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5 **Experimental signal dissection and method sensitivity analyses reaffirm**  
6 **the potential of fossils and morphology in the resolution of seed plant**  
7 **phylogeny**

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20 **Abstract [273 words]:**

21 The phylogeny of seed plants remains one of the most enigmatic problems in evolutionary  
22 plant biology, with morphological phylogenies (which include fossils) and molecular  
23 phylogenies pointing to very distinct topologies. Almost all morphology-based phylogenies  
24 support the so-called anthophyte hypothesis, grouping the angiosperms with Gnetales and  
25 several extinct seed plant lineages, while most molecular phylogenies link Gnetales with  
26 conifers. In this study, we investigate the phylogenetic signal present in seed plant  
27 morphological datasets. We use maximum parsimony and Bayesian inference, combined  
28 with a number of experiments with all available seed plant morphological matrices to  
29 address the morphological-molecular conflict. First, we ask whether the lack of association  
30 of Gnetales with conifers in morphological analyses is due to an absence of signal or to  
31 the presence of competing signals, and second, we compare the performance of  
32 parsimony and Bayesian approaches with morphological datasets. Our results imply that  
33 the grouping of Gnetales and angiosperms is largely the result of long branch attraction,  
34 consistent across a range of methodological approaches. Thus, the signal for the grouping  
35 of Gnetales with conifers in morphological matrices was swamped by convergence  
36 between angiosperms and Gnetales, both situated on long branches, in previous analyses.  
37 However, this effect becomes weaker in more recent analyses, as a result of addition and  
38 critical reassessment of characters. Bayesian inference proves to be more resistant to  
39 long branch attraction, and the use of parsimony is largely responsible for persistence of  
40 the anthophyte topology. Our analyses finally reconcile morphology with molecules in the  
41 context of the seed plant phylogeny, and show that morphology may therefore be useful in  
42 reconstructing other aspects of the phylogenetic history of the seed plants.

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44

## 45 INTRODUCTION

46 The use of morphology as a source of data for reconstructing phylogenetic relationships  
47 has lost most of its ground since the advent of molecular phylogenetics, except in  
48 paleontology. However, in more recent times there has been renewed interest in  
49 morphological phylogenetics (Pyron 2015; Lee and Palci 2015). A major impetus for this  
50 renaissance has been an increased interest in the phylogenetic placement of fossil taxa in  
51 trees of living organisms, stimulated by the growing necessity of accurate calibrations for  
52 dating the molecular trees that represent the main basis for modern comparative  
53 evolutionary studies. Other factors have been by the development of new methods for  
54 dating phylogenies that can integrate phylogenetic inference of the placement of fossils in  
55 the dating process, i.e., tip-dating (Pyron 2011; Ronquist et al. 2012; Zhang et al. 2016),  
56 as well as renewed interest in the application of statistical phylogenetics to morphological  
57 data both on a theoretical (Wright et al. 2014, 2015; O'Reilly et al. 2016) and an empirical  
58 level (Lee and Worthy 2012; Godefroit et al. 2013; Cau et al. 2015). To these motivations  
59 may be added the long-recognized value of fossils for elucidating the homologies of novel  
60 structures (such as the seed plant ovule and eustele) and the order of origin of the  
61 morphological synapomorphies of extant (crown) groups. This is critical because major  
62 groups, such as angiosperms, are often separated from their closest living relatives by  
63 major morphological gaps (numbers of character changes), even if the incorporation of  
64 fossils does not affect inferred relationships among living taxa (Doyle and Donoghue 1987;  
65 Donoghue et al. 1989).

66 Many phylogenies based on morphology have been recently published for important  
67 groups with both living and fossil representatives, including mammals (O'Leary et al. 2013),  
68 squamate reptiles (Gauthier et al. 2012), arthropods (Legg et al. 2013), and the genus  
69 *Homo* (Dembo et al. 2016). However, the validity and use of morphological data in

70 reconstructing phylogeny have been severely criticized, notably by Scotland et al. (2003),  
71 based on supposed diminishing returns in the discovery of new morphological characters  
72 and the prevalence of functional convergence. The painstaking acquisition of  
73 morphological characters, which requires a relatively large amount of training and time,  
74 could turn out to be systematically worthless if the phylogenetic signal present in these  
75 data is either insufficient or misleading. Indeed, the number of characters that can be  
76 coded for morphological datasets represents a major limit to the use of morphology and its  
77 integration with molecular data, especially in the age of phylogenomics, where the ever-  
78 increasing amount of molecular signal could simply “swamp” the weak signal present in  
79 morphological datasets (Doyle and Endress 2000; Bateman et al. 2006). Morphological  
80 data may also be afflicted to a higher degree than molecules by functional convergence  
81 and parallelism (Givnish and Sytsma 1997), which could lead a morphological dataset to  
82 infer a wrong phylogenetic tree. Even though the confounding effect of convergence has  
83 been formally tested only in a few studies (Wiens et al. 2003), it seems to be at the base of  
84 one of the deepest cases of conflict between molecules and morphology in the  
85 reconstruction of evolutionary history, namely the phylogeny of placental mammals (Foley  
86 et al. 2016). In this case, the strong effect of selection on general morphology caused by  
87 similar lifestyle seems to hinder attempts to use morphology to reconstruct phylogenetic  
88 history in this group (Springer et al. 2007), and it affects even large “phenomic” datasets  
89 (Springer et al. 2013).

90 Another example of conflict between morphology and molecular data involves the  
91 relationships among seed plants. Before the advent of cladistics, some authors proposed  
92 that angiosperms were related to the highly derived living seed plant order Gnetales, while  
93 others argued that these two groups were strictly convergent and Gnetales were instead  
94 related to conifers (for a review, see Doyle and Donoghue 1986). However, the view that  
95 angiosperms are related to Gnetales and fossil Bennettitales, called the anthophyte

96 hypothesis, is one of the oldest and seemingly most stable results of the morphologically  
97 based parsimony analyses of seed plant phylogeny. Since Hill and Crane (1982) and  
98 Crane (1985), the grouping of Bennettitales, Gnetales, the fossil *Pentoxylon*, and  
99 angiosperms (sometimes with the fossil *Caytonia* as the closest outgroup of angiosperms)  
100 was retrieved in almost all successive analyses (Doyle and Donoghue 1986, 1992; Nixon  
101 et al. 1994; Rothwell and Serbet 1994; Doyle 1996, 2006, 2008; Hilton and Bateman 2006;  
102 Friis et al. 2007; Rothwell et al. 2009; Rothwell and Stockey 2016; Fig. 1). Some analyses  
103 associated anthophytes with “Mesozoic seed ferns” (glossopterids, corystosperms, and  
104 *Caytonia*), others with “coniferophytes” (conifers, *Ginkgo*, and fossil cordaites). By contrast,  
105 since the advent of molecular phylogenetics, the anthophyte hypothesis has lost most of  
106 its support among plant biologists. Although molecular analyses cannot directly evaluate  
107 the status of presumed fossil anthophytes, they can address the relationship of  
108 angiosperms and Gnetales. Molecular data from different genomes analyzed with different  
109 approaches do not yield a Gnetales plus angiosperm clade, with the exception of few  
110 maximum parsimony (MP) and neighbor joining analyses of nuclear ribosomal RNA or  
111 DNA (Hamby and Zimmer 1992; Stefanovic et al. 1998; Rydin et al. 2002) and one MP  
112 analysis of *rbcL* (Rydin and Källersjö 2002). The majority of molecular trees retrieve a  
113 clade of Gnetales plus Pinaceae (Bowe et al. 2000; Chaw et al. 2000; Gugerli et al. 2001;  
114 Qiu et al. 2007; Zhong et al. 2011), conifers other than Pinaceae (cupressophytes)  
115 (Nickrent et al. 2000; Rydin and Källersjö 2002), or conifers as a whole (Wickett et al.  
116 2014), which we refer to collectively as “Gnetales-conifer” trees. In most of these trees  
117 angiosperms are the sister group of all other living seed plants (acrogymnosperms). The  
118 main exceptions are “Gnetales-basal” trees, in which Gnetales are sister to all other living  
119 seed plants (e.g., Albert et al. 1994; Rydin and Källersjö 2002).

120         Several potential issues have been identified with both sorts of data. Regarding  
121 molecules, these include limited taxonomic sampling resulting from extinction the majority

122 of seed plant lineages, loss of phylogenetic signal due to saturation (particularly at third  
123 codon positions), strong rate heterogeneity among sites across lineages and conflict  
124 between gene trees (Mathews 2009), composition biases among synonymous  
125 substitutions (Cox et al. 2014) as well as systematic errors and biases (Magallón and  
126 Sanderson 2002; Burleigh and Mathews 2007; Zhong et al. 2011), leading to a plethora of  
127 conflicting signals. In analyzing datasets that yielded Gnetales-basal trees, studies that  
128 have attempted to correct for these biases have generally favored trees in which Gnetales  
129 are associated with conifers (Magallón and Sanderson 2002; Burleigh and Mathews 2007).  
130 Regarding morphology, it has been shown that different taxon sampling strategies,  
131 particularly regarding choice of the closest progymnosperm outgroup of seed plants (Hilton  
132 and Bateman 2006), can lead to different results concerning the rooting of the seed plants.

133         The conflict between molecules and morphology has led to different attitudes  
134 toward morphological data within the botanical community (Donoghue and Doyle 2000;  
135 Bateman et al. 2006; Rothwell et al. 2009). Following suggestions of Donoghue and Doyle  
136 (2000), Doyle (2006, 2008) reconsidered several supposed homologies between  
137 angiosperms and Gnetales in the light of the molecular results. These studies and the  
138 analysis of Hilton and Bateman (2006) also incorporated newly recognized similarities  
139 between Gnetales and conifers, for example in wood anatomy (Carlquist 1996), as well as  
140 improved evidence on the morphology of the seed-bearing cupules in fossil taxa. When  
141 building a morphological matrix, dissecting a character into more character states may  
142 represent an improvement by distinguishing convergent states during primary homology  
143 assessment (Jenner 2004; Zou and Zhang, 2016), although it may also lead to a lack of  
144 resolution when the number of states becomes excessive. In the phylogeny of seed plants,  
145 there are many special factors that complicate character coding. Among living taxa, the  
146 assessment of homology is complicated by the plastic and modular nature of plant  
147 development (Mathews and Kramer 2012). Among fossil taxa, the mode of preservation of

148 many key fossils has critical consequences for the amount of data available. This affects  
149 not only the number of missing characters, but also the process of primary homology  
150 assessment and character coding. Although these issues with coding are most severe in  
151 fossils preserved as compressions, such as *Caytonia* (Doyle 2008; Rothwell et al. 2009)  
152 and *Archaeofructus* (Sun et al. 2002; Friis et al. 2003; Doyle 2008; Rudall and Bateman  
153 2008; Endress and Doyle 2009), even fossil groups that are exquisitely preserved as  
154 permineralizations (e.g., Bennettiales) are not immune to conflicting interpretations (Friis  
155 et al. 2007; Rothwell et al. 2009; Crepet and Stevenson 2010; Doyle 2012; Pott 2016).  
156 Indeed, even after careful reconsideration of potentially convergent traits between  
157 Gnetales and angiosperms, maximum parsimony seemed to continue to favor the  
158 anthophyte hypothesis (Doyle 2006; Hilton and Bateman 2006; Rothwell et al. 2009). The  
159 possibility that morphological data are inadequate to resolve the phylogeny of seed plants  
160 would represent a severe hindrance, especially in the light of the small number of extant  
161 lineages that survived extinction during the Paleozoic and Mesozoic (Mathews 2009) and  
162 the great morphological gaps among these surviving lineages. However, there have been  
163 signs that the conflicts with molecular data are weakening: Doyle (2006) found that trees in  
164 which Gnetales were nested in conifers were only one step less parsimonious than  
165 anthophyte trees, and in Doyle (2008) trees of the two types became equally parsimonious.

