1   **PhyKIT: a UNIX shell toolkit for processing and analyzing phylogenomic data**

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3   Jacob L. Steenwyk[1,*], Thomas J. Buida III[2], Abigail L. Labella[1], Yuanning Li[1], Xing-Xing

4   Shen[3], & Antonis Rokas[1,*]

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6   [1] Vanderbilt University, Department of Biological Sciences, VU Station B #35-1634, Nashville,

7   TN 37235, United States of America

8   [2] 9 City Place #312, Nashville, TN 37209, United States of America

9   [3] Ministry of Agriculture Key Lab of Molecular Biology of Crop Pathogens and Insects, Institute

10   of Insect Sciences, Zhejiang University, Hangzhou 310058, China

11

12   *Correspondence should be addressed to: jacob.steenwyk@vanderbilt.edu or

13   antonis.rokas@vanderbilt.edu

14

15   **ORCiDs**

16   J.L. Steenwyk: 0000-0002-8436-595X

17   T.J. Buida III: 0000-0001-9367-6189

18   Abigail L. Labella: 0000-0003-0068-6703

19   Y. Li: 0000-0002-2206-5804

20   X.-X. Shen: 0000-0001-5765-1419

21   A. Rokas: 0000-0002-7248-6551

22

23   **Running title:** PhyKIT: a toolkit for examining phylogenomic datasets

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25   signal, network, gene-gene covariation, evolutionary rate, polytomy, tree distance

26

27   <u>Abstract</u>

28   Diverse disciplines in biology process and analyze multiple sequence alignments (MSAs) and

29   phylogenetic trees to evaluate their information content, infer evolutionary events and processes,

30   and predict gene function. However, automated processing of MSAs and trees remains a

31   challenge due to the lack of a unified toolkit. To fill this gap, we introduce PhyKIT, a toolkit for

32   the UNIX shell environment with 30 functions that process MSAs and trees, including but not

33   limited to estimation of mutation rate, evaluation of sequence composition biases, calculation of

34   the degree of violation of a molecular clock, and collapsing bipartitions (internal branches) with

35   low support. To demonstrate the utility of PhyKIT, we detail three use cases: (1) summarizing

36   information content in MSAs and phylogenetic trees for diagnosing potential biases in sequence

37   or tree data; (2) evaluating gene-gene covariation of evolutionary rates to identify functional

38   relationships, including novel ones, among genes; and (3) identify lack of resolution events or

39   polytomies in phylogenetic trees, which are suggestive of rapid radiation events or lack of data.

40   We anticipate PhyKIT will be useful for processing, examining, and deriving biological meaning

41   from increasingly large phylogenomic datasets. PhyKIT is freely available on GitHub

42   (https://github.com/JLSteenwyk/PhyKIT) and documentation including user tutorials are

43   available online (https://jlsteenwyk.com/PhyKIT).

44 **<u>Introduction</u>**

45 Multiple sequence alignments (MSAs) and phylogenetic trees are widely used in numerous

46 disciplines, including bioinformatics, evolutionary biology, molecular biology, and structural

47 biology. As a result, the development of user-friendly software that enables biologists to process

48 and analyze MSAs and phylogenetic trees is an active area of research (Kapli *et al.* 2020).

49

50 In recent years, numerous methods have proven useful for diagnosing potential biases and

51 inferring biological events in genome-scale phylogenetic (or phylogenomic) datasets. For

52 example, methods that evaluate sequence composition biases in MSAs (Phillips and Penny

53 2003), signatures of clock-like evolution in phylogenetic trees (Liu *et al.* 2017), phylogenetic

54 treeness (Lanyon 1988; Phillips and Penny 2003), taxa whose long branches may cause variation

55 in their placement on phylogenetic trees (Struck 2014), and others have assisted in summarizing

56 the information content in phylogenomic datasets and improved phylogenetic inference

57 (Felsenstein 1978; Philippe *et al.* 2011; Salichos and Rokas 2013; Doyle *et al.* 2015; Liu *et al.*

58 2017; Smith *et al.* 2018; Walker *et al.* 2019).

59

60 Other methodological innovations include identifying significant gene-gene covariation of

61 evolutionary rate, which has been shown to accurately and sensitively identify genes that have

62 shared functions, are co-expressed, and/or are part of the same multimeric complexes (Sato *et al.*

63 2005; Clark *et al.* 2012). Furthermore, gene-gene covariation serves as a powerful evolution-

64 based genetic screen for predicting gene function (Brunette *et al.* 2019). Lastly, a recently

65 developed method has enabled the identification of unresolved internal branches or polytomies in

66 species trees (Sayyari and Mirarab 2018; One Thousand Plant Transcriptomes Initiative 2019);

67   such branches can stem from rapid radiation events or from lack of data (Rokas and Carroll

68   2006).

