Phylogenetic comparative methods are problematic when applied to gene trees with speciation and duplication nodes: correcting for biases in testing the ortholog conjecture

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Abstract

Most comparative studies of functional genomics have used pairwise comparisons. Yet it has been shown that this can provide biased results, since genes, like species, are phylogenetically related. Phylogenetic comparative methods should allow to correct for this, but they depend on strong assumptions, including unbiased tree estimates relative to the hypothesis being tested. An ongoing trend in comparative genomic studies is to adopt phylogenetic comparative method to answer a wide range of biological questions including evolutionary hypotheses testing. Notably among them is the recently controversial "Ortholog Conjecture" that assumes the functional evolution is faster in paralogs than orthologs. Using pairwise comparisons of tissue specificity index (τ), earlier we provided support for the ortholog conjecture. In contrast, a recent publication suggested that the ortholog conjecture, is not supported by gene expression tissue-specificity using phylogenetic independent contrasts. We find that the gene trees used suffer from important biases, due to the inclusion of trees with no duplication nodes, to the relative age of speciations and duplications, to systematic differences in branch lengths, and to non-Brownian motion of tissue-specificity on many trees. We find some support for the ortholog conjecture, but especially that incorrect implementation of phylogenetic method in empirical gene with duplications can be problematic.

Author Summary

There is a lot of interest in understanding the evolution of gene function. Comparing functional genomics results offers a way to do this. In most cases, it is done by comparing pairs of genes, and notably pairs of orthologs (homologs derived by speciation) and pairs of paralogs (homologs derived by duplication). A drawback of such pairwise comparisons is that they neglect the evolutionary history of the genes, and thus violate the statistical presumption of independence of observations. Phylogenetic comparative methods have been suggested to correct for this. Recent results indicate that whereas pairwise comparisons support stronger functional divergence of paralogs than of orthologs, phylogenetic analyses would not. Re-analyses of these data show that gene trees are biased in such a way as to cause biases in phylogenetic methods when there are gene duplication. This can led to erroneous results, and have serious consequences in biological data interpretation.

Introduction

The "Ortholog Conjecture", a cornerstone of phylogenomics, has become a topic of debate in recent years [1–9]. Dealing with the roles of gene duplications in functional evolution, the ortholog conjecture is routinely used by both experimental and computational biologists in predicting or understanding gene function. According to this model, orthologs (i.e. homologous genes which diverged by a speciation event) retain equivalent or very similar functions, whereas paralogs (i.e. homologous genes which diverged by a duplication event) share less similar functions [1]. This is linked to the hypothesis that paralogs evolve more rapidly. This hypothesis was challenged by results suggesting that paralogs would be functionally more similar than orthologs [2]. Such findings not only raised questions on the evolutionary role of gene duplication but also questioned the reliability of using orthologs to annotate unknown gene functions in different species [10]. Several more recent studies [3–6, 8] found support for the ortholog conjecture, mostly based on comparisons of gene expression data.

While all previous studies of the ortholog conjecture had used pairwise comparisons of orthologs and paralogs, a recent article suggested that this was flawed, and that phylogenetic comparative methods should be used [7]. Ignorance of phylogenetic structure has been reported to underestimate the fundamental assumption of independent observations in statistics [11, 12]. A solution is to use phylogeny-based methods to investigate such evolutionary patterns [11–17]. There are three main such phylogenetic methods: Phylogenetic Independent Contrast (PIC) [11], Phylogenetic Generalized Least-Square (PGLS) [16], and Monte Carlo computer simulation [17]. PIC is widely adopted for its relative simplicity, and its applicability to a wide range of statistical

73 procedures [7, 18]. The performance of PIC relies on three basic assumptions: a correct
74 tree topology; accurate branch lengths; and trait evolution following a Brownian model
75 (where trait variance accrues as a linear function of time) [12–15, 18–20]. If any of these
76 assumptions is incorrect, this can lead to incorrect interpretation of results. This is
77 probably why the application of such phylogenetic methods is still limited, and debated
78 even after being introduced about three decades ago [12, 13, 15, 18, 19]. Dunn et al. [7]
79 took an innovative approach in applying PIC to compare the divergence rates between
80 two different events ("speciation" and "duplication") to test the ortholog conjecture.
81 However, such an application might be problematic since the time of occurrence of gene
82 duplication, one of the two types of events compared, is unknowable by external
83 information (e.g. no fossil evidence). Therefore, further study is required to understand
84 why Dunn et al. [7] obtained results which are inconsistent with most studies by using the
85 phylogenetic method. It is possible that all the conclusions drawn by previous studies on
86 gene duplication are incorrect due to overlooking phylogenetic tree structure. If so, it
87 should be well supported.

88 We re-examined the data of Dunn et al., after reproducing it using the resources and
89 scripts provided by the authors [7]. Our reanalysis highlights potential problems
90 associated with phylogenetic independent contrasts when applied to the impact of gene
91 duplication. Finally, with proper controls, the phylogenetic method supports the ortholog
92 conjecture.

93

Results

Issues with naive use of Phylogenetic Independent Contrasts (PICs)

To understand their results, we first reanalyzed the data of Dunn et al. [7]. We were able to reproduce all the results published in their article by running the code, which they clearly supplied. Dunn et al. reported a non-significant result for the PIC under the null simulations, using a Wilcoxon one-tailed rank test to check if the contrasts of duplication events are higher than the contrasts of speciation events ($P = 1$). Surprisingly, the PIC rejects the null hypothesis on the null simulations with a Wilcoxon two-tailed rank test (Fig 1A), with significant support for higher contrasts after speciation than duplication. This was robust to repeating the simulations with different random seed numbers (data not shown). This indicates that neither of the methods, PIC or pairwise, worked properly for these calibrated trees, since both reject the null when simulations are performed under the null.

