Acanthodian dental development and the origin of gnathostome dentitions

Martin Rücklin^{1,2*}, Benedict King^{1,3}, John A. Cunningham², Zerina Johanson⁴, Federica Marone⁵, Philip C. J. Donoghue^{2*}

¹Naturalis Biodiversity Center, Postbus 9517, 2300, RA, Leiden, The Netherlands.

²School of Earth Sciences, University of Bristol, Life Sciences Building, Tyndall Avenue, Bristol BS8 1TQ, UK.

³Department of Linguistic and Cultural Evolution, Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, 04103 Leipzig, Germany.

⁴Department of Earth Sciences, Natural History Museum, Cromwell Road, South Kensington, London SW7 5BD, UK.

⁵Swiss Light Source, Paul Scherrer Institut, CH-5232 Villigen, Switzerland.

*authors for correspondence: martin.rucklin@naturalis.nl; phil.donoghue@bristol.ac.uk

ORCID: MR, 0000-0002-7254-837X; BK, 0000-0002-9489-8274; JC, 0000-0002-2870-1832; ZJ, 0000-0002-844-6776; FM, 0000-0002-3467-8763; PCJD, 0000-0003-3116-7463

Chondrichthyan dentitions are conventionally interpreted to reflect the ancestral gnathostome condition but interpretations of osteichthyan dental evolution in this light have proven unsuccessful, perhaps because chondrichthyan dentitions are equally specialized, or else independently evolved. Ischnacanthid acanthodians are stem-Chondrichthyes; as phylogenetic intermediates of osteichthyans and crown-chondrichthyans, the nature of their enigmatic dentition may inform homology and the ancestral gnathostome condition. Here we show that ischnacanthid marginal dentitions were statodont, composed of multicuspidate teeth added in distally-diverging rows and through proximal superpositional replacement, while their symphyseal tooth whorls are comparable to chondrichthyan and osteichthyan counterparts. Ancestral state estimation indicates the presence of oral tubercles on the jaws of the gnathostome crown-ancestor; tooth whorls and/or tooth rows evolved independently in placoderms, osteichthyans, ischnacanthids, other acanthodians and crown-chondrichthyans. Crown-chondrichthyan dentitions are derived relative to the gnathostome crown-ancestor which possessed a simple dentition and lacked a permanent dental lamina which evolved independently in Chondrichthyes and Osteichthyes.

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40 The dentitions of most modern chondrichthyans (Elasmobranchii: sharks, rays) are organized
41 into files of replacement teeth arrayed side-by-side along the jaw. The simplicity of this
42 conveyor-belt system has long been interpreted to reflect the ancestral condition for the
43 dentitions of jawed vertebrates and theories of dental developmental evolution have
44 invariably attempted to rationalize the dentitions of osteichthyans on such a model^{1,2}. Critical
45 to this model is the presence of a permanent dental lamina along the jaw which is
46 responsible for tooth development and replacement, which can be observed in living jawed
47 vertebrates³⁻⁶ and inferred from evidence of comparable tooth replacement patterns in extinct
48 relatives. However, the first fossil evidence of crown-chondrichthyan divergence is from the
49 end-Middle Devonian, later than the first crown-osteichthyans which are late Silurian⁷.
50 Furthermore, recent fossil discoveries have decisively overturned the view that
51 chondrichthyan morphology is representative of the ancestral gnathostome condition^{8,9}. The
52 extinct acanthodians are recognized as a paraphyletic lineage of stem chondrichthyans⁸ and,
53 as phylogenetic intermediates of the crown-chondrichthyans and osteichthyans, they have
54 the potential to inform the nature of the dentition in the ancestral crown-gnathostome and,
55 indeed, address the question of whether it possessed a dentition at all¹⁰. Acanthodians
56 exhibit variation in their dentitions, from acanthodids and diplacanthids which lack teeth
57 entirely, climatiids whose dentition was comprised wholly of statodont tooth whorls, to
58 ischnacanthids that possessed symphyseal tooth whorls, a marginal dentition^{11,12} and tooth-
59 like scales around the jaw margins^{13,14}. Here we focus on the nature of the dentition in
60 ischnacanthids which manifest the diversity of dentitions seen in dentate acanthodians.

