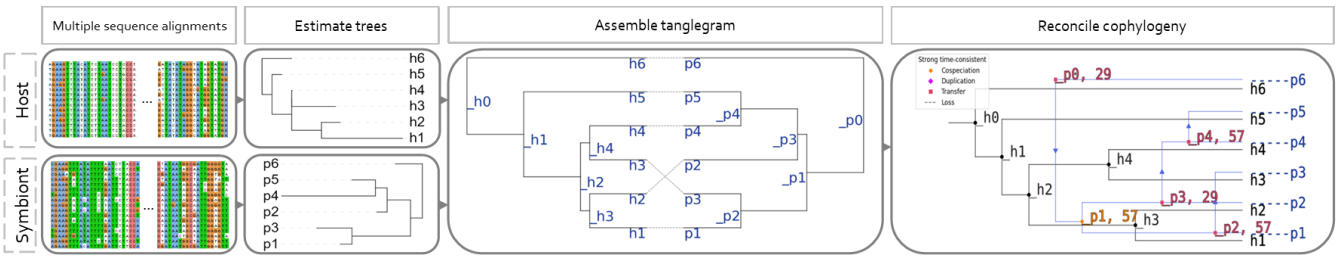


Figure 1: *A typical workflow for cophylogenetic reconstruction.* (1) Biomolecular sequence data for host taxa and symbiont taxa are aligned. (2) A host species tree and symbiont species tree are independently estimated using each multiple sequence alignment as input. (3) The input to cophylogenetic reconstruction consists of the estimated host tree, estimated symbiont tree, and known host/symbiont associations. All three can be visualized using a tanglegram. (4) Finally, a cophylogeny is reconstructed using the two species trees and host/symbiont associations as input. The cophylogeny maps topological structure in the host tree to corresponding topological structure in the symbiont tree based on shared coevolutionary history, where each element in the mapping corresponds to a coevolutionary event (e.g., a cospeciation event, a host shift event, etc.). Example dataset from [14].

to be computationally difficult [32]. Substantial research effort has been devoted to developing scalable and practical algorithms for this problem, which include eMPRess [41], Jane [9], Treemap [7], COALA [2], CoRe-PA [28], and TALE [27]. Event-based methods typically account for multiple types of coevolutionary events [6]: cospeciation (or codivergence or codifferentiation) involving both host and symbiont lineages, duplication of a symbiont lineage within a host lineage, loss of a symbiont lineage within a host lineage, and host shift (or host switch or host transfer) where a symbiont lineage's association transfers to a different host lineage. In this study, we focus on event-based cophylogenetic reconstruction methods to investigate a finer granularity of evolutionary and coevolutionary event reconstructions.

The multi-stage pipeline design requires a critically important assumption: the estimated species trees in the first stage are used directly in the second stage under the assumption that they are correct. However, it is well understood in traditional phylogenetics that many factors can cause phylogenetic estimation methods to return some degree of estimation error, and estimation errors introduced in upstream computational tasks are important factors to consider. For example, numerous studies have investigated the strong impact that upstream multiple sequence alignment error can have on subsequent gene tree estimation [23, 29]. But this insight conflicts with the prevailing assumption made by cophylogenetic reconstruction pipelines, as noted by [4].

The implications of this conflict must be carefully assessed. A rigorous examination of inter-related estimation error across a multi-stage cophylogenetic reconstruction pipeline is needed, and there is a lack of relevant experimental studies today [12]. The high-level findings can be qualitative (e.g., whether major effects occur or if downstream estimation is largely robust to upstream estimation error) or, more usefully, quantitative assessments (e.g., quantification of the relationship between estimation error in different pipeline stages.) Going further, more nuanced implications arise from context dependence (e.g., the extent to which the combination of estimation errors is modulated by evolutionary divergence) and other experimental factors (e.g., dataset size, taxon sampling density, etc.). Finally, these outcomes will point to important practical consequences for systematists and other researchers that study cophylogenies. As with other topics in phylogenetics, recommendations regarding best practices are needed to reconstruct accurate phylogenies and cophylogenies. Any reconstruction errors invite misinterpretation and incorrect conclusions in dependent analyses. For example, incorrectly estimated cophylogenetic relationships among a set of model organisms may yield spurious conclusions about the evolutionary processes under study (e.g., the relative frequency and significance of different coevolutionary processes). Another practical matter is empirical study design. Ideally, computational resources and research effort should concentrate on computational and experimental bottlenecks. Clarification of the above questions will help illuminate whether some, all, or none of traditional cophylogeny reconstruction pipelines merit careful consideration relative to other aspects of study design (e.g., taxon sampling, sequencing technology, sequencing effort, etc.).

To address this gap, we have undertaken a study to examine the relationship between upstream phylogenetic estimation error and downstream cophylogeny reconstruction accuracy. Our performance study utilizes both simulated and empirical datasets that span a range of dataset sizes, evolutionary divergence, and evolutionary scenarios.

2 METHODS

Our performance study included a comprehensive suite of simulated benchmarking datasets that spanned a range of evolutionary conditions. The simulation conditions differed in terms of number of taxa, sequence length, evolutionary divergence, and distribution of coevolutionary event types.

The simulation study experiments utilized two different procedures to simulate synthetic datasets (Supplementary Figure S1). First, the "mixed" simulations utilized an empirically estimated cophylogeny and its constituent species trees and host/symbiont associations as the models for *in silico* simulation of biomolecular sequence evolution. Second, a fully *in silico* set of simulations were

run using the forward-time cophylogeny model proposed by [13], which we refer to as the "forward" simulations. Cophylogenetic and phylogenetic method performance on each simulated dataset was then assessed with respect to model/reference cophylogenies and phylogenies.

