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## Main Manuscript for

### Fossilized cell structures identify an ancient origin for the teleost whole-genome duplication.

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## Abstract

Teleost fishes comprise half of all vertebrate species, and possess a duplicated genome. This whole-genome duplication (WGD) occurred on the teleost stem-lineage, in an ancient common ancestor of all living teleosts, and is hypothesized as a trigger of their exceptional evolutionary radiation. Genomic and phylogenetic data indicate that WGD occurred in the Mesozoic, after the divergence of teleosts from their closest living relatives, but before the origin of the extant teleost groups. However, these approaches cannot pinpoint WGD among the many extinct groups that populate this 50- to 100-million-year lineage, preventing tests of the evolutionary effects of WGD. We infer patterns of genome size evolution in fossil stem-group teleosts using high-resolution synchrotron X-ray tomography to measure the bone cell volumes, which correlate with genome size in living species. Our findings indicate that WGD occurred very early on the teleost stem-lineage, and that all extinct stem-group teleosts known so far possessed duplicated genomes. WGD therefore substantially pre-dates both the origin of proposed key innovations of the teleost skeleton and the onset of substantial morphological diversification in the clade. Moreover, the early occurrence of WGD allowed considerable time for post-duplication reorganization prior to the origin of the teleost crown-group. This suggests at most an indirect link between WGD and evolutionary success, with broad implications for the relationship between genomic architecture and large-scale evolutionary patterns in the vertebrate Tree of Life.

## Significance Statement

Some lineages of organisms have undergone major evolutionary radiations, while others have not. Establishing why is a central goal of evolutionary research. Whole-genome duplication (WGD) is often proposed as having caused the spectacular evolutionary radiation of teleost fishes. However, due to the absence of genetic data for fossil species, it has been impossible to pinpoint precisely when WGD occurred during teleost history. We use 3D measurements of fossilized bone cell spaces to estimate genome sizes in extinct species, observing a near-doubling of size during earliest teleost ancestry. This suggests that WGD occurred very early, substantially pre-dating the dramatic radiation of teleosts. These findings suggest at most an indirect link between WGD and teleost diversification.

## Main Text

### Introduction

Whole-genome duplication (WGD) has occurred independently in multiple lineages of plants, fungi, and animals (1–3). This represents a major change to genomic architecture, with hypothesized impacts on evolutionary diversification (4, 5), caused by the origin of new gene functions from duplicate copies, expanding the genetic toolbox available for evolutionary ‘tinkering’ (6). However, in spite of its mechanistic plausibility, this hypothesis is so far supported by only limited and contradictory empirical evidence (7–10). Teleost fishes – comprising more than half of modern vertebrates – are a key example, with their spectacular variety of form and kind (ranging from eels to seahorses) often viewed as *prima facie* evidence for the role of WGD in triggering evolutionary diversification (6, 11). Teleost also show an incredible diversity of genome biology, demonstrating particularly high rates of evolution of protein-coding genes (12) and non-

coding elements (13), a broad range of genome sizes including the smallest known in vertebrates (14), and multiple polyploid lineages (15).

The genome of all living teleosts derives from an ancient WGD event that occurred before the last common ancestor of modern species (16). Additional duplication events occurred more recently in several teleost subgroups (9, 17), but are not generally proposed as drivers of diversification (9). Studies of the role of WGD in contributing to teleost diversity so far have analyzed the distribution of species richness among extant lineages and morphometric data for fossil phenotypes, with potentially conflicting results: extant teleosts have high rates of lineage diversification compared to other ray-finned fishes (7), but early fossil members of the teleost crown-group do not show increased rates of morphological evolution (18).

Molecular phylogenetic studies indicate that WGD occurred on the teleost stem-lineage: after the divergence of teleosts from their extant sister taxon (Holostei), but before the most recent common ancestor of all living teleosts (19, 20). However, these bounds encompass a large phylogenetic diversity of extinct groups that diverged during an interval of 50–100 million years, from the initial divergence of the teleost total-group from the holostean total-by the earliest Triassic (21), up to the first appearance of crown-group teleosts in the Late Jurassic (18, 22). Molecular-clock estimates provide only broad constraints on the precise timing of duplication (316–226 Ma (23); ~310 Ma (24)), and offer no information on its phylogenetic position on the teleost stem lineage. The imprecision of these estimates and the sometimes considerable incongruence of molecular clocks with the teleost fossil record questions the reliability of these inferences in the absence of further evidence.

Patterns of genome size evolution on the teleost stem lineage could provide alternative and independent evidence on the timing and phylogenetic position of the teleost WGD. However, stem lineages by definition comprise entirely extinct species that are known only from fossils, for which genomic data are absent. Nevertheless, some information about vertebrate genome size is preserved within fossil bone (25–27). Living organisms show a positive correlation between cell size and genome size (28–30), such that the volumes of bone cell spaces (osteocyte lacunae) allow estimates of genome size. This relationship has been demonstrated in ray-finned fishes, including teleosts, and is predictive for large-scale variation in genome size (31). The precision of this approach is sufficient for inferring the large change (presumably, doubling) of genome size involved in WGD (31). Here, we use this relationship to trace the evolution of genome size in extinct ray-finned fishes, using osteocyte lacuna volumes as a proxy for genome size. Our sample includes a broad range of stem- and crown-group teleosts, providing information on patterns of teleost genome-size evolution during the deep evolutionary history of the teleost total-group.

Three-dimensional measurement of fossil bone cell spaces with  $\mu\text{m}$ -scale diameters presents considerable technical challenges. We used propagation phase contrast synchrotron radiation X-ray micro-computed tomography (PPC-SR $\mu$ CT) to address this, collecting standardized measurements of osteocyte lacuna volumes for 61 fossil ray-finned fish species ranging from 2.5 to 252 million years in age (*SI Appendix, Part I*). This fossil evidence is complemented by data from a previous study including 34 modern ray-finned fish species with known genome sizes (31). Our fossil sample includes all major groups of stem-group teleosts, members of both living and extinct lineages within the teleost crown group, and several non-teleost ray-finned fishes. This sample allows us to estimate relative genome sizes in extinct groups, providing information on the absolute timing and specific phylogenetic position of the teleost WGD as well as the timescale of post-duplication reductions in genome size (24). Both statistical analysis and qualitative observations demonstrate the effectiveness of lacuna size for inferring large evolutionary increases in genome size: known polyploid lineages such as catostomids and salmonids, which

underwent additional rounds of WGD, both show large osteocyte lacuna volumes compared to their close relatives (31).

## Results

Our results suggest that WGD occurred early on the teleost stem lineage. Osteocyte lacuna volumes of early to mid-Mesozoic (Early Jurassic-Early Cretaceous) teleosts overlap with those of extant polyploid taxa and exceed values for non-polyploid taxa (Figs. 1-2, S1-3). Phylogenetic ancestral trait estimates attribute this to a substantial increase in lacuna volume (from 148 to 240  $\mu\text{m}^3$ ) immediately following the split of teleosts from holosteans, and therefore before the deepest-known divergences of the teleost total group (Fig. 1). The oldest stem-group teleosts in our sample with measurable lacuna volumes hail from the Early Jurassic (~195 Ma) and have large lacuna volumes similar to those of recent polyploids such as salmonids and catostomids (Fig. 2). Late Jurassic and Early Cretaceous members of the teleost crown-group generally also have large lacunae compared to extant teleosts. In contrast, late Mesozoic and Cenozoic teleosts mostly have lacuna volumes comparable to those of their closest modern relatives (Figs. 1–2, S1). Osteocyte lacuna volumes therefore decrease through time along the teleost stem-lineage, with further reductions within major lineages of crown teleosts (such as elopomorphs and clupeocephalans) or within some members of the stem group (such as ichthyodectiforms). Aspects of these patterns of lacuna size evolution are consistent with those inferred from molecular data. Nevertheless, our study provides valuable additional evidence, placing important constraints on the timing and phylogenetic placement of WGD on the extinct teleost stem lineage as a test of its relationship to phenotypic diversification.

## Discussion

A large, almost two-fold increase in osteocyte sizes at the base of the teleost stem lineage suggests a phylogenetically-early occurrence of WGD. Large genome sizes can also occur via other genomic mechanisms such as the accumulation of transposable elements, introns, or tandem repeats (32–34) (e.g. within vertebrates, lungfishes and salamanders). Nevertheless, the timing, phylogenetic distribution (spanning early members of the teleost stem-lineage and their crown group), and observation of subsequent decreases in cell size all suggest that the patterns of genome size variation inferred here result from WGD and not from other forms of evolutionary genome size expansion. Furthermore, our previous work has demonstrated that polyploidy provides a strong statistical explanation for large osteocyte size in actinopterygians, on par with genome size (31).

The oldest stem-group teleosts in our sample (*Pholidophoroides* and *Pholidophoropsis*) are of Early Jurassic age. These taxa represent lineages that diverged much earlier from the teleost stem-group, and therefore constrain the timing of the teleost WGD to no later than the Late Triassic (~235 Ma) using phylogenetic ancestral state estimation (Fig. 1). Direct sampling of Triassic stem teleosts will provide a further test of this hypothesis (35), but this has not so far been possible in spite of multiple attempts, due to poor preservation of bone microstructure (*SI Appendix, Part II*). Our phylogenetically-inferred age for the teleost WGD falls within the timeframe estimated by some molecular studies (316–226 Ma (23); ~310 Ma (24)). The subsequent decrease in lacuna volumes toward the crown and within individual crown-group lineages indicates post-duplication genome-size reduction, spanning tens of millions of years. This confirms previous inferences of post-duplication genome size reduction based on clock analysis of paralogous genes, mathematical modelling, and the phylogenetic distribution of genome size in extant teleosts (24). Ichthyodectiforms, a long-lived clade of Mesozoic stem-teleosts, show a similar pattern of smaller volumes in more recent species compared to earlier

ones, providing evidence of parallel reduction in an extinct lineage closely related to the crown (Fig. 1).

Our findings are inconsistent with hypotheses that propose that spectacular evolutionary diversification of teleosts is an immediate consequence of the WGD. Because of the phylogenetically-early occurrence of WGD, all currently recognized members of the teleost stem-group have inferred duplicate genomes. Therefore, WGD did not coincide with the origin of celebrated teleost functional innovations like the mobile premaxilla, symmetrical caudal-fin and aspects of vertebral geometry implicated in their later evolutionary success (36, 37) (Fig. 1). These traits arose later — conceivably many millions of years after the inferred origin of WGD (35). WGD occurred during or before the Triassic, and therefore also predates more general phenotypic diversification, which occurred from the Late Jurassic onwards (38). The earliest known (i.e., Triassic and Early Jurassic) stem teleosts conform to a conservative bodyplan, being small, fusiform fishes with ganoid scales. Quantitative analyses show consistently low levels of morphological diversity for at least ~50 Myr after the origin of the teleost total-group (38). The conservative early history of teleosts cannot be attributed to inadequacies of the Triassic record, which yields an abundance of fish fossils (39) including anatomically diverse holosteans (38). The morphological diversity of teleosts increased only gradually in the later stages of the Mesozoic, especially in the Cretaceous with the origin of fundamentally new bodyplans (e.g., eel-like, deep-bodied (40, 41)) and amplified by prolific morphological diversification in the early Paleogene (42, 43). Triassic-Early Cretaceous teleosts, including early crown-group taxa, show comparable or lower rates of body-shape evolution than contemporary holosteans (18), which are known to lack duplicated genomes (20). Early teleosts also show delayed patterns of taxonomic and ecological diversification compared to the timing of WGD inferred here: teleosts comprise only a minority of actinopterygian genera in marine and freshwater settings throughout the Triassic (39) and much of the Jurassic (44), even after the origin of their crown-group. Only in the Late Jurassic — at least 80 Myr after the WGD — does the taxonomic diversity of teleosts approach or exceed that of other actinopterygians, as evidenced by the marine fossil assemblages of famous *Lagerstätten* like Cerin and Solnhofen (43).

