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2 Evolution of transmissible spongiform encephalopathy and the prion 3 protein gene (*PRNP*) in mammals

4

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23 # Abstract

24 Wildlife managers are concerned with transmissible spongiform encephalopathies (TSEs) as they
25 are currently incurable, always fatal, and have the potential to cross species boundaries.
26 Although a wide range of mammals exhibit TSEs, it is currently unclear whether they are
27 evolutionarily clustered or if TSE+ species are randomly distributed phylogenetically. We tested
28 whether mammalian species with TSEs are phylogenetically underdispersed on a tree derived
29 from 102 PRNP sequences obtained from the Orthologous Mammalian Markers database. We
30 determined that the PRNP tree was topologically congruent with a species tree for these same
31 102 taxa constructed from 20 aligned gene sequences, excluding the PRNP sequence. Searches
32 in Google Scholar were done to determine whether a species is known to have expressed a TSE.
33 TSEs were present in a variety of orders excluding Chiroptera, Eulipotyphyla, and Lagomorpha
34 and no marine mammals (Artiodactyla) were recorded to have a TSE. We calculated the
35 phylogenetic signal of binary traits (D-Value) to infer if the phylogenetic distribution of TSEs
36 are conserved or dispersed. The occurrence of TSEs in both trees is non-random (Species tree
37 D-value = 0.291; PRNP tree D-value = 0.273), and appears to have arisen independently in the
38 recent history of different mammalian groups. Our findings suggest that the evolution of TSEs
39 develops in groups of species irrespective of PRNP genotype. The evolution of TSEs merits
40 continued exploration at a more in-depth phylogenetic level, as well as the search for genetic
41 combinations that might underlie TSE diseases.

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45 # Introduction

46 Wildlife managers are concerned with transmissible spongiform encephalopathies (TSEs)
47 as they are currently incurable, always fatal, and have the potential to cross species boundaries.
48 Known TSEs include chronic wasting disease (CWD) in cervids, scrapie in sheep, bovine
49 spongiform encephalopathy (BSE, also known as mad cow disease), transmissible mink
50 encephalopathy (TME), feline spongiform encephalopathy (FSE) and Creutzfeld-Jacob in
51 humans [1-3]. In response to health concerns of livestock and humans, research has focused on
52 learning how species contract TSEs, how they are spread, causes of immunity, and prevention or
53 cures [4].

54 Most researchers accept the hypothesis that resistance to TSEs in mammals results from
55 certain genotypes found at the highly conserved prion protein gene (PRNP)[5] . TSEs are
56 thought to be caused by the misfolding of the host's prion protein (*PrP*) whose primary
57 physiological function is not entirely clear. When correctly folded the prion protein has been
58 theorized to localize at synaptic membranes and be related to normal synaptic functioning, signal
59 transduction, and copper binding [6-9]. When misfolded the protein induces other prion proteins
60 to misfold as well, followed by ultimately fatal accumulation in the central nervous system
61 within the host [1, 5, 10]. Misfolded prion proteins can be spontaneously generated [4, 11] or
62 introduced to the host by inoculation from the environment or through direct contact with
63 infected individuals [5, 12]. Differences in mammalian prion proteins might reduce
64 transmissibility between species because they function as a species barrier [5, 13].

65 A wide range of mammalian species exhibit TSEs, and it is currently unclear whether
66 they are evolutionarily clustered, or whether TSE+ species are randomly distributed
67 phylogenetically. If a species barrier inhibits horizontal transfer of TSEs, one might predict that