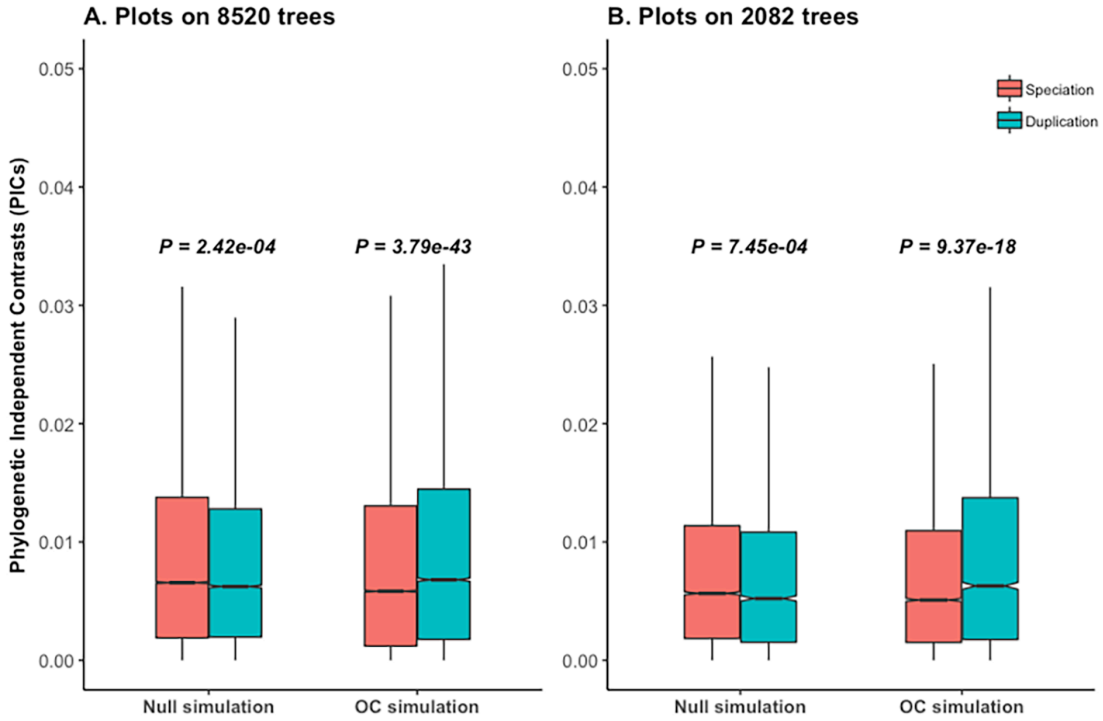


Fig 1: **Reanalyses of phylogenetic simulation data of Dunn et al. [7]**. P values are from Wilcoxon two-tailed tests. In null simulations, there should be no difference in contrasts between events. In OC (Ortholog Conjecture) simulations, contrasts are expected to be higher for duplication than for speciation. (A) An excess of contrasts for speciation than duplication reject the null hypothesis under null simulation scenario for all empirical time calibrated gene trees. (B) Results are similar with a subset of trees with strong phylogenetic signal for τ.

PIC is standardized by the expected variance of daughter branch lengths for each node of event [12–15]. Therefore, accurate branch length estimation in absolute time (e.g. in Million Years – My) is needed [12–15, 18]. Since the accuracy and performance of the PIC method largely depend on proper branch length calibration [13, 14, 21] we investigated possible biases created during calibration of gene trees, especially concerning duplication times for which we have no external reference (e.g. no fossils) (S1 Fig).

The purpose of applying a phylogenetic method is to generate independent statistical data points for traits, which are otherwise not independent because of their phylogenetic relatedness. Such statistical non-independence among species trait values can be measured by "phylogenetic signal" [21–25]. Blomberg's K and Pagel's λ are widely used methods to capture such phylogenetic signal [21, 22]. Dunn et al. [7] used Blomberg's K in their study [23]. Blomberg's K value ranges from 0 to ∞, where a value of 0 indicates no phylogenetic signal, a value of 1 indicates trait evolution according to a Brownian

model (BM), values between 0 and 1 indicate that distant relatives are more similar than expected under BM, and values larger than 1 indicate that close relatives are more similar than expected under BM [21–25]. With a cutoff of $K > 0.551$, Dunn et al. [7] obtained only 2082 out of 8520 calibrated trees with strong phylogenetic signal. Using a cut-off of $P < 0.05$ on this K statistic leads to 2896 trees, which produce similar results (data not shown) to that set of 2082 trees. We continued analyses with the 2082 trees of Dunn et al. [7] for consistency. In any case, it is notable that trait (τ) values are independent of phylogeny for the majority of the gene trees. For these 5624 trees, the use of PIC to produce statistically independent data points might not be necessary. Moreover, it can be misleading if the contrasts are not checked for adequate standardization as per BM after applying PIC (discussed later). The phylogenetic method still rejects the null hypothesis under null simulations for those 2082 trees (Fig 1B), showing that the problem is not simply due to low phylogenetic signal.

