Linking development with generation of novelty in mammalian teeth

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The evolution of mammalian teeth is characterized by the frequent and convergent evolution of new cusps. The evolution of new cusps can be linked to tooth development via population-level variation. This allows testing whether development increases the capacity to evolve, or evolvability, by facilitating and even directing morphological change. In a population sample of living seals, variation in cusp number of individual teeth is from three to five cusps, the variably present cusps being the shortest ones that also develop last. By factoring in recent evidence on development, I show that the variation in cusp number can be explained by a patterning cascade mode of cusp development that cumulatively increases and directs height variation in short cusps. The biased variation in seal tooth cusps supports the recognition of teeth as highly evolvable because only small developmental changes are needed to produce large changes in size and number of small cusps. This evolvability of tooth cusps may have facilitated the fast and independent acquisition of new cusps in mammalian evolution. In phylogenetic studies, small cusps may be unreliable as phylogenetic signals. Population level variation can be a powerful tool in testing and generating hypotheses in developmental evolution studies.

Because even large morphological differences among species or higher taxa stem from population-level processes, critical examination of roles of development in macroevolution benefit from the inclusion of population-level variation. For example, when hypothesized classifications of macroevolutionary traits and novelties are derived directly from molecular-level developmental models, mechanisms of how natural selection might produce these evolutionary changes are not easily testable. Eventually, developmental evolution studies must address three hierarchical components: development, population-level variation (or microevolution), and macroevolution. Structures that provide ample opportunities to examine population-level variation linking development to macroevolution are mammalian molar teeth, organs that have a good fossil record with high morphological diversity.

Mammals have evolved to eat a wide variety of foods, which is reflected in the high diversity of cusp patterns on their molar teeth (1, 2). The repeated convergent evolution of cusps, most notably the hypocone, has been considered to be important for evolution of new adaptations and subsequent diversification of many mammalian lineages, thus representing a key innovation in mammalian evolution (3). The evolution of the hypocone also has been suspected to manifest the capacity of tooth development to promote the evolution of novel morphologies (2, 4), often called evolvability (4–8). The acquisition of cusps in mammalian lineages is stereotypic in that novel cusps are initially small although they may evolve to be larger (3). If there were no differences in variation among tooth cusps in a population, natural selection against size variation in tall main cusps should also, as a byproduct, constrain variation in small cusps. Consequently, this should prevent the evolution of new cusps and tooth morphologies, which, however, appears not to be the case (1–3). Therefore, models generating population-level variation (9) are

needed to explain evolution of new cusps and diverse tooth shapes.

Lake Ladoga seal (*Phoca hispida ladogensis*) postcanine dentition provides a good basis to study subtle variation in tooth cusps because the dentition is simple. In this species, the cusps are aligned in a row and lack occlusal contact with opposing teeth (Fig. 1*A*). These seals have been isolated in Lake Ladoga (in western Russia) ever since the end of last glaciation (approximately 9,500 years B.P.) (10). The analysis of a single, isolated Recent population ascertains the sampling of a broad range of morphological variation. Indeed, intact, large postcanine teeth of Lake Ladoga seals possessed from three to five cusps (Fig. 1*B*), a difference easily justifying a species distinction if recovered as fossil specimens.