We also performed comparative analyses of two empirical genomic sequence datasets. One empirical dataset consists of cephalopod hosts and their bacterial symbionts, which serve as a well-studied model of open symbiosis (i.e., partnerships arising from horizontal transmission between hosts and/or the environment); the other dataset was sampled from fungal hosts and their bacterial endosymbionts, which are an emerging model of closed symbiosis (i.e., partnerships whose coevolution involves strictly vertical descent over time). The two systems thus provide a comparative contrast along a spectrum of symbiotic partnership flexibility [34].

The combination of experimental approaches is a design choice in our study. Taken together, the simulation study and empirical dataset experiments represent an array of natural symbiotic systems – by design and by definition, respectively. Some differences between the experimental approaches are worth noting. The forward simulations provide ground truth coevolutionary histories that enable the analysis of cophylogenetic reconciliation accuracy, whereas the mixed simulation experiments use an estimated cophylogeny reconciliation as reference to analyze cophylogenetic reconciliation precision in the context of phylogenetic inference error. On the other hand, our study uses empirical datasets to assess cophylogenetic reconciliation reproducibility without prior knowledge of the true coevolutionary history.

2.1 Definitions

We now introduce mathematical background needed to describe the experimental procedures. Some of the notation and definitions follow [50].

A rooted phylogenetic tree $T_{\mathcal{N}} = (V_{\mathcal{N}}, E_{\mathcal{N}})$ is a rooted evolutionary history for a set of taxa $\mathcal{N}$. We note that many cophylogenetic reconstruction algorithms require rooted binary phylogenetic trees as input. The rooted binary tree $T_{\mathcal{N}}$ has a root ρ with in-degree zero and out-degree two, leaves $\mathcal{N} \subseteq V_{\mathcal{N}}$ where each leaf has out-degree zero and in-degree one, and inner nodes $v \in V_{\mathcal{N}} \backslash \mathcal{N}$ where each inner node has out-degree two and in-degree one. For each directed edge $(u, v) \in E_{\mathcal{N}}$, v is a child of u. Each edge is also denoted by e_v with branch length $bl(e_v) \in \mathbb{R}^+$. For vertices $u, v \in V_n$, u is an ancestor of v, $u \in anc(v)$, v is a descendent of u, and $u \in desc(v)$ if and only if u lies on the unique path from root ρ to v.

For a pair of rooted phylogenetic trees T_H and T_S denoting the evolutionary history of a set H of hosts and a set S of symbionts, respectively, T_H is the host tree and T_S is the symbiont tree. A mapping function $\phi(s, h) : S \times H \rightarrow \{0, 1\}$ denotes known interactions between the extant species of T_H and T_S, where $\phi(s, h) = 1$ means a symbiont is associated with a host, and otherwise $\phi(s, h) = 0$. The tuple (T_H, T_S, ϕ) serves as the input to cophylogenetic methods, and can be nicely visualized using a tanglegram. A cophylogenetic reconciliation or reconstruction is defined as the set of event associations $\mathcal{R} \subset V_S \times V_H$ between the internal nodes of the symbiont tree T_S and the internal nodes of the host tree T_S. For a symbiont s, an event association $(s, h) \in \mathcal{R}$ means h is one of the host species known to have been associated with s.

The unrooted version $U_{\mathcal{N}}$ of a rooted phylogenetic tree $T_{\mathcal{N}}$ can be obtained by converting all directed edges into undirected edges, deleting the root, and connecting its two outgoing edges into a single remaining edge. Equivalently, an unrooted binary tree $U_{\mathcal{N}}$ on the leaf set $\mathcal{N}$ has internal nodes with degree three and leaves with degree one, and each leaf represents a distinct taxon in the taxon set $\mathcal{N}$.

In our study, tree topology differences were evaluated with normalized Robinson-Fould (nRF) distances [38]. For two unrooted trees U_1 and U_2 with the same set of leaf nodes $\mathcal{N}$ and having bipartition sets B_1 and B_2 respectively, the Robinson-Fould (RF) metric is the cardinality of the symmetric difference between the sets of bipartitions that appear in U_1 and U_2, which is $|B_1 - B_2| + |B_2 - B_1|$. (Note that bipartitions corresponding to leaf edges are trivial since the latter must always appear, and trivial bipartitions do not contribute meaningfully to the RF calculation.) The normalized RF distance is calculated by dividing RF distance by the maximum RF distance between two trees with $|\mathcal{N}|$ taxa, which is $\frac{|B_1-B_2|+|B_2-B_1|}{2|\mathcal{N}|-6}$. We note that the RF distance is a de facto standard for topological comparisons of phylogenetic trees involving the same set of taxa. Generalizations of the RF distance have been proposed for comparing phylogenetic trees with overlapping but non-identical sets of taxa (e.g., [24]), although we note that the issue does not arise in the context of our study due to the nature of our simulation and empirical dataset analysis procedures.

Reconciled cophylogenies were compared based on the calculation proposed by [50], which we refer to as cophylogenetic precision. We now define this calculation. Let $\mathcal{R}_A$ and $\mathcal{R}_B$ be the reconstructed event associations of all internal vertices from cophylogenetic reconciliations A and B, respectively. Then, the proportion of reconciled events in $\mathcal{R}_A$ that were also found in $\mathcal{R}_B$ is $\frac{|\mathcal{R}_B \cap \mathcal{R}_A|}{|\mathcal{R}_A|}$. Cophylogenetic precision factors in all coevolutionary event types that are accounted for by the cophylogenetic reconstruction methods in this study – i.e., cospeciation, duplication, loss, and host switch events.