This temporal pattern suggests an alternative hypothesis: that reorganization and integration of genomic architecture after the WGD, and not WGD itself, drove the phenotypic diversification and origin of key morphological traits during later stages of teleost evolution. Consistent with this hypothesis, the 50 Myr-interval between the origin of the teleost total-group and their observed increase in morphological diversity (38) is similar to the estimated duration of post-WGD genome size reductions to modern teleost-like levels (20)(Fig. 2). Decrease in genome size is associated with profound changes in genome organization, through gene loss, neo-functionalization, and changes in expression.

Because of recent focus on genomic hypotheses, relatively little attention has been given to alternative explanations for the phenomenal diversity of extant teleosts. However, the temporal incongruence between a Triassic WGD and the late Mesozoic and early Cenozoic events of teleost diversification (18, 38, 43) also demands broader consideration of non-genomic hypotheses. One possibility is that teleosts were the beneficiaries of biotic turnover during major environmental changes of the late Mesozoic. For example, the mid-Cretaceous interval saw substantial environmentally-driven alterations to marine ecosystems induced by large igneous province volcanism (45), regional ocean anoxic events (46), and other climatic variations. Among these, the Turonian thermal maximum c. 92 million years ago (47) was a major climate perturbation associated with biotic turnover at all levels of the marine trophic chain (46, 48). The early Late Cretaceous coincided with a major burst of cladogenesis in marine fishes, the diversity of which is strongly and positively correlated with sea surface temperature during the Jurassic and Cretaceous (44, 49). Although teleosts dominate this mid-Cretaceous radiation, other ray-finned fish groups show similar patterns (e.g., pycnodonts; (50)), arguing for a general—rather than clade-specific—mechanism. The Cretaceous concluded 66 million years ago with a major

mass extinction event. This induced a major reorganization of aquatic ecosystems, particularly in higher trophic levels of marine environments (51, 52). Paleontological and molecular data indicate major morphological diversification among marine teleosts in the early Paleogene (42, 53). This was associated with the colonization of different environments and the refilling of vacated functional roles (54–57), and included short-lived “evolutionary experiments” found alongside familiar living lineages (58, 59). As with the mid-Cretaceous, the early Paleocene represented a “hothouse” period in Earth’s climate history, with evidence that higher temperatures might have led to increased fish production in some marine settings (60). Collectively, these observations suggest a role for environmental change and ecosystem reorganization in facilitating the diversification of teleosts, either in addition to or instead of the effects from WGD.

Our results provide direct fossil evidence of phylogenetic placement of the teleost WGD, and a more robust window for the timing and consequences of teleost genome duplication. WGD occurred early on the teleost stem-lineage, with considerable time for post-duplication reorganizations of genomic architecture before the morphological and taxonomic radiation of teleosts. This suggests an indirect and temporally-offset link between WGD and evolutionary diversification, if any, and is consistent with genomic studies of recent polyploids among fishes (e.g. salmonids (9)) and vascular plants (10). More broadly, our data demonstrate the potential of the fossil record of cell structures (26) as an independent source of evidence on genomic events that may have underpinned major diversification events, not just in fishes and other vertebrates, but also in plants and across the Tree of Life (2, 3).

## Materials and Methods

- **Specimen collection**

Our taxon sample builds on a previous study (31) that collected osteocyte lacuna volumes for 34 species of extant actinopterygians (including 28 teleost species). The present study adds 61 fossil species displaying osteocytic bone (61) to this sample: 8 non-teleost actinopterygians, 26 stem-group teleosts, and 27 crown teleosts. They range from 2.5 (Pliocene) to 252 (Early Triassic) million years in age (*SI Appendix, Part I, Dataset S1*). However, no representative of the teleost lineage is older than 200 million years (Early Jurassic; *SI Appendix, Part II*). As fossil specimens have a higher density than extant ones, capturing a decent signal of transmitted X-ray beam could be challenging should the specimens be too large. Thus, we took small (~ 1-5 mm) bone samples from the fossils, targeting areas that reduced the loss of anatomical information from the specimens (e.g., areas affected by pre-existing cracks or broken bone). Since lacunae vary in volume from one bone to another (30, 31), the same skeletal element has to be used consistently across the sample. Therefore, all samples were taken from the dentary bone (lower jaw) as it is found in all target taxa and is often preserved in the fossil record. Moreover, the already-existing dataset of extant actinopterygians (31), used dentary bones for each included species.

- **Data acquisition**

The data presented in this study were acquired using propagation phase contrast synchrotron radiation X-ray micro-computed tomography (PPC-SR $\mu$ CT). Acquisition was performed over the course of three sessions in two synchrotrons: two sessions were done at the ID19 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and the remaining session was done at the I13-2 Diamond Manchester Imaging beamline of the Diamond Light Source (DLS, Didcot, UK). The setups on the two beamlines were generally similar, the main difference being that much higher energies were available at the ID19 beamline (up to 112 keV in this study) compared to I13-2 beamline (here, 21.39 keV). Both setups produced high-resolution data, with recorded voxel sizes of 0.357  $\mu$ m at the I13-2 beamline and near 0.7  $\mu$ m at the ID19 beamline. A voxel size of 0.7  $\mu$ m or smaller is empirically adequate for imaging osteocyte lacunae (31, 62–64). Detailed information on the synchrotron experimental setups is found in the *SI Appendix, Part III* and Dataset S2. The tomograms (digital ‘slices’) obtained from our specimens

were processed with the software VGSTUDIO MAX v. 3.0 and 3.1 (Volume Graphics, Heidelberg, Germany), to segment osteocyte lacunae from the fossil bone matrix. We used specimen-specific grey-value thresholds to segment osteocyte lacunae from a selected region of interest, focusing on well-defined lacunae from primary bone, excluding objects such as specimen edges, cracks and abiotic inclusions manually. Grey-value thresholds were selected to ensure that the boundaries of segmented osteocytes coincided with the boundaries of osteocytes in the image volume, attempting to maximize fit of the segmented object to the underlying data. We did not segment the canaliculi that accommodate cytoplasmic projections of the osteocytes, as they were not visible in every tomogram and thus could bias our estimates of the osteocyte lacuna volumes. We used the 'Porosity/Inclusion' module of VGSTUDIO MAX, to measure the individual lacuna volumes. This module also provides visualization, coloring lacunae according to their volume with a consistent color range across our sample (Figs. 1-2, S1). We analyzed median osteocyte volumes computed from the entire population of segmented objects, excluding objects smaller than 25  $\mu\text{m}^3$ , which did not generally represent osteocyte lacunae. The whole protocol followed the one we applied previously for extant actinopterygians (31), allowing us to incorporate extant and fossil samples together in downstream analyses. The complete measurement and positional data for populations of osteocyte lacunae in all specimens analyzed are available in Dryad (<https://doi.org/10.5061/dryad.bcc2fqzcc>), and our scan image volumes are available on MorphoSource (<https://www.morphosource.org/projects/0000C1125>). We used objective quality control criteria to exclude some specimens from the analysis after obtaining PPC-SR $\mu$ CT tomograms (*SI Appendix, Part I.3*). Datasets were excluded based on the following criteria: [1] When the data clearly showed the absence of osteocyte lacunae from the sample, either because the taxon has anosteocytic bone (61, 65), or because osteocytes were not fossilized. [2] When osteocyte lacunae were present but not sufficiently well-resolved to allow an accurate segmenting of the lacunae or measurement of their volume. [3] After segmenting the osteocyte lacunae, we excluded more specimens in which the number of osteocyte lacunae we were able to measure was too small to warrant confident estimates of average size ( $n < \sim 50$ ). Prior to the segmentation and measurement, our sample of tomograms included 77 fossil specimens in total representing 71 species, and notably included five stem-group teleosts from the Triassic. Of these, 16 specimens were excluded based on quality control criteria (see the *SI Appendix, Part I.1 and I.2* for the complete list of specimens).

- **Reference tree**

We used a composite phylogeny based on a consensus of published hypotheses on the interrelationships of fossil actinopterygians, and divergence ages inferred from earliest known occurrences in the fossil record (*SI Appendix, Part I.2, Dataset S1*). The framework for extant taxa is a recently published molecular timetree (66) that has the best species sample and taxonomic coverage of any actinopterygian phylogeny so far published. Fossil taxa were stitched to the resulting tree using a custom script that makes extensive use of the R package ape version 5.0 (67) and is available in Dryad (<https://doi.org/10.5061/dryad.bcc2fqzcc>). Fossil age ranges, specifier taxa for their sister clades, and minimum divergence times from those sister clades based on fossil occurrences are available as Dataset S1. Phylogenetic position and age data for the fossil taxa and their divergence times from other species were taken from the most recent available paleontological and stratigraphic information (see the *SI Appendix, Part I.2* for the justification of phylogenetic and stratigraphic placement for each species in the sample). Extant terminals were pruned, retaining the 34 extant species for which we measured osteocyte volumes, prior to analysis. The resulting tree is available as Fig. S1 and Dataset S3.

- **Data analysis**

To visualize patterns of genome size evolution, we mapped osteocyte lacuna volume (Dataset S4) to our composite phylogeny with branch lengths of units in time, using ancestral character state estimation (68) via the ace function of ape version 5.0 (67). Osteocyte lacuna volume was  $\log_{10}$ -transformed prior to analysis and is used as a proxy for genome size (31). WGD predicts an approximate doubling of osteocyte size somewhere on the teleost stem-lineage.



## Data availability

The SR $\mu$ CT data (tomograms and 3D reconstructions) generated during this study have been deposited online in MorphoSource: [www.morphosource.org/projects/0000C1125](http://www.morphosource.org/projects/0000C1125) (fossil specimens) and [www.morphosource.org/projects/00000C959](http://www.morphosource.org/projects/00000C959) (extant specimens). The median osteocyte lacuna volumes for each specimen are available in Dataset S4. The complete volume measurement data for populations of osteocyte lacunae in all specimen analysed, as well as the custom R script used to build the reference tree and reconstruct osteocyte lacuna volume evolution are available in Dryad (<https://doi.org/10.5061/dryad.bcc2fqzcc>). All other data files are included in the SI Appendix and Datasets S1-S4.