Because mammalian teeth change shape after formation only by wear, the variation in cusp number of seal teeth has to result from differences in development. The development of the tallest cusp begins first, and continued downward growth and folding in the epithelium forms the smaller cusps (Fig. 2). The height differences among cusps in an erupted tooth closely correspond to their height differences when their development began (2, 14). Thus, because the variably present seal cusps were always the smallest cusps, they were also the last-developing ones (Fig. 1*B*).
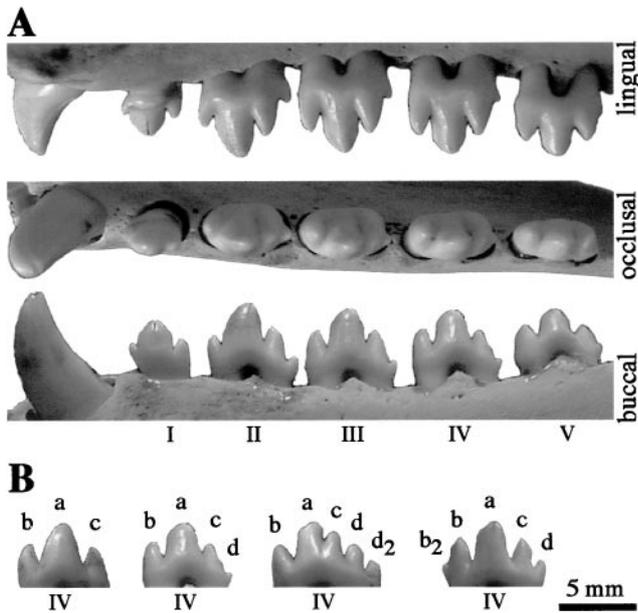
Current evidence on development shows that tooth morphogenesis is punctuated by transient signaling centers in the epithelium corresponding to the initiation of tooth crown and individual cusps (refs. 11 and 12 and references therein). These signaling centers, the primary enamel knot and the secondary enamel knots, resemble other embryonic signaling centers, such as the notochord and the apical ectodermal ridge in limbs (11). The enamel knots may direct the differential growth and subsequent folding of the dental epithelium, and members of the fibroblast growth factor signal family have been implicated in the control of cell proliferation around the nondividing cells of the enamel knots (15). The secondary enamel knots are the first embryological signs of species-specific cusp patterns (16). However, genes involved in cusp development appear to be the same among all the individual cusps, and at the level of molecular signaling, all the cusps are alike (11, 16). Therefore, it is unlikely that cusp-specific positional information determines the identity of each cusp separately. Rather, cusp positions appear to be determined sequentially as a patterning cascade and individual teeth may differ only in the timing of cusp initiation (Fig. 2) (11, 12). Developmentally, the patterning cascade can be defined as iterative activation and silencing of a set of conserved signaling pathways. Spacing differences in the activation of the iterated

EVOLUTION

Fig. 1. Lower teeth of the ringed seal (*Phoca hispida ladogensis*). (*A*) The postcanine dentition consists of five teeth, four of which (II–V) are roughly the same size and have at least three cusps. (*B*) The range of cusp patterns in the fourth postcanine and the cusp nomenclature used. Anterior is toward the left.

Fig. 2. Patterning cascade mode of tooth cusp development. The formation of each cusp begins by the appearance of the enamel knot (circle with thick line) that consists of nonproliferative epithelial cells expressing several molecular signals, including growth factors, and is surrounded by strongly proliferative epithelium (gray) and mesenchyme underneath (11). After the formation of the cusp tip, the enamel knot disappears and the epithelial and mesenchymal cells begin to differentiate into enamel-forming ameloblasts (lighter shading) and the dentin-forming odontoblasts (below the epithelium, not shown), respectively. The height difference between cusp a (Δ_a) of the left and right tooth is 0, but the subsequent enamel knots and cusps are at a distance that is determined by the previous enamel knot (hatched circle). This distance can be regulated by long-range inhibitors or by a decline in activators diffusing from the enamel knot (2, 11, 12). A small difference in the inhibitory field among teeth has a small effect in the position of the second set of cusps (c and b), but the effect is cumulative in the lower cusp d, resulting in teeth that are sharper (on the left) or blunter (on the right) in their cusp configurations. The actual number of cusps realized in each tooth crown is also determined by the termination of crown morphogenesis and the initiation of root formation (thick, hatched line). In the tooth on the left, the last developing cusp (d) would be initiated below the threshold and no cusp forms (marked with X). The anterior cusp b can have slightly different cusp spacing from the distal cusps. Note that the effects of inhibition and activation also can be realized by cell-to-cell-mediated propagation of the signal (13) and that both growth rate and the rate of spreading of the inhibitor can affect cusp spacing. Δ_a, Δ_c, and Δ_d denote the height differences between cusps a, c, and d, respectively, of the left- and right-hand side teeth.
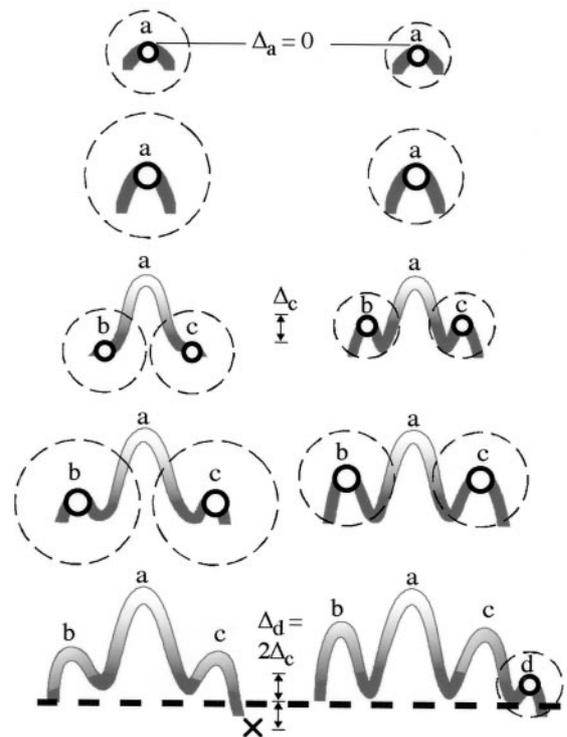
signaling pathways result in cumulative morphological differences in each iteration (Fig. 2). In the case of teeth, the activation of conserved signaling pathways is manifested by the generation of the enamel knots.

