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1 Biting into the Genome to Phenome Map: Developmental
2 Genetic Modularity of Cichlid Fish Dentitions

3

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22 **Synopsis**

23 Within vertebrates, teleost fishes provide a rich evolutionary context for studying the
24 mechanisms of dental divergence because of the numerous axes along which their teeth
25 have diverged phenotypically and presumably developmentally. Using both a review of
26 teleost *in situ* hybridization and *de novo* transcriptome sequencing in a cichlid fish, we
27 examined whether 341 gene homologs thought to play a role in developing mice teeth are
28 expressed in the tooth-bearing jaws of teleosts. The similarities and putative differences
29 in gene expression documented between the two most commonly used models, zebrafish
30 and cichlids, highlight what can be learned from using a greater diversity of teleost model
31 systems in studies of tooth development. Both types of gene expression analysis also
32 provide substantial evidence for conservation of tooth gene expression from teleosts to
33 mammals as well as between initial and replacement teeth. Additionally, we found that
34 the cichlid oral and pharyngeal jaws share expression for a large percentage of genes that
35 influence tooth development. Our transcriptome analyses also suggest sub-
36 functionalization between gene paralogs expressed in teeth and paralogs expressed in
37 other structures is likely a common pattern across teleost diversity. Teleost dentitions will
38 continue to provide a potent system in which to examine the importance of both gene
39 duplication as well as the conservation of gene expression for phenotypic diversification.

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45 **Introduction**

46 Teeth provide a powerful phenotype for integrating across biological disciplines ranging
47 from ecology to genomics. For instance, teeth are used to identify extant and fossil
48 species (Dieleman et al. 2015), to document ancient (Purnell et al. 2007) as well as recent
49 (Cuozzo et al. 2014) ecologies, and to understand tissue (Lumsden 1988; Mitsiadis et al.
50 1998; Tucker and Sharpe 2004), cell (Jernvall and Thesleff 2000; Sharpe 2001), and gene
51 interactions (Thesleff and Sharpe 1997; Jernvall and Thesleff 2012; Jackman et al. 2013).
52 Because human and teleost fish teeth are homologous and derived from mineralized
53 tooth-like structures present in a common early vertebrate ancestor (Smith and Coates
54 1998, 2000; Smith 2003; Fraser and Smith 2011; Rasch et al. 2016), teeth provide an
55 ideal organ system for determining how multiple levels of biological complexity have
56 comparatively contributed to vertebrate diversification. Additionally, since a wide array
57 of serially homologous but differentiated tooth phenotypes can co-occur within the same
58 trophic apparatus, we can also assess how independent mechanisms of tooth formation
59 contribute to differentiation within the same individual organism (Fraser et al. 2009;
60 Hlusko et al. 2011; Ellis et al. 2015). Furthermore, because well-studied mammalian
61 dentitions represent only a small subset of vertebrate dental diversity (Stock 2007;
62 Jernvall and Thesleff 2012), comparative studies in new vertebrate models will continue
63 to provide insights into the mechanisms structuring dental diversification (Tucker and
64 Fraser 2014).

65 Modularity, or the degree to which traits evolve independently, is often invoked
66 as a critical mechanism during phenotypic diversification. Phenotypic ‘modules’, units
67 that are semi-autonomous in evolution and potentially so in function, are therefore

68 important to delineate mechanistically (Wagner and Altenberg 1996; Bolker 2000;
69 Hulsey et al. 2005). One potential advantage of unit autonomy is that the pleiotropic
70 effects of change in one component of the genotype to phenotype map, such as the
71 presence or absence of the expression of a particular gene, tend to fall to a greater degree
72 within modules than between modules (Wagner 1996). Generally, the degree to which
73 structural modules like teeth change independently during evolution is thought to be
74 enhanced if there is a corresponding modular organization, a qualitative as well as
75 quantitative difference, in the genetic pathways controlling the development of these
76 structures (Arone and Davidson 1997). Recently, we have come to appreciate that there is
77 a core set of genes that unites the development of all vertebrate teeth that includes
78 members of the *bmp*, *fgf*, *hh* and *wnt*/ β -catenin signaling pathways (Rasch et al. 2016;
79 Fraser et al. 2009). Intriguingly, although every vertebrate tooth likely utilizes this core
80 developmental set of genes, these genes are not uniquely expressed in teeth. Indeed,
81 many other ectodermal appendages in addition to teeth e.g. hair, feathers, scales and
82 various ectodermal glands develop via signaling interactions that involve these same
83 developmental genes (Wu et al. 2004; Pummila et al. 2007; Sadier et al. 2013).
84 Therefore, a deep developmental homology unites many putative phenotypic modules
85 emerging from the ectoderm that like teeth exhibit reciprocal signaling involving the
86 underlying mesenchymal cells. Understanding what developmental genetic mechanisms
87 allow teeth to phenotypically differentiate during both ontogeny and evolution will
88 demand extending our comparative knowledge of what genes are shared with other
89 ectodermally derived modules as well as what genes are commonly expressed during the
90 formation of different types of vertebrate teeth.