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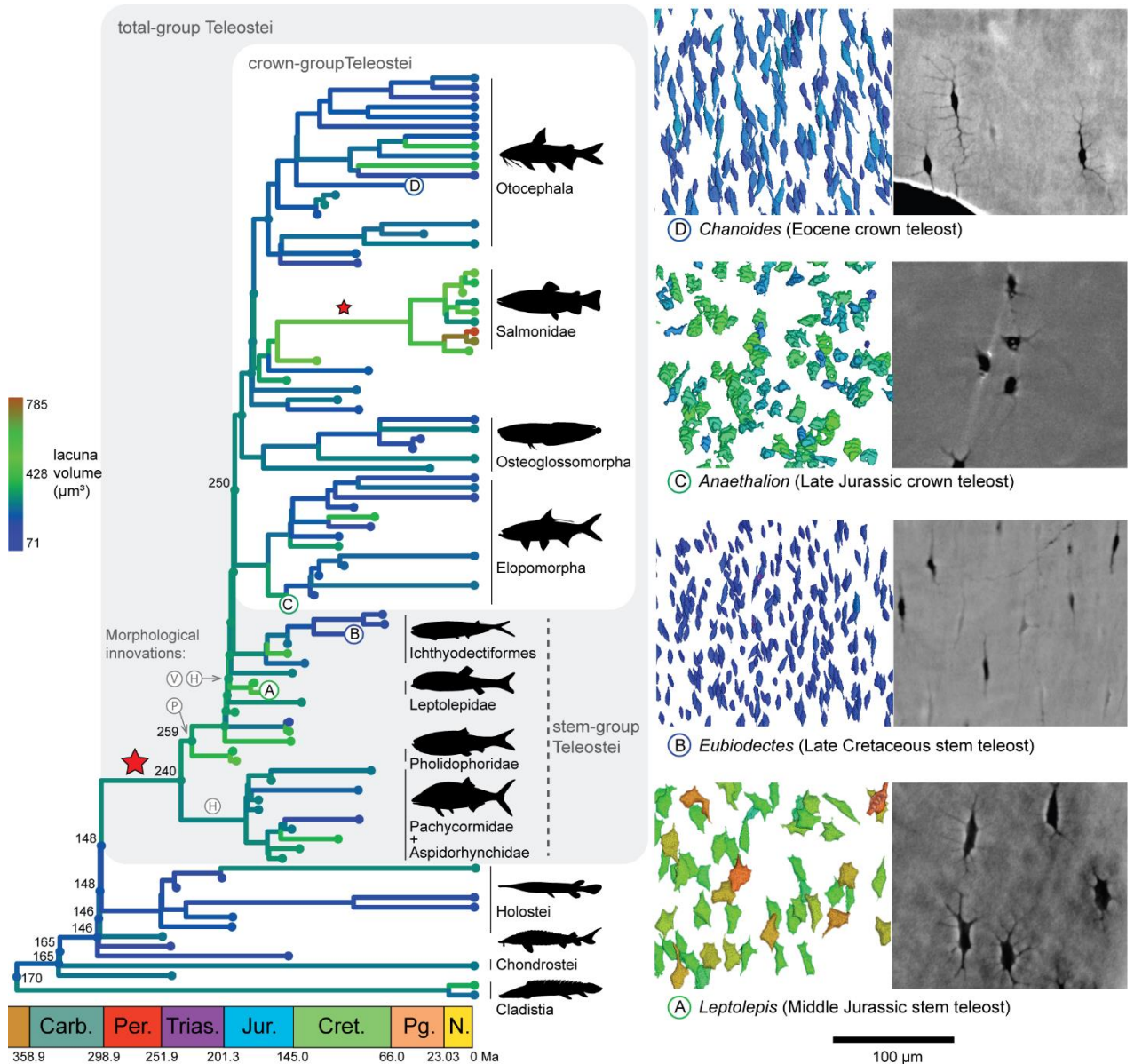
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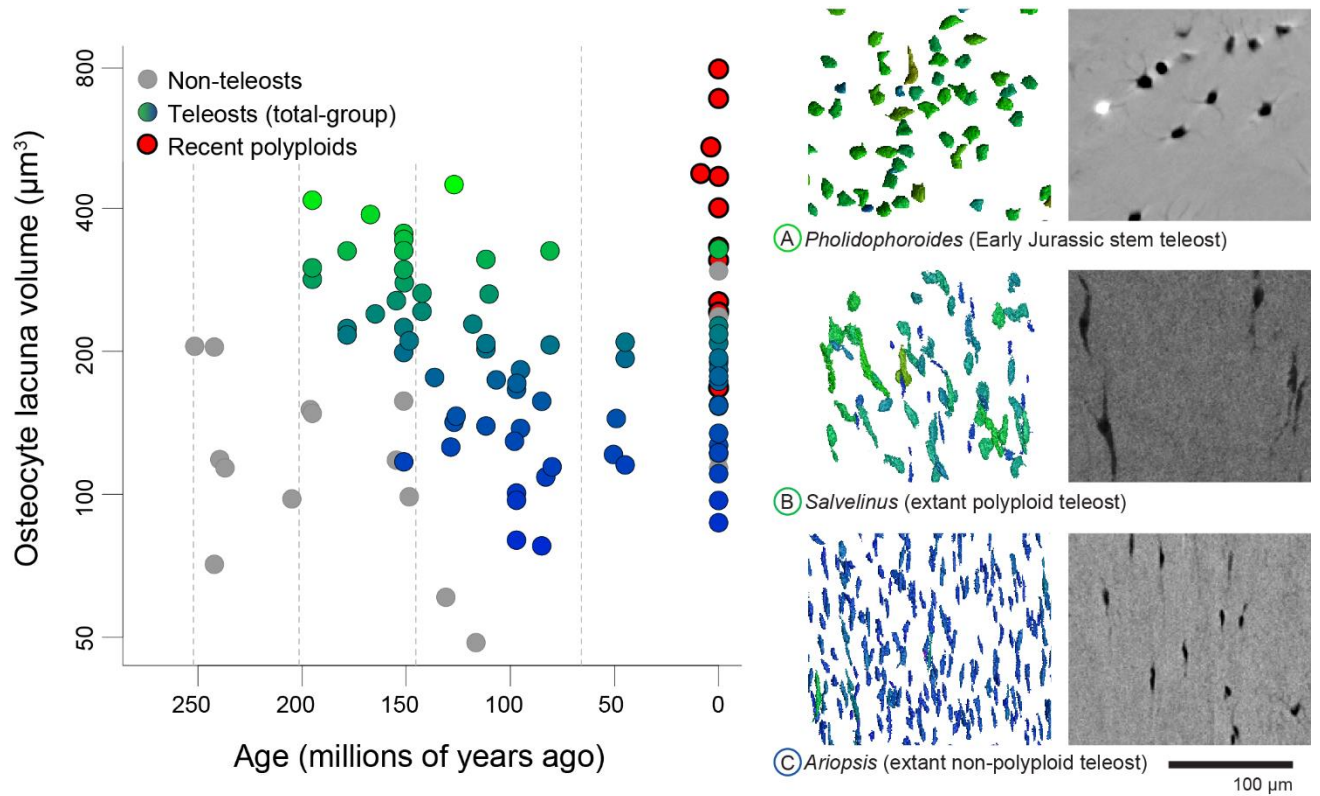
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## Figures



**Figure 1. Evolution of osteocyte lacuna volume in fossil and modern teleosts revealed by synchrotron microtomography.** Timescaled composite phylogeny of actinopterygians with mapped cell-size volumes for fossil and modern lineages. Red stars visualise the inferred occurrences of whole-genome duplications: the teleost WGD (large star), and the salmonid-specific WGD (small star). Renderings of osteocyte lacunae shown for highlighted fossil taxa branching close in time to the inferred teleost-specific WGD (A, C) and those substantially postdating it (B, D). Letters correspond to key teleost synapomorphies, mapped to the corresponding node based on morphological phylogeny (35): P, mobile premaxilla; H, homocercal caudal fin; A, vertebral autocentra.



**Figure 2. Evolution of osteocyte lacuna volume in fossil and modern teleosts revealed by synchrotron microtomography, plotted against time.** We show cell size in an Early Jurassic stem-group teleost (A), within the range of modern polyploid species (exemplified by the salmonid *Salvelinus*; B), and indicative of a WGD. A steady decrease in cell size through time is evident in the fossil record, and is reflected by the relatively small cells of many extant teleosts, which lack polyploidy events subsequent to the ancestral teleost WGD (e.g. the catfish *Ariopsis*; C). Note semi-log axes (osteocyte lacuna volume values are log-transformed). Color scale follows Fig. 1.

## Supplementary Information for

### Fossilized cell structures identify an ancient origin for the teleost whole-genome duplication.

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#### **This file includes:**

Supplementary text  
Figures S1 to S3  
Legends for Datasets S1 to S4  
SI References

#### **Other supplementary materials for this manuscript include the following:**

Datasets S1 to S4



## Supplementary Information Text

### I. Complete list of specimens used in the study

This list includes all fossil specimens that we SR $\mu$ CT-scanned for the present study. Some of the specimens were not included in the downstream analysis because they did not yield exploitable data in the form of osteocyte lacuna volumes.

#### I.1. Institutional abbreviations for fossil specimens

IRSNB – Institut Royal des Sciences Naturelles de Belgique, Brussels, Belgium; MB.f – Museum für Naturkunde, Berlin, Germany; MCSNB – Museo Civico di Scienze Naturali, Bergamo, Italy; MNHN – Muséum National d'Histoire Naturelle, Paris, France; NHMUK – Natural History Museum, London, UK; OUMNH – Oxford University Museum of Natural History, Oxford, UK; PIMUZ – Paläontologisches Institut und Museum, Zurich, Switzerland; UMMP – University of Michigan Museum of Paleontology, Ann Arbor, USA.

#### I.2. Justification of the phylogenetic and stratigraphic placement of the fossil species used in the analyses

We used the Rabosky *et al.* (1) phylogeny as our reference tree for extant taxa, since it was the most complete molecular tree of actinopterygians available at the date of our study. Moreover, it provides estimates of divergence times that are amongst the closest to what is suggested by the fossil record (i.e. 192 Ma for the origin of crown-group teleosts).

##### - †*Paleolox larsoni* Kimmel, 1975

**Specimen number:** UMMP V74157

**Position:** stem-group *Salvelinus* according to (2, 3)

**Position in the reference tree:** sister to the clade (crown *Salvelinus*) comprising *Salvelinus leucomaenis leucomaenis*, *Salvelinus confluentus*, their most recent common ancestor and all its descendants

**Age / Locality:** Miocene / Chalk Hills Formation, USA – 9.8-7.4 Ma (2, 4)

**Minimum divergence time:** †*Paleolox larsoni* itself is the oldest representative of the total-group *Salvelinus*.

##### - †*Prosopium prolixus* Smith, 1975

**Specimen number:** UMMP 21728

**Position:** in total-group *Prosopium* according to (2)

**Position in the reference tree:** sister to the clade (crown *Prosopium*) comprising *Prosopium coulterii*, *Prosopium spilonotus*, their most recent common ancestor and all its descendants

**Age / Locality:** Pliocene / Glens Ferry Formation, USA – 5.0-2.5 Ma (2, 4)

**Minimum divergence time:** †*P. prolixus* itself is the oldest representative of the total-group *Prosopium*.

##### - †*Spaniodon elongatus* Pictet, 1850

**Specimen number:** NHMUK PV OR 44831

**Position:** crown Euteleostei, sister to “Osmeroidei” (i.e. Osmeriformes in the reference phylogeny) according to (5)

**Position in the reference tree:** sister to the clade (crown Osmeriformes) comprising *Retropinna retropinna*, *Osmerus eperlanus*, their most recent common ancestor and all its descendants

**Age / Locality:** Late Santonian / Sahel Alma, Lebanon – 86.3-83.6 Ma (6, 7)

**Minimum divergence time:** †*Spaniodon* itself is the oldest representative of the total-group Osmeriformes.

**- †*Pattersonella formosa* (Traquair, 1908)**

**Specimen number:** IRSNB Vert-01680-00240 ce

**Position:** crown Euteleostei, is an “argentinoid” (i.e. Argentiniformes in the reference phylogeny) according to (8)

**Position in the reference tree:** sister to the clade (crown Argentiniformes) comprising *Argentina silus*, *Bathylagus antarcticus*, their most recent common ancestor and all its descendants

**Age / Locality:** Barremian-Aptian / Bernissart, Belgium – 128.0-126.0 Ma (9, 10)

**Minimum divergence time:** †*Pattersonella* itself is the oldest representative of the total-group Argentiniformes.

**- †*Leptolepides sprattiformis* (Blainville, 1818)**

**Specimen number:** MB.f.18203

**Position:** total-group Euteleostei according to (11–14)

**Position in the reference tree:** sister to the clade (crown Euteleostei) comprising *Lepidogalaxias salamandroides*, *Oncorhynchus mykiss*, their most recent common ancestor and all its descendants

**Age / Locality:** Kimmeridgian-Tithonian / Solnhofen Limestone, Germany - 150.94 Ma (15)

**Minimum divergence time:** †*Leptolepides* itself is the oldest representative of the total-group Euteleostei.

**- †*Chanoides macropoma* Agassiz, 1843**

**Specimen number:** NHMUK PV P 37226

**Position:** stem Otophysi according to (16, 17)

**Position in the reference tree:** sister to the clade (crown Otophysi) comprising *Gymnotus carapo*, *Danio rerio*, their most recent common ancestor and all its descendants

**Age / Locality:** late Ypresian / Bolca, Italy – 49.11 Ma (15)

**Minimum divergence time:** †*Santanichthys diasi*, oldest representative of the total-group Otophysi, probably within the clade formed by †*Chanoides* and crown Otophysi – 113.4-110 Ma (18).

**- †*Tharrias araripis* Jordan & Branner, 1908**

**Specimen number:** NHMUK PV P 54675b

**Position:** stem Chanidae (crown Gonorhynchiformes), sister to *Chanos chanos* according to (10)

**Position in the reference tree:** sister to *Chanos chanos*

**Age / Locality:** early Albian / Romualdo Member of the Santana Formation, Brazil – 113.4-110 Ma (10)

**Minimum divergence time:** †*Tharrias* itself is the oldest representative of the clade it forms with *Chanos*.

**- †*Dastilbe elongatus* Silva-Santos, 1947**

**Specimen number:** NHMUK PV P 63381

**Position:** stem Chanidae (crown Gonorhynchiformes), sister to [†*Tharrias* + *Chanos*] according to (10)

**Position in the reference tree:** sister to the clade comprising †*Tharrias araripes*, *Chanos chanos*, their most recent common ancestor and all its descendants

**Age / Locality:** late Aptian / Nova Olinda Member of the Crato Formation, Brazil – 123.0-113.0 Ma (10)

**Minimum divergence time:** †*Dastilbe* itself is the oldest representative of the clade it forms with †*Tharrias* and *Chanos*.