166 In this study, we attempt to elucidate the phylogenetic signal present in published  
167 morphological datasets of the seed plants. We test whether the potential convergence  
168 between angiosperms and Gnetales represents a major issue in morphological datasets of  
169 seed plants by reanalyzing the matrices that were driven by earlier homology assumptions  
170 concerning characters of the two groups (i.e., the matrices compiled before the incoming  
171 of the molecular results) as well as the matrices that revised such assumptions (the  
172 matrices of Doyle 2006 and Hilton and Bateman 2006, and datasets derived from them),  
173 and testing whether the signal and support for the anthophytes changes between these

174 two sets of matrices. Then we investigate whether the fact that these analyses did not  
175 place Gnetales in or near the conifers was due to the absence of signal or the presence of  
176 competing signals by investigating the relative support for the anthophytes and the  
177 Gnetales-conifer clade in all the matrices. After revealing a more coherent signal  
178 supporting a Gnetales-conifer clade in the latest matrices, we investigate whether the  
179 retrieval of an anthophyte topology by maximum parsimony was affected by  
180 methodological biases that could be overcome by using model-based Bayesian methods.

181

## 182 **MATERIALS AND METHODS**

### 183 **Matrices**

184 The Crane (1985), Doyle and Donoghue (1986, 1992), Nixon et al. (1994), Rothwell  
185 and Serbet (1994), and Doyle (1996, 2006, 2008) matrices were manually coded from the  
186 respective articles. The Hilton and Bateman (2006) matrix was kindly provided by Richard  
187 Bateman. The matrices from Analysis 3 of Rothwell et al. (2009) and from Rothwell and  
188 Stockey (2016) were downloaded from the supplementary materials of the respective  
189 articles.

### 190 **Parsimony analyses**

191 We performed maximum parsimony analyses of all matrices with PAUP 4.0a136  
192 (Swofford 2003), using the heuristic search algorithm with random addition of taxa and  
193 1000 replicates. Bootstrap analyses were conducted using 10,000 replicates, using the  
194 “asis” addition option and keeping one tree per replicate (Müller 2005).

195 We also conducted analyses with a topological constraint, forcing the Gnetales into  
196 a clade with the extant conifers. Significant differences between the constrained and



197 unconstrained topologies were tested using the Templeton test (Templeton 1983) as  
198 implemented in PAUP v. 4.0a136 (Swofford 2003). We investigated the effects of recoding  
199 characters by Doyle (2006, 2008) in more detail by using MacClade (Maddison and  
200 Maddison 2003) to compare the number of steps in each character on trees with Gnetales  
201 nested in anthophytes and associated with conifers.

202

### 203 **Bayesian inference (BI)**

204 Bayesian analyses relied on MrBayes v. 3.2.3 (Ronquist et al. 2012), under the  
205 Markov k-states (Mk) model (Lewis 2001).

206 For each matrix, we conducted two analyses, one with an equal rate of evolution  
207 among characters and another with gamma-distributed rate variation. In both cases, we  
208 used the  $MK_{pr-inf}$  correction for parsimony informative characters. The analyses were run  
209 for 5,000,000 generations, sampling every 1000<sup>th</sup> generation. The first 10,000 runs were  
210 discarded as burn-in. Posterior traces were inspected using Tracer (Rambaut and  
211 Drummond 2007).

212

### 213 **Model testing and rate variation**

214 We also conducted stepping stone analyses (SS) (Xie et al. 2011; Ronquist et al.  
215 2012) in order to evaluate the most appropriate model of rate variation among characters  
216 (equal rates vs. gamma-distributed rates). We used 4 independent runs with 2 chains with  
217 the default MrBayes parameters, run for 5,000,000 generations and sampling every 1000<sup>th</sup>  
218 generation. Using the marginal likelihoods from the SS analysis, we then calculated the  
219 support for the two models using Bayes factors (BF) (Kass and Raftery 1995).

220

## 221 **Exploring conflict in the data**

222 To explore phylogenetic conflict in the data, we employed the software SplitsTree 4  
223 (Huson and Bryant 2006). We used this program to visualize conflicts among the bootstrap  
224 replicates from the MP analysis and among the posterior tree samples from the BI analysis.  
225 A consensus network (Holland et al. 2004) was built using the “count” option. The cut-off  
226 for visualizing the splits was set at 0.05.

227

## 228 **Long branch attraction tests**

229 We modified the matrices to perform tests for long branch attraction (LBA), following  
230 the suggestions of Bergsten (2005). Two matrices were created to test the potentially  
231 destabilizing effect of the two long-branched groups suspected to create this artifact,  
232 angiosperms and Gnetales, by successively removing them (long branch extraction  
233 analysis, LBE). To test further the hypothesis of an LBA artifact exerted by angiosperms,  
234 we followed a similar approach to the sampling experiment in Rota-Stabelli et al. (2010):  
235 another matrix was created to elongate the branch subtending angiosperms by removing  
236 non-angiospermous fossil outgroups (*Pentoxylon*, Bennettitales, and *Caytonia*) (branch  
237 elongation analysis, BE). To test the effect of including fossil data in the matrices, we  
238 created a set of matrices in which all fossil taxa were removed (extant experiment, EX).

## 239 **Morphospace analysis**

240 To visualize morphological patterns in the different matrices, we conducted  
241 principal coordinates (PCO) analyses using the R package Claddis (Lloyd 2016).  
242 The taxa were then plotted on the first two PCO axes.

243

## 244 RESULTS

245

246 Our re-analyses of the historical morphological matrices of seed plants resulted in  
247 trees identical to the published trees (Table 1). The MP trees and the consensus trees  
248 always show an anthophyte clade (with or without *Caytonia*), except trees based on the  
249 Doyle (2008) matrix, in which anthophyte and Gnetales-conifer topologies are equally  
250 parsimonious. However, bootstrap analysis shows that the anthophyte clade is not strongly  
251 supported in any of the matrices, with the exception of the Nixon et al. (1994) matrix (Fig.  
252 2).

253 Constraining Gnetales and conifers to form a clade always results in trees longer  
254 than the most parsimonious trees, except in the trees based on the Doyle (2008) matrix  
255 (Table 2). The Templeton test of the best trees against the worst of the constrained trees  
256 (i.e., the most parsimonious constrained tree that is statistically most different from the  
257 most parsimonious unconstrained tree) does however show that this difference is only  
258 significant in the Nixon et al. (1994) matrix.

259 The stepping stone analysis shows strong support for rate variation among  
260 characters in all matrices except those of Crane (1985) and Doyle and Donoghue (1986)  
261 (Table 3). The strength of the support seems to be correlated with both the number of  
262 characters and the number of taxa (Supplementary Fig. 1), which were lowest in the oldest  
263 analyses.

264 The trees obtained from the BI analyses show a much sharper differentiation  
265 between early and late matrices. With the pre-2006 matrices, support and topology are  
266 mostly in agreement with the MP analyses. However, with the post-2000 matrices we  
267 observe a shift in support from the anthophytes to a clade of Gnetales and coniferophytes  
268 (Fig. 2, 3).

269 To test the whether the anthophyte topology could be the result of LBA, we first  
270 performed removal experiments. The removal of the angiosperms has different effects on  
271 the pre- and post-2000 matrices. With the Crane (1985) matrix, a topology with  
272 Bennettitales, *Pentoxylon* and the Gnetales diverging after *Lyginopteris* and before the  
273 other taxa becomes as parsimonious as the topology with the anthophytes nested among  
274 Mesozoic seed ferns that was retrieved with the full matrix. With the Doyle and Donoghue  
275 (1986) matrix, Bennettitales, *Pentoxylon*, and Gnetales are nested within coniferophytes.  
276 With the Doyle and Donoghue (1992) and Rothwell and Serbet (1994) matrices, the  
277 consensus tree is identical to the trimmed consensus of the full matrix. With the Nixon et al.  
278 (1994) matrix, cordaites and *Ginkgo* are successive outgroups to a conifer + anthophyte  
279 clade, whereas with the full matrix they are equally parsimoniously placed as successive  
280 outgroups to the conifers, in a clade that is sister to monophyletic anthophytes. The  
281 inverse happens with the Doyle (1996) matrix, where the position of *Ginkgo* and cordaites  
282 is destabilized by the removal of the angiosperms, with these taxa being either successive  
283 outgroups to extant and fossil conifers or sister to a clade composed of anthophytes,  
284 conifers, *Peltaspermum*, and *Autunia*. The position of the Gnetales in an anthophyte clade  
285 is maintained in all matrices.

286 With the post-2000 matrices, the effect of removal of the angiosperms is consistent  
287 among different matrices (Fig. 4d-f). With the Hilton and Bateman (2006), Doyle (2006),  
288 and Doyle (2008) datasets, the resulting trees see the Gnetales nested within the  
289 coniferophytes, with or without Bennettitales. With the Rothwell et al. (2009) matrix, a  
290 topology with a clade of Gnetales and conifers that excludes Bennettitales becomes most  
291 parsimonious (Fig. 4e). With the Rothwell and Stockey (2016) matrix, Gnetales are sister  
292 to *Taxus* in a coniferophyte clade that also includes *Doylea*.

293 The removal of the Gnetales has no impact at all on trees based on the Crane

294 (1985), Doyle and Donoghue (1986), and Doyle and Donoghue (1992) matrices, in which  
295 the topology is identical to the trimmed topology of the consensus in the full analysis. With  
296 the Nixon et al. (1994) matrix, the removal of the Gnetales results in a coniferophyte clade  
297 (including *Ginkgo* and Cordaitales) becoming the most parsimonious topology. With the  
298 Rothwell and Serbet (1994) matrix, the removal of Gnetales results in a breakup of the  
299 *Caytonia-Glossopteris-corystosperm* clade. With the Doyle (1996) matrix, the only  
300 difference lies in the placement of the corystosperms, *Autunia*, and *Peltaspermum*, which  
301 are sister to a coniferophyte clade in the analysis without Gnetales.

302 With the post-2000 matrices, the removal of the Gnetales results in a shift of the  
303 anthophyte clade to a position outside a coniferophyte clade (Fig. 4f). With the Doyle  
304 (2006) and Doyle (2008) matrices, an extended anthophyte clade including Cycadales and  
305 glossopterids is sister to a clade of *Callistophyton*, *Peltaspermum*, *Autunia*, and  
306 corystosperms plus coniferophytes. The analysis of the Rothwell and Stockey (2016)  
307 matrix represents an exception, where the placement of the anthophytes is not affected by  
308 the removal of the Gnetales. The removal of *Doylea* in addition to Gnetales results in a  
309 similar pattern to the other post-2000 matrices.

310 In the branch elongation experiment, we observed that MP bootstrap support for the  
311 angiosperm plus Gnetales clade increases with decreasing taxon sampling in all matrices  
312 (Fig. 4g). This effect is even stronger in the extant experiment matrices, where a split  
313 including angiosperms plus Gnetales is strongly supported by the MP bootstrap in all  
314 matrices.

315 BI analysis of the BE and EX matrices shows a less linear pattern (Fig. 4h, i). In the  
316 BE analyses, the signal for the anthophytes decreases in the Doyle and Donoghue (1986,  
317 1992) matrices, reaching less than 0.5 posterior probability (pp) in the analysis with  
318 gamma-distributed rate variation. In the Nixon et al. (1994), Rothwell and Serbet (1994)

319 and Doyle (1996) matrices, the pp of the anthophytes in the BE matrices is comparable to  
320 that from the full matrices. In the post-2000 BE matrices, BI support for the anthophytes is  
321 almost null in the Hilton and Bateman (2006) and Doyle (2006) matrices (<0.07 pp) and  
322 increases in the Doyle (2008) and Rothwell et al. (2009) matrices analyzed using gamma-  
323 rate variation (0.55 and 0.51 respectively) and in the Rothwell and Stockey (2016) matrix  
324 (0.23 for the equal-rate analysis, 0.37 for the gamma analysis) .

325         The analyses of the EX matrices all show high to moderate support (1-0.75 pp) for  
326 the split containing angiosperms plus Gnetales. With the post-2000 matrices, the use of  
327 the gamma-distributed model recovers a higher pp for the anthophytes.

328         The morphospace analyses (Fig. 5) provide a graphic confirmation of the  
329 morphological separation of both Gnetales and angiosperms from other seed plants and  
330 the impression that Gnetales share competing morphological similarities with both  
331 angiosperms and conifers. In the morphospace generated from most of the pre-2000  
332 matrices, Gnetales lie closer to angiosperms (data not shown). With the Doyle (1996)  
333 matrix and the post-2000 matrices, the first axis of the PCO appears to separate  
334 angiosperm-like and non-angiosperm-like taxa, whereas the second axis seems to  
335 represent a tendency from a seed fern-like towards a conifer-like morphology. The  
336 placement of the Gnetales is always closer to the conifers than to the angiosperms (Fig. 5).  
337 However, in all cases, Gnetales seem to have higher levels of “angiosperm-like”  
338 morphology than do conifers, represented by their rightward placement on the first PCO  
339 axis. This is shared by *Doylea* in the Rothwell and Stockey (2016) matrix. Between the  
340 analyses of the Doyle (1996) and Doyle (2008) matrices (Fig. 5a, b), there is a modest  
341 shift of Gnetales away from angiosperms and towards conifers.