91 Serially homologous systems such as the leaves of plants, arthropod limbs, or
92 vertebrate teeth clearly contribute to organismal diversification, and the degree of genetic
93 independence among these iterative structures is likely to have substantial evolutionarily
94 consequences (Bateson 1894; Wagner 1989; Streelman and Albertson 2006; Smith et al.
95 2009). The teeth of teleost fish provide a rich evolutionary system for understanding how
96 the independence of developmental genetic modules contributes to phenotypic
97 divergence. There are numerous axes along which teleost teeth have diverged
98 phenotypically and presumably developmentally to meet the astonishing array of trophic
99 challenges their prey presents in aquatic environments (Figs. 1,2). For instance, many
100 teleost fishes can exhibit a large number of teeth in multiple rows on two independent
101 sets of jaws (oral and pharyngeal), differentially shaped teeth within a row (heterodonty),
102 and the production of replacement tooth germs throughout life (polyphyodonty) (Fryer
103 and Illes 1972; Motta 1984; Huysseune and Thesleff 2004; Huysseune 2006; Zhang et al.
104 2009; Fraser et al. 2009).

105 Among the many lineages of teleosts, cichlid fishes likely represent one of the
106 best groups for examining modularity in the dentition. Cichlids, like most fish, have two
107 toothed jaws (Fig. 1). They have oral jaws that are largely homologous to our jaws and
108 are used primarily to capture prey, and they also have pharyngeal jaws, modified gill
109 arches, that process prey (Liem 1973, Schaeffer and Rosen 1961). However, unlike any
110 other group of fish, cichlids exhibit an incredible amount of divergence in tooth
111 morphology, and the putative functional independence of their two toothed jaws could
112 have promoted both their trophic divergence as well as their unparalleled species richness
113 (Fryer and Iles 1972; Liem 1973; Hulsey et al. 2006).

114 Generally, the degree that teeth in different regions of the teleost trophic
115 apparatus are evolutionarily or developmentally decoupled remains unclear. However,
116 several aspects of tooth morphology are conserved between vertebrates as divergent as
117 cichlids and humans (Kerr 1960; Sire et al. 2002). Additionally, tooth number is
118 correlated on the oral and pharyngeal jaws of cichlids, tooth size is associated with
119 variation in tooth number on their pharyngeal jaws, and the teeth on the two jaws of
120 cichlids do share a core network of gene expression (Fraser et al. 2009; Hulsey et al.
121 2015; Fig. 3). Cichlid tooth phenotypes could therefore be highly integrated at multiple
122 levels of biological design and constrained to diverge in concert. Alternatively, the
123 capacity of the cichlid dentition to diversify independently could be substantial as their
124 oral and pharyngeal jaw mechanics have been shown to diverge in a completely
125 independent fashion (Hulsey et al. 2006). Furthermore, cypriniform fish such as *Danio*
126 *rerio*, the most commonly used genetic model system the zebrafish, have lost their oral
127 jaw dentition while retaining teeth on only their lower pharyngeal jaw (Huysseune and
128 Sire 1998; Stock 2001; Aigler et al. 2014). Teeth on the two jaws of fish can also
129 diversify independently within populations. In cichlids, single polymorphic species like
130 *Herichthys minckleyi* show no apparent variation in their oral jaw teeth but are highly
131 polymorphic even among interbreeding individuals in the size and number of their
132 pharyngeal jaw teeth (Hulsey et al. 2005; Hulsey et al. 2015; Fig. 2G,H). Therefore, the
133 developmental genetic systems underlying the formation of teeth on the two jaws of
134 teleosts might be expected exist as highly distinct modules and often diverge
135 independently during evolution.

136 Studies of gene expression during the formation of teeth in cichlids and other
137 teleost fishes have produced at least two generalizable results. First, an extensive number
138 of genes are conserved in their expression during the formation of teeth from fish to
139 tetrapods (Stock 2001; Fraser et al. 2006; Wise and Stock 2006; Cleves et al. 2014).
140 Although these findings have not been extensively reviewed, many genes like *bmp2*,
141 *bmp4*, *fgf8*, *pitx2*, *shh*, *dlx2*, as well as *runx2* are all present during tooth development in
142 cichlids as well as in mice (Fraser et al. 2008, 2009). Second, there is likely substantial
143 conservation in the presence of the same basic set of genes wherever teeth are formed in
144 the trophic apparatus (Fraser et al. 2009). Both of these results support the ideas that all
145 vertebrate teeth are evolutionarily homologous structures, that they are ancient in origin,
146 and that they only evolved once (Smith and Johanson 2003; Ellis et al. 2015). Therefore,
147 much of the genome-to-phenome map governing tooth diversification in one clade of
148 vertebrates or in one part of the trophic apparatus could provide insight into how teeth
149 have diverged in other vertebrate lineages.