61

62 The development of the marginal dentition of ischnacanthids has been interpreted based on
63 its external morphology^{12,15}, broken surfaces¹⁶ and a few traditional destructive studies
64 e.g.^{14,17}. These data have led to divergent interpretations of the development of the marginal
65 dentition (and therefore its homologies). It has been argued that acanthodian marginal
66 dentitions were shed and replaced *in toto*¹⁸ or that they grew episodically with the teeth
67 developing as continuous projections of the underlying bony plate¹⁹. Confirming either of
68 these hypotheses would reveal tooth development mechanisms without parallel in other
69 gnathostomes, thereby expanding our knowledge of the disparity of early dentitions. A third
70 hypothesis is that each of the cusps (regardless of size) represent distinct teeth that were
71 added sequentially, extending the tooth row distally²⁰. This invites comparisons with
72 arthrodire placoderms (and to a lesser extent osteichthyans), raising the possibility that such
73 dentitions are ancestral for gnathostomes as a whole, or that similar dentitions appeared
74 multiple times through convergent evolution. To discriminate among these interpretations we
75 used SRXTM²¹ to study the structure and infer the development of the marginal and

symphyseal dentitions of ischnacanthid acanthodians based on exceptionally-preserved material from the Lower Devonian (Lochkovian) of Prince of Wales Island, Arctic Canada.

Acanthodian jaws consist of paired upper palatoquadrate cartilages and paired lower Meckel's cartilages that were only rarely ossified (perichondrally) and therefore, preserved. The marginal dentition of ischnacanthids is associated with the oral side of these upper and lower jaw cartilages and comprises a more or less extensively developed ossification including oral tubercles. The tubercles are organized into two or more rows that diverge distally at about 20° within a horizontal plane, the first approximately parallel to the jaw margin and the second extending lingually in a distal direction (relative to the jaw joint, Fig. 1c, d, f). A ridge occurs between the rows of tubercles, increasing in prominence distally (Fig. 2c, d).

Tomographic data demonstrate that these oral, tooth-like tubercles developed separately from the bony base to which they are ankylosed (cf. ¹⁶). The bony base is comprised of cancellar cellular bone exhibiting frequent spheritic mineralisation (Fig. 1d,e), overlying a layer of compact lamellar bone. Each of the overlying tubercles has a prominent conical central cusp and a number of smaller accessory cusplets (Fig. 1c, d; 2c); these increase in size distally. A thin (5- 70µm) surface layer of highly attenuating hypermineralised tissue that we interpret as enameloid, extends across the large central cusp and the smaller marginal cusplets, evidencing their formation as a single morphogenetic unit (Fig. 1i), rather than as separately developing tubercles (cf. ¹²). The tubercles are otherwise composed of dentine with tubuli extending from a large central pulp-cavity to near the tubercle surface, into the hypermineralised enameloid layer (Fig. 1i). The tubercles in the marginal dentition are therefore compositionally, developmentally and topologically compatible with teeth.

The overlapping relationships of the teeth, delimited by growth arrest lines, allow the development of the dentition to be reconstructed (Figs 1d, f, 2, 3; Extended Data Fig 1). The teeth were added sequentially along a proximal to distal vector within each row, as revealed by their overlapping relationship, with each tooth added onto the distal margin of the predecessor. This proximal to distal sequence is also evidenced by the differing degree to which the pulp cavities have been infilled by centripetal layers of dentine. Teeth within the lateral row are overlapped marginally by teeth within the lingual row (Fig. 1g), indicating that the lateral teeth developed earlier and more distally. This arrangement breaks down proximally where teeth exhibit considerable wear and are replaced through superpositional apposition, though they cannot be assigned to any particular row with confidence (Figs 1f, h, 2, 3; Extended Data Fig 1). We find no evidence for tooth resorption and our data allow us to

reject hypotheses that (a) the dentigerous jawbones of ischnacanthids were episodically shed and replaced in toto¹⁸, (b) the teeth developed episodically as elaborations of the underlying bone¹⁹, and (c) each cusp and cusplet constitutes a developmental unit distinct from the principal cusp²⁰.