69

70   Despite the wealth of information in MSAs and phylogenetic trees, there is a dearth of tools,

71   especially ones that allow to conduct these analyses in a unified framework. For example, to

72   utilize the functions mentioned in the previous paragraphs, a combination of web-server

73   applications, 'hard-coded' scripts available through numerous repositories and supplementary

74   material, standalone software, and/or extensive programming in languages including R, Python,

75   or C is currently required (Cock *et al.* 2009; Junier and Zdobnov 2010; Revell 2012; Talevich *et*

76   *al.* 2012; Struck 2014; Kück and Longo 2014; Wolfe and Clark 2015; One Thousand Plant

77   Transcriptomes Initiative 2019). As a result, integrating these functions into bioinformatic

78   pipelines is challenging, reducing their accessibility to the scientific community.

79

80   To facilitate the integration of these methods into bioinformatic pipelines, we introduce PhyKIT,

81   a UNIX shell toolkit with 30 functions (Table 1) with broad utility for analyzing and processing

82   MSAs and phylogenetic trees. Current functions implemented in PhyKIT include measuring

83   topological similarity of phylogenetic trees, creating codon-based MSAs, concatenating sets of

84   MSAs into phylogenomic datasets, editing and/or viewing alignments and phylogenetic trees,

85   and identifying putatively spurious homologs in MSAs. We highlight three uses of PhyKIT: (1)

86   calculating diverse statistics that summarize the information content and potential biases (e.g.,

87   sequence- or phylogeny-based biases) in MSAs and phylogenetic trees; (2) creating a gene-gene

88   covariation network of evolutionary rates; and (3) inferring the presence of polytomies from

89   phylogenomic data. The diverse functions implemented in PhyKIT will likely be of interest to

90   bioinformaticians, molecular biologists, evolutionary biologists, and others.

91

92   **Materials and Methods**

93   PhyKIT is a command line tool for the UNIX shell environment written in the Python

94   programming language (https://www.python.org/). PhyKIT requires few dependencies

95   (Biopython (Cock *et al.* 2009) and SciPy (Virtanen *et al.* 2020)) making it user-friendly to install

96   and integrate into existing bioinformatic pipelines. Furthermore, the online documentation of

97   PhyKIT comes complete with tutorials that detail how to use various functions. Lastly, PhyKIT

98   is modularly designed to allow straightforward integration of additional functions in future

99   versions.

100

101   PhyKIT has 30 different functions that help process and analyze MSAs and phylogenetic trees

102   (Table 1). The 30 functions can be grouped into broad categories that assist in conducting

103   analyses of MSAs and phylogenies or in processing/editing them. For example, "analysis"

104   functions help examine information content biases, gene-gene covariation, and polytomies in

105   phylogenomic datasets; "processing/editing" functions help prune tips from phylogenies,

106   collapse poorly supported bipartitions in phylogenetic trees, concatenate sets of MSAs into a

107   single data matrix, or create codon-based alignments from protein alignments and their

108   corresponding nucleotide sequences.

109

110   Detailed information about each one of PhyKIT's functions and tutorials for using the software

111   can be found in the online documentation (https://jlsteenwyk.com/PhyKIT). Here, we focus on

112   three specific groups of functions implemented in PhyKIT that enable researchers to summarize

113   information content in phylogenomic datasets, create gene-gene evolutionary rate covariation

114   networks, and identifying polytomies in phylogenomic data.

115

116   **Evaluating information content and biases in phylogenomic datasets**

117   MSAs and phylogenetic trees are frequently examined to evaluate their information content and

118   potential biases in characteristics such as sequence composition or branch lengths (Phillips and

119   Penny 2003; Philippe *et al.* 2011; Struck 2014; Doyle *et al.* 2015; Shen *et al.* 2016a; Liu *et al.*

120   2017; Smith *et al.* 2018). PhyKIT implements numerous functions for doing so. Here, we

121   demonstrate the application of 14 functions:

122   (1) *Alignment length.* The length of a multiple sequence alignment, which is associated with

123   robust bipartition support and tree accuracy (Shen *et al.* 2016a; Walker *et al.* 2019);

124   (2) *Alignment length with no gaps*. The length of a multiple sequence alignment after excluding

125   sites with gaps, which is associated with robust bipartition support and tree accuracy (Shen *et al.*

126   2016a);

127   (3) *Degree of violation of a molecular clock (DVMC).* A metric used to determine the clock-like

128   evolution of a gene using the standard deviation of branch lengths for a single gene tree (Liu *et*

129   *al.* 2017). DVMC is calculated using the following formula:

$$DVMC = \sqrt{\frac{1}{N-1}\sum_{j=1}^{N}\left(i_j - \bar{\imath}\right)^2}$$

130   where *N* represents the number of tips in a phylogenetic tree, i_j being the distance between the

131   root of the tree and species *j*, and $\bar{\imath}$ represents the average root to tip distance. DVMC can be

132   used to identify genes with clock-like evolution for divergence time estimation (Liu *et al.* 2017);

133   (4) *Internal branch lengths*. Summary statistics of internal branch lengths in a phylogenetic tree

134   are reported including mean, median, 25th percentile, 75th percentile, minimum, maximum,

135   standard deviation, and variance values. Examination of internal branch lengths is useful in

136   evaluating phylogenetic tree shape;

137   (5) *Long branch score*. A metric that examines the degree of taxon-specific long branch

