

Evolution of Novelty in the Cichlid Dentition

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ABSTRACT The shape of teeth occupies a central position in various biological disciplines, from paleo-ecology to molecular biology to cosmetic and reconstructive dentistry. Despite a long tradition of study in mammals, important questions remain regarding the genetic and developmental basis of differences in tooth shape. Here, we use natural mutants of cichlid fish from East Africa, which exhibit tremendous dental diversity, to help fill the gaps in our understanding of vertebrate odontogenesis. We employ an expanded genetic linkage map to demonstrate that cusp number segregates as a gene of major effect, which explains ~40% of the phenotypic variance, on cichlid chromosome 5. Furthermore, we examine patterns of *Bmp4* expression in early odontogenesis to address and refine predictions of models linking tooth shape and tooth number. Mutations in the *Bmp4* cistron do not control tooth shape in this mapping cross. Our data suggest that the evolution of novelty in the cichlid dentition is galvanized by a small number of genetic changes, echoing similar conclusions from recent studies of other vertebrate adaptive morphologies. *J. Exp. Zool. (Mol. Dev. Evol.)* 306B, 2006. © 2006 Wiley-Liss, Inc.

The shape of teeth occupies a central position in various sciences, from paleontology to ecology to molecular biology. Tooth shape is used to identify species or describe new fossils (Carpenter et al., '98; Sereno et al., '99), to test biogeographic hypotheses (Krause et al., '97), and to decipher ancient (MacFadden et al., '99; Dean et al., 2001) as well as recent (MacLeod, 2000) ecologies. Molecular and developmental biologists consider tooth development a prime model of tissue (Lumsden, '88), cell (Chai et al., 2000), and gene interaction (Peters and Balling, '99). Despite a long tradition of study in mammals, important questions remain regarding the genetic and developmental basis of differences in tooth shape. Less than a decade ago, Stock and colleagues began the conclusion of their review of mammalian teeth by claiming, "We do not yet have any unambiguous candidates for genes controlling tooth shape differences within a dentition (Stock et al., '97, p 488)." Most genetic knock-outs of candidate genes result in the lack of teeth, instead of changes in tooth shape. Notably, variation in the "number, location, and size of cusps on the molars and premolars [has] been among the most common evolutionary modifications (Stock et al., '97)" of the mammalian dentition (e.g., Luo et al., 2001).

Models of mammalian tooth shape

In 1998, Thomas and Sharpe argued, by analogy to other organ systems, that the mouse dentition was patterned by an odontogenic homeobox code (OHC, Thomas and Sharpe, '98). Key to this model (further elaborated by Tucker and Sharpe, 2004) is the early specification of molar (multicuspid) vs. incisor (unicuspid) regions of the jaw. According to the OHC, *Bmp4* is expressed distally in dental epithelium and specifies incisor identity. *Fgf8* is expressed more proximally and specifies the molar tooth field. These respective tooth types (molar and incisor) then develop under the control of distinct homeobox genes expressed in the mesenchyme (i.e., incisors—*Msx1/2*; molars—*Barx1*, *Dlx1/2*, *Lhx6/7*). Support for this model comes from the work of Tucker et al. ('98) who used noggin beads to knock down BMP function in explants from the mouse incisor region. The researchers showed that molars developed in place

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of incisors, accompanied by expanded expression of *Barx1* in dental mesenchyme. Similarly, Mitsiadis et al. (2003) demonstrated that ectopic expression of *Islet1* could induce *Bmp4* in the proximal epithelium (molar region) and subsequently inhibit *Barx1* and the development of molars.

Jernvall et al. (2000) showed that correlated gene expression patterns of *Fgf4*, *Lef1*, *P21*, and *Shh* could predict differences in molar cusp shapes between mice and voles. Likewise, Jernvall (2000) and Salazar-Ciudad and Jernvall (2002) presented general developmental and morphogenetic models to explain the “evolvability” of cusps in mammalian evolution. The models accurately reproduced the diversity of mammalian tooth shapes within and between individuals by varying the concentration of molecular activators and inhibitors expressed from singular or multiple signaling centers, called enamel knots (EKs).

Taken together, this body of work is consistent with the hypothesis that tooth shape is controlled, in part, by antagonistic actions of extracellular signaling ligands (e.g., fibroblast growth factors, FGFs; and BMPs) secreted from transitory EKs (Vaahtokahari et al., '96; Jernvall et al., '98; Jernvall and Thesleff, 2000). The expression of *Bmp4* from the primary EK may inhibit secondary EKs from forming (Jernvall and Jung, 2000) and/or regulate the development of subsequent EKs (Salazar-Ciudad and Jernvall, 2002). Control of EK number and spacing ultimately determines cusp number and the sharpness of teeth (Jernvall, 2000; Salazar-Ciudad and Jernvall, 2002).