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68 related species would exhibit greater susceptibility to TSE expression. The reasoning for this
69 prediction is that phylogenetically more distant relatives would be less similar genetically and,
70 therefore, less susceptible to horizontal (cross-species) transmission. A phylogenetic test
71 involves constructing a tree from PRNP sequences and testing whether species with TSEs are
72 phylogenetically underdispersed, or clumped within clades [14]. In addition, because the PRNP
73 gene might be under strong selection, it is important to document that the PRNP tree was
74 topologically congruent with one that was not constructed with PRNP data. If the topology of
75 the two trees differ significantly, it would suggest that selection has constrained the evolution the
76 PRNP gene. If the presence of TSEs is phylogenetically clustered, and the two trees are more
77 similar than one would expect by chance, it can be inferred that some lineages are predisposed,
78 perhaps by their genetics, to acquiring this class of diseases. If TSEs are phylogenetically
79 dispersed, and the two trees are similar, it would suggest that factors other than shared history
80 explain the distribution of TSEs in mammalian taxa. Therefore, we have two objectives: 1)
81 Determine if a mammalian species tree and PRNP gene tree have similar topologies and, 2)
82 Determine if the presence of TSEs are phylogenetically dispersed in a species tree.
83

84 Materials and methods

85 We used 102 aligned mammal sequences (Table S1) obtained from the Orthologous
86 Mammalian Markers database (OrthoMam)[15]. In all phylogenic analyses, the platypus
87 (*Ornithorhynchus anatinus*) was used as the outgroup. To determine whether a species is known
88 to have a TSE, searches in Google Scholar were done using the scientific and common name of
89 species combined with “TSE”, “transmissible spongiform encephalopathy”, “prion disease”,
90 “CWD”, “chronic wasting”, “BSE”, “bovine spongiform”, “FSE”, “feline spongiforme”, “MSE”,

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91 and "mink spongiform". Specific prion diseases were included in our search to broaden our list
92 of taxa (Table S1). Many species appeared to have ambiguous evidence for TSE presence (Table
93 S1), and we conservatively scored them as absent. Some species known to express TSEs lacked
94 gene sequences that would have permitted including them in the species tree (e.g., moose, *Alces*
95 *alces*, caribou, *Rangifer tarandus*, elk, *Cervus canadensis*, mule deer *Odocoileus hemonius*).

96 In addition to analyzing the aligned sequences available on Orthamam, we computed
97 alternative alignments on the nucleotide data. We aligned sequences three separate ways in
98 MEGA X [16] the default MUSCLE settings, the default MUSCLE settings followed with the
99 program Gblocks [17-18] under stringent conditions to eliminate poorly aligned positions, and
100 running the available Orthomam alignments only through Gblocks. The results of performing
101 these alignment methods are the same as using aligned Orthomam sequences as-is. In addition,
102 we constructed a phylogenetic tree using sequences of amino acids to determine if particular
103 protein structures were associated with TSE+ species.

104

105 Phylogeny construction

106 To construct a species tree independent of the PRNP gene, we selected 20 aligned gene
107 sequences of coding regions (Table S2) for 102 species of mammals spanning 20 orders, 58
108 families, and 85 genera, and for which evidence of TSE presence/absence was available (Table
109 S1). Using the aligned nucleotide coding regions, partitioned (by gene) analyses were run using
110 the Bayesian Evolutionary Analysis by Sampling Trees 2 (BEAST 2) package [19]. The
111 sequences were analyzed using the best fit model (HKY + G) identified using MEGA X [16].
112 We ran the analyses for 75,000,000 generations while sampling every 5,000 chains under a strict
113 clock model and Yule speciation model. The first 10% of sampled trees were discard as burn-in.

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114 Two independent runs were performed with these specifications, and log files were combined in
115 LogCombiner [20] to address low Effective Sample Size (ESS) values of parameters. Resulting
116 trees were re-rooted to the platypus and exported as Nexus and Newick files. The PRNP gene
117 tree was constructed using the same procedures as the species tree, with the best fit model
118 identified as TN93 + G + I. To construct the PRNP gene tree, analyses ran for 10,000,000
119 generations while sampling every 5,000 chains under a relaxed log normal clock model and Yule
120 model, along with three independent runs that were combined in LogCombiner [20]. The
121 phylogenetic analysis of amino acids residues, obtained from Orthomam, followed the same
122 protocol as the two preceding analyses. The amino acid PRNP gene tree had the same
123 specifications as the nucleotide species tree with the best fit model identified as JTT + G and had
124 three independent runs that were combined.

