

# 1 Differences in developmental potential predict the contrasting 2 patterns of dental diversification in characiform and cypriniform 3 fishes

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10

## 11 Abstract

12 Morphological diversification during adaptive radiation may depend on factors external or  
13 internal to the lineage. We provide evidence for the latter in characiform fishes (tetras and  
14 piranhas), which exhibit extensive dental diversity. Phylogenetic character mapping supported  
15 regain of lost teeth as contributing to this diversity. To test for latent potential for dentition that  
16 would facilitate its evolutionary expansion, we overexpressed a tooth initiation signal, the tumor  
17 necrosis factor pathway ligand ectodysplasin, in a model characiform, the Mexican Tetra  
18 (*Astyanax mexicanus*). This manipulation resulted in extensive ectopic dentition, in contrast to  
19 its previously-reported limited effect in the Zebrafish (*Danio rerio*). Tooth location in the Order  
20 Cypriniformes, to which the Zebrafish belongs, is much more restricted than in characiforms, a  
21 pattern that may be explained by differences in the retention of ancestral developmental  
22 potential. Our results suggest that differences in evolvability between lineages may lead to  
23 contrasting patterns of diversification.

24

## 25 Introduction

26 The morphological diversity present in a clade of organisms is influenced both by the  
27 environments encountered by the included species, as well as their evolvability – the capacity to  
28 generate adaptive variation (Wagner & Altenberg 1996, Gerhart & Kirshner 2003; Hendrikse *et al.*  
29 *et al.* 2007, Pigliucci 2008, Erwin 2017). One manner in which evolvability might be manifest is the  
30 biasing of phenotypic variants toward those that were adaptive in the past (Watson *et al.* 2014,  
31 Watson & Szathmáry 2016). An example is provided by the retention and re-expression of  
32 “ancestral developmental potential” for a specific caste morphology in the evolution of ants  
33 (Rajakumar *et al.* 2012). The degree to which such potential differs among clades and whether

34 these differences are responsible for differing patterns of morphological diversification remains  
35 largely unknown, however.

36 In a previous study (Aigler *et al.* 2014), we used overexpression of a tooth initiation signal  
37 encoded by the *ectodysplasin (eda)* gene to show that the Zebrafish (*Danio rerio*), a species  
38 with highly reduced dentition, retains limited potential to re-express teeth in ancestral locations.  
39 This limited potential is consistent with the pattern of dental diversification of the order  
40 Cypriniformes, to which the Zebrafish belongs. Cypriniform fishes, which include carps, loaches,  
41 minnows, suckers and over 4200 species, are dominant elements of the freshwater fish faunas  
42 of North America, Africa and Eurasia (Nelson *et al.* 2016). Despite exploiting a diversity of food  
43 sources, ranging from detritus to plants to insects to other fishes (Howes 1991), teeth in this  
44 group are restricted to a single pair of bones (fifth ceratobranchials) in the lower posterior  
45 pharynx (Stock 2007).

46 In contrast to the limited extent and evolutionary conservatism of tooth location in the  
47 Cypriniformes, the members of the related order Characiformes (tetras, piranhas, and relatives)  
48 generally exhibit a more extensive dentition; in addition, considerable variation in tooth location  
49 exists among species. The order Characiformes is actually smaller than the Cypriniformes  
50 (approximately 2300 species), exhibits a comparable diversity of diets (Guisande *et al.* 2012)  
51 and while co-occurring with cypriniforms in North America and Africa, is a dominant element of  
52 the freshwater fish fauna of South America, which lacks cypriniforms (Nelson *et al.* 2016). Teeth  
53 in characiforms may be found on marginal bones of the oral jaws (including their surfaces  
54 outside of the mouth), bones of the palate, paired bones and gill rakers of the upper and lower  
55 pharynx, and midline bones of the floor of the mouth and pharynx (Fink & Fink 1981;  
56 Novakowski *et al.* 2004; Oyakawa & Mattox 2009, Roberts 1969; 1973; Toledo-Piza 2000; 2007;  
57 Weitzman 1962; Weitzman & Fink 1985).

58 In the present study, we tested the hypothesis that the greater variability of tooth location in  
59 characiforms relative to cypriniforms is the result of a difference in evolvability, and specifically a  
60 greater retention of ancestral potential for dentition in the former group. This hypothesis is  
61 based on the commonly-held view that the ancestral condition of dentition in bony fishes  
62 consisted of teeth on virtually all of the bones lining the oral and pharyngeal cavities, as can be  
63 seen in the extant bowfin (*Amia calva*) (Grande & Bemis 1998; Stock 2001). The toothless  
64 bones of the mouth and pharynx of any characiform (or cypriniform) species therefore bore  
65 teeth at some point in its ancestry. An alternative to our hypothesis on the cause of variability in  
66 tooth location in characiforms that does not involve retention of ancestral potential is that  
67 variability in tooth location among species arises simply from loss of teeth from the extensive

68 dentition of the common ancestor of this group. We used phylogenetic character mapping to  
69 show that while teeth have indeed been lost within the characiforms in this manner, there have  
70 also been instances of re-expansion of the dentition, a phenomenon that might involve the  
71 realization of latent developmental potential. We next tested for the existence of such potential  
72 in the characiform Mexican Blind Cave Tetra (*Astyanax mexicanus*) by overexpression of  
73 ectodysplasin. We found that such expression was capable of greatly expanding both the larval  
74 and adult dentitions of this species. Bones bearing ectopic teeth included several that have  
75 regained lost teeth in characiform evolution, as well as others from which teeth are absent in all  
76 characiforms but have been regained in other lineages outside of this group. In addition to  
77 supporting our specific hypothesis that dental evolution in characiforms has resulted from the  
78 realization of retained latent potential for dentition, our results suggest that differences in  
79 morphological outcomes in related groups radiating in similar environments may result from  
80 differences in evolvability.

81

## 82 **Results**

### 83 *Distribution of teeth in the Characiformes*

84 Because of the absence of a concise summary of all of the bones that may bear teeth in  
85 characiforms, we surveyed the osteological and taxonomic literature of this group (Weitzman  
86 1962; Roberts 1969; 1973; Fink & Fink 1981; Weitzman & Fink 1985; Toledo-Piza 2000; 2007;  
87 Novakowski *et al.* 2004; Oyakawa & Mattox 2009) to produce our own (Fig. 1J, L). Teeth may  
88 be found on all of the bones of the jaw margins – the premaxillaries and maxillaries of the upper  
89 jaw and the dentaries of the lower jaw (Fig. 1B-D). In some genera, such as *Tytocharax* and  
90 *Roeboides*, teeth are present on the surfaces of these bones that extend outside of the mouth  
91 (Fig. 1G).

92 The roof of the mouth (loosely the palate) of teleost fishes is lined medially by bones of the  
93 ventral braincase and laterally by bones comprising the hyopalatine arch or suspensorium  
94 (Hilton 2011). In characiforms, palatal teeth are limited to the suspensorium, and may be  
95 present on the ectopterygoids, endopterygoids and metapterygoids (Figure 1H, J-L). An  
96 additional tooth plate anterior to the ectopterygoid that is present in members of the families  
97 Erythrinidae (Fig. 1H) and Hepsetidae has been considered neomorphic (an accessory  
98 ectopterygoid) rather than a dermopalatine, which is absent in characiforms but occupies a  
99 similar position in some teleosts (Fig 1H) (Roberts 1969, 1973, Fink & Fink 1981).

100 As in cypriniforms, teeth may be found in the lower pharynx on the fifth ceratobranchials  
101 (last gill arch) (Fig 1F, J-L). Unlike cypriniforms, characiforms may have teeth on upper

102 pharyngeal tooth plates supported by the second and third pharyngobranchials and third and  
103 fourth epibranchials (Fig. 1E, J-L). Teeth may also be found on gill rakers attached to all five gill  
104 arches (Fig. 1F). In the midline of the mouth and pharynx, teeth may be found (rarely) on tooth  
105 plates attached to the basihyal (“tongue”) and basibranchials (Fig 1I-J, L).

