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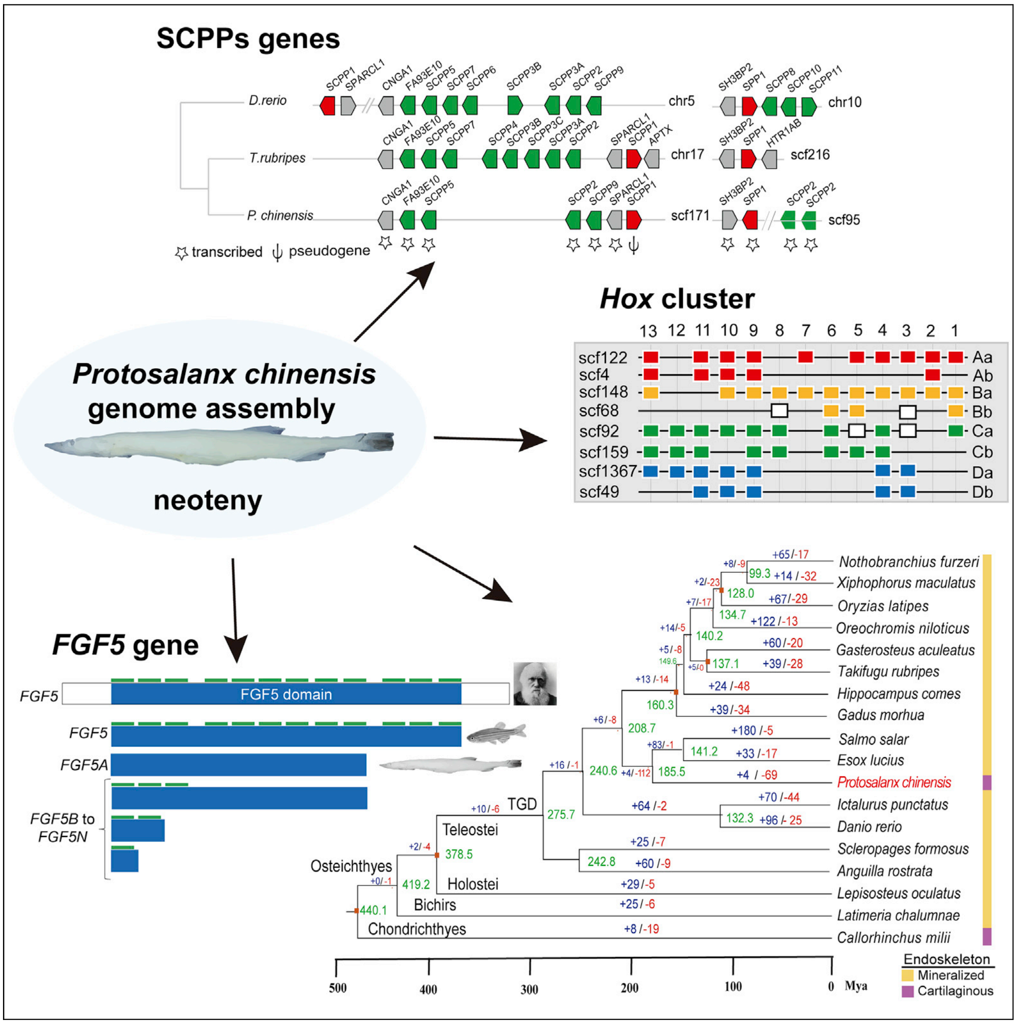

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HIGHLIGHTS

Generated chromosome-level genome of the Asian icefish *Protosalanx chinensis*

Larval features present in adult *P. chinensis* (neoteny)

Identified genes underlying icefish neoteny, including an adult cartilaginous skeleton

Valuable resource for wet-lab and genome research on icefishes

Zhang et al., iScience *23*, 101267
July 24, 2020 © 2020 The Author(s).
https://doi.org/10.1016/j.isci.2020.101267

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Insights into the Evolution of Neoteny from the Genome of the Asian Icefish *Protosalanx chinensis*