**- †*Aethalionopsis robustus* (Traquair, 1911)**

**Specimen number:** IRSNB Vert-01680-00556

**Position:** stem Chanidae (crown Gonorhynchiformes), sister to [†*Dastilbe* + †*Tharrias* + *Chanos*] according to (10, 19)

**Position in the reference tree:** sister to the clade comprising †*Dastilbe elongatus*, *Chanos chanos*, their most recent common ancestor and all its descendants

**Age / Locality:** Barremian-Aptian / Bernissart, Belgium – 128.0-126.0 Ma (9, 10)  
**Minimum divergence time:** †*Aethalionopsis* itself is the oldest representative of the clade it forms with †*Tharrias*, †*Dastilbe* and *Chanos*.

- †*Knightia* sp.

**Specimen number:** UMMP Tmp-1008

**Position:** crown Clupeidae (crown Clupeiformes), Pellonulinae according to (20)

**Position in the reference tree:** sister to the clade (crown Pellonulinae) comprising *Sierrathrissa leonensis*, *Pellonula vorax*, their most recent common ancestor and all its descendants

**Age / Locality:** Eocene / Green River Formation, USA – 55.8-33.9 Ma (20)

**Minimum divergence time:** †*Knightia* itself is the oldest representative of the total-group Pellonulidae.

- †*Armigatus namourensis* Forey et al., 2003

**Specimen number:** NHMUK PV P 63151a

**Position:** stem Clupeiformes according to (21)

**Position in the reference tree:** sister to the clade (crown Clupeiformes) comprising *Denticeps clupeoides*, *Pellonula vorax*, their most recent common ancestor and all its descendants

**Age / Locality:** early to middle Cenomanian / Namoura, Lebanon – 99.7-94.4 Ma (7)

**Minimum divergence time:** †*Ezkutuberezi carmeni* from the Valanginian, oldest †Ellimichthyiformes (within the clade that †*Armigatus* forms with crown Clupeiformes) – 132.9-139.8 Ma (21)

- †*Ornategulum sardinioides* (Pictet, 1850)

**Specimen number:** NHMUK PV P 52508

**Position:** stem Clupeiformes according to (20, 22), possibly as sister to [†*Armigatus* + crown Clupeiformes]

**Position in the reference tree:** sister to the clade comprising †*Armigatus namourensis*, *Denticeps clupeoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Cenomanian / Hakel and Namoura, Lebanon (7) – 99.7-94.4 Ma

**Minimum divergence time:** †*Ezkutuberezi carmeni* from the Valanginian, oldest †Ellimichthyiformes (within the clade that †*Ornategulum* forms with †*Armigatus* and crown Clupeiformes) – 132.9-139.8 Ma (21)

- †*Plethodus expansus* (Dixon, 1850)

**Specimen number:** NHMUK PV P 49895

**Position:** member of †Tselfatiiformes, which are crown Teleostei, sister to crown Clupeocephala according to (23)

**Position in the reference tree:** sister to the clade (crown Clupeocephala) comprising *Denticeps clupeoides*, *Oncorhynchus mykiss*, their most recent common ancestor and all its descendants

**Age / Locality:** Cenomanian-Turonian / English Chalk, UK – 100.5-89.8 Ma (24)

**Minimum divergence time:** †*Leptolepides* from the Kimmeridgian-Tithonian Solnhofen Limestone is the oldest representative of crown Clupeocephala - 150.94 Ma (15)

- †*Rhacolepis buccalis* Agassiz, 1841

**Specimen number:** UMMP 101952

**Position:** within the †Crossognathiformes clade of (25), sister to crown Clupeocephala (that includes Clupeomorpha, Ostariophysi and Euteleostei). According to (23), †Crossognathiformes are sister to [†Tselfatiiformes + crown Clupeocephala]. Sister to Euteleostei (represented by *Salmo salar*) according to (26)

**Position in the reference tree:** We adopt the phylogenetic position proposed by (25), as it is a recent phylogeny that incorporates many †crossognathiform and crown teleost taxa: sister to the clade (crown Clupeocephala + †Tselfatiiformes) comprising †*Plethodus expansus*, *Oncorhynchus mykiss*, their most recent common ancestor and all its descendants

**Age / Locality:** Albian / Santana Formation, Brazil – 113.4-110 Ma (10, 27)

**Minimum divergence time:** †*Leptolepides sprattiformis* from the Kimmeridgian-Tithonian Solnhofen Limestone is the oldest representative of crown Clupeocephala - 150.94 Ma (15)

**- †*Apsopelix anglicus* (Dixon, 1850)**

**Specimen number:** NHMUK PV P 4246

**Position:** within the †Crossognathiformes clade of (25)

**Position in the reference tree:** sister to †*Rhacolepis buccalis*

**Age / Locality:** Cenomanian-Turonian / English Chalk, UK – 100.5-89.8 Ma (24)

**Minimum divergence time:** †*Bavarichthys incognitus* (13) from the Kimmeridgian-Tithonian Solnhofen Limestone is the oldest representative of the †Crossognathiformes clade of (25) - 150.94 Ma (15)

**- †*Phareodus encaustus* Cope, 1871**

**Specimen number:** NHMUK PV P 64636I

**Position:** stem Osteoglossidae according to (28)

**Position in the reference tree:** sister to the clade (crown Osteoglossidae) comprising *Osteoglossum bicirrhosum*, *Arapaima gigas*, their most recent common ancestor and all its descendants

**Age / Locality:** Eocene / Laney Member, Green River Formation, USA - 55.8-33.9 Ma

**Minimum divergence time:** †*Cretophareodus alberticus* from the Campanian is the oldest representative of the clade formed by †*Phareodus* and crown Osteoglossidae – 83.6-72.1 Ma

**- †*Brychaetus muelleri* Woodward, 1901**

**Specimen number:** NHMUK PV OR 28424

**Position:** sister to †*Phareodus* according to (29)

**Position in the reference tree:** sister to †*Phareodus encaustus*

**Age / Locality:** Ypresian / Sheppey, London Clay, UK – 50.5 Ma (24)

**Minimum divergence time:** †*Brychaetus* itself is the oldest representative of the clade it forms with †*Phareodus*.

**- †*Eohiodon falcatus* Grande, 1979**

**Specimen number:** NHMUK PV P 61245

**Position:** stem Hiodontidae according to (28, 30, 31)

**Position in the reference tree:** sister to the clade (crown Hiodontidae) comprising *Hiodon alosoides*, *Hiodon tergisus*, their most recent common ancestor and all its descendants

**Age / Locality:** Eocene / Green River Formation, USA – 55.8-33.9 Ma

**Minimum divergence time:** †*Eohiodon* itself is the oldest representative of the clade it forms with *Hiodon*.

**Elopomorpha** have two alternative topologies in the reference phylogenetic study of (6). Here we chose the hypothesis presented in the Fig. 3 of (6) as it is better resolved for the fossil taxa of our sample.

**- †*Hayenchelys germanus* (Hay, 1903)**

**Specimen number:** NHMUK PV P 62726

**Position:** stem Anguilliformes according to (32)

**Position in the reference tree:** sister to the clade (crown Anguilliformes) comprising *Diastobranchius capensis* + *Anguilla anguilla*, their most recent common ancestor and all its descendants

**Age / Locality:** under the †*Mantilliceras mantelli* Ammonite zone, Cenomanian / Hadjula, Lebanon (6) – 98.0 Ma

**Minimum divergence time:** †*Hayenchelys* itself is the oldest representative of the total-group Anguilliformes.

**- †*Istieus grandis* Agassiz, 1842**

**Specimen number:** NHMUK PV P 3886

**Position:** stem Pterothrissidae according to (6)

**Position in the reference tree:** sister to *Pterothrissus gissu*

**Age / Locality:** younger than the †*Scaphites hippocrepis* III Zone but older than the †*Baculites mclearnii* Zone (6) / Coesfeld Member of Westphalia, Germany – 81.28-80.67 Ma

**Minimum divergence time:** †*Istieus* is the oldest representative of the total-group Pterothrissidae.

**- †*Lebonichthys gracilis* (Davis, 1887)**

**Specimen number:** NHMUK PV P 49076

**Position:** stem Albulidae according to (6)

**Position in the reference tree:** sister to the clade (crown Albulidae) comprising *Albula glossodonta*, *Albula vulpes*, their most recent common ancestor and all its descendants

**Age / Locality:** Late Santonian / Sahel Alma, Lebanon – 86.3-83.6 Ma (6, 7)

**Minimum divergence time:** †*Lebonichthys* itself is the oldest representative of the clade it forms with crown Albulidae.

**- †*Brannerion vestitum* Jordan, 1920**

**Specimen number:** NHMUK PV P 60923

**Position:** sister to [Anguilliformes + Notacanthiformes] according to (6)

**Position in the reference tree:** †*Brannerion* is found as sister to [Anguilliformes + Notacanthiformes] in (6), but this clade does not exist in the reference phylogeny, unless Albuliformes are also included. We chose the conservative approach to position it as sister to [Anguilliformes + Notacanthiformes + Albuliformes]: the clade comprising *Anguilla anguilla*, *Albula vulpes*, their most recent common ancestor and all its descendants

**Age / Locality:** early Albian / Romualdo Member of the Santana Formation, Brazil – 113.4-110 Ma (6)

**Minimum divergence time:** †*Baugeichthys caeruleus*, earliest representative of the total-group Albuliformes – 134.5-129.4 Ma (1, 6)

**- †*Osmeroides* sp. (presumably †*Osmeroides lewesiensis*)**

**Specimen number:** OUMNH K.64151

**Position:** sister to [Albuliformes + Notacanthiformes + Anguilliformes] according to (6)

**Position in the reference tree:** sister to the clade comprising †*Brannerion vestitum*, *Albula vulpes*, their most recent common ancestor and all its descendants [following the proposed position of †*Brannerion*]

**Age / Locality:** †*Hoplites dentatus* through †*Mortoniceras inflatus* zones of the Albian / Gault Clay Formation, UK – 110.22 Ma

**Minimum divergence time:** †*Baugeichthys caeruleus*, earliest representative of the total-group Albuliformes – 134.5-129.4 Ma (1, 6)

**- †*Flindersichthys denmaedi* Longman, 1932**

**Specimen number:** NHMUK PV P 3886

**Position:** stem Megalopidae (crown Elopiformes) according to (33)

**Position in the reference tree:** sister to the clade (crown Megalopidae) comprising *Megalops cyprinoides*, *Megalops atlanticus*, their most recent common ancestor and all its descendants

**Age / Locality:** Albian / Stewart Creek, Rolling Downs Formation, Australia – 113.0-100.5 Ma

**Minimum divergence time:** †*Flindersichthys* itself is the oldest representative of the clade it forms with *Megalops*.

**- †*Arratiaelops vectensis* (Woodward, 1890)**

**Specimen number:** NHMUK PV P 42013

**Position:** stem Megalopidae (crown Elopiformes) according to (33)

**Position in the reference tree:** sister to the clade comprising †*Flindersichthys denmaedi*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Barremian-Aptian / Bernissart, Belgium – 126.0 Ma

**Minimum divergence time:** †*Arratiaelops* itself is the earliest representative of the total-group Megalopidae.

**- †*Ichthyemidion vidali* Poyato-Ariza, 1995**

**Specimen number:** MNHN.F.MSE356a

**Position:** stem Elopiformes according to (6)

**Position in the reference tree:** sister to the clade (crown Elopiformes) comprising *Elops saurus*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Late Berriasian – Early Valanginian / El Montsec, Spain – 131.3-125.9 Ma (6)

**Minimum divergence time:** †*Ichthyemidion* itself is the oldest representative of the clade it forms with crown Elopiformes.

