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RESEARCH ARTICLE

Distinct developmental genetic mechanisms underlie convergently evolved tooth gain in sticklebacks

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ABSTRACT

Teeth are a classic model system of organogenesis, as repeated and reciprocal epithelial and mesenchymal interactions pattern placode formation and outgrowth. Less is known about the developmental and genetic bases of tooth formation and replacement in polyphyodonts, which are vertebrates with continual tooth replacement. Here, we leverage natural variation in the threespine stickleback fish *Gasterosteus aculeatus* to investigate the genetic basis of tooth development and replacement. We find that two derived freshwater stickleback populations have both convergently evolved more ventral pharyngeal teeth through heritable genetic changes. In both populations, evolved tooth gain manifests late in development. Using pulse-chase vital dye labeling to mark newly forming teeth in adult fish, we find that both high-toothed freshwater populations have accelerated tooth replacement rates relative to low-toothed ancestral marine fish. Despite the similar evolved phenotype of more teeth and an accelerated adult replacement rate, the timing of tooth number divergence and the spatial patterns of newly formed adult teeth are different in the two populations, suggesting distinct developmental mechanisms. Using genome-wide linkage mapping in marine-freshwater F2 genetic crosses, we find that the genetic basis of evolved tooth gain in the two freshwater populations is largely distinct. Together, our results support a model whereby increased tooth number and an accelerated tooth replacement rate have evolved convergently in two independently derived freshwater stickleback populations using largely distinct developmental and genetic mechanisms.

KEY WORDS: Convergent evolution, Odontogenesis, Stickleback, Polyphyodonty, Tooth replacement

INTRODUCTION

Teeth are a classic model system for studying organogenesis in vertebrates, as repeated epithelial-mesenchymal interactions orchestrate odontogenesis (Ahn, 2015; Biggs and Mikkola, 2014; Jernvall and Thesleff, 2012). The developmental genetic basis of tooth formation has been most intensively studied in the mouse (Tucker and Sharpe, 2004), which has revealed detailed genetic networks that specify primary tooth formation and placement (Bei, 2009; Jernvall and Thesleff, 2000; Lan et al., 2014; O'Connell et al., 2012; Tummers and Thesleff, 2009). However, since mice are monophyodont rodents that do not replace their teeth, other vertebrate models are needed to study the developmental basis of tooth replacement. The ancestral vertebrate dental phenotype is polyphyodonty, or continuous tooth replacement, a trait retained in sharks, fish and reptiles (Jernvall and Thesleff, 2012; Tucker and Fraser, 2014). These replacement teeth may appear adjacent or beneath primary teeth and are often retained in the tooth field before the primary tooth is shed. In sharks, up to 200 teeth can develop as replacements for a single position (Reif, 1984).

Recent studies on tooth replacement have supported the hypothesis that genetic networks controlling primary tooth formation are redeployed during tooth replacement in both polyphyodont vertebrates (Fraser et al., 2006, 2013; Handrigan and Richman, 2010) and diphyodont mammals (which have two successive sets of teeth) (Jussila et al., 2014). Genetic studies in humans also demonstrate that shared genetic networks pattern primary and replacement teeth (Nieminen, 2009; van den Boogaard et al., 2012). Induction of tooth replacement has been proposed to be regulated by odontogenic stem cells, and candidate pathways, stem cell niches and markers have been proposed (Abduweli et al., 2014; Gaete and Tucker, 2013; Handrigan et al., 2010; Huysseune and Thesleff, 2004; Juuri et al., 2013; Smith et al., 2009b; Wu et al., 2013).

Fish retain the polyphyodont mode of tooth replacement and offer several advantages for developmental genetic studies, including external development and large numbers of offspring per mating. Many fish also have two sets of tooth-covered jaws – an oral jaw in their mandibular arch primarily for grasping prey, as well as a pharyngeal jaw in the posterior branchial segments in their throat for manipulation and mastication (Hulsey et al., 2005; Lauder, 1983; Sibbing, 1991; Wainwright, 2006). Oral and pharyngeal teeth form via highly similar developmental genetic mechanisms and are developmentally homologous (Fraser et al., 2009). For example, the Eda/Edar pathway is required for the proper formation of oral teeth in mammals (Mikkola and Thesleff, 2003), both oral and pharyngeal teeth in medaka fish (Atukorala et al., 2011), and for the only teeth that form in zebrafish – ventral pharyngeal teeth (Harris et al., 2008).

To study the developmental genetic basis of tooth formation and replacement, we leveraged natural variation in dental patterning in threespine stickleback fish (*Gasterosteus aculeatus*). Sticklebacks have undergone a dramatic adaptive radiation in which ancestral marine populations have repeatedly colonized and rapidly adapted to thousands of freshwater lakes and creeks throughout the Northern Hemisphere (Bell and Foster, 1994). Colonization of freshwater environments is accompanied by a variety of adaptations to the head skeleton, many of which are likely to be due to a major shift in diet from small zooplankton in the ocean to larger prey in freshwater (Schluter and McPhail, 1992). Recent studies (Miller et al., 2014; Cleves et al., 2014) have identified a major dental patterning polymorphism: a near twofold increase in ventral pharyngeal tooth number in a derived freshwater benthic (adapted to live on the bottom of a lake)

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population from Paxton Lake in Canada, possibly adaptive for crushing larger prey in the benthic niche. The increase in tooth number is accomplished by both expanding the size of the tooth field and by decreasing intertooth spacing (Cleves et al., 2014). Marine and freshwater sticklebacks can be intercrossed and their F1 hybrids are fertile, allowing forward genetic mapping of genomic regions controlling morphological differences. Genetic mapping revealed that tooth plate area (field size) and intertooth spacing are genetically separable, being controlled by largely non-overlapping genomic regions (Cleves et al., 2014). One genomic region with the largest effects on tooth number, tooth plate area and intertooth spacing maps to chromosome 21 and contains a *cis*-regulatory allele of the *Bone morphogenetic protein 6* (*Bmp6*) gene (Cleves et al., 2014).

Little is known about the developmental mechanisms underlying this evolved tooth gain. Additionally, whether increased tooth number has evolved in other freshwater populations and, if so, whether similar or different developmental genetic mechanisms are used remain open questions. Here we identify a second derived freshwater stickleback population with convergently evolved tooth gain. In both freshwater populations, increased tooth number arises late in development and is associated with increased rates of new tooth formation in adults. However, the two freshwater populations have different timing of tooth number divergence, strikingly different spatial patterns of tooth addition in adults, and mostly non-overlapping genomic regions controlling tooth number. Thus, convergently evolved tooth gain in the two freshwater populations arises via largely distinct underlying developmental and genetic bases.