Here, I tested whether the patterning cascade mode of cusp development can explain the population level variation in seal tooth cusps and whether this may promote the generation of novel cusps and tooth morphologies.

Methods

The cranial material was collected between 1910 and 1929 and is located in the Zoology Division, Finnish Museum of Natural History, Helsinki. Because seal postcanines are essentially two-dimensional, only the cusp positions along the longitudinal axis and in height were recorded from lateral digital images of mandibles ($n = 132$). Morphologically, *Phoca* postcanines resemble the teeth of Mesozoic triconodont mammals (e.g., ref. 17).
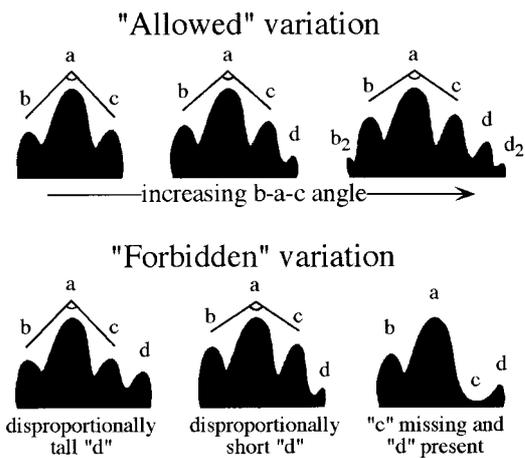
Two types of data manipulations were used before analysis of cusp size variation. I report here the results from using the third postcanine teeth (largest number of intact teeth, $n = 116$), although analysis when using other teeth gave equivalent results. First, the third postcanines were superimposed vertically along a central axis and the tallest, first-developing, cusp. The vertical central axis was perpendicular to a horizontal baseline connecting the anterior and distal ends of a tooth crown at the junction of the crown and the roots. Second, cusps a to d were optimally superimposed by rescaling and rotating each tooth to a common size and orientation by using a generalized least-squares (GLS) fit on cusp tips (18). This was performed by using MORPHOMETRICA (by J. A. Walker at http://life.bio.sunysb.edu/morph/). Both Pearson's and Spearman's correlation coefficients were calculated except when variables were distributed nonnormally (based on Shapiro–Wilk W test and normal quantiles plots), and only the latter coefficient was used. The parametric and nonparametric tests gave equivalent results. Departure of GLS-fitted cusp tips from their mean was tested by comparing average

deviation of points from their means (19), using a nonparametric multiple-comparisons test (20).

To test specifically the patterning cascade mode of tooth development, a measure of cusp height differences was needed because the spacing of adjacent cusps can be hypothesized to be correlated within a tooth (Fig. 2). To avoid the alignment of teeth affecting the measurements, an angle of the tallest three cusps (angle b-a-c) was used (Fig. 3). The correlation between angle and cusp height (from cusp a) ranged from $r = 0.51$ to 0.69 ($P < 0.001$) and between angle and cusp distance from cusp a along the longitudinal axis ranged from $r = -0.10$ to -0.31 (not significant to $P < 0.01$). Therefore, the angle measure is affected mostly by the height differences among cusps, and an increase in the b-a-c angle, i.e., tooth bluntness, can be used to test whether

Jernvall

"Allowed" variation

————increasing b-a-c angle———→

"Forbidden" variation

disproportionally tall "d"   disproportionally short "d"   "c" missing and "d" present

