Evolutionary phenome-genome analysis of cranial suture closure in mammals

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1   **ABSTRACT**

2

3   Cranial sutures are growth and stress diffusion sites that connect the bones protecting the brain. The

4   closure of cranial suture is a key feature of mammalian late development and evolution, which can

5   also lead to head malformations when it occurs prematurely (craniosynostosis). To unveil the

6   phenotypic and genetic causes of suture closure in evolution, we examined 48 mammalian species

7   searching for (i) causal links between suture patency, brain size, and diet using phylogenetic path

8   analysis; and (ii) instances of genome-phenome convergence amino acid substitutions. Here we

9   show that brain size and the anteroposterior order of ossification of the skull are the two main

10   causes of sutures patency in evolution. We also identified three novel candidate genes for suture

11   closure in evolution (HRNR, *KIAA1549*, and *TTN*), which have never been reported in clinical

12   studies of craniosynostosis. Our results suggest that different genetic pathways underlie cranial

13   suture closure in evolution and disease.

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15   **Keywords:** Evolution; Anatomy; Brain Size; Craniosynostosis; Phylogenetic Path Analysis;

16   Convergent amino acid substitution

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19 **INTRODUCTION**

20 Cranial sutures separate the bones of the skull and function as sites of bone growth and stress

21 diffusion (Herring, 2008; Opperman, 2000). They are necessary to develop a healthy, functioning

22 head in mammals. Interestingly, while many sutures remain open through life, some cranial sutures

23 will naturally close by turning into bone. The closure of suture is a key feature of the mammalian

24 skull late development (**Figure 1**), growth, functioning, and evolution (Cray et al., 2014; Oh et al.,

25 2017; Roston & Roth, 2019). However, a premature closure of sutures (craniosynostosis) can also

26 lead to head malformations in newborns (Cohen & MacLean, 2000).

27

28 In mammals, cranial sutures closure evolves in coordination with the rest of the body. In fact, suture

29 closure is positively correlated with skull size and body size (Bärmann & Sánchez-Villagra, 2012;

30 Wilson & Sánchez□Villagra, 2009). Cranial sutures and the brain are also tightly integrated by

31 physical contiguity and shared signaling pathways during development (Lieberman, 2010;

32 Richtsmeier & Flaherty, 2013). Thus, changes of brain size could affect suture closure in evolution.

33 Likewise, diet can cause suture closure as a secondary effect of the mechanical loads generated

34 during feeding (Rafferty & Herring, 1999; Sun et al., 2004; Rafferty et al., 2019; Byron et al.,

35 2004). Biomechanical studies show that compression on a suture creates an environment that favors

36 suture closure (e.g., enhances osteogenesis, narrows suture space, immobilizes bones), whereas

37 tension favors suture patency (Herring, 2008). Therefore, we could expect suture closure to be a by-

38 product of the evolution of these traits, particularly brain size and diet. To our knowledge, there are

39 no studies on the impact of these phenotypic traits to suture closure in evolution.

40

41 Anatomical constraints can also bias which sutures close and which remain open (Moss, 1975;

42 Lieberman, 2011; Esteve-Altava & Rasskin-Gutman, 2014; Rasskin-Gutman & Esteve-Altava,

43 2018). Theoretical models predict that the whole arrangement of sutures in the skull—as a

44 network—acts itself as an anatomical constraint that influences which sutures will close and which

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45   ones will remain open (Esteve-Altava et al., 2017), for example, by directing the signaling pathways

46   that promote osteogenesis through mechanosensors (Khonsari et al., 2013; Katsianou et al., 2016).

47   Additionally, the timing of ossification of skull bones, from face to vault (Koyabu et al., 2014), and

48   of suture closure, from vault to face (Rager et al., 2014), have also the potential to explain biases in

49   suture closure, since one suture closure may influence the closure of a neighboring suture.

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51   The genetic causes of suture closure in evolution remain largely unknown. Most of our knowledge

52   comes from medical studies of genetic syndromes causing premature suture closure in humans

53   (Lattanzi et al., 2017; Morriss-Kay & Wilkie, 2005; Poot, 2019; Twigg & Wilkie, 2015; Wilkie et

54   al., 2017) and animal models (Cornille et al., 2019; Grova et al., 2012). These studies have revealed

55   a complex network of genes involving many signaling pathways (e.g., *BMP*/*TGF*-β, *FGF*, and

56   *WNT*). However, about 80% of craniosynostosis cases are nonsyndromic: they typically affect only

57   one suture and are not associated with other body malformations (Dempsey et al., 2019; Garza &

58   Khosla, 2012). There is little information on the genetic causes of nonsyndromic craniosynostosis

59   (Sewda et al., 2019). Evolutionary genomics offers a powerful tool to explore the genetic causes of

60   natural variation (Alföldi & Lindblad-Toh, 2013; de Magalhães & Wang, 2019; Smith et al., 2020),

61   as shown by studies on skull shape evolution (Roosenboom et al., 2018) and marine adaptations

62   (Foote et al., 2015; Zhou et al., 2015). Evolutionary studies have shown that some of the genes

63   regulating suture closure (e.g., *BMP3*, *MSX2*, *RUNX2*) have evolved under positive selection in

64   humans and other primates (Green et al., 2010; Magherini et al., 2015; Twigg et al., 2015; Wu et al.,

65   2010, 2012), which suggests that the same processes favoring suture closure at evolutionary scale

66   could be causing craniosynostosis conditions. Similarities of potential genetic factors and

67   phenotypes between skull evolution and craniosynostosis (e.g., fewer bones, same sutures

68   frequently affected, related shape changes) could indicate that analogous mechanisms underlie

69   suture closure in evolution and disease (Esteve-Altava et al., 2017; Richtsmeier, 2018; Richtsmeier

70   et al., 2006).

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71

72   Here we assessed the evolutionary factors determining the closure of the metopic, coronal, and

73   sagittal suture in mammals. To this end, we analyzed the cranial anatomy of 48 species of

74   mammals, for which their reference genomes were aligned at UCSC (100-way) and for which there

75   was information on their diet and brain mass in the literature. First, we tested 12 alternative

76   evolutionary hypotheses of how brain size, diet, and developmental constraints may determine

77   suture closure in evolution. Then, we looked for convergent amino acid substitutions in multiple-

78   sequence alignments of proteins, comparing species with sutures closed or open. Our hypotheses

79   are that (1) brain size, diet hardness, and constraints, together, determine suture patency and closure

80   in evolution; (2) species with a given suture closed will share mutations in the same key genes that

81   will be absent in closely related species with the same suture open; (3) these genes will be enriched

82   in biological functions relevant to cranial suture formation and maintenance, brain growth, and

83   biomechanical performance; and (4) they will overlap with genes previously associated to

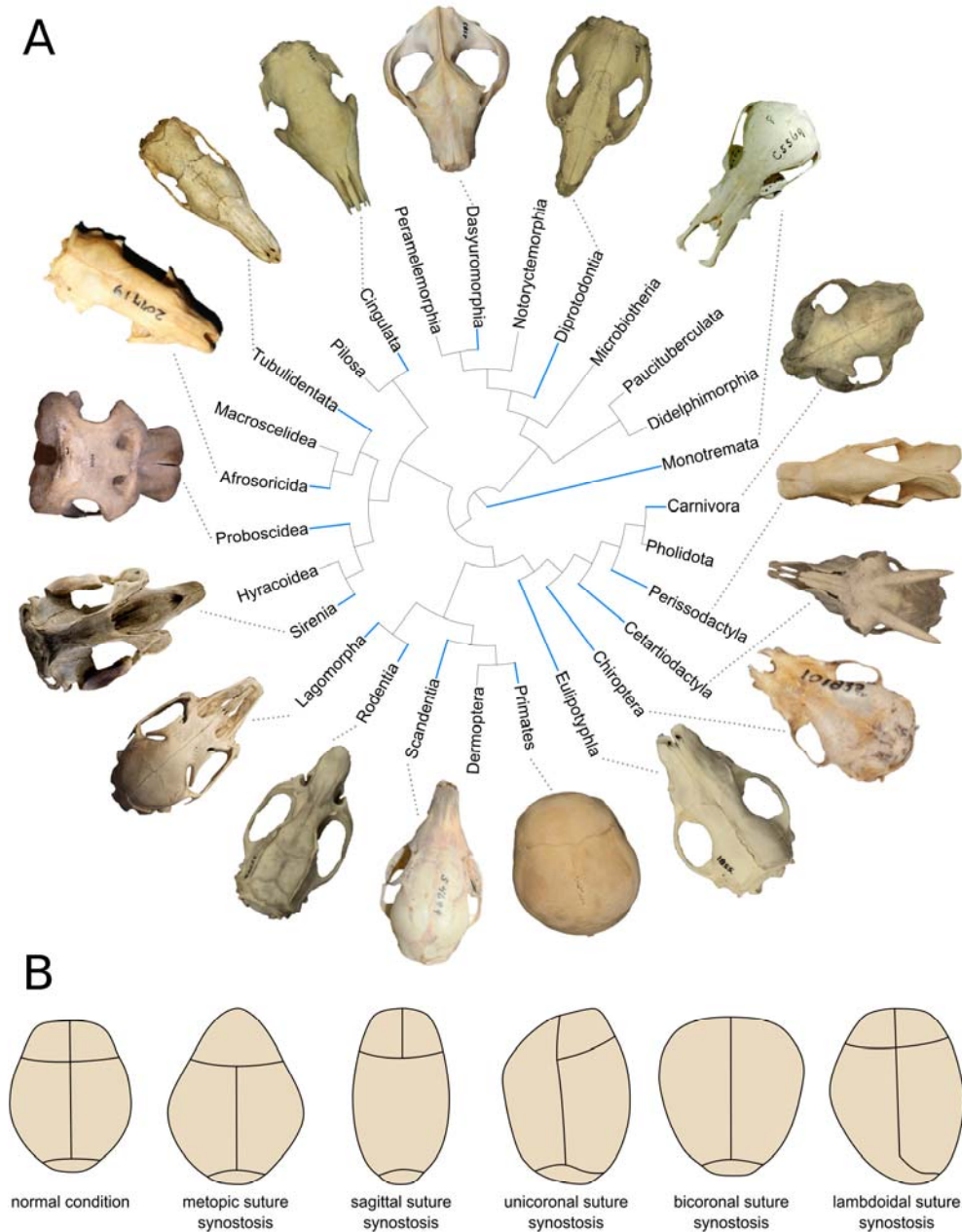84   craniosynostosis in clinical studies.