138   attraction (Struck 2014; Weigert *et al.* 2014). Long branch scores of individual taxa are

139   calculated using the following formula:

$$LB_i = \left(\frac{\overline{PD_i}}{\overline{PD_{all}}} - 1 \right) \times 100$$

140   where $\overline{PD_i}$ represents the average pairwise patristic distance of taxon *i* to all other taxa, $\overline{PD_{all}}$

141   represents the average patristic distance across all taxa, and LB_i represents the long branch score

142   of taxon *i*. Long branch scores can be used to evaluate heterogeneity in tip-to-root distances and

143   identify taxa that may be susceptible to long branch attraction;

144   (6) *Pairwise identity*. Pairwise identity is a crude approximation of the evolutionary rate of a

145   gene and is calculated by determining the average number of sites in an MSA that are the same

146   character state between all pairwise combinations of taxa. This can be used to group genes based

147   on their evolutionary rates (e.g., faster-evolving genes vs. slower-evolving ones) (Chen *et al.*

148   2017);

149   (7) *Patristic distances*. Patristic distances refer to all distances between all pairwise combinations

150   of tips in a phylogenetic tree (Fourment and Gibbs 2006), which can be used to evaluate the rate

151   of evolution in gene trees or taxon sampling density in species trees;

152   (8) *Parsimony-informative sites*. Parsimony-informative sites are those sites in an MSA that have

153   a least two character states (excluding gaps) that occur at least twice (Kumar *et al.* 2016); the

154   number of parsimony-informative sites is associated with robust bipartition support and tree

155   accuracy (Shen *et al.* 2016a; Steenwyk *et al.* 2020);

156   (9) *Variable sites*. Variable sites are those sites in an MSA that contain at least two different

157   character states (excluding gaps) (Kumar *et al.* 2016); the number of variable sites is associated

158   with robust bipartition support and tree accuracy (Shen *et al.* 2016a);

159   (10) *Relative composition variability*. Relative composition variability is the average variability

160   in the sequence composition among taxa in an MSA. Relative composition variability is

161   calculated using the following formula:

$$Relative\ composition\ variability = \sum_{i=1}^{c} \sum_{j=1}^{n} \frac{\left|c_{ij} - \bar{c}_\iota\right|}{s \times n}$$

162   where c is the number of different character states per sequence type, n is the number of taxa in

163   an MSA, c_{ij} is the number of occurrences of the *i*th character state for the *j*th taxon, $\bar{c}_\iota$ is the

164   average number of the *i*th c character state across n taxa, and s refers to the total number of sites

165   (characters) in an MSA. Relative composition variability can be used to evaluate potential

166   sequence composition biases in MSAs, which in turn violate assumptions of site composition

167   homogeneity in standard models of sequence evolution (Phillips and Penny 2003);

168   (11) *Saturation.* Saturation refers to when an MSA contains many sites that have experienced

169   multiple substitutions in individual taxa. Saturation is estimated from the slope of the regression

170   line between patristic distances and pairwise identities. Saturated MSAs have reduced

171   phylogenetic information and can result in issues of long branch attraction (Lake 1991; Philippe

172   *et al.* 2011);

173   (12) *Total tree length*. Total tree length refers to the sum of internal and terminal branch lengths

174   and is calculated using the following formula:

$$total\ tree\ length = \sum_{i=1}^{a} l_i + \sum_{j=1}^{b} l_j$$

175

176   Where l_i is the branch length of the ith branch of a internal branches and l_j is the branch length of

177   the jth branch of b terminal branches. Total tree length measures the inferred total amount or rate

178   of evolutionary change in a phylogenetic tree;

179   (13) *Treeness*. Treeness (also referred to as stemminess) is a measure of the inferred relative

180   amount or rate of evolutionary change that has taken place on internal branches of a phylogenetic

181   tree (Lanyon 1988; Phillips and Penny 2003) and is calculated using the following formula:

$$treeness = \sum_{u=1}^{b} \frac{l_u}{l_t}$$

182   where l_u is the branch length of the uth branch of b internal branches, and l_t refers to the total

183   branch length of the phylogenetic tree. Treeness can be used to evaluate how much of the total

184   tree length is observed among internal branches;

185   (14) *Treeness divided by relative composition variability*. This function combines two metrics to

186   measure both composition bias and other biases that may negatively influence phylogenetic

187   inference. High treeness divided by relative composition variability values have been shown to

188   be less susceptible to sequence composition biases and are associated with robust bipartition

189   support and tree accuracy (Phillips and Penny 2003; Shen *et al.* 2016a).

190

191   **Calculating gene-gene evolutionary rate covariation or coevolution**

192   Genes that share similar rates of evolution through speciation events (or coevolve) tend to have

193   similar functions, expression levels, or are parts of the same multimeric complexes (Sato *et al.*

194   2005; Clark *et al.* 2012). Thus, identifying significant coevolution between genes (i.e.,

195   identifying genes that are significantly correlated in their evolutionary rates across speciation

196   events) can be a powerful evolution-based screen to determine gene function (Brunette *et al.*

197   2019).