**- †*Anaethalion angustus* (Münster, 1839)**

**Specimen number:** NHMUK PV OR 37926

**Position:** stem-Elopidae (crown Elopiformes) according to (34), but may be a stem Elopiformes according to (12, 25)

**Position in the reference tree:** since the position of †*Anaethalion* varies from one phylogenetic analysis to the other, we chose the conservative approach to place it as a stem Elopiformes: sister to the clade comprising †*Ichthyemidion vidali*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** †*Neochetoceras rebouletianum* Horizon within the †*Lithacoceras ulmense* Subzone of the †*Hybonotoceras beckeri* Ammonite Zone, Late Kimmeridgian / Eichstätt, Germany – 151.2 Ma (6)

**Minimum divergence time:** †*Anaethalion* itself is the oldest representative of the total-group Elopiformes, and of crown Elopomorpha.

†*Ichthyodectiformes* are a clade of stem Teleostei, which position varies from one study to another. Some studies find them as the sister to crown Teleostei (12, 25) while others find some stem Teleostei closer to the crown group than †*Ichthyodectiformes* (26, 35). We chose to follow (25) since it is a recent phylogenetic analysis including several †*ichthyodectiform* representatives alongside a comprehensive sample of stem- and crown Teleostei. Other recent large-scale studies either did not include any †*ichthyodectiform* representative (34, 36) or only one †*ichthyodectiform* (26). We thus interpret †*Ichthyodectiformes* as sister to crown Teleostei.

**- †*Xiphactinus audax* Leidy, 1870**

**Specimen number:** UMMP 11003

**Position:** in †*Ichthyodectiformes*, family †*Ichthyodectidae* (37)

**Position in the reference tree:** sister to the clade (crown Teleostei) comprising *Megalops cyprinoides*, *Oncorhynchus mykiss*, their most recent common ancestor and all its descendants

**Age / Locality:** Turonian-Maastrichtian / USA – 93.9-66 Ma (37)

**Minimum divergence time:** †*Occithrissops wilsoni* (38) is the oldest representative of †*Ichthyodectiformes*, and of the clade they form with crown Teleostei – Bathonian, 168.3-166.1 Ma.

**- †*Ichthyodectes ctenodon* Cope, 1870**

**Specimen number:** UMMP V56318

**Position:** in †*Ichthyodectiformes*, family †*Ichthyodectidae* (37)

**Position in the reference tree:** sister to †*Xiphactinus audax*

**Age / Locality:** Turonian-Campanian / USA – 93.9-72.1 Ma (37)

**Minimum divergence time:** †*Ichthyodectes* and †*Xiphactinus* themselves are the oldest representatives of the clade (†*Ichthyodectidae*) they form together.

**- †*Eubiodectes libanicus* (Pictet & Humbert, 1866)**

**Specimen number:** NHMUK PV P 62691a

**Position:** in †*Ichthyodectiformes*, family †*Cladocyclidae* (37)

**Position in the reference tree:** sister to the clade (†*Ichthyodectidae*) comprising †*Xiphactinus audax*, †*Ichthyodectes ctenodon*, their most recent common ancestor and all its descendants

**Age / Locality:** Cenomanian / Hakel, Lebanon (7) – 99.7-94.4 Ma

**Minimum divergence time:** †*Chiromystus mawsoni* is the oldest representative of †*Cladocyclidae* (and of the clade †*Eubiodectes* forms with †*Ichthyodectidae*) – Hauterivian-Barremian, 132.9-125 Ma (37).

**- †*Thrissops formosus* Agassiz, 1833**

**Specimen number:** NHMUK PV OR 35013

**Position:** in †*Ichthyodectiformes*, outside of the clade that includes †*Cladocyclidae* and †*Ichthyodectidae* (37)

**Position in the reference tree:** sister to the clade comprising †*Xiphactinus audax*, †*Eubiodectes libanicus*, their most recent common ancestor and all its descendants

**Age / Locality:** Kimmeridgian / Eichstätt, Germany – 151.2 Ma (6)

**Minimum divergence time:** †*Thrissops* itself is the oldest representative of the clade it forms with †*Ichthyodectidae* and †*Cladocyclidae*.

**- †*Allothrissops regleyi* (Thiollière, 1854)**

**Specimen number:** NHMUK PV P 921

**Position:** in †*Ichthyodectiformes*, outside of the clade that includes †*Thrissops*, †*Cladocyclidae* and †*Ichthyodectidae* (37)

**Position in the reference tree:** sister to the clade comprising †*Thrissops formosus*, †*Xiphactinus audax*, their most recent common ancestor and all its descendants

**Age / Locality:** Kimmeridgian / Eichstätt, Germany – 151.2 Ma (6)

**Minimum divergence time:** †*Occithrissops wilsoni* (38) is the oldest representative of the clade †*Allothrissops* forms with other †*Ichthyodectiformes* (37) – Bathonian, 168.3-166.1 Ma.

**- †*Pachythrissops laevis* Woodward, 1919**

**Specimen number:** NHMUK PV P 41859

**Position:** (37) considers the inclusion of †*Pachythrissops* inside †*Ichthyodectiformes* to be dubious. However, several analyses, including the extensive one of (25) find it to be an †*ichthyodectiform*, sister to all other members of the clade.

**Position in the reference tree:** sister to the clade comprising †*Allothrissops regleyi*, †*Xiphactinus audax*, their most recent common ancestor and all its descendants

**Age / Locality:** Valanginian / Purbeck Group, UK – 139.8-132.9 Ma

**Minimum divergence time:** †*Occithrissops wilsoni* (38) is the oldest representative of the clade †*Pachythrissops* forms with other †*Ichthyodectiformes* – Bathonian, 168.3-166.1 Ma (37).

**Stem-group teleosts (outside of †*Ichthyodectiformes*):** Several recent phylogenetic studies have tried to resolve the relationships between stem teleost taxa (25, 26, 34, 36, 39). They all recover different topologies for our sampled taxa, but there are several areas of agreement, most notably:

- 1) a clade including †*Leptolepis coryphaenoides*, †*Tharsis*, †*Ascalabos*, †*Ichthyodectiformes* (when included) and crown Teleostei;
- 2) a clade including the above, plus †*Dorsetichthys*, †*Siemensichthys* (and †*Ankylophorus* when included), and †*Eurycormus*;
- 3) †*Pholidophoridae sensu stricto*, as defined by (34) as sister to this clade;
- 4) †*Aspidorhynchidae* and †*Pachycormiformes* outside of the clade formed by all of the above. †*Ichthyokentema* is a wildcard taxon and has a different position in every study where it is included.

Since the positions of the different taxa constitutive of these various clades vary from one study to another, we cannot choose which topology is the most likely to reflect reality. In that context, we have chosen to follow the study that has the most comprehensive taxon sample and the best resolution for each portion of the total-group teleost tree: (25) for the clade described in (1), and (34) for the rest of the tree.

- †*Tharsis dubius* (Blainville, 1818)

**Specimen number:** NHMUK PV OR 37852b

**Position:** sister to [†*Ascalabos* + †*Varasichthyidae* + †*Ichthyodectiformes* + crown Teleostei] according to (25)

**Position in the reference tree:** sister to the clade comprising †*Xiphactinus audax*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Kimmeridgian-Tithonian / Solnhofen, Germany –157.3-145 Ma

**Minimum divergence time:** †*Occithrissops wilsoni* (38) is the oldest representative of the clade †*Ascalabos* forms with crown teleosts (25) – Bathonian, 168.3-166.1 Ma.

- †*Leptolepis coryphaenoides* (Bronn, 1830)

**Specimen number:** NHMUK PV P 947c

**Position:** sister to [all of the above, including crown Teleostei] according to (25, 34, 36, 39)

**Position in the reference tree:** sister to the clade comprising †*Tharsis dubius*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Toarcian / Curcy, France – 182.7-174.1 Ma (26)

**Minimum divergence time:** †*Leptolepis coryphaenoides* itself is the oldest representative of the clade it forms with crown teleosts.

- †*Leptolepis disjectus* Woodward, 1895

**Specimen number:** NHMUK PV P 3793a

**Position:** †*Leptolepis disjectus* is not included in any phylogenetic analysis, therefore we tentatively consider it to be closely related to the congeneric †*L. coryphaenoides*

**Position in the reference tree:** sister to †*Leptolepis coryphaenoides*

**Age / Locality:** Bathonian / Thaynton Limestone, UK – 168.3-166.1 Ma

**Minimum divergence time:** †*Leptolepis coryphaenoides* is the oldest representative of the clade †*Leptolepis* forms with crown teleosts – 182.7-174.1 Ma.

- †*Ichthyokentema purbeckensis* (Davies, 1887)

**Specimen number:** NHMUK PV P 7807

**Position:** sister to [†*Leptolepis coryphaenoides* + all of the above, including crown Teleostei], according to (34)

**Position in the reference tree:** sister to the clade comprising †*Leptolepis coryphaenoides*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Berriasian / Purbeck Group, UK – 145-139.8 Ma

**Minimum divergence time:** †*Leptolepis coryphaenoides* is the oldest representative of the clade †*Ichthyokentema* forms with crown teleosts – 182.7-174.1 Ma.

- †*Dorsetichthys bechei* (Agassiz, 1837)

**Specimen number:** OUMNH J.3369

**Position:** sister to [†*Ichthyokentema* + all of the above, including crown Teleostei] according to (34)

**Position in the reference tree:** sister to the clade comprising †*Ichthyokentema purbeckensis*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Sinemurian / Lyme Regis, UK – 199.3-190.8 Ma (26, 34)

**Minimum divergence time:** †*Dorsetichthys* itself is the oldest representative of the clade it forms with crown teleosts.

- †*Siemensichthys macrocephalus* (Agassiz, 1834)

**Specimen number:** NHMUK PV P 9135

**Position:** sister to [†*Dorsetichthys* + all of the above, including crown Teleostei] according to (34)

**Position in the reference tree:** sister to the clade comprising †*Dorsetichthys bechei*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Kimmeridgian-Tithonian / Solnhofen, Germany –157.3-145 Ma

**Minimum divergence time:** †*Dorsetichthys bechei* is the oldest representative of the clade †*Siemensichthys* forms with crown teleosts – 199.3-190.8 Ma.



- †*Ankylophorus similis* (Woodward, 1895)

**Specimen number:** NHMUK PV P 1083

**Position:** sister to †*Siemensichthys* according to (34, 36)

**Position in the reference tree:** sister to †*Siemensichthys macrocephalus*

**Age / Locality:** Kimmeridgian-Tithonian / Solnhofen, Germany –157.3-145 Ma

**Minimum divergence time:** †*Ankylophorus* and †*Siemensichthys* themselves are the oldest representatives of the clade they form together (†Ankylophoridae).

- †*Eurycormus speciosus* Wagner, 1863

**Specimen number:** MB.f.7016

**Position:** sister to [†Ankylophoridae + all of the above, including crown Teleostei] according to (34)

**Position in the reference tree:** sister to the clade comprising †*Siemensichthys macrocephalus*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Kimmeridgian-Tithonian / Solnhofen, Germany –157.3-145 Ma

**Minimum divergence time:** †*Dorsetichthys bechei* is the oldest representative of the clade †*Eurycormus* forms with crown teleosts – 199.3-190.8 Ma.

- †*Pholidophoropsis caudalis* (Woodward, 1895)

**Specimen number:** OUMNH J.3363

**Position:** according to (34), part of †Pholidophoridae *sensu stricto*, a clade of Triassic and Early Jurassic stem Teleostei, represented in the phylogenetic analysis by Triassic genera and not by †*Pholidophoropsis*. The clade is sister to [†*Eurycormus* + all of the above, including crown Teleostei], according to (26, 34)

**Position in the reference tree:** sister to the clade comprising †*Eurycormus speciosus*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Sinemurian / Lyme Regis, UK – 199.3-190.8 Ma

**Minimum divergence time:** †*Pholidophoretes salvus* and †*Knerichthys bronni* are the oldest representatives of the †Pholidophoridae *sensu stricto*, and thus of the clade †*Pholidophoropsis* forms with crown teleosts – early Carnian, 237-227 Ma (34).