RESULTS

Two freshwater stickleback populations exhibit evolved tooth gain

To test the hypothesis that independently derived freshwater stickleback populations have repeatedly evolved increases in pharyngeal tooth number, we compared pharyngeal tooth morphology of three adult laboratory-reared stickleback populations: a marine population from Rabbit Slough (RABS) in Alaska, USA; a freshwater benthic population from Paxton Lake (PAXB) in British Columbia, Canada [previously shown to have evolved tooth gain (Cleves et al., 2014)]; and a second freshwater population from Cerrito Creek (CERC) in California, USA (supplementary material Fig. S1).

Sticklebacks have three sets of bilateral pharyngeal tooth plates near the back of the throat (Fig. 1A) (Anker, 1974): a ventral pair on the fifth ceratobranchials, hereafter referred to as ventral tooth plates (VTP); a small dorsal pair on the anterior pharyngobranchials, hereafter referred to as dorsal tooth plates 1 (DTP1); and a large dorsal pair on the posterior pharyngobranchials, hereafter referred to as dorsal tooth plates 2 (DTP2). Relative to ancestral marine fish, both freshwater populations have evolved increased ventral and dorsal DTP1 pharyngeal tooth number (Fig. 1B,D; supplementary material Fig. S2A-C). By contrast, no significant differences in DTP2 tooth number were found. As increased tooth number could result from a larger field of teeth and/or reduced intertooth spacing (Cleves et al., 2014), we quantified tooth plate area and intertooth spacing on the ventral pharyngeal tooth plate, which is the tooth plate with the largest magnitude difference in tooth number (Fig. 1C). Both freshwater populations have also evolved increased tooth plate area and decreased intertooth spacing compared with ancestral marine fish

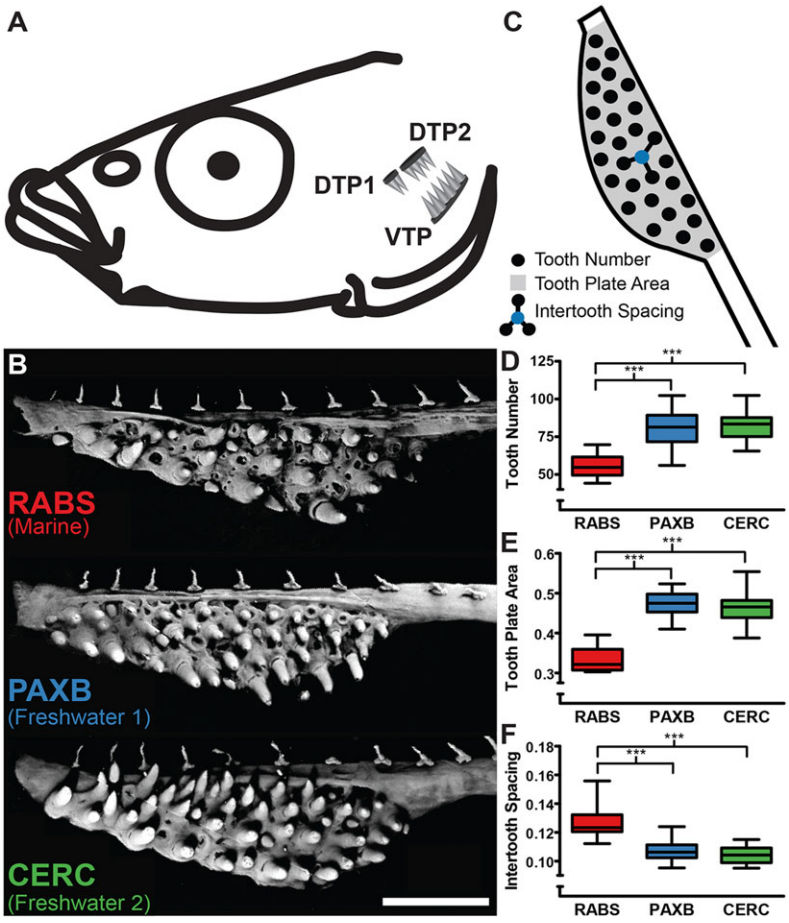

Fig. 1. Two freshwater stickleback populations exhibit evolved tooth gain. (A) Location of three pharyngeal tooth plates in the stickleback head: VTP, ventral tooth plate on the fifth ceratobranchial; DTP1, dorsal tooth plate 1 (on the anterior pharyngobranchial); DTP2, dorsal tooth plate 2 (on the posterior pharyngobranchial); all three are bilaterally paired (only a unilateral set is shown). (B) Representative 3D projections of adult stickleback unilateral ventral tooth plates from three populations. Scale bar: 500 µm. (C) Depiction of tooth number, tooth plate area and intertooth spacing phenotypes. (D-F) Quantification of size-corrected total tooth number (D), tooth plate area (mm^2) (E), and intertooth spacing (mm) (F) in ventral pharyngeal tooth plates of laboratory-reared adults. ***P<0.001 (one-way ANOVA using a Tukey-Kramer post-hoc test). (D-F) Respective sample size for each trait: RABS, n=19, 18, 18; PAXB, n=35, 32, 33; CERC, n=29, 29, 30.

Development (2015) 142, 2442-2451 doi:10.1242/dev.124248

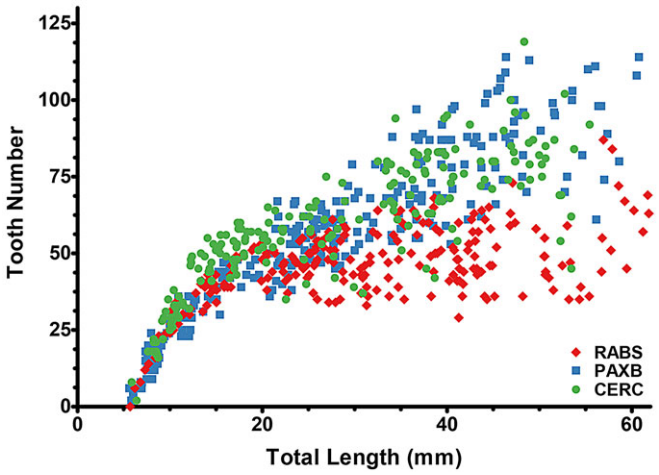

Fig. 2. In both freshwater populations, evolved tooth gain manifests late in development. Developmental timecourse of total ventral pharyngeal tooth number in the marine (RABS) and two freshwater (PAXB and CERC) populations. Total length is used as a proxy for age. CERC tooth number diverges at ~10-15 mm [binned CERC versus PAXB, and CERC versus RABS: $P<0.01$; PAXB versus RABS: not significant (ns); one-way ANOVA using a Tukey-Kramer post-hoc test], whereas PAXB tooth number diverges at ~20-25 mm (PAXB versus RABS, and CERC versus RABS: $P<0.01$; PAXB versus CERC: ns). Previously published points for RABS and PAXB are shown in gray in supplementary material Fig. S3.