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Figure 1. Cranial suture close in evolution and disease. (**A**) Mammalian orders studied (blue lines), with representative skulls in dorsal view showing the differences in patency for the metopic, coronal, and sagittal sutures. Some mammals close their cranial sutures after birth, and we see them closed in adults, while others keep their sutures open through life or until an old age. (**B**) Outlines showing the consequences in the shape of the skull by a premature suture closure in humans, a condition called craniosynostosis.

86

87   **RESULTS**

88   The patency of the metopic, coronal, and sagittal sutures varied in the sample set of mammals

89   analyzed, with some taxonomic groups showing a consistent pattern of closure for some sutures

90   (**Figure 2**). We used the frequency of suture patency (i.e., specimens with the suture open/total

91   specimens) to infer the causal links between suture closure and other phenotypic traits of interest in

92   a phylogenetic path analysis. The high degree of conservation of suture patency within species

93   enabled us to categorize sutures phenotype, as either open or closed, and to carry out a search for

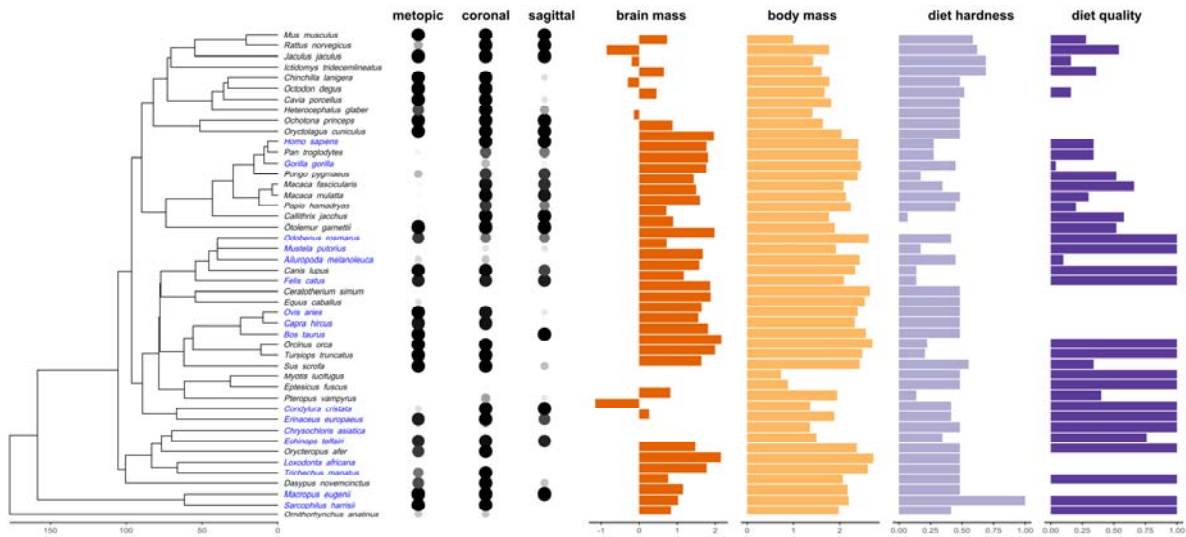94   convergent amino acid substitutions in the protein-coding genes.

Figure 2. Variation in suture patency and potentially related traits in mammals. In blue, species used for the pair-wise convergent amino acid substation analysis. Note that different pairs of species were used for each suture and that only suture patency below/above the threshold of 0.25/0.75 were considered. Dot size and gray scale of suture patency ranges between 0 to 1, corresponding to 0% to 100% of specimens with the suture open in our sample. Brain and body mass shown as log-transformation of weight in grams. Diet hardness and quality are normalized. See **Supplementary File 1** for the exact values of each variable.

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Suture patency and life-history traits evolution

96

97   The best supported model for the patency of the metopic, coronal, and sagittal sutures in evolution

98   agrees with the hypothesis of an anteroposterior direction in sutures' closure following the timing of

99   skull ossification and with the influence of brain size after correcting for body size (CICc = 56.9, *p*-

100   *value* = 0.196, *w* = 0.829). (N/A: CICc is a modified version of AICc for path analysis, significant

101   p-values, < 0.05, mean that the model is rejected, see *Methods*). **Figure 3** summarizes the results of

102   the phylogenetic path analysis. The best model includes mid-to-high effects of one suture on

103   another in an anteroposterior direction and low-to-mid effects of brain size on sutures. Larger brains

104   (after correcting for body size) tend to favor the maintenance of the coronal and sagittal sutures

105   open, and to a lesser extent, the closure of the metopic. In contrast, diet quality has a negligible

106   positive effect on brain size. Regardless of the support of each model (ΔCICc), only those

107   hypotheses that included a causal relation between sutures and brain size were supported by

108   evidence (*p-value* > 0.05). See **Supplementary File 1** for details.
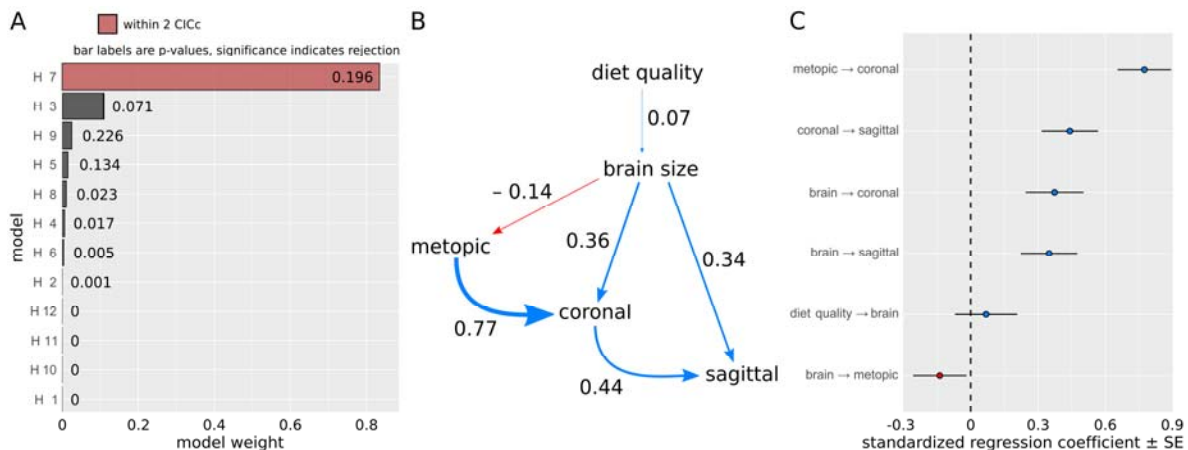

Figure 3. Summary of the phylogenetic path analysis. (**A**) Comparison of statistics for each model. There is only one best supported causal model (i.e., within 2 CICc and *p-value* > 0.05). (**B**) The best model shows a causal relation for cranial sutures patency following an anteroposterior direction, with larger brain sizes causing the sutures coronal and sagittal to remain open and the metopic suture to close at an evolutionary scale. An arrow indicates the direction of the causal

relation, its width corresponds to the standardized regression coefficient (i.e., how much the causal variable affects the effect variable), and its color represents a positive (blue) or a negative (red) effect. (**C**) Amount of change (with standard error) produced by causal variables on effect variables for the best supported model.