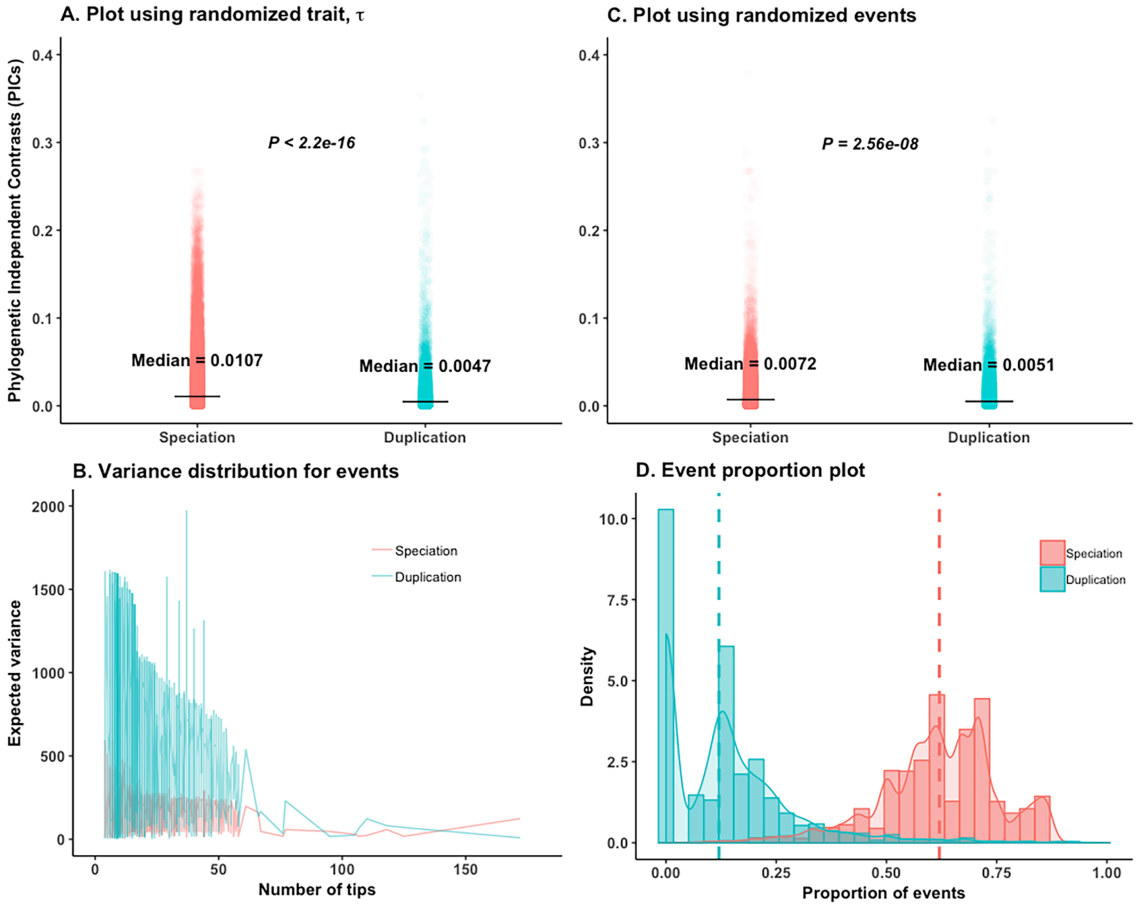
Dunn et al. [7] used 7 speciation time points to calibrate gene trees. The oldest speciation calibration was at 296 My. All other calibrated nodes were duplication nodes, leading to ~652 unique duplication time points for the 8520 calibrated trees [7]. Out of these, 359 time points preceded the oldest speciation node age. Surprisingly, the calibrated node age of the oldest duplication event was found to be 11799977 My, that is, 2600 times older than the Earth. This is indicative of the difficulty of estimating the age of ancient duplications by phylogenetic methods. Those 359 high duplication node ages eventually led to much larger expected variances for gene duplication events (median expected variance for duplication events preceding the oldest speciation events: 828, median expected variance for speciation events: 184). This explains why the mean paralog

distance was ~5.6 times higher than that of the mean ortholog distance (S2A Fig). This data distribution also makes it appear as if no speciation happened before 296 My, a problem shared with the pairwise analysis of the same data [6]. There are also branch length issues for duplication events younger than the oldest speciation events, including very short branches and negative branch lengths. All this led to obtain abnormally high duplication contrasts for the empirical data. To limit such problems, Dunn et al. [7] removed node contrasts higher than 0.5 on the empirical data (this does not impact Fig 1, as the simulation data never has such high contrasts). Following this practice, there are still higher expected variances following gene duplication events (S2A and S2B Figs). In the null simulations only the τ values are simulated, while the branch lengths (hence the variances) are taken from the empirical data, and thus share its biases. This explains why contrasts are lower for duplications than for speciations under null simulations as well as with empirical data.

Randomization tests to assess the performance of phylogenetic method

We used randomization tests to assess whether the results of different analyses of the empirical dataset are reliable and unbiased. In a first randomization test, we randomized the trait values (i.e. randomly permuting the τ values) across the tips of each tree without altering the node events of the trees. We then computed the contrasts for the speciation and duplication events of the trees when there is no relation between trait values and phylogenetic relationships. When we compared the nodes contrasts of speciation and duplication events of these randomized trees (Fig 2A), we found the same pattern as