2.2 Simulation study

Mixed simulations. The mixed simulations utilized empirically-based phylogenetic estimates to perform parametric sampling of synthetic biomolecular sequence data. The simulation procedure begins with the former: obtaining a pair of species trees and cophylogeny via empirical dataset analysis. Six empirical datasets were obtained from literature to sample a range of evolutionary scenarios and dataset types: from single-locus datasets with sequence length under 1 kb to next-generation-sequencing (NGS) multi-locus datasets with sequence length well over 1 Mb (Table 1). The sequence data were preprocessed and aligned using MAFFT v7.221 with default settings [19]. Species phylogenies were reconstructed from concatenated multiple sequence alignments under the General Time Reversible (GTR) model of nucleotide substitution with Γ model of rate heterogeneity [51] and midpoint rooted using RAxML v8.2.12 [45]. As methods eMPRess [41] and COALA [2] were limited to one-to-one host/symbiont associations; symbiont

taxa were subsampled as needed to address this limitation. Cophylogenetic events were estimated with eMPRess [41] from the host and symbiont phylogenies and host-symbiont associations.

Next, the resulting phylogenetic estimates for each empirical dataset served as the statistical model for downstream *in silico* simulation of biomolecular sequence data. Specifically, the reconstructed species trees (including branch lengths) and associated substitution model parameter estimates served as generative models from which multiple sequence alignments were simulated using Seq-Gen [37]; accordingly, we refer to the species trees as model trees. Note that the above cophylogeny was used for assessing methodological performance (see "Phylogenetic and cophylogenetic reconstruction and assessment" below) but was not directly used for simulations.

As noted above, our study is motivated by more nuanced questions beyond establishing the impact of upstream phylogenetic estimation error on downstream cophylogeny reconstruction. We also investigated how this relationship is modulated by two key contextual factors – the evolutionary divergence and number of taxa under study – via two additional simulation experiments. In simulations with varying evolutionary divergence, model tree branch lengths were multiplied by a scaling parameter h. We explored a range of settings for the parameter h where each set of experiments selected a setting from the set $\{0.1, 0.5, 1, 2, 5, 10\}$. The simulations with varying dataset size were conducted by modifying alignment lengths (as listed in Table 1) to 400,228 bp and 1,455,978 bp for host and symbiont, respectively. The modified lengths were adapted from the concatenated MSA lengths of the avian host dataset [35] and the avian feather lice parasite dataset [11].

Forward simulations. The forward simulations utilized the R-based [36] implementation of the Treeducken [13] version 1.1.0 software and its forward-time coalescent model to sample a model cophylogeny, along with its associated species trees and host/symbiont associations. The model cophylogeny and model trees served as the reference cophylogeny and reference trees, respectively, during subsequent performance assessments (see "Performance assessments" below). Model parameter settings (Table 2) were based on estimates from selected empirical datasets. The resulting five model conditions included a range of dataset sizes (i.e., number of taxa and sequence length), substitution rates, base frequency distributions, and coevolutionary event distributions (Table 3). Model trees were deviated away from ultrametricity using Moret et al. [30]'s approach with deviation factor $c = 2.0$ [31]. We used custom scripts to perform the ultrametricity deviation calculations. Sequence evolution was then simulated on each model tree using the same approach as in the mixed simulation procedure, resulting in host and symbiont MSAs.

Additional experiments varying evolutionary divergence were performed with the forward simulation procedure, where the scaling parameter h was assigned a value from $\{0.1, 0.5, 1, 2, 5, 10\}$.

Experimental replication. For each model condition, the procedure to simulate biomolecular sequence evolution was repeated to obtain 100 replicate datasets. Results are reported across all replicate datasets in each model condition.

Phylogenetic and cophylogenetic reconstruction and assessment. On each simulated dataset, phylogenetic trees were reconstructed under the GTR+Γ model and midpoint rooted using RAxML v8.2.12. The resulting phylogenetic estimates and host/symbiont associations were used by eMPRess [41] to perform cophylogenetic reconciliation using default settings. We also conducted additional eMPRess analyses using alternative cophylogenetic event costs that were estimated using COALA [2] and CoRe-PA [28]; the additional estimated cophylogenies were used in the random forest-based variable importance analyses described below (and additional experiments in the Supplementary Online Materials). (Also see Supplementary Online Materials section S7 for an additional experiment that uses TALE to perform statistical cophylogenetic reconstruction).

In each simulation study experiment, the topological error of an estimated tree was compared to its corresponding model tree based on nRF distance. Each estimated cophylogeny was compared to the reference cophylogeny based on [50]'s precision calculation. Scatterplots and linear regression analysis were used to characterize the relationship between upstream phylogenetic estimation error and downstream cophylogenetic reconstruction error, where phylogenetic estimation error was assessed based on average topological error of host and symbiont trees, and cophylogenetic reconstruction error was assessed using cophylogenetic precision. The linear regression analyses were performed using R version 4.2.2 [36].

Variable importance analysis. In mixed and forward simulation experiments, the relative importance of species tree topology and other factors that can impact cophylogenetic reconciliation accuracy was assessed using the randomForest package [10] implemented in R [36]. The following variables were assessed for their impact on cophylogenetic reconciliation: tree topology (true species trees versus reconstructed trees in nRF distance), cophylogenetic software (eMPRess versus CoRe-PA), dataset size (default versus modified alignment lengths), event cost parameter (default versus alternative), and evolutionary divergence (tree height scaling factor $h = 0.1$ versus 10).