342

## 343 **DISCUSSION**

### 344 *Morphology and the phylogeny of the seed plants*

345       The results of our analyses help to unravel some of the main issues regarding the  
346 phylogenetic signal for the anthophyte clade in morphological matrices of seed plants. MP  
347 bootstrap analyses, the Templeton test on constrained topologies, and BI analyses all  
348 agree in showing that support for assignment of Gnetales to an anthophyte clade did not  
349 increase with increasing taxon or character sampling, as noticed by Donoghue and Doyle  
350 (2000). One of the most interesting results is the switch in support between matrices  
351 compiled before the main molecular analyses of seed plant phylogeny (pre-2000) and  
352 afterwards (i.e., Doyle 2006 and Hilton and Bateman 2006). These two matrices, which  
353 both used Doyle (1996) as a starting point but were modified independently, with only  
354 limited discussion at later stages of the two projects, and made different choices regarding  
355 character coding, taxon sampling, and splitting of higher-level taxa, both show a very  
356 similar pattern. If under the MP criterion an anthophyte topology was more parsimonious,  
357 although without significant support, the Bayesian criterion favors a grouping of Gnetales  
358 and conifers. This phenomenon was already reported by Mathews et al. (2010), who  
359 reanalyzed the matrix of Doyle (2008) using BI, but their result passed mostly unnoticed.  
360 The matrices descended from Doyle (2006) (i.e., Doyle 2008) and Hilton and Bateman  
361 (2006) (i.e., Rothwell et al. 2009, 2016) exhibit a similar pattern.

362       Examination of the behavior of characters on anthophyte and Gnetales-conifer  
363 trees illustrates how changes in character analysis between the studies of Doyle (1996)  
364 and Doyle (2006, 2008) increased support for Gnetales-conifer trees. Some changes were  
365 the result of doubts concerning the homology of anthophyte characters. For example,  
366 character 14 of Doyle (1996), which contrasted the absence of a tunica layer in the apical

367 meristem in cycads, *Ginkgo*, and most conifers with its presence in Gnetales, angiosperms,  
368 and Araucariaceae, underwent one less step on anthophyte trees. However, the tunica  
369 consists of one layer of cells in Gnetales, but two layers in angiosperms, suggesting that it  
370 may not be homologous in the two groups. Doyle (2006, 2008) therefore split presence of  
371 a tunica into two states, and the resulting character (4) underwent the same number of  
372 steps with Gnetales in both positions. The same is true for redefinition of the megaspore  
373 membrane character (120), from thick vs. reduced to present vs. absent; the megaspore  
374 membrane is thin in Gnetales, but absent in angiosperms, *Caytonia*, and probably  
375 Bennettitales. Other changes involved newly recognized conifer-like features of Gnetales.  
376 For example, Doyle (2006, 2008) added a character for presence of a torus in the pit  
377 membranes of xylem elements in conifers and Gnetales (character 12, based on Carlquist  
378 1996) and rescored Gnetales as having a tiered proembryo (character 130), as in conifers;  
379 both characters undergo one less step on Gnetales-conifer trees than on most anthophyte  
380 trees (except some with major rearrangements elsewhere in seed plants). Doyle (1996)  
381 scored Gnetales as having as pinnate/paddle-shaped microsporophylls (character 37,  
382 state 0), which favored an anthophyte tree by one step, but when Doyle (2008) rescored  
383 microsporophylls in Gnetales as simple and one-veined (character 55, state 1), as in  
384 conifers, based on developmental studies by Mundry and Stützel (2004), the character  
385 favored the Gnetales-conifer topology by one or two steps. The shift of Gnetales away  
386 from angiosperms and towards conifers in the morphospace analyses based on Doyle  
387 (1996) and Doyle (2008) (Fig. 5a, b) is presumably the result of these changes in  
388 character analysis.

389         These trends show that reconsideration of potentially convergent characters  
390 between angiosperms and Gnetales and recognition of previously overlooked similarities  
391 between Gnetales and conifers succeeded in generating a matrix containing a signal that  
392 agreed with the molecular signal associating Gnetales with extant conifers. This result



393 clearly contradicts the view that morphology and molecules are in strong conflict with each  
394 other (Bateman et al. 2006, Rothwell et al. 2009) and validates the arguments to this effect  
395 advanced by Doyle (2006, 2008) on a parsimony basis. Indeed, in all post-2000 matrices a  
396 topology with Gnetales linked with conifers requires the addition of only a few steps to the  
397 length of the anthophyte trees, and in the Doyle (2008) matrix both topologies became  
398 equally parsimonious. The common focus on the MP consensus tree and the lack of  
399 exploration of almost equally parsimonious alternatives may have tended to inflate the  
400 perceived conflict between molecules and morphology (e.g., Rothwell et al. 2009). Our  
401 analyses show that the signal retrieved using MP is more correctly characterized as  
402 ambiguous.

403         On the other hand, our BI analyses of all post-2000 matrices converge on a similar  
404 result. The placement of Gnetales in an extended coniferophyte clade including  
405 Ginkgoales, cordaites, and extant and extinct conifers becomes favored in all BI analyses,  
406 with stronger support obtained in analyses with gamma rate variation among sites  
407 implemented in the model. A signal for linking Gnetales and angiosperms in an anthophyte  
408 clade seems to be much weaker, especially compared with the results of the MP analyses.  
409 The presence of a coherent signal in the BI analyses of post-2000 morphological matrices  
410 of seed plants favoring the placement of Gnetales in or near conifers has interesting  
411 implications regarding stem relatives of the angiosperms. Indeed, most post-2000 matrices  
412 are broadly congruent in attaching *Pentoxylon*, glossopterids, Bennettitales, and *Caytonia*  
413 to the stem lineage of the angiosperms (Fig. 3).

414

#### 415 *Parsimony and Bayesian inference perform differently with seed plant datasets*

416         Our results also add new empirical evidence on the debate concerning the  
417 usefulness of morphological data in reconstructing phylogenetic relationships, as well as  
418 discussion of the best method to analyze such data (Wright and Hillis 2014; O'Reilly et al.

419 2016; Puttick et al. 2017). One of the causes of the incompatibility between MP and BI  
420 could be the presence of long branches in the tree, which could lead to LBA phenomena  
421 (Felsenstein 1978; Bergsten 2005). Analyses based on simulated matrices and real data  
422 have repeatedly shown that probabilistic, model-based approaches are more robust to  
423 LBA than MP (Swofford et al. 2001; Brinkmann et al. 2005, and references therein). The BI  
424 trees show that both angiosperms and Gnetales are situated on very long morphological  
425 branches, especially in the post-2000 matrices. After following some of the suggestions by  
426 Bergsten (2005) and other methodologies (Rota Stabelli et al. 2011), we conclude that  
427 LBA is responsible at least in part for the continuing support for the anthophyte clade in  
428 MP analyses of the post-2000 matrices. We base this conclusion on several lines of  
429 evidence. First, BI recovers a Gnetales-conifer topology with higher probability than a  
430 topology with Gnetales in anthophytes, thus favoring a topology that separates the long  
431 branches over a topology that unites them. Second, more complex and better-fitting  
432 models recover a higher posterior probability for the topology in which angiosperms and  
433 Gnetales are separated (Figs. 2, 3). Third, removing Gnetales or angiosperms results in a  
434 rearrangement of the MP topologies in which the other long branch “flies away” from its  
435 original position. Fourth, support for the Gnetales plus angiosperms increases with  
436 decreased taxon sampling on the branch leading to the angiosperms (Fig. 4g-i). However,  
437 relationships in many other parts of the trees obtained with MP and BI are similar,  
438 suggesting that MP is not necessarily misleading where long branch effects are lacking. To  
439 our knowledge, this represents the first reported case of LBA in a morphological analysis  
440 that is supported by multiple tests (Bergsten 2005), with much stronger support than  
441 previously reported cases (Lockhart and Cameron 2001; Wiens and Hollingsworth 2000).  
442 The nature of this phenomenon can be easily visualized using a principal coordinates  
443 analysis, where the presumed close relationship between Gnetales and conifers and the  
444 convergence of the former with the angiosperms are effectively congruent with the

445 positions of the three taxa in the plot of the first two PCO axes (Fig. 5). Such a tool could  
446 represent an interesting option for exploring the structure of the data in future phylogenetic  
447 analyses.

448 In conclusion, our analyses show that morphological data agree in broad lines with  
449 the results of the molecular analyses regarding the position of the Gnetales in seed plant  
450 phylogeny. This strongly suggests that morphology carries a phylogenetic signal that is  
451 consistent with molecular data, and may therefore be useful in reconstructing other  
452 aspects the phylogenetic history of the seed plants, especially the position of fossils  
453 relative to living taxa. The supposed conflict between the two sorts of data on the  
454 phylogeny of seed plants (Bateman et al. 2006; Rothwell et al. 2009) seems therefore less  
455 deep than previously thought, and due partially to methodological issues. Since data from  
456 the fossil record are particularly important for resolving the evolutionary history of seed  
457 plants, because of the wide gaps that separate extant groups and the potential biases in  
458 analysis of such sparsely sampled taxa (Burleigh and Mathews 2007; Mathews 2009;  
459 Magallón et al. 2013), our results give new hope for the possibility of integrating fossils and  
460 molecules in a coherent way. This is even more important in light of new fossil discoveries  
461 (e.g., Rothwell and Stockey 2013, 2016) and the reconstruction of new species-level taxa  
462 that show similarities to fossils previously associated with angiosperms (e.g., the Triassic  
463 *Petriellaea* plant, which shares leaf and cupule features with *Caytonia*: Bomfleur et al.  
464 2014).

465 Another aspect that emerges from our study is the importance of signal dissection  
466 in all phylogenetic analyses involving morphology. Although most phylogenetic analyses  
467 based on morphology are still conducted in a parsimony framework, some authors have  
468 already underlined the potential of model-based approaches in this field (Lee and Worthy  
469 2012; Lee et al. 2014). Our analyses show that BI yields more robust results under  
470 different taxon sampling strategies, and is particularly promising for correcting errors due

471 to long branch effects. Our study converges with previous work indicating that the use of  
472 model-based techniques could allow the successful integration of taxa with a high  
473 proportion of missing data (Wiens 2005; Wiens and Tiu 2012), which would be extremely  
474 useful given the nature of the paleobotanical record.

475

## 476 **SUPPLEMENTARY MATERIAL**

477 The supplementary material is available as an online appendix.

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720

721 **Figure 1:** a) Relationships among extant seed plants. On the left an anthophyte topology,  
722 and on the right a Gnetales-conifer topology. Relationships between Cycadales and  
723 *Ginkgo* vary among analyses of both sorts. b) Relationships among the matrices  
724 reanalyzed in this paper.

725 **Figure 2:** Support for the anthophytes or Gnetales-conifers in the different matrices and  
726 using different methods. Solid lines represent BI results, dashed lines results from the  
727 MP analysis.

728 The difference between the pre-2000 and post-2000 matrices is clearly underlined by a  
729 shift in support from anthophytes to Gnetales-conifers in the BI analyses, and a drop in  
730 support for the anthophytes in the MP analyses.

731 **Figure 3:** Split network consensus of the posterior tree sample of the BI analysis of the  
732 Rothwell and Stockey (2016) matrix using gamma-distributed rate variation. Only splits

733 with more than 0.15 pp are shown, and support is shown only for splits with more than  
734 0.50 pp.