reported for the empirical gene trees by Dunn et al. [7]. This shows that the PIC applied to these data is not measuring phenotypic evolution. It confirms that results are driven by their large differences in branch lengths (i.e. in expected variances) (Fig 2B), as on simulated null data. Any effect of trait divergence rates of speciation and duplication events is masked by this branch length difference. This violates the basic assumption of applicability of the PIC method to Brownian trait evolution. To remove the problem of difference in expected variances of the two events, we performed a second randomization test: we kept the original τ value for tips but randomly shuffled the events (duplication or speciation) of internal nodes of the empirical gene trees to maintain the original proportions of speciation and duplication events. The resulting trend (Fig 2C) still resembled the empirical gene trees data of Dunn et al. [7]. This appears due to the fact that the majority of the tree events are speciation events (Fig 2D) with speciation node ages $\leq$ 296 My. Most of the trees with many duplication events on the other hand have ancient duplication events (duplication node age > 296 My) for which the evolutionary rates of duplication are often masked by the effect of longer branch lengths. Hence, opposite to our expectation, the calibrated trees with no or few duplications have higher overall nodes contrast (apparent rapid evolution) than trees with many duplications (apparent slow evolution). This might be due to greater difficulty in detecting paralogs for fast evolving genes. Therefore, reshuffling of the events may not change the observed pattern of higher speciation than duplication contrasts.



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196 **Fig 2: Analyses on calibrated empirical gene trees of Dunn et al. [7].** P values are
197 from Wilcoxon two-tailed tests. (A) Randomly shuffling the τ values of the tips for 8520
198 gene trees does not alter the trend of empirical result [7] showing an opposite trend to the
199 ortholog conjecture. (B) The figure indicates the expected variance is much higher for
200 duplication than speciation events irrespective of the number of tips considered for the
201 study. (C) Using the original τ data, if we permute the events
202 (Speciation/Duplication/NA) of the nodes, the trend of result still resembles the empirical
203 result obtained by the recent study [7]. (D) The proportions of speciation events are much
204 higher than duplication events for all time-calibrated trees. The dotted line represents the

median proportion of both events. This plot shows a high frequency of trees have no/few duplication events.

Out of 8520 calibrated trees, 2990 were species-only trees with no duplication event. For these 2990 trees, random shuffling of events had no impact. To avoid this bias, we removed those 2990 speciation trees as well as trees with negative branch lengths, and randomized the trait or the internal node events 100 times on the remaining 5479 trees. However, we still always obtained significantly higher contrasts of speciation than of duplication (S3A and S3B Figs). The randomization tests pattern is the same when we used 2082 trees with strong phylogenetic signals (S3C and S3D Figs). All these analyses indicate that the results reported by Dunn et al. [7] are biased by the available gene trees, and that this bias is not easy to correct.

The ortholog conjecture when τ evolution follows a Brownian model

Following a Brownian model (BM), small and large values of standardized contrasts should be equally likely to occur on any node of the tree [19]. Diagnostic plot tests (details in the Methods) for each tree can indicate whether trait evolution follows BM for that tree [13–15, 18–20]. Since BM is intrinsic to the implementation of PIC method, we selected trees that passed diagnostic plot tests for τ evolution. Hence, we used a subset of 2545 gene trees, by removing trees with negative branch lengths, pure speciation trees, and trees that failed diagnostic tests for BM (S4 Fig). Moreover, to meet the correct branch length assumption of PIC applicability on trees with gene duplications, we used

227 limitations of node age on gene duplication. It is necessary to put such a limitation to
228 avoid biases due to inaccurate calibration of very old duplication nodes. Analyses on
229 these 2545 trees, with a node age limit of 296 My (i.e. node age ≤ maximum speciation
230 age), provided support for the ortholog conjecture (Fig 3), in contrast to previous results.
231 We repeated the analysis including slightly older duplication events (duplication age ≤
232 370 My, i.e. maximum 25% older than the oldest speciation). We still were able to detect
233 higher contrasts for duplication than for speciation (Fig 3). Randomization tests on these
234 trees indicated no bias in the inference (S5A-S5D Figs), supporting the relevance of the
235 inferences on empirical data.