(Fig. 1E,F). Thus, both derived freshwater populations have convergently evolved increased ventral pharyngeal tooth number, increased tooth plate area and decreased intertooth spacing.

Evolved tooth gain manifests late in development

Previous work showed that, compared with marine tooth number, the increased tooth number in PAXB fish manifests late in development (Cleves et al., 2014). We hypothesized that the independently derived freshwater population CERC also gains an increase in tooth number late in development. In support of this hypothesis, in sets of laboratory-reared developmental timecourse fish, both freshwater populations had similar tooth numbers to marine fish early in development. Later in development, however, CERC diverges earliest by ~10-15 mm, whereas PAXB diverges slightly later than CERC at ~20-25 mm (Fig. 2). Post 25 mm, both freshwater populations continue to increase total tooth number while marine tooth number plateaus.

As fish replace their teeth continuously, the differences in tooth number could result from an increase in new tooth formation and/or from a difference in tooth shedding dynamics. We hypothesized that the increased tooth number in freshwater fish results from a developmentally late increase in the tooth replacement rate, with more newly forming replacement teeth retained on the tooth plate. This model predicts that tooth germ number would differ at late but not early developmental stages. To test this hypothesis, we cut serial sections across different time points of each population to compare developing tooth germs over time. Similar to other vertebrates, stickleback teeth form at the interface of the epithelium and mesenchyme following stereotypic tooth development stages (these stages are reviewed by Thesleff, 2003) (Fig. 3A). At 15 mm and 25 mm, the populations do not have significantly different germ numbers, but by 25 mm both freshwater populations are trending towards having more developing germs. By 40 mm, both freshwater populations had significantly more tooth germs than marine fish, showing that both high-toothed freshwater populations form more teeth late in development and that the change in tooth number cannot only be attributed to differential tooth loss rates (Fig. 3B).

During the development of teeth and other epithelial appendages that develop from placodes, lateral signals from placodal cells inhibit interplacodal cells from adopting placode fates (Chuong et al., 2013; Jung et al., 1998; Mou et al., 2011; Noramly and Morgan, 1998). We hypothesized that the derived increase in tooth number in freshwater fish might result from smaller tooth germs, which may generate a reduced lateral inhibition signal, resulting in smaller intertooth spaces and more teeth. To test this, we measured tooth germ size by quantifying the area of individual developing tooth germs at early to mid-bell stage between populations at three time points: 15, 25 and 40 mm. Contrary to this hypothesis, tooth germ area was not significantly different between populations

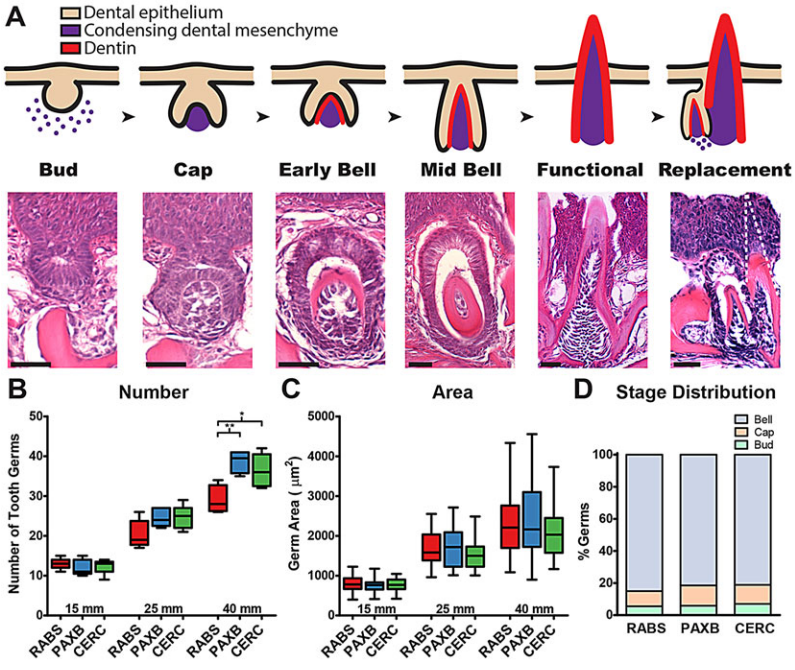

Fig. 3. Tooth germ number, but not area, differs between marine and freshwater fish late in development. (A) Stereotypic stages of tooth development from initial budding to tooth replacement, with schematic representations above and 6 μm stickleback sections beneath. Scale bars: 25 μm. The white dotted line outlines the adult tooth in an adjacent section. (B) Number of developing tooth germs from serially sectioned animals at 15, 25 and 40 mm across populations. $n=5$ for each population, except $n=6$ for 25 mm RABS and $n=4$ for 40 mm RABS and PAXB. (C) Tooth germ area at early to mid-bell stage. 15 mm: $n=37$ RABS, $n=23$ PAXB, $n=28$ CERC; 25 mm: $n=33$ RABS, $n=36$ PAXB, $n=44$ CERC; 40 mm: $n=70$ RABS, $n=85$ PAXB, $n=72$ CERC. No pairwise comparisons are significant. Average germ size for each animal between populations was also not significant. (D) Distribution of tooth germs by developmental stage from post tooth number divergence. Pooled 25 and 40 mm; $n=200$ RABS, $n=254$ PAXB, $n=244$ CERC. $^{**}P<0.01$, $^{*}P<0.05$ (one-way ANOVA using a Tukey-Kramer post-hoc test).

Development (2015) 142, 2442-2451 doi:10.1242/dev.124248

at each time point, although CERC trended towards exhibiting smaller germ size (Fig. 3C). Alternatively, the increase in tooth number could result from an additional wave of late-forming primary teeth, which would predict a shift towards younger germ stages in the freshwater populations. However, comparing the distribution of germ stages in all three populations revealed no significant differences (Fig. 3D), arguing against a model in which an additional single wave of teeth is added late, but instead suggesting differential replacement dynamics.