The structure of the tooth whorls is quite distinct, comprised of monocuspid conical teeth that project from a concave oval base and exhibit an ordered increase in height and width lingually. One row of teeth and paired marginal teeth may occur within any one tooth whorl, reducing in height laterally (Fig. 4). The teeth are distinct from the underlying bony base which is composed of a layer of cancellar bone on a thin base of compact lamellar bone (Fig. 4c). Each tooth is composed largely of dentine surrounding a central pulp cavity and a thin (10-50 µm) capping hypermineralised enameloid layer that does not extend to encompass adjacent (successional and marginal) teeth (Fig. 4b). Cancellar bone attaches each tooth to the bony base and the lingual margin of the preceding tooth. Successive teeth are distinguished by a growth arrest line indicating that the largest teeth were added last (Fig. 4b). There is no evidence of apposition of the tooth and its underlying bony base indicating that the two developed synchronously. A network of vascular canals connects the teeth and the dental pulp cavities exhibit polarised pattern of infilling, with the earliest being completely infilled (Fig. 4b).

Ischnacanthid tooth whorls are comparable to the tooth families of living chondrichthyans to which they have long been compared, but they are even more similar to the statodont tooth whorls of other acanthodian stem-chondrichthyans (e.g. *Climatius*, *Ptomacanthus*) and the symphyseal tooth whorls of stem- and early crown-osteichthyans (e.g. *Onychodus*)^{22,23} which also possess a unifying bony base²⁴ and multiple rows of cusps. However, osteichthyan tooth whorls exhibit distinct growth of the teeth and bony base²³.

The marginal dentitions of ischnacanthids find no counterpart in living chondrichthyans in terms of their association with an ossified mandibular plate, their pattern of addition along the jaw rather than across it, or their pattern of dental replacement. They may be compared to the marginal dentitions of arthrodiran placoderms²⁵ and osteichthyans (e.g. *Onychodus*, *Moythomasia*) in being arranged in marginal rows. In contrast to arthrodiran placoderms and osteichthyans in which tooth addition occurs in both a proximal and distal direction^{26,27}, the ischnacanthid marginal dentition shows only distal extension of the tooth rows. Our data evidences tooth replacement at the proximal end of the row but, unlike in osteichthyans, this occurs superpositionally and without resorption in ischnacanthids. Thus, while the tooth whorls of dentate acanthodians support inference of a permanent dental lamina, the pattern

of superpositional replacement in the dentigerous jaw bones is incompatible with tooth development within a permanent dental lamina, similarly inferred for stem-osteichthyans^{25,28}.

Inferring the nature of the ancestral crown-gnathostome dentition requires resolution of homology among diverse gnathostome dentitions, including those of ischnacanthids. This is a question not merely of structural and developmental similarity, but of phylogenetic congruence²⁹, which is complicated by the uncertainty of phylogenetic relationships among early gnathostomes and acanthodians, in particular. Accounting for this uncertainty, we estimated ancestral states for dental characteristics on the posterior distribution trees from a tip-dated Bayesian analysis of early gnathostome relationships (Figs 5, 6). This recovered strong support for the presence of oral tubercles on jaw cartilages in the ancestral crown-gnathostome (Fig. 5, pp=0.99), and homology of osteichthyan and (conventionally-defined) chondrichthyan⁸ teeth as oral tubercles (pp=0.95). Loss of oral tubercles is inferred several times in acanthodians (Fig. 5). Testing homology of arthrodiran, osteichthyan and ischnacanthid dentitions, there is evidence for the convergent evolution of marginal tooth rows (Fig. 6a) and tooth whorls among gnathostomes (Fig. 6b). The highest posterior density interval for the number of independent tooth whorl origins was 6-15, and 3-7 for marginal tooth rows. These results are robust to the phylogenetic position of 'psarolepid' osteichthyans (Extended Data Fig 2), the status of placoderms as paraphyletic or monophyletic and different divergence dating methodologies (Extended Data Fig 3).