109

Convergent amino acid substitutions in species with cranial sutures closed

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111   We compared the amino acid sequences of 10,922 genes in pairs of closely related species that vary

112   in their suture patency (open *versus* closed). We recovered an aggregate of 6,158 putatively

113   convergent amino acid substitutions (CAAS) in 3,250 unique genes. These genes were mutated in at

114   least one suture, meaning that they were identified in three pair-wise comparisons of closely related

115   species with different suture patency. The number of unique genes found is significantly lower than

116   expected at random (1,000-bootstrap: $\alpha_{0.05}$ = 5,759.8; see *Methods* for more details). We recovered a

117   total of 28 candidate genes that overlap in the three sutures (i.e., identified in nine pair-wise

118   comparisons), which were used to perform set enrichment analyses. This is a significant overlap

119   between the genes identified for each suture compared to that expected if the three sets were

120   independent (*Fold Enrichment* = 15, p-value = 5.04E-25). Out of the 28 candidates, three genes

121   were also internally validated in the whole sample of mammals: Hornenin (*HRNR*), *KIAA1549*, and

122   Titin (*TTN*). We focused our discussion on these three gene and their potential role in suture

123   closure. See **Supplementary File 1** for the complete list of CAAS.

124

Functional and pathological enrichment

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126   Only one GO functional enrichment was found for the list of 28 candidate genes shared between the

127   three sutures: a molecular function related to limpid transport (GO:0005319, ER = 20.97, FDR =

128   0.013). For comparison, we also performed functional enrichment analyses for genes identified in at

129   least two sutures (i.e., found in six pair-wise comparisons). This shows that other well supported

130   GO enrichments for these genes include, for example, biological adhesion (GO:0022610, ER =

131 2.39, FDR = 0.005) and components of the extracellular matrix (GO:0031012, ER = 2.47, FDR =

132 0.006). See more details in **Supplementary File 1**. Overall, these results provide little support to

133 our initial working hypothesis that candidate genes would be enriched in functions commonly

134 associated with suture biology.

135

136 Moreover, neither of the 28 candidate genes overlaps with craniosynostosis genes as ascertained

137 from the study of human disease. None of the candidate genes is present in the lists of genes from

138 the literature (set 1), from upregulated in nonsyndromic craniosynostosis osteoblast cultures (set 2),

139 or from GWAS of nonsyndromic metopic and sagittal cases (set 3). As a reference, we also

140 estimated the overlap between these three gene sets. One-sided Fisher's exact tests show that only

141 one overlap for the list of literature genes and the list of GWAS (set 1 vs set 2: Odds Ratio = 1.616,

142 FDR = 0.106; set 1 vs set 3: Odds Ratio = 93.29, FDR = 2.05E-30; set 2 vs set 3: Odds Ratio =

143 1.08, FDR = 0.807). In contrast to candidate genes, genes associated to human craniosynostosis are

144 enriched in biological functions more related to suture biology (see **Supplementary File 1**). This

145 result rejects our starting hypothesis that candidate genes, with mutations linked to suture closure in

146 evolution, would overlap with genes associated to craniosynostosis.

147

148   **DISCUSSION**

149   Our results suggest that cranial suture closure in evolution is regulated by two developmental

150   processes: the order of ossification of skull bones and the growth of the brain relative to body size.

151   In contrast, we found no support for diet hardness (as a proxy for chewing biomechanics) as a cause

152   for differences in suture patency among species. From a genetic point of view, we identified 28

153   candidate genes for suture closure in evolution, out of which three show the strongest support:

154   *HRNR*, *KIAA1549*, and *TTN*. These genes have never been causally linked to craniofacial

155   development, suture biology, or craniosynostosis. However, they are expressed in tissues adjacent

156   to cranial sutures, such as the brain and skeletal muscles, which affect suture biology.

157

158   **Brain size and ossification timing determine suture patency in evolution**

159   Brain growth is tightly integrated with cranial suture maintenance and closure in normal

160   development and in pathological conditions (Richtsmeier, 2018; Richtsmeier et al., 2006;

161   Richtsmeier & Flaherty, 2013). The traditional idea is that suture closure is a passive consequence

162   of the growth of the brain (Moss & Young, 1960; Moss, 1975), with dura mater triggering a

163   signaling cascade that promotes osteoblast activity and regulates cell proliferation and apoptosis

164   (Opperman, 2000; Spector et al., 2002). Our results support a direct influence of brain size on suture

165   patency in mammals, acquired by an increased brain growth relative to body size. However, each

166   suture responds differently to brain size. Larger brains cause the coronal and sagittal sutures to

167   remain open, while they cause the closure of the metopic suture. Why sutures respond in a different

168   way to brain size is unclear, but it could be a consequence of how the growing brain interacts

169   differently with each of the enclosing bones and sutures (Barbeito-Andrés et al., 2020).

170

171   Our evolutionary analysis shows that the patency of a suture depends also on the patency of other

172   sutures, following the most common anteroposterior order of ossification of the skull in mammals

173   (Koyabu et al., 2011, 2014). This result agrees with recent theoretical findings proposing that the

174   organization of the skull, as a network of bones connected by sutures, can bias suture closure

175   (Esteve-Altava et al., 2017; Esteve-Altava & Rasskin-Gutman, 2015). For example, by directing

176   mechano-transduction and morphogenetic signals (Khonsari et al., 2013; Katsianou et al., 2016). If

177   that is true, this means that sutures are not only passive subjects of their underlying functional

178   matrices (Moss, 1975), instead they can constrain each other's closure. However, the exact relation

179   between the timing of ossification and suture closure during development is still unclear and may

180   depend on other species-specific anatomical constraints. In humans, for example, the later closure

181   of the metopic suture compared to our extinct relatives and other primates is thought to be an

182   adaptive response to pelvic constraints on the birth canal (Falk et al., 2012).

183

184   **Mechanical stress and suture closure**

185   Biomechanical studies on vertebrate skulls as disparate as lizards and mammals have shown that

186   cranial sutures relieve strain locally in response to mechanical loads, for example, from chewing

187   (Herring & Teng, 2000; Moazen et al., 2009; Rafferty & Herring, 1999). However, using diet

188   hardness as a proxy, we found no support for an effect of mechanical stress on sutures patency or

189   closure in mammals. This is either because there is no evolutionary relation between them or

190   because diet hardness is not a good proxy for the mechanical stress supported by the skull. If diet

191   hardness is not a good proxy, the most accurate alternative would be to carry out biomechanical

192   studies on every species and measure each suture response individually, for example, using Finite

193   Elements Analysis (Bright, 2012). This would be a feasible albeit challenging empirical work.

194

195   **New candidate genes for suture closure in mammals**

196   Following a comparative analysis of protein-coding regions, we identified 28 candidate genes that

197   may have a potential role in determining cranial suture closure in mammalian evolution. Out of this

198   list, three genes—*HRNR*, *KIAA1549*, and *TTN*—are the most likely candidates, because they also

199   show convergent mutations across the whole sample of mammals. These three genes have never

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200   been associated with neither normal nor pathological craniofacial development. Thus, we can only

201   speculate about their relation to suture closure based on indirect evidence, such as the tissues where

202   they are expressed, their biological function, and their relations to other proteins.

203

204   *HRNR* encodes for a profilaggrin-like protein that functions as an ion binder for calcium and other

205   metals in different tissues (mostly the skin, but also in the brain), organizing the cell envelope and

206   extracellular keratinization. Although *HRNR* was tentatively reported as a risk factor for

207   craniosynostosis in a study of twins (Rymer, 2015), this result has not been validated (*personal*

208   *communication*). *HRNR* is not known to play a role in cranial development, but other proteins

209   related to keratinization have been reported to participate in developing calvarial bone and sutures

210   (Atsawasuwan et al., 2013). A total of 59 different mutations were found along the entire protein of

211   *HRNR*, none of which targets a functionally active region for calcium binding. However, *HRNR*-

212   coding region is enriched in methylation sites that undergo modifications during human

213   development from newborn to adult (Salpea et al., 2012), the time when cranial sutures close. We

214   identified 58 convergent substitutions for *HRNR* in our evolutionary study (the most of all candidate

215   genes), which could be interacting with methylation sites that determine cranial phenotypes during

216   postnatal development.

217

218   *KIAA1549* encodes for a protein component of the cellular membrane that is highly expressed in the

219   brain. Eleven different mutations were found for *KIAA1549*, none within the transmembrane region.

220   *KIAA1549* has never been associated to craniofacial morphology or premature suture closure, but

221   through a fusion with the B-Raf proto-oncogene (*BRAF*) it has been associated to developing

222   pilocytic astrocytoma, a benign brain tumor (Yamashita et al., 2019). Interestingly, *de novo*

223   mutations in *BRAF* has been recently discovered in patients with isolated sagittal synostosis

224   (Armand et al., 2019; Davis et al., 2019). Whether *KIAA1549* can affect suture development

225   through its effect on *BRAF* is not known.