106

#### 107 *Loss and reappearance of teeth in the evolution of characiforms*

108 We next searched for evidence that teeth had reappeared during the evolution of the  
109 Characiformes. Teeth on the metapterygoid bones of the suspensorium are extremely rare in  
110 ray-finned fishes, being found only in the non-teleost families Amiidae (the Bowfin) and  
111 Polypteridae (bichirs), as well as the characiform genera *Hydrolycus* and *Raphiodon* of the  
112 Cynodontinae (Toledo-Piza 2000). This subfamily is nested within the Characiformes in  
113 molecular (Oliveira *et al.* 2011; Arcila *et al.* 2017; Betancur-R *et al.* 2019), morphological  
114 (Mirande 2009, 2010) and combined (Mirande 2019) phylogenies, providing strong support for  
115 the reappearance of these teeth after an absence of 200-300 million years (Irisarri *et al.* 2017;  
116 Hughes *et al.* 2018). Mirande (2009, 2010) compiled a morphological dataset for 160  
117 characiform species that allows mapping the presence or absence of teeth on the premaxillaries  
118 outside of the mouth, maxillaries, ectopterygoids, endopterygoids, fourth basibranchial, gill  
119 rakers, fifth ceratobranchials, and pharyngobranchials (third, fourth, and fifth) on his phylogeny  
120 (Fig. S1, S2). Premaxillary teeth outside of the mouth are not present in non-teleostean ray-  
121 finned fishes (Nelson *et al.* 2016) but appeared in multiple characiform lineages. Maxillary teeth  
122 are reconstructed as having appeared within the Characiformes, but use of alternative  
123 outgroups would likely change this interpretation (Stock 2007). Reappearance of teeth within the  
124 Characiformes is supported for ectopterygoid, endopterygoid, and basibranchial bones, as well  
125 as gill rakers. To test the robustness of a subset of these results, we mapped presence and  
126 absence of ectopterygoid and endopterygoid teeth onto the molecular phylogeny of Oliveira *et*  
127 *al.* (2011) (Fig. 2). We chose these teeth because of the necessity of compiling a character  
128 matrix (Table S1; Supplementary References) for taxa not present in Mirande’s (2009) analysis,  
129 which was facilitated by the fact that these teeth are commonly mentioned in taxonomic studies  
130 of characiform species. Our analysis suggested that ectopterygoid teeth were present in the  
131 common ancestor of characiforms and were regained after loss four times within the group (Fig.  
132 2A, S3A). Endopterygoid teeth were reconstructed as absent in the characiform common  
133 ancestor and were gained five times within the group (Fig. 2B, S3B). Reappearance of  
134 ectopterygoid and endopterygoid teeth during characiform evolution was also supported by a

135 similar analysis using the phylogeny of Betancur-R *et al.* (2019) (Fig. S3C, D; Table S2;  
136 Supplementary References).

137

### 138 *Larval dentition of eda-overexpressing A. mexicanus*

139 The developmental genetic basis of the loss and reappearance of teeth in characiform  
140 evolution remains unknown. A candidate cause is modification of the ectodysplasin signaling  
141 pathway, which has been shown in the Zebrafish to be both necessary for tooth development  
142 (Harris *et al.* 2008) as well as sufficient for expanding tooth-bearing locations (Aigler *et al.*  
143 2014). We tested the ability of altered *eda* signaling to induce ectopic teeth in a model  
144 characiform species, the Mexican Blind Cave Tetra, *Astyanax mexicanus* (Jeffery 2009; Casane  
145 & Rétaux 2016), by injection of an *eda* overexpression construct into one-celled embryos.  
146 Injection of a similar construct in the Zebrafish expanded dentition along the dorsal-ventral axis,  
147 but not along the anterior-posterior axis (Aigler *et al.* 2014). Specifically, teeth in wild type  
148 zebrafish are found only in the posterior ventral pharynx, while overexpression of *eda*-induced  
149 ectopic teeth in this location, as well as the posterior dorsal pharynx.

150 The wildtype dentition of *A. mexicanus* is similar to that of numerous characiforms, with  
151 teeth being found on the premaxillary, maxillary and dentary bones of the oral jaw margins, the  
152 fifth ceratobranchial bones of the lower pharynx, and dorsal pharyngeal tooth plates attached to  
153 the second and third pharyngobranchials, as well as the third and fourth epibranchials (Figure  
154 1B-F, J-K) (Valdéz-Moreno & Contreras-Balderas 2003). In addition, teeth are present on gill  
155 rakers attached to dorsal and ventral elements of the anterior four gill arches, as well as the  
156 ventral fifth ceratobranchials (Atukorala & Franz-Odenaal 2014). In our initial injections of the  
157 *eda*-overexpression construct, we examined larvae stained for calcified structures with alizarin  
158 red at 6 days post-fertilization (dpf). In wild type larvae of this age, teeth are limited to the  
159 premaxillary and dentary bones of the oral jaws, the fifth ceratobranchials and the posterior-  
160 most upper pharyngeal toothplate (Trapani *et al.* 2005; Atukorala & Franz-Odenaal 2014), *i.e.*  
161 dorsally and ventrally at the anterior and posterior margins of the oropharyngeal cavity (Fig. 3A-  
162 H). We found ectopic teeth in 47 of 397 (11.8%) larvae surviving to 6 dpf following injection with  
163 the *eda*-overexpression construct and none of the 33 surviving control larvae injected with a  
164 similar construct for expressing green fluorescent protein (*gfp*) ( $p = 0.0372$ , Fisher's exact test).  
165 In contrast to our previous results with the zebrafish (Aigler *et al.* 2014), we found that *eda*  
166 overexpression was capable of expanding the dentition into the central part of the  
167 oropharyngeal cavity, including laterally on anterior ceratobranchials and medially in the ventral  
168 basibranchial area (Fig. 3E-H).

169

170 *Ectopic palatal teeth in eda-overexpressing A. mexicanus*

171 Most of the bones on which teeth have reappeared in characiform evolution are not present  
172 in 6 dpf larvae and the description of dentition is largely restricted to adult specimens.

173 Therefore, in order to compare the dentition of *eda*-overexpressing *A. mexicanus* with that of  
174 other characiform species, we examined specimens at juvenile stages (25-274 dpf) in which all  
175 adult ossifications are present. We focused our analysis on the palate, both for its accessibility,  
176 as well as the fact that many of the likely reappearances of teeth in characiform evolution  
177 occurred in this region. The specific bones we scored for the presence of teeth were the  
178 palatine, ectopterygoid, endopterygoid, and metapterygoid bones of the suspensorium and the  
179 vomer and parasphenoid bones of the ventral braincase. None of these bones is toothed in wild  
180 type *A. mexicanus* (Valdéz-Moreno & Contreras-Balderas 2003).

181 We found ectopic teeth in 51 of 195 (26.2%) of juveniles injected with the *eda* expression  
182 construct and none of the 25 control *gfp*-injected juveniles ( $p = 0.0017$ , Fisher's exact test).  
183 These ectopic teeth were located on the ectopterygoid (Fig. 4E, F, H) ( $n = 35$  individuals,  
184 17.9%), endopterygoid (Figure 4H) ( $n = 12$ , 6.2%), the boundary between the ectopterygoid and  
185 endopterygoid (Figure 4G) ( $n = 4$ , 2.1%), the parasphenoid (Fig. 5B) ( $n = 3$ , 1.5%) and the  
186 vomer (Fig. 5E, F) ( $n = 3$ , 1.5%). No teeth were found on the palatine (lacking teeth in all  
187 characiforms) or metapterygoid (toothed in a few characiform lineages). Interestingly, the rank  
188 order ectopterygoid > endopterygoid > parasphenoid, vomer parallels the frequency of these  
189 teeth in the order Characiformes (with parasphenoid and vomerine teeth being completely  
190 absent).

191

192 **Discussion**

193 *Contrasting patterns of dental diversification in cypriniforms and characiforms*

194 Cypriniforms and characiforms are members of the Superorder Ostariophysi (Nelson *et al.*  
195 2016), which has been considered one of nine exceptional radiations in the history of jawed  
196 vertebrates (Alfaro *et al.* 2009). Within this radiation, both groups have diversified to fill similar  
197 trophic niches, with changes in tooth shape and organization thought to have played a major  
198 role in the process (Howes 1991; Guisande *et al.* 2012, Burns & Sidlauskas 2019). The pattern  
199 of dental diversification is strikingly different between the two groups, however (Gosline 1973).  
200 Cypriniforms lack teeth in the mouth cavity but exhibit extensive variation in shape, number, and  
201 arrangement of teeth in the pharynx (Sibbing 1991; Stock 2007; Pasco-Viel *et al.* 2010).  
202 Variation in tooth location is limited to the simple presence (most cypriniforms) or absence

203 (Gyrinocheilidae – algae eaters) of teeth on the fifth ceratobranchial bones of the lower posterior  
204 pharynx (Stock 2007; Nelson *et al.* 2016). Characiforms also exhibit extensive variation in tooth  
205 shape, number, and arrangement (Guisande *et al.* 2012), but this variation is largely limited to  
206 the teeth of the oral jaw margins – those of the pharynx have a simple conical shape in most  
207 groups (Roberts 1969). In addition, and in contrast to cypriniforms, characiforms exhibit  
208 substantial variation in tooth location, particularly on bones of the palate (Weitzman 1962;  
209 Roberts 1969; 1973; Fink & Fink 1981; Weitzman & Fink 1985; Toledo-Piza 2000; 2007;  
210 Novakowski *et al.* 2004; Oyakawa & Mattox 2009).