198

199   To measure gene-gene evolutionary rate covariation, PhyKIT implements the mirror tree method

200   (Pazos and Valencia 2001; Sato *et al.* 2005), which examines whether two trees have correlated

201   branch lengths. Specifically, PhyKIT calculates the Pearson correlation coefficient between

202   branch lengths in two phylogenetic trees that share the same tips and topology. To account for

203   differences in taxon representation between the two trees, PhyKIT first automatically determines

204   which taxa are shared and prunes one or both such that the same set of taxa is present in both

205   trees. PhyKIT requires that the two input trees have the same topology, which is typically the

206   species tree topology inferred from whole genome or proteome data. Thus, the user will typically

207   first estimate a gene's branch lengths by constraining the topology to match that of the species

208   tree. When running this function, users should be aware that many biological factors, such as

209   horizontal transfer (Doolittle and Bapteste 2007), incomplete lineage sorting (Degnan and Salter

210   2005), and introgression / hybridization (Sang and Zhong 2000), can lead to gene histories that

211   deviate from the species tree. In these cases, constraining a gene's history to match that of a

212   species may lead to errors in the covariation analysis.

213

214   Due to factors including time since speciation and mutation rate, correlations between

215   uncorrected branch lengths result in a high frequency of false positive correlations (Sato *et al.*

216   2005; Clark *et al.* 2012; Chikina *et al.* 2016). To ameliorate the influence of these factors,

217   PhyKIT first transforms branch lengths into relative rates. To do so, branch lengths are corrected

218   by dividing the branch length in the gene tree by the corresponding branch length in the species

219   tree. Previous work revealed that one or a few outlier branch length values can be responsible for

220   false positive correlations and should be removed prior to analysis (Clark *et al.* 2012). Thus,

221   PhyKIT removes outlier data points defined as having corrected branch lengths greater than five

222   (i.e., removing gene tree branch lengths that are five or more times greater than their

223   corresponding species tree branch lengths). Lastly, values are converted into relative rates using

224   a Z-transformation. The resulting relative rates are used when calculating Pearson correlation

225   coefficients.

226

227   **Identifying polytomies in phylogenomic data**

228   Rapid radiations or diversification events have occurred throughout the tree of life including

229   among mammals, birds, plants, and fungi (Jarvis *et al.* 2014; Liu *et al.* 2017; One Thousand

230   Plant Transcriptomes Initiative 2019; Li *et al.* 2020). Polytomies correspond to internal branches

231   whose length is 0 (or statistically indistinguishable from 0) and can be driven either by biological

232   (e.g., rapid radiations) or analytical (e.g., low amount of data) factors. Thus, polytomies are

233   useful for inferring rapid radiation or diversification events and exploring incongruence in

234   phylogenies (Sayyari and Mirarab 2018; One Thousand Plant Transcriptomes Initiative 2019; Li

235   *et al.* 2020).

236

237   To identify polytomies, a modified approach to a previous strategy was implemented (Sayyari

238   and Mirarab 2018). More specifically, the support for three alternative topologies is calculated

239   among all gene trees from a phylogenomic dataset. For example, in species tree *((A,B),C), D);*, if

240   examining the presence of a polytomy at the ancestral bipartition of tips *A, B,* and *C*, PhyKIT

241   will determine the number of gene trees that support *((A,B),C);*, *((A,C),B);*, and *((B,C),A);* using

242   the rooted gene trees provided by the user. Equal support for the three topologies (i.e., the

243   presence of a polytomy) among a set of gene trees is assessed using a Chi-squared test. Failing to

244   reject the null hypothesis is indicative of a polytomy (Sayyari and Mirarab 2018). Note that this

245   approach is distinct from the approach of Sayyari and Mirarab to identify polytomies because

246   PhyKIT uses a gene-based signal rather than a quartet-based signal. The difference between the

247   two methods is that each gene contributes equally to the inference of a polytomy when a gene-

248   based signal is used, whereas genes with greater taxon representation (which contain a greater

249   number of quartets) will contribute a greater signal during polytomy identification when a

250   quartet-based signal is used.

251

252   **Results and Discussion**

253   We outline three example uses of PhyKIT: 1) summarizing information content and identifying

254   potential biases in animal, plant, yeast, and filamentous fungal phylogenomic datasets (Shen *et*

255   *al.* 2016b; Steenwyk *et al.* 2019; Laumer *et al.* 2019; One Thousand Plant Transcriptomes

256   Initiative 2019), 2) constructing a network of significant gene-gene covariation, which reveals

257   genes of shared functions from empirical data spanning ~550 million years of evolution among

258   fungi (Shen *et al.* 2020), and 3) illustrating how to identify polytomies using simulated and

259   empirical data (Steenwyk *et al.* 2019).