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227   *TTN* encodes for the largest human protein, a common type of filament present in cardiac and

228   skeletal muscles that is essential for muscle contraction. Thirty-two mutations were found for *TTN*,

229   none within its active sites. Although *TTN* has not specifically been associated with craniofacial

230   development or dysmorphologies, we known that head muscles activity is necessary for the correct

231   formation and maintenance of cranial sutures (Byron et al., 2004; Herring & Teng, 2000; Moss &

232   Young, 1960). For example, osteoblast in the sutures respond to muscle tension by increasing the

233   formation of bone (Herring, 2008), which is something also observed in craniosynostosis (Al-

234   Rekabi et al., 2017). We can only speculate on whether small changes in *TTN* proteins modulate

235   head muscles contraction during cranial development, and by doing so, can alter cranial sutures

236   maintenance and closure.

237

238   The 28 candidate genes for cranial suture closure in evolution are not enriched in biological

239   functions and cellular components often associated with skeletal development (e.g., osteogenesis,

240   growth factor binding, cell proliferation), which are those dysregulated in pathological cases of

241   suture closure (Rojas-Peña et al., 2014). Instead, we found that candidate genes are functionally

242   enriched in proteins for the transport of lipids across the membrane. Lipids play an important role in

243   skeletal metabolism, for example, by limiting permeability of the bone surface and by regulating

244   biomineralization through the transport of essential fat-soluble vitamins D and K (Tintut & Demer,

245   2014). In the context of cranial sutures, a relationship between vitamin D deficiencies (congenital or

246   nutritional) and craniosynostosis has been known for a long time (Imerslund, 1951; Jaszczuk et al.,

247   2016; Wang et al., 2015). This result suggests that mechanisms of cranial suture closure in

248   evolution could evolve through changes in the regulation of vitamin D transport, rather than acting

249   directly on osteological regulation pathways. Finally, for genes differentially mutated in two out of

250   three of the sutures, we did find enrichments for biological adhesion and components of the

251   extracellular matrix, which are essential for the maintenance and closure of cranial sutures

252   (Opperman & Rawlins, 2005; Stamper et al., 2011).

253

Are evolution and pathological development decoupled for cranial suture closure?

255   Candidate genes from our evolutionary analysis show a complete lack of overlap with genes linked

256   to pathological suture closure (Adhikari et al., 2016; Justice et al., 2012, 2020; Rojas-Peña et al.,

257   2014). There are many plausible reasons that could explain this mismatch, from methodological

258   limitations to biological causes. On the one hand, the list of genes compared could be incomplete.

259   This is because either (1) our evolutionary analysis fails to identify candidate genes for suture

260   closure or because (2) we only know a limited number of genes which mutation lead to premature

261   suture closure. The first reason would imply that our approach does not work for this phenotype,

262   because it cannot capture mutations affecting the timing of closure (only the mechanism performing

263   the closure), whereas pathological conditions maybe occur due to mutations affecting timing

264   exclusively (e.g., via ectopic gene expression, Poot, 2019). The second reason would mean that our

265   current knowledge of the genetic origins of nonsyndromic craniosynostosis is limited and, therefore,

266   the lists of genes compared fail to capture the complete genetic landscape of this complex disease

267   (Magalhães & Wang, 2019; Lattanzi et al., 2017). On the other hand, there may be biological

268   reasons that explain this mismatch. As mentioned before, changes in brain growth rates or vitamin

269   D regulation in evolution could be one of such underlying causes, acting differentially for each

270   suture (Barbeito-Andrés et al., 2020).

271

272   Another possibility is that evolutionary mechanisms are decoupled from developmental

273   mechanisms commonly disrupted in disease, so that analogous phenotypic changes (i.e., closing a

274   suture) can proceed through different paths. Decoupling evolutionary mechanisms of phenotypic

275   variation from those genetic pathways whose disruption is most likely to be detrimental for the

276   individual could be a way to maintain evolvability without compromising fitness, bypassing

277   pleiotropic or epistatic constraints (Payne & Wagner, 2019). The fact that candidate evolutionary

278   genes for suture closure show no enrichment in any disease set supports this hypothesis. However,

279   it is unclear whether macroevolutionary genetic changes should involve the same loci or mutations

280   uncovered by microevolutionary and clinical studies (Smith et al., 2020). For example, the Runt-

281   related transcription factor 2 (*RUNX2*) is a strongly supported candidate to drive facial

282   morphological and suture closure in human evolution (Adhikari et al., 2016; Magherini et al., 2015)

283   and which mutation causes craniosynostosis (Cuellar et al., 2020; Maeno et al., 2011). However,

284   *RUNX2* takes no part in marsupial craniofacial diversity (Newton et al., 2017). This suggests that

285   different mammalian clades can use alternate pathways to control the exact same phenotypic traits.

286

287   **Conclusion**

288   Our study dissected the phenotypic and genetic causes of cranial suture patency in evolution,

289   highlighting developmental and evolutionary factors for suture closure in mammals. From a

290   phenotypic point of view, we found two main factors: (1) brain growth, which was a known cause

291   of suture patency in normal and pathological development, and (2) sutures self-regulation, which

292   was previously suggested only by theoretical models. From a comparative genomics approach, we

293   identified candidate genes involved in lipid transport, cell adhesion, and the formation of the

294   extracellular matrix. The best supported candidate genes to play a role in cranial suture closure in

295   evolution are *HRNR*, *KIAA1549*, and *TTN*. If validated by additional comparative analyses, and

296   experimentally in model organisms for suture closure (e.g., zebrafish, mice, or rabbit), they could

297   provide new ways to study the genetic basis of suture closure in evolution and disease. To our

298   knowledge, this study is the first attempt to search for the genetic causes of cranial suture closure

299   and associated pathologies at a macroevolutionary scale. Our findings highlight the importance of

300   evolutionary approaches to make new discoveries and test hypothesis on development and disease.

301

302   **METHODS**

303   **Sampling**

304   We surveyed an initial sample of 53 species of mammals with multiple sequence alignments of their

305   reference genomes available at UCSC (Kent et al., 2002) and with reliable information on their diet,

306   brain mass, and body mass (Burger et al., 2019). We examined adult skull specimens in vivo and in

307   digital images from museums and online collections with an ID catalog number. A total of 48

308   species had more than two well-preserved specimens available for examination and were included

309   in the present study. Details for specimens ID, suture patency, life traits, phylogeny, and analysis

310   code are available at https://figshare.com/projects/Cranial_Suture_Closure/81209.

311

312   **Suture patency**

313   For each specimen, we coded the state of the metopic, coronal, and sagittal sutures as either open or

314   closed, depending on whether they were visible (patent) or not (obliterated). Ambiguous cases (e.g.,

315   when a suture is half closed) were rare and we excluded them from the study. Suture patency was

316   quantified as the ratio of the number of specimens with the suture open to the total number of

317   specimens examined, ranging from 1 (all open) to 0 (all closed). This continuous measure provided

318   an amenable variable to perform the phylogenetic path analysis. We omitted from our analyses the

319   sagittal suture in the orca and the dolphin, because cetaceans never form this suture in the first place

320   due to the expansion of the occipital bone (Roston & Roth, 2019).

321

322   To later search for convergent amino acid substitution (CAAS), suture patency was binarized by

323   thresholding it between 0.75 and 0.25. A suture with a patency above 0.75 was counted as open and

324   below 0.25 was counted as closed. A binarization of suture patency was necessary because CAAS

325   comparisons require as input two discrete groups of species (Muntané et al., 2018). Because suture

326   patency is a highly conserved trait, most species ranked well above or below these thresholds. Only

17

327   15 sutures out of 144 observations were left uncategorized, and we omitted them when selecting

328   pairs of species to compare their protein-coding sequences.

329

330   **Life traits and phylogenetic path analysis**

331   We tested 12 alternative causal models for the closure of the metopic, coronal, and sagittal sutures

332   using a phylogenetic confirmatory path analysis (von Hardenberg & Gonzalez□Voyer, 2013). To

333   this end, we downloaded a calibrated phylogeny for all mammals from TimeTree

334   (http://www.timetree.org/) and pruned off the species not sampled. The analysis was carried using

335   the Pagel's lambda model of evolution, which is estimated internally by the function *phylo_path*

336   (Bijl, 2018) in R (R Core Team, 2019). **Figure 4** shows the models evaluated. As potential relations

337   we modeled the mutual causation between sutures due to their development (Koyabu et al., 2014;

338   Rager et al., 2014), the effect of hard diet on sutures due to the stress involving in chewing

339   (Herring, 2008; Rafferty et al., 2019; Sun et al., 2004), and the effect of brain size on sutures due to

340   the influence of the brain on the growth of the bones of the vault and the maintenance of sutures

341   (Richtsmeier, 2018; Richtsmeier et al., 2006; Richtsmeier & Flaherty, 2013). Finally, we included

342   the effect of diet quality on brain size as an indirect link on sutures (Aiello & Wheeler, 1995; Allen

343   & Kay, 2012). To reduce the number of variables in the models, body size was included as a

344   corrector for brain size, instead of as an independent variable.