Integrating tooth number and tooth shape

In 2003, Streelman and colleagues demonstrated that oral jaw tooth number was correlated with cusp number in natural populations of cichlid fish from Lake Malawi, East Africa (Streelman et al., 2003a). Given simple genetic control of tooth shape in this system (Albertson et al., 2003a,b) and the iterative role of certain genes in the stages of tooth development (Peters and Balling, '99), these authors suggested that variation in the expression of a single activating or inhibitory factor (i.e., *Bmp4*) might integrate tooth and cusp number (Streelman et al., 2003a; also see Plikus et al., 2005). Subsequently, multiple labs have reported that ectodysplasin (*Eda*, Kangas et al., 2004) and its receptor (*Edar*, Tucker et al., 2004)

have pleiotropic effects on mouse molar tooth number and cusp number. Tucker et al. (2004) further argued that variation in tooth number is rate-limited by the ligand *Eda* but that changes in cusp number and shape are determined later in odontogenesis by the receptor *Edar*. It is unknown if and how *Eda* and *Edar* interact with BMPs and FGFs during tooth development, but Pispas et al. ('99) showed that *Eda* defects can be partially rescued by FGF in *tabby* mice. Similarly, Houghton et al. (2005) presented evidence that BMPs function upstream of *Edar* in feather development. Kassai et al. (2005) demonstrated that *Ectodin*, a BMP inhibitor, is expressed as a “negative image” of mouse EKs and that *Ectodin*-deficient mice exhibit modified cusp patterns and extra teeth. The evolutionary implications of pleiotropic function for *Bmp4*, members of the ectodysplasin signaling pathway and the *Ectodin* gene are clear; these molecules integrate changes in tooth number with changes in tooth shape.

The evolution of novelty in the cichlid dentition

Cichlid fishes are the most species-rich group of vertebrates and are textbook exemplars of evolutionary diversification (Kocher, 2004). Cichlids are tractable genetic models for a variety of traits (e.g., jaw and tooth shape (Albertson et al., 2003a,b; Streelman et al., 2003a; Albertson et al., 2005; Hulsey et al., 2006, submitted), pigmentation (Streelman et al., 2003b; Lee et al., 2005), visual sensitivity (Carleton and Kocher, 2001; Parry et al., 2005), sex determination (Lee et al., 2003, 2004, 2005)) because (i) species can be crossed in the laboratory to produce viable and fertile hybrids, (ii) species have relatively large broods and short generation times, (iii) embryos are easily manipulated, (iv) genetic linkage maps exist for multiple species (Albertson et al., 2003b; Lee et al., 2005), and (v) species segregate for phenotypes not found in other vertebrate models (Streelman et al., 2003a). These characteristics provide a rich, relatively untapped genetic and morphological resource for the study of craniofacial development.

Cichlid teeth are key components of the trophic machinery on both oral and pharyngeal jaws (Fig. 1). Morphologies range from widely spaced, sharply pointed unicuspid in piscivorous, planktivorous and insectivorous species (e.g., *Cynotilapia afra* (CA); *Rhamphochromis esox*, 1a) to closely