Fig. 3. Angle measurement used to measure the patterning cascade mode of cusp development. The b-a-c angle should predict the height and number of short cusps (d, d_2, and b_2) because sharp teeth (small angle) should have fewer cusps and cusps that are more unequal in height than blunt teeth (large angle). The patterning cascade also makes some morphological variation rare or "forbidden," such as a cusp missing in the middle of a cascade or a disproportionally large or small cusp, requiring a change in the patterning cascade parameters. The kinds of teeth (Fig. 1B) and variation in cusp positions (Figs. 4 and 5) encountered support the division between "allowed" and "forbidden" variation. Note that in many mammals, differential growth between cusps can alter cusp positions along the crown after their developmental initiation (19) and that the cascading pattern also can be obscured by conules (small cusps) that form between main cusps late in development.

it relates to an increase in the height and number of short, later-developing, cusps (Figs. 2 and 3). The sidewise departure of tooth cusps from their longitudinal axis affected the angle measure less than 2°. Principal component analysis [on covariance matrix, SYN-TAX 5.02 (http://ramet.elte.hu/~podani/)] was used to ordinate the GLS-fitted tooth cusps to measure what proportion of the total variance among GLS-fitted teeth the angle measure explained.

Results and Discussion

First, I tested whether the small, last-developing cusps possess more variation in height because they are further away in time and space from the developmental initiation of the first cusp. The results show that variation in height is greater in the small cusps (Fig. 4A), and variation appears to increase as a function of advancing development. However, because these analyses were made from teeth aligned by using the first cusp (a), this interpretation could be too simplistic if the distances from each cusp to the first cusp are not taken into account. Indeed, the SDs increase steadily as a function of average distances among cusp tips (Fig. 4A Inset), suggesting that, for example, the small cusp d is not more variable than would be expected based on its distance from the first cusp. Therefore, to examine further whether short cusps might posses more variation, I removed differences resulting from location, orientation, and size among teeth by using a GLS fit (18). The superimposed teeth show that the cusp positions are remarkably similar (Fig. 4B), suggesting that, from the point of view of morphology *per se*, the cusps show very little variation.
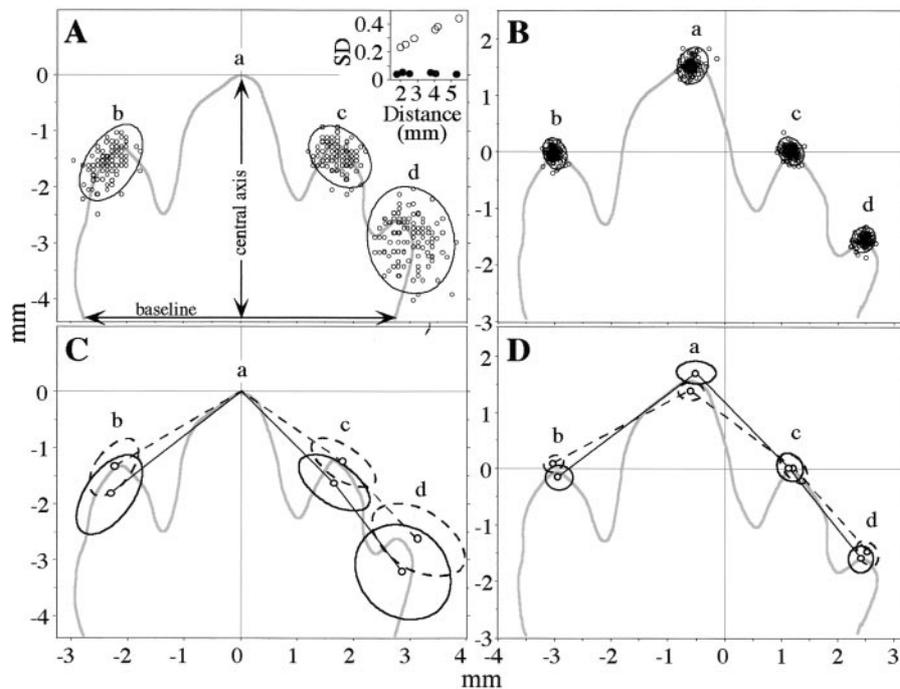
Fig. 4. Variation in main cusp position in a sample of 116 third postcanines. All the ellipses are 95% density ellipses. (A) The teeth were aligned on the tallest cusp and along a central axis (perpendicular to the baseline). Note the high variability of the short cusp d. *Inset* shows the SD as a function of average distance among any two cusps for the sample (○) and corresponding measurement errors for 20 times repeatedly imaged and measured tooth (●). (B) GLS fit showing that the cusps are varying only approximately 0.5 mm in their position. The distributions around the means differ between cusps a and d ($U = 8180$, $P < 0.05$). Other differences are not significant. (C) Density ellipses of the upper (hatched lines) and lower (solid lines) quartiles of teeth grouped based on the b-a-c angle. Teeth are aligned as in A. Notice that the variation in cusp d position is related to the b-a-c angle. (D) Similar groupings as in C but on the GLS fit of B. The sharp and blunt teeth are superimposed in a scissors-like manner in that the tallest cusp of the blunt teeth (hatched line) is below the tallest cusp of the sharp teeth (solid line) whereas the lowest cusps of the blunt teeth are above the corresponding cusps of the sharp teeth. The first principal component factor accounts for 46.0% of the total variance among the GLS-fitted teeth and correlates substantially with the b-a-c angle ($r = -0.84$, $P < 0.001$) and less so with tooth height ($r = 0.21$, $P < 0.05$) and tooth length ($r = -0.41$, $P < 0.001$). Partial correlation is $r = -0.72$, $P < 0.001$, between the first factor and b-a-c angle when height and length are kept constant.