345

346   Brain and body mass information was gathered from a recent study on brain size allometry in

347   mammals (Burger et al., 2019). We noticed an error in the body mass for *Bos taurus* (46,100 g) and

348   fixed using the correct value (461,000 g) from the original reference (Isler & van Schaik, 2012). We

349   favor Burger's dataset because measurements were systematically compiled (e.g., two cross-

350   reference check to assess authenticity of measurements, female-male averages except for dimorphic

351   species), and brain and body mass for each species come from the same study, which minimizes

18

352   potential errors. Brain size was calculated as the residual of a phylogenetic generalized least square

353   regression of the log-transformation of brain mass against body mass.

354

355   Diet information was extracted from EltonTraits 1.0 database on species-level foraging attributes

356   (Wilman et al., 2014). Data includes the percentage of the type of food consumed for each species.

357   Diet quality was measured using Sailer and colleagues' equation (Sailer et al., 1985) as, $DQ =$

358    $plants + 2 \times (fruit + seed + nectar) + 3.5 \times (meat)$, ranging from 100 to 350. Because there

359   is not a similar measure for diet hardness based on the relative amount of food consumed, we

360   followed an approach similar to that used for diet quality, measuring diet hardness as, $DH =$

361    $(fruit + meat) + 2 \times (plants + invertebrates) + 3.5 \times (seed + scanveing)$, ranging from 0

362   to 350 (however, only species with a nectar-based diet will rank between 0 and 100). This relative

363   division of food types by hardness (i.e., 1x, 2x, 3.5x) agrees with the division of hard foods used in

364   experimental studies (Marcé-Nogué et al., 2017; Williams et al., 2005). Diet quality and hardness

365   were both normalized between 0 and 1.

366
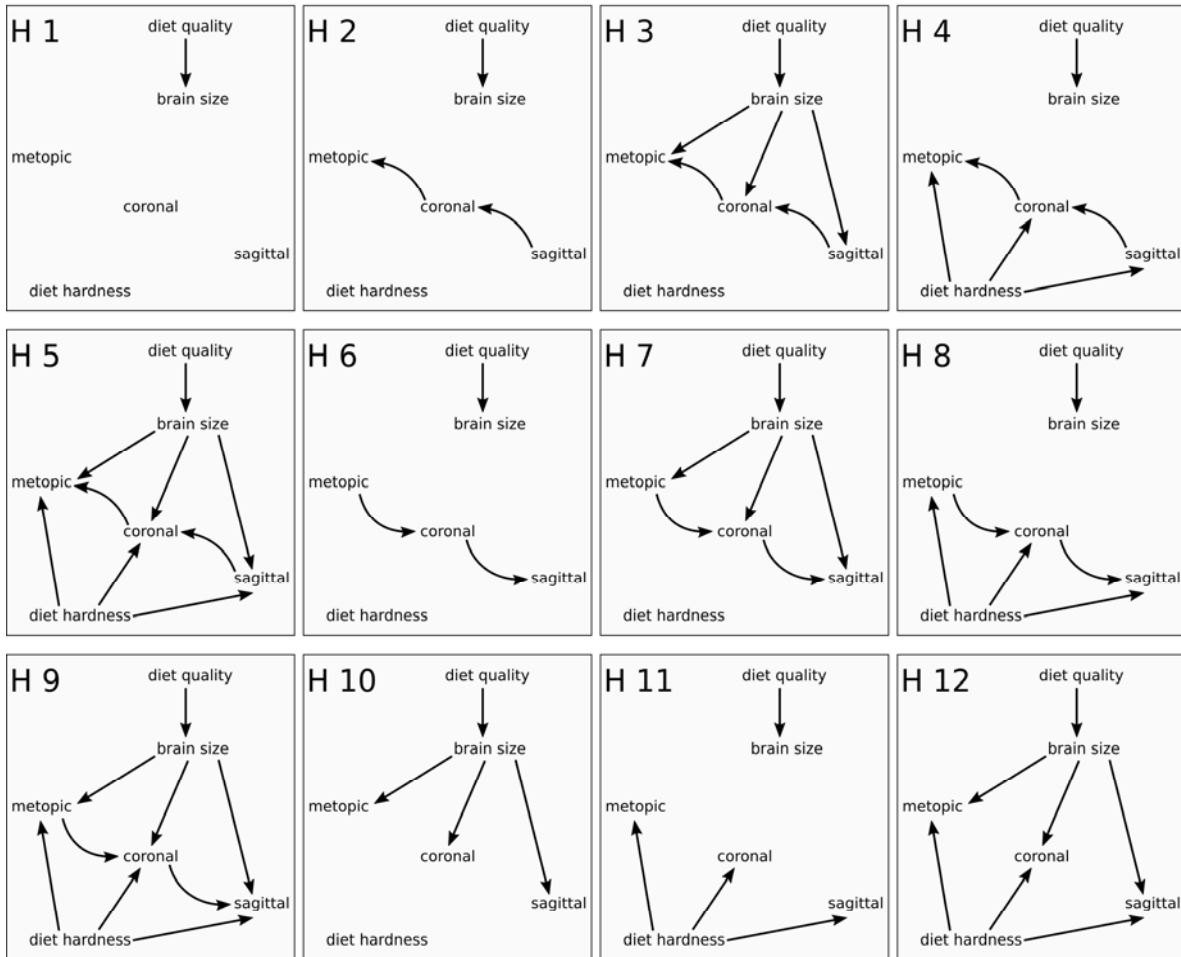
Figure 4. Alternative causal models compared with the phylogenetic path analysis. Model H1 is the null hypothesis of no causal relation among brain size, diet hardness, and sutures patency. H2 to H5 evaluate the causation of brain and/or diet when suture closure has a causal directionality following the relative order of closure in mammals (Rager et al., 2014). H6 to H9 evaluate the causation of brain and/or diet when suture closure follows the anteroposterior timing of ossification of the skull (Koyabu et al., 2014). H10 to H12 evaluate the influence of brain and diet in the absence of any type of developmental causation between sutures.

367

368 **Convergent amino acid substitutions (CAAS)**

369 Multiple sequence alignments (MSA) were downloaded from the University of California Santa

370 Cruz (UCSC) Genome Browser (Kent et al., 2002). We kept the 18533 MSA corresponding to the

371 longest transcript of each gene. We then filtered out those sequences having more than 30% gaps or

372 ambiguous amino acid definitions in any of the species analyzed. The final background pool of

373 genes included 10922 MSA. On this gene pool, we searched for CAAS that co-occur in three pairs

374 of closely related species with an opposite suture patency (open/closed) for the metopic, coronal,

375 and sagittal sutures (**Figure 5**), for a total of nine pair-wise comparisons. Using an in-house script

376 from a past study (Muntané et al., 2018), we retrieved all positions in which an amino acid differed

377 between species with the suture open and species with the suture closed. We kept those cases in

378 which an amino acid differed between the two groups and was shared by all the species of at least

379 one group, discarding any case with a gap in that position. The final list of candidate genes includes

380 only those genes that had convergent amino acid substitutions in the compared pairs for the three

381 sutures.

382

383 We considered three scenarios or types of convergent substitution. Scenario 1 captures the same,

384 single amino acid substitution for all pairs compared between species with the suture open and

385 closed (e.g., open = {asparagine} → closed = {histidine}). Scenario 2 captures substitutions of a

386 same fixed amino acid in species with the suture open to a variable set of different amino acids in

387 species with the suture closed (e.g., open = {alanine} → closed = {proline, glutamate, lysine}).

388 Scenario 3 is the reverse case of scenario 2: a variable set of amino acids in species with the suture

389 open changed to a same amino acid in species with the suture closed (e.g., open = {glutamine,