- †*Pholidophoroides crenulata* Egerton, 1843

**Specimen number:** NHMUK PV OR 36313

**Position:** according to (34), part of †Pholidophoridae *sensu stricto*, a clade of Triassic and Early Jurassic stem Teleostei, represented in the phylogenetic analysis by Triassic genera and not by †*Pholidophoroides*. The clade is sister to [†*Eurycormus* + all of the above, including crown Teleostei], according to (26, 34)

**Position in the reference tree:** sister to †*Pholidophoropsis caudalis*

**Age / Locality:** Sinemurian / Lyme Regis, UK – 199.3-190.8 Ma

**Minimum divergence time:** as †*Pholidophoroides* and †*Pholidophoropsis* were never included in a formal phylogenetic analysis, their position within †Pholidophoridae is unknown. Since they are both of the same age and locality, we chose the conservative approach to give their own age as the minimum divergence time for the clade they form together.

- †*Protosphyraena* sp.

**Specimen number:** NHMUK PV P 9644b

**Position:** in †Pachycormiformes (40, 41), the latter forming a clade with †Aspidorhynchiformes that is sister to all other total-group Teleostei, according to (26, 34, 36)

**Position in the reference tree:** sister to the clade comprising †*Prohalecites porroi*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Coniacian-Campanian / Smoky Hill Member, Niobrara Group, USA – 89.8-72.1 Ma

**Minimum divergence time:** †*Prohalecites porroi* is the oldest representative of the total-group Teleostei – Ladinian-Carnian, 237 Ma (34, 42).

- †*Hypsocormus* sp.

**Specimen number:** NHMUK PV P 46348b

**Position:** in †Pachycormiformes, sister to the 'macrocarnivore clade' comprising †*Protosphyraena* (40, 41, 43)

**Position in the reference tree:** sister to †*Protosphyraena* sp.

**Age / Locality:** Callovian / Oxford Clay, UK – 166.1-163.6 Ma

**Minimum divergence time:** †*Hypsocormus* itself is the oldest representative of the clade it forms with †*Protosphyraena* (40, 43).

- †*Rhinconichthys taylori* Friedman *et al.*, 2010

**Specimen number:** NHMUK PV OR 33219

**Position:** in †Pachycormiformes, sister to the clade comprising †*Hypsocormus* and †*Protosphyraena* (40, 41, 43)

**Position in the reference tree:** sister to the clade comprising †*Protosphyraena* sp., †*Hypsocormus* sp., their most recent common ancestor and all its descendants

**Age / Locality:** Lower Chalk, Cenomanian / Burnham, UK – 100.5-93.3 Ma (41)

**Minimum divergence time:** †*Pachycormus*, †*Euthynotus* and other taxa are the oldest representatives of the clade †*Rhinconichthys* forms with †*Protosphyraena* and †*Hypsocormus* (40, 43) – late Toarcian, 182.7-174.1 Ma.

- †*Pachycormus macropterus* (Blainville, 1818)

**Specimen number:** MNHN.F.STC103 (as. †*P. curtus*)

**Position:** in †Pachycormiformes, sister to the 'filter-feeding clade' comprising †*Rhinconichthys* (40, 41, 43). †*Pachycormus curtus* is considered a junior synonym of †*P. macropterus* by (43)

**Position in the reference tree:** sister to †*Rhinconichthys taylori*

**Age / Locality:** late Toarcian / Curcy, France – 182.7-174.1 Ma

**Minimum divergence time:** †*Pachycormus* itself is the oldest representative of the clade it forms with †*Rhinconichthys* (40, 43).

- †*Euthynotus incognitus* (Blainville, 1818)

**Specimen number:** NHMUK PV P 2044

**Position:** in †Pachycormiformes, sister to all other members of the clade (40, 41, 43)

**Position in the reference tree:** sister to the clade comprising †*Protosphyraena* sp., †*Pachycormus macropterus*, their most recent common ancestor and all its descendants

**Age / Locality:** Toarcian / *Posidonia* shale, Holzmaden, Germany – 182.7-174.1 Ma

**Minimum divergence time:** †*Euthynotus* itself is the oldest representative of †Pachycormiformes (40, 43).

- †*Vinctifer comptoni* (Agassiz, 1841)

**Specimen number:** UMMP 101950

**Position:** in †Aspidorhynchiformes, themselves sister to †Pachycormiformes according to (26, 34, 36)

**Position in the reference tree:** sister to the clade (†Pachycormiformes) comprising †*Euthynotus incognitus*, †*Pachycormus macropterus*, their most recent common ancestor and all its descendants

**Age / Locality:** early Albian / Romualdo Member of the Santana Formation, Brazil – 113.4-110 Ma (44)

**Minimum divergence time:** †*Euthynotus incognitus* is the oldest representative of the [†Pachycormiformes + †Aspidorhynchiformes] clade (26, 40) – Toarcian, 182.7-174.1 Ma.

- †*Belonostomus* sp.

**Specimen number:** NHMUK PV P 4029

**Position:** in †Aspidorhynchiformes, sister to †*Vinctifer* according to (44)

**Position in the reference tree:** sister to †*Vinctifer comptoni*

**Age / Locality:** early to middle Cenomanian / Namoura, Lebanon – 99.7-94.4 Ma (7, 44)

**Minimum divergence time:** †*Belonostomus tenuirostris* and †*B. dorsetensis* are the oldest representatives of †*Belonostomus* – Kimmeridgian, 157.3-152.1 Ma (44).

**- †*Belonostomus muensteri* (Agassiz, 1834)**

**Specimen number:** NHMUK PV OR 37796

**Position:** in †*Belonostomus* according to (44)

**Position in the reference tree:** sister to †*Belonostomus* sp.

**Age / Locality:** Tithonian / Solnhofen Limestone, Germany – 152.1-145 Ma (44)

**Minimum divergence time:** †*Belonostomus tenuirostris* and †*B. dorsetensis* are the oldest representatives of †*Belonostomus* – Kimmeridgian, 157.3-152.1 Ma (44).

**- †*Aspidorhynchus fisheri* Egerton, 1854**

**Specimen number:** NHMUK PV P 17317

**Position:** in †Aspidorhynchiformes, sister to [†*Belonostomus* + †*Vinctifer*] according to (44)

**Position in the reference tree:** sister to the clade comprising †*Vinctifer comptoni*, †*Belonostomus* sp., their most recent common ancestor and all its descendants

**Age / Locality:** Berriasian / Purbeck Group, UK – 145-139.8 Ma

**Minimum divergence time:** †*Aspidorhynchus crassus* is the oldest representative of †Aspidorhynchiformes – Bathonian, 168.3-166.1 Ma (44).

**- †*Aspidorhynchus acutirostris* (Blainville, 1818)**

**Specimen number:** NHMUK PV P 97

**Position:** in †*Aspidorhynchus* according to (44)

**Position in the reference tree:** sister to †*Aspidorhynchus fisheri*

**Age / Locality:** Kimmeridgian / Kimmeridge Clay, UK – 157.3-152.1 Ma

**Minimum divergence time:** †*Aspidorhynchus crassus* is the oldest representative of †Aspidorhynchiformes – Bathonian, 168.3-166.1 Ma (44).

**- †*Semionotus elegans* Newberry, 1888**

**Specimen number:** UMMP 13664

**Position:** stem Gynglimodi according to (26)

**Position in the reference tree:** sister to the clade (crown Gynglimodi) comprising *Atractosteus tropicus*, *Lepisosteus osseus*, their most recent common ancestor and all its descendants

**Age / Locality:** Hettangian-Sinemurian / Newark Supergroup, USA – 201.3-190.8 Ma (26, 45)

**Minimum divergence time:** †*Semionotus bergeri* is the oldest representative of the clade †*S. elegans* forms with crown Gynglimodi – Carnian, 237-227 Ma (26).

**- †*Heterolepidotus dorsalis* Kner, 1866**

**Specimen number:** NHMUK PV P 10290

**Position:** stem Halecomorphi, potentially close to †*Ophiopsis* according to (46) but never included in a phylogenetic analysis

**Position in the reference tree:** sister to *Amia calva* (crown Halecomorphi)

**Age / Locality:** Rhaetian / Hallein, Germany – 208.5-201.3 Ma

**Minimum divergence time:** †*Heterolepidotus* itself is the oldest representative of the clade it forms with crown Halecomorphi.

**- †*Eoeugnathus megalepis* Brough, 1939**

**Specimen number:** PIMUZ T344

**Position:** stem Halecomorphi, close to †*Panxianichthys* and thus outside of the clade formed by †*Ophiopsis* (and thus †*Heterolepidotus*) and *Amia* according to (26, 47)

**Position in the reference tree:** sister to the clade comprising †*Heterolepidotus dorsalis*, *Amia calva*, their most recent common ancestor and all its descendants

**Age / Locality:** Ladinian / Monte San Giorgio, Switzerland – 242-237 Ma

**Minimum divergence time:** †*Panxianichthys imparilis* is the oldest representative of the clade †*Eoeugnathus* forms with crown Halecomorphi – Anisian, 247.2-242 Ma.

- †*Dapedium* cf. *politum* Leach, 1822

**Specimen number:** OUMNH J.3323

**Position:** in †Dapediiformes, stem Holostei according to (26, 48)

**Position in the reference tree:** sister to the clade (crown Holostei) comprising *Lepisosteus osseus*, *Amia calva*, their most recent common ancestor and all its descendants

**Age / Locality:** Sinemurian / Lyme Regis, UK – 199.3-190.8 Ma (49)

**Minimum divergence time:** †*Watsonulus eugnathoides* is the oldest representative of crown Holostei, and of the clade †*Dapedium* forms with them – Induan-Olenekian, 251.9-247.2 Ma (26, 50).

- †*Perleidus* sp.

**Specimen number:** NHMUK PV P 26456

**Position:** stem Neopterygii, according to (51, 52)

**Position in the reference tree:** sister to the clade (crown Neopterygii) comprising *Amia calva*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Proptychites beds, Induan / Stensjö Plateaus, Greenland – 251.9-251.2 Ma

**Minimum divergence time:** †*Perleidus* itself is the oldest representative of the clade it forms with crown Neopterygii (53).

- †*Pholidopleurus typus* Bronn, 1858

**Specimen number:** NHMUK PV P 19314

**Position:** in †Pholidopleuridae, stem Neopterygii, outside of [†*Perleidus* + crown Neopterygii] according to (51, 52)

**Position in the reference tree:** sister to the clade comprising †*Perleidus* sp., *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Anisian-Ladinian / Besano, Italy – 247.2-237 Ma

**Minimum divergence time:** †*Perleidus* is the oldest representative of the clade †*Pholidopleurus* forms with crown Neopterygii – 251.9-251.2 Ma.

- †*Proscinetes elegans* (Agassiz, 1833)

**Specimen number:** NHMUK PV P 1626

**Position:** in †Pycnodontiformes, that are stem Neopterygii according to (48). †Pycnodontiformes, †*Pholidopleurus* and †*Perleidus* have never been included in the same phylogenetic analysis, so we choose the conservative approach and place †*Proscinetes* outside of the clade [†*Perleidus* + †*Pholidopleurus* + crown Neopterygii]

**Position in the reference tree:** sister to the clade comprising †*Pholidopleurus typus*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** lower Tithonian / Solnhofen, Germany – 152.1-145 Ma (54)

**Minimum divergence time:** †*Perleidus* is the oldest representative of the clade †*Proscinetes* forms with crown Neopterygii – 251.9-251.2 Ma.

- †*Birgeria stensioei* Aldinger, 1931

**Specimen number:** PIMUZ T2188

**Position:** traditionally viewed as a stem Chondrostei (50), †*Birgeria* is a stem Actinopteri according to (55)

**Position in the reference tree:** sister to the clade (crown Actinopteri) comprising *Acipenser transmontanus*, *Megalops cyprinoides*, their most recent common ancestor and all its descendants

**Age / Locality:** Anisian-Ladinian / Besano, Italy – 247.2-237 Ma (56)

**Minimum divergence time:** †*Discoserra pectinodon* is the oldest representative of the total-group Neopterygii, and crown Actinopteri (50, 55) – Bashkirian, 323.2-315.2 Ma.