We further tested the hypothesis that tooth size might differ between populations, and that smaller teeth in freshwater fish might result in a smaller zone of lateral inhibition (Osborn, 1971, 1978), by comparing tooth size from serial sections of erupted adult functional teeth. We found the adult teeth in both freshwater populations to be significantly narrower, but not shorter, than marine teeth (supplementary material Fig. S4). In addition to possibly creating a smaller zone of lateral inhibition, decreased tooth width might indicate reduced time retained on the tooth plate in freshwater fish, further suggesting a difference in tooth cycling dynamics.

Increased rate of new tooth formation late in development underlies evolved tooth gain

To further test the hypothesis that increased tooth number in freshwater fish results from increased replacement rates, we quantified the rate of tooth replacement in adult fish using a pulse-chase method with vital dyes to mark new tooth formation. By pulsing first with Alizarin, waiting 2 weeks, then chasing with Calcein, ossifying teeth are marked at two points in time with red and green fluorescence, respectively, allowing the visualization of new teeth that formed between the two dye soaks (Calcein-positive, Alizarin-negative teeth; Fig. 4A). Using this method, we found that both freshwater populations have an increased number of new teeth compared with marine fish (Fig. 4B). To account for the total difference in tooth number between populations, we divided the number of new teeth by the total tooth number to quantify a normalized rate of tooth gain. Both freshwater populations have a similarly increased normalized rate of tooth gain as compared with marine fish, both in adults and in ∼20 mm juveniles (Fig. 4C; supplementary material Fig. S5).

As tooth loss rates during replacement could also affect tooth number, we tested the hypothesis that freshwater fish also have differential tooth shedding rates. Because we quantified the number of teeth observed in the developmental timecourses, as well as the new tooth gain rates quantified by pulse-chase experiments at both early and late stages, the number of teeth shed in each population could be inferred. We found that tooth shedding rates also differ between marine and freshwater populations (Table 1). These data show that two freshwater populations not only gain teeth late at an increased rate, but also shed teeth at a different rate, suggesting that the entire tooth replacement program has been sped up.

Localization of new teeth varies between freshwater populations

To examine whether marine and freshwater populations add new teeth in similar spatial patterns, we marked the position of newly formed teeth in adults. Surprisingly, we found that adult PAXB fish preferentially form new teeth on the medial edge or off the tooth plate medially, whereas CERC fish and marine RABS fish form most new teeth on the tooth plate, without an apparent medial bias (Fig. 5A). Comparing the number of new teeth off the tooth plate medially, PAXB is significantly different to CERC (Fig. 5B). However, the number of new teeth on the tooth plate is not significantly different between the two freshwater populations (Fig. 5C). Comparing total numbers of teeth on and off the tooth plate for all three populations (supplementary material Table S1) shows that although PAXB makes more new teeth off the tooth plate than either CERC or RABS, the number of total teeth on the tooth plate is still significantly larger in PAXB compared with CERC and RABS. Therefore, distinct developmental mechanisms in the two high-toothed freshwater populations result in different timing of tooth number divergence and different spatial patterns of new tooth formation, supporting a different mechanism of evolved tooth gain.

Unique genetic basis of evolved tooth gain

To begin to understand the genetic basis of evolved tooth gain in the CERC population, we performed genome-wide linkage mapping of tooth patterning traits. Previous work on a large PAXB×marine F2 cross identified five genomic regions controlling evolved tooth gain in PAXB fish (Miller et al., 2014). We generated a CERC×marine F2 cross and determined genome-wide genotypes using Genotyping-by-Sequencing (GBS)

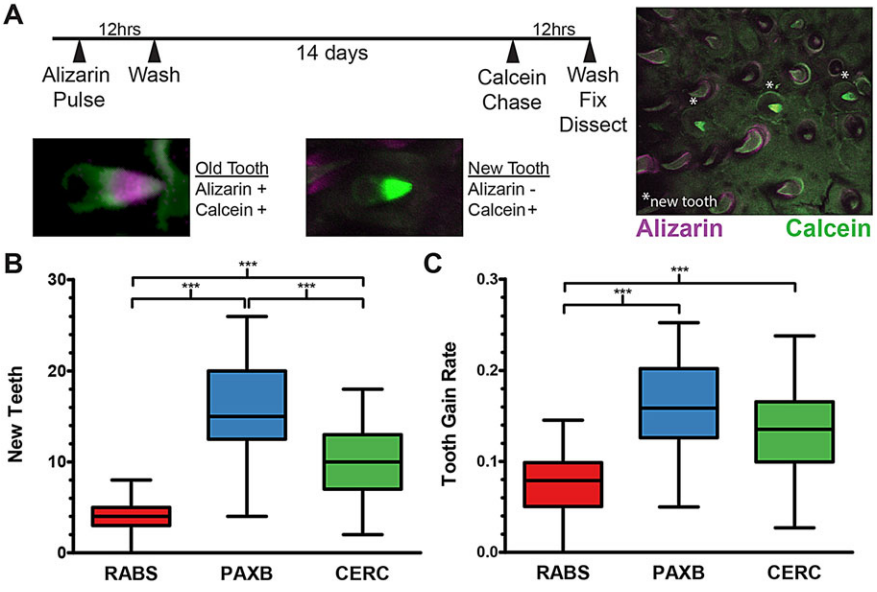

Fig. 4. Pulse-chase reveals that both freshwater populations have elevated rates of new tooth formation. (A) Schematic of pulse-chase method using Alizarin and Calcein to mark developing teeth in living fish. Examples of a field of teeth (right) and individually classified teeth (beneath) are included. Alizarin is false colored magenta. White asterisks denote new teeth. (B) Number of new teeth. (C) Normalized rates of tooth gain (new teeth divided by total teeth). ***P<0.001 (one-way ANOVA using a Tukey-Kramer post-hoc test). (B,C) Sample size: n=33 RABS, n=25 PAXB, n=22 CERC.

Development (2015) 142, 2442-2451 doi:10.1242/dev.124248

Table 1. Tooth cycling dynamics

Trait	Population		
	RABS	PAXB	CERC
20 mm average tooth number (~2 months)	51.8	54.8	55.6
40 mm average tooth number (~6 months)	54.4	98.2	75.4
Average new teeth per 2 weeks	3.8	16.2	10.2
Expected gain in tooth number	30.5	129.3	81.8
Actual gain in tooth number	2.6	43.4	19.8
Teeth shed (expected minus actual)	28	85.9	62.1
Inferred teeth shed per 2 weeks	3.5	10.7	7.8
Net gain in tooth number per 2 weeks	0.3	5.4	2.6
Tooth gain rate (%)	7.3	16.4	13.9
Inferred tooth shedding rate (%)	6.4	10.9	10.3
Net gain rate (%)	0.8	5.5	3.6

Average 20 mm tooth number sample size: RABS, *n*=31; PAXB, *n*=24; CERC, *n*=30. Average 40 mm tooth number sample size: RABS, *n*=33; PAXB, *n*=25; CERC, *n*=22 (see Fig. 1). Average new teeth per 2 weeks is derived from the pulse-chase (see Fig. 4). Expected gain in tooth number is the average new teeth per 2 weeks multiplied by 8 to represent the 4 month period. Actual gain in tooth number is the 20 mm tooth number minus the 40 mm tooth number. Net gain in tooth number per 2 weeks is the average new teeth per 2 weeks minus the teeth shed per 2 weeks. All rates are new teeth/teeth shed/net gain divided by the 40 mm tooth number to correct for overall tooth number differences.