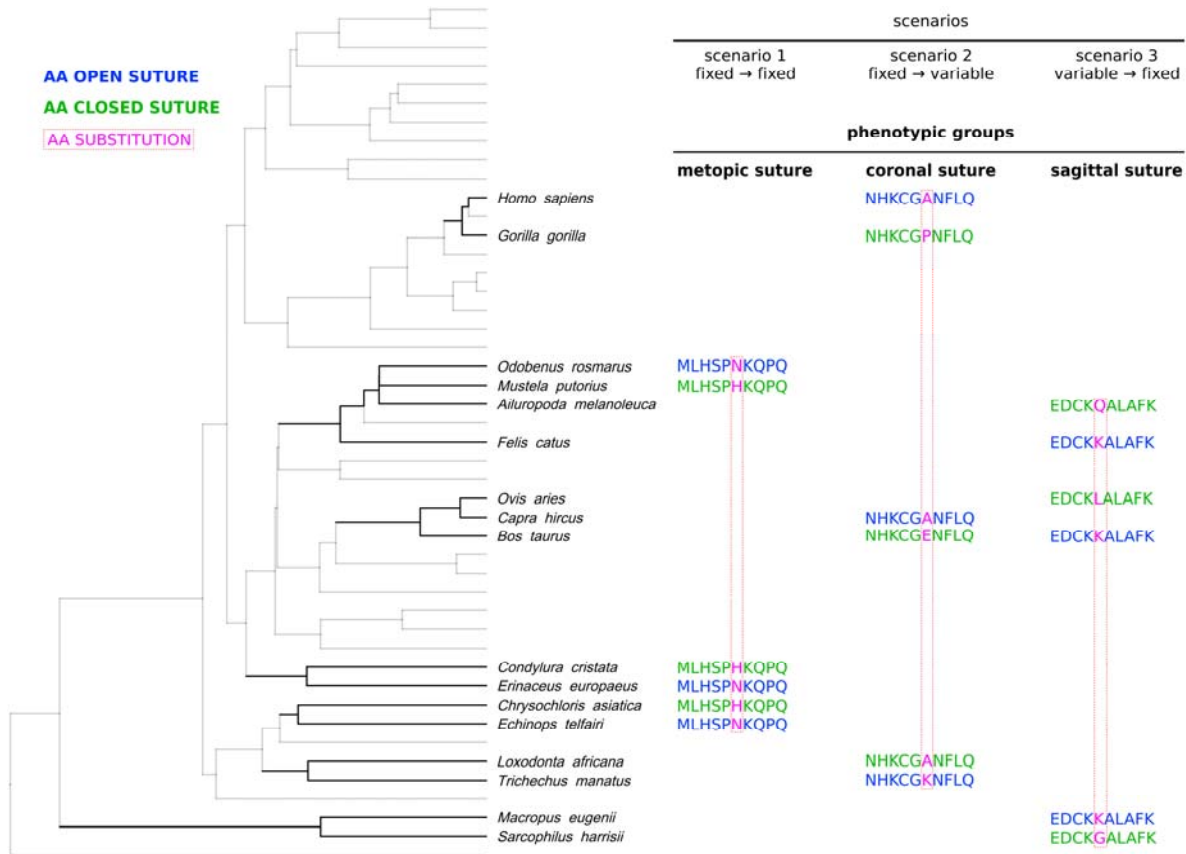390 leucine, glycine} → closed = {lysine}).

391

Figure 5. Structure of the convergent amino acid substitution analysis. Pairs of closely related species were selected for comparison based on their suture status as either open (blue) or closed (green), producing three phenotypic groups with three pairs of species each. Three scenarios of substitution were searched for every phenotypic group (only one is shown here for each suture).

392

Statistical and internal validation of candidate genes

394   We performed a statistical validation of candidate genes using bootstrapping to assess whether the

395   number of genes carrying CAAS were different than expected at random. For 1000 iterations, we

396   sorted the 17 species analyzed (see **Figure 6**) into two random groups and scanned the background

397   pool of genes for genes carrying at least one non-gapped CAAS. The bootstrap results span from

398   3,274 to 10,681 hits with a median of 8,898 hits, and 5% and 95% intervals are 5,759.8 and 10,082

399   genes hit, respectively. Finally, we tested the significance of the genes overlapping for the three

400   sutures in R using the *SuperExactTest* package (Wang et al., 2015).

22
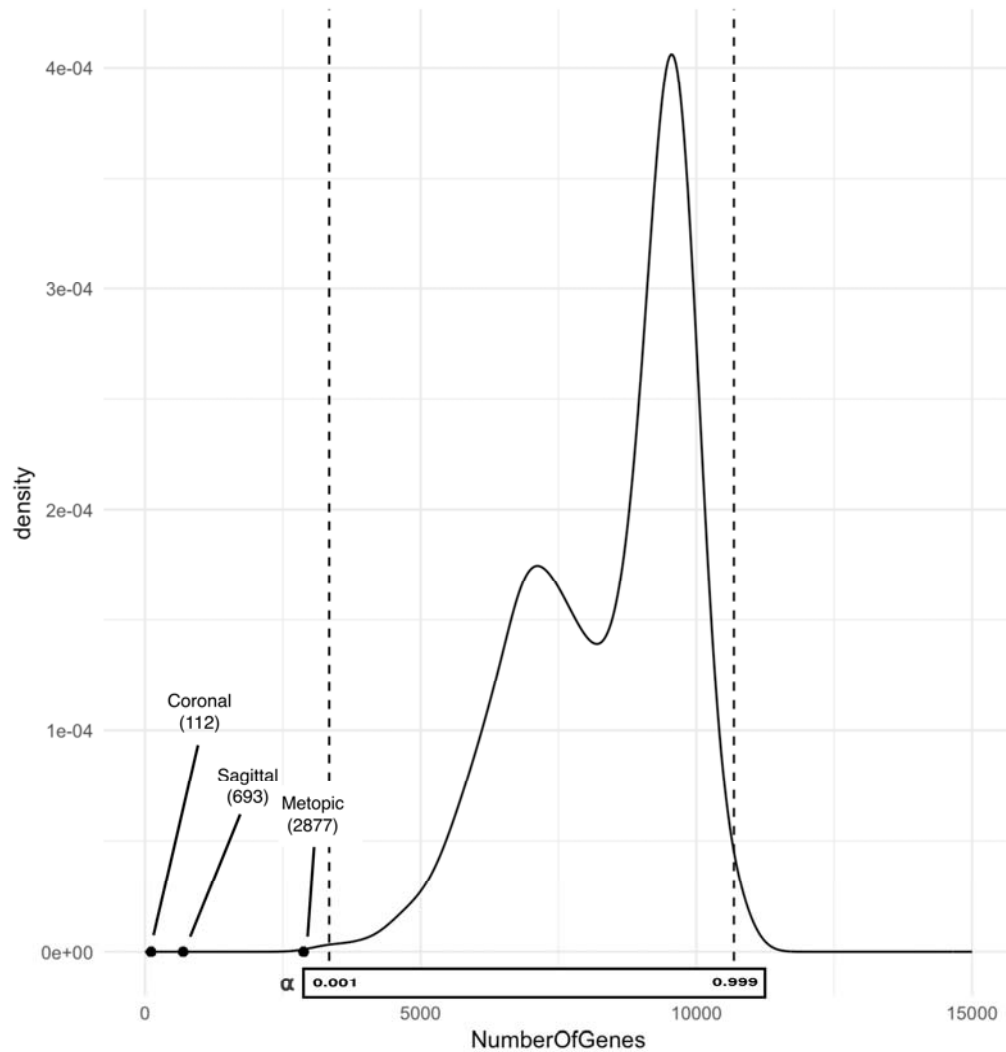
Figure 6. Results of the bootstrap comparisons of CAAS. Red lines show the 0.001 and 0.999 probability limits. Dots mark the total number of genes identified for each suture.

401

402 To assess biological significance, we performed an internal validation of candidate genes within the

403 whole sample of mammals, following the same procedure described before. We considered a

404 candidate gene as validated when it is also identified carrying open-versus-closed CAAS in the

405 whole sample. Validated genes are discussed in the context of suture biology in more detail.

406

Functional and pathological enrichment

407
408   We performed set enrichment analyses for the list of candidate gens using GOATOOLS

409   (Klopfenstein et al., 2018) with the background pool of 10,922 protein-coding genes as reference.

410   First, we performed a functional enrichment for biological processes, cellular components, and

411   molecular functions of the Gene Ontology (GO) database (Ashburner et al., 2000;

412   The Gene Ontology Consortium, 2019) using in-house scripts. Enrichments are based on GO

413   definitions present in the go-basic.obo file available on Gene Ontology public database (Accessed

414   February 2020). Then, we performed an enrichment analysis of candidate genes for three sets of

415   genes associated to premature cranial suture closure (craniosynostosis) using Fisher's exact test.

416   The first set comprises 97 genes linked to (mostly) syndromic craniosynostosis conditions in the

417   Human Phenotype Ontology (HP:0001363). The second set comprises 959 genes with differential

418   gene expression profiles in RNA-Seq data for human osteoblast cultures derived from bone biopsy

419   of nonsyndromic craniosynostosis cases (Rojas-Peña et al., 2014). The third set comprises 53 genes

420   from two GWAS studies on nonsyndromic craniosynostosis: one for the sagittal suture (Justice et

421   al., 2012) and one for the metopic suture (Justice et al., 2020). Note that we only included genes

422   that are also present in our background pool of genes.

423

Methodological limitations

424
425   *Suture patency and sample size*. The number of specimens examined of each species is uneven, and

426   for some species only a few individuals were available. This is either because the species is rare and

427   was not available in the natural history museums visited or in the online repositories consulted or

428   because the covid-19 outbreak prevented us visiting additional museum collections. However, the

429   conserved nature of suture patency gave us confidence that the suture patency measurements

430   represent the general, highly conserved pattern of each species, and that its categorization as open

431   or closed is valid. A broad sampling would provide stronger support to our conclusions. Moreover,

24

432   it would make possible to include intraspecific variation within the analysis, which was not possible

433   now because some species (perhaps due to the small sample size) showed little variation.