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SUMMARY

Salangids, known as Asian icefishes, represent a peculiar radiation within the bony fish order Protacanthopterygii where adult fish retain larval characteristics such as transparent and miniaturized bodies and a cartilaginous endoskeleton into adulthood. Here, we report a *de novo* genome of *Protosalanx chinensis*, the most widely distributed salangid lineage. The *P. chinensis* genome assembly is more contiguous and complete than a previous assembly. We estimate that *P. chinensis*, salmons, trouts, and pikes diverged from a common ancestor 185 million years ago. A juxtaposition with other fish genomes revealed loss of the genes encoding ectodysplasin-A receptor (*EDAR*), SCPP1, and four *Hox* proteins and likely lack of canonical fibroblast growth factor 5 (*FGF5*) function. We also report genomic variations of *P. chinensis* possibly reflecting the immune system repertoire of a species with a larval phenotype in sexually mature individuals. The new Asian icefish reference genome provides a solid foundation for future studies.

INTRODUCTION

Bony vertebrates develop a mineralized endoskeleton from a cartilaginous larval scaffold (endochondral ossification), whereas chondrichthyans (chimeras, sharks, skates, and rays) retain a cartilaginous endoskeleton throughout life (Hirasawa and Kuratani, 2015). The two bony fish lineages, lobe-finned fishes (lungfishes and coelacanths) and ray-finned fishes, are collectively also known as teleosts, derived from the Greek *teleios* + *osteon*, "complete bone" (Brazeau and Friedman, 2015). A peculiar radiation is observed in order Osmeriformes of Protacanthopterygii, a teleost superorder that also includes Esociformes (e.g., pikes) and Salmoniformes (e.g., salmons and trouts). Osmeriformes comprises the six-genera (~17 species) family Salangidae of short-lived (lifespan ~12 months; sexual maturity at 7 months of age), morphologically similar fishes endemic to East Asia and mainly distributed in China (Zhang et al., 2007). Members of Salangidae are known by many colloquial names (e.g., [Asian] icefishes, salangids, whitefishes, and noodlefishes) (Roberts, 1984). Adult Asian icefishes are small, transparent, and scaleless. They possess several larval features, including a cartilaginous endoskeleton and notochords throughout life (Nelson, 2006; Wu and Lin, 1965; Roberts, 1984). Such morphological and structural variants, or "developmental deviations," are thought to be of great significance in fish (Wu and Lin, 1965). The present study aimed to explore the genetic features the enigmatic Asian icefish *Protosalanx chinensis* (Figure 1A). *P. chinensis* is one of the most ecologically plastic Asian icefish species (Roberts, 1984; Kang et al., 2015) and has the broadest geographical distribution (China, Korea, and Vietnam) (Kang et al., 2015). Freshwater stocks of *P. chinensis* occur in the inland lakes, reservoirs, and out-flowing rivers in China, whereas marine stocks are distributed in estuarine and coastal areas in east Asia (Zhang et al., 2007). We report an improved genome assembly and transcriptomes of *P. chinensis* and identify genomic variations that may be associated with its unusual features.

RESULTS AND DISCUSSION

Genome Assembly and Annotation

By integrating PacBio technology, 10X Genomics linked-read sequencing, and Illumina short-read sequencing, we constructed a 484.1 Mb *P. chinensis* genome assembly with a contig N50 size of 103 kb and a scaffold N50 size of 5.1 Mb (Tables S1–S3, Figures S1A and S1B). Our long-read assembly is superior to a previously published assembly (genome size 525 Mb; contig N50 17.2 kb, scaffold N50 1.16 Mb)

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https://doi.org/10.1016/j.isci.2020.101267