(Elshire et al., 2011; Glazer et al., 2015) to test whether the convergently evolved tooth gain in freshwater fish (PAXB and CERC) occurs through a similar genetic architecture. Since, during development, CERC fish gain teeth earlier than PAXB fish (Fig. 2) and form new teeth in adults in a different spatial pattern (Fig. 5), we hypothesized that the genetic basis controlling evolved tooth gain would differ in at least some respects between the two high-toothed freshwater populations.

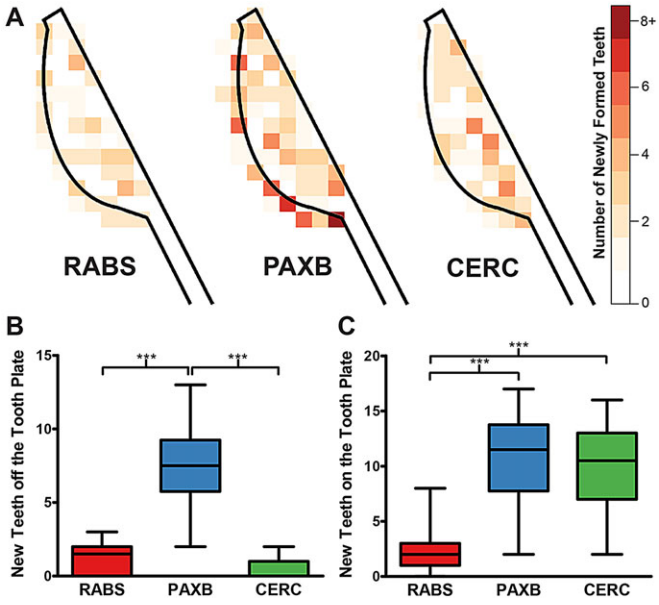

Fig. 5. Distinct spatial patterns of new teeth in two high-toothed freshwater populations. (A) Heat maps of the spatial location of newly formed teeth on an idealized tooth plate by population. (B) New teeth off the tooth plate. (C) New teeth on the tooth plate. ***P<0.001 (one-way ANOVA using a Tukey-Kramer post-hoc test). (A-C) Sample size: n=14 for each population (see supplementary material Table S1).

Supporting this hypothesis, we identified six genomic regions (quantitative trait loci, or QTL) controlling ventral pharyngeal tooth patterning in the CERC cross (Fig. 6; supplementary material Fig. S7, Tables S2 and S3), including five tooth number QTL, two intertooth spacing QTL, and no area QTL. One tooth number and one spacing QTL map to the same region on chromosome 18. In these CERC×marine F2 fish, as was found for a PAXB×marine F2 cross (Cleves et al., 2014), tooth number is highly correlated with tooth plate area and intertooth spacing, yet tooth plate area and intertooth spacing were not significantly correlated with each other (supplementary material Fig. S6), further supporting the idea that tooth plate area and intertooth spacing are genetically separable. We also detected two DTP1 tooth number QTL and a single DTP2 tooth number QTL (supplementary material Fig. S8, Tables S2 and S3). Of all identified CERC cross QTL, only one QTL on chromosome 21 overlaps the QTL previously identified in the PAXB cross (Miller et al., 2014; Cleves et al., 2014) (Fig. 6). However, the peak marker for the PAXB QTL is not in the CERC interval, suggesting that these might be two distinct loci.

The QTL on chromosome 21 has the largest effect on tooth number in each cross, and the PAXB QTL has recently been associated with *cis*-regulatory changes in an excellent candidate gene, *Bmp6* (Cleves et al., 2014). Candidate genes within the CERC cross QTL include another BMP pathway member, *Bmp7a*, and a downstream effector of BMP signaling, *Msxe*, both located on chromosome 17. The mammalian homologs of *Bmp7a* and *Msxe* cause tooth agenesis when deleted in mice (Satokata and Maas, 1994; Zouvelou et al., 2009). Another candidate gene, *Pitx2*, maps near the peak marker of the chromosome 4 QTL and is expressed early in the tooth field and later in the epithelium of both primary and replacement teeth in other fish (Smith et al., 2009b).

Overall, the largely non-overlapping sets of genomic regions controlling dental patterning in PAXB and CERC suggest that the convergent evolution of tooth gain in these two freshwater populations is controlled by largely distinct genetic mechanisms.

DISCUSSION

Evolved tooth gain occurs through a developmentally late increased rate of new tooth formation in two independently derived freshwater populations

Here we identify a second derived freshwater stickleback population (Cerrito Creek, CERC) that has evolved more teeth than ancestral marine fish. This increase in tooth number, like the increased tooth number in the Canadian Paxton benthic (PAXB) population (Cleves et al., 2014), occurs at least in part through an expansion of the tooth plate area (tooth field) as well as a decrease in intertooth spacing. In both freshwater populations, the increased tooth number occurs late in development, is associated with an increased number of tooth germs in juveniles and adults, and an increased rate of new tooth formation in adults. Thus, evolved differences have resulted in the convergent evolution of changes in dental patterning in the two independently derived freshwater populations. In freshwater×marine F2 crosses from both freshwater populations, tooth number is highly correlated with tooth plate area (field size) and intertooth spacing, whereas tooth plate area and intertooth spacing are not correlated. This lack of correlation between tooth plate area and intertooth spacing suggests separable mechanisms for specifying the area of the tooth plate and the spacing of the teeth, which is supported by findings in mice that tooth field size is specified by *Osr2* without any reported tooth spacing phenotype (Zhang et al., 2009).