125 We mapped the presence or absence of TSE on the two trees using stochastic character
126 mapping [21], which samples character histories based on their posterior probability distribution;
127 we reconstructed the ancestral states using the equal rates model (run in Program R; version
128 3.5.2, R Development Core Team, 2018, Code S4). All analyses were done using the R package
129 *phytools* version 0.6-99 [22]. The function *cophylo* was used to compare the species and gene
130 tree. We also calculated the phylogenetic signal of binary traits (D-value) to infer if the
131 phylogenetic distribution of TSEs are conserved or dispersed [23]. D-values close to 0 are not
132 randomly distributed and are conserved, where if the value is close to 1 then the presence of the
133 state is considered randomly distributed on the tree.

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Results

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137 ## Basic genetic results

138 The number of aligned base pairs ranged from 324 (*Monodelphis domesticus*) to 783 (*Bos*
139 *taurus*, *Bos mutus*, *Bison bison*), and the total alignment included 861 base pairs, of which 486
140 were variable. Of the 287 total amino acids (no stop codons were noted), 166 were variable.
141 Nucleotide composition differed little between species with and without TSE (Table S3). No
142 amino acid positions separated TSE+ from TSE- species.
143

144 ## Species and PRNP trees

145 Most of the internal nodes in the species tree (Fig. 1) were well supported with posterior
146 probabilities over 0.90, with a few exceptions close to the terminal tips (Fig 1). The topology is
147 consistent with current taxonomy, at least to the extent that species from the same orders are
148 supported as clades. In contrast, the PRNP gene tree has relatively few strongly supported nodes
149 (Fig 1), although the topology is also consistent with current mammalian ordinal taxonomy.
150 Both trees are topologically congruent, with most of the discrepancies occurring at poorly
151 supported nodes deep in the trees (Fig 1). The tree constructed from amino acids (Fig 2) is
152 congruent with both the species and PRNP trees.
153

154 **Fig 1. Species Tree (left) and PRNP Gene Tree (right) Comparison using Nucleotides.**
155 Compiled using 20 autosomal genes and rooted with platypus (excluded from figure). Positive
156 TSE presence (red) and absence TSE (blue) shown at tips with orders that contain 2 or more
157 species labeled. Posterior probabilities less than 0.90 and stochastic character mapping
158 probabilities (as pies) displayed at nodes. Congruent tips are connected by solid lines, whereas
159 topological differences between the trees are connected by dashed lines.
160

161 **Fig 2. PRNP Gene Tree Created using Amino Acids.** Compiled using the translated PRNP
162 gene and rooted with platypus (excluded from figure). Positive TSE presence (red) and absence
163 TSE (blue) shown at tips with orders that contain 2 or more species labeled. Posterior

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164 probabilities less than 0.90 and stochastic character mapping probabilities (as pies) displayed at
165 nodes.
166

167 Reconstruction of TSE evolution

168 Because of the congruence of the two trees, we focused on the results from the species
169 tree. TSEs are present in a variety of orders excluding Chiroptera, Eulipotyphyla, and
170 Lagomorpha. No marine mammals (Artiodactyla) have been recorded to have a TSE. According
171 to the ancestral reconstruction, TSEs appear to have arisen relatively recently in TSE+ groups,
172 with the basal condition being absence of TSEs. The reconstruction of TSE evolution is also
173 notable in that there was only one hypothesized transition from TSE presence to absence
174 (Tibetan antelope, *Pantholops hodgsonii*). The presence of TSEs is non-random (D-value =
175 0.291), suggesting that TSE presence is relatively conserved. Therefore, the distribution of TSEs
176 is not randomly distributed across the phylogeny (Fig. 3). The results for the PRNP gene alone
177 were identical to the results inferred from the species tree.
178

179 **Fig 3. Density plot of scaled observed value of D for the species tree.** The observed value of
180 D for the species tree (D = 0.291) in black compared to simulated values of D = 0 (blue),
181 representing the traits being phylogenetically conserved as expected under a Brownian threshold
182 model (p = 0.115), and D = 1 (red) as the traits being phylogenetically random under a Brownian
183 threshold (p = 0). PRNP tree has similar results (not shown) with observed value of D = 0.273. P
184 = 0.135 for the simulated value of D = 0, and a p = 0 for the simulated value of D = 1.
185