150 Yet, one of the problems with using the mouse, the most ubiquitously used
151 vertebrate genetic model, and its dental developmental network as a standard for all
152 vertebrate teeth is that unlike both humans and cichlid fishes, mice do not replace their
153 teeth (Fraser et al. 2004). Therefore, we know relatively little about whether the genes
154 responsible for phenotypic differentiation of vertebrate replacement teeth are generally
155 the same genes utilized in the formation of the initial dentition (Fraser et al. 2013). An
156 example of differential expression between first generation and the replacement dentition
157 is that of the single gene sonic hedgehog (*shh*). It appears that *shh* is necessary for tooth
158 initiation and the establishment of the odontogenic band in vertebrate dentitions but is not

159 redeployed to initiate the replacement dentition across vertebrate taxa ranging from fish
160 (Fraser et al. 2006; Fraser et al. 2013) to reptiles (Handrigan and Richman, 2010). Thus,
161 there could be substantial differences in the genes generating replacement teeth as first
162 generation cichlid teeth are generally homogenous, simple, and are not generally as
163 phenotypically differentiated as replacement teeth (Fryer and Iles 1972; Streelman et al.
164 2003). Importantly, unlike mammals that replace their teeth at most a single time, cichlids
165 and most teleost fishes can replace their teeth once every 100 days repeatedly throughout
166 their life (Tuisku and Hildebrand 1994; Huysseune and Sire 1997; Stock et al. 1997;
167 Streelman et al. 2003). Much of the phenotypic diversity in the teleost dentition is also set
168 up during the time between when tooth replacement begins and the onset of reproductive
169 activity (Ellis et al. 2015; Hulsey et al. 2015). Therefore, teleost fish, including cichlids,
170 offer a system that could be used to determine what genes are conserved not only during
171 initial vertebrate tooth formation but also what genes are expressed as these structures are
172 replaced and differentiate phenotypically into adult dentitions.

173 The developmental genetic redundancy that follows whole genome duplication
174 has potentially played a major role in vertebrate diversification (Ohno 1970; Braasch et
175 al. 2016). Genome duplication could also have been fundamental to the diversification of
176 teeth because as compared to their distant relatives like tunicates or amphioxus, the clade
177 uniting jawed vertebrates from sharks to tetrapods have had two rounds of genome
178 duplication (Van de Peer and Meyer 2005). These genome duplications effectively gave
179 organisms like mice and humans four paralogous copies of many important craniofacial
180 genes that play a role in fundamental processes such as tooth development (Sharpe 2001).
181 Additionally, following their split from other vertebrate groups, the ancestor of most

182 teleost fishes underwent another round of genome duplication approximately 350 million
183 years ago that gave them an additional copy of many genes when compared to tetrapods
184 (Amores et al. 1998; Wittbrodt et al. 1998; Meyer and Schartl 1999; Taylor et al. 2001;
185 Braasch et al. 2006, 2007; Arnegard et al. 2010; Opazo et al. 2013). When contrasted
186 with their sister group that contains only the seven species of gar and one species of
187 *Amia*, the success and unparalleled adaptive divergence of the over 28000 teleost species
188 is thought to be partly a consequence of this further genome duplication (Taylor et al.
189 2003; Santini et al. 2009). However, the mechanistic significance of this teleost specific
190 genome duplication during ontogeny and across phylogeny is only now being fully
191 appreciated as a diversity of fish species like the Mexican tetra (*Astyanax mexicanus*),
192 medaka (*Oryzias latipes*), pufferfishes (Tetraodontidae), stickleback (*Gasterosteus*
193 *aculeatus*), and cichlids have had their whole genome sequenced (Jones et al. 2012;
194 Hulsey 2009; McGaugh et al. 2014; Brawand et al. 2014; ; Braasch et al. 2016). It is
195 exciting that the genomic resources are now available to allow us to examine the role of
196 processes like gene duplication in the adaptive diversification of a species rich group like
197 teleosts.

198 One of the most widely proposed mechanisms whereby duplicate genes, or
199 paralogs, might contribute to diversification is through a process known as sub-
200 functionalization (Force et al. 1999; Yu et al. 2003; Postlethwait et al. 2004). Sub-
201 functionalization occurs when a gene that was ancestrally expressed in a number of
202 tissues is duplicated, and then over time, the functions of these paralogs evolve to become
203 subdivided in where or when they are expressed. For instance, immediately following
204 duplication both paralogs might be expressed in all tissues (i.e. both the oral and

205 pharyngeal jaw teeth) where the originally unduplicated gene was expressed. But,
206 subsequently these paralogs could evolve to become narrowly expressed in a
207 complementary subset of the tissues in which they were originally found (i.e. one paralog
208 present only in oral teeth and one paralog present in only pharyngeal teeth). This
209 subdivision of gene function could thereby reduce pleiotropy between gene expression
210 modules and facilitate adaptive divergence in different tissues without the potentially
211 constraining effects of shared gene expression (Force et al. 1999; Guillaume and Otto
212 2012).