Both freshwater populations have an increased number of teeth through an increase in tooth germ number, ruling out the possibility

Development (2015) 142, 2442-2451 doi:10.1242/dev.124248

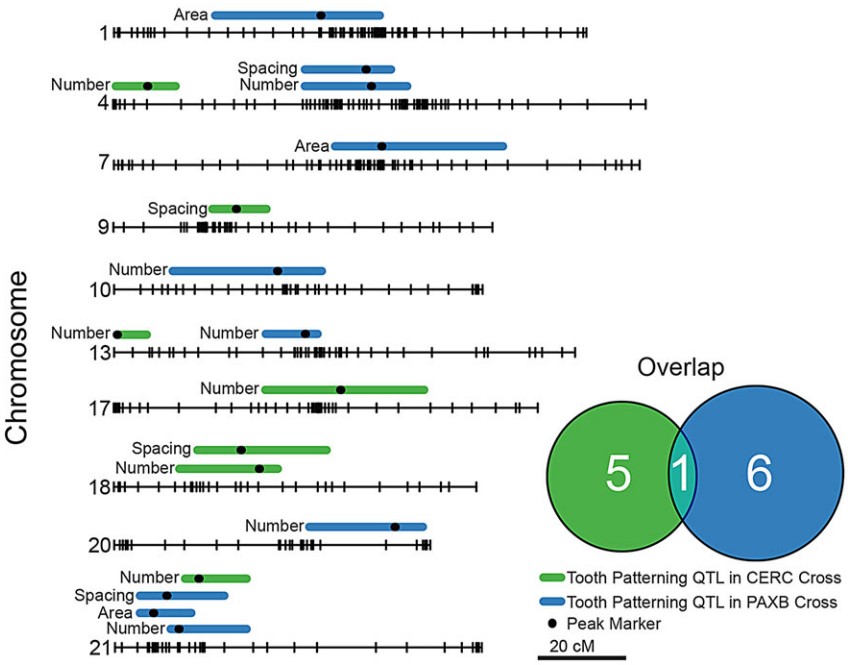

Fig. 6. Largely distinct genetic bases underlie evolved tooth gain in two high-toothed freshwater populations. Summary of identified tooth patterning QTL from a CERC×marine F2 cross (this study) compared with a PAXB×marine F2 cross (Cleves et al., 2014; Miller et al., 2014). Each black horizontal line represents a chromosome and each vertical line is a genetic marker. Each colored bar represents the 1.5 LOD interval with the black dot denoting the peak marker. The overlap between CERC and PAXB QTL is depicted in the Venn diagram.

that the differences in adult tooth number arise solely through differential shedding dynamics. Despite having more developing tooth germs, the germs are not smaller in area in the high-toothed freshwater populations, arguing against an increase in tooth number via reduced lateral inhibition signals due to a smaller tooth germ. Unlike in cichlids, where developing tooth germ size has been shown to correlate with intertooth spacing (Fraser et al., 2008), we found no significant difference in germ sizes between populations despite different intertooth spacing. However, this cichlid study measured spacing of the first few *Shh*-expressing germs in the oral jaws of wholemount embryos, whereas we measured pharyngeal tooth germ size in histological sections and pharyngeal tooth spacing in juveniles and adults by ossified tooth pattern. Nevertheless, both freshwater populations have adult teeth that are narrower than marine teeth, which could result in a reduced zone of inhibition from adult teeth, perhaps consistent with the observed activation of the tooth replacement process in alligators upon adult tooth removal (Wu et al., 2013). The increased number of tooth germs combined with the increased new tooth formation rates in adults support a model (Fig. 7) whereby the tooth replacement program has been sped up in the two independently derived freshwater populations, and this increased replacement rate underlies evolved tooth gain.

Most polyphyodonts, unlike diphyodont humans, have replacement teeth adjacent to the functional teeth they replace and that do not physically dislodge the older primary tooth. In basal polyphyodont vertebrates, like sharks, tooth replacement occurs in tooth families, where discrete tooth positions contain a developmentally staggered series of replacement teeth (Reif, 1984; Smith et al., 2009a). Although a few fish species, such as zebrafish, medaka and gobies, have been reported to have tooth families (Abduweli et al., 2014; Huysseune, 2006; Huysseune et al., 1998; Moriyama et al., 2010; Van der heyden and Huysseune, 2000), most teleosts lack obvious multigerm tooth families, instead appearing to replace teeth on an individual basis – as has been termed one-for-one replacement, such as in rainbow trout, Lake Malawi cichlids, Mexican tetra, among others (Atukorala and Franz-Odendaal, 2014; Bemis et al., 2005; Fraser et al., 2006, 2013; Kerr, 1960; Motta, 1984; Wakita et al., 1977). In both cases, replacement teeth can be present on the tooth plate before the previous tooth is shed and can contribute to the functional dentition. Recently, Tucker and Fraser (2014) proposed that dental diversity could arise in a system of continuous tooth replacement by shifting the replacement program to produce an adaptive advantage. Supporting that proposal, here we find that the rate of new tooth formation is significantly higher in both freshwater populations than in their marine counterpart, resulting in increased tooth number late in development. Although it is formally possible that newly formed teeth in adults could be late-forming primary teeth, we interpret the majority of late-forming new teeth to be replacement teeth based on their frequent proximity to large old teeth or craters of recently shed teeth.

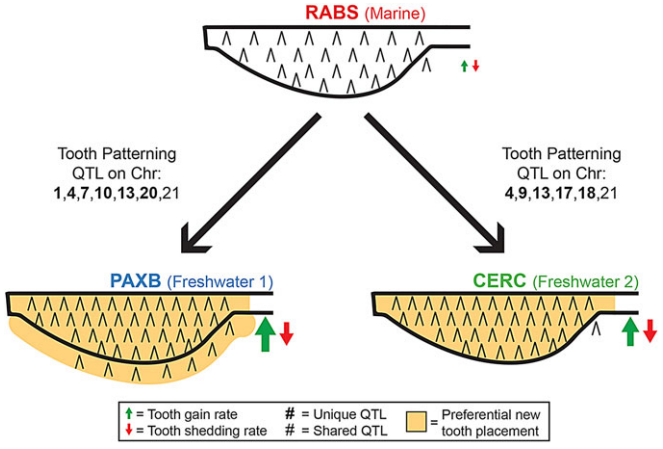

Fig. 7. Model for convergent evolution of tooth gain in two freshwater populations. Evolved tooth gain in two independently derived freshwater stickleback populations (PAXB and CERC) occurs through increased tooth replacement rates and multiple distinct mechanisms, such as the different spatial placement of new teeth and a largely non-overlapping genetic basis. Shading indicates preferred regions of new tooth placement. Green and red arrow size is representative of the relative levels of tooth gain and shedding rates between populations. The PAXB and CERC QTL on chromosome 21 overlap, whereas the PAXB and CERC QTL on chromosome 4 do not.