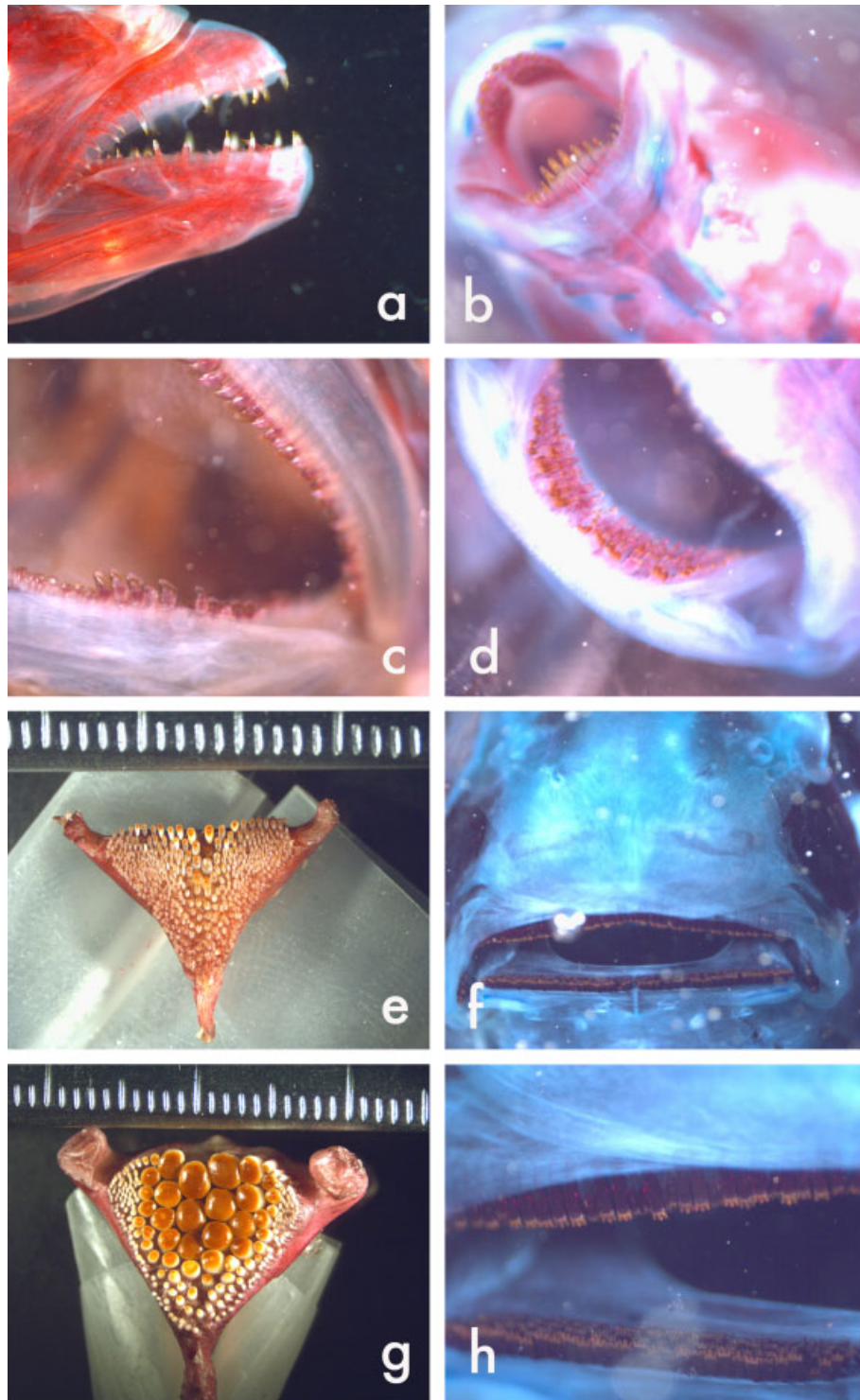


Fig. 1. Cichlid natural mutants segregate for evolutionary novelty in tooth shape. Examples of oral jaw teeth include (a) *Rhamphochromis esox* (unicuspid, piscivore, Lake Malawi), (b) *Labidochromis vellicans* (unicuspid, insectivore, Lake Malawi), (c) *Hemilapia oxyrhynchus* (recurved unicuspid, epiphyte scraper from grass blades, Lake Malawi), (d) *Maravichromis incola* (bicuspid, omnivore, Lake Malawi), (f) *Labeotropheus fuelleborni* (LF, tricuspid, algal scraper, Lake Malawi) and (h) close-up of the tricuspid dentition of LF. (e) Papilliform, plant shredder and (g) molariform, snail crusher depict a pharyngeal tooth polymorphism found among individuals of *Herichthys minckleyi* (Cuatro Ciénegas, Mexico).

packed tricuspid teeth in algal scrapers (e.g., *Labeotropheus fuelleborni* (LF), 1f,h). The majority of African cichlids have unicuspid or bicuspid dentitions of the oral jaw; many species like *Metriaclima zebra* (MZ) (below) possess an outer row of bicuspid teeth with tricuspid teeth in posterior rows. Very few species have exclusively, or primarily, tricuspid teeth. The addition of a third cusp on teeth of the outer row is an evolutionary novelty among cichlids, shared by a single species in Central America (*Herotilapia multispinosa*), a few morphospecies of soda lake tilapia (Tichy and Seegers, '99), and one to a few lineages in each of Lakes Malawi (*Labeotropheus* [below], *Petrotilapia*), Tanganyika (*Petrochromis*), and Victoria (*Tridontochromis*). The shape of cichlid teeth may respond rapidly to natural selection; tooth shape characterizes diverging morphs (Tichy and Seegers, '99; Hulsey et al., 2005), and evolves replicatively (Rüber et al., '99).

A great deal is known about the development of cichlid teeth (Huyseune, '90; Huyseune and Sire, '92a,b; Streelman et al., 2003a; Vandervennet and Huyseune, 2005). Like many teleost fishes, adult cichlids have (1) multiple rows of teeth on two sets of jaws (oral and pharyngeal); (2) similarly shaped teeth within a row (homodonty), and (3) tooth replacement throughout life via de novo formation of tooth germs (polyphyodonty, Huyseune and Thesleff, 2004). In addition, cichlids (and most teleosts) possess a set of first-generation teeth, which are distinguished from replacement teeth by their small size, simple shape, and rudimentary organization (Huyseune and Sire, '97; Sire et al., 2002). Cichlid teeth develop similarly to those of mammals (Huyseune and Sire, '97; Huyseune and Thesleff, 2004).

Here, we report progress in our understanding of evolutionary novelty, or discontinuous and dramatic phenotypic change (Jernvall, 2000), in the dentition of Lake Malawi cichlid fishes. Phenotypic variants produced by natural selection provide the context in which to understand the genetic and developmental basis of differences in tooth shape. Using a genetic linkage map with greater coverage of the genome from previous reports (i.e., Albertson et al., 2003b), we show that genetic variance in cusp number between LF (tricuspid) and MZ (bicuspid) segregates as a gene of major effect. In addition, we present data on the expression of *Bmp4* in the developing dentition to revisit and refine models of cichlid odontogenesis (Streelman et al., 2003a).