213 Only a few studies of gene expression during the development of teleost teeth
214 have examined gene expression in paralogous duplicates (Wise and Stock 2006; Gibert et
215 al. 2015). Importantly, sub-functionalization of gene duplicates could occur in a number
216 of ways spatially between different phenotypic modules. Each complementary paralog
217 could be differentially expressed in one of the two original structures as suggested above.
218 For instance, one paralog of a duplicated *wnt10* gene could retain its expression in both
219 structures while the complementary paralog becomes sub-functionalized to a single
220 structure. Alternatively, expression of *wnt10a* might be isolated to the pharyngeal jaw
221 teeth while its paralog *wnt10b* might be isolated to the cichlid oral jaws. Another
222 possibility is that only one paralog, *wnt10a*, could be isolated to all forms of a particular
223 structure such as teeth on both the oral and pharyngeal jaws, while *wnt10b* could be
224 isolated to another deeply homologous structure such as the scales that cover the fish
225 externally (Fraser et al. 2010). The teeth on the two jaws of cichlids represent a set of
226 serially homologous but evolutionarily divergent structures that could provide a rich

227 system for investigating the role of gene sub-functionalization during vertebrate
228 phenotypic divergence.

229 Using both a review of *in situ* hybridization studies in teleosts as well as
230 transcriptome sequencing of the oral and pharyngeal jaws of a cichlid, we examined
231 several questions concerning the conservation and independence of gene expression in
232 teleost dentitions. First, we detailed a large number of genes expressed during tooth
233 development that are conserved in tooth bearing regions from mice to teleosts. Then, we
234 investigated the overlap of tooth gene expression between the oral and pharyngeal jaws.
235 Finally, we documented patterns of sub-functionalization in gene paralogs to understand
236 how this process might be generally playing a role in differentiating teleost oral and
237 pharyngeal dentitions.

238

239 **Methods**

240 To determine which genes have previously been found to show expression in teleost
241 teeth, we reviewed the literature and web-based resources (e.g. www.zfin.org) for studies
242 of *in situ* hybridization, the primary method used prior to RNA-seq to establish
243 localization of gene expression. We tabulated the gene name, taxon of teleost fish used in
244 the study, whether the *in situ* hybridization was performed on initial or replacement teeth,
245 and if oral or pharyngeal teeth were examined. We also compared these studies to our
246 analyses of tooth gene expression in the transcriptomes of juvenile cichlid oral and
247 pharyngeal jaws.

248 To further explore the mouse tooth gene homologs expressed in teleost tooth
249 bearing regions, we separately assembled two transcriptome libraries: one for the oral and

250 one for the pharyngeal jaws of the cichlid *Herichthys cyanoguttatus*. This cichlid was
251 utilized because it belongs to the sister group of the endangered and polymorphic cichlid
252 *Herichthys minckleyi* (Hulsey et al. 2010; Hulsey et al. 2016), that shows substantial
253 phenotypic divergence in teeth on the pharyngeal jaws but little variation in oral jaw teeth
254 (Hulsey et al. 2005, 2006, 2015). To generate the oral jaw library, we dissected the
255 toothed premaxilla and dentary from an ontogenetic series of 65 fish ranging in size from
256 20 mm to 70 mm standard length and pooled their jaws. Using these same individuals, we
257 removed the toothed lower pharyngeal jaw to generate a single pharyngeal jaw
258 transcriptome. Because these species are polyphyodont with tooth replacement
259 continuously occurring at these sizes and since teeth should be one of the most
260 transcriptionally active structures in these bony regions (Schneider et al. 2014), we
261 assumed that we would be capturing primarily RNA that is expressed in developing
262 cichlid replacement teeth. In the closely related species *H. minckleyi*, tooth numbers are
263 generally not increasing at the body sizes examined (Hulsey et al. 2015). Although we
264 cannot rule out that some initial teeth are forming in the sizes of fish examined here, this
265 suggests that the teeth forming in the fish we examined were likely primarily
266 replacements for teeth lost from previously formed tooth crypts.

267 Once the jaws were dissected, we placed these tissues immediately into RNAlater
268 and shipped them on dry ice to LC Sciences (Houston, Texas, USA) for sequencing. Our
269 two RNA-seq libraries were generated using Illumina Truseq RNA Sample Preparation
270 Kits. Sequencing of the resulting cDNA libraries was carried out with an Illumina HiSeq
271 2000. The resulting Illumina libraries were then filtered and only paired-end reads were
272 used for further assembly. *De novo* transcript assembly was conducted using Trinity

273 release_20130216 that consists of three successive software programs: Inchworm,
274 Chrysalis, and Butterfly (Grabherr et al. 2011).

275 We utilized a custom comparative genomics pipeline to isolate putative tooth
276 genes from the transcriptome of the cichlid *H. cyanoguttatus*. To isolate these loci, we
277 first documented all the genes and their paralogs that have been examined in teleost tooth
278 *in situ* hybridization studies (Table 1). Then, we augmented this list with genes annotated
279 in the “bite-it” tooth gene expression database (<http://bite-it.helsinki.fi/>) that catalogues
280 genes that have been screened for roles in mouse tooth development. From this database,
281 we isolated 268 genes and their currently accepted abbreviations. Individual gene
282 abbreviations were then queried against the annotated Tilapia (*Oreochromis niloticus*)
283 ensembl genome database (Cunningham et al. 2015) resulting in 341 cichlid homologs to
284 genes known to be expressed in mouse teeth. For these loci, 146 genes, or 73 pairs,
285 represented two duplicated paralogs.