236

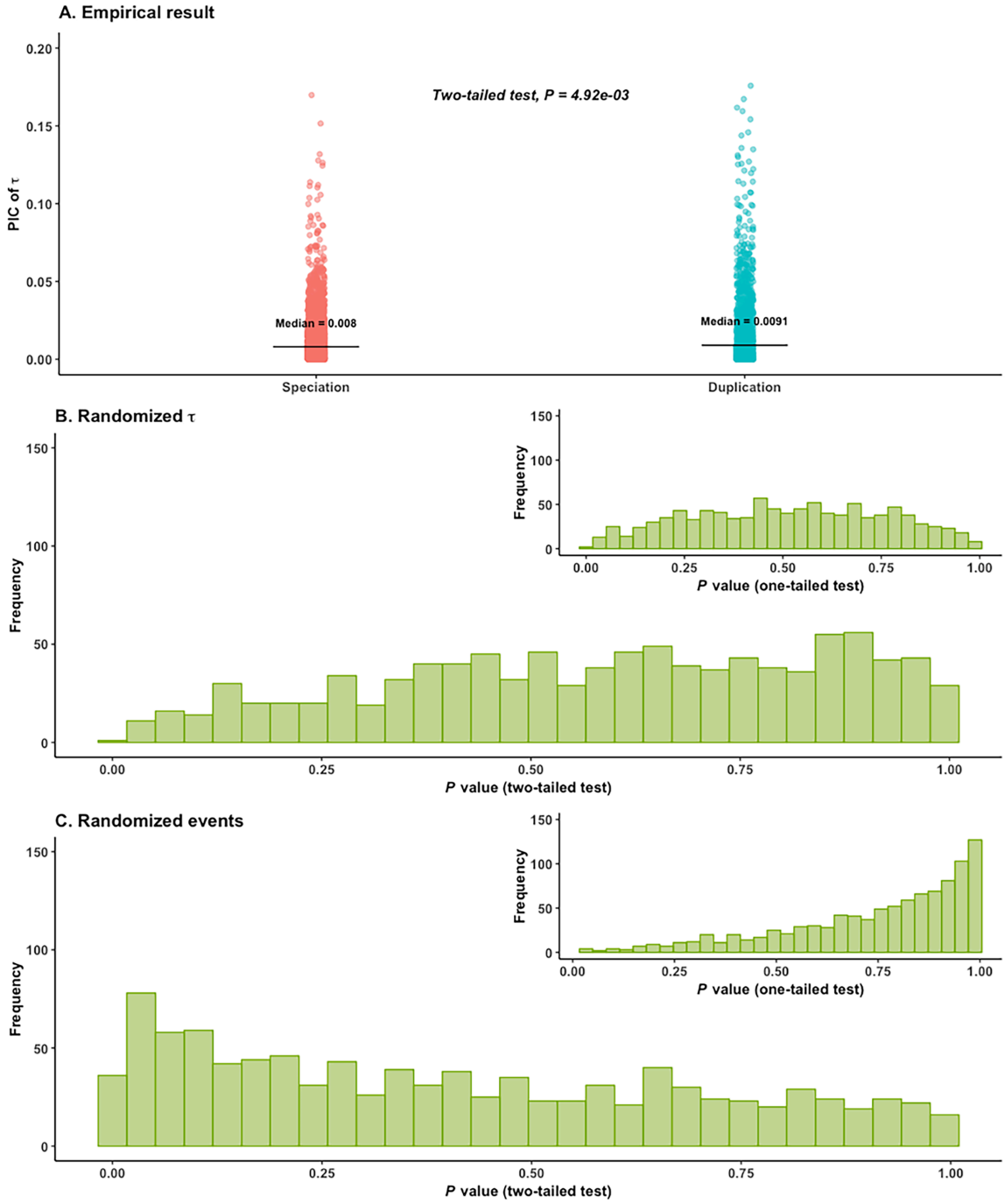
237 **Fig 3**: **The ortholog conjecture test on τ for trees passing diagnostic plot tests with**
238 **proper controls.** P values are from Wilcoxon two-tailed tests. 'PICs' refers

239 'Phylogenetic Independent Contrasts'. Contrasts of speciation and duplication events
240 reject the null hypothesis with different node age limits for the trees following Brownian
241 model of τ evolution, and provide support for the ortholog conjecture.

242

243 Among these 2545 trees (S4 Fig), very old duplication node contrasts were also involved
244 in identifying BM trees of τ, which might bias the analysis. Hence, we repeated the
245 identification of BM trees by standard diagnostic plot tests considering only 5479 trees
246 with at least one duplication event, after removing very old duplication nodes
247 (duplication age > 370 My) (S4 Fig). We still were able to find support for the ortholog
248 conjecture on these identified 2682 BM trees (S6 Fig).

249 To avoid calibration bias of older duplication events, we also considered a more restricted
250 set of 806 trees passing more strict conditions (S4 Fig), i.e. BM trees in which there is at
251 least one duplication, and a speciation event older than all duplications. Calibration bias
252 for duplication events is expected to be very limited for such trees. Support for the
253 ortholog conjecture still holds with these 806 trees (Fig 4A). Randomization tests on
254 these 806 trees also support the biological relevance of these results (Figs 4B and 4C).
255 These observations confirm that the ortholog conjecture is supported for adequately
256 standardized BM trees.



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258 **Fig 4**: **Results on real and randomized data for 806 BM trees with ancient speciation**
259 **events**. *P* values are from two-tailed Wilcoxon tests. (A) The jitter plot on empirical gene
260 tree data supports the ortholog conjecture. Main plots in (B) and (C) show the

distributions of two-tailed test P values estimated for the whole set of 806 BM trees by permuting τ (B), and by permuting the internal node events (C) ('Speciation', 'Duplication', and 'NA') for 1000 independent runs respectively. Inset plots in (B) and (C) show *P* value distributions plots of one-tailed test to check if there was an excess of duplication contrasts over speciation contrasts by randomizing τ, and events respectively. The proportions of speciation (median = 0.62), and NA events (median = 0.2) are much higher than that of duplication events (median = 0.14) in these trees (S7 Fig). Hence, higher duplication contrasts are more likely to be replaced by speciation events by permutations of events. Due to this reason, we found greater shifts towards left in the main plot of C, and towards P value 1 in the inset plot of C to depict larger contrasts for speciation events. Comparisons of plot A to plot B, and plot A to plot C show that randomization results in both the cases actually differ from the empirical result.

Instead of using standard diagnostic plot tests [13–15, 18–20], Dunn et al. [7] considered model-fitting criteria to identify BM trees from their list of 8520 calibrated trees. They contrasted the BM model (neutral drift model with no selection) to the Ornstein-Uhlenbeck (OU) model (i.e. an extended BM model with stabilizing selection) [26, 27] for this purpose. After computing the difference in Akaike Information Criterion (ΔAIC) [28] scores of the models for each tree, they identified 3151 BM trees, out of which only 8 trees purely favored the BM model ($\Delta AIC_{BM} < 2$ and $\Delta AIC_{OU} \geq 2$). The other 3143 trees favored both the BM and OU models ($\Delta AIC_{BM} < 2$ and $\Delta AIC_{OU} < 2$). A Wilcoxon two-tailed test on those 3151 trees provided similar results to the 8520 calibrated trees, i.e. higher τ evolution for orthologs than for paralogs ($PIC_{speciation} = 0.0063$, $PIC_{duplication} =$