MATERIALS AND METHODS

Species

We compared three Lake Malawi rock-dwelling cichlid species with distinct feeding behaviors, jaw and tooth morphologies (Albertson et al., 2003a,b; Streelman et al., 2003a; Albertson et al., 2005). LF is a specialized biting species, characterized by a short, stout lower jaw, and multiple rows of tightly packed tricuspid teeth. LF feeds by cropping attached algae from the substrate. MZ, or *M. benetos* (MB) are generalized feeders, characterized by gracile, relatively elongate lower jaws, a first row of bicuspid teeth, with tricuspid teeth in multiple rows behind. MZ and MB are two of a few rock-dwelling species that feed from the substrate by brushing, and feed on plankton in the water column by suction. CA is an obligate suction feeding planktivore with widely spaced unicuspid teeth. The breeding and rearing of cichlids for quantitative trait locus (QTL) analysis has been described previously (Albertson et al., 2003b). LF, MB and CA embryos were obtained for developmental studies by natural matings in 40-gallon tanks.

Linkage analysis

A Lake Malawi cichlid linkage map was constructed using JoinMap 3.0 (van Ooijen and Voorrips, 2001). The locus file consisted of genotypes for 173 F₂ hybrid progeny (LF × MZ) at 170 marker loci. The grouping module of JoinMap assigned 160 of 170 loci to 24 linkage groups using a logarithm of odds (LOD) score threshold of 3.0. The mapping module of JoinMap built the genetic map for each linkage group using the Kosambi mapping function, a LOD threshold of 1.0, a recombination threshold of 0.450, and a jump threshold of 5.0. A ripple function was performed after each locus was added to ensure optimal marker order. Linkage groups have been renamed and extended from those reported in Albertson et al., 2003b (~30 new markers have been added), with reference to a more complete linkage map of the Nile tilapia (Lee et al., 2005) and a comparative genetic map of East African cichlids (Streelman, unpublished).

QTL analysis of tooth shape

Interval mapping analysis was performed in MapQTL 5.0 (van Ooijen, 2004) with tooth shape phenotypes described previously (Albertson et al., 2003a,b; Streelman et al., 2003a). MQM mapping

(effectively, composite interval mapping) did not further resolve QTL signal (i.e., did not change QTL number and did not significantly change LOD or PVE (percent of phenotypic variance explained)), likely because only two QTL were detected (see results, below). Genome-wide significance of QTL (at $\alpha = 0.05$, 0.01, and 0.001) was determined by 1,000 permutations of phenotype, with fixed marker data (i.e., the permutation test of MapQTL 5.0).

Association of tooth number and tooth shape in natural populations

We counted the number of teeth per mm of jaw width for closely related species with different number of cusps (Streelman et al., 2003a). Here we include data from 11 individuals of MZ (from natural populations in the southeast arm of Lake Malawi) with abnormal tooth patterning. These individuals (*Metriaclima* 2 of Fig. 3) had a mixture of unicuspid and bicuspid teeth in the first tooth row.

Whole-mount in situ hybridization (WISH) analysis

WISH analyses were performed on staged embryos using an adapted protocol and riboprobes synthesized from cichlid *Bmp4* cDNA (Albertson et al., 2005).

RESULTS

QTL analysis of tooth shape

Interval mapping revealed two regions of the genome segregating for tooth shape in this cross, which together account for ~50% of the phenotypic variance (Table 1, Fig. 2). As noted previously, tooth shapes on the upper and lower jaws

are highly correlated (Albertson et al., 2003a; Streelman et al., 2003a) and map to the same genomic intervals (Albertson et al., 2003b). For simplicity, we refer to upper jaw and lower jaw tooth shape as “tooth shape” and provide data in Table 1 for the lower jaw dentition only. The tooth shape QTL on linkage group 15 segregates with genes contributing to skull shape and jaw width, implying pleiotropy or linkage in the genetic architecture of these traits (Albertson et al., 2003b, linkage group 16). The QTL on linkage group 5 segregates as a gene of major effect. It accounts for ~40% of the phenotypic variance and phenotypic means of F₂ genotypic classes approach values exhibited by F₀ (Table 1). The MQM method (i.e., composite interval mapping) does not further resolve QTL number or signal (i.e., LODs and PVEs do not change significantly). The MQM method does suggest a nearly significant genetic effect (genome-wide $\alpha = 0.05$) of linkage group 9, near marker UNH2191 (formerly linkage group 7, Albertson et al., 2003b), when markers on linkage groups 5 and 15 are specified as cofactors.

Association of tooth number and tooth shape in natural populations

In accordance with previous results, there is a relationship between tooth number and tooth shape among closely related Lake Malawi species (Streelman et al., 2003a). Individuals of LF averaged 4.8 (± 0.74 SD) tricuspid teeth per mm of jaw width, in the first tooth row. Notably, MZ with variable tooth shapes exhibited distinct tooth spacing. Individuals with normal dentitions averaged 3.3 (± 0.52) bicuspid teeth per mm of jaw width in the first tooth row (Fig. 3). Conversely, fishes with a mixture of bicuspid and unicuspid teeth in the first row averaged 1.52 (± 0.34) teeth per mm of jaw width. The spacing of teeth in these individuals is similar to species with unicuspid dentitions (e.g., CA, 1.3 [± 0.20]).