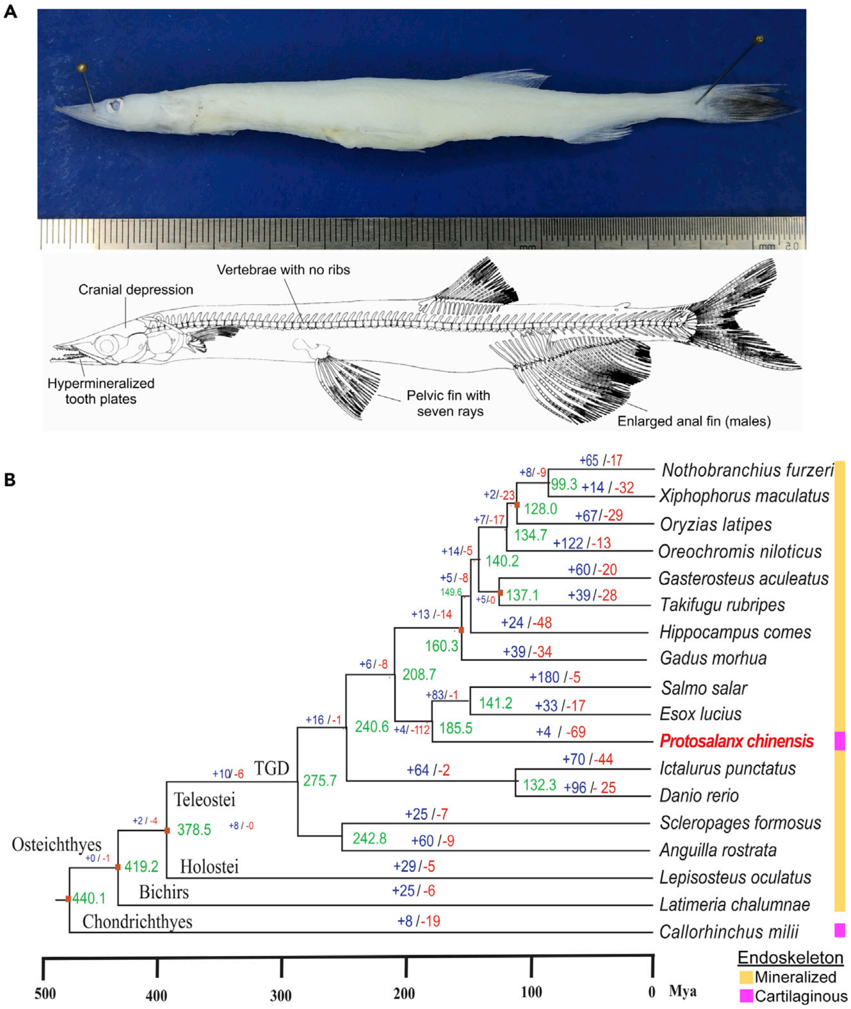

Figure 1. *P. chinensis* and a Phylogenetic Tree Showing Gene/Family Expansions/Contractions Analysis Compared with 17 Representative Fish Species

(A) Top: an adult female *P. chinensis*, an Asian icefish, collected from Chaohu lake, Anhui province. Bottom: skeletal features of *P. chinensis*. In contrast to other bony fishes, and similar to distantly related cartilaginous fishes such as the elephant fish (*Callorhinchus milii*), *P. chinensis* cannot produce a mineralized endoskeleton (e.g., neurocranium, vertebrae) from larval cartilage precursors. Prominent exoskeletal features (dermal bones, such as fin rays and teeth) of *P. chinensis* are indicated.

(B) Consensus phylogenetic tree of 18 teleost fishes. The tree was generated from 627 single-copy genes. The divergence times (million years ago; mya; shown in green) for all nodes were estimated based on the six red nodes with fossil records as calibration times and are marked in each node with error ranges. Gene family expansion events are marked in blue, and gene family contraction events in red. A gene duplication event at the base of teleosts (TGD) is indicated. The type of adult endoskeletal bone (cartilaginous or mineralized) is indicated in pink and yellow, respectively.

See also Figures S1–S3 and Tables S1–S14.