286 The transcript sequence for each gene from Tilapia was then used to query an un-
287 annotated transcriptome database for the model Central American cichlid *Amphilophus*
288 *citrinellus* using ‘blastn’ algorithms run using default parameters as implemented in
289 Viroblast (Deng et al. 2007). The transcriptome and genome of this cichlid have been
290 well-characterized using genomic and transcriptomic analyses of multiple life-stages and
291 multiple tissue types (Henning et al. 2013; Elmer et al. 2014; Franchini et al. 2014;
292 Kratochwil et al. 2015), and the species is relatively closely related to *H. cyanoguttatus*
293 (Hulsey et al. 2010, 2016). Only Tilapia tooth gene sequences that returned an
294 unambiguous single best match and *A. citrinellus* sequences that subsequently generated
295 a reciprocal best blast hit to the same gene in Tilapia were used in further analyses.

296 The assembled oral jaw transcriptome was composed of 182,230 contigs and had
297 a mean contig size of 657 base pairs. The assembled pharyngeal jaw transcriptome was
298 composed of 156,892 contigs and had a mean contig size of 585 base pairs. Subsequently,
299 all *H. cyanoguttatus* transcriptome contigs produced for each jaw were aligned against
300 individual *A. citrinellus* transcripts of each gene. Using the program Sequencher 4.8
301 (Genecodes, Ann Arbor, MI), we isolated tooth gene homologs in the *H. cyanoguttatus*
302 transcriptome using an initial cutoff of 90% sequence similarity that permitted large
303 alignment gaps. This sequence similarity ensured that homologs would align but paralogs
304 that diverged before the last common ancestor with *Tilapia* would not align. We
305 constrained the searches to only return sequences with a minimum alignment of 40
306 nucleotides with *A. citrinellus* genes. Then, the alignments for these genes were
307 individually inspected visually to ensure protein-coding alignment of at least 200 base
308 pairs thereby providing high confidence in the homology of our annotations.

309 Genes recovered were sorted into four categories: 1) those that appeared in the
310 transcriptome of both jaws, 2) the transcriptome of the oral jaw only, 3) the transcriptome
311 of the pharyngeal jaw only, and 4) putative tooth genes that were not present in either
312 transcriptome. We also annotated the 73 pairs of paralogs based on three potential kinds
313 of differential expression and putative sub-functionalization. The first group contained
314 one tooth gene paralog that was expressed in both jaws but another paralog that was
315 isolated to a single jaw. The second group examined were complementary paralogs that
316 were alternatively expressed in the two jaw transcriptomes. The third group we
317 demarcated contained genes that have one paralog expressed in the jaws but another
318 paralog presumably expressed in other tissues since the protein retains an open reading

319 frame in the cichlid genomes.

320

321 **Results and Discussion**

322 We documented several general patterns concerning the presence and absence of teleost
323 tooth gene expression. Both *in situ* hybridization and RNA-seq transcriptomes provided
324 substantial evidence for conservation of tooth gene expression from teleosts to mammals
325 and between initial and replacement teeth. Additionally, we found that the oral and
326 pharyngeal jaws share expression in a substantial percentage of genes that influence tooth
327 development indicating that the dentitions on these two jaws are not exceptionally
328 independent at the level of the presence or absence of genes expressed. Our transcriptome
329 analyses of paralog expression also suggest sub-functionalization between gene paralogs
330 expressed in teeth and paralogs expressed in other structures is likely a common pattern
331 across teleost diversity.

332

333 **Teleost teeth and *in situ* hybridization**

334 There are 76 genes that have been implicated in mouse tooth development that have also
335 been verified via *in situ* hybridization to play a role in the formation of teleost dentitions
336 (Table 1). The reviewed studies further support the idea that there is extensive
337 conservation in the genetic underpinnings of tooth development from mice to teleosts.
338 Additionally, eleven of these genes have been shown via *in situ* hybridization to be
339 expressed in both the oral and pharyngeal teeth of teleosts suggesting there might be
340 substantial similarity in the developmental genetic basis of tooth formation on both jaws
341 (Fraser et al. 2009). However, 34 of the tooth markers have only been studied in the oral

342 jaws and 31 genes have been exclusively examined in the pharyngeal dentition.
343 Therefore, whether the proportion of genes shared between the dentition on the two jaws
344 is as low as 10% or is much greater is unclear from the *in situ* hybridization studies.
345 Because most pharyngeal tooth gene expression has been performed in zebrafish, which
346 only houses teeth on their lower fifth ceratobranchial element (Stock et al. 2006; Stock
347 2007) and because most of the remaining studies have examined expression in cichlid
348 teeth but on only the oral jaw, the degree of developmental genetic independence of the
349 dentitions on these two jaws requires further investigation.