211 While diversification of tooth location in characiforms could simply be the result of loss from  
212 a more extensive ancestral dentition, it has been suggested that expansion of dentition has also  
213 occurred within the group, particularly for the ectopterygoid and endopterygoid teeth of the  
214 palate (Roberts 1973; Weitzman & Kanazawa 1976). We used phylogenetic character mapping  
215 to confirm these early proposals that were not based on explicit phylogenetic hypotheses.  
216 Specifically, we found evidence that ectopterygoid teeth, which were likely to have been present  
217 in the common ancestor of characiforms, were regained at least four times after being lost, while  
218 endopterygoid teeth, likely absent in this common ancestor, were gained at least five times (Fig.  
219 2, S1, S3).

220  
221 *Potential functional explanations for the regain of palatal teeth in characiform but not cypriniform*  
222 *evolution*

223 An ancestral condition from which both cypriniform and characiform dentitions likely evolved  
224 is the presence of teeth throughout the oral and pharyngeal cavities (Gosline 1973). Palatal  
225 teeth in such predatory forms, represented by the modern day *Elops* (Ladyfish), serve to grasp  
226 struggling prey and facilitate its transport posteriad toward the pharynx and esophagus (Gosline  
227 1973). A common trend in the evolution of the teleost fish dentition is its reduction in the central  
228 portion of the oral and pharyngeal cavities (including the palate) and its concentration anteriorly  
229 in oral and posteriorly in pharyngeal jaws (Gosline 1985). In characiforms, this trend is manifest  
230 in many species through specialization of oral jaw dentition for biting and shearing (Gosline  
231 1973). A notorious example is provided by piranhas (Serrasalminidae), in which blade-like teeth  
232 allow biting pieces from animals too large to ingest. Interestingly, ectopterygoid teeth, which occur  
233 in some members of this family, exhibit a similar flattened shape to the teeth of the oral jaw  
234 margins (Roberts 1969) and may also function as part of the shearing bite. Palatal teeth in other  
235 characiforms are simple cones in shape (Figure 1H, 4D) (Roberts 1969), and as they appear to

236 be limited to insect and fish-eating species (Roberts 1973), are likely to serve the ancestral  
237 function of gripping and transporting prey.

238 The feeding apparatus of cypriniforms has evolved in a quite different direction from that of  
239 characiforms (Gosline 1973; Sibbing *et al.* 1986; Sibbing 1991). The posterior pharynx  
240 constitutes a powerful apparatus for mastication of food items, with teeth on hypertrophied lower  
241 pharyngeal bones biting against a dorsal keratinized pad braced by the basioccipital bone of the  
242 braincase. The mouth has become specialized for suction feeding, with a protrusible upper jaw  
243 (premaxilla) that serves a variety of functions, such as controlling the direction of water flow  
244 (Sibbing *et al.* 1986). Because teeth are absent from the mouth, palate, and upper pharynx, the  
245 function of transporting food has been assumed by muscular (dorsal) palatal and (ventral)  
246 postlingual organs that provide a peristaltic action sufficient to transport small food particles  
247 posteriorly to the masticatory apparatus (Sibbing *et al.* 1986). It is thought that the cypriniform  
248 feeding apparatus is particularly effective for feeding on plant and animal matter in bottom  
249 deposits (Gosline 1973; Sibbing *et al.* 1986; Sibbing 1991), and in such a role, palatal teeth  
250 might serve no useful role. Several lineages of cypriniforms have secondarily evolved the habit  
251 of feeding on other fishes, however, with modifications to the typical cypriniform condition  
252 including reduction of premaxillary protrusion to allow a firm grip on prey between the oral jaws  
253 (Gosline 1973; Sibbing 1991), reduction of the palatal and postlingual organs (Doozey and Bart  
254 2011) and specialization of the pharyngeal teeth for laceration and transport of prey (Sibbing  
255 1991). A number of authors have speculated that fish-eating cypriniforms might be more  
256 efficient predators with more extensive dentition (Nichols 1930; Weisel 1962; de Graaf *et al.*  
257 2000; 2008) and indeed, radiation of such forms has occurred only in situations lacking  
258 competitors that retain oral teeth (de Graaf *et al.* 2000; 2008). We therefore suggest that the  
259 absence of palatal teeth in cypriniforms is not simply the result of absence of selection for them.

260

261 *Retention of ancestral developmental potential for palatal dentition in characiforms but not*  
262 *cypriniforms*

263 Well before methods existed to test their hypothesis, Weitzman and Kanazawa (1976)  
264 proposed that “teeth and bony tooth patches remain a genetic potential for nearly any oral  
265 surface in characoids [characiforms].” We have demonstrated the existence of such potential  
266 through the overexpression of *eda* in the characiform *Astyanax mexicanus*. Some of this  
267 potential has been realized in characiform evolution in the form of reappearance of teeth on the  
268 ectopterygoid and endopterygoid bones (Fig. 6). We also found that *eda* is capable of inducing  
269 teeth in locations that are toothed in some teleosts but not in any characiform, namely the



270 vomer and the parasphenoid on the midline of the oral cavity (Fig. 5, 6). Interestingly, vomerine  
271 teeth are likely to have reappeared in the evolution of the ostariophysan order Siluriformes  
272 (catfishes) (Fink & Fink 1981), and in an analogous situation, have been discovered as an  
273 atavism in a single individual of the Black Drum, *Pogonias cromis* (Cione & Torno 1987). It has  
274 also been suggested that teeth on the parasphenoid have reappeared in the evolution of spiny-  
275 rayed fishes (Fig. 5) (Gosline 1985); we have provided more explicit evidence that this is the  
276 case by mapping parasphenoid teeth on a phylogeny of ray-finned fishes (Fig. S4; Table S3,  
277 Supplementary References).

278 The developmental potential for dentition that we have demonstrated in *Astyanax* does not  
279 appear to have been retained in the cypriniform Zebrafish. In a previous study (Aigler *et al.*  
280 2014), we found that overexpression of ectodysplasin was capable of inducing ectopic upper  
281 pharyngeal teeth, but no teeth appeared on the palate or other regions of the oral cavity. If this  
282 restricted potential is characteristic of cypriniforms in general, it may explain the “failure” to  
283 regain palatal teeth during the radiation and trophic diversification of this group.

284

285 *The nature of the difference in retained potential for dentition between characiforms and*  
286 *cypriniforms*

287 The nature of the difference in competence to produce teeth in the oral cavity between  
288 characiforms and cypriniforms remains unknown. Aigler *et al.* (2014) showed that the oral  
289 epithelium of the zebrafish retains broad competence to respond to ectodysplasin signaling with  
290 activation of NF-kappaB, a transcriptional effector of this pathway. The transcription factor *pitx2*  
291 and signaling ligand *shh* are considered markers of dental competence (Fraser *et al.* 2008) and  
292 both are present in the oral region of developing zebrafish larvae (Stock *et al.* 2006). It has been  
293 reported that *Astyanax mexicanus* has two *eda* co-orthologs, while the Zebrafish retains only  
294 one (Braasch *et al.* 2009), but how this might relate to competence to respond to the ligand with  
295 tooth initiation is unclear.

296 Competence to form teeth on anterior gill arches at early larval stages in *Astyanax*  
297 *mexicanus* might be maintained because of the later development of teeth in these locations on  
298 gill rakers (at approximately 40 dpf - Atukorala & Franz-Odenaal 2014). Not all characiforms  
299 possess such teeth and it would be interesting to determine whether species without toothed gill  
300 rakers also retain competence to form teeth in the anterior pharynx.

301

302 *Retention of ancestral developmental potential as a component of evolvability*

303 Evolvability has been argued to be the central focus of Evolutionary Developmental Biology  
304 (Hendrikse *et al.* 2007) but what constitutes evolvability has differed widely among authors  
305 (Pigliucci 2008; Brown 2014; Payne & Wagner 2019). Features that have been proposed to  
306 contribute to evolvability include standing genetic variation in populations (Barrett & Schluter  
307 2008; Huang 2015), key (morphological or physiological) innovations (Hunter 1998), gene or  
308 genome duplication (Ohno 1970; Cuypers & Hogeweg 2014), and features of developmental  
309 systems, such as modularity and integration (Hendrikse *et al.* 2007; Le Pabic *et al.* 2016; Fish  
310 2019). We suggest that in the case of characiform fishes, evolvability is enhanced by retention  
311 of competence to respond to tooth induction signals in a much broader region of the oropharynx  
312 than such signals are normally produced. Such retention of ancestral developmental potential  
313 has been documented in the case of ant castes (Rajakumar *et al.* 2012); we further demonstrate  
314 that this type of evolvability differs among lineages and may have contributed to differences in  
315 morphological diversification during parallel adaptive radiations. If so, these radiations have  
316 been sculpted by “developmental push” in addition to “environmental pull” (Erwin 2017).