Superficially, the results in Fig. 4 *A* and *B* appear paradoxical. How can variation in tooth cusp position increase in small cusps during development when, morphometrically, the small cusps are not more variable? Below, I show a solution that links the development and evolution of tooth shapes and proposes that mammalian teeth are highly evolvable.

To test whether a sequential patterning cascade might control cusp development (Fig. 2), I examined whether the positioning of the first cusps limits or directs the variation of small cusps down the crown. As a proxy for differences in cusp development, I measured cusp configuration of the three tallest cusps by using angle measure, where small and large angles correspond to sharp and blunt cusp configurations, respectively (Fig. 3). In sharp teeth, cusps are initiated further apart from each other than in blunt teeth during development (Fig. 2). The results show that difference in cusp configuration among blunt and sharp teeth has a cumulative effect on the location of the small cusp d (Fig. 4*C*) and also that the major difference among GLS-fitted teeth is the bluntness or sharpness of their cusp configuration (Fig. 4*D*). The angle measure correlates more strongly ($r = -0.84$) with the first principal component factor, explaining 46% of the total variance among GLS-fitted teeth, than the maximum height or length of the teeth ($r = 0.21$ and $r = -0.41$, respectively (Fig. 4*C*). Thus, the absolute size of a seal tooth appears to play a small role in its cusp configuration and may indicate other factors affecting the final growth of teeth apart from the cusp patterning. No tooth has very blunt configuration of the first three cusps with a very short cusp d, and no tooth has very sharp configuration of the first three cusps with a very tall cusp d (see the "forbidden" shapes in Fig. 3). Furthermore, individual teeth that are blunter (cusp height differences are smaller) are more likely to possess extra cusps than teeth that are sharper (cusp height differences are larger; Figs. 3 and 5*A*). The same pattern of variation is repeated along the tooth row but with increasing bluntness in the distal teeth. Thus, distal seal postcanine cusps are initiated closer to each other during development and they are more likely to have five cusps (Fig. 5*A*). An exception is the last tooth, where the frequency of three cusped teeth is high. However, the heights of the tooth crowns decrease in the distal teeth (Fig. 5*B*), pointing to an earlier termination of cusp morphogenesis, resulting in a paedomorphic last tooth (Fig. 2). Therefore, among teeth, interplay between the spacing of cusp initiation and the global termination of crown development seem to determine the potential cusp pattern and the degree of the pattern realization, respectively. These two factors may form the basis of developmental fields that have been proposed to produce the gradual differences among mammalian teeth along a tooth row (refs. 14 and 21, but see also ref. 22). Considering evolution of more complex tooth morphologies, no seal teeth with extra cusps on both sides of the tallest cusp were found, suggesting slightly variable control of patterning around the initial cusp. This partly decoupled control of patterning around the tallest cusp may have increased the evolutionary versatility (23) of mammalian teeth.