(Liu et al., 2017). The *P. chinensis* genome has an average GC content of 47.25%, higher than most other sequenced fishes (Table S4, Figures S1C and S1D). We mapped short-insert (250–500 bp) reads to the *P. chinensis* genome and found that 97.89% could be aligned (Table S5). The *P. chinensis* genome contains 31.97% repeat elements, the majority (10.77%) DNA transposons (Tables S6 and S7, Figure S1E). The assembly is of high quality, as >98% of *de novo* assembled transcripts could be mapped (Table S8). Moreover, CEGMA (Parra et al., 2007) and BUSCO (Waterhouse et al., 2017) completeness scores are 93% and 94%, respectively. The scores of the previous *P. chinensis* assembly (Liu et al., 2017) are 87% CEGMA and 85% BUSCO (Tables S9 and S10). We predicted 23,645 genes in the genome of *P. chinensis* by combining *ab initio* gene prediction, protein-based homology, and transcript-mapping strategies

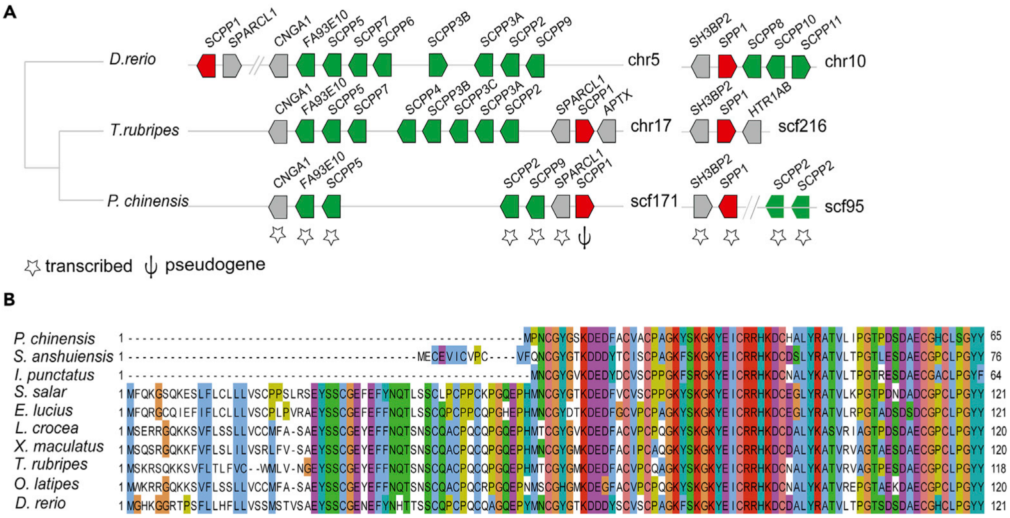
A
D.rerio
SCPP1
SPARCL1
CNGA1
FA93E10
SCPP5
SCPP7
SCPP6
SCPP3B
SCPP3A
SCPP2
SCPP9
chr5
SH3BP2
SPP1
SCPP8
SCPP10
SCPP11
chr10
T.rubripes
CNGA1
FA93E10
SCPP5
SCPP7
SCPP4
SCPP3B
SCPP3C
SCPP3A
SCPP2
SPARCL1
SCPP1
APTX
chr17
SH3BP2
SPP1
HTR1AB
scf216
P. chinensis
CNGA1
FA93E10
SCPP5
SCPP2
SCPP9
SPARCL1
SCPP1
scf171
SH3BP2
SPP1
SCPP2
SCPP2
scf95
transcribed
pseudogene
B
P. chinensis 1 --MPNCGYGSKDEDFACVACPAGKYSKGKYEICRRHKDCHALYRATVLIPGTPDSDAECGHCLSGYY 65
S. anshuiensis 1 ---MECEVICVPC----VFQNCGYGTKDDDYTCISCPAGKFSKGKYEICRRHKDCDSLYRATVLTPGTLESDAECGPCLPGYY 76
I. punctatus 1 ---MNCGYGVKDEDYDCVSCPPGKFSRGKYEICRRHKDCNALYKATVLTPGTRESDAECGACLPGYF 64
S. salar 1 MFQKGSQKESLFLCLLLVSCPPSLRSEYSSCGEFEFYNQTLSSCLPCPPCKPGQEPHMNCGYGTKDEDFVCVSCPPGKYSKGKYEICRRHKDCEGLYRATVLKPGTPDNDADCGPCLPGYY 121
E. lucius 1 MFQRGCQIEFIFLCLLLVSCPLPVRAEYSSCGEFEFFNQTLSSCQPCPPCQPGHEPHMNCGYDTKDEDFGCVPCPAGKYSKGKYEICRRHKDCEGLYRATVLRPGTADSDSDCGPCLPGYY 121
L. crocea 1 MSERRGQKKSVFLSSLLVCCMFA-SAEYSSCGEYEFFNQTSNSCQACPQCQPGQEPHMTCGYGVKDEDFACVPCPQGKYSKGKYEICRRHKDCDALYKASVRIAGTPDSDAECGPCLPGYY 120
X. maculatus 1 MSQSRGQKKFVFLSLILVSRLFV-SAEYSSCGEYEFFNQTSNSCQACPQCQPGQEPHMNCGYGMKDEDFACIPCAQGKYSKGKYEICRRHKDCDALYKATVRVAGTAESDAECGPCLPGYY 120
T. rubripes 1 MSKRSQKKSVFLTLFVC--WMLV-NGEYSSCGEYEFFNQTSNSCQACPQCQPGQEPHMTCGYGMKDEDFACVPCQAGKYSKGKYEICRRHKDCNALYKATVRVAGTPESDAECGPCLPGYY 118
O. latipes 1 MWKRRGQKKSVFLSSLLVCCMFA-SAEYSSCGEYEFFNQTSNSCQACPQCRPGQEPNMSCGHGMKDEGFACVPCPQGKYSKGKYEICRRHKDCNALYKATVREPGTAEKDAECGPCLPGYY 120
D. rerio 1 MGHKGGRTPSFLLHFLLVSSMSTVSAEYSSCGENEFYNHTTSSCQPCPQCQAGQEPYMNCGYGTKDDDYSCVSCPAGKFSKGKYEICRRHKDCDALYRATVLTPGTSDSDAECGHCLPGYY 121