186 Discussion

187 Our species tree and the tree inferred solely from the PRNP gene (Fig. 1) closely match
188 accepted mammalian phylogenetic trees [24-28]. Therefore, our species tree provides a glimpse
189 into the evolution of TSEs. The occurrence of TSEs is not randomly distributed across the
190 mammal phylogeny. TSEs appear to have arisen independently and recently in several major

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191 mammalian groups whereas they are absent in others; had information for the 20 genes been
192 available, a group of cervid species (e.g., moose, caribou, elk), all which exhibit TSEs, would
193 have been clustered with *Odocoileus virginianus*. The lack of amino acid substitutions unique to
194 all TSE+ or TSE- species suggests there is not a particular set of amino acid substitutions that
195 either provides resistance or susceptibility. In sheep amino acids positions relevant to scrapie
196 resistance are 136 (A/V), 154 (R/H) and 171 (Q/R/H), with the 136A/154R/171R genotype
197 conferring complete or nearly complete resistance [29]. We did not find this genotype in any
198 other mammalian taxa. Our ancestral reconstructions included only one instance of a reversal
199 from TSE presence to absence, suggesting that mutations conferring resistance are relatively
200 rare. The nonrandom occurrence of TSEs in some mammalian orders (e.g., rodents, bovids,
201 felines, cervids) suggest that TSEs are a recently evolved class of mammalian disease, which
202 could explain why TSEs are nearly always fatal. Rongyan et al. (2008:650) suggested that "no
203 dramatic sequence changes have occurred to avoid cross-species TSE infectivity." Why TSEs
204 are not more widespread across mammals is unclear at this time. It seems possible that the
205 evolution of TSEs is independent of PRNP genotype.
206
207 Given the high fatality rates of TSEs, one might expect strong selection on the PRNP gene.
208 Balancing selection in sheep [30] and strong purifying selection for PRNP CDS has been
209 implicated in cattle [31]. In contrast, the congruence between trees reconstructed from
210 nucleotides (Fig. 1) and amino acid residues (Fig. 2) suggests that selection has not yet played a
211 major role in the evolution of the PRNP gene. That is, if there were a common PRNP genotype at
212 the amino acid level that conferred resistance to TSEs, those species ought to have been grouped
213 together on the amino acid tree in a way that conflicts with the species tree.

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214

215 Reconstruction of TSE evolution suggests the ancestral state is the absence of TSEs (Fig. 1),
216 and that certain orders of mammals are apparently at greater risk of developing or contracting
217 these diseases. Alternatively, it is possible that our knowledge of the occurrence of TSEs is
218 incomplete, and one interpretation of our analysis is that all mammalian orders are susceptible,
219 which could be confirmed by more extensive testing. If TSEs are a relatively recent
220 phenomenon in mammals, perhaps enough time has not passed for crossing of species-group
221 barriers. Rongyan et al. (2008) noted that scrapie has been endemic in the United Kingdom for
222 more than 200 years and yet has not crossed the species barrier into humans.

223

224 # Conclusions

225 Understanding how diseases have evolved plays a crucial part in determining which
226 species are currently most at risk, and if the possibility for others to become at risk is an
227 immediate concern. Our findings show that the evolution of TSE+ species is localized, non-
228 random, and recently developed in groups of species irrespective of PRNP genotype. As the
229 PRNP gene has been associated with varying susceptibility to TSE diseases in past studies [11,
230 32-34], future studies should focus on other genes. For example, in some cattle breeds and the
231 gayal (*Bos frontalis*), a 23-bp deletion in the PRNP promoter region is associated with
232 susceptibility to bovine spongiform encephalopathy (BSE) [35-36], although this was not found
233 for white-tailed deer and mule deer (Zink et al. *in review*). Most research into TSEs has involved
234 species such as cows, sheep, deer, rodents, and select primates, which could illuminate how
235 TSEs could cross the species barrier into humans. However, as shown by our list of TSE+
236 species (Table S1) there are many species that are as yet unstudied. Therefore, our list of TSE+

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237 species could be incomplete. The evolution of TSEs merits continued exploration at a more in-
238 depth phylogenetic level, as well as the search for genetic combinations that might underlie TSE
239 diseases.
240