434

435   *Quality of referenced genomes, alignments, and number of gaps*. The quality of the mammalian

436   reference genomes available for comparison is also uneven; specially compared to human and some

437   model organisms. The consequence is that some alignments of protein-coding genes have a high

438   number of gaps (i.e., not comparable positions because of unknown amino acid, deletion/insertion).

439   These gapped positions complicate comparing simultaneously many species. In this study, we

440   decided to take the most conservative approach: we excluded genes with more than 30% of

441   positions with a gap from the background pool and also positions with gaps for each pairwise

442   CAAS. The side effect of this strategy is that some genes are inevitably excluded because they

443   show many gaps, among them genes linked to craniofacial development, such as ALX4, ERF,

444   SMAD6. In addition, protein lengths may exert some bias in the genes excluded as well as the

445   number of CAAS. Nevertheless, we adopted this conservative approach because the laxer

446   alternative added an additional layer of ambiguity in the results. We hope that soon more and better

447   referenced genomes will be available that allow expanding our comparative study.

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COMPETING INTERESTS

Authors declare no competing interests.

DATA AND CODE AVAILABILITY

Data and code used in this manuscript is available at

https://figshare.com/projects/Cranial_Suture_Closure/81209

AUTHOR CONTRIBUTIONS

BE-A designed the study, collected the data, and wrote the manuscript.

BE-A and FB analyzed the data.

FB, XF, GM, and AN conceived and designed the in-house scripts for CAAS.

All authors interpreted the results and wrote the manuscript.

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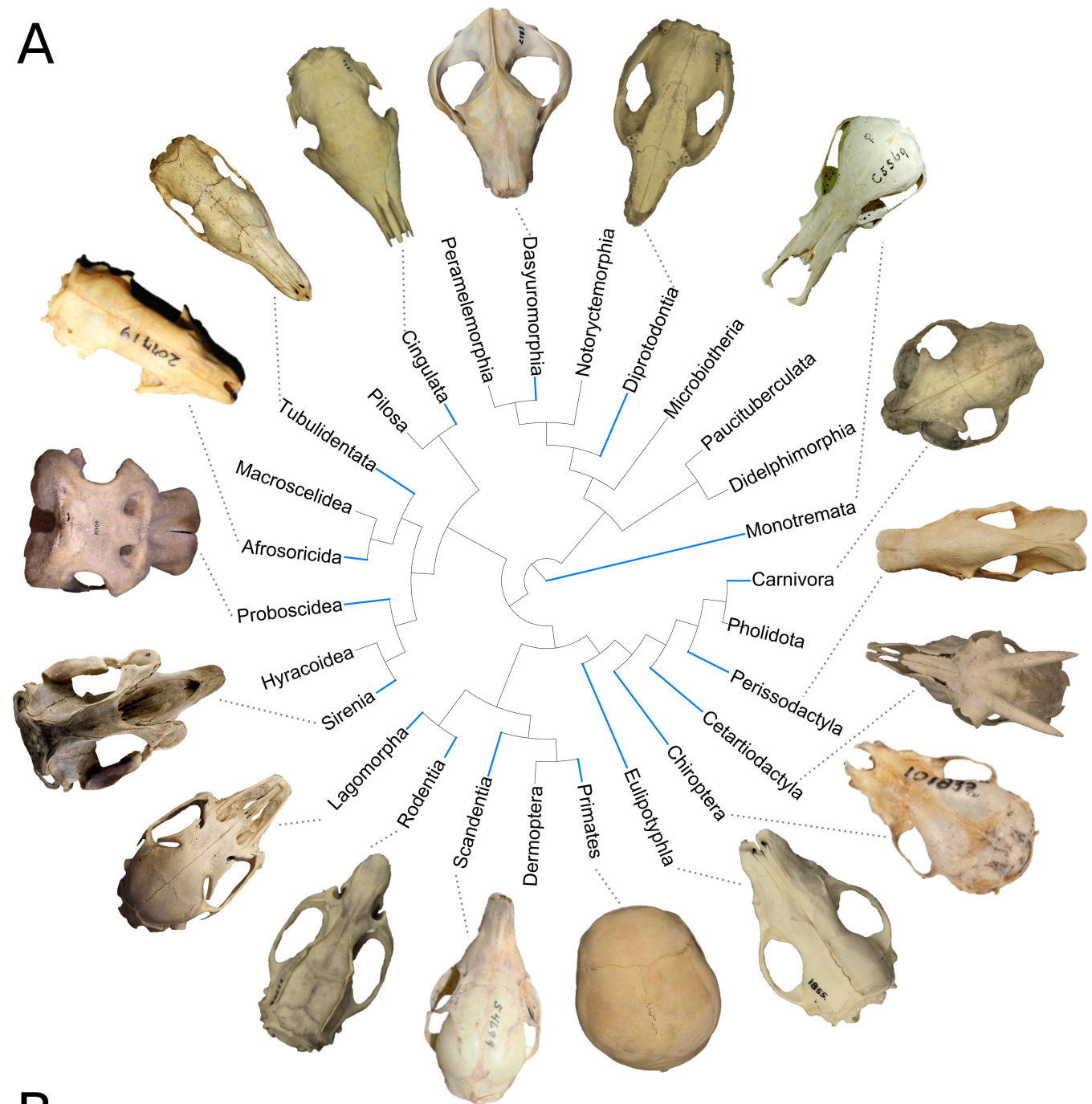
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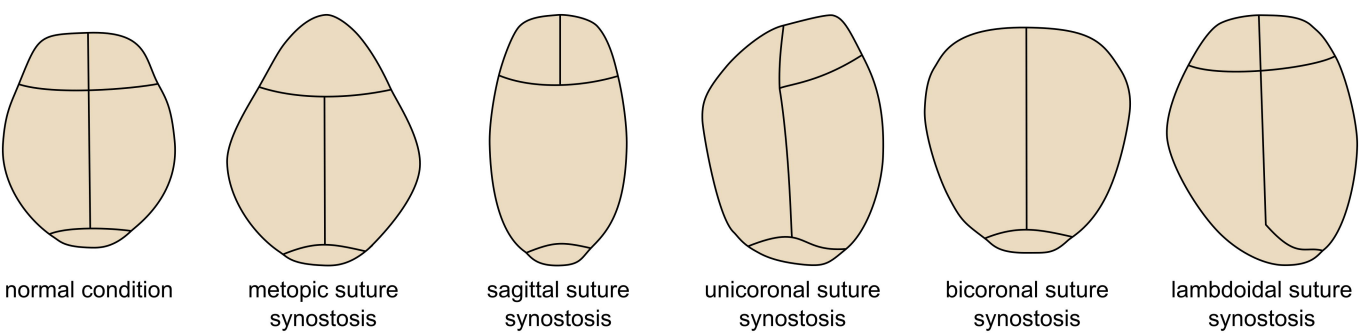
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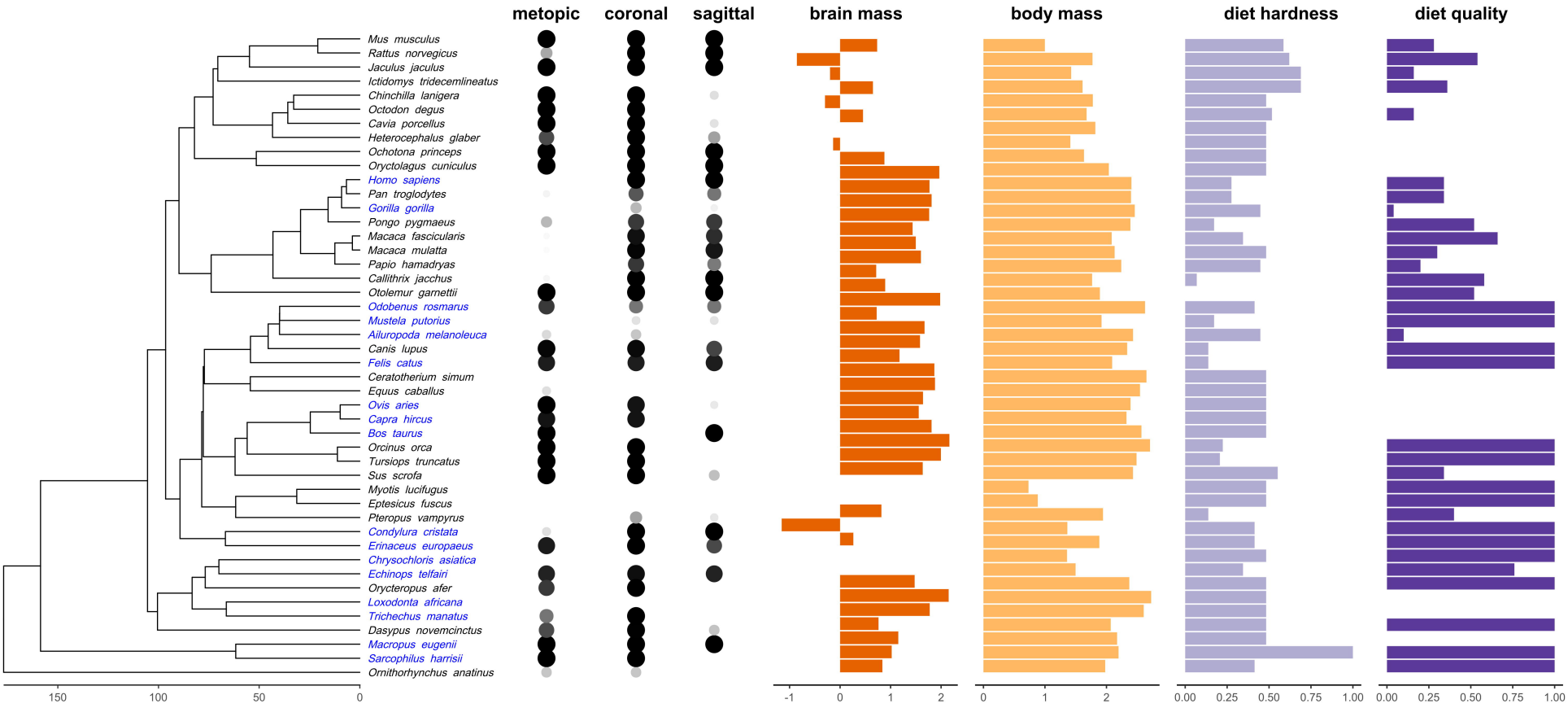
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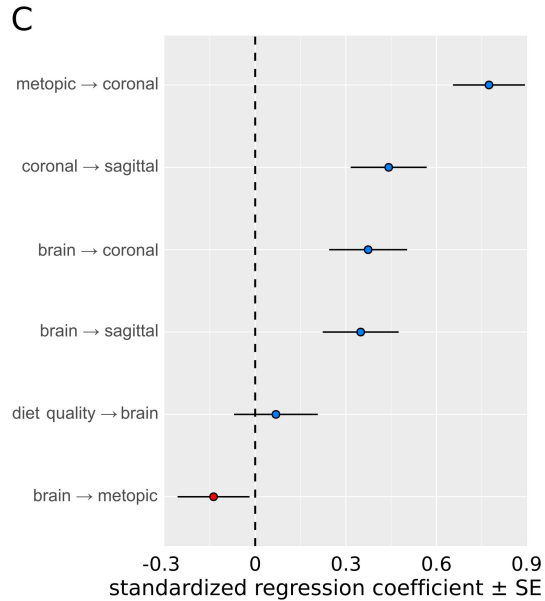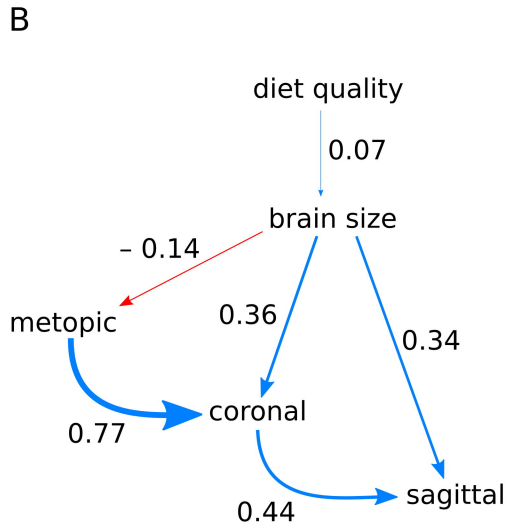associated with distinct substitutions in common genes. *Scientific Reports*, *5*(1), 16550.
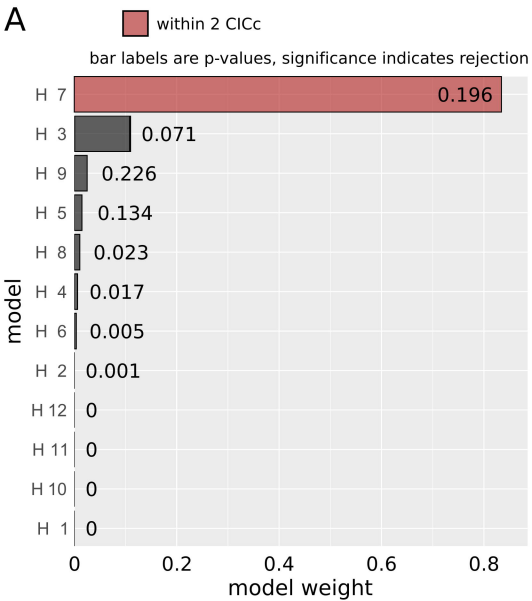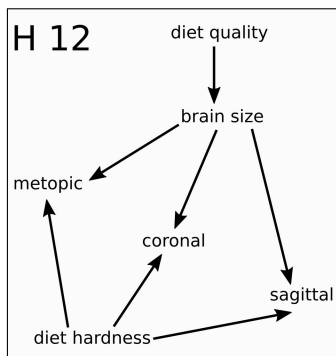
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A