317

318 **Materials and Methods**

319 *Ancestral state reconstruction*

320 We used the character states in Mirande’s (2009) matrix of morphological features to map  
321 the presence or absence of teeth in multiple locations (premaxillaries outside of the mouth,  
322 maxillaries, ectopterygoids, endopterygoids, fourth basibranchial, gill rakers, fifth  
323 ceratobranchials, and third, fourth and fifth pharyngobranchials) on the characiform tree  
324 topology from his weighted parsimony analysis (implied weighting scheme - his Figures 1-2).  
325 Our analysis (Fig. S1, S2) was conducted with the parsimony option of Mesquite (Maddison &  
326 Maddison 2019).

327 Ancestral states for ectopterygoid and endopterygoid teeth were also reconstructed using  
328 the characiform molecular phylogenies of Oliveira *et al.* (2011) and Betancur-R *et al.* (2019).  
329 The tree topology and branch lengths that we used from the study of Oliveira *et al.* (2011) were  
330 based on its maximum likelihood analysis of partial sequences of two mitochondrial and three  
331 nuclear genes from 213 specimens. We assigned presence or absence of ectopterygoid teeth to  
332 128 of these taxa and endopterygoid teeth to 94 using statements from the literature about the  
333 species, or in some cases, the genus or family to which it belonged (Table S1; Supplementary  
334 References). The Betancur-R *et al.* (2019) topology and branch lengths were from the maximum  
335 likelihood analysis of 1051 exons from 206 characiform species presented in their Figure 4. We

336 assigned presence or absence of ectopterygoid teeth to 135 of these species and  
337 endopterygoid teeth to 126 using statements from the literature about the species or about one  
338 or more congeners (Table S2; Supplementary Reference). In both cases, the original trees were  
339 ultrametricized using the penalized likelihood with `chronos` command in the ape R library (Kim &  
340 Sanderson 2008) and then pruned to fit the character dataset using the `drop.tip` function in  
341 `phytools` (Revell 2012). Ancestral states were then estimated by maximum likelihood using a  
342 continuous time Markov model of binary character evolution (Mk2) with the `asr.marginal` function  
343 in the R package `diversitree` (FitzJohn 2012).

344 The evolution of parasphenoid teeth in ray-finned fishes was reconstructed on the relaxed  
345 molecular clock phylogeny of Farina *et al.* (2015), which they inferred from Bayesian analysis of  
346 sequences of nine nuclear genes from 285 taxa representing 284 families. We assigned  
347 presence or absence of parasphenoid teeth to each of these taxa using statements in the  
348 literature about the species or the genus, family, suborder, or order to which it belonged (Table  
349 S3; Supplementary References). When this was not possible, we used character states  
350 reported for congeneric or confamilial species. Ancestral state reconstruction was carried out as  
351 described above for characiform molecular phylogenies.

352

### 353 *Transient transgenic overexpression of eda in A. mexicanus*

354 The *pEF1 $\alpha$ :EDA* plasmid described by Aigler *et al.* (2014) contains the zebrafish *eda* coding  
355 region under the control of the *Xenopus laevis ef1 $\alpha$*  promoter, which is expected to drive  
356 ubiquitous and continuous expression throughout development (Johnson & Krieg 1994). We  
357 modified this plasmid to allow screening injected embryos for DNA incorporation by adding an  
358 *mCherry* coding sequence with a separate *ef1 $\alpha$*  promoter to produce  
359 *pEF1 $\alpha$ :EDA/EF1 $\alpha$ :mCherry*.

360 *Astyanax mexicanus* embryos were collected from natural spawning of laboratory  
361 populations originating from either La Cueva Chica (San Luís Potosí, Mexico) or La Cueva de El  
362 Pachón (Tamaulipas, Mexico) (Jeffery & Martasian 1998). 0.5 nl of a solution containing 30 ng  
363 *pEF1 $\alpha$ :EDA/EF1 $\alpha$ :mCherry* and 30 ng mRNA encoding *tol2* transposase was injected into the  
364 blastomeres of one-celled embryos. Preliminary experiments suggested that the modified  
365 plasmid produced similar results to the original *pEF1 $\alpha$ :EDA*. Injection of *pTAL200R150G*  
366 (Urasaki *et al.* 2006), which contains an *egfp* coding region under the control of the *ef1 $\alpha$*   
367 promoter, served as a negative control.

368

### 369 *Histology*

370        Injected individuals exhibiting mCherry fluorescence as embryos were raised to a variety of  
371 larval and juvenile stages, sacrificed, and cleared and stained for calcified structures with  
372 Alizarin red S and, in some cases, cartilage matrix with Alcian blue. These procedures followed  
373 Wise & Stock (2010) for larvae and Hanken & Wassersug (1981) for juveniles. Intact larvae  
374 were imaged in bright field with a Zeiss Axiovert 135 inverted compound microscope equipped  
375 with a Zeiss AxioCam digital camera, while juveniles were dissected before imaging in bright  
376 field or fluorescence with Zeiss Discovery V8 or Leica MZ FLIII stereomicroscopes. The former  
377 stereomicroscope was equipped with a Zeiss AxioCam MRc5 camera and the latter with a Leica  
378 DFC7000 T camera.