350 The examination of multiple lineages of teleosts can clearly provide interesting
351 insight into the conservation and divergence of dental developmental networks. For
352 instance, six orthologous genes that are shared during dental development between
353 zebrafish and mouse (*bmp2a*, *dkk1b*, *dlx2b*, *lhx7*, *scpp1*, and *scpp9*) have likely been lost
354 from the genomes of cichlids and medaka (Table 1). In some cases, paralogs of these
355 genes are known to be involved during tooth development and this developmental
356 redundancy leading to loss of paralogs might be a general feature of teleost evolution.
357 However, only the paralogs of *bmp2*, *dlx2*, *dlx4*, and *rara* have been documented through
358 *in situ* hybridization to both be expressed in teleost teeth. Additionally, only for *bmp2* in
359 medaka have the two paralogs of any duplicated gene been recorded from both the oral
360 and pharyngeal dentitions (Wise et al. 2006). Interestingly, the *Tilapia* genome appears to
361 have lost the *bmp2a* paralog making the redundancy in *bmp2* ortholog expression for
362 cichlids likely dispensable as has been suggested for *bmp2* paralogs in zebrafish (Wise
363 and Stock 2010). Although teleosts such as the Mexican tetra, medaka, pufferfishes, and
364 stickleback have only been used in a comparatively few studies, more extensive

365 examinations of tooth gene expression in these and additional lineages of fish will likely
366 continue to shed important light on the conservation and divergence of vertebrate dental
367 development. It is also clear that many studies of *in situ* hybridization have not
368 adequately detailed which paralog of duplicated genes they have studied during tooth
369 development (Table 1). Further analyses of the presence and absence of paralogs within
370 the developing dentitions of teleosts could provide a more general understanding of the
371 importance of redundancy, neo-functionalization, and sub-functionalization, as well as
372 whether the same genes are involved in forming teeth during different stages of ontogeny.

373 Our understanding of the genes involved in teleost tooth replacement is primarily
374 confined to studies of the teeth on the oral jaws of cichlids. There are only seven genes
375 that teleost *in situ* hybridization studies have shown to be involved in both initial tooth
376 formation as well as tooth replacement (Table 1). However, because we know that a
377 substantial number of genes are involved in tooth initiation from *in situ* studies and that
378 many of these genes are present in the transcriptomes analyses of primarily replacement
379 teeth examined here, the combination of these two techniques suggest the majority of
380 genes that are involved in the formation of initial teeth are likely to be involved in the
381 formation of replacement teeth (Table 1). A total of 91% of the genes that have been
382 examined in teleost *in situ* studies and that are present in the Tilapia genome are present
383 in at least one of the cichlid jaw transcriptomes. Some notable exceptions include *eve1*
384 and several Hox genes. These genes have been implicated in the formation of initial teeth
385 in the oral and pharyngeal jaws (Laurenti et al. 2004; Debiais-Thibau et al. 2007; Fraser
386 et al. 2009), but they are absent from the transcriptome of the jaws. Combining single
387 gene studies using methods such as *in situ* hybridization with high throughput analyses of

388 expression as provided via RNA-seq will continue to provide synergistic insight into the
389 genes underlying dental diversification.

390

391 **Cichlid Oral and Pharyngeal Jaw Transcriptomes**

392 Using transcriptome sequences, we were able to more than double the list of genes
393 expressed in mouse teeth that are also expressed in the toothed jaws of teleosts.
394 Approximately 80% of the genes we screened are present in the oral and/or pharyngeal
395 jaw tooth transcriptomes. This supports the idea that a substantial number of the genes
396 that function to generate vertebrate tooth phenotypes are likely to be conserved in that
397 role in the over 60,000 vertebrates descended from the last common ancestor of mammals
398 and teleosts. This extensive conservation in gene expression might represent a general
399 pattern for many types of organismal structures like eyes and hearts that have a single
400 ancient origin but have been maintained across much of vertebrate diversity (Meng et al.
401 2013; Richards et al. 2013; McGaugh et al. 2014).

402 The oral and pharyngeal jaw transcriptomes indicate that there is shared
403 expression for a large number, 137, of the tooth genes between the two jaws of cichlids.
404 Although there are a number of interesting exceptions, many of the genes that have only
405 been examined in one jaw using *in situ* hybridization tended to also be present in the
406 transcriptomes from both jaws (Table 1). This sharing of over 1/3 of the genes examined
407 between both toothed components of the cichlid trophic apparatus indicates that
408 pleiotropy could commonly constrain tooth differentiation on the two jaws of cichlids.
409 The morphological correlations that have been observed among species in phenotypes

410 like oral and pharyngeal jaw tooth number could well be a result of this substantial
411 sharing of conserved gene expression during tooth formation (Fraser et al. 2009).