Peramelemorphia
Dasyuromorphia
Notoryctemorphia
Diprotodontia
Microbiotheria
Paucituberculata
Didelphimorphia
Monotremata
Carnivora
Pholidota
Perissodactyla
Cetartiodactyla
Chiroptera
Eulipotyphla
Primates
Dermoptera
Scandentia
Rodentia
Lagomorpha
Sirenia
Hyracoidea
Proboscidea
Afrosoricida
Macroscelidea
Tubulidentata
Pilosa
Cingulata

B

normal condition

metopic suture synostosis

sagittal suture synostosis

unicoronal suture synostosis

bicoronal suture synostosis

lambdoidal suture synostosis

	metopic	coronal	sagittal	brain mass	body mass	diet hardness	diet quality

Mus musculus
Rattus norvegicus
Jaculus jaculus
Ictidomys tridecemlineatus
Chinchilla lanigera
Octodon degus
Cavia porcellus
Heterocephalus glaber
Ochotona princeps
Oryctolagus cuniculus
Homo sapiens
Pan troglodytes
Gorilla gorilla
Pongo pygmaeus
Macaca fascicularis
Macaca mulatta
Papio hamadryas
Callithrix jacchus
Otolemur garnettii
Odobenus rosmarus
Mustela putorius
Ailuropoda melanoleuca
Canis lupus
Felis catus
Ceratotherium simum
Equus caballus
Ovis aries
Capra hircus
Bos taurus
Orcinus orca
Tursiops truncatus
Sus scrofa
Myotis lucifugus
Eptesicus fuscus
Pteropus vampyrus
Condylura cristata
Erinaceus europaeus
Chrysochloris asiatica
Echinops telfairi
Orycteropus afer
Loxodonta africana
Trichechus manatus
Dasypus novemcinctus
Macropus eugenii
Sarcophilus harrisii
Ornithorhynchus anatinus

150   100   50   0   -1   0   1   2   0   1   2   0.00 0.25 0.50 0.75 1.00   0.00 0.25 0.50 0.75 1.00

A

within 2 CICc

bar labels are p-values, significance indicates rejection

B

diet quality

0.07

brain size

− 0.14

0.36

0.34

metopic

0.77

coronal

0.44

sagittal

C

scenarios

| | scenario 1
fixed → fixed | scenario 2
fixed → variable | scenario 3
variable → fixed |

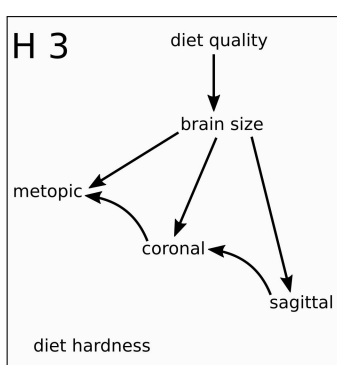**AA OPEN SUTURE**
AA CLOSED SUTURE
AA SUBSTITUTION

phenotypic groups

| | **metopic suture** | **coronal suture** | **sagittal suture** |

Homo sapiens — NHKCGANFLQ

Gorilla gorilla — NHKCGPNFLQ

Odobenus rosmarus — MLHSPNKQPQ

Mustela putorius — MLHSPHKQPQ

Ailuropoda melanoleuca — EDCKQALAFK

Felis catus — EDCKKALAFK

Ovis aries — EDCKLALAFK

Capra hircus — NHKCGANFLQ

Bos taurus — NHKCGENFLQ — EDCKKALAFK

Condylura cristata — MLHSPHKQPQ

Erinaceus europaeus — MLHSPNKQPQ

Chrysochloris asiatica — MLHSPHKQPQ

Echinops telfairi — MLHSPNKQPQ

Loxodonta africana — NHKCGANFLQ

Trichechus manatus — NHKCGKNFLQ

Macropus eugenii — EDCKKALAFK

Sarcophilus harrisii — EDCKGALAFK