735 **Figure 4:** a-c) Scheme of the long branch attraction tests; a and b represent the long  
736 branch extraction experiment, c represents the branch elongation experiment. Null  
737 hypotheses are in the right upper corner. d-f) Results of the LBE experiment on the  
738 Rothwell et al. (2009) matrix. All trees are MP consensus trees. Fossil taxa diverging  
739 below the most recent common ancestor of extant seed plants removed for ease of  
740 comparison. d) Untrimmed matrix, showing an anthophyte topology and paraphyletic  
741 conifers. e) Angiosperm removal matrix, showing Gnetales nested in the conifers and  
742 other anthophytes removed from the coniferophyte clade. f) Gnetales removal matrix,  
743 with monophyletic conifers nested in a large coniferophyte clade. g-i) Results of the BE  
744 and EX experiment. g) Results of the MP analyses. h-i) Results of the BI analyses  
745 under the Markov k-states (Mk) model (Lewis 2001) with equal rates (h) and with  
746 gamma-distributed rate variation (i).

747 **Figure 5:** Plot of the first two principal coordinate axes for four of the matrices analyzed.  
748 The first PCO axis mainly separates the angiosperms and the other seed plants, while  
749 the second PCO axis separates more conifer-like and more fern-like groups. These  
750 plots illustrate the effect of the reassessment of gnetalean characters between the two  
751 Doyle matrices (a, b), and the similar structure of the data in the Hilton and Bateman  
752 (2006) (c) and Rothwell and Stockey (2016) (d) matrices.

753

754 **Table 1.** Statistics for the maximum parsimony analyses of fossil matrices.

	<i>Number of trees</i>	<i>Length</i>	<i>Ci</i>	<i>Ri</i>
<b>Crane 1985</b>	8	50	0.600	0.730
<b>Doyle and Donoghue</b>	36	123	0.504	0.674

<b>1986</b>				
<b>Doyle and Donoghue</b>	94	112	0.545	0.658
<b>1992</b>				
<b>Nixon et al. 1994</b>	225	332	0.392	0.788
<b>Rothwell and Serbet</b>	8	191	0.529	0.721
<b>1994</b>				
<b>Doyle 1996</b>	123	247	0.494	0.782
<b>Hilton and Bateman</b>	480	313	0.457	0.801
<b>2006</b>				
<b>Doyle 2006</b>	8	321	0.514	0.753
<b>Doyle 2008</b>	16	346	0.503	0.744
<b>Rothwell et al. 2009</b>	66	330	0.503	0.776
<b>Rothwell and Stockey</b>	6	363	0.466	0.754
<b>2016</b>				

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759 **Table 2: Results from the MP analysis of constrained Gnetales-conifer trees**

	<i>Length unconstrained</i>	<i>Length Gnetales+Conifer</i>	<i>Length difference</i>	<i>Templeton Test p- value (Best value)</i>
<b>Crane 1985</b>	50	54	4	0.1573
<b>Doyle and Donoghue 1986</b>	123	130	7	0.1266
<b>Doyle and Donoghue 1992</b>	112	118	6	0.1088



<b>Nixon et al. 1994</b>	332	348	16	0.0131*
<b>Rothwell and Serbet 1994</b>	191	197	6	0.2252
<b>Doyle 1996</b>	247	257	10	0.0679
<b>Hilton and Bateman 2006</b>	313	317	4	0.4595
<b>Doyle 2006</b>	321	322	1	0.8474
<b>Doyle 2008</b>	346	346	0	0.9888
<b>Rothwell et al. 2009</b>	330	334	4	0.3458
<b>Rothwell and Stockey 2016</b>	363	369	6	0.1336

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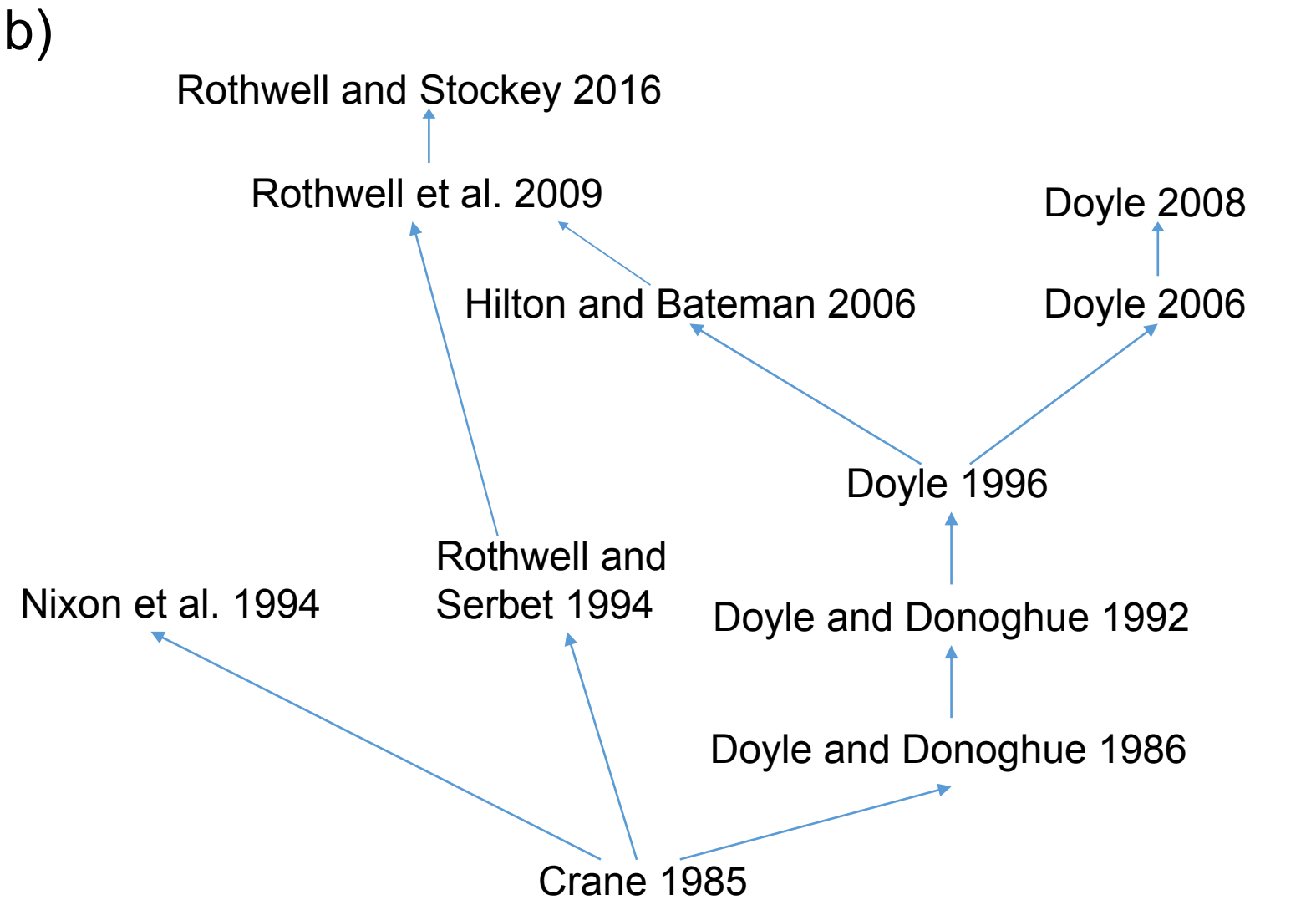
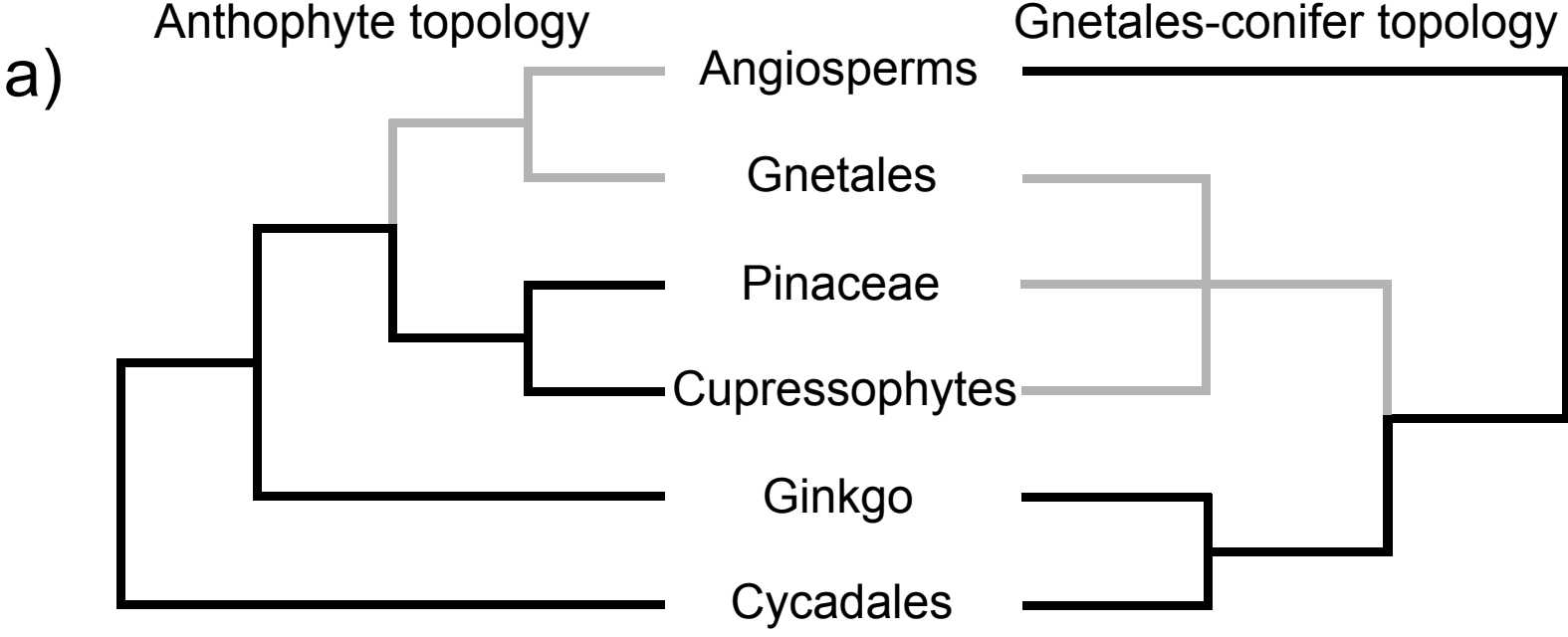
762 **Table 3.** Model-testing statistics for the Bayesian inference analyses.

	$Mk_{prinf}$	$Mk_{prinf} + \Gamma$	$\ln BF$	$2 \times \ln BF$
<b>Crane 1985</b>	-223.03	-223.01	0.02	0.04
<b>Doyle and Donoghue 1986</b>	-473.68	-473.70	-0.02	-0.04
<b>Doyle and Donoghue 1992</b>	-432.38	-431.00	1.38	2.76
<b>Rothwell and Serbet 1994</b>	-861.53	-854.14	7.39	14.78
<b>Nixon et al. 1994</b>	-1555.76	-1538.27	17.49	34.98
<b>Doyle 2006</b>	-1383.60	-1365.27	18.33	36.66
<b>Hilton and Bateman 2006</b>	-1559.87	-1532.70	27.17	54.34

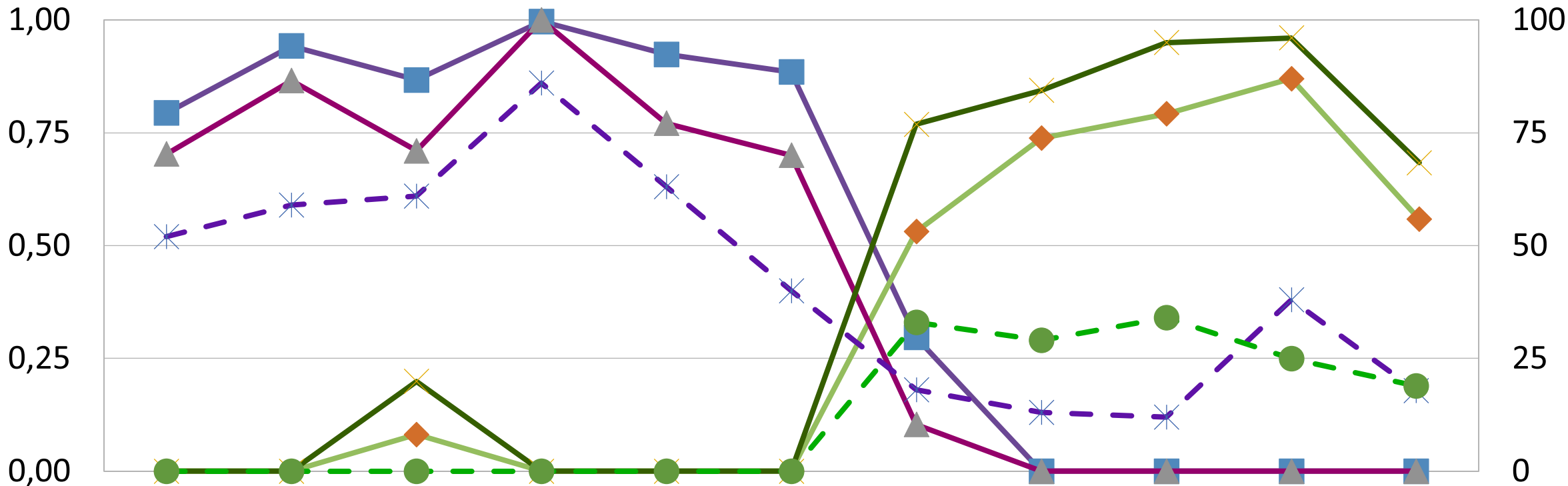
<b>Doyle 2008</b>	-1481.46	-1455.09	26.37	52.74
<b>Rothwell et al. 2009</b>	-1541.68	-1527.09	14.59	29.18
<b>Rothwell and Stockey 2016</b>	-1511.73	-1493.78	17.95	35.90