412 We recovered a higher proportion of the mouse tooth genes homologs from the
413 oral jaw transcriptome (Table 2). There were 136 genes, almost the same number that
414 present in both jaw transcriptomes, that were recovered exclusively from the oral jaw
415 transcriptome. However, only 11 genes were isolated exclusively from the pharyngeal
416 transcriptome. This bias between the two jaws in observed expression could be due in
417 part to the fact that mouse tooth development takes place on one of the same bones, the
418 dentary, that is toothed in the oral jaws of cichlids (Smith and Coates 1998; Fraser et al.
419 2004, 2008). However, this pattern could also be due to the vagaries of RNA-seq or the
420 fact that only the lower pharyngeal jaw was examined whereas both the upper as well as
421 the lower jaw were analyzed in the oral jaw transcriptome. However, if the tooth genes
422 shared across vertebrates do show a bias towards expression only in the oral jaw, then
423 teleost fishes like cichlids, that do have teeth on their oral jaws, might provide greater
424 insight into human and mammalian tooth development when compared to teleosts such as
425 zebrafish that only have teeth on their lower pharyngeal jaw (Stock 2007; Fraser et al.
426 2009). These data also suggest that cichlids with their two toothed jaws could provide a
427 framework in which to uncover developmental discrepancies between teeth from what
428 are seemingly the disparate structural units of the oral and pharyngeal jaws (Fraser et al.
429 2009). Because distinct developmental programs could even define anterior (incisors) to
430 posterior (premolars) teeth in the oral jaw of mammals (Hlusko et al. 2011), expression
431 differences among tooth bearing regions like the jaws of cichlids could provide intriguing
432 insights into the origins and evolution of the vertebrate dentition.

433 A substantial number of mouse tooth genes were not recovered in either cichlid
434 jaw transcriptome. Of the 57 genes that we screened that were not recovered in the
435 transcriptomes of cichlid tooth-bearing regions, 20 of these genes were represented by the
436 paralogs of the genes *bcl2*, *cspg5*, *dab1*, *foxf2*, *foxf1*, *lrrn3*, *ngfr*, *nrp2*, *ntrk3*, and *wt1*.
437 Although all of these genes could be absent from developing teeth, caution might be
438 warranted in completely excluding their presence from developing cichlid dentitions. As
439 in any transcriptome study, genes that show low transcript abundance, as important
440 morphogens and transcription factors often do, could have been missed (García-Ortega
441 and Martínez 2015). Additionally, many of these genes might be expressed only in the
442 formation of first generation teeth that develop during the first few weeks following
443 hatching. The transcriptomes presented here were generated from fish that ranged from a
444 month to several months old making our inferences about gene expression primarily
445 relevant to the formation of replacement teeth (Fraser et al. 2009; Kratochwil et al. 2015).
446 The absence of many of these genes during the development of teeth in cichlids could
447 also reflect a lack of conservation across vertebrate tooth development. Because of their
448 morphological differentiation, mammalian teeth as represented by the mouse dentition
449 could readily have a suite of genes that are not expressed in the teeth of other vertebrate
450 groups. The monophyodont mouse dentition is also unusual compared to most mammals
451 that possess a diphyodont dentition characterized by a round of tooth replacement.
452 Furthermore, gene expression from the mouse dental model has been predominantly
453 compiled from their non-replacing molars (Miletich and Sharpe, 2003). As gene
454 expression is investigated in more non-model organisms, the presence and absence of

455 genes unique to the teeth of particular lineages will undoubtedly become apparent (Rasch
456 et al. 2016).

457

458 **Tooth Gene Paralog Expression**

459 The expression patterns of paralogs provide several interesting insights into the potential
460 role of gene duplicates in dental diversification. In approximately 12% of the paralogs
461 examined, both paralogs were conserved and expressed in both the oral and pharyngeal
462 jaw transcriptomes. The retained duplicates included the paralogs of *collan1*, *col4a*,
463 *ctnbn1*, *nfkbia*, *pstpip1*, *timp2*, *tjp1*, and *tufi1*. In all these cases where both paralogs are
464 present, it would be interesting to know if the duplicates have somehow diverged in
465 function in time or space among different morphological components of individual teeth.
466 It is also possible that the co-expression of the duplicates might have been conserved
467 simply to ensure functional redundancy in critical aspects of tooth development (Wagner
468 2008; Chen et al. 2013). Cichlid teeth could provide a powerful replicated framework on
469 multiple levels to examine how co-expressed paralogs become temporally or spatially
470 differentiated within serially homologous structures.

471 Sub-functionalization of putative tooth gene paralogs has occurred in a number of
472 ways in the jaws of cichlids. Notably, in about 16% of the paralogs examined, one
473 paralog was present in both jaw transcriptomes but the other paralog appeared to be sub-
474 functionalized to a particular jaw. Examples of this included *crabp1b*, *jag2b*, and
475 *sema3aa* in the oral jaw transcriptome as well as *baxb*, *fn1a*, and *oclna* that were found in
476 the pharyngeal jaw transcriptome. There were only a few genes that displayed a pattern
477 of alternative transcription with one paralog expressed exclusively in the oral jaw and one

478 paralog expressed exclusively in the pharyngeal jaw (Table 2). The paralogs of *col2a1* as
479 well as *wnt10* exhibited this pattern. In the oral jaws, *col2a1b* and *wnt10b* were
480 recovered, but in the pharyngeal jaws *col2a1a* and *wnt10a* were expressed.
481 Complementary sub-functionalization is clearly not a major axis of developmental
482 genetic divergence of the tooth genes examined. Interestingly, approximately 20% of the
483 genes we screened and were not recovered in either transcriptome did have paralogs that
484 were expressed in at least one of the jaw transcriptomes. Some notable examples of this
485 type of sub-functionalization included the paralogs of *bmp7*, *fgf1*, and *ndrg1*.
486 Importantly, these tooth genes that show jaw specific expression could provide candidate
487 loci for the dental divergence of polymorphic cichlid species like *Herichthys minckleyi*
488 that show exceptional phenotypic differentiation in teeth on only one jaw (Hulsey and
489 García de León 2013; Hulsey et al. 2015).