260

261   **Summarizing information content and biases in phylogenomic data**

262   Examining information content in phylogenomic datasets can help diagnose potential biases that

263   stem from low signal-to-noise ratios, multiple substitutions, non-clocklike evolution, and other

264   biological or analytical factors. To demonstrate the utility of PhyKIT to summarize the

265   information content in phylogenomic datasets, we calculated 14 different metrics known to help

266   diagnose potential biases in phylogenomic datasets or be associated with accurate and well

267   supported phylogenetic inferences (Felsenstein 1978; Phillips and Penny 2003; Philippe *et al.*

268   2011; Struck 2014; Doyle *et al.* 2015; Shen *et al.* 2016a; Liu *et al.* 2017; Smith *et al.* 2018) using

269   four empirical phylogenomic datasets from animals (201 tips; 2,891 genes) (Laumer *et al.* 2019),

270   budding yeast (332 taxa; 2,408 genes) (Shen *et al.* 2018), filamentous fungi (93 taxa; 1,668

271   genes) (Steenwyk *et al.* 2019), and plants (1,124 taxa; 403 genes) (One Thousand Plant

272   Transcriptomes Initiative 2019) (Figure 1, Table 1).

273

274   Examination of the distributions of the values of the 14 different metrics revealed inter- and

275   intra-dataset heterogeneity (Figure 1). For example, inter-dataset heterogeneity was observed

276   among animal and plant datasets, which had the lowest and highest average pairwise identity

277   across alignments, respectively; intra-dataset heterogeneity was observed in the uniform

278   distribution of pairwise identities in the budding yeast datasets. Similarly, inter-dataset

279   heterogeneity was observed in estimates of saturation where the budding yeast and filamentous

280   fungal MSAs were less saturated by multiple substitutions than the plant and animal datasets;

281   intra-data heterogeneity was also observed in all four datasets. Varying degrees of inter- and

282   intra-dataset heterogeneity was observed for other information content statistics, which may be

283   due biological (e.g., mutation rate) or analytical factors (e.g., taxon sampling, distinct alignment,

284   trimming, and tree inference strategies).

285

286   In summary, PhyKIT is useful for examining the information content of phylogenomic datasets.

287   For example, the generation of different phylogenomic data submatrices by selecting subsets of

288   genes or taxa with certain properties (e.g., retention of genes with the highest numbers of

289   parsimony-informative sites or following removal of taxa with high long branch scores) can

290   facilitate the exploration of the robustness of species tree inference or estimating time since

291   divergence (Salichos and Rokas 2013; Liu *et al.* 2017; Shen *et al.* 2018, 2020; Steenwyk *et al.*

292   2019; Walker *et al.* 2019; Li *et al.* 2020).

293

294   **A network of gene-gene covariation reveals neighborhoods of genes with shared function**

295   Genes with similar evolutionary histories often have shared functions, are co-expressed, or are

296   parts of the same multimeric complexes (Sato *et al.* 2005; Clark *et al.* 2012). Using PhyKIT, we

297   examined gene-gene covariation using 815 genes spanning 1,107 genomes and ~563 million

298   years of evolution among fungi (Shen *et al.* 2020). By examining 331,705 pairwise combinations

299   of genes, we found 298 strong signatures of gene-gene covariation (defined as r > 0.825). The

300   two genes with the strongest signatures of covariation were *SEC7* and *TAO3* (r = 0.87),

301   suggesting that their protein products have similar or shared functions. Supporting this

302   hypothesis, Sec7p contributes to cell-surface growth in the model yeast *Saccharomyces*

303   *cerevisiae* (Novick and Schekman 1979) and genes with the Sec7 domain are transcriptionally

304   coregulated with yeast-hyphal switches in the human pathogen *C. albicans* (Song *et al.* 2008).

305   Similarly, Tao3p in both *S. cerevisiae* and *C. albicans* is part of a RAM signaling network,

306   which controls hyphal morphogenesis, polarized growth, and cell-cycle related processes

307   including cell separation, cell proliferation, and phase transitions (Bogomolnaya *et al.* 2006;

308   Song *et al.* 2008).

309

310   Complex relationships of gene-gene covariation can be visualized as a network (Figure 2).

311   Examination of network neighborhoods identified groups of genes that have shared functions and

312   are parts of the same multimeric complexes. For example, the proteins encoded by *NDC80* and

313   *NUF2* are part of the same kinetochore-associated complex termed the NDC80 complex—which

314   is required for efficient mitosis (Sundin *et al.* 2011)—and significantly covary with one another

315   (r = 0.84). Similarly, multiple genes that encode proteins involved in DNA replication and repair

316   (i.e., *POL2, MSH6, RAD26, CDC9,* and *EXO1*) were part of the same network neighborhood,

317   consistent with previous work suggesting an intimate interplay between DNA replication and

318   multiple DNA repair pathways (Tsubouchi and Ogawa 2000; Lujan *et al.* 2012; Boiteux and

319   Jinks-Robertson 2013). Similarly, network neighborhoods of genes involved in ribosome

320   biogenesis, Golgi apparatus-related transport, and control of DNA replication were identified

321   (Figure 2).

322

323   Taken together, these results indicate PhyKIT is a useful tool for evaluating gene-gene

324   covariation and predicting genes' functions (Sato *et al.* 2005; Clark *et al.* 2012; Brunette *et al.*

325   2019). Thus, we anticipate PhyKIT will be helpful for evaluating gene-gene covariation and

326   conducting evolution-based screens for gene functions across the tree of life.