379

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386

### 387 **Competing interests**

388        The authors have no competing interests to declare.

389

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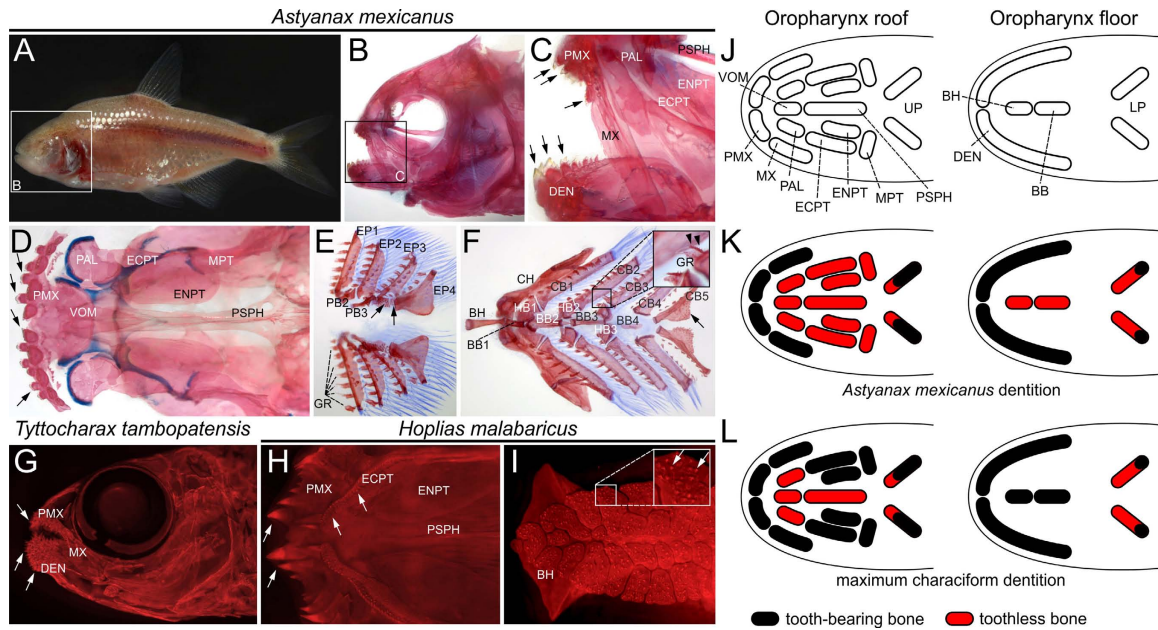
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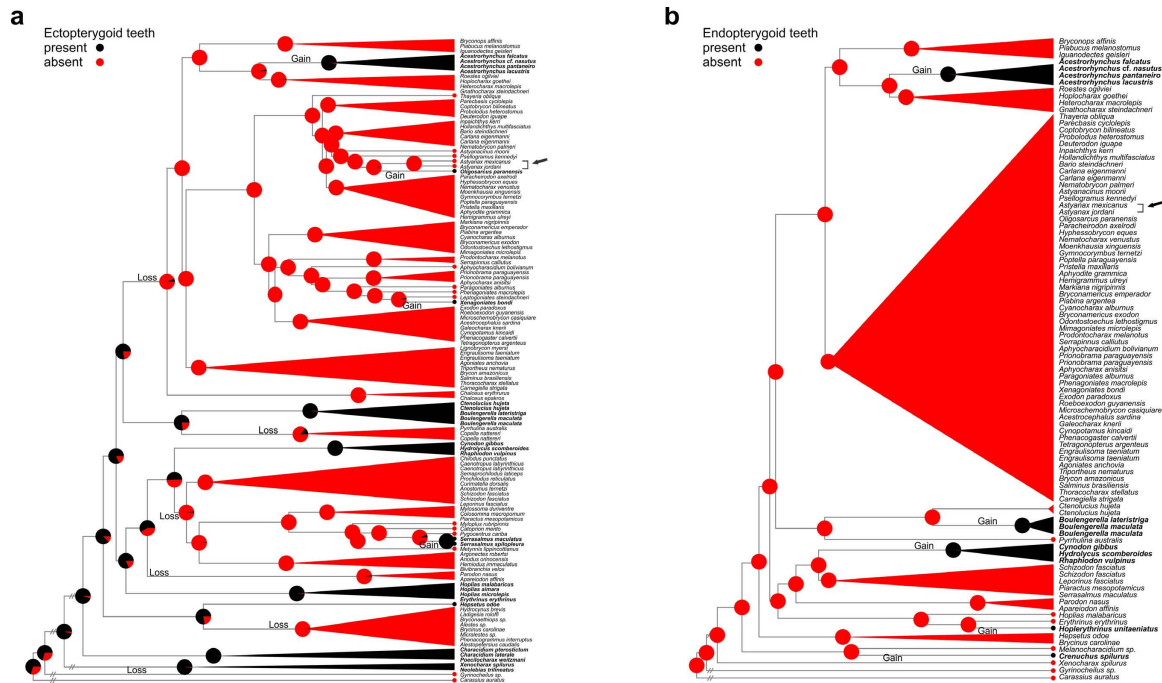
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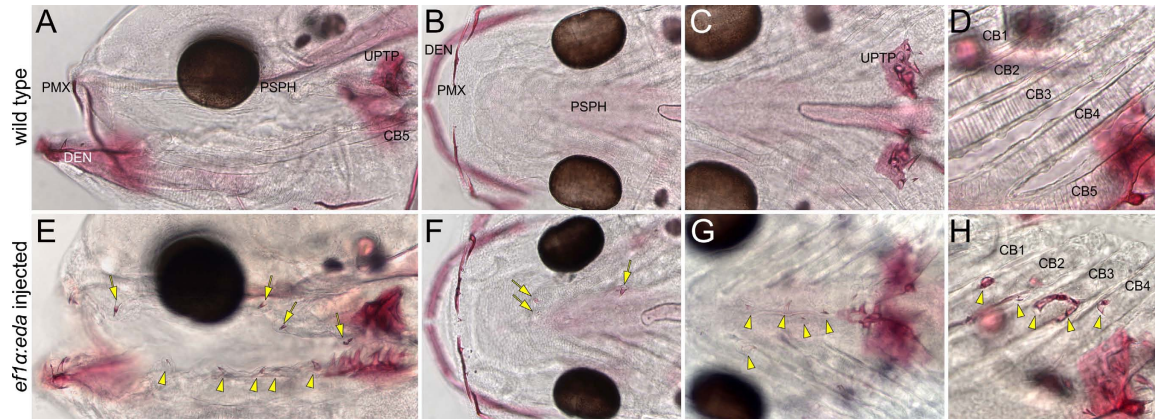
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**Figure 1.** Distribution of teeth in wild type *Astyanax mexicanus* and other members of the Order Characiformes. A) Lateral view of adult cave-dwelling morph of *A. mexicanus*. B-F) Alizarin red and Alcian blue-stained intact (B, C) and dissected (D-F) head skeletons of adult *A. mexicanus*. Lateral view of jaw margins (B, C), ventral view of palate (D), ventral view of dorsal gill arches (E) and dorsal view of ventral hyoid and gill arches (E, F). Teeth (arrows) are present on premaxillary (C, D), maxillary (C) and dentary (C) bones (C), of the jaw margins, are absent from the palate (D), and are present on tooth plates supported by pharyngobranchials and epibranchials of the dorsal gill arches (E), as well as the fifth ceratobranchials of the lower gill arches (F). Teeth are additionally present on gill rakers attached to epibranchials (E), hypobranchials and ceratobranchials (arrowheads in F). G) Lateral view of cleared and alizarin-stained head skeleton of *Tyttocharax tambopatensis* (Characiformes: Characidae) showing teeth (arrows) outside of the mouth on the premaxillary and dentary bones. H-I) Cleared and alizarin-stained palate (H) and basihyal (I) of *Hoplias malabaricus* (Characiformes: Erythrinidae). Anterior arrows in H indicate premaxillary teeth and middle and posterior arrows indicate accessory ectopterygoid and ectopterygoid teeth, respectively. Arrows in I indicate fine teeth attached to tooth plates supported by the basihyal (“tongue”). J-L) Schematic of bones lining the roof (left drawing) and floor (right drawing) of the oropharynx modelled after Figure 16 of Gosline (1971). Gill arches have been simplified as a single element with anterior and posterior ends corresponding to the position of individual arches along the anterior-posterior axis. Bones that bear teeth in *A. mexicanus* (K) and may bear teeth when the entire Order Characiformes is considered (L) are indicated in black, and bones without teeth in red. Abbreviations: BB, basibranchial; BH, basihyal; CB, ceratobranchial; CH, ceratohyal; DEN, dentary; ECPT, ectopterygoid; ENPT, endopterygoid; EP, epibranchial; GR, gill raker; HB, hypobranchial; MPT, metapterygoid; MX, maxillary; PAL, palatine; PB, pharyngobranchial; PMX, premaxillary; PSPH, parasphenoid; UP, upper pharyngeal elements; LP, lower pharyngeal elements; VOM, vomer.



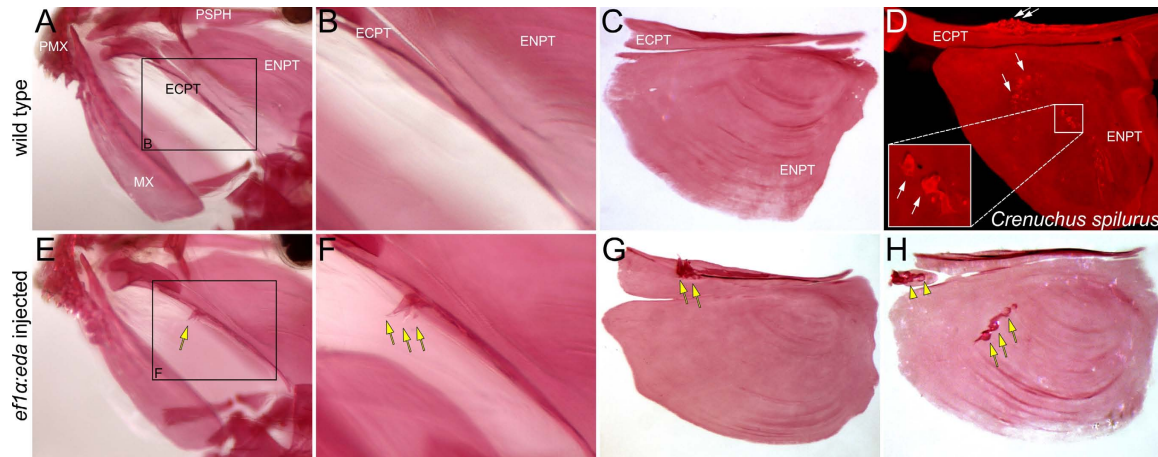
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**Figure 2.** Teeth on the ectopterygoid and endopterygoid bones have been re-gained following loss in multiple characiform lineages. Maximum likelihood ancestral state reconstruction of presence (black) or absence (red) of teeth on the ectopterygoid (A) and endopterygoid (B) bones. Tree topologies and branch lengths are from Oliveira *et al.* (2011) and character states were compiled from the literature (Supplementary References and Table S1). Hashmarks indicate branches not drawn to scale. Pie charts at nodes represent the relative probabilities of each character state. Clades with no internal character change were collapsed; full versions of the tree are presented in Figure S3(A, B) and reconstructions based on alternative phylogenies in Figures S1 and S3(C, D). *Carassius auratus* and *Gyrinocheilus* sp. are outgroups within the Order Cypriniformes, while the position of *A. mexicanus* (and its cave morph, sometimes designated *A. jordani*) is indicated with an arrow. Ectopterygoid teeth are reconstructed with highest probability as being present in the characiform common ancestor, with six losses and four re-gains occurring within the group. Endopterygoid teeth (lacking in the characiform common ancestor) were gained at least five times in the Characiformes.