Figure 2. Loss of Bone Formation and Maintenance Genes in *P. chinensis*

(A) Secretory calcium-binding phosphoproteins (SCPPs) genes in *P. chinensis*, *D. rerio* (zebrafish), and *T. rubripes* (fugu). *SPARCL1*, the ancestral SCPP gene, is shown in gray; P/Q-rich SCPP genes in green; acidic SCPP genes in red. In *P. chinensis*, *SCPP1* is a pseudogene (denoted by ψ).

(B) *EDAR* (ectodysplasin-A receptor) protein sequences from ten fish species were aligned by MUSCLE. The coding sequence of the *EDAR* signal peptide region and parts of the extracellular region is lost in *P. chinensis*, the scaleless channel catfish *I. punctatus*, and the cavefish *S. anshuiensis*.

See also Figures S4 and S5 and Tables S15 and S16.

(Table S11, Figure S1F). The gene length in *P. chinensis* assembly averages 8,530 kb. Average exon and intron sizes are 0.17 and 0.91 kb, respectively, similar to other teleost fish (Table S12, Figure S2). Non-coding RNA annotation revealed 95 rRNA, 1,382 tRNA, 1,327 miRNA, and 1,025 snRNA genes (Table S13).

Phylogenetic Placement of *P. chinensis*

Protosalanx is a monotypic genus, with reported species in addition to *P. chinensis* (e.g., *P. hyalocranius* and *P. anderssoni*) attributed to reports of *P. chinensis* under different species names and misclassification with other Asian icefishes (Roberts, 1984). *P. hyalocranius* and *P. chinensis* are synonyms (Zhang et al., 2007). Thus, the previously reported (Liu et al., 2017) Asian icefish genome of *P. hyalocranius* is the same species sequenced in our study. By examining 627 single-copy gene families from 18 sequenced fish genomes, we generated a phylogenetic tree in agreement with the fossil record (Benton et al., 2009; Bian et al., 2016; Schartl et al., 2013; Yang et al., 2016) (Figure 1B). The phylogeny places Osmeriformes (*P. chinensis*) as a sister order to Salmoniformes (*Salmo salar*) and Esociformes (*Esox lucius*). The divergence time between these orders was estimated to be about 185.5 million years ago (mya), the Jurassic period (Figures 1B and S3; Table S14).