Development (2015) 142, 2442-2451 doi:10.1242/dev.124248

Distinct developmental bases underlie convergently evolved tooth gain

A second main finding of this study is that despite converging on the same adult phenotype of increased tooth number through an increased rate of new tooth formation late in development, two independently derived freshwater stickleback populations have increased tooth number via distinct developmental mechanisms, with different timing of divergence and spatially different patterns of new tooth formation.

First, comparing the developmental timecourse trajectories shows that although both high-toothed freshwater populations increase tooth number late, but not early, the increase in tooth number appears to occur earlier in development in CERC (10-15 mm) than in PAXB (20-25 mm) fish. Second, although both freshwater populations add new teeth on and off the tooth plate late, many new teeth in PAXB fish form medially off the edge of the tooth plate whereas most new teeth in CERC fish form on the tooth plate. We hypothesize that newly formed teeth off the tooth plate in the PAXB population function to expand the area of the tooth plate, which is larger in PAXB than in marine or CERC fish, which add fewer teeth off the tooth plate. The different spatial patterns of new tooth formation could result from different programs of primary tooth placement (e.g. the PAXB population but not the CERC population might add a medial row of primary teeth late during development). Arguing against this model is the lack of any difference in the distribution of germ sizes in the three populations, instead suggesting a model of altered replacement dynamics. Together, our findings suggest that some features of tooth development appear more constrained (e.g. the size of early tooth germs, early larval tooth number) than others (e.g. the rate and the spatial pattern of new tooth formation in adults).

Distinct genetic bases underlie convergently evolved tooth gain

A third main finding of this study is that two independently derived freshwater stickleback populations have evolved more teeth via largely distinct genetic mechanisms. These different genetic mechanisms are perhaps surprising as previous work in sticklebacks has shown that the genetic basis of many derived traits, including loss of armored plates (Colosimo et al., 2005), reduced pelvis (Chan et al., 2010; Shapiro et al., 2004), reduced pigmentation (Miller et al., 2007), reduced gill raker number (Glazer et al., 2014) and increased branchial bone length (Erickson et al., 2014), have similar genetic bases in multiple freshwater populations. Largely distinct genetic bases of evolved tooth gain could result from differences in available genetic variation in the two freshwater populations, pleiotropy of tooth patterning with other traits that differ in the two freshwater populations, and/or different diets in the two freshwater niches being better processed by differently patterned pharyngeal jaws. Additionally, serially repeated structures, such as teeth, that might be functionally redundant could be less constrained genetically to evolve changes than previously studied traits.

Despite the polygenic nature of evolved tooth gain, our genome-wide linkage mapping using GBS identified eight unlinked genomic regions that control pharyngeal tooth patterning in the high-toothed CERC freshwater population. For ventral pharyngeal tooth patterning, we identified five tooth number QTL and two intertooth spacing QTL. Only one genomic region on chromosome 21 is shared between the two high-toothed freshwater populations. This QTL also has the largest effect on tooth number in each cross. However, the peak marker for the PAXB QTL is not included in the CERC QTL interval, suggesting the two chromosome 21 QTL might have distinct genetic bases as well. Thus, it appears that similar changes in morphology (more teeth) have evolved via largely distinct developmental genetic mechanisms, as has been found in sex comb patterning (Tanaka et al., 2009) and wing size (Zwaan et al., 2000) in *Drosophila*, trunk elongation in salamanders (Parra-Olea and Wake, 2001) and eye loss in different cavefish species (Stemmer et al., 2015).

A largely distinct genetic basis of evolved tooth gain was also detected for dorsal pharyngeal tooth number. We detected two genomic regions controlling tooth number on DTP1 and only a single genomic region controlling tooth number on DTP2, although DTP2 tooth number was not significantly different between marine and freshwater fish. However, CERC freshwater fish trended towards having more DTP2 teeth than marine fish (P=0.09). Also, detecting QTL does not require phenotypic differences between populations, as different populations can have the same quantitative phenotype due to different combinations of positive and negative allelic effects. None of the three detected QTL from the CERC cross had any significant effect on dorsal pharyngeal tooth number in the PAXB cross.

The genetic basis of dorsal and ventral tooth patterning is also largely modular, as two of the three dorsal tooth number QTL have no significant effects on ventral pharyngeal tooth number in either the CERC or PAXB cross. Similar modularity for evolved differences in dorsal and ventral pharyngeal tooth number was also previously reported in sticklebacks (Miller et al., 2014). Thus, tooth number is highly modular at a genetic level, with different loci controlling dorsal and ventral pharyngeal tooth number in both crosses. Modularity of the dentition can be seen across vertebrate lineages, as in Cypriniformes such as zebrafish, which have uncoupled tooth patterning in the dorsal and ventral pharynx, completely losing dorsal pharyngeal teeth while retaining ventral pharyngeal teeth (Stock, 2007). In zebrafish, the addition of a single transgene driving ubiquitous *Ectodysplasin* is sufficient to drive the formation of ancestrally lost dorsal pharyngeal teeth (Aigler et al., 2014). In mice, strong support for genetic modularity of the dentition has also been found. *Dlx1/Dlx2* double mutants lack dorsal (maxillary) molars but other teeth are unaffected (Qiu et al., 1997; Thomas et al., 1997), and activin βA mutants lack incisors and ventral (mandibular) molars whereas dorsal molars are unaffected (Ferguson et al., 1998). Similarly, in $Gli2^{-/-}; Gli3^{+/-}$ mice dorsal (maxillary) incisors are more severely affected than ventral (mandibular) incisors (Hardcastle et al., 1998).

Several outstanding candidate genes lie within the QTL detected in the CERC×marine cross. *Pitx2* lies close to the peak marker on the chromosome 4 QTL and is required for tooth development in mice (Lu et al., 1999) and humans (Childers and Wright, 1986; Semina et al., 1996). *Pitx2* is also expressed in the epithelium connecting the primary tooth to the replacement tooth in some polyphyodonts (Fraser et al., 2013; Smith et al., 2009b) and has been hypothesized to be important for the tooth replacement process. Pitx homeodomain proteins have been shown to bind a mouse *Bmp4* tooth enhancer, with this binding site required for *Bmp4* enhancer activity (Jumlongras et al., 2012). *Pitx2* has also been shown to inhibit the BMP antagonists *Bmper* and *Nog* through miR200c in dental epithelium in mice (Cao et al., 2013) and to regulate the Wnt signaling pathway (Vadlamudi et al., 2005). *Msxe*, on chromosome 17, is a downstream effector of BMP signaling, and mutations in the mammalian ortholog, *Msx1*, cause tooth agenesis in mice (Satokata and Maas, 1994) and humans (Nieminen, 2009;

Development (2015) 142, 2442-2451 doi:10.1242/dev.124248

Vastardis et al., 1996). *Bmp7a*, a close paralog of *Bmp6*, also lies on chromosome 17 in a region controlling tooth number in CERC, and has been shown to promote dental ossification when added exogenously (Sloan et al., 2000) and to be required for tooth development in mice (Zouvelou et al., 2009).