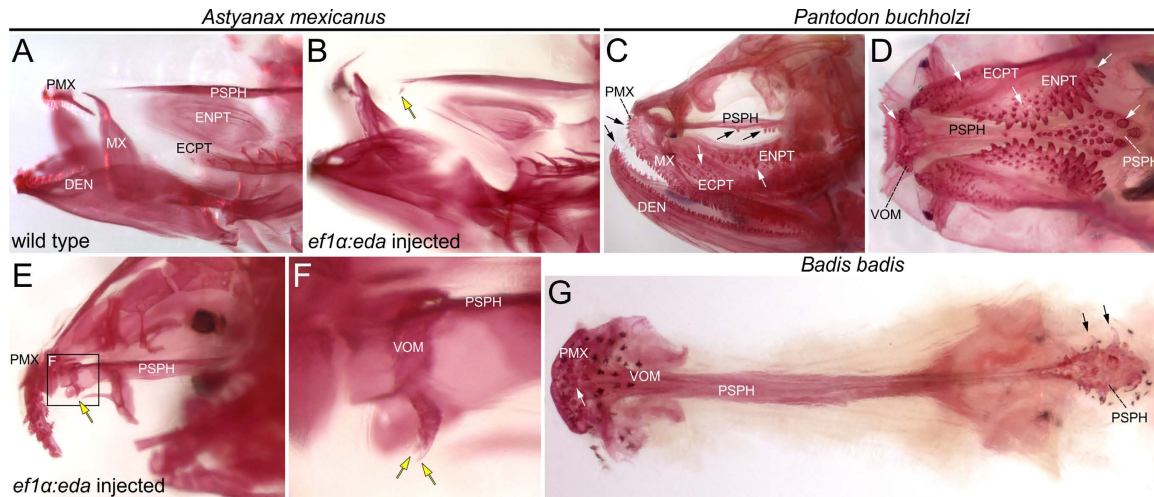
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**Figure 3.** Ectopic expression of *eda* expands the larval dentition of *A. mexicanus* into the central oropharynx. Cleared and alizarin-stained wild type (A-D) and *pEF1α:EDA*-injected larvae (E-H) at six dpf in lateral (A, E), dorsal (B-D, F-G) and ventral (H) views. Teeth in wild type larvae at this stage are restricted anteriorly to the premaxillaries and dentaries of the jaw margins (A, B), and posteriorly to upper pharyngeal toothplates and the (lower) fifth ceratobranchials (C, D). Dorsal ectopic teeth in *pEF1α:EDA*-injected individuals are indicated with yellow arrows and ventral ones with arrowheads. Ectopic teeth appear in the region of the parasphenoid (arrows immediately anterior and posterior to the eye in E and F), the basibranchials (five arrowheads in E and four in midline of G) and the second through fourth ceratobranchials (arrowheads in H). One individual is represented in A-D, one in E and G, and one in F and H. Abbreviations: CB, ceratobranchial; DEN, dentary; PMX, premaxillary; PSPH, parasphenoid; UPTP, upper pharyngeal tooth plate.



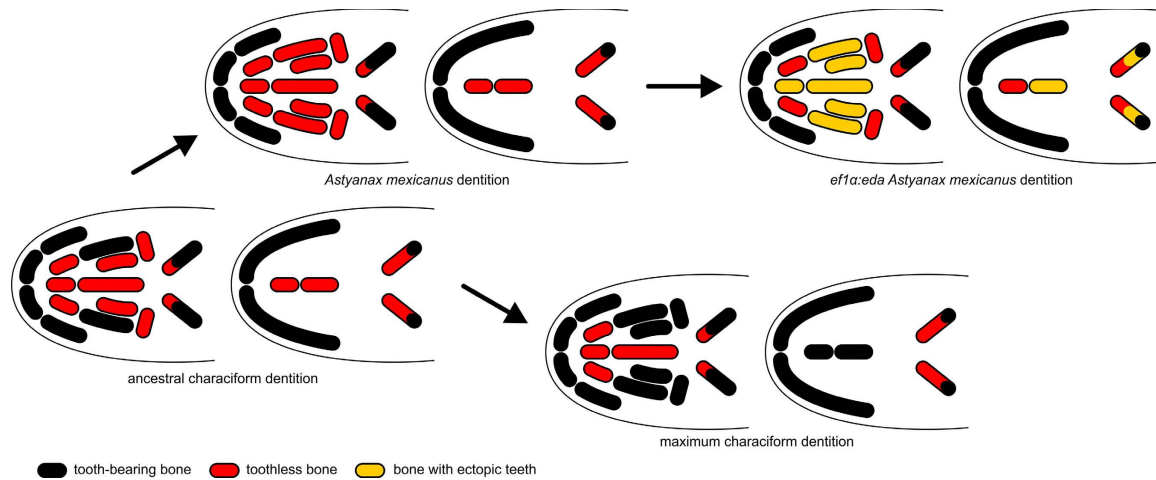
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620 **Figure 4.** Ectopic expression of *eda* induces teeth on the ectopterygoid and endopterygoid of  
621 adult *A. mexicanus*. Cleared and alizarin-stained wild type (A-C) and *pEF1α:EDA*-injected *A.*  
622 *mexicanus* (E-H) in left lateral (A-B, E-F) and ventral (C, G-H) views. Teeth may be present on  
623 either the ectopterygoid (arrows in E-G), the endopterygoid (arrows in H), or an ectopic bone  
624 anterior to the ectopterygoid (arrowheads in H) in *pEF1α:EDA*-injected specimens but are  
625 absent from both bones in wild type *A. mexicanus* (A-C). Teeth (arrows in D) are present on the  
626 ectopterygoid and endopterygoid of wild type *Crenuchus spilurus* (Characiformes: Crenuchidae)  
627 for comparison. Abbreviations: ECPT, ectopterygoid; ENPT, endopterygoid; MX, maxillary;  
628 PMX, premaxillary; PSPH, parasphenoid.  
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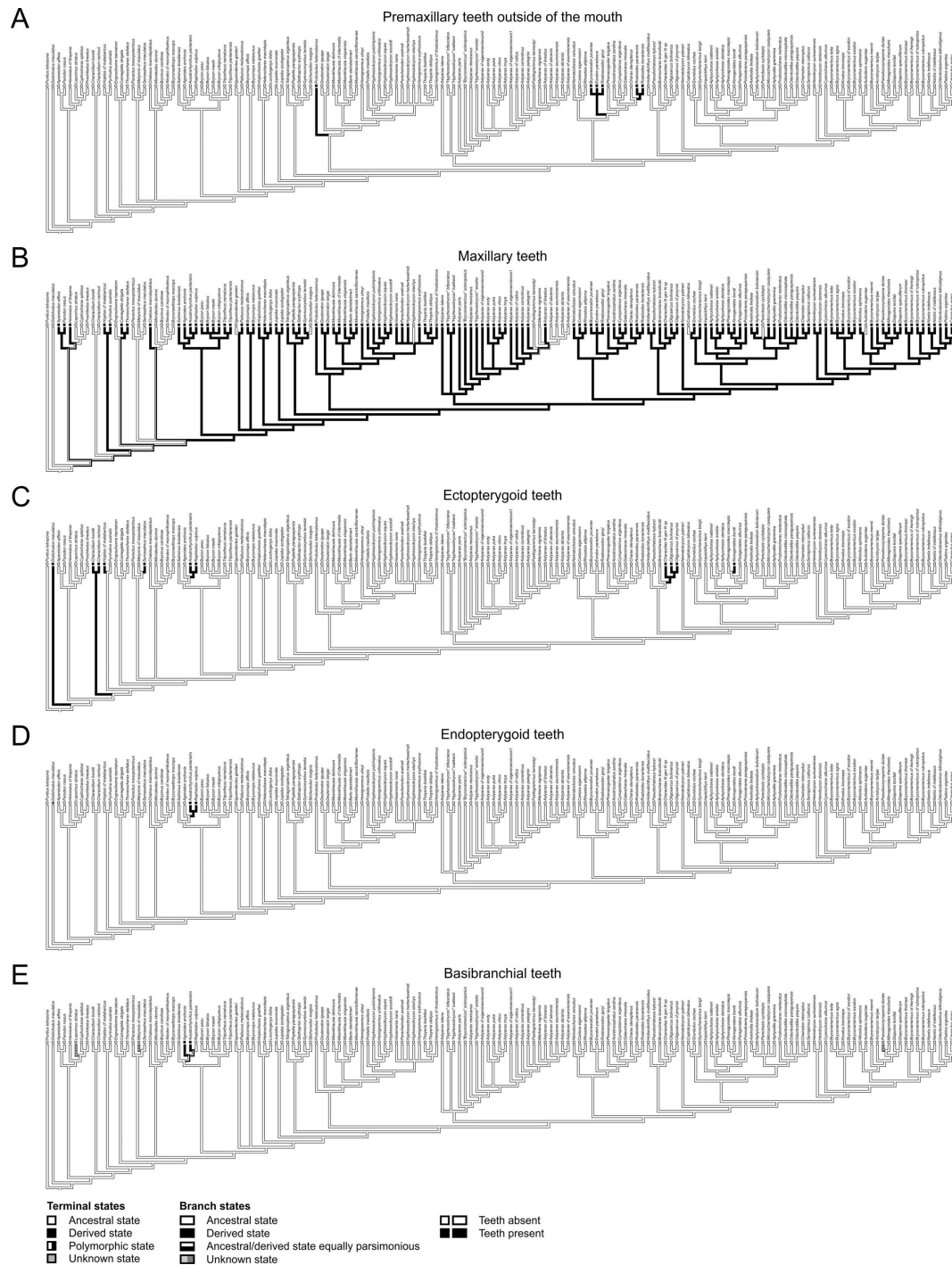