Molecular Basis for Bone and Scale Formation

P. chinensis belongs to the bony fishes (Osteichthyes), but its endoskeleton is composed of cartilage (Figure S4). In this sense, it is more similar to cartilaginous fishes such as sharks. In order to understand the genetic mechanism underlying the cartilaginous skeleton of *P. chinensis*, we next identified genes involved in bone formation and maintenance from a set of 166 genes (Venkatesh et al., 2014). We found that *P. chinensis* possesses intact orthologs for most genes involved in bone formation (Table S15). However, genes encoding matrix Gla protein (*MGP*) and osteocalcin (*BGLAP*, also known as bone Gla protein), and several secretory calcium-binding phosphoproteins (SCPPs) are absent in *P. chinensis*. *MGP* and osteocalcin are important regulators of calcium metabolism and skeletal development (Kawasaki et al., 2009). *MGP* and osteocalcin control bone mineralization, whereas SCPP genes have crucial functions in the mineralization of bone, dentin, enamel, and enameloid (Kawasaki and Weiss, 2003). The SCPP gene family arose by gene duplication from a common ancestor, *SPARCL1*. The SCPP family has two subclasses: acidic SCPPs and Pro/Gln (P/Q)-rich SCPPs (Kawasaki, 2009, 2011). Interrogation of the *P. chinensis* genome and transcriptomes showed that it has two acidic SCPP genes (*SPARCL1* and *SPP1*) and four P/Q-rich SCPP genes (*SCPP2*, *SCPP5*, *SCPP9*, and *FA93E10*) (Figure 2A). We found three copies of *SCPP2* (located on different scaffolds) in the *P. chinensis* genome. Further analysis of gene synteny—comparing *P. chinensis*, fugu (*T. rubripes*), and zebrafish (*D. rerio*) genomes—revealed that *SCPP4*, *SCPP3A*, *SCPP3B*, *SCPP3C*, *SCPP6*, *SCPP7*, and *SCPP8* are absent in the *P. chinensis* genome (Figure 2A). *SCPP1* sequence was identified in a highly syntenic region. However, only two of eight exons could be identified, and we could not detect its expression, indicating that it is a pseudogene. We found that two other species with cartilaginous skeletons, the elephant fish (*Callorhinchus milii*) (Venkatesh et al., 2014) and ocean sunfish (*Mola mola*) (Pan et al., 2016), have also lost SCPP genes. Loss of *SCPP1* or *SCPP5*, or both, may result in a scaleless phenotype in bony fishes. A scaleless three-spine stickleback (*Gasterosteus aculeatus*) has intact *SCPP1* but lacks *SCPP5*; a scaleless electric eel (*Electrophorus electricus*) (Gallant et al., 2014) has *SCPP5* but lost *SCPP1*; and a scaleless channel catfish (*Ictalurus punctatus*) has lost both *SCPP5* and *SCPP1* (Liu et al., 2016). The gene encoding the ectodysplasin-A receptor (*EDAR*) has deletions in the signal peptide and extracellular regions in *P. chinensis* (Figure 2B). Similarly, the gene lacks a signal peptide in the scaleless channel catfish (*Ictalurus punctatus*) (Kellogg, 1975) and cavefish (*Sinocyclocheilu asanshuiensis*) (Yang et al., 2016), and *EDAR* mutations in the gene exon region lead to complete scale loss in medaka (Kondo et al., 2001) and zebrafish (Harris et al., 2008). To complement the genome analysis, we profiled the *P. chinensis* transcriptome at four development stages (pharyngula, hatching, larva, and adult). The expression of most genes involved in ossification showed different levels among the stages. At the adult stage, highly expressed genes included proteoglycans and bone differentiation gene families (Figure S5; Table S16). Furthermore, although *SCPP2*, *SCPP5*, *SCPP9*, and *FA93E10* are intact in *P. chinensis*, their expression is low at all development stages. Taken together, we speculate that the cartilaginous skeleton of *P. chinensis* is manifested by various gene variations, with loss of *EDAR* and *SCPP1* emerging as the leading cause of complete scale loss.