284 0.0059, $P = 4.68e^{-03}$). A similar trend was observed for the recent duplicates (node age ≤
285 296 My) (S8A Fig). The pattern of systematic increase of duplication PICs with node age
286 (S8A Fig) plausibly implies that the evolution of τ does not in fact follow Brownian
287 motion for these trees. Randomization tests on those trees (S8B and S8C Figs) confirmed
288 that contrasts were not appropriately standardized as per BM, and thus the inference
289 drawn on empirical data was not supported. When we performed diagnostic plot tests on
290 those 3151 trees, we found support for the ortholog conjecture for 2412 trees passing the
291 diagnostic tests (S9 Fig). All these analyses suggest that Dunn et al. did not identify
292 accurately BM trees, and that such misidentification leads to violating the assumptions of
293 phylogenetic comparative methods, and to erroneous results.

294

Discussion

We agree with Dunn et al. [7] that evolutionary comparisons should be done considering a phylogenetic framework when possible. However, this does not imply that phylogenetic independent contrasts can be applied easily to phylogenomics data and questions. To get a clear picture, we limited our study to the same phylogenetic gene trees used by Dunn et al. [7]. Our reanalysis identified problems generated during the time calibration of duplication nodes of pruned trees for which no external time references are available, and with non respect of the hypothesis of Brownian motion. The strongest bias was for duplication nodes preceding the oldest speciation nodes. This, in turn, introduced several biases in their analyses, and influenced their results. When we identified and controlled for such biases, PIC results changed to support the ortholog conjecture, consistent with our previous pairwise approach [6] on the same τ data.

To our knowledge, no one before Dunn et al. [7] applied a phylogenetic approach in comparative biology to study the effect of gene duplication on functional evolution, despite an early call to do so [29]. In line with previous studies [13, 30], we explored whether the application of a phylogenetic method might inflate errors (e.g. rejection of the null hypothesis in null condition, Figs 1A and 1B) if applied without thorough testing for the fundamental assumptions of the method. Assumptions of proper branch length information and of Brownian model of trait evolution are related, so that modifications of branch lengths can change the evolutionary model [30]. We find that branch length calibration is mostly inaccurate for old duplication events (duplication events prior to the oldest speciation event in the data) (S1 Fig). Use of such node contrasts causes a strong rise in expected variance for duplication events compared to the speciation events (S2

318 Fig). This may bring about lack of statistical power to detect the signal of ortholog
319 conjecture, when in reality the signal is present, and even bias towards a pseudo-signal.
320 As a remedial measure, we limited our analysis to the trees for which Brownian motion
321 of trait evolution has been identified with the aid of standard diagnostic plot tests [13–15,
322 18–20]. To remove issues with branch length inaccuracies for older duplication events,
323 we used limitation of node ages for duplication events. Using all such measures to control
324 for biases, we found support for the ortholog conjecture (Figs 3 and 4A). The reliability
325 of our inference was validated by two different randomization tests, which confirmed the
326 fact that our result was not due to biases in the data or analysis (S5A-S5D Figs, Figs 4B
327 and 4C).

328 Being aware of the fact that branch lengths are fundamental to phylogenetic comparative
329 methods, Dunn et al. [7] added random noise in the speciation calibration time point, and
330 extended terminal branch lengths to test sensitivity of their observations. While extending
331 terminal branch can reduce error rate due to the presence of negative branch lengths in 54
332 trees, none of the modifications appear to avoid biases when there are so vast differences
333 in branch lengths [31–37]. These can also lead to violating the Brownian model of trait
334 evolution [30]. Diagnostic plot tests appear necessary to assess adequate standardization
335 of contrasts as per BM in such cases [13–15, 18–20].

336 The importance of proper BM tree selection should not be underestimated as it can
337 impact the contrast analysis. There exist no fixed protocols to select BM trees. We used
338 standard diagnostic plots recommended in earlier studies for PIC [13–15, 18–20, 38–40]
339 to rule out significant departure from BM for each tree individually at 95% confidence
340 level. In contrast, Dunn et al. [7] used both BM and OU as null models for each tree,

leading them to identify "BM trees" for which the two models were equally good fits. These do not appear to be proper BM trees according to several observations, since duplication contrasts increased with node age (S8A Fig). Moreover, the inferences based on such trees with empirical data were not different from that based on randomized trees (S8B and S8C Figs). When we applied diagnostic tests on those 3151 "BM trees", 734 trees failed the diagnostic tests, and rest of the trees provided support for the ortholog conjecture (S9 Fig).

Empirical support for the ortholog conjecture has been mixed, with most studies supporting, and a few failing to do so [1–9], our results provided support for the ortholog conjecture using large-scale genome wide tissue specificity data in a phylogenetic framework after controlling for bias. Due to lack of detailed functional information, many studies are still limited to gene expression data as a proxy of function. Recently, using functional replaceability assay, experimental studies [41, 42] have shown that orthologous genes can be swapped between essential yeast genes and human, although this is rarely the case for all the members of expanded human gene families [42], validating one prediction of the ortholog conjecture.

These analyses are mainly based on gene trees dominated by small scale duplication events. In these trees, the age of the same duplication clade is never fixed. Use of time calibrated whole genome duplication trees could be beneficial in this regard. Since the approximate time period of whole genome duplications are known, we can test the hypothesis more conveniently by avoiding use of any node age limits on whole genome duplication events for those trees.