Random forests are used in machine learning to perform regression, classification, and other statistical analyses. To evaluate the relative importance of each variable, the out-of-bag (OOB) data for the tested variable was randomly shuffled and then this shuffled OOB data was used to construct 1000 regression trees. The original OOB data was used to construct another 1000 regression trees. On each regression tree, a mean squared error (MSE) is calculated based on the regression tree's prediction error rate. The variable importance is the difference in MSE between the random forest constructed on original OOB values and the random forest constructed on the shuffled OOB values, divided by standard error [10]. We scaled the importance of each factor to the most important variable to generate partial dependence plots.

2.3 Empirical study of soil-associated fungi and their bacterial endosymbionts

Sample acquisition and sequencing. A total of 13 metagenomic samples of *Mortierella spp.* and their associated endobacteria were collected and sequenced. Next-generation sequencing reads were assembled into contigs, which were then used to call single-nucleotide polymorphism variants (SNVs). The SNV MSAs for fungi and their bacterial endosymbionts had total length of 4,607,802 bp and 215,165 bp, respectively.

Model conditions	Source	Taxa	# taxa	Aln length	ANHD Avg	ANHD SE	Height Avg	Height SE	# cospec	# dup	# switch	# loss
mixed-gopher	[14]	Host	15	379	0.2241	0.0007	0.4024	0.0042	8	0	8	2
		Symbiont	17	379	0.5249	0.0007	3.0598	0.0359				
mixed-stinkbug	[17]	Host	7	1,745	0.2371	0.0016	0.2651	0.0016	5	5	1	0
		Symbiont	12	1,583	0.0661	0.0006	0.1349	0.0011				
mixed-primate	[46]	Host	55	696	0.2599	0.0002	0.6079	0.0046	24	0	14	22
		Symbiont	41	425	0.3376	0.0004	0.8169	0.0050				
mixed-damselfly	[25]	Host	24	1,051	0.1734	0.0004	0.4919	0.0036	4	3	15	4
		Symbiont	23	3,297	0.1327	0.0004	0.2643	0.0010				
mixed-moth	[52]	Host	82	1,404	0.1021	0.0001	0.2147	0.0013	13	0	27	12
		Symbiont	53	4,326	0.0250	0.0000	0.0486	0.0003				
mixed-bird	[35] [11]	Host	37	5,000	0.1087	0.0001	0.1526	0.0009	15	12	29	17
		Symbiont	57	5,000	0.3562	0.0001	0.5459	0.0011				

Table 1: *Summary statistics for mixed simulation datasets.* Each mixed simulation condition ("Model conditions") is based on a previously published cophylogenetic study ("Source"). For each dataset type (either host or symbiont, as denoted by "Taxa"), the number of taxa ("# taxa"), true MSA length ("Aln length"), average and standard error of normalized Hamming distance of true MSAs ("ANHD Avg" and "ANHD SE", respectively), and average and standard error of model tree height ("Height Avg" and "Height SE", respectively) are reported. The number of cospeciation, duplication, host switch, and loss events in the reference cophylogeny are reported as "# cospec, "# dup", "# switch", and "# loss", respectively.

Model condition	H_{tips}	S_{tips}	λ_H	λ_C	λ_S	μ_H	μ_S	time
forward-gopher	35	55	0.3104	1.2000	0.0290	0	0	2.2
forward-stinkbug	35	55	0.2104	1.2000	0.0290	0	0	2.0
forward-primate	203	50	0.3374	0.6246	0.0452	0	0	4.8
forward-damselfly	25	25	0.1843	0.8846	0.2920	0	0	2.0
forward-bird	27	134	0.0544	0.6000	0.4520	0	0	4.0

Table 2: *Treeducken parameters used in forward simulations.* Treeducken was used to simulate cophylogenies and their constituent species phylogenies under a forward-time coalescent-based model [13]. Treeducken's model specifies the following parameters: the symbiont speciation rate λ_S, the symbiont extinction rate μ_S, the cospeciation rate λ_C, the host speciation rate λ_H, the host extinction rate μ_H, the expected number of host taxa H_{tips}, and the expected number of symbiont taxa S_{tips}.

Reconstruction and comparison of phylogenies and cophylogenies. Maximum likelihood tree estimation was performed using RAxML v8.2.12 [45] under finite-sites models of nucleotide sequence evolution. The latter consisted of the GTR+Γ [48] and nested models – specifically the HKY [15], K80 [20], and Jukes-Cantor [18] models; these substitution models span a range of model complexity from simplest (in the case of Jukes-Cantor) to more complex (i.e., GTR, HKY, and K80). PAUP* [47] was used to conduct additional phylogenetic reconstructions using neighbor-joining (NJ) [39] and the unweighted pair group method with arithmetic mean (UPGMA) algorithms [44]. Multispecies coalescent model-based species tree reconstruction was performed using SVDquartet [8]. If SVDquartet produced a tree with polytomies, the matrix rank was set to 1, 4, and 5 to produce three different tree topologies. Reconstructed phylogenetic trees were midpoint rooted. Finally, the estimated phylogenetic trees were reconciled to obtain a cophylogeny using either CoRe-PA [28] or eMPRess [41].