Although convergent evolution of increased tooth number has occurred using largely distinct sets of genes, different components of the same genetic circuitry might be altered in the two high-toothed populations (e.g. *Bmp6*, *Bmp7*, *Msx1*). Future work will test the hypothesis that the convergently evolved tooth gain presented here occurs through modulating different components of the BMP signaling pathway to altertooth replacement stem cell dynamics.

MATERIALS AND METHODS

Stickleback husbandry

Fish were raised at 18°C in 110 l aquaria in a common brackish salinity (3.5 g/l Instant Ocean salt, 0.217 ml/l 10% sodium bicarbonate). Fish were fed a common diet of live *Artemia* as young fry, live *Artemia* and frozen *Daphnia* as juveniles and frozen bloodworms and *Mysis* shrimp as adults. All experiments were performed with approval of the Institutional Animal Care and Use Committees of the University of California-Berkeley (protocol # R330).

Skeletal staining and visualization

Laboratory-reared fish were fixed in 10% neutral buffered formalin (NBF) overnight at 4°C, washed in water, and stained with 0.008% (>20 mm) or 0.004% (<20 mm) Alizarin Red S in 1% KOH for 24 h. Fish were rinsed again in water and cleared in 50% glycerol and 0.25% KOH. Branchial skeletons were dissected and mounted as described (Miller et al., 2014). Tooth number was scored on a Leica DM2500 under a TX2 filter (with PAXB adult tooth number in Fig. 1D from Cleves et al., 2014). Area and spacing measurements were performed as described (Cleves et al., 2014). Phenotype quantifications are left and right combined for tooth number and the average of left and right for area and spacing. Representative tooth plate *z*-stack projections were collected on a Zeiss 700 confocal microscope. See supplementary material Methods for details of statistical analysis and phenotype corrections.

Live vital dye bone staining was adapted from Kimmel et al. (2010) and performed by pulsing fish with 100 µg/ml Alizarin Red S buffered with 1 mM HEPES (pH 7.0) in tank water for 12 h in the dark. After replacing Alizarin with clean tank water, fish were returned to tanks for 14 days, and then chased with 50 µg/ml Calcein buffered with 1 mM sodium phosphate (pH 8.0) in tank water for 12 h in the dark. After replacing Calcein with clean tank water, fish were fixed overnight in 10% NBF at 4°C and stored in 100% ethanol in the dark at 4°C until dissection and clearing as described above. Preparations were phenotyped on a Leica DM2500 using GFP and TX2 filter sets. Two month pulse-chase was performed as described above with 50 µg/ml Alizarin Red S, 25 µg/ml Calcein, and 6 h dye soaks.

Histology

Fish were fixed in 10% NBF overnight at 4°C, dissected, and decalcified as required in Humason's formic acid A (Humason, 1962). Tissue was processed pre-embedding as described (Schulte-Merker, 2002) using Histoclear (National Diagnostics) in place of xylene. Tissue was transferred, oriented, and embedded in Paraplast (Fisher) using plastic molds. Serial sections were collected using a Microm HM340E (Thermo Scientific). Sections were baked on slides overnight at 50°C then stained with Hematoxylin and Eosin using a Varistain Gemini ES automated stainer (Thermo Scientific) and cover-slipped with Permount (Fisher) mounting media. Stained sections were imaged under brightfield optics on a Leica DM2500. See supplementary material Methods for details of tooth germ quantification.

Heat maps

Small, newly erupted teeth that (1) were in a deeper focal plane than adult teeth, (2) had a translucent enameloid cap and (3) had a clearly visible dental pulp (i.e. a cone within a cone visible with DIC optics) were counted and their position marked on an idealized tooth plate in ImageJ (Schneider et al., 2012). A custom R script generated bins across the tooth plate and assigned a color score based on the number of teeth within each bin per population using color schemes from ColorBrewer (http://colorbrewer2.org). Individual teeth were scored as off the tooth plate if >90% of the base of the tooth failed to overlap with the underlying tooth plate.

Preparation of Genotyping-by-Sequencing (GBS) libraries

DNA was isolated by phenol-chloroform extraction or with a DNeasy 96 Blood and Tissue Kit (Qiagen). Genomic DNA concentration was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and by Quant-iT PicoGreen Assay (Invitrogen). GBS Illumina sequencing libraries were constructed as described (Elshire et al., 2011; Glazer et al., 2015). Briefly, genomic DNA was digested with *Ape*KI, ligated to Y-shaped adapters, and PCR amplified. Libraries were analyzed on an Agilent 2100 Bioanalyzer High-Sensitivity Chip for quality control and sequenced with 100 base pair, paired-end sequencing on an Illumina HiSeq 2000 sequencer. 174 F2 fish were barcoded into a single lane of Illumina sequencing together with 190 samples not used in this study. See supplementary material Methods for detail on read processing.

Quantitative trait loci (QTL) mapping

QTL mapping was initially performed with *stepwiseqtl* using Haley-Knott regressions in R/qtl (Broman and Sen, 2009). *Scantwo* penalty scores for tooth number, area and spacing were 3.9, determined via 1000 permutations at α=0.05. The top three models for each phenotype were identified and further explored using *scanone* and *addqtl* adjusting for QTL found using *stepwise* scanning. Genome-wide LOD (logarithm of the odds) significance thresholds for each phenotype were determined with *scanone* via 10,000 permutations at α=0.05 resulting in a median threshold of 3.9. QTL peaks, LOD scores and percent variance explained were calculated with *refineqtl* and *fitqtl*. PAXB×marine QTL were previously identified and included for comparison (Miller et al., 2014; Cleves et al., 2014). Genotypes, phenotypes and map used are listed in supplementary material Table S4.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

N.A.E., A.M.G., P.A.C., C.T.M. conceived and designed the experiments; N.A.E., A.M.G., N.N.D., P.A.C. and R.M.A. performed the experiments; N.A.E., A.M.G., N.N.D., P.A.C. and C.T.M. analyzed the data; N.A.E. and C.T.M. wrote the manuscript, with input from all authors.

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Supplementary material

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