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**Figure 5.** Ectopic expression of *eda* induces teeth on the parasphenoid and vomer of adult *A. mexicanus*. Cleared and alizarin-stained wild type (A) and *pEF1α:EDA*-injected *A. mexicanus* (B, E-F)) in left lateral. Teeth may be present on either the parasphenoid (arrow in B) or the vomer (arrows in E-F) in *pEF1α:EDA*-injected specimens but are absent from both bones in wild type *A. mexicanus* (A). C-D) Parasphenoid and vomerine teeth are ancestrally present in teleost fishes such as *Pantodon buchholzi* (Osteoglossiformes: Pantodontidae). Teeth in additional locations are indicated by arrows. G) According to our ancestral state reconstructions (Figure S4), parasphenoid (black arrows) and vomerine (white arrow) teeth have re-evolved in the lineage leading to *Badis badis* (Perciformes: Badidae). Abbreviations: DEN, dentary; ECPT, ectopterygoid; ENPT, endopterygoid; MX, maxillary; PMX, premaxillary; PSPH, parasphenoid; VOM, vomer.



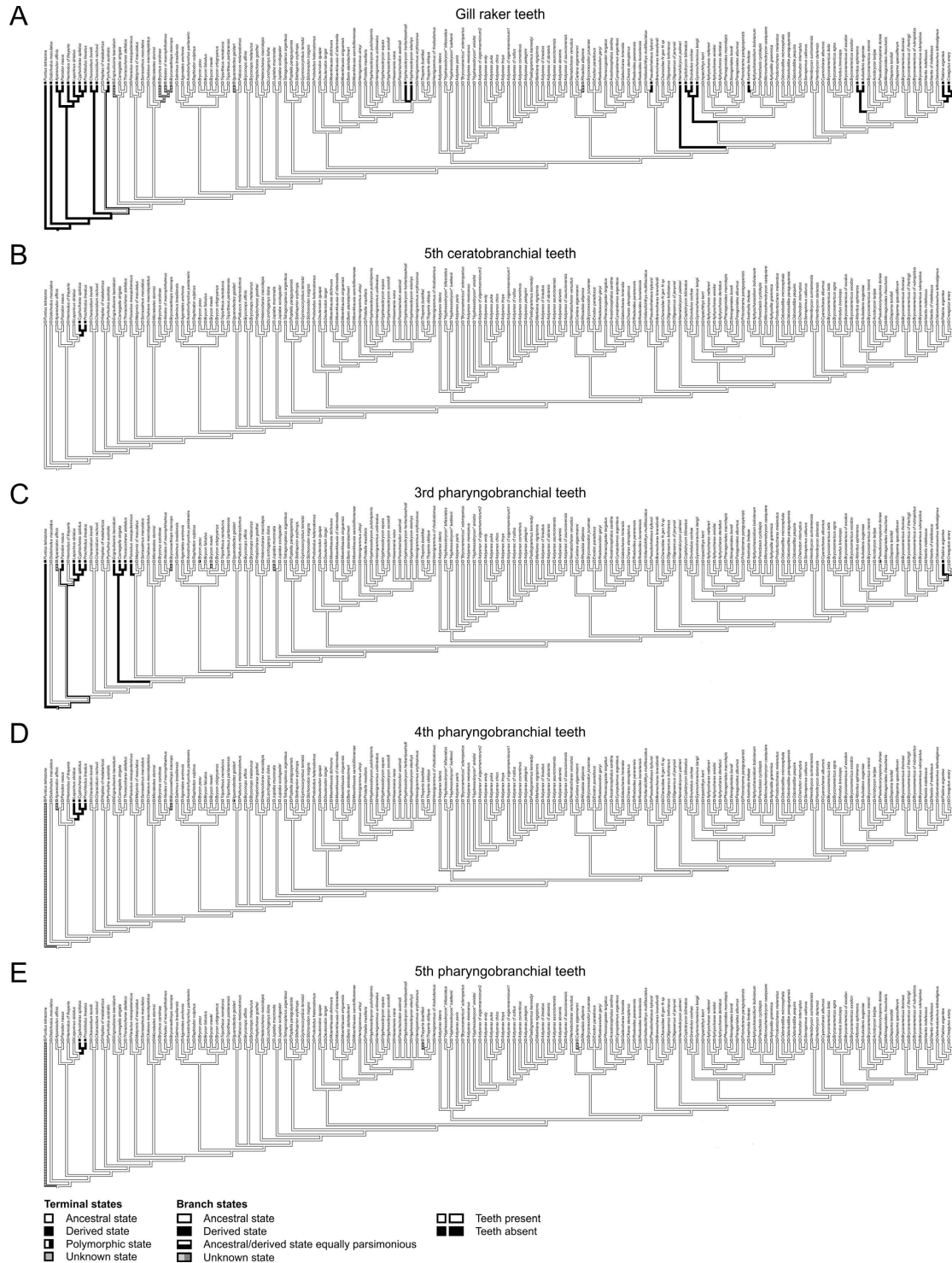
**Figure 6.** Expansion of dentition within characiforms may have been facilitated by retention of ancestral developmental potential, as seen in *A. mexicanus*. Schematic representation of dentition of the upper (left) and lower (right) oropharynx as in Fig. 1. The dentition of *A. mexicanus* is slightly reduced (loss of ectopterygoid teeth) relative to the ancestral characiform dentition (leftmost arrow). Nevertheless, this species retains the potential to form teeth in many additional locations (yellow) in response to ectopic expression of *eda* (upper right arrow). Some of these locations have experienced gain of teeth in characiform evolution (lower right arrow), while others have not.