Loss of *Hox* Cluster *Ca* Genes in Distantly Related Fish Species with a Larval Phenotype

Homeobox (*Hox*) genes are highly conserved transcription factors organized into chromosomal clusters (Mallo, 2018). Extensive *Hox* gene loss (10 genes) was recently reported in two species of the Southeast Asian dwarf minnow genus *Paedocypris* (*P. carbunculus* and *P. micromegethes*) (Malmstrom et al., 2018). Similar to *P. chinensis*, adult fish in this genus retain larval features (developmental truncation). Four of 62 *Hox* genes are pseudogenized in *P. chinensis* (validated by PCR and Sanger sequencing) (Figure 3; Table S17). Two of the genes are also lost in *Paedocypris* (*HOXC5a* and *HOXC3a*) and are located in the same *Hox* cluster (*HOXCa*). These genes are expressed in the neural tube of fish embryos (Davis and Stellwag, 2010; le Pabic et al., 2009; Lyon et al., 2013). *P. chinensis* (Roberts, 1984) and *Paedocypris* (Kottelat et al., 2006) have a roofless skull (posterior portions are open throughout life); however, we are not aware of studies that have examined the effect of *HOXC5a* and *HOXC3a* loss on the development of the skeletal system of teleost fish. An assignment of function, and whether *HOXC5a* acts alone or in concert with *HOXC3a*, awaits further investigation.

No Canonical Fibroblast Growth Factor 5 in *P. chinensis*

The gene family history analysis software CAFE (Computational Analysis of gene Family Evolution) cannot identify families created after the most recent common ancestor of the analyzed species (De Bie et al., 2006), that is, gene families that are lineage specific and created in a particular lineage and that may contribute to unique traits (Martin et al., 2010). We identified homologous gene families across 18 fish species using OrthoMCL (Li et al., 2003). When we compared the gene families of 18 fish species (see Figure 1B), we identified 86 single-copy gene families unique to *P. chinensis* (Figure S6; Table S18). Pfam (El-Gebali et al., 2019) and BLAST (Johnson et al., 2008) searches revealed that one of these gene families contain a fibroblast growth factor (FGF) domain (Pfam PF00167) with sequence similarity to *FGF5*. Further manual inspection of the genome assembly and Sanger sequencing of PCR amplicons showed that *P. chinensis* has two distinct *FGF5* gene types (Figure 4A). A three-exon ortholog to *FGF5* to the other fish species was found on scaffold189 (i.e., the canonical *P. chinensis FGF5* gene, denoted *FGF5A*). An *FGF5* gene tree reflected the expected phylogenetic relationship between species (Figure S7). In total, we detected 13 additional copies of *FGF5* in *P. chinensis* (Data S1). Intron sizes of the duplicates ranged from 53 to 6,313 bp. Our data provide an estimate for the number of *FGF5* duplicates in *P. chinensis*; there is a possibility that our analyses did not recover all copies. To determine whether the *P. chinensis FGF5* duplicates are transcribed, we interrogated 14.14Gb RNA sequencing data from a whole animal. Although the number of reads matching *FGF5* was low (2–12 reads), we identified reads corresponding to the exon-intron junction of *FGF5A*, as well as several gene duplicates (Table S19). Therefore, we conclude that *P. chinensis FGF5* duplicates can be transcribed. All *P. chinesis FGF5* genes, including the canonical *P. chinensis FGF5*, would encode