Methods

Resource details for reanalyses

Our reanalyses are based on 8520 annotated (with the events: speciation, duplication or NA), pruned, and time calibrated ENSEMBL Compara v.75 [43] gene trees, having at least 4 tips with non null trait data. They were obtained from Dunn et al. [7]. Like them [7], we used a subset of precomputed τ data (as trait) of 8 vertebrate species from the study of Kryuchkova-Mostacci and Robinson-Rechavi [6]. Kryuchkova-Mostacci and Robinson-Rechavi [6] computed τ and mean gene expression levels by following the method of Yanai et al. [44]. The subset of data was based on the RNA-seq data of Brawand et al. for 6 tissues [45]. We used the same random seed number as in [7] to reproduce the simulation results for reanalysis. All reproduced data of Dunn et al. were stored in the "manuscript_dunn.RData" file (https://doi.org/10.5281/zenodo.3354285). We used the results stored in the 'data' or 'phylo' slot of the trees for further analyses. To differentiate our own function from theirs [7], we renamed the original function script of Dunn et al. from "functions.R" to "functions_Dunn.R". Some of the analyses were time consuming, so we made a separate script "Premanuscript_run_TM.R" to run before knitting the markdown file. We stored the outputs in "Data_TMRR.rda" file (https://doi.org/10.5281/zenodo.3354285) and loaded it during our analyses. All the details of different functions were provided inside the script. We supply all the previously stored data (to reduce computation time during reproduction of result) and function files

385 including our own ("function_TM_new.R") with this manuscript. All scripts are available
386 on GitHub: https://github.com/tbegum/Testing_the_ortholog_conjecture.

387 We used the phylosig function() of the "phytools" package [46] to identify all trees with
388 phylogenetic signal ($P < 0.05$) using Blomberg's K [22, 23,46].

389 Other analyses and plotting were performed in R version 3.5.1 [40] using treeio [47],
390 geiger [48], stringr [49], digest [50], dplyr [51], tidyverse [52], ggrepel [53], gtools [54],
391 ggplot2 [55], cowplot [56], easyGgplot2 [57], gridExtra [58], and png [59] libraries.

392 **Randomization test of τ values**

393 For each tree, we used τ data (column name "Tau" in each tree 'data' object) across the
394 tips to carry out our randomization test. To randomize we permuted the actual τ data
395 without altering internal node events. The pic() function of the "ape" package [60] was
396 used to compute PIC of nodes for each tree using permuted τ of tips. For each run, we
397 compared the contrasts of speciation and duplication events of the whole set of
398 randomized trees to estimate difference in event contrasts based on Wilcoxon signed rank
399 test. For 100 or 1000 runs, we repeated the above process 100 or 1000 times to obtain a
400 distribution plot of 100 or 1000 independent *P* values.

401 **Randomization test of internal node events**

402 Some of the speciation nodes had daughters with same clade names in the gene trees we
403 used for our study. Dunn et al. changed such node events to "NA" to avoid problems
404 during time calibration of the trees. Such annotated node event information ("Speciation",
405 "Duplication", "NA") for each tree was available as "Event" in the tree 'data' slot. To

406 randomize, we permuted the internal node events (added as column name "event_new" in
407 the 'data' slot) by maintaining the actual proportion of events for each tree. Then, we
408 used the PIC of actual τ at tips to estimate contrasts difference between newly assigned
409 speciation and duplication node events by Wilcoxon rank tests. For 100 or 1000
410 independent runs, we repeated the same procedure to obtain 100 or 1000 independent *P*
411 values.

412 **Checking for Brownian model of τ evolution**

413 There exists no established protocol to identify BM (Brownian model) trees. Dunn et al.
414 used model-fitting with $\Delta AIC_{BM} = AIC_{BM} - \min(AIC_{BM}, AIC_{OU})$ and $\Delta AIC_{OU} = AIC_{OU} -$
415 $\min(AIC_{BM}, AIC_{OU})$. They applied $\Delta AIC_{BM} < 2$ as the relative support for the BM model.
416 We used several other diagnostic tests to test BM behavior, as recommended in several
417 studies [13–15, 18, 19, 30]. Among diagnostic tests, the most usual method for contrasts
418 standardization is to check a correlation between the absolute values of standardized
419 contrasts and their expected standard deviations [13, 18, 20]. Under Brownian motion,
420 there should be no correlation. This test and the correlation between the absolute values
421 of standardized contrasts and the logarithm of their node age are model diagnostic plot
422 tests in the caper ("Comparative Analyses of Phylogenetics and Evolution in R") package
423 [18, 38–40]. We used both of them in our study by using the "crunch" algorithm of the
424 "caper" package in R, which implements the methods originally provided in CAIC [18,
425 38–40]. Correlation of node heights with absolute values of contrasts has also been
426 reported to be a reliable indicator of deviation from the Brownian model [19]. Hence, we
427 computed node height for each node in a tree using the ape package [60]. We also used
428 the correlations of node height and node depth to the absolute value of nodes contrasts to

429 rule out significant trend in any of the 4 tests. We used $P < 0.05$ to assess a significant
430 correlation for the diagnostic plot tests. A significant trend (positive or negative) reliably
431 indicates a deviation from the BM of trait evolution for that tree [13–15, 18–20], and we
432 removed those trees from our analysis. Contrast calculation on negative branch lengths is
433 not desirable, so we removed trees with negative branch lengths before applying the
434 crunch() function. Trees passing all 4 diagnostic tests were considered as BM trees for
435 our study.