### I.3. Quality control criteria and specimens excluded from the analysis

We performed an initial quality-control evaluation of our scan data based on a list of three objective criteria. Specimens that did not pass any one of these criteria were not included in our analysis of evolution of osteocyte volumes and inferred genome size through time:

- (1) It was possible to confidently identify osteocyte lacunae in the PPC-SR $\mu$ CT tomograms. Specimens lacking osteocytes, or in which osteocytes could not be confidently identified were not included in the analysis;
- (2) The osteocyte lacunae were well-preserved or well-defined enough in the PPC-SR $\mu$ CT tomograms to guarantee that their volumes would be accurately measured. Poor definition or preservation could result from taphonomic factors (e.g. infilling of the lacunae by mineral-rich fluids) or issues with the scans themselves causing low contrast between fossil bone and its lacunae;
- (3) Sufficiently large numbers of osteocyte lacunae were present in the scan (count > ~50) to provide quantitative confidence in estimates of their average volumes. Specimens that had fewer osteocyte lacunae, even if they were well-defined, were excluded from the analysis.

**- †*Oncorhynchus lacustris* (Cope, 1870)**

**Specimen numbers:** UMMP 47839, UMMP 52619, UMMP 21756.

**Position:** in Salmonidae, close to *Oncorhynchus mykiss* according to (2, 3)

**Age / Locality:** Miocene / Chalk Hills Formation, USA – 9.8-4.8 Ma (2, 4)

**Failed criterion 2.**

**- †*Paleolox larsoni* Kimmel, 1975**

**Specimen number:** UMMP 50352.

**Failed criterion 2.**

**- †*Lycoptera middendorffi* Müller, 1848**

**Specimen number:** NHMUK PV P 6728.

**Position:** stem Osteoglossomorpha according to (28)

**Age / Locality:** Early Cretaceous / Mt Uksuk, Mongolia – 136.4-125.5 Ma

**Failed criterion 2.**

**- †*Anaethalion angustus* (Münster, 1839)**

**Specimen number:** NHMUK PV P 3575.

**Failed criteria 2 and 3.**

**- †*Cladocycclus gardneri* Agassiz, 1841**

**Specimen number:** UMMP 117484.

**Position:** in †Ichthyodectiformes, family †Cladocyclidae (37)

**Age / Locality:** Aptian-Albian / Crato and Santana Formations, Brazil – 123-110 Ma (37)

**Failed criteria 2 and 3.**

**- †*Ascalabos voithii* Münster, 1839**

**Specimen number:** NHMUK PV P 3673a.

**Position:** sister to [†Ichthyodectiformes + crown Teleostei] according to (25)

**Age / Locality:** Kimmeridgian / Kelheim, Germany – 157.3-152.1 Ma

**Failed criteria 2 and 3.**

**- †*Lombardichthys gervasuttii* (Zambelli, 1980)**

**Specimen number:** MCSNB 6662.

**Position:** part of †Pholidophoridae *sensu stricto* (36)

**Age / Locality:** Norian / Ponte Giurino, Italy – 210 Ma (34, 36)

**Failed criterion 1.**

**- †*Pholidorhynchodon malzannii* Zambelli, 1980**

**Specimen number:** MCSNB 11015.

**Position:** part of †Pholidophoridae *sensu stricto* (34, 36)

**Age / Locality:** Norian / Cene, Italy – 210 Ma (34, 36)

**Failed criterion 1.**

- †*Parapholidophorus nybelini* Zambelli, 1975

**Specimen number:** MCSNB 6530c.

**Position:** part of †Pholidophoridae *sensu stricto* (34, 36)

**Age / Locality:** Norian / Cene, Italy – 210 Ma (34, 36)

**Failed criterion 1.**

- †*Pholidoctenus serianus* Zambelli, 1977

**Specimen number:** MCSNB 4827.

**Position:** part of †Pholidophoridae *sensu stricto* (34, 36)

**Age / Locality:** Norian / Cene, Italy – 210 Ma (34, 36)

**Failed criterion 1.**

- †*Prohalecites porroi* (Bellotti, 1857)

**Specimen number:** NHMUK PV P 19471.

**Position:** sister to [†Pholidophoridae *sensu stricto* + all other total-group Teleostei] according to (34, 36)

**Age / Locality:** Ladinian-Carnian boundary / Perledo, Italy – 237 Ma (42)

**Failed criteria 2 and 3.**

- †*Pachycormus macropterus* (Blainville, 1818)

**Specimen number:** NHMUK PV P 32433.

**Failed criterion 2.**

- †*Aspidorhynchus acutirostris* (Blainville, 1818)

**Specimen number:** NHMUK PV P 56466.

**Failed criterion 2.**

- †*Cheirolepis canadensis* Whiteaves, 1881

**Specimen number:** UMMP 3453.

**Position:** stem Actinopterygii according to (48, 55, 57, 58)

**Age / Locality:** Escuminac Formation, Frasnian / Miguasha, Canada – 382.7-372.2 Ma (59)

**Failed criterion 2.**

## II. Comments on Triassic stem-group teleosts

Stem-group teleosts are known since the Middle Triassic, the oldest ones being the pholidophorids †*Malingichthys nimaiguensis* and †*M. wanfenglinensis* from the late Ladinian (239-238 Ma) of southern China (60). These species are slightly older than †*Prohalecites porroi* from the latest Ladinian (237 Ma) of the Perledo-Varenna Formation (Grigna Mountains, Italy) and Kalkschieferzone (Meride Limestone) of Monte San Giorgio (42). In order to reflect as accurately as possible the temporal distribution of total-group teleosts, we included five Triassic stem-group teleosts in our sample: †*Prohalecites porroi* (NHMUK PV P 19471), as well as the Norian †*Lombardichthys gervasuttii* (MCSNB 6662), †*Pholidorhynchodon malzannii* (MCSNB 11015), †*Parapholidophorus nybelini* (MCSNB 6530c) and †*Pholidoctenus serianus* (MCSNB 4827) from Northern Italy (34, 36). All of these five Triassic taxa were represented by one sample from the dentary (lower jaw), and SRμCT-scanned at the Diamond Light Source I13-2 beamline, U.K. There were no issues with the SRμCT data acquisition itself, however, none of the four Norian specimens apparently preserved osteocyte lacunae. The apparent absence of preserved osteocyte lacunae may be related to the presence of abundant syndimentary pyrite in fossils from these localities (61), which may have affected the skeletal tissue obliterating any evidence of cellular structure, but additional data on the taphonomy of the localities would be needed to substantiate this hypothesis. There is no reason to believe that osteocytes were absent in these taxa (i.e. anosteocytic bone (62, 63)), because other histological structures (e.g., growth lines, lines of reversal) were not visible either. Conversely, osteocyte lacunae have been detected and segmented in †*Prohalecites porroi*. However, only very few of them were preserved in the examined sample for this species and their preservation was poor, limiting their statistical power and potentially introducing a bias. For this reason, and because of the strong influence of this taxon on the results, we chose not to include it in the analyses. Due to the impossibility to obtain information from Triassic stem-group teleosts, the oldest total-group teleosts present in our sample are the Early Jurassic (Sinemurian) †*Pholidophoroides crenulata* and †*Pholidophoropsis caudalis* from Lyme Regis, U.K. Future investigations should aim to include Triassic stem-teleosts in order to more precisely define the age of the teleost WGD. Additional taxa not sampled in this study which could be targeted are †*Annaichthys pontegiuriniensis*, †*Eopholidophorus forojuliensis*, †*Jianglichthys* sp., †*Knerichthys bronni*, †*Parapholidophorus caffii*, †*Pholidophorettes salvus*, †*Pholidophorus gervasuttii*, †*Pholidophorus latiusculus* and †*Zambellichthys bergamensis* (34, 64). In addition, a few enigmatic Middle Triassic neopterygians may represent the earliest stem-group teleosts, including the middle Anisian (about 242 Ma) †*Marcopoloichthys ani* from the Upper Member of the Guangling Formation, Yunnan, South China (65). This taxon is characterized by a unique combination of features, including an apparently mobile premaxilla, a dorso-ventrally symmetrical caudal fin with a reduced number of hypurals, and an extreme reduction of the cover of ganoid scales (65, 66). A reevaluation of the phylogenetic position of this taxon would be desirable to address key questions about the earliest stages of teleost evolution, the origin of teleost morphological innovations and of their WGD.

## III. Synchrotron beamline setups

### III.1. DLS I13-2 Diamond-Manchester Imaging beamline setup

The X-ray beam was generated by a 2 m long in vacuum undulator (gap 5 mm), filtered with pyrolytic graphite (1340 μm), aluminium (2100 μm) and molybdenum (84 μm). Additionally, a rhodium mirror was used to cut high energies. It resulted in a weighted mean detected energy of 21.39 keV. Radiographs were recorded using an indirect detector comprising a 34 μm GGG scintillator, a 10x microscope lens, a 2x eyepiece and a PCO.edge 5.5 sCMOS camera (PCO AG, Kelheim, Germany). The effective recorded pixel size for this system was 0.357 μm. Based on the pixel size and the detected energy, the sample-detector propagation distance was set to 17 mm. The acquisition consisted of 2500 projections recorded over a 180° rotation of the sample, with an exposure time of 0.5 s per projection. Additionally, 20 flatfield images (sample out

of the beam) were recorded before and after the scan as well as 20 dark images before the beginning of the scan (X-ray beam off to record the noise of the camera). Tomographic reconstruction was performed using the Savu processing pipeline (67) and the I13 analysis Python package. The workflow consisted of a dezing correction (i.e. removing the scattering spikes), an optical distortion correction, ring removal on sinogram, single distance phase retrieval using the Paganin filter (68), tomographic reconstruction using TomoPy (69), and a conversion of the 32-bits HDF5 volume as a 16-bits tiff stack.