Whole-mount in situ hybridization

We observed a distinct pattern of *Bmp4* expression in the developing dentitions of cichlid species with adult tricuspid (LF), bicuspid (MB), and unicuspid (CA) teeth, respectively (Fig. 4c). At 10 days post fertilization (dpf), *Bmp4* is expressed bilaterally, marking the position of tooth families in the three species. We did not generate histological sections from these preparations, and so we are unsure of the developmental stage of individual teeth; it is unlikely that each tooth is at the

TABLE 1. Summary of quantitative trait locus (QTL) data for tooth shape in the cross between *Labeotropheus fuelleborni* (LF) and *Metriaclima zebra* (MZ)

Position	LOD	PVE	LF/LF (3)	LF/MZ (2.5)	MZ/MZ (2)
LG 5	9.92***	38.9	2.88	2.6	2.25
LG 15	4.15*	11.5	2.74	2.59	2.44

The table lists the position (linkage group (LG)) of each QTL, the maximum LOD score (* indicates significance at $\alpha = 0.05$; *** indicates significance at $\alpha = 0.001$), the percent variance explained by each QTL (PVE), and the phenotypic mean of F₂ fishes (i.e., the number of cusps on teeth in the first tooth row) inheriting two alleles from LF (LF/LF), one allele from each parent (LF/MZ) and two alleles from MZ (MZ/MZ). Parental values are included within parentheses for comparison.