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Posterior Probability



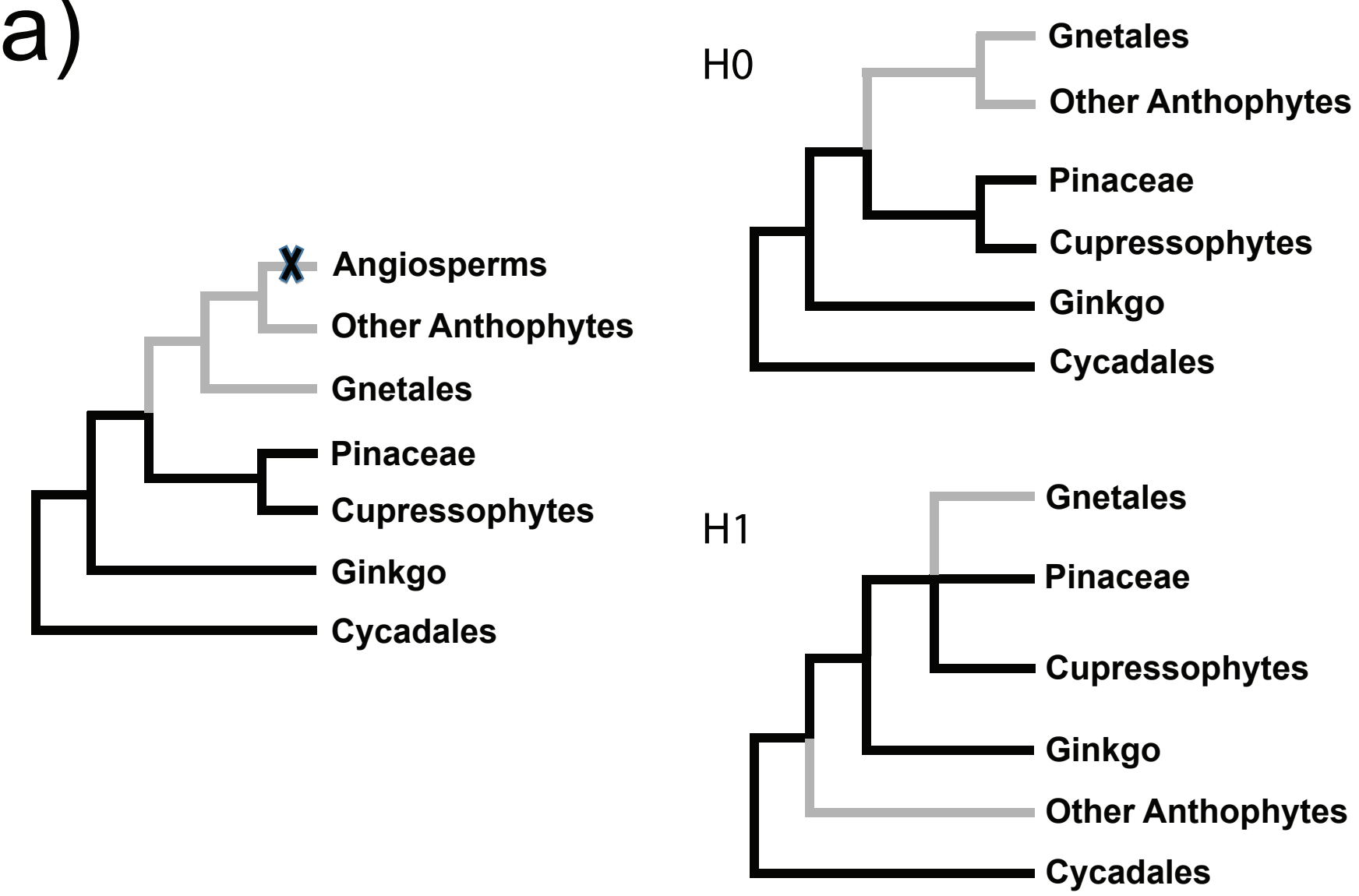
Parsimony Bootstrap

Crane 1985  
Doyle and Donoghue 1986  
Doyle and Donoghue 1992  
Nixon et al.1994  
Rothwell and Serbet 1994  
Doyle 1996  
Hilton and Bateman 2006  
Doyle 2006  
Doyle 2008  
Rothwell et al.2009  
Rothwell and Stockey 2016

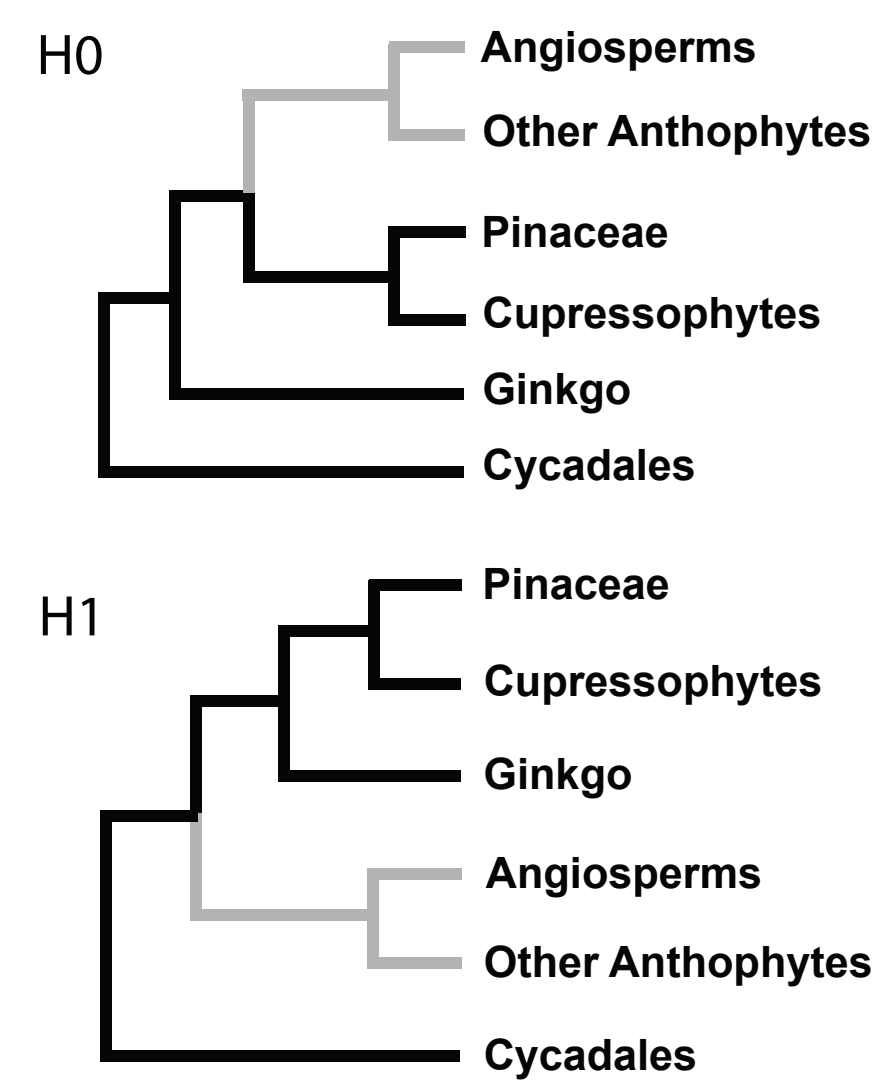
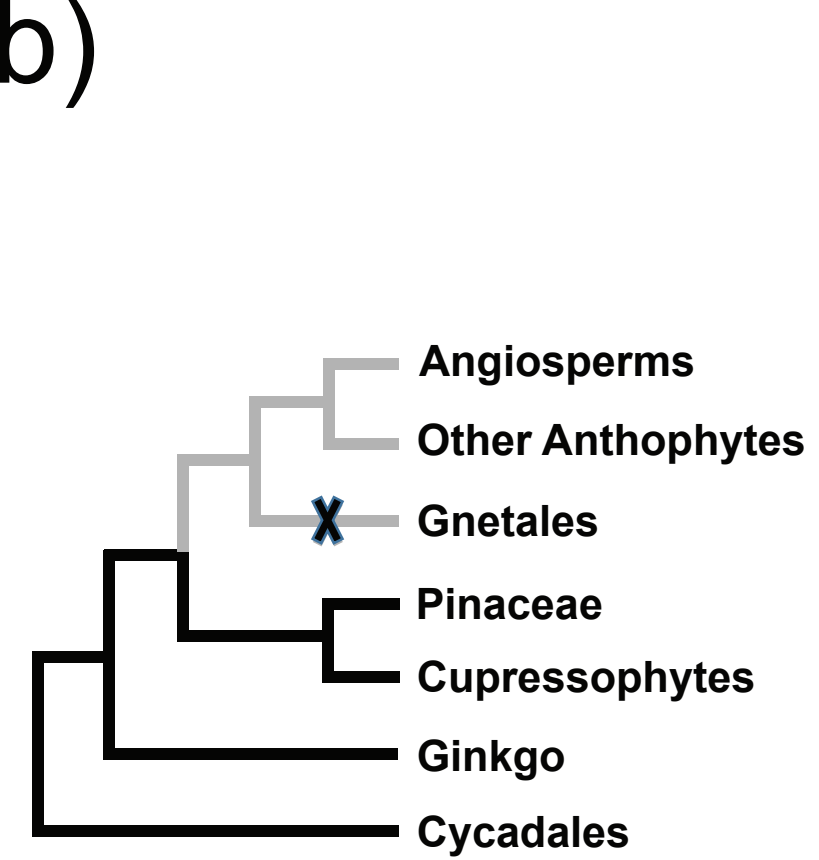
- Anthophyte pp equal
- Anthophyte pp gamma
- Gnetifer pp equal
- Gnetifer pp gamma
- Anthophyte MP bootstrap
- Gnetifer MP bootstrap