For each dataset, phylogenetic and cophylogenetic estimates obtained using any phylogenetic estimation method and eMPRess, respectively, were compared on a pairwise basis using the calculations described below; CoRe-PA-based results were evaluated similarly. For each pairwise comparison, phylogenetic tree estimation agreement was assessed using the average of the nRF distance between the two host trees and the nRF distance between the two symbiont trees. Then, for each pairwise comparison, cophylogenetic estimation agreement was assessed using the precision of [50]. Linear regression analyses were also performed to assess the relationship between phylogenetic tree estimation agreement and cophylogenetic estimation agreement, using the same procedures as in the simulation study experiments.

2.4 Empirical study of bobtail squids and their symbiotic bioluminescent bacteria

Sample acquisition and sequencing. Genomic sequence data for 22 samples of bobtail squids from the study of Sanchez et al. [40], and metadata for 37 *Vibrio* samples from the study of Bongrand et al. [5] were downloaded. The concatenated squid MSA had total length of 37,512 bp. Sanchez et al. [40] sequenced the former via genome skimming to identify more than 5000 ultraconserved loci. Host-symbiont association data came from the study of Bongrand et al. [5].

Reconstruction and comparison of phylogenies and cophylogenies. We reconstructed a phylogenetic tree for host taxa using the same approach as in the fungal/endobacterial dataset analysis. The bacterial symbiont phylogeny consisted of the *Vibrio* phylogeny reported by Bongrand et al. [5]. Cophylogenetic reconciliation and comparison of estimated phylogenies and cophylogenies followed the same procedures as in the other empirical dataset analysis.

3 RESULTS

3.1 Simulation study

The impact of upstream phylogenetic estimation error on downstream cophylogenetic reconciliation accuracy. Across the mixed simulation conditions, phylogenetic tree estimation returned average

Model conditions	Source	Taxa	# taxa	Aln len	ANHD Avg	ANHD SE	Height Avg	Height SE	# cosp	# dup	# switch	# loss
forward-gopher	[14]	Host	17	300	0.5664	0.0010	2.3260	0.0313	16	0	1	0
		Symbiont	16	300	0.5426	0.0009	2.5639	0.0403				
forward-stinkbug	[17]	Host	16	1,000	0.5672	0.0012	4.2617	0.0707	14	0	2	0
		Symbiont	14	1,000	0.5825	0.0016	3.9159	0.0326				
forward-primate	[46]	Host	48	400	0.6030	0.0002	8.0586	0.0791	31	3	17	0
		Symbiont	34	400	0.7017	0.0004	10.7577	0.2931				
forward-damselfly	[25]	Host	24	1,000	0.3437	0.0003	0.5804	0.0031	12	9	12	0
		Symbiont	21	1,000	0.4233	0.0007	1.1334	0.0066				
forward-bird	[35] [11]	Host	31	5,000	0.6953	0.0004	4.1329	0.0023	21	33	10	0
		Symbiont	54	5,000	0.7125	0.0002	5.0964	0.0027				

Table 3: *Summary statistics for forward simulation datasets.* For each model condition ("Model conditions"), Treeducken was used to perform forward simulations based on a previously published cophylogenetic study ("Source"). Each simulated dataset consisted of a model cophylogeny, its constituent model species trees and host/symbiont associations, and true MSAs. Table layout and description are otherwise identical to Table 1.

topological error of 7% and cophylogenetic reconstruction returned average precision of 66%. (Supplementary Figure S2 reports average topological errors of estimated species trees and cophylogenies for each model condition.)

Random forest-based variable importance analyses confirmed that tree topology inference error was the most important contributor to cophylogenetic reconciliation accuracy, while the second most important was evolutionary divergence at 70% of the variable importance of tree topology (Table 4). In our experiments, the choice of cophylogenetic reconciliation software and the choice of default versus statistically estimated event cost vectors contributed the least to cophylogenetic reconciliation accuracy.

The relationship between phylogenetic and cophylogenetic estimation error was examined using linear regression: Figure 2a shows the regression models fitted to observed topological errors across replicate datasets in each model condition. The regression analyses were statistically significant in all cases ($\alpha = 0.05$; $n = 100$), as shown in Supplementary Table S1. Increasing topological error during upstream estimation was clearly associated with reduced cophylogenetic accuracy, as evidenced by consistently negative regression coefficients and average regression coefficient of -1.96 across model conditions. We also observed varying scatter around fitted models: the coefficient of determination was highest in the mixed-gopher, mixed-stinkbug, and mixed-primate model conditions – ranging between 0.47 and 0.89 – and lower in others.

As in the mixed simulations, the partial dependence scores from random forest-based variable importance analysis showed that tree topology inference error was the most important contributor to cophylogenetic reconciliation accuracy in forward simulations, with evolutionary divergence having 82% of the relative importance of tree topology (Table 4). Similar to mixed simulations, the choice of cophylogenetic reconciliation software and the choice of default versus statistically estimated event cost vectors contributed the least to cophylogenetic reconciliation accuracy in forward simulation experiments. Topological error of estimated phylogenies and cophylogenies varied among forward simulation conditions. The observation is due in part to heterogeneity among the empirical estimates that served as the basis for the forward simulation conditions. On the other hand, topological errors were somewhat higher than in the other simulation experiments: the forward simulation experiments returned average tree topology error of 13% and average cophylogenetic precision of 35% (Supplementary Figure S4). As shown in Figure 2b, correlation between upstream tree estimation error and downstream cophylogeny reconstruction precision yielded similar findings as in the rest of simulation study. We observed significant and negative correlation in all forward simulation conditions (Supplementary Table S2). Furthermore, the coefficient of determination varied across forward simulation conditions in a similar pattern to the mixed simulation conditions, based on shared empirical dataset estimates. The largest values were seen on forward-gopher, forward-stinkbug, and forward-primate model conditions – ranging between 0.585 and 0.744; smaller values were seen on the other model conditions.