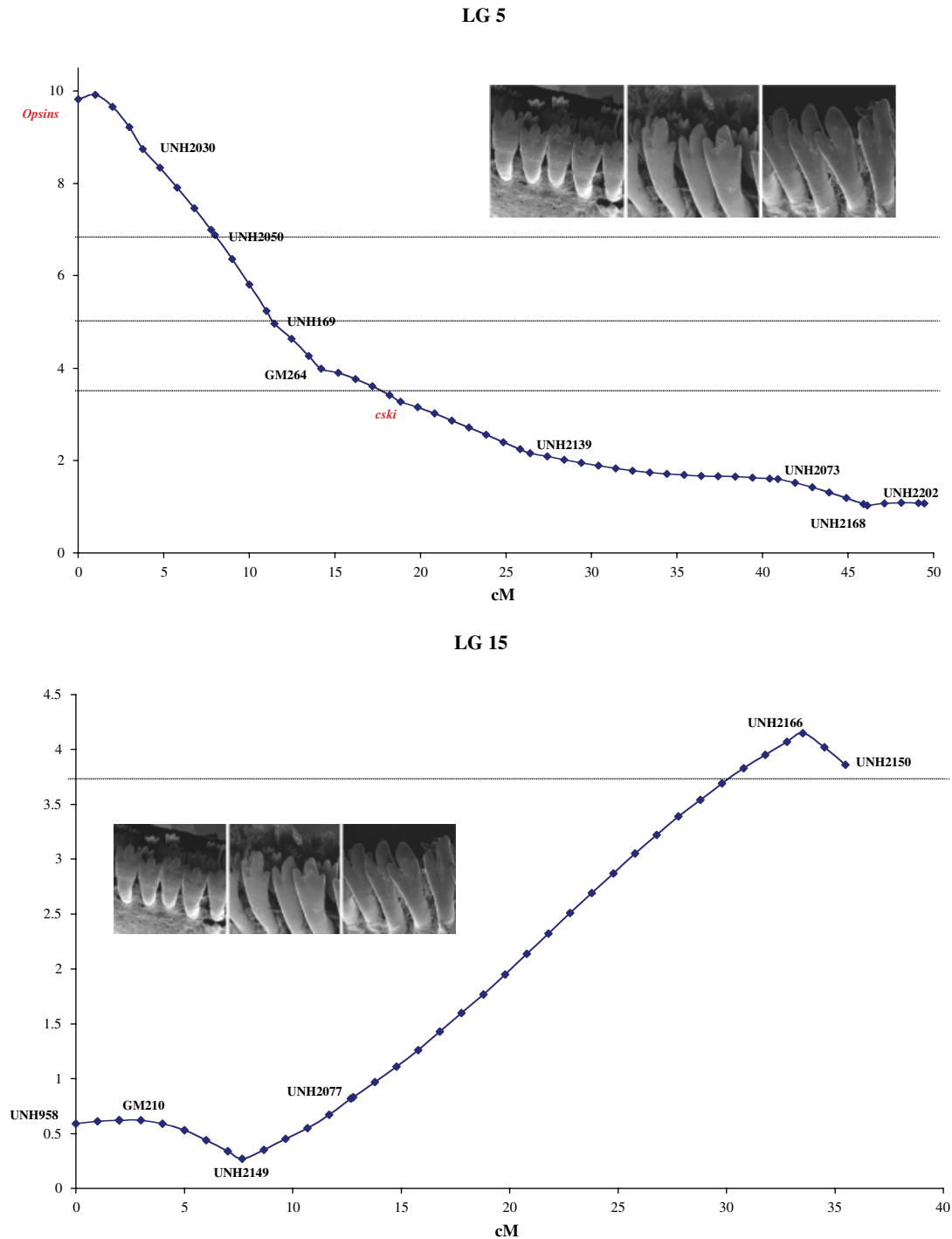


Fig. 2. Two regions of the cichlid genome segregate for variance in cichlid cusp number (see also Table 1). We plot the LOD score (y-axis) vs. map position in centimorgans (cM, x-axis) for quantitative trait loci identified on linkage groups (LG) 5 (top) and 15 (bottom). For the QTL on LG5, we show statistical thresholds of LOD scores corresponding to $\alpha = 0.05, 0.01$ and 0.001 , respectively. For the QTL on LG 15, we show the statistical threshold of LOD scores corresponding to $\alpha = 0.05$. The positions of microsatellite markers are shown in black and those of genes are shown in red. The inset image on each plot depicts scanning electron micrographs of the adult teeth from LF (left, tricuspid), the F₁ hybrid (middle, intermediate), and MZ (right, bicuspid).

same developmental stage. In LF (closely packed tricuspid teeth as an adult), there are more and smaller foci of *Bmp4* expression (Fig. 4c, arrows)

than in MB (bicuspid teeth as an adult), which exhibit more and smaller foci than CA (widely spaced unicuspid teeth as an adult). Notably, all

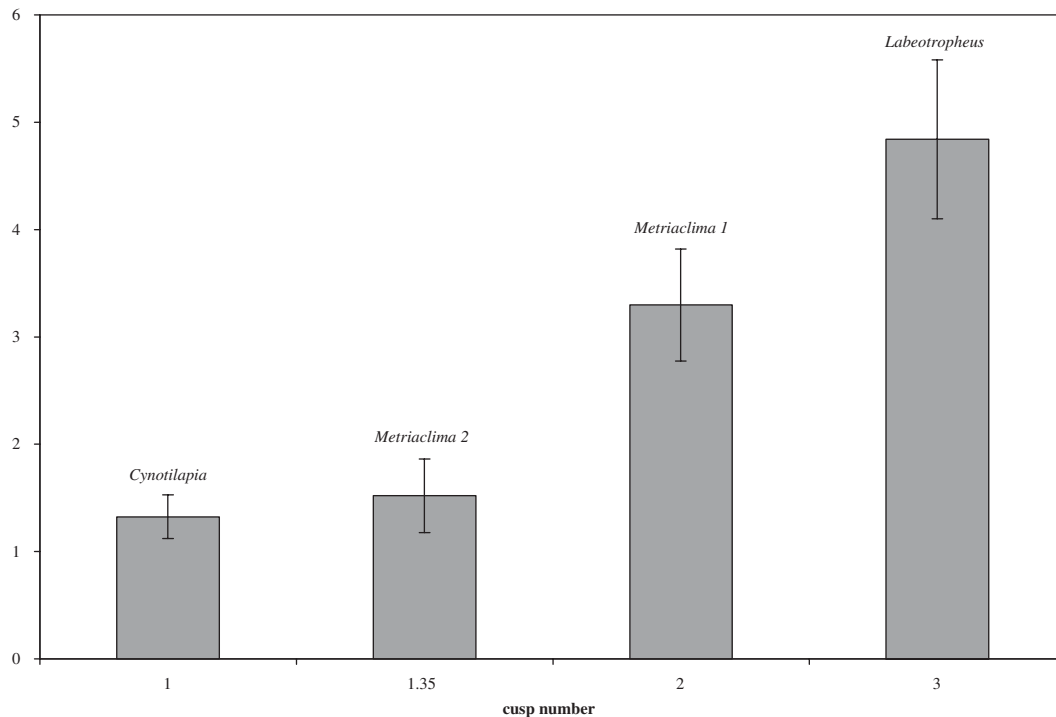


Fig. 3. Tooth shape is associated with tooth number in natural populations of cichlid fish from Lake Malawi, East Africa. We plot cusp number (x-axis) vs. teeth per mm of jaw width (y-axis), following Streelman et al. (2003a), for individuals of *Cynotilapia afra* (unicuspid), *Metriaclima zebra* and *Labeotropheus fuelleborni* (tricuspid). *Metriaclima 1* represents individuals with normal, wild-type dentitions (bicuspid teeth); *Metriaclima 2* represents individuals with a mixture of unicuspid and bicuspid teeth in the first tooth row.

three species possess unicuspid teeth at 10 dpf; multicuspid teeth appear in waves of replacement a few weeks later (Streelman et al., 2003a).