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245

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343 # Supporting information

344 **S1 Table. List of species with a record of contracting a TSE or not with related references.**

345 **S2 Table. List of genes used for BEAST analysis, all gene sequences obtained from**
346 **Orthomam.**

347 **S3 Table: Nucleotide composition for the PRNP gene averaged by TSE+ and TSE- species**

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348 **S4 Code: R Software (version 3.5.2) code used to visualize phylogenies and run analyses**

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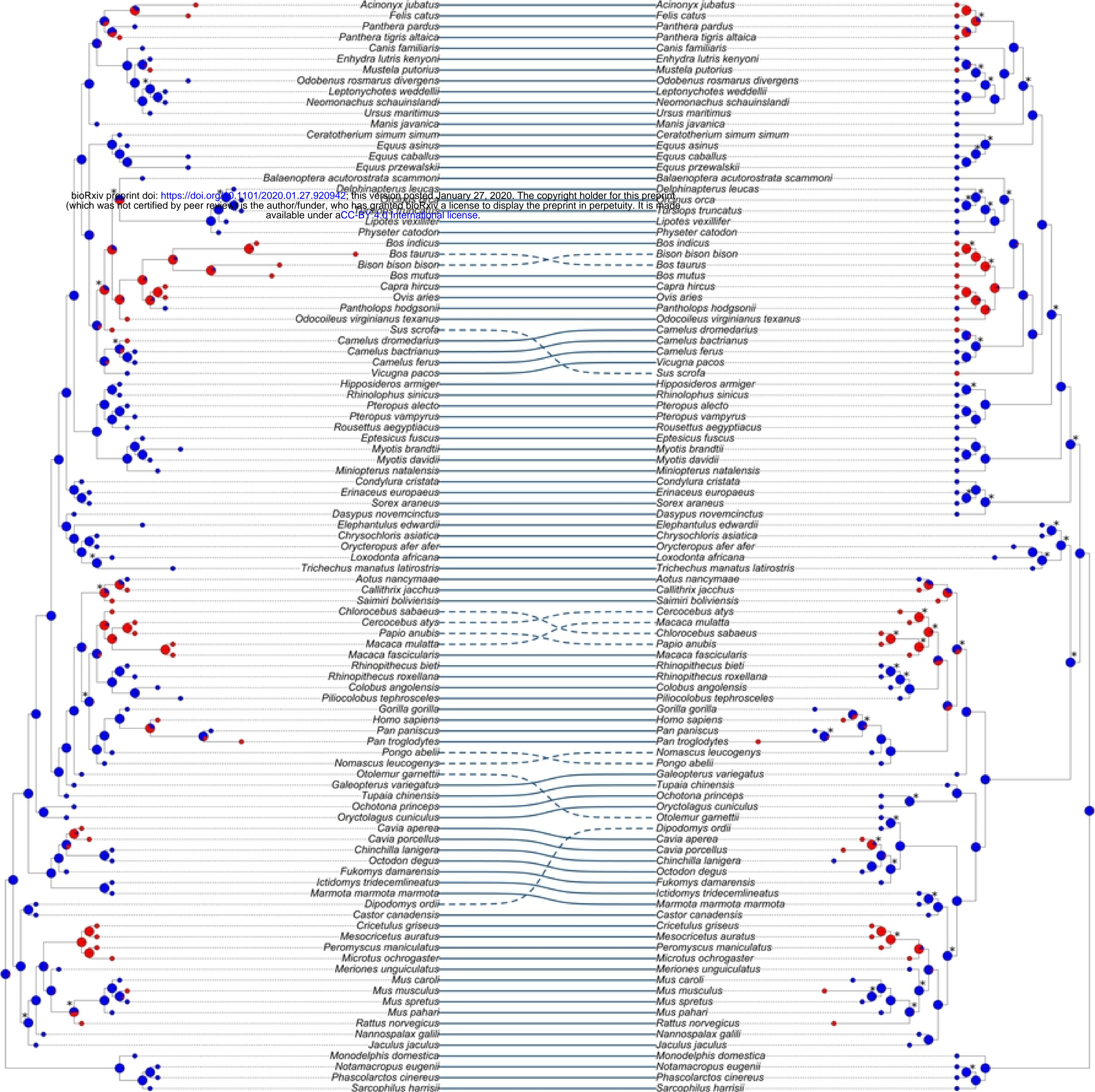