Our results suggest that the ancestral crown-gnathostome possessed teeth. However, complex dentitions, a permanent dental lamina and coordinated tooth replacement, all evolved multiple times; teeth were also lost multiple times among acanthodians (Figs 5, 6). The similarities reported here between tooth rows in ischnacanthid dentitions and those of arthrodiran placoderms and osteichthyans, are inferred to reflect convergence rather than homology (contra ²⁵). The diversification of crown-gnathostomes is associated with an extremely rapid burst of phenotypic evolution³⁰ manifest in the diversity of early crown-gnathostome dentitions. This may go some way to explain why models of tooth replacement based on crown-chondrichthyans are such a poor explanatory model for the dentitions of crown-osteichthyans, as well as differences which at least in part inspired the hypothesis that teeth evolved independently within these and other lineages of jawed vertebrates^{10,31}.

Methods

Museum repository abbreviation: Naturhistoriska Riksmuseet, Stockholm (NRM) and National History Museum London (NHMUK).

Material: Fossil material comprises specimens of an ischnacanthid acanthodian from the Lochkovian, Early Devonian, Prince of Wales Island, Canada. Mandibles with tooth rows: NRM-PZ P. 9449: labeled model (Figs 1b-h, 2). Tooth whorls: specimen figured in Rücklin et al. (2011)³² from the same locality (NRM-PZ P. 15908, Fig. 3a-c). Ischnacanthid acanthodian jaw from the Downtonian, Upper Silurian, Baggeridge Colliery, South Staffordshire, UK (NHMUK PV P.15362, Fig. 1a)³³.

Tomography: Material from Canada was acid prepared and scanned using SRXTM²¹ at the TOMCAT (X02DA) beamline³⁴ of the Swiss Light Source (SLS), Paul-Scherer Institut, Switzerland. Using a 10x objective 1501 projections were acquired equi-angularly over 180°. Projections were post-processed and rearranged into flat- and darkfield-corrected sinograms, and reconstruction was performed on a Linux PC farm resulting in isotropic voxel dimensions of 0.74 µm. The complete jaw BMNH P. 15362 was scanned using an x-tex XTH 225ST scanner at Nikonmetronics, Tring. 3142 projections were acquired and were post-processed resulting in isotropic voxel dimensions of 100 µm. Slice data were analysed and manipulated using Avizo 8.01 (www.fei.com). Sectional images were studied and three-dimensional models of the different growth stages were derived segmenting following lines of arrested growth.

Phylogeny and ancestral state reconstruction: The phylogenetic data matrix was based on King et al.³⁰, with a revised taxon and character list incorporating new information on stem chondrichthyans^{14,35,7}, and improved sampling of sarcopterygian osteichthyans. The analysis was a tip-dated approach performed in BEAST2.5.2³⁶ with BEAGLE likelihood calculation library³⁷. Characters were partitioned according to the number of states. We applied the Mkv model³⁸, gamma distributed among-character rate variation, the sampled ancestor birth-death model³⁹ and the Lognormal relaxed clock⁴⁰. Fossil ages were assigned uniform priors across the range of uncertainty. Analyses were run for 200 million generations with 2000 trees saved. Convergence was assessed in Tracer⁴¹ and RWTY⁴². The analysis strongly supports a sarcopterygian position for the ‘psarolepid’ osteichthyans, but as previously discussed, this may be an artefact of the relatively sparse coding for the characters supporting a stem osteichthyan position for these taxa⁴³. Therefore a second analysis was performed in which they were constrained to be stem osteichthyans. We utilised a backbone constraint, so that *Ligulalepis*, *Dialipina* and *Janusiscus* were free to move into or out of the crown. To additionally assess the robustness of results to different phylogenetic and timescaling methods, an additional undated Bayesian analysis was performed in MrBayes3.2.6⁴⁴, and the post-burnin sample of trees was time-scaled using the “equal” method in the R function timePaleoPhy, package paleotree⁴⁵. All three sets of trees

(BEAST2, BEAST2 constrained and MrBayes timescaled) were used for ancestral state reconstruction.