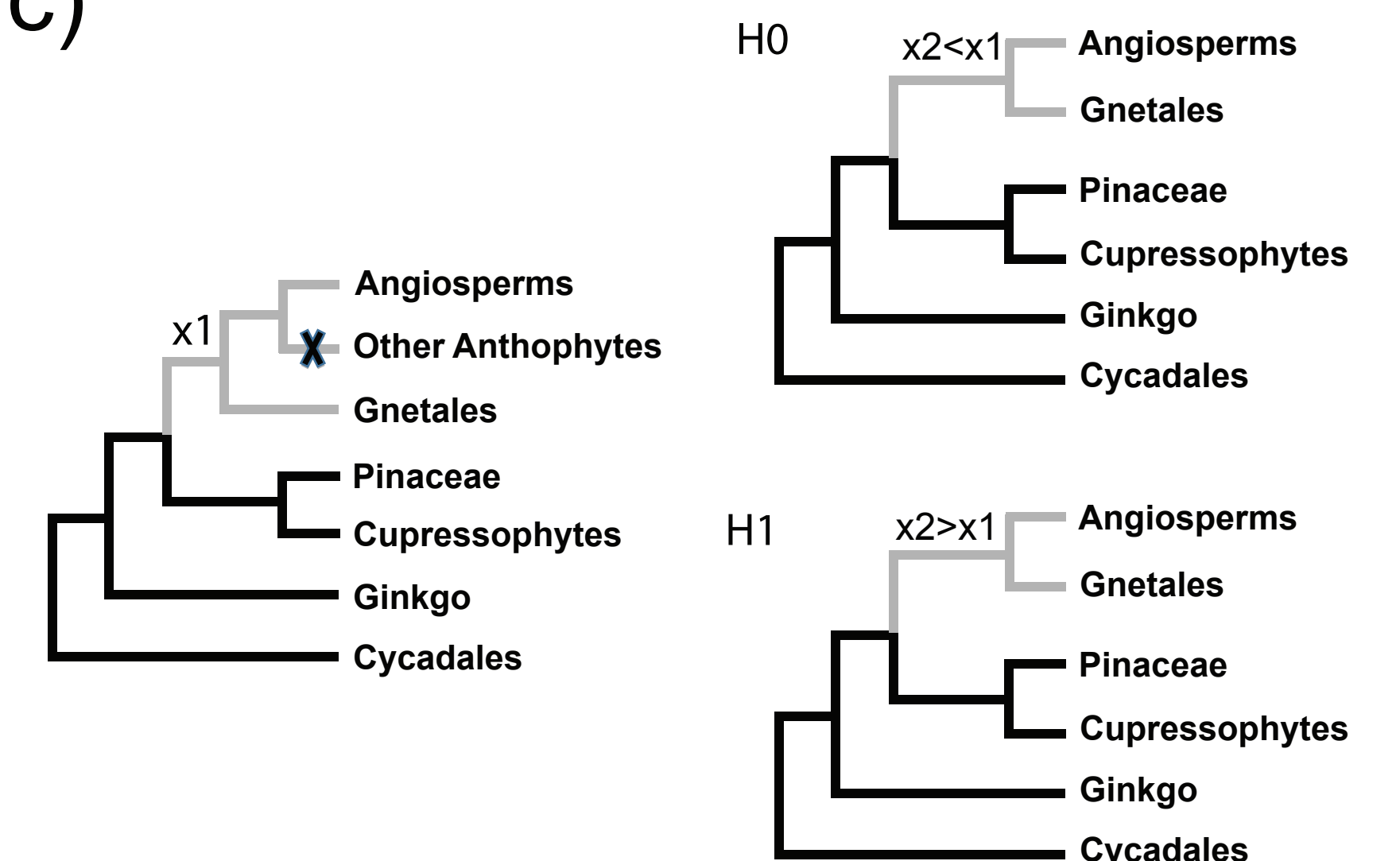
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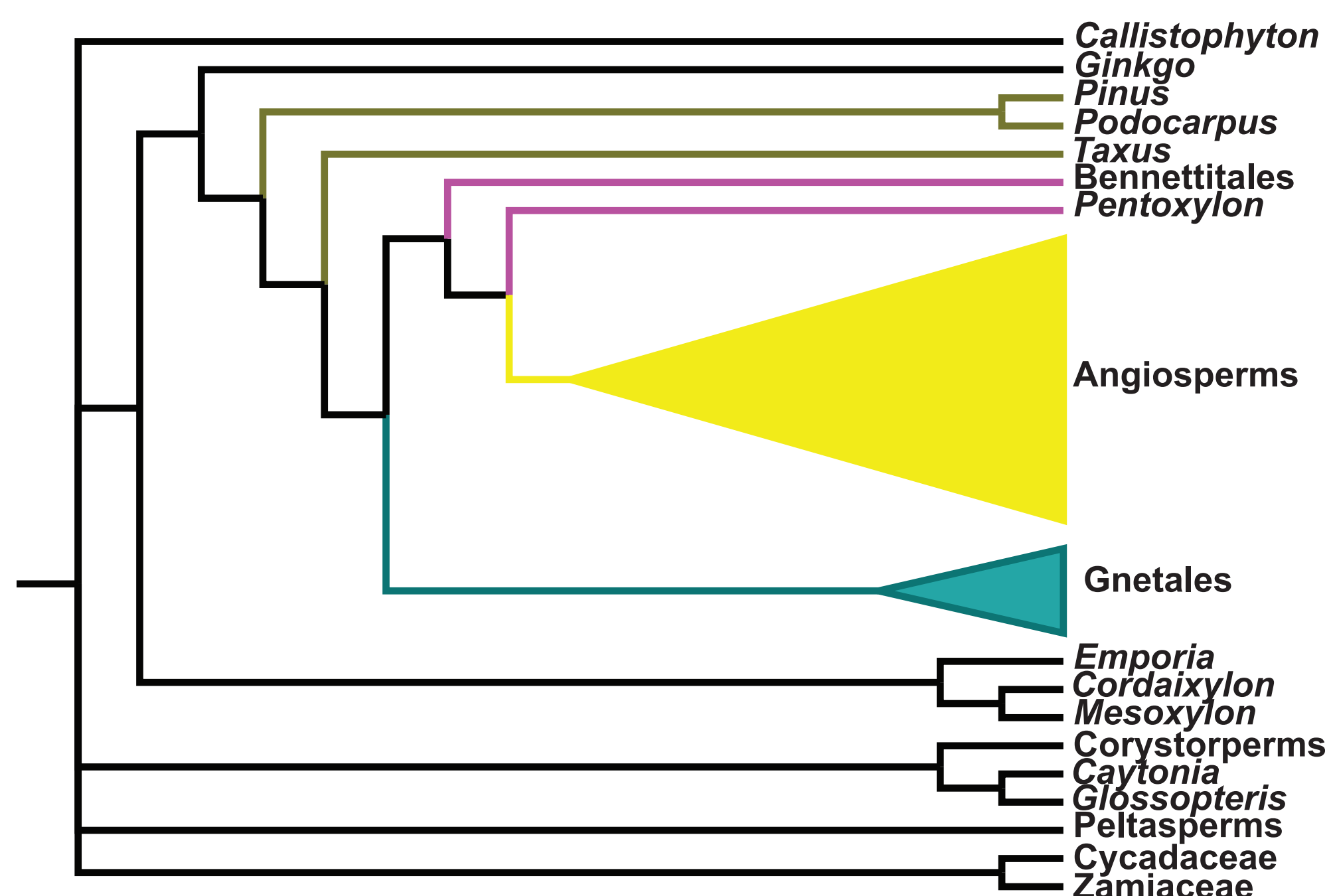
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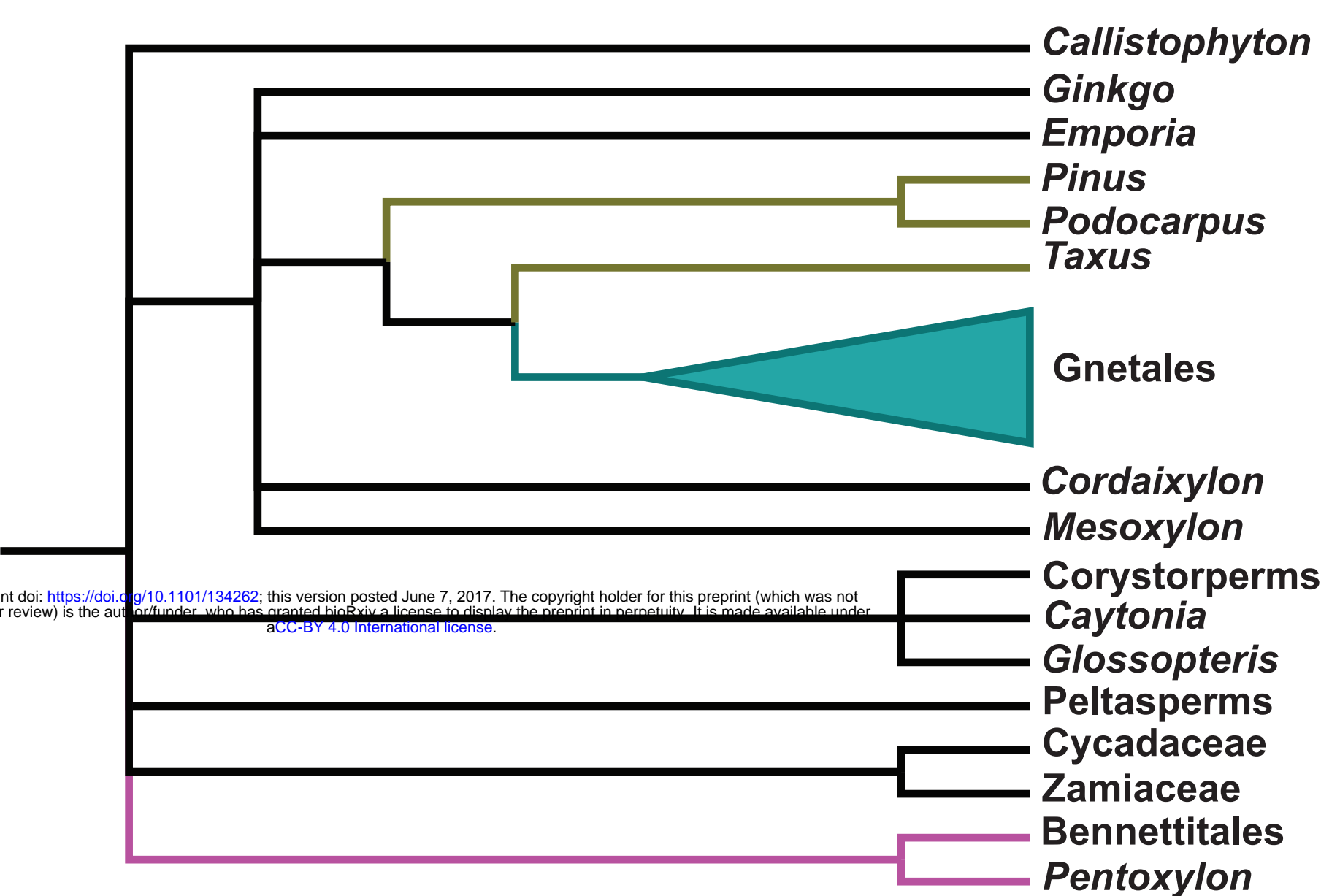
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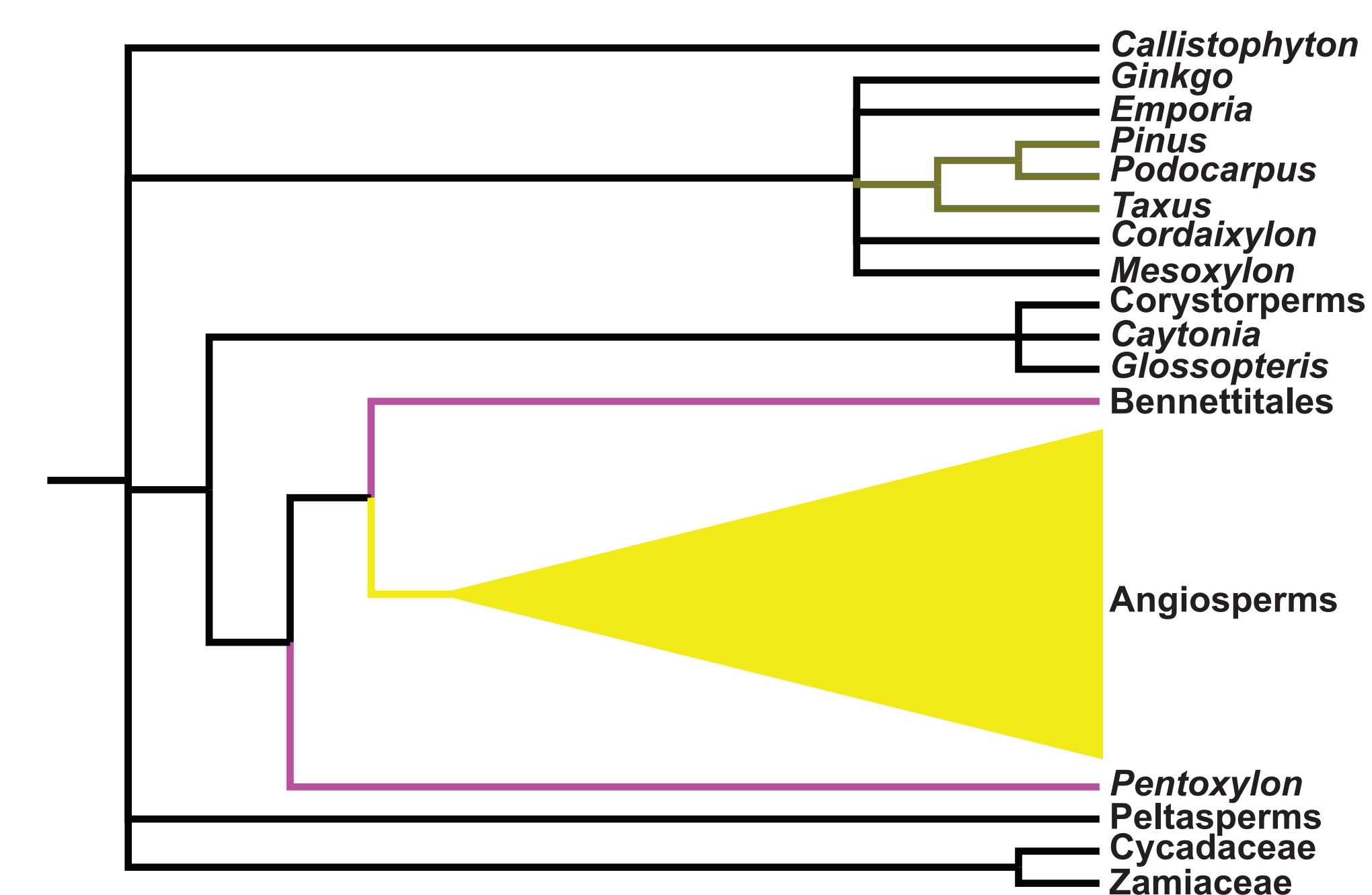