The impact of evolutionary divergence on the relationship between phylogenetic and cophylogenetic reconstruction accuracy. For each set of forward simulation conditions (Figure 3b), we found that phylogenetic and cophylogenetic estimation error was negatively and significantly correlated as the tree height parameter h varied between 0.1 and 10. Regression analysis returned regression coefficients between -0.899 and -0.220, and coefficients of determination between 0.668 and 0.222 (Supplementary Table S4). Both upstream and downstream topological error was lowest for the smallest h settings (i.e., 0.1, 0.5, and 1.0). As the height h increased, both topological errors increased in tandem, and both were largest on simulations with height $h = 10$. The latter was likely at saturation, as topological errors tended to be maximal. Similar outcomes were observed in the corresponding mixed simulation experiments with varying tree height h, as shown in Figure 3a. The effect of increasing h on topological error was more complicated and non-linear in some cases. This was in part due to heterogeneity of empirical estimates used for parametric resampling, unlike the fully *in silico* simulations used elsewhere in the simulation study.

3.2 Empirical study

Soil-associated fungi and their bacterial endosymbionts. Topological disagreements among estimated phylogenies were higher than in the simulation study (Supplementary Figure S5); a similar outcome was observed among estimated cophylogenies. This is by

Simulations	Tree topology	Evolutionary divergence	Dataset size	Cophylogenetic software	Event cost parameter
Mixed	1.0000	0.7029	0.5511	0.3513	0.0611
Forward-time	1.0000	0.8160	N/A	0.7786	0.3144

Table 4: *Simulation study: variable importance assessment for mixed and forward simulations.* A random forest model was used to determine the mean importance of each variable. Results are reported as an average across all mixed simulation conditions and scaled relative to the most importance variable ($n = 100$), and similarly for the forward simulation conditions.

design: the empirical study utilized a wide array of phylogenetic reconstruction methods with varying estimation accuracy. The design choice provides an indirect means to vary the topological accuracy of input phylogenies and then observe its effects on downstream cophylogenetic estimation, in contrast to the direct control and model/reference comparisons enabled by *in silico* simulations. We analyzed the relationship between phylogenetic and cophylogenetic estimation error using linear regression (Figure 4a). Consistent with the simulation study, we observed that greater topological agreement in the former set of inputs was significantly associated with greater topological agreement of the latter output, as assessed using an F-test with Benjamini-Hochberg [3] correction for multiple tests ($\alpha = 0.05$; $n = 114$). The full assembly dataset analysis returned a regression coefficient of -2.067 and coefficient of determination of 0.672, which is also in line with the simulation study.

Bobtail squids and their symbiotic bioluminescent bacteria Topological disagreements among species cophylogenies and resulting cophylogenetic reconciliations were somewhat smaller than those observed on the fungal/endosymbiont dataset (Supplementary Figure S6). Another key difference concerns host/symbiont associations: relatively few squid hosts were associated with most bacterial symbionts. Still, we observed a similar relationship between upstream phylogenetic estimation agreement and downstream cophylogeny precision (Figure 4b). Linear regression analyses returned significant and negative correlation ($\alpha = 0.05$; $n = 216$), along with a regression coefficient of -0.449, intercept of 0.841, F-test p-value $< 10^{-12}$, coefficient of determination of 0.213, and residual standard error of 0.109.

4 DISCUSSION

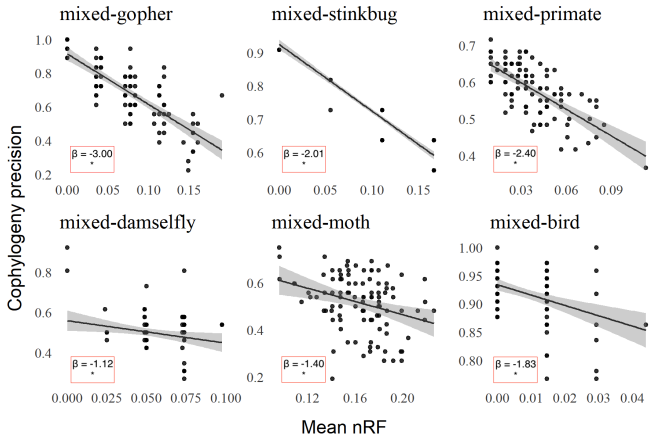
Across all forward simulation experiments, correlation between upstream phylogenetic estimation error and downstream cophylogenetic estimation accuracy was significant and consistently negative. As the former increased, the latter would degrade. The mixed simulation experiments and empirical dataset analyses involving eMPRess-estimated cophylogenies (as well as a supplementary simulation experiment involving TALE, as described in the Supplementary Online Materials) also returned a consistent outcome: namely, a significant and negatively correlated relationship between upstream phylogenetic reconstruction error and downstream cophylogenetic estimation reproducibility. The expanded simulation experiments that focused on varying evolutionary divergence (while fixing other experimental factors) refined our study's primary finding and demonstrated that evolutionary divergence plays a key role in modulating upstream and downstream estimation error in tandem. Of course, other factors also play a role (e.g., taxon sampling, choice of phylogenetic and cophylogenetic reconstruction method(s), coevolutionary event distribution, evolutionary and coevolutionary model mis-specification, etc.), and the relationship between phylogenetic and cophylogenetic reconstruction is complex [12]. Heterogeneity among simulation conditions due to these factors helps to explain some of the more minor differences among experimental outcomes. Nevertheless, our primary finding – that phylogenetic estimation error strongly impacts downstream cophylogenetic reconciliation accuracy – was robust to these factors. Furthermore, variable importance analyses revealed that phylogenetic tree estimation error was the most important experiment factor associated with cophylogenetic reconciliation accuracy, compared to the other factors.