Four characters were used for ancestral state reconstructions, three of which were essentially the same as those found in the data matrix. These were *Oral dermal tubercles borne on jaw cartilages*, *Oral dermal tubercles in patterned rows (teeth)* and *Tooth whorls*. The latter two characters were changed from the form in the phylogenetic data matrix by recoding inapplicable (-) taxa as absent (0). This prevents illogical results (in particular the reconstruction of tooth whorls as present but oral tubercles as absent, even though tooth whorls are a form of oral tubercle). A fourth character was introduced for ancestral state reconstructions to assess the homology of osteichthyan, arthrodiran and ischnacanthid tooth rows. This character was formulated as *teeth, made of dentine, in organised rows and ankylosed to dermal jaw bones*. Due to its compound formulation it was not included in the original phylogenetic data matrix, which includes each of these aspects as a separate character. Brazeau & Friedman⁴⁶ demonstrated the importance of phylogenetically constrained comparative analysis, suggesting that oral tubercles and tooth whorls are ancestral for crown-gnathostomes. Our phylogenetic analysis corroborates the ancestral condition of oral tubercles, but disagrees with the conclusion that tooth whorls are ancestral.

Ancestral state reconstructions were performed in BEAST1.10.2⁴⁷ with BEAGLE likelihood calculation library³⁷, using the post-burnin sample of trees from the three analyses detailed above. Characters were analysed with a strict clock, and a separate evolutionary rate was calculated for each of the four characters. An exponential prior with mean 0.1 was placed on the evolutionary rate. The analysis produced ancestral state reconstructions mapped onto the sample of trees⁴⁸ and a count of the number of state changes⁴⁹. The analysis was run for 10 million generations, with 1000 trees saved. We tested symmetrical and asymmetrical models of trait evolution using Bayes factors. Marginal likelihoods were calculated using the stepping-stone method⁵⁰ with 100 steps, a chain length of 100,000 per step and alpha 0.3. The Bayes factor⁵¹ support for asymmetrical models was 0.53 ("not worth more than a bare mention"), and we therefore chose the symmetrical model for interpretation. Results using the asymmetrical models are included in Extended Figure 3 for comparison.

Post-analysis processing was performed in R using the packages OutbreakTools⁵², ape⁵³ and phangorn⁵⁴. The state for each character at the crown gnathostome node in each tree of the post burn-in sample was assessed, producing posterior probabilities. We also assessed the homology of characters between osteichthyans and chondrichthyans (characters were said to be homologous if they were present at every node linking the two clades).

Transition counts are output by the BEAST analysis⁴⁹, but detailed inspection of the results reveals that some transitions are reconstructed incorrectly (for example a transition to a state on a branch leading to a taxon which lacks that state, and no reversal reconstructed on the same branch). Therefore the transition counts were also analysed in R using the ancestral state reconstruction at each node. Transitions were counted when a node had a state different to the immediately ancestral node. This provides a good estimate of the number of transitions, although it will be a slight underestimate as occasional double hits (i.e. two transitions in a single branch) will be missed.

Data availability

The data matrix is available in the Dryad data supplement. Sources for taxa and age ranges and the phylogenetic character list are available as supplementary information. Tomograms and surface files are archived in the University of Bristol Research Data Storage Facility at publication.

Code availability

Xml BEAST2 files, MrBayes nexus files, BEAST1 xml files and R scripts are available in the Dryad data supplement.