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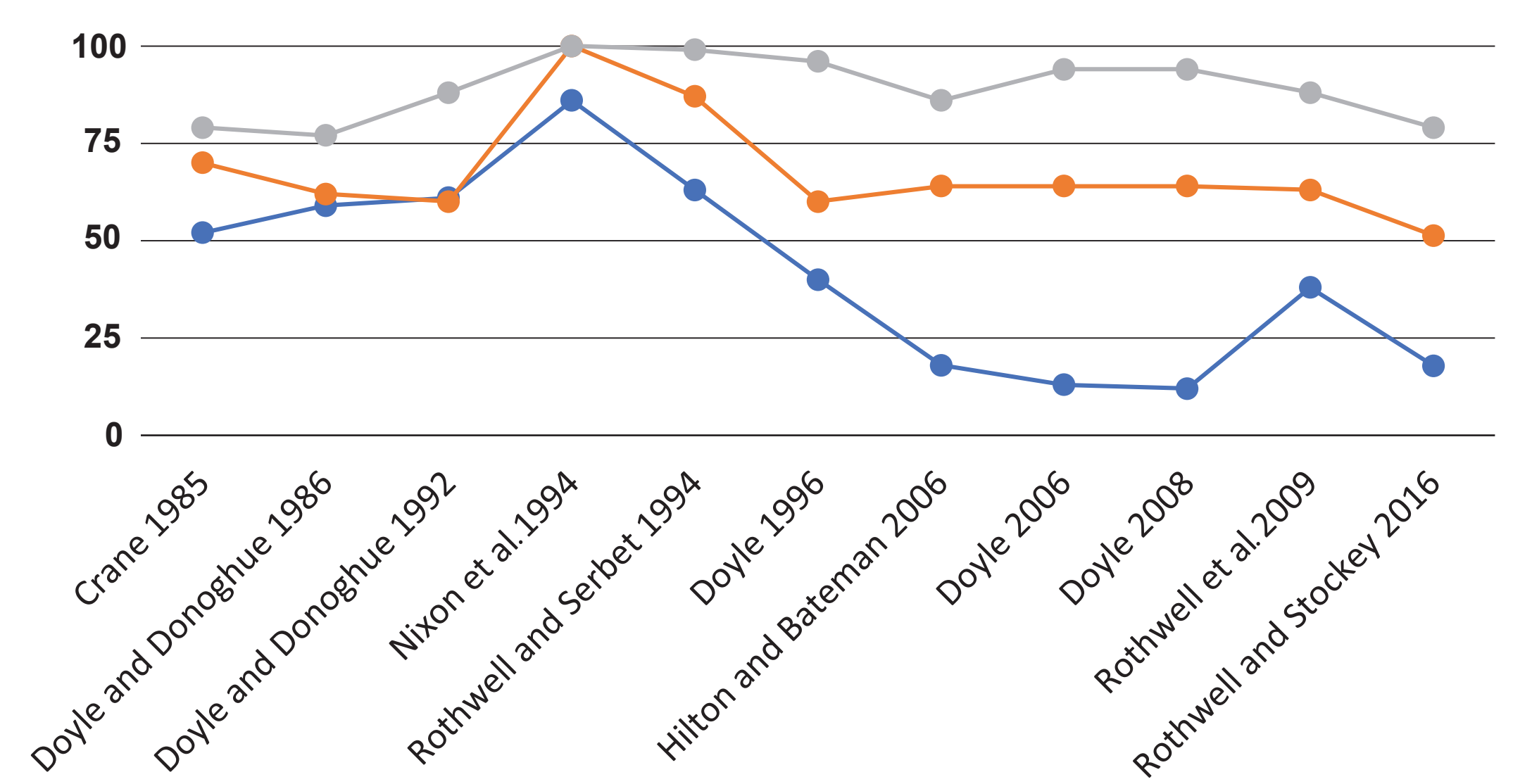
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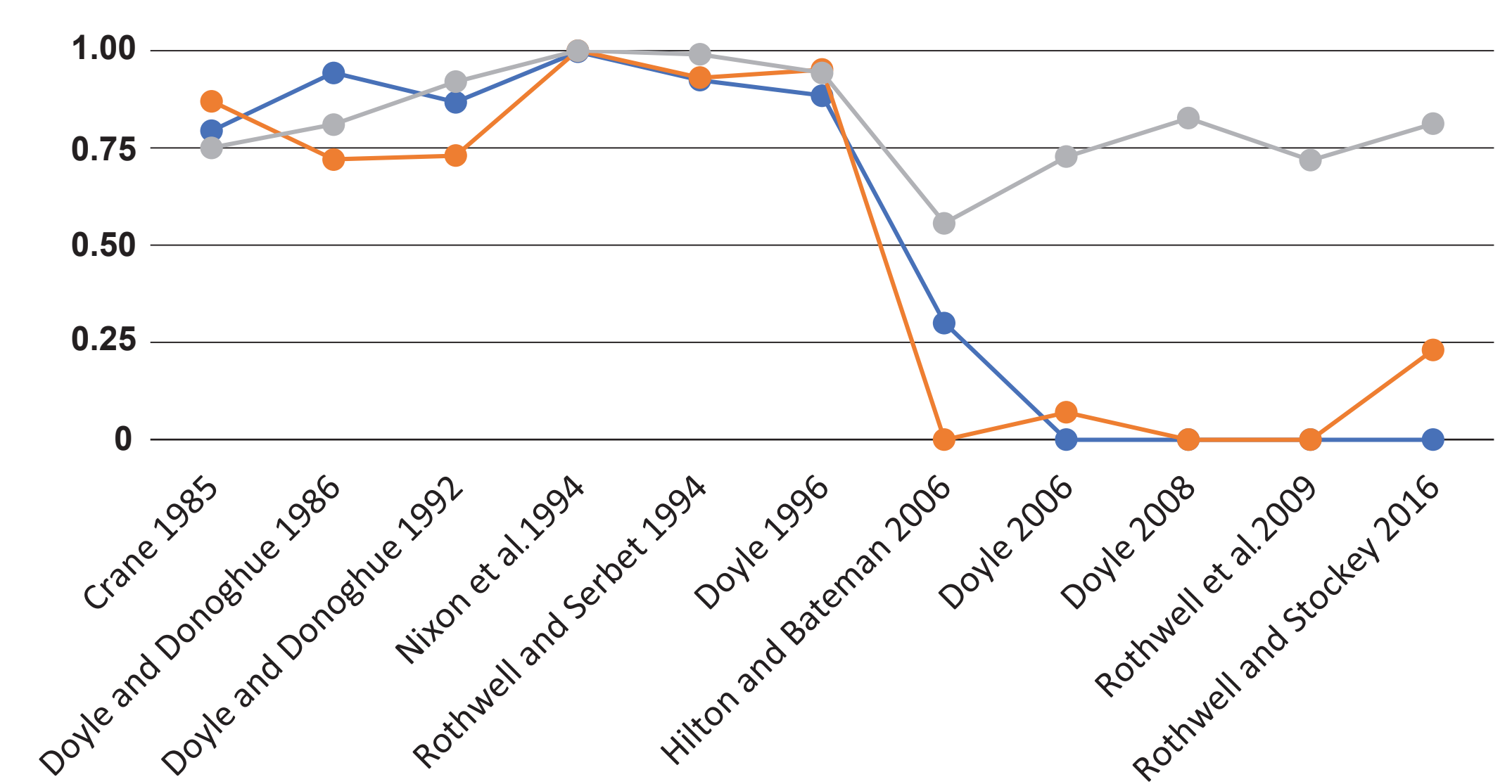
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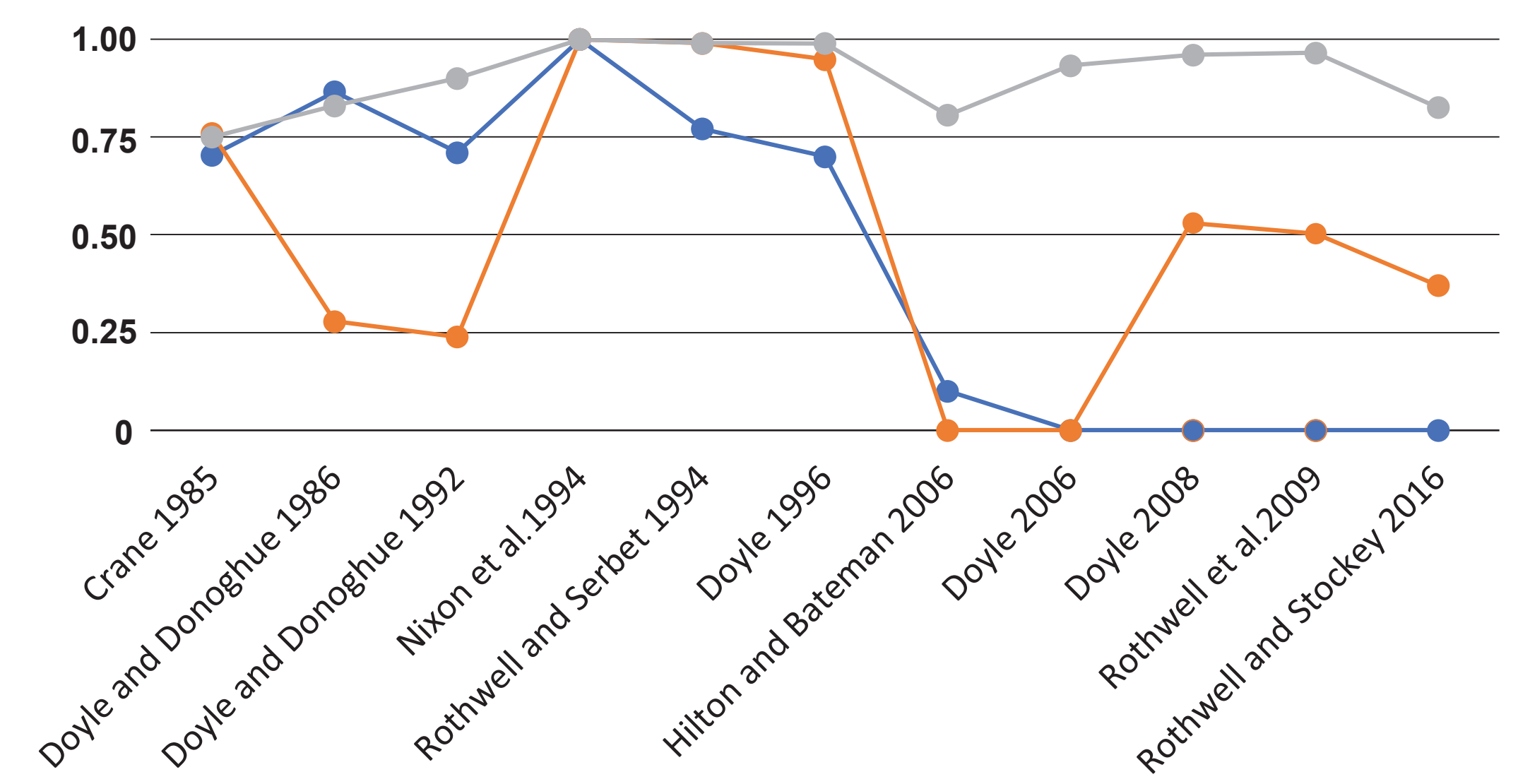
g)