We note a key difference between the simulation study and the empirical study. A primary advantage of the former is the ability to benchmark against model/reference phylogenies and cophylogenies. But the latter is inherently more complex and nuanced than the former. For example, the two systems in our empirical study are models sampled along a continuum of symbiotic coevolution modes [34]: from open – as in the case of bobtail squids and their bioluminescent symbionts [34] – to mixed to closed – as in the case of early diverging fungi and their endosymbionts [33]. Where a system exists along this continuum is thought to strongly influence the probabilities of different coevolutionary events: for example, host shifts occur more frequently in an open system, and cospeciation predominates in a closed system. Depending on the taxa under study, it is plausible that symbiotic coevolution may switch between different modes along a phylogeny (e.g., from closed to mixed). But we are not aware of any suitable non-homogeneous cophylogenetic models and we also lack a basic understanding of their theoretical properties (e.g., statistical identifiability). The gap between natural symbiotic coevolution and current statistical cophylogenetic modeling represents an opportunity for advanced model and methods development; for now, this study is constrained by the limitations of the state of the art.

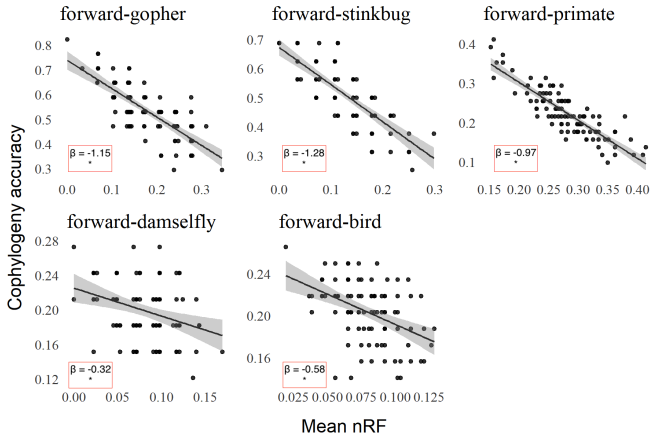
5 CONCLUSIONS

This study demonstrated the major effect that phylogenetic estimation error has on downstream cophylogenetic reconstruction accuracy. The finding was consistently observed throughout the simulation study experiments. Empirical analyses of two genomic sequence datasets for models of symbiosis also revealed that variable phylogenetic tree estimation quality decreased reproducibility of cophylogenetic estimation.

We propose the following strategies to put the key findings of our study into practice. One ideal solution would be to develop and utilize a new generation of cophylogenetic reconstruction methods

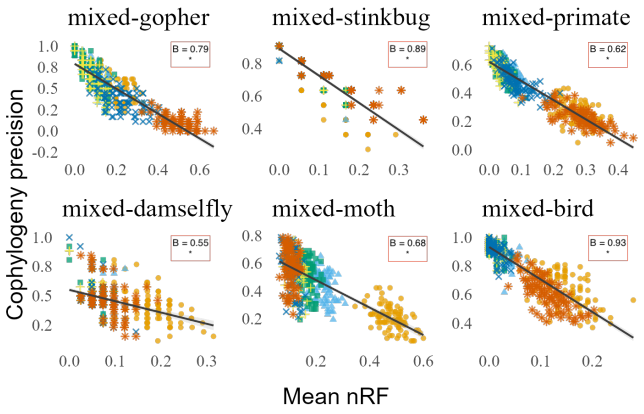


(a) Mixed simulation conditions.

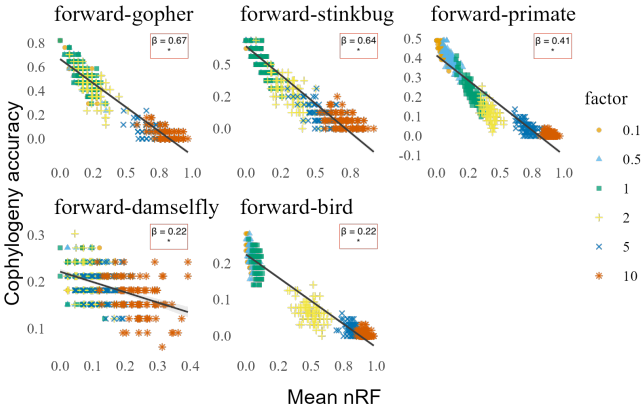


(b) Forward simulation conditions.

Figure 2: *Simulation study: the relationship between phylogenetic and cophylogenetic estimation error.* For each model condition, the topological error returned by phylogenetic tree estimation (averaged across the pair of host and symbiont datasets) and the precision returned by cophylogenetic reconstruction are shown for each replicate dataset ($n = 100$). A fitted linear regression model is shown for each model condition as well, and the 95% confidence interval is shown in grey around the regression line. A red box inside each plot shows the regression coefficient β and an asterisk (*) denoting statistical significance ($\alpha = 0.05$; $n = 100$) using an F-test with Benjamini-Hochberg multiple test correction [3]. The linear regression analyses were statistically significant in all cases. (Supplementary Tables S1 and S2 provide additional regression analysis results.)