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Author contributions

M.R. and P.C.J.D. designed initial research; M.R., J.A.C., P.C.J.D. and F.M. performed scans, M.R. and J.A.C. segmented tomograms, B.K. produced the phylogenetic data matrix performed the phylogenetic analysis and ancestral state reconstruction. M.R. and P.D. drafted the manuscript to which all authors contributed.

Competing interests statement

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper. Correspondence and requests for materials should be addressed to M.R. (martin.rucklin@naturalis.nl) and P.D. (phil.donoghue@bristol.ac.uk). Reprints and permissions information is available at www.nature.com/reprints.

Figure Legends

Figure 1 | Jaw bones and marginal dentition of ischnacanthid acanthodians. Mandible of *Ischnacanthus kingi* lateral view of complete specimen in rock (**a**) NHMUK PV P.15362. Mandible of ischnacanthid acanthodian NRM-PZ P. 9449 lateral view (**b**) and dorsal view (**c**) of complete ossified bone and teeth. Detailed lateral view showing the tooth addition in the lateral row, indicated by arrows (**d**), and detail of the spheritic mineralisations (**e**). Detailed dorsal view with teeth separated by growth arrest line, indicated by arrows (**f**). Distal most tooth of the lingual row overlaps tooth within the lateral row (**g**). Overgrowth of teeth at the centre of ossification and initial sequential addition, indicated by arrow (**h**). Largest and last added medial tooth showing a hypermineralised layer, we interpret as enameloid, forming the proximal ridge and the smaller marginal cusplets and dentine infilling the pulp cavity (**i**). Scale bar in (**a**) represents 4.3 mm, 270 μm (**b, c**), 107 μm (**d**), 61 μm (**e**), 156 μm (**f**), xx μm (**g**), 21 μm (**h**) and 50 μm (**i**).

Figure 2 | Surface and reconstructed growth of marginal tooth rows on an ischnacanthid acanthodian jawbone. Jawbone NRM-PZ P. 9449 Early Devonian, Canada. Lateral view of the surface (**a**) and reconstructed addition of teeth (**b**). Occlusal view of the surface (**c**) and reconstructed addition of teeth (**c**). Colours of the nested boxes reflect the successive stages of tooth development. Scale bar represents 220 μm , prox, proximal; dist, distal; ling, lingual; lab, labial.

Figure 3 | Virtual development of teeth on an ischnacanthid acanthodian jawbone. Marginal tooth rows of NRM-PZ P. 9449 Early Devonian, Canada. Labelled sclerochronology of the teeth in possible sequence of addition in oral (**a**) and labial view (**b**). Colours of the nested boxes reflect the successive stages of tooth development. Scale bar represents 150 μm . Arrow indicates sequence of addition; prox, proximal; dist, distal; ling, lingual; lab, labial.

Figure 4 | Tooth whorl of an ischnacanthid acanthodian. Tooth whorl NRM-PZ P. 15908 (**a - c**), lateral view complete bone and teeth (**a**), virtual section showing tooth and base developing synchronous separated from successive teeth by a growth arrest line (**b**) and virtual section through second tooth and side teeth (**c**). Scale bar equals 120 μm in (**a**), 60 μm in (**b**) and 42 μm in (**c**).

Figure 5 | 50% majority rule consensus tree from a tip-dated Bayesian analysis, annotated with ancestral state reconstructions for oral tubercles. Yellow represents absent

and blue represents present; branch widths proportional to posterior probability for reconstructed state. Arrows indicate taxon ages that extend beyond the range displayed on the figure.

Figure 6 | 50% majority rule consensus tree from a tip-dated Bayesian analysis, annotated with ancestral state reconstructions for ankylosed tooth rows (**a**) and tooth whorls (**b**). Branch widths proportional to posterior probability of reconstructed state. Arrows indicate taxon ages that extend beyond the range displayed on the figure.

a

b

c

d

f

h

i

Lingual tooth row

Lateral tooth row

e

Spheritic mineralisations

g

Hypermineralised layer

Tubuli

Main cusp

Marginal cusplet

Pulp cavity