h)



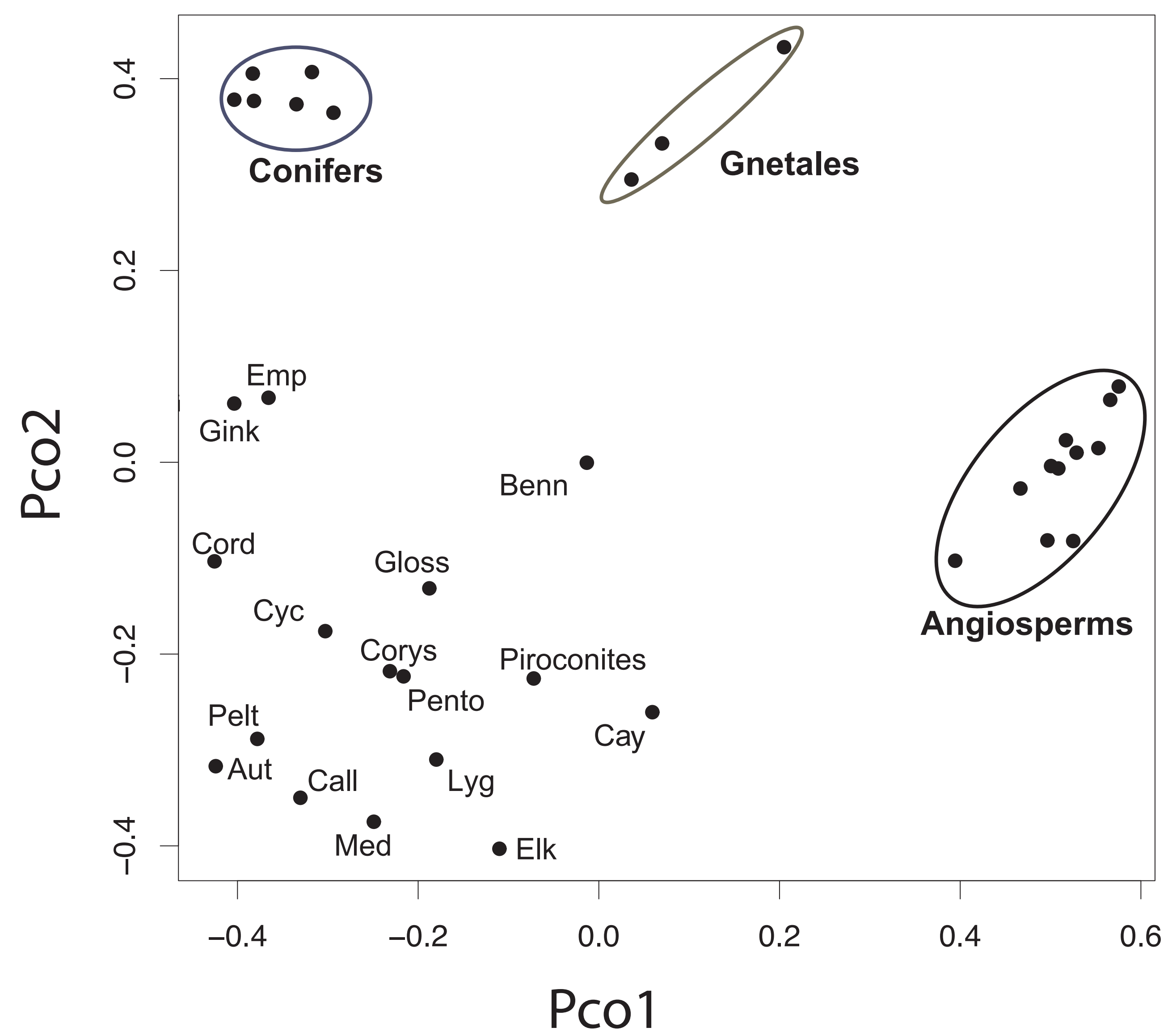
i)



● Total matrix  
● Branch extraction  
● Extant only

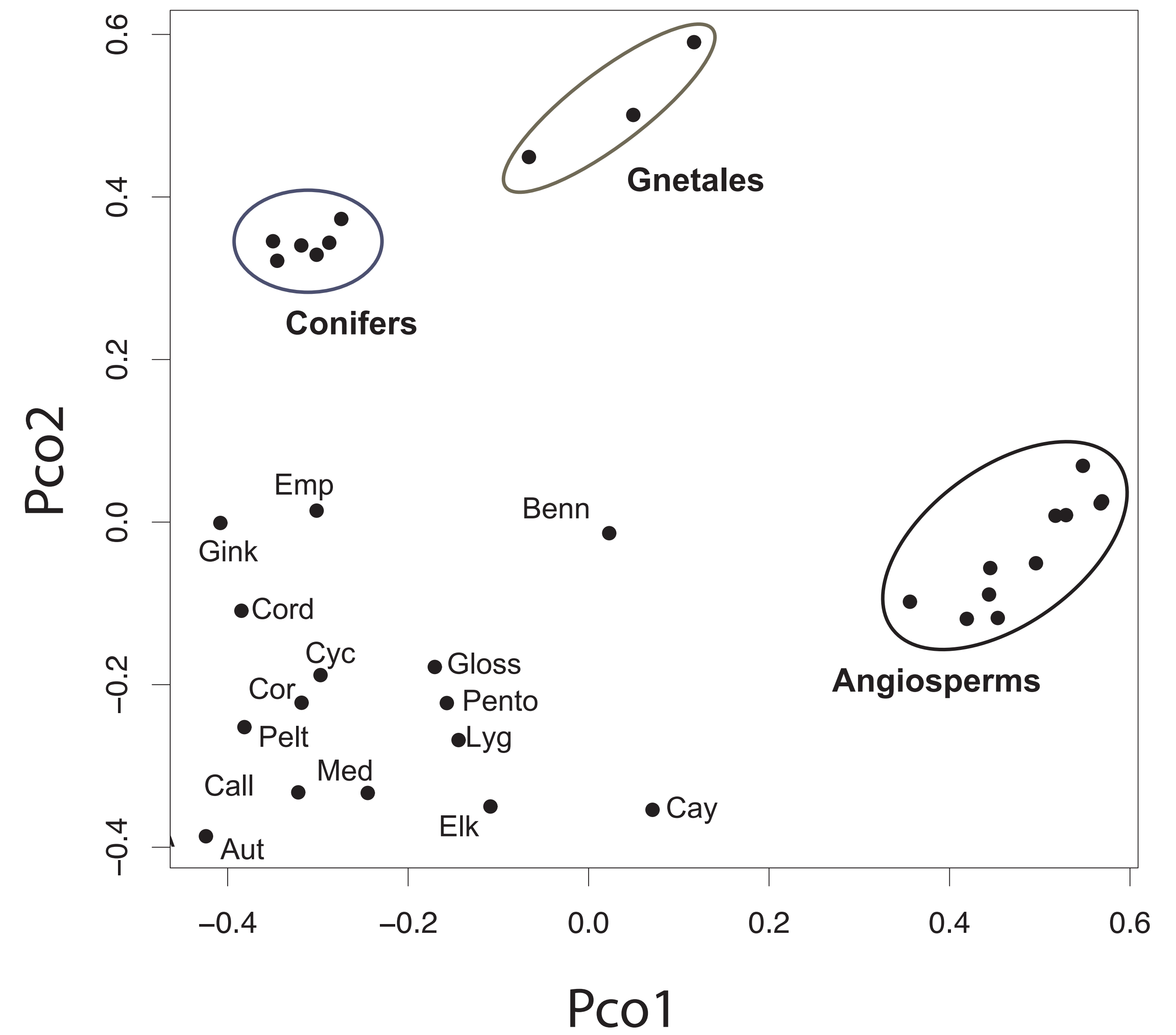
a)

Doyle 1996



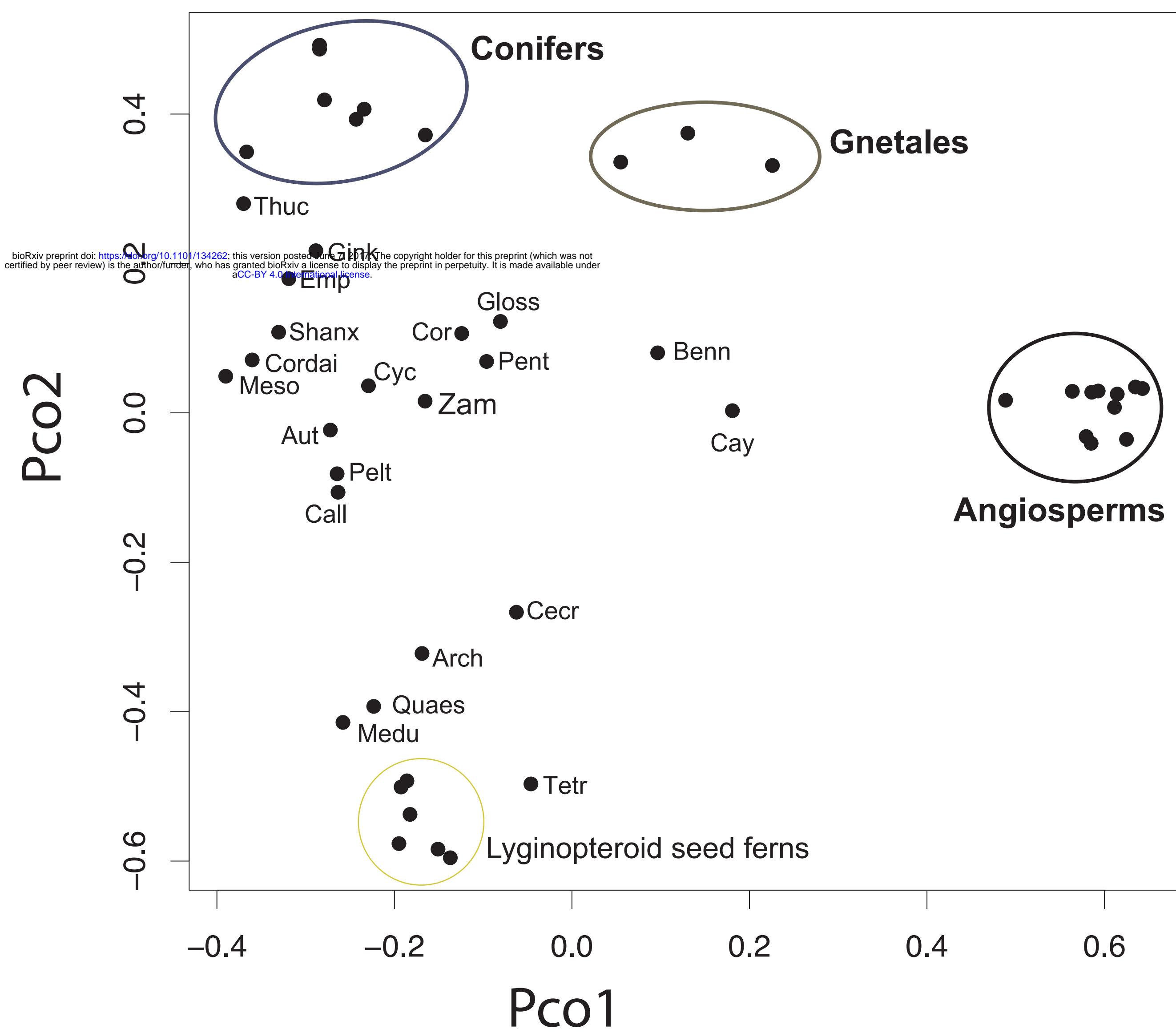
b)

Doyle 2008



c)

Hilton and Bateman 2006



d)

Rothwell and Stockey 2016