Figure 1

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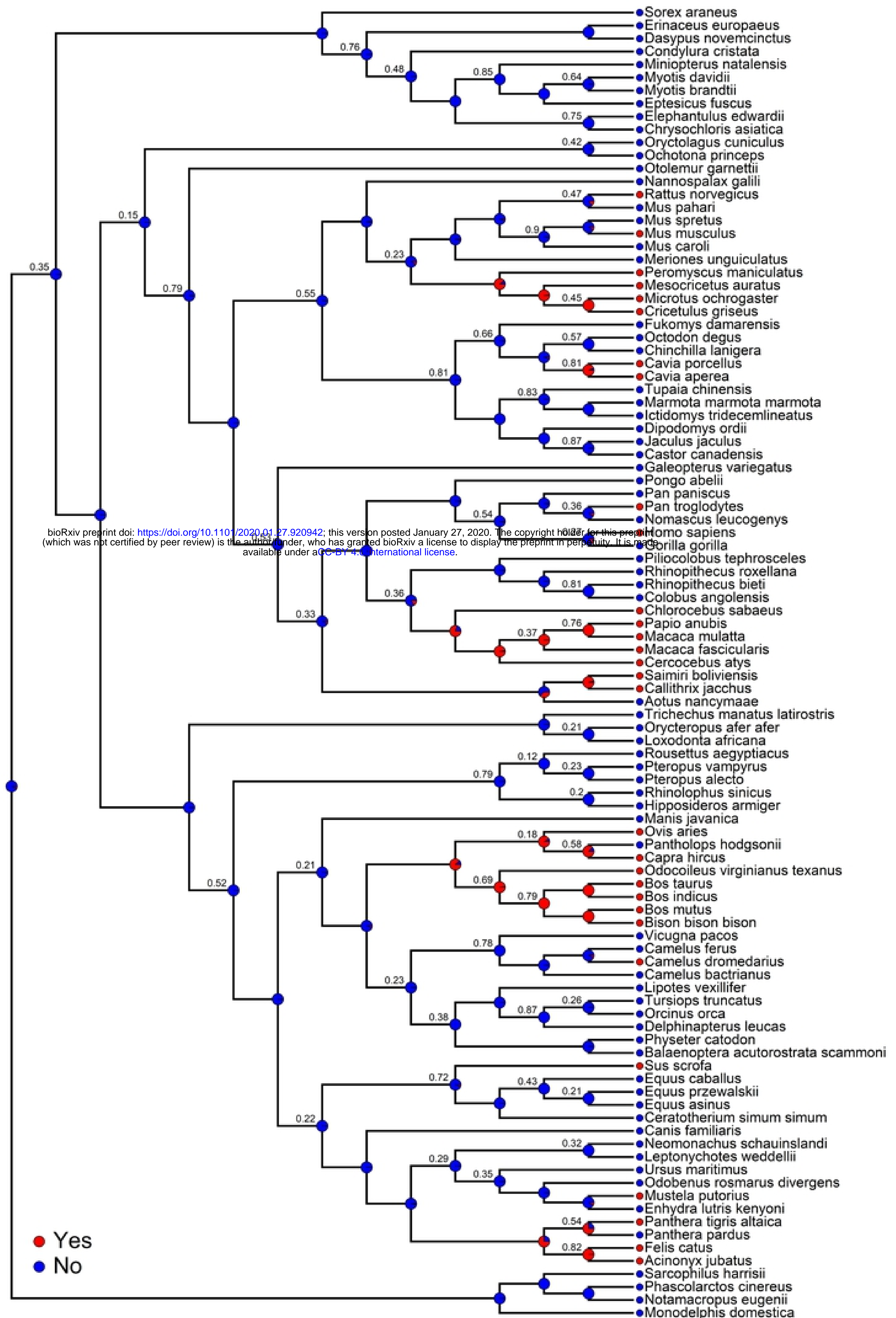