327

328   **Identifying polytomies in phylogenomic datasets**

329   Rapid radiations or diversification events have occurred throughout the tree of life (Jarvis *et al.*

330   2014; Liu *et al.* 2017; One Thousand Plant Transcriptomes Initiative 2019; Li *et al.* 2020). One

331   approach to identifying rapid radiations is by testing for the existence of polytomies in species

332   trees (Sayyari and Mirarab 2018; One Thousand Plant Transcriptomes Initiative 2019; Li *et al.*

333   2020). Polytomies can also arise when the amount of data at hand is insufficient for resolution

334   (Walsh *et al.* 1999). To demonstrate the utility of PhyKIT to identify polytomies, we tested it

335   using a simulated set of phylogenies that had a branch whose length was extremely small (Figure

336   3A). We found that PhyKIT was able to conservatively identify the simulated polytomy. Our

337   results demonstrate PhyKIT can accurately identify a polytomy and provide further support that

338   equal support among alternative topologies can be used as a means to identify a rapid radiation.

339

340   We next examined if there is evidence of polytomies in the evolutionary history of filamentous

341   fungi from the genera *Aspergillus* and *Penicillium*. We examined three branches. The first two

342   branches—one dating back ~110 million years ago (Figure 3B), and another dating back ~25

343   million years ago (Figure 3C)—were not polytomies. In contrast, examination of a ~60 million-

344   year-old branch involving *Lanata-divaricata, Citrina,* and *Exilicaulis* (Figure 3D), which are

345   major lineages (or sections) in the genus *Penicillium*, was consistent with a polytomy. Given the

346   large number of gene trees used in our analysis (n=1,668), these results are consistent with a

347   rapid radiation or diversification event in the history of *Penicillium* species.

348

349   In summary, these results suggest that PhyKIT is useful in identifying polytomies in simulated

350   and empirical datasets. PhyKIT can also be useful for exploring incongruence in phylogenies by

351   calculating gene support frequencies for alternative topologies. Calculations of gene-based

352   support for various topologies can be used in diverse applications, including identifying putative

353   introgression / hybridization events and conducting phylogenetically-based genome-wide

354   association (PhyloGWAS) studies (Pease *et al.* 2016; Steenwyk *et al.* 2019).

355

Conclusion

357 We have developed PhyKIT, a comprehensive toolkit for processing and analyzing MSAs and

358 trees in phylogenomic datasets. PhyKIT is freely available on GitHub

359 (https://github.com/JLSteenwyk/PhyKIT) with extensive documentation and user tutorials

360 (https://jlsteenwyk.com/PhyKIT). PhyKIT is a fast and flexible toolkit for the UNIX shell

361 environment, which allows it to be easily integrated into bioinformatic pipelines. We anticipate

362 PhyKIT will be of interest to biologists from diverse disciplines and with varying degrees of

363 experience in analyzing MSAs and phylogenies. In particular, PhyKIT will likely be helpful in

364 addressing one of the greatest challenges in biology, building, understanding, and deriving

365 meaning from the tree of life.

366

Data Availability

368 All data used will become available in figshare (doi: 10.6084/m9.figshare.13118600) upon

369 publication.

370

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377

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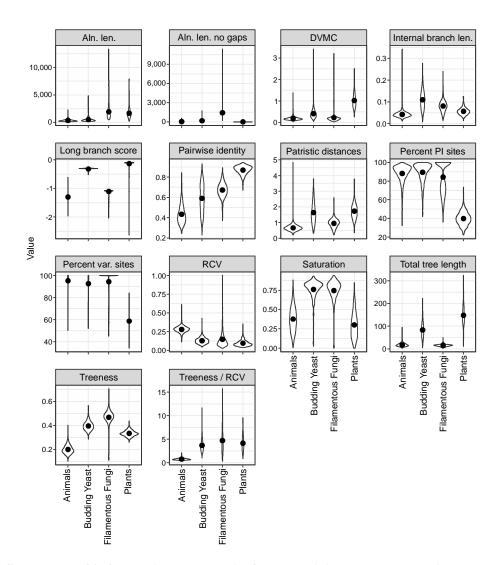528   https://doi.org/10.1093/bioinformatics/btv454

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Figure 1. Summary of information content in four empirical phylogenomic datasets. 14

metrics implemented in PhyKIT help summarize the information content and identify potential

biases in phylogenomic datasets. Each graph displays a violin plot with a black point

representing the mean. Error bars indicate one standard error above and below the mean;

however, these are difficult to see in nearly all graphs because they were often near the mean.