(a) Mixed simulation experiments.



(b) Forward simulation experiments.

Figure 3: *Simulation study: the impact of evolutionary divergence on phylogenetic and cophylogenetic estimation error.* Estimation error was assessed based upon average topological error of estimated trees (averaged across the pair of host and symbiont datasets) and cophylogenetic precision. Model tree branch lengths were scaled by height parameter h ("factor"); data points for a given setting of h are distinguished by a distinct color. A fitted linear regression model is shown for each simulation condition. A red box inside each plot shows the regression coefficient β and an asterisk (*) denoting statistical significance ($\alpha = 0.05$; $n = 600$) using an F-test with Benjamini-Hochberg multiple test correction [3]. The linear regression analyses were statistically significant in all cases. (Supplementary Tables S3 and S4 provide additional regression analysis results.)

that account for upstream phylogenetic estimation error and perform statistical inference and learning directly from biomolecular sequence data inputs. To our knowledge, the choices are very limited for now. We are aware of one option that represents a partial first step towards this goal: a new method called TALE [27] which accepts distributions of symbiont species trees and gene trees as input, but only a point estimate for the host species tree (as of this writing). However, given the outcome of a supplementary experiment involving TALE as well as other considerations regarding TALE's design (see Supplementary Online Materials section S7), our study underscores the need for continued research, modeling, and computational methods development in this direction. By far the most widely used options for cophylogenetic reconstruction remain the current generation of methods which require fixed species trees

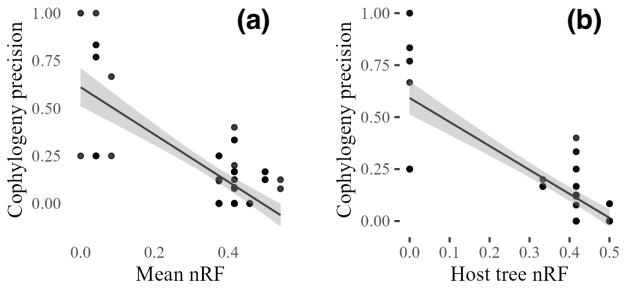


Figure 4: *Empirical study: topological discordance among phylogenetic and cophylogenetic estimates.* The scatterplots show topological discordance between each pair of different phylogenetic tree estimation methods – averaged across the host dataset and symbiont dataset – versus disagreement among the resulting cophylogenetic reconciliations produced using either eMPRess or CoRe-PA. (a) In the fungal dataset, a range of different phylogenetic tree estimation methods were used to estimate phylogenetic trees on the host dataset and the symbiont dataset. Along with the known host/symbiont associations, each estimated host tree and symbiont tree pair returned by a given phylogenetic tree estimation method was reconciled into a cophylogeny using either eMPRess or CoRe-PA. Topological discordance between different host trees estimated by different methods was assessed on a pairwise basis using nRF distance. Disagreement among cophylogenies estimated using eMPRess reconciliation of different phylogenetic tree estimates was assessed on a pairwise basis using cophylogenetic precision; disagreement among CoRe-PA estimates was evaluated similarly. A fitted linear regression model is shown ($n = 114$). (b) The squid dataset analyses used a symbiont tree that was fixed to the estimate of [40] and $n = 216$; analyses and results are otherwise reported similarly to the fungal dataset.

as input. In lieu of an ideal solution, we provide the following practical guidance as temporary workarounds. First, we propose that researchers adopt more intensive species tree reconstruction as best practices in a cophylogenetic study. For example, we recommend that researchers select more intensive local optimization heuristic settings for addressing the computationally difficult tree reconstruction problems in this study and in the state of the art. Second, more intensive sequencing effort to obtain additional high-quality biomolecular sequence data can also help, assuming that suitable methods can be used to account for the complex interplay of evolutionary processes – substitutions, sequence insertion and deletion, genetic drift and incomplete lineage sorting, and more – that arises in this setting. A new generation of phylogenomic inference and learning methods are now used to better address species phylogeny reconstruction using large-scale multi-locus and/or genomic sequence data, and they may also pay dividends when reconstructing cophylogenies using genomic sequence analysis.

We conclude with thoughts on future research directions. First, we have already mentioned the need for richer coevolutionary models. Our study's empirical models of open symbiosis (Hawaiian bobtail squid and its bioluminescent bacterial symbiont) and closed symbiosis (soil-associated fungi and its bacterial endosymbionts) bookend a rich spectrum of symbiotic lifestyles and coevolution modes. Richer statistical models are urgently needed to better account for the dynamic interplay of different coevolutionary processes that can shift over time. Second, new methods that jointly reconstruct species trees, gene trees, and a cophylogeny from multi-locus sequence data are needed. While TALE represents an important partial step in this direction, more methodological research and development is needed. But important prerequisites must be addressed first: realistic models of coevolution that also permit tractable statistical calculations, as well as statistically efficient inference and learning algorithms under the new models. Scalability-enhancing algorithmic techniques such as phylogenetic divide-and-conquer [16, 23, 29] may prove fruitful here.

6 DATA AVAILABILITY

Updated versions of the study data and software scripts underlying this article are available in the public GitLab repository at https://gitlab.msu.edu/liulab/cophylogeny-species-tree-quality-performance-study-data-scripts. An archival snapshot of the study data and software scripts has been uploaded to Figshare and can be accessed at https://doi.org/10.6084/m9.figshare.21713996.v1.

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