Figure 2

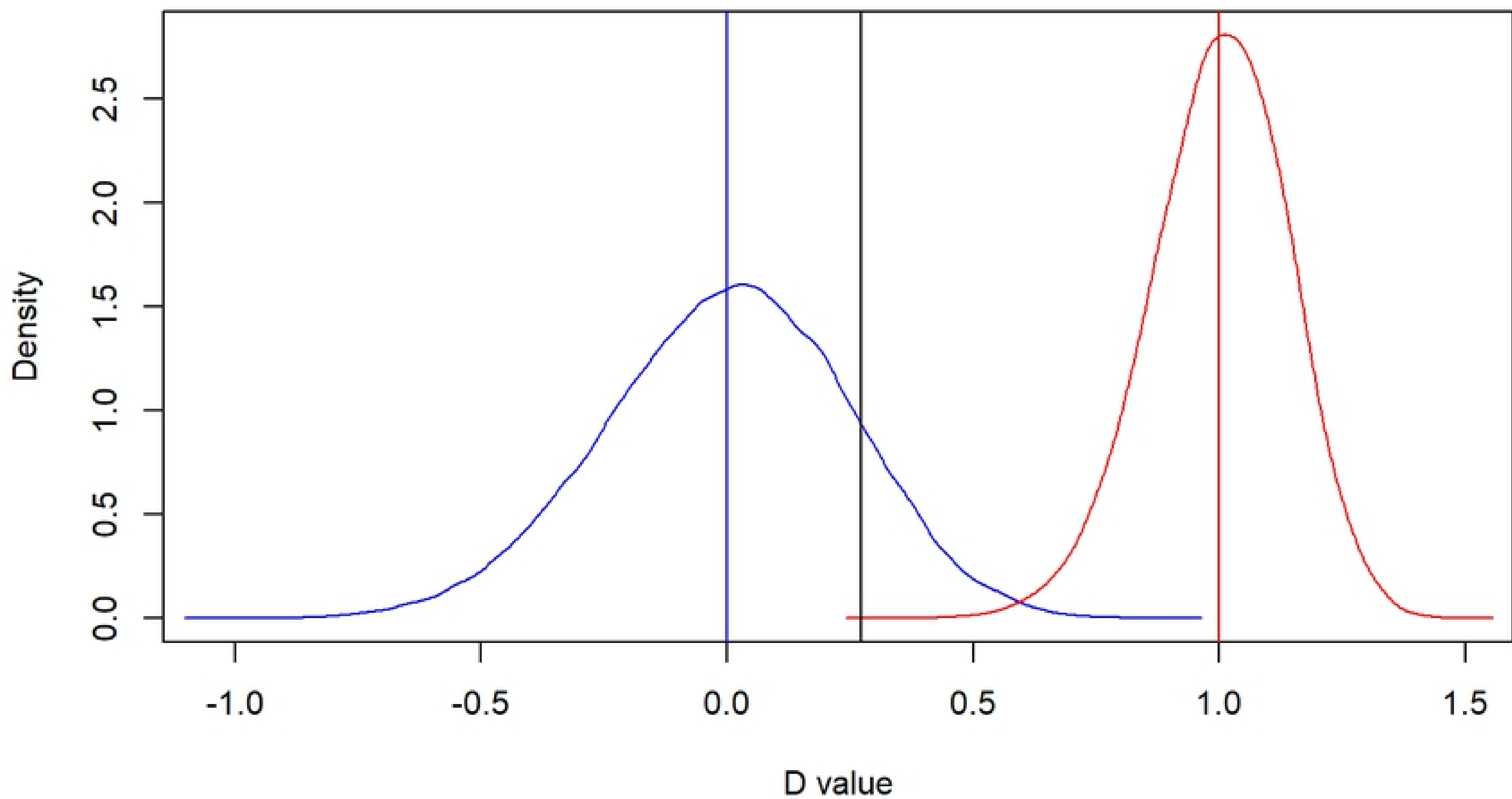

Figure 3