DISCUSSION

The vertebrate dentition is an evolutionary enigma. It is a critical organ system for survival, and yet it is among the most variable characters in vertebrate history (Carroll, '88). Great progress has been made in the last decade to understand the origin of evolutionary novelty in mammalian teeth, and yet the corresponding focus on the mouse as a model organism has suggested the need for experimentation in other systems (Stock, 2001). Recent work in fishes demonstrates the complexity of dental patterning in vertebrates, with key similarities and differences to the mouse. Fraser et al. (2004) demonstrated that oral jaw first-generation teeth of rainbow trout express *Pitx2*, *Shh*, and *Bmp4* in similar spatio-temporal patterns to the mouse, suggesting the conservation of these molecules in odontogenesis since the common ancestor of fish and mammals (~450 million years ago). Notably, Fraser et al. (2004)

describe differences in *Pitx2* expression during the morphogenesis of trout teeth on the oral (presence of *Pitx2*) vs. pharyngeal jaws (absence). Working with zebrafish, Laurenti et al. (2004) likewise demonstrate differences between pharyngeal first-generation teeth and the oral teeth of mammals (zebrafish lack teeth on the oral jaw so no direct comparison is possible). Specifically, the gene *Eve1*, a member of the homeobox-containing *evx* gene family, not expressed during tooth development in mammals, is expressed during tooth initiation and morphogenesis of the first pharyngeal tooth. Jackman et al. (2004) used chemical knockdown (SU5402) of FGF signaling to demonstrate that FGFs are required for zebrafish first-generation tooth development. Furthermore, they showed that *Fgf8* and *Pax9* are not expressed under normal conditions in zebrafish tooth germs (unlike in mouse) and that both *Dlx* and *Lhx* genes are expressed in dental mesenchyme (as in mouse molars). This work in fishes is significant but incomplete. For instance, first-generation teeth are morphologically unlike replacement teeth (Huysseune and Sire, '97; Sire et al., 2002) and exhibit divergent gene expression programs

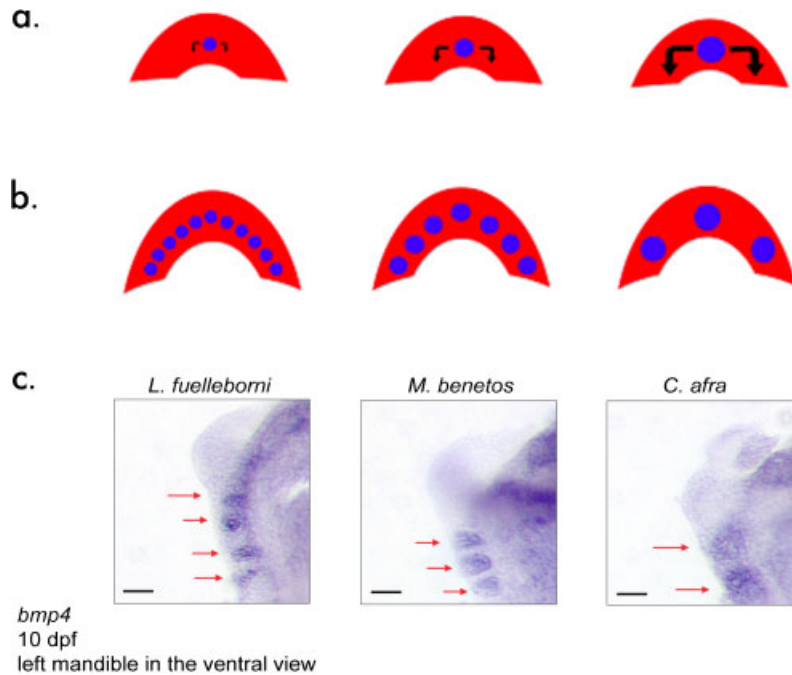


Fig. 4. Patterns of *Bmp4* expression in early cichlid odontogenesis refine models linking tooth number with tooth shape (Streelman et al., 2003a). (a) and (b) Qualitative model wherein tooth spacing is regulated by foci of activator or inhibitor (blue circles) within a region permissive for teeth (red). The tip of the jaw is towards the top of the page. The model links tooth number and tooth spacing via the concerted action of activator/inhibitor (Streelman et al., 2003a for details) to produce widely-spaced unicuspid vs. tightly-packed tricuspid dentitions. (c) Expression pattern of *Bmp4* at 10 dpf in the left mandible of species with tightly-packed tricuspid teeth as adults (*Labeotropheus fuelleborni*, LF), moderately-spaced bicuspid teeth as adults (*Metriaclima benetos*, MB) and widely-spaced unicuspid teeth as adults (*Cynotilapia afra*, CA). Species express *Bmp4* in divergent patterns, days before tooth shapes differentiate (see text for discussion). Scale bars = 100 μ m.

(Fraser et al., this issue). Similarly, the pharyngeal dentition develops in a different (Hox-positive) environment than oral teeth. Therefore, differences between tooth generations (first-generation vs. replacement) or local environments (pharyngeal vs. oral) may explain some of the variation noted between fish and mouse.