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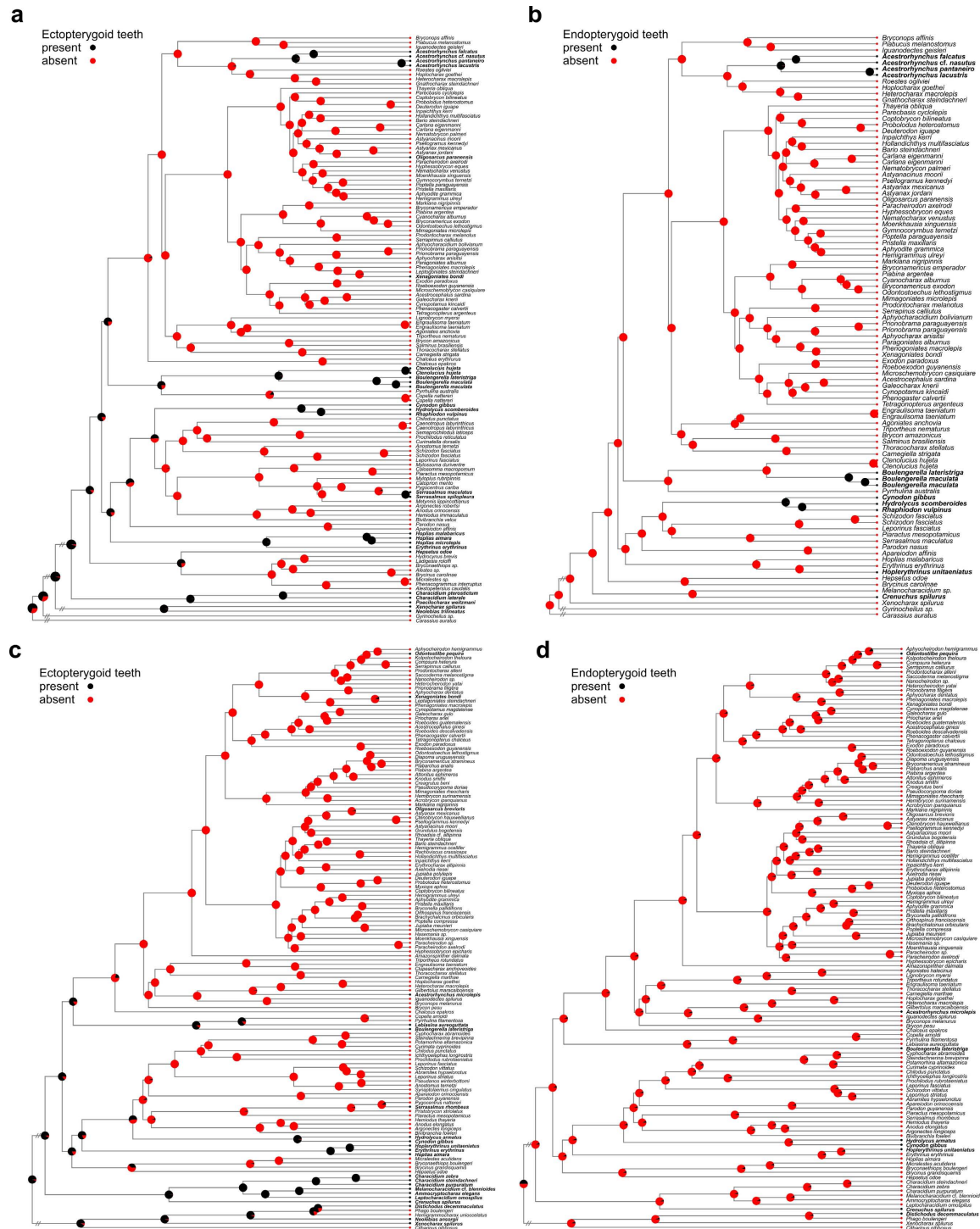
**Figure S1.** Teeth on the premaxillaries outside of the mouth, maxillaries, ectopterygoids, endopterygoids, and basibranchials have been gained in multiple characiform lineages. Parsimony reconstruction of presence (black) or absence (white) of teeth using tree topologies and character state matrices from Mirande (2009). *Puntius tetrazona* is an outgroup within the Order Cypriniformes, For the locations in this figure, presence of teeth was coded as the derived state by Mirande (2009).





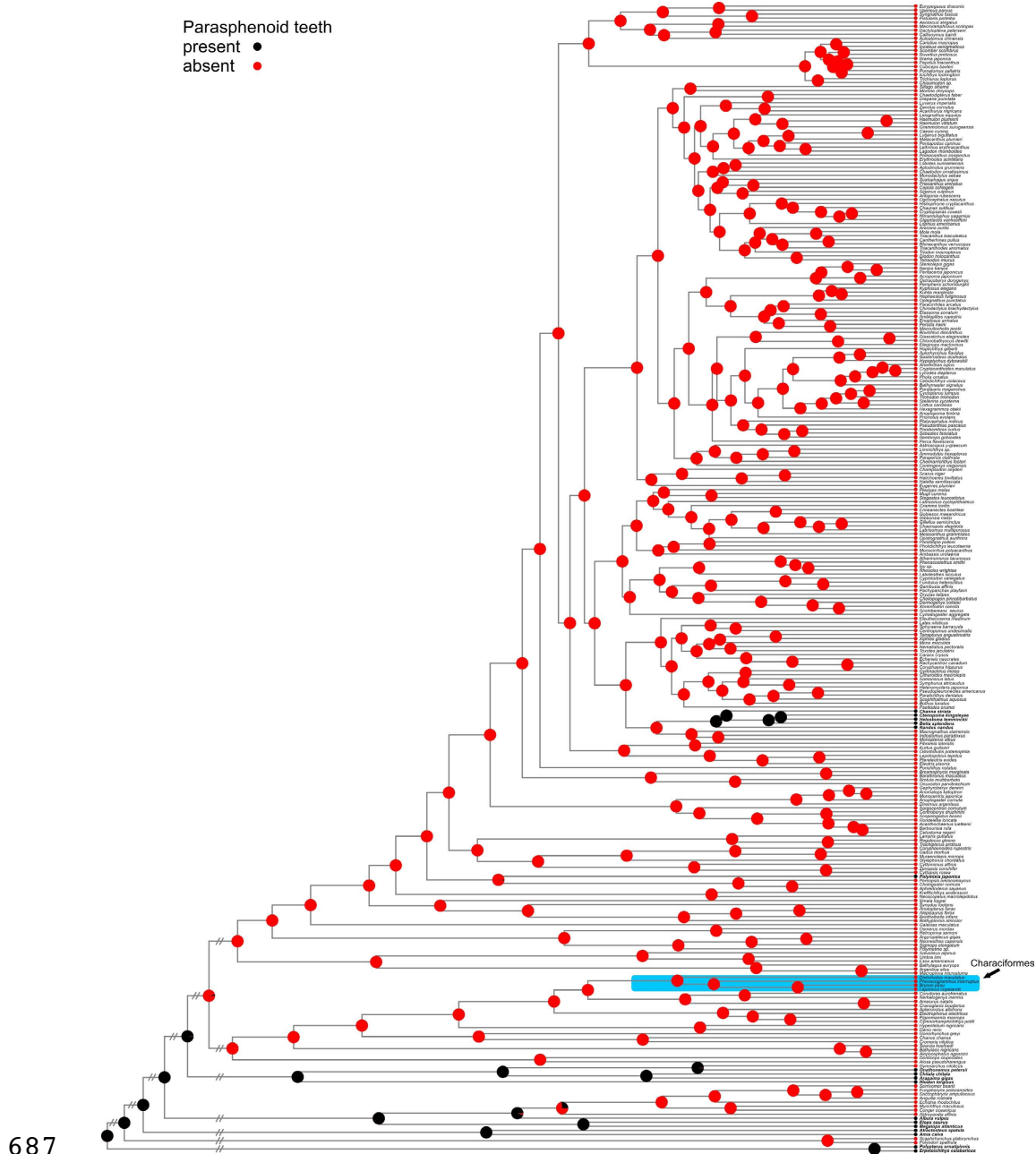
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**Figure S2.** Teeth on the gill rakers have been gained within the Order Characiformes. Parsimony reconstruction of presence (white) or absence (black) of teeth using tree topologies and character state matrices from Mirande (2009). *Puntius tetrazona* is an outgroup within the Order Cypriniformes, For the locations in this figure, absence of teeth was coded as the derived state by Mirande (2009).



**Figure S3.** Teeth on the ectopterygoid and endopterygoid bones have been re-gained following loss in multiple characiform lineages. A-B) Trees from Fig. 2A, B depicted with full taxonomic representation. C-D) Maximum likelihood ancestral state reconstruction of presence (black) or absence (red) of teeth on the ectopterygoid (C) and endopterygoid (D) bones. Tree topologies and branch lengths are from Betancur-R *et al.* (2019) and character states were compiled from the literature (Table S1, Supplementary References). Hashmarks indicate branches not drawn to scale. Pie charts at nodes represent the relative probabilities of each character state. All taxa

681 in S3C, D are members of the Order Characiformes. Ectopterygoid teeth (C) are reconstructed  
682 with highest probability of being present in the characiform common ancestor, with five potential  
683 regains following losses occurring within the group. Endopterygoid teeth (D) are reconstructed  
684 with equal probability of being present or absent in the characiform common ancestor, with  
685 seven potential gains occurring within the group.  
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**Figure S4.** Parasphenoid teeth were present in the common ancestor of ray-finned fishes and were regained following loss twice within teleost fishes. Maximum likelihood ancestral state reconstruction of presence (black) or absence (red) of teeth on the parasphenoid bone. Tree topologies and branch lengths are from Farina *et al.* (2015) and character states were compiled from the literature (Table S3; Supplementary references). Hashmarks indicate branches not drawn to scale. Pie charts at nodes represent the relative probabilities of each character state. Lineages in which parasphenoid teeth were regained following loss are the Suborder Anabantoidei (*C. striata*, *C. kingsleyae*, *H. temminckii*, *B. splendens* and *N. nandus*) and the Order Polymyxiiformes (*P. japonica*). The order Characiformes is indicated with an arrow and blue shading.

700 Table S1. (not included in pdf). Ectopterygoid and endopterygoid tooth character states for Fig.  
701 2A, B; S3A, B.  
702  
703 Table S2. (not included in pdf). Ectopterygoid and endopterygoid tooth character states for Fig.  
704 S3C, D.  
705  
706 Table S3. (not included in pdf). Parasphenoid tooth character states for Fig. S4.  
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708 Supplementary References (not included in pdf)