490 Gene duplication is a common phenomenon and appears to be playing a
491 substantial role in developmental differentiation of cichlid teeth. Importantly, whole
492 genome duplications are only the most obvious and large-scale manifestation of genetic
493 duplication. Gene copy number variation is now recognized as ubiquitous in most
494 populations and its influence on micro-evolutionary divergence is receiving increasing
495 attention (Cheng et al. 2005; Hastings et al. 2009). This potential for individual genes to
496 duplicate means that for many of the genes examined we cannot unambiguously ascribe
497 their duplication to the initial teleost whole genome duplication event. Detailing the
498 patterns and timescale over which tooth genes become sub-functionalized will demand a
499 much better understanding of the homology and origin of many of these genes. As our
500 knowledge of teleost genomics and gene duplication increases, it will be interesting to

501 evaluate whether gene expression changes in structures such as teeth following macro-
502 evolutionary events like whole genome duplication mirror those consequences found on a
503 more micro-evolutionary level when individual genes are duplicated.

504

505 **Future Directions**

506 The presence and absence of particular tooth genes as we examined here only provides an
507 initial window into the qualitative divergence that characterizes the developmental
508 genetics of dental diversity of cichlids and other vertebrates. Quantitative variation in
509 many layers of developmental genetic mechanisms are critical to how phenotypes are
510 shaped and undoubtedly are playing a large role in cichlid dental modularity. For
511 instance, alternative enhancers on the same gene that influence the abundance of gene
512 transcripts, the presence of alternative transcripts of the same proteins, as well as the
513 timing and patterning of micro-RNAs are all likely to be modified substantially during
514 the differentiation of serially homologous structures like teeth (Jackman and Stock 2006;
515 Kratochwil and Meyer 2015). With the ever-increasing availability of genomic resources,
516 it is now also feasible to extensively manipulate gene expression and perform functional
517 assays to experimentally test the independence of gene networks in different structures
518 like the jaws of cichlids. Coupling these experimental approaches with modeling of the
519 potential interactions among genes will further allow us to test the distinctiveness of
520 individual dental modules. As our understanding of the genome to phenome map
521 continues to expand for conserved structures like teeth, we will be able to increasingly
522 appreciate how the organization of developmental genetic modules influences vertebrate
523 phenotypic diversification.

524

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528

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839

840 Fig. 1 Cichlids, like most fishes, have two sets of toothed jaws: the oral (A) and
841 pharyngeal (B) jaws. The oral jaw is fairly homologous to our jaw and the premaxilla and
842 dentary bones are both toothed in cichlids. The pharyngeal jaws are modified gill arches.
843 In cichlids, the fused 5th ceratobranchials form the toothed lower pharyngeal jaw.

844

845 Fig. 2. Axes of cichlid fish tooth diversity. The dentition of different cichlid species
846 varies extensively in whether it is heterodont (A), with variation in tooth shape and
847 number in the many rows that can occur on the same jaw, or homodont (B), fairly
848 uniformly shaped teeth throughout a jaw. Cichlids commonly vary in whether their teeth
849 or tricuspid (C), bicuspid (D), or unicuspid (E). The lines depict where teeth with these
850 shapes are located in the heterodont and homodont cichlid dentitions. Cichlids also vary
851 extensively in patterns of tooth replacement (F) as is shown in the lateral CT-scan of a
852 cichlid lower pharyngeal jaw. Substantial variation in cichlid tooth morphology that is
853 only seen after several rounds of tooth replacement can also occur within populations as
854 well as in radiations of species that have diverged over very short timeframes. For
855 instance, the papilliform (G) and molariform (H) lower pharyngeal jaw dental phenotypes
856 depicted represent morphological variants that interbreed within populations of the
857 cichlid *Herichthys minckleyi*.

858

859 Fig. 3. Testing for evolutionary independence of phenotypes among species. The
860 evolutionary independence of any two phenotypes (A) can be tested explicitly using
861 phylogenies and correlations of independent contrasts. In the example shown, the number
862 of teeth on the pharyngeal jaw (Trait X) and the number of teeth on the oral jaw (Trait Y)
863 are evolving independently. Effectively, when there is lots of change in the pharyngeal
864 jaw teeth number, there is very little change in oral jaw tooth number. Conversely, when
865 there is lots of change in the oral jaw tooth number there is very little change in
866 pharyngeal jaw tooth number. This is the kind of macro-evolutionary change we would
867 expect if these traits evolve independently during evolution (B). If trait evolution is
868 alternatively highly correlated, we would expect change in trait X and trait Y to change in
869 concert and show a correlation (C). In Malawi cichlids at least, changes in tooth number
870 on the two jaws evolve in a surprising integrated manner. These phenotypic correlations
871 characterizing this classic adaptive radiation suggest there are likely shared mechanistic
872 forces, such as the shared presence of the same tooth genes, structuring phenotypic
873 evolution of teeth on the two distinct jaws.