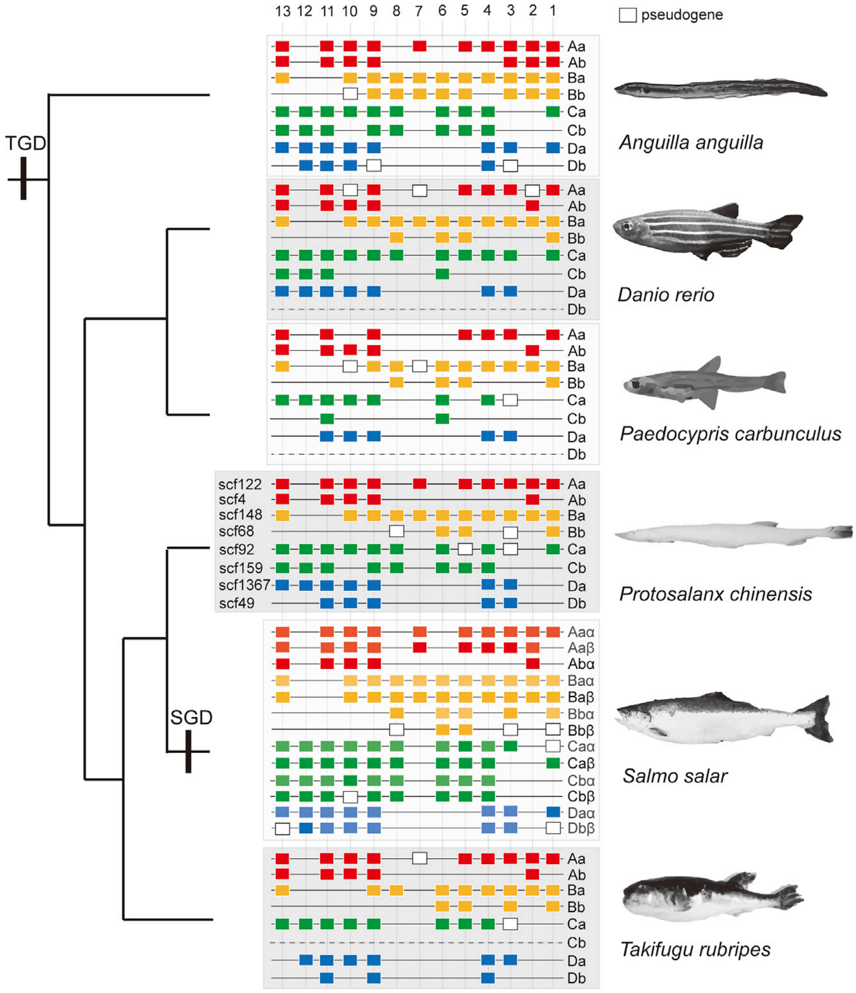

Figure 3. Phylogeny of *Hox* Gene Clusters of Six Teleosts

Overview of the *Hox* gene clusters of *Anguilla anguilla* (European eel) (Minegishi et al., 2005), *Danio rerio* (zebrafish) (Bian et al., 2016), *Paedocypris carbunculus* (a dwarf minnow; common name yet to be assigned) (Malmstrom et al., 2018), *Protosalanx chinensis* (Asian icefish), *Salmo salar* (Atlantic salmon) (Mungpakdee et al., 2008), and *Takifugu rubripes* (fugu) (Bian et al., 2016). Each horizontal black line refers to a *Hox* cluster. Solid rectangles represent complete *HoxA* (red), *HoxB* (orange), *HoxC* (green), and *HoxD* (blue) genes, whereas hollow rectangles indicate pseudogenes or partial genes. Paralogs generated by TGD (a teleost whole-genome duplication event) are denoted "a" and "b," whereas paralogs produced by lineage-specific SGD (a salmonid whole-genome duplication event) are denoted "α" and "β." See also Table S17.

C-terminally truncated peptides missing three to eleven of the highly conserved β strands involved in the interaction between *FGF5* and its receptor (see Mohammadi et al., 2005) (Figure 4B). Interestingly, the *P. chinensis FGF5* duplicates are conceptually similar to *FGF5*-short (also known as *FGF5-S*), a mammalian exon 2-deleted isoform that encodes a peptide that prevents *FGF1R* activation by wild-type *FGF5* (Daverio et al., 2017; He et al., 2016; Higgins et al., 2014; Ozawa et al., 1998). *FGF5* is broadly expressed in embryonic, but not adult, tissues of vertebrates. *FGF5* and its receptor play a role in zebrafish development, including neural development during the transition from a larva to an adult (Leerberg et al., 2019; Vemaraju et al., 2012). It is also plausible that *FGF5* is required for scale development, given that there is evidence to suggest that shared development pathways regulate the scales of bony fishes and the hair of