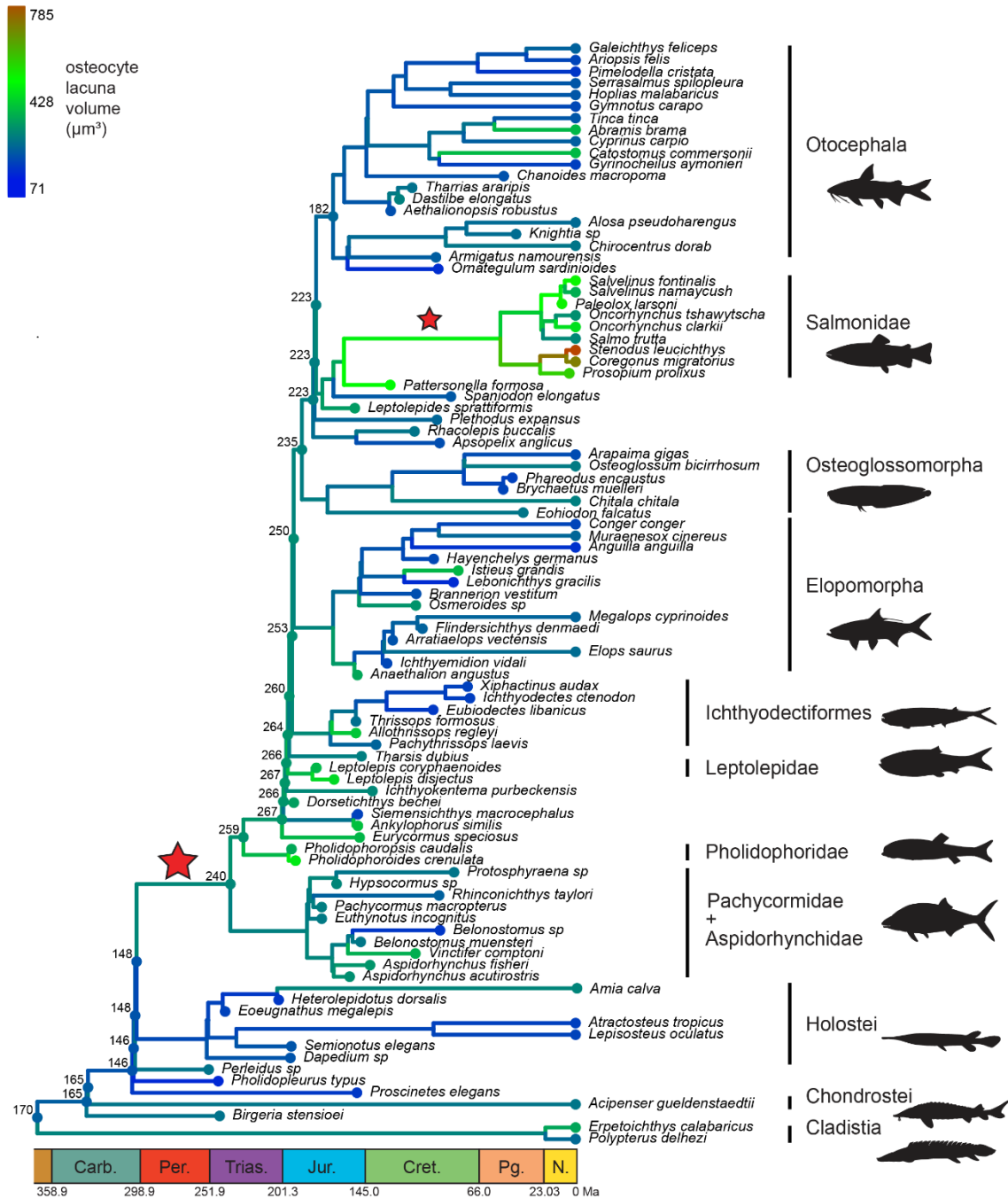
### **III.2. ESRF ID19 beamline setup**

The ID19 beamline could be tuned for a much broader range of energies and the setup was regularly changed to adjust to the sample (see Supplementary Data). The X-ray beam was generated either by a wiggler or a single harmonic undulator, depending on the required energy. Filters were also adjusted depending on the energy. Beryllium X-ray lenses were used to focus the beam and increase flux on the sample (number of lenses adjusted with energy). Finally, we used a 1 mm graphite rotating disk as a decoherer and reduce potential ring artefact (70). Radiographs were recorded using an indirect detector comprising a scintillator (10  $\mu\text{m}$  GGG or 25  $\mu\text{m}$  LuAG), a 10x microscope lens and a PCO.edge camera (5.5 or 4.2  $\mu\text{m}$  gold). The effective pixel size for this system was ca. 0.7  $\mu\text{m}$  (as small variations were induced by changing the propagation distance). Based on the pixel size and the detected energy, the sample-detector propagation distance was adjusted, ranging from 20 to 220 mm. The acquisition consisted of 2999 projections recorded over a 360° rotation of the sample. Exposure time was ranging from 0.03 to 0.2 seconds, as transmitted signal was getting lower as the energy increased. Additionally, 21 flatfield images (sampled out of the beam) were recorded before and after the scan as well as 20 dark images before the beginning of the scan (X-ray beam off to record the noise of the camera). Tomographic reconstruction was performed using PyHST2 (71). The workflow consisted of a tomographic reconstruction using the single distance phase retrieval approach of PyHST2, followed by a conversion of the 32-bits EDF stack as a 16-bits tiff stack. Additional processing included ring correction (72).

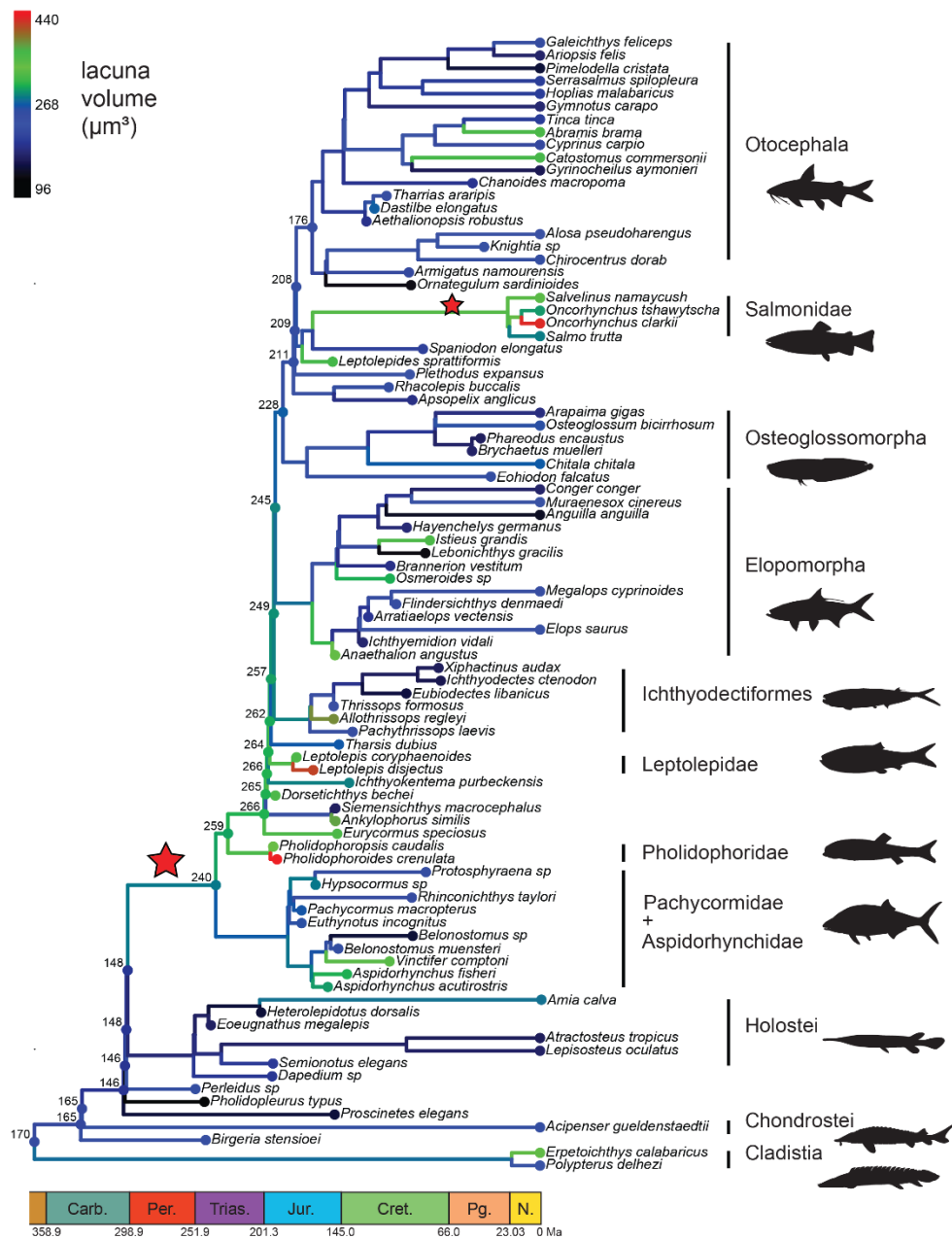
### **III.3. Note on the resolution**

Outside of the higher energies available at the ESRF ID19 beamline, the second main difference between both beamlines was the magnification setups, generating data with a recorded voxel size of 0.357  $\mu\text{m}$  at the I13-2 beamline and near 0.7  $\mu\text{m}$  at the ID19 beamline. It is important to note that these values indicate the magnification only and not the resolution, i.e. the smallest feature observable in the resulting tomographic data. Several parameters will influence the resolution, including notably the brilliance and spatial coherence of the incoming X-ray beam, the thickness and composition of the scintillator, the optical lenses and the digital camera. As the aim of this project is to measure osteocyte volumes, the variation in magnification and possibly in resolution could affect: 1) the smallest osteocyte detected; 2) the volume of osteocytes by adding or removing voxels at the periphery of the volume. The smallest osteocytes measured have a volume of ca. 70  $\mu\text{m}^3$ ; such a volume would be defined by ca. 1500 voxels and 195 voxels for voxel sizes of 0.357  $\mu\text{m}$  and 0.7  $\mu\text{m}$  respectively. The variation in voxel size hence could not influence the detection of the smallest osteocytes in our dataset.

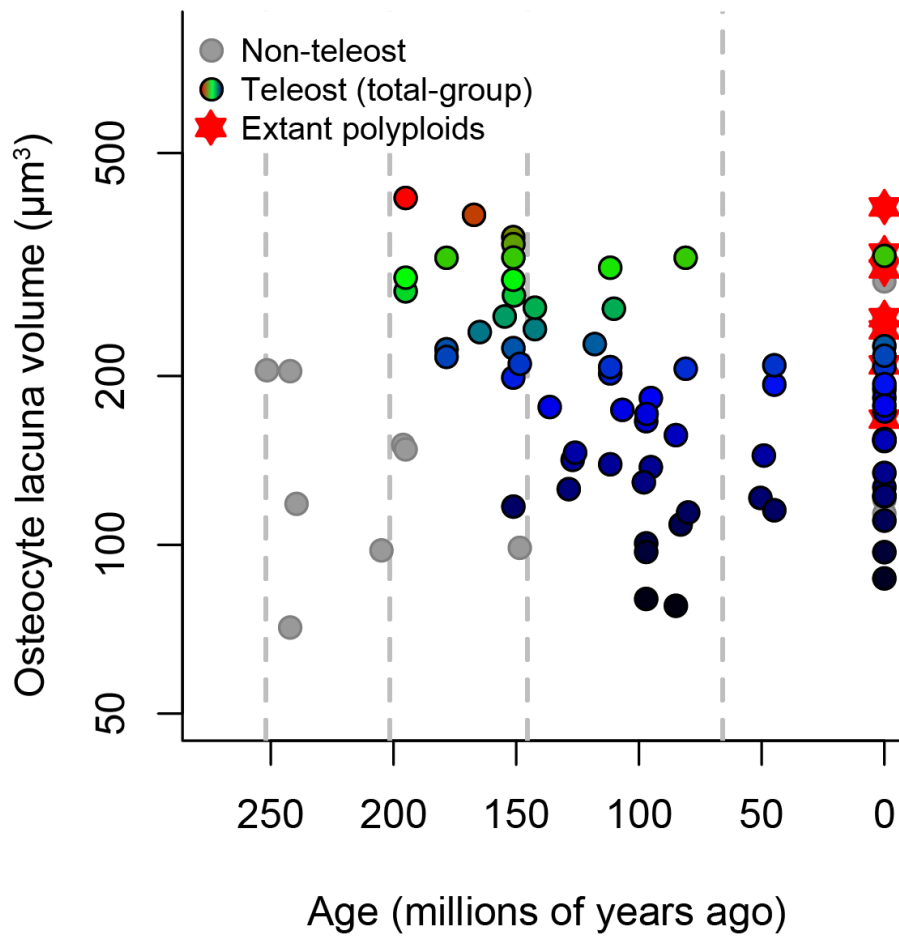




**Figure S1.** Evolution of osteocyte lacuna volume in fossil and modern teleosts revealed by synchrotron microtomography. Timescaled composite phylogeny of actinopterygians with mapped cell-size volumes for fossil and modern lineages. This version includes terminal taxon names.



**Figure S2.** Evolution of osteocyte lacuna volume in fossil and modern teleosts revealed by synchrotron microtomography. Alternative version of Fig. 1 where *Salvelinus fontinalis*, *Coregonus migratorius*, *Stenodus leucichthys*, *Prosopium prolixus*, *Paleolox larsoni*, and *Pattersonella formosa* were omitted due to their very large osteocyte lacuna volumes. Therefore, the color scale distinguishes better the variation in volume of most of the taxon sample. This scale is different to that of Figs. 1-2.



**Figure S3.** Evolution of osteocyte lacuna volume in fossil and modern teleosts revealed by synchrotron microtomography, plotted against time. Alternative version of Fig. 2, omitting the same taxa and using the same color scale as Fig. S2.

**Dataset S1 (separate file).** Full list of specimens used in the study, with their age ranges, the specifier taxa for their sister clades, and minimum divergence times from those sister clades based on fossil occurrences.

**Dataset S2 (separate file).** Full list of specimens for which PPC-SRμCT data were obtained, with the corresponding detailed experimental setups.

**Dataset S3 (separate file).** Tree file used in our analysis, obtained from stitching fossil taxa to the reference tree (1).

**Dataset S4 (separate file).** Full list of specimens, with the corresponding median osteocyte volumes using the minimum and maximum realistic grey value thresholds. We only used the “max” values for the analyses.

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