Abbreviations are as follows: Aln. len.: alignment length; Aln. len. no gaps: alignment length

excluding sites with gaps; DVMC: degree of violation of a molecular clock; Internal branch len.:

average internal branch length; Patristic distances: average patristic distance in a gene tree;

541 Percent PI Sites: percentage of parsimony-informative sites in an MSA; Percent var. sites:

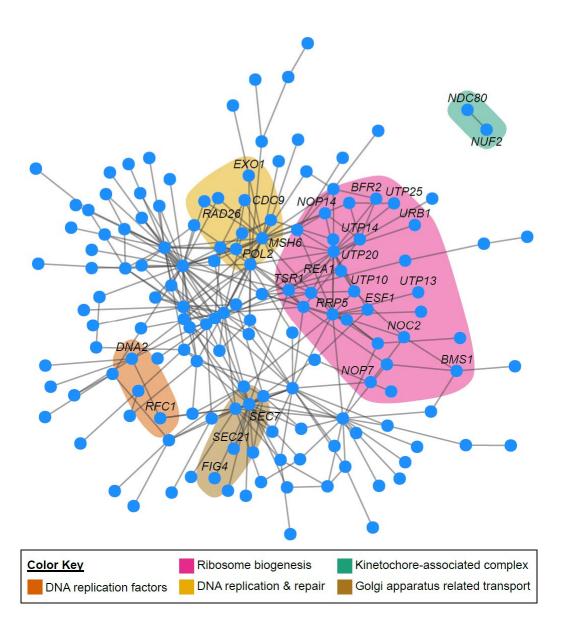542 percentage of variable sites in an MSA; RCV: relative composition variability.

543

Figure 2. Gene-gene covariation network inferred from ~550 million years of evolution

across 1,107 fungi. A network of significant gene-gene coevolution identifies network

neighborhoods representative of associated functional categories. For example, the *NDC80* and

NUF2 genes (toward the top right of the network) were identified to be significantly coevolving

with one another (r = 0.84, p < 0.01, Pearson's correlation test); they both encode proteins that

are part of the same multimeric kinetochore-associated complex (green). Similarly, genes that are

DNA replication factors (orange), contribute to DNA replication and repair processes (yellow),

551   participate in Golgi apparatus-related transport (brown), or ribosome biogenesis (pink) were

552   found to be neighbors in the network. Network visualization was done with the igraph package,

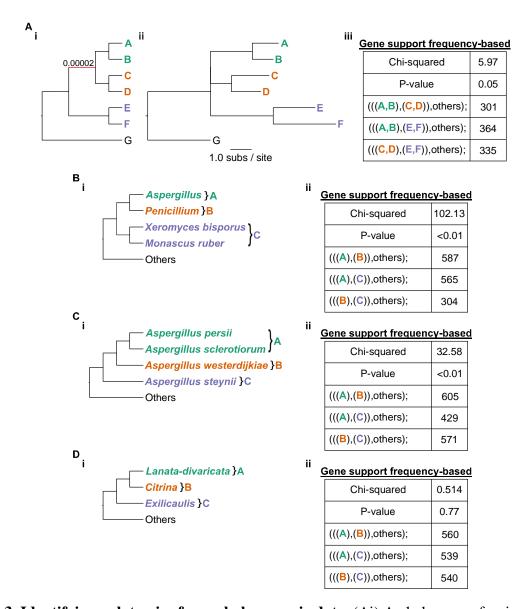553   v1.2.4.2 (Hunter and Cohen 2007), in R, v3.6.2 (https://www.r-project.org/).

554

A

i

0.00002

A
B
C
D
E
F
G

ii

A
B
C
D
E
F
G

1.0 subs / site

iii

Gene support frequency-based

Chi-squared	5.97
P-value	0.05
(((A,B),(C,D)),others);	301
(((A,B),(E,F)),others);	364
(((C,D),(E,F)),others);	335

B

i

Aspergillus }A
Penicillium }B
Xeromyces bisporus }C
Monascus ruber
Others

ii

Gene support frequency-based

Chi-squared	102.13
P-value	<0.01
(((A),(B)),others);	587
(((A),(C)),others);	565
(((B),(C)),others);	304

C

i

Aspergillus persii }A
Aspergillus sclerotiorum
Aspergillus westerdijkiae }B
Aspergillus steynii }C
Others

ii

Gene support frequency-based

Chi-squared	32.58
P-value	<0.01
(((A),(B)),others);	605
(((A),(C)),others);	429
(((B),(C)),others);	571

D

i

Lanata-divaricata }A
Citrina }B
Exilicaulis }C
Others

ii

Gene support frequency-based

Chi-squared	0.514
P-value	0.77
(((A),(B)),others);	560
(((A),(C)),others);	539
(((B),(C)),others);	540

555

556 **Figure 3. Identifying polytomies from phylogenomic data.** (Ai) A cladogram of a simulated

557 species phylogeny with tip names *A-G*. The red branch has a very short branch length of 2×10^{-5}

558 substitutions per site. (Aii) Phylogram of the same phylogeny shows that all other branches are

559 much longer ($\geq$ 1.0 substitutions per site). (Aiii) After reconstructing the evolutionary history

560 from 1,000 alignments simulated from the phylogeny in *Aii*, the hypothesis of a polytomy was

561 tested using gene support frequencies for three alternative rooted topologies defined by the

562 clades of green, orange, and purple taxa. Failure to reject the null hypothesis of equal support

563 among genes for each topology is indicative of a polytomy (χ^2 = 5.97, p-value = 0.05, Chi-

564   squared test). (B-D) The same approach was then used to examine if there is evidence for a

565   polytomy at three different branches in a phylogeny of filamentous fungi. (D) Support for a

566   polytomy (χ^2 = 0.514, p-value = 0.77, Chi-squared test) was observed for the relationships

567   between three different sections of *Penicillium* fungi. These results demonstrate the utility of

568   gene-support frequencies for evaluating polytomies and examining incongruence in

569   phylogenomic datasets.