Simple genetic basis of evolutionary novelty in the cichlid dentition

Cichlid fishes offer the opportunity to identify the genetic basis of differences in tooth shape, a phenotype difficult to study in most vertebrate models (Streelman et al., 2003a). The data presented here support earlier suggestions that cusp number is a simple genetic trait in cichlids (Albertson et al., 2003a; Streelman et al., 2003a), and add to a growing list of vertebrate phenotypes whose microevolutionary divergence is controlled by genes of large effect (Nachman et al., 2003; Streelman et al., 2003b; Abzhanov et al., 2004; Albertson et al., 2005; Colosimo et al., 2005;

Kimmel et al., 2005). Cichlid tooth shapes show the greatest degree of variation in lineages from Lakes Tanganyika and Malawi, where differences in cusp number are associated with trophic ecology (Fryer and Iles '72). Tooth shape characterizes diverging morphs of soda lake tilapias (Tichy and Seegers, '99), and evolves replicatively in Tanganyikan eretmodine cichlids (Rüber et al., '99). Our genetic mapping results suggest that tooth shape evolves rapidly and replicatively in cichlids because a small number of genetic changes can drive dramatic phenotypic evolution. Hence, the "evolvability" of novelty in the cichlid dentition, which approaches that observed across orders of mammals (Jernvall, 2000), may be elicited by ecological selection on a small set of genes.

Cichlid linkage group 5 is a speciation chromosome

A major QTL for tooth shape (~40% PVE) is located in a 3.7 centimorgan (cM) interval on

cichlid linkage group 5, between three cone opsin genes linked in tandem (*LWS*, *SWS2a*, *SWS2b*; Carleton and Kocher, 2001) and marker UNH2030. Linkage group 5 has previously been shown to segregate for the oligogenic orange-blotch color pattern (Streelman et al., 2003b) and a strong female sex determiner *W* (Kocher, 2004). This puts genes for (i) visual sensitivity and color opponency, (ii) trophic morphology, (iii) color pattern, and (iv) sex determination, on the same chromosome. Given recent models of speciation, which require strong disruptive selection to create gametic association among suites of ecological, marker, sex determining, and preference genes (Dieckmann and Doebeli, '99; Seehausen et al., '99), we are very interested in the role of this chromosome in cichlid evolution.

Refining models of variation in cichlid tooth shape

Our previous model of cichlid tooth shape proposed that the concerted action of an activating or inhibitory molecule, acting at multiple stages of odontogenesis, could explain the empirical relationship between tooth number and tooth shape (Streelman et al., 2003a; Fig. 3). The model was based primarily on the observation that genes like BMPs and FGFs are active during tooth initiation and tooth morphogenesis (Peters and Balling, '99) and may therefore affect both the sites in the jaw where teeth will develop, as well as the shape of individual teeth. The role of BMPs in the morphoregulation of the dentition has recently been demonstrated by Plikus et al. (2005), who used tissue-specific knockdown of BMP signaling to alter the number, size, and shape of mouse teeth.

We speculated that *Bmp4* might be divergently expressed during the development of cichlid tricuspid, bicuspid, and unicuspid dentitions (Streelman et al., 2003a). Notably, *Bmp4* expression is associated with tooth site patterning early in odontogenesis (Fig. 4c). Its expression differentially marks the developing tooth families of each dentition, but does so in a manner not predicted by our model (Fig. 4a,b). We suggested that all first-generation and early-replacement dentitions, regardless of adult tooth patterning, would resemble the model for CA (Streelman et al., 2003a). We proposed that the pattern of *Bmp4* expression would change later in ontogeny for species with bicuspid or tricuspid dentitions; that differences in gene expression would become apparent as adult

multicuspid teeth replaced unicuspid pioneer dentitions. By contrast, our data show that *Bmp4* is already expressed divergently at 10 dpf, even though all three species share unicuspid teeth at this age (Streelman et al., 2003a). Thus, tooth spacing is specified by molecular interactions before differences in tooth shape are evident. These data support the notion of Fraser et al. (2006) that replacements are pre-patterned by the position of pioneer and primary teeth. Mutations in the *Bmp4* cistron are not responsible for tooth shape differences in the cross between LF and MZ; *Bmp4* maps to cichlid linkage group 19, independent of tooth shape QTL.

We are interested to identify the genes that control tooth shape and tooth number in Lake Malawi cichlids. Members of the ectodysplasin signaling pathway, *Eda* and *Edar*, as well as the gene *Ectodin*, which integrate both tooth number and tooth shape (Kangas et al., 2004; Tucker et al., 2004; Kassai et al., 2005) are obvious functional candidates. The major QTL for tooth shape is located in a 3.7 cM interval on cichlid linkage group 5, between three cone opsin genes and marker UNH2030. Notably, genes mapped to cichlid linkage group 5 (opsins and *c-ski1*, roughly 15 cM from one another, Fig. 2) are found approximately 5 Mb apart on *Tetraodon* (pufferfish) chromosome 11 (Jaillon et al., 2004). These data suggest that it is reasonable to attempt to generate a physical map spanning the critical interval for tooth shape (Katigiri et al., 2005). The *Tetraodon* data are interesting for another reason; they can be used to identify comparative positional candidate genes for cichlid tooth shape. Positional candidates (genes located near the opsins on *Tetraodon* chromosome 11), include *wnt4* (Sarkar and Sharpe, '99), *sema3c* (Løes et al., 2001), *bmp7* (Helder et al., '98), and *rarg-1* (Bloch-Zupan et al., '94). Continued genetic mapping, and analysis of gene expression in replacement dentitions, both in vivo and in explant culture systems (Van der heyden et al., 2005), will be key to this understanding.

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