436

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Supporting Information

S1 Fig: An example of a calibrated gene trees used by Dunn et al. in their study. The blue nodes represent the speciation nodes while the red nodes are duplication nodes. For nodes with no number the event is assigned as NA by the authors [7]. Since, the duplication time is unknown, the tree is calibrated based on speciation node ages. Notice that this gives an unreasonably large estimated date for the ancient duplication, resulting in higher expected variance and abnormally low contrast for the corresponding node event. This example is to demonstrate that it is often risky to use such nodes to infer the result of phylogenetic method that intends to study the effect of gene duplication.

S2 Fig: Re-analyses of expected variances of calibrated trees considered by Dunn et al. The expected variance plots of (A) all calibrated trees, and (B) trees with strong phylogenetic signal. The dotted line represents the mean expected variance of the events. These plots suggest that the use of duplication nodes preceding ancient speciation nodes for calibration is often erroneous and should be avoided.

S3 Fig: *P* value distribution plots after 100 independent runs on each set of trees. Wilcoxon two-tailed test with 95% confidence interval was used to compare the speciation and duplication contrasts after randomization tests. (A) and (B) applied to trees with at least one speciation and one duplication event. (C) and (D) applied to trees with strong phylogenetic signal. (A) and (C) randomization of trait (τ) over the trees. (B) and (D) randomization of internal node events. The inset plots show *P* values adjusted with Benjamini-Hochberg [61, 62]. Supporting our observations of Figs 2A and 2C, all the

643 plots confirm that the empirical result of Dunn et al. [7] is not different from randomized
644 test results.

645 **S4 Fig: Pipeline to generate different subsets of contrasts standardized BM trees of**
646 **τ.** The symbol '-' indicates removal of trees with the corresponding mentioned criteria.
647 Diagnostic plot tests (discussed in details in Methods) refer to the tests recommended in
648 prior studies [13–15,18–20] to identify trees where trait (in this case τ) evolution follows
649 BM. Old duplication nodes refers to duplication events older than 370 My. Pure
650 speciation trees indicate gene trees with no duplication event. Removal of trees without
651 old speciation means that we keep only trees where all duplication events are more recent
652 than the oldest speciation event, i.e. trees with age ≤ 296 My.

653 **S5 Fig: Randomization test results on contrasts standardized 2545 BM trees for τ.**
654 Wilcoxon tests were used to generate *P* value distribution plots by 1000 independent runs
655 with a node age limit of 296 My. (A) For randomized τ, Wilcoxon two-tailed tests
656 unexpectedly showed all the values were significant. (B) *P* values of 1 in all cases of
657 Wilcoxon one-tailed test (as performed in Dunn et al.) on randomized τ. This may be due
658 to the fact that there is an excess proportions of speciation and NA events compared to
659 the duplication events in these trees. By permuting τ, higher τ values of duplication
660 events are more likely to be assigned to the speciation and NA events. Hence, this
661 produced higher speciation contrasts, although we controlled the bias in expected
662 variance of older duplication events by node age limits. (C) and (D) Wilcoxon two-tailed
663 and one-tailed tests were performed by randomization of events. After randomization, the
664 smaller proportions of duplication events were more likely to be replaced by speciation
665 and NA events, showing greater shifts towards excess speciation contrasts. These plots

show that randomization results are different from the empirical results of Fig 3, as expected.

S6 Fig: Result on 2682 BM trees using maximum age of oldest duplication event as 370 My. Wilcoxon two-tailed test was used to estimate the significance level at 95% confidence interval. With different node age limits (as used in Fig 3), we found support for the ortholog conjecture. The difference between S6 Fig and Fig 3 is that in Fig 3we used the PICs of all the old duplication nodes to identify BM trees, and then we used different node age limits to test the ortholog conjecture, whereas in S6 Fig we removed the old duplication nodes (duplication age > 370 My) before identifying BM trees, and tested for the ortholog conjecture with different node age limits.

S7 Fig: Distribution of proportions of events in 806 BM trees. These trees have a median of 8 internal nodes. Most of the trees have speciation nodes whose descendants have the same clade name. Hence, Dunn et al. [7] changed such nodes from speciation events to NA to avoid problem with tree calibration. From the figure, it is clear that the proportion of speciation events is much higher than that of the duplication events. For these trees, the proportions of NA events (median: 0.2) are even higher than the proportion of duplication events (median: 0.14). The dotted line represents the median proportions of the events.

S8 Fig: Re-analyses of 3151 trees called as following a BM model by Dunn et al. (A) Wilcoxon two-tailed test was used to obtain the significance level using empirical data. The box plot shows that there is no support for the ortholog conjecture for recent duplicates (age ≤ 296 My), but that there is support for the older duplicates. (B) and (C) *P* values estimated using randomized τ (B), and using randomized events (C), with a node

689 age limit of 296 My to test if there was any difference in contrasts between speciation and
690 duplication events, and plotted based on 100 independent *P* values. The main plots in (B)
691 and (C) demonstrate that speciation and duplication contrasts differ significantly in each
692 randomization test. The inset plots of (B) and (C) with Wilcoxon one-tailed test to check
693 if duplication contrasts was higher than the speciation contrasts, confirm the opposite
694 trend, as was observed with empirical data with node age ≤ 296 My. This suggests that
695 contrasts are not adequately standardized for these trees as per BM, and that the inference
696 based on these trees can be misleading.

697 **S9 Fig: The ortholog conjecture test on Dunn et al. identified BM trees after**
698 **diagnostic tests.** Among 3151 trees, 2412 trees passed the diagnostic tests of BM.
699 Wilcoxon two-tailed test was used to estimate the significance level at 95% confidence
700 interval. We did not exclude pure speciation trees before the contrasts analyses. With
701 node age limit (age ≤ 296 My) as well as without, we found support for the ortholog
702 conjecture.

703