570

571 **Table 1. Summary of 30 functions implemented in PhyKIT**

Description	Name	Function Alias(es)	Type of function	Input data	Citation
Calculate MSA length	alignment_length	aln_len; al	analytic	alignment	NA
Calculate MSA length after removing sites with gaps	alignment_length_no_gaps	aln_len_no_gaps; alng	analytic	alignment	NA
Combine numerous MSAs into one concatenated matrix	create_concatenation_matrix	create_concat; cc	processing/editing	alignment	NA
Calculate guanine/cytosine content in an MSA	gc_content	gc	analytic	alignment	NA
Calculate summary statistics* for pairwise identity among sequences in an MSA	pairwise_identity	pairwise_id; pi	analytic	alignment	(Chen *et al.* 2017)
Calculate the number and percentage of parsimony-informative sites in an MSA	parsimony_informative_sites	pis	analytic	alignment	NA
Calculate relative composition variability in an MSA	relative_composition_variability	rel_comp_var; rcv	analytic	alignment	(Phillips and Penny 2003)
Rename FASTA entries in an MSA file	rename_fasta_entries	rename_fasta	processing/editing	alignment	NA
Thread DNA sequences over a protein MSA	thread_dna	pal2nal; p2n	processing/editing	alignment	NA
Calculate the number and percentage of variable sites in an MSA	variables_sites	vs	analytic	alignment	NA
Calculate summary statistics* for bipartition support values from a set of phylogenetic trees	bipartition_support_stats	bss	analytic	phylogeny	NA
Multiply all branch lengths of a phylogenetic tree by a specific number	branch_length_multiplier	blm	processing/editing	phylogeny	NA

Collapse bipartitions with bipartition support lower than a user-specified value in a phylogenetic tree	collapse_branches	collapse; cb	processing/editing	phylogeny	NA
Calculates the correlation of evolutionary rates between two genes trees with the same topology	covarying_evolutionary_rates	cover	analytic	phylogeny	(Clark *et al.* 2012)
Calculate the degree a gene tree violates a molecular clock-like rate of evolution	degree_of_violation_of_a_molecular_clock	dvmc	analytic	phylogeny	(Liu *et al.* 2017)
Calculate summary statistics* for internal branch lengths in a phylogenetic tree	internal_branch_stats	ibs	analytic	phylogeny	NA
Create numeric labels for bipartitions in a phylogenetic tree	internode_labeler	il	processing/editing	phylogeny	NA
Calculate summary statistics* for long branch scores for taxa in a phylogenetic tree	long_branch_score	lb_score; lbs	analytic	phylogeny	(Weigert *et al.* 2014)
Calculate the total length of a phylogenetic tree	total_tree_length	tree_len	analytic	phylogeny	NA
Calculate summary statistics* for all patristic distances (or all tip-to-tip distances) in a phylogenetic tree	patristic_distances	pd	analytic	phylogeny	(Fourment and Gibbs 2006)
Use gene support frequencies to test for the existence of a polytomy	polytomy_test	polyt_test; polyt; ptt	analytic	phylogeny	(Sayyari and Mirarab 2018)
Print a phylogenetic tree using ASCII text in the UNIX shell environment	print_tree	print; pt	processing/editing	phylogeny	NA

Prune tips from a phylogenetic tree	prune_tree	prune	processing/editing	phylogeny	NA
Rename tip names in a phylogenetic tree	rename_tree_tips	rename_tree; rename_tips	processing/editing	phylogeny	NA
Calculate raw and normalized Robinson-Foulds distance scores between two phylogenetic trees	robinson_foulds_distance	rf_distance; rf_dist; rf	analytic	phylogeny	(Robinson and Foulds 1981)
Identify putatively spurious sequences by identifying terminal branches with an outlier branch length	spurious_sequence	spurious_seq; ss	analytic	phylogeny	(Jarvis *et al.* 2014)
Print all tip names in a phylogenetic tree	tip_labels	tree_labels; labels; tl	processing/editing	phylogeny	NA
Calculate treeness or stemminess of a phylogenetic tree	treeness	tness	analytic	phylogeny	(Lanyon 1988)
Calculate saturation, a measure of multiple substitutions across many sites in an MSA	saturation	sat	analytic	alignment and phylogeny	(Philippe *et al.* 2011)
Calculate treeness divided by relative composition variability, a measure of composition bias susceptibility	treeness_over_rcv	toverr	analytic	alignment and phylogeny	(Phillips and Penny 2003)

572 *Summary statistics reported are mean, median, 25th percentile, 75th percentile, minimum, maximum, standard deviation, and variance

573 values. All functions that calculate summary statistics also have verbose options that print out the raw data used to calculate summary

574 statistics.