In conclusion, the increase in variation of height and number of small cusps can be interpreted to result from only slight changes in the developmental system parameters spacing subsequent cusps (Fig. 2). This mode of development results in biased variation because even when natural selection limits the variation in tall cusps, it will not eliminate the cumulative variation in small cusps caused by the patterning cascade. Because this variation in small cusp size does not appear to result from increasing "sloppiness" or "noise" in developmental control (Fig. 4*B*), but instead from parameter differences controlling cusp spacing, natural selection can act efficiently on short cusps (3, 24). With a very small increase in height of tall cusps, the short cusps can become taller and also increase in number.

This link between development and evolution has several new implications. Considering morphological evolution, this
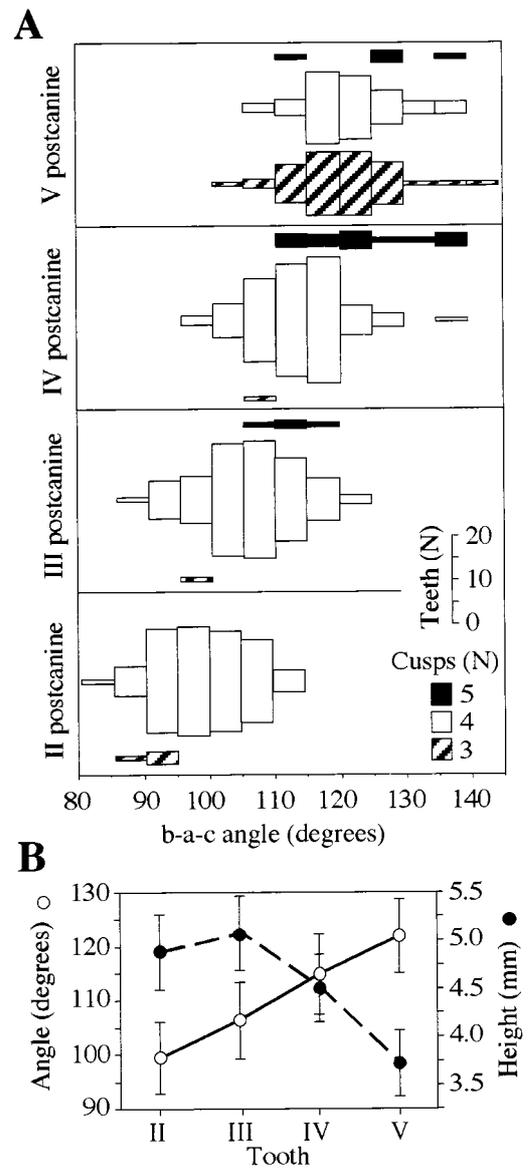
Fig. 5. The angle of the three first-developing cusps (b-a-c) and the number of cusps on the postcanine teeth. (*A*) A plot showing the frequency distribution of three-, four-, and five-cusped teeth along the tooth row. Teeth that are blunter (b-a-c angle is large) have more five-cusped teeth except for the last postcanine (V). (*B*) Average angle of the first three cusps and crown height (with SD) along the tooth row. Note how the average angle increases in distal teeth (solid line) whereas the crown height (hatched line) decreases starting in postcanine IV, which has the highest frequency of five-cusped teeth. The last postcanine (V) is short, and the fourth cusp (d) often is missing, representing the situation shown in Fig. 2 *Left*.

evolvability (4–8) of teeth may have facilitated the fast and independent acquisition of the hypocone in several mammalian lineages in response to ecological opportunity (3, 25). Furthermore, the rarity or lack of certain morphological variants can be due to developmental rather than ecological causes (e.g., Fig. 3). Another implication of these results is that small cusps may be unreliable as phylogenetic signals. Small cusps are not, however, redundant characters, but, rather, smaller cusps can be useful for separating lower taxa whereas taller cusps can be used for separating higher taxa (24). Also, commonly used linear tooth measures, such as lengths and

widths, may be only indirect measures of dental complexity. Developmentally, the sequential activation of the secondary enamel knots requires only small developmental changes to produce large morphological changes. This suggests that there is no one-to-one relationship between molecular signaling and morphological change but, rather, a proficient program regulating morphogenesis. Thus, the generation of mammalian cheek tooth complexity may have required very little increase in developmental complexity. This developmental program is likely to have evolved early in the history of mammals, something that should be testable with the fossil record. The use of population-level variation to link development and macroevolution can be a powerful tool in testing and generating hypotheses in developmental evolution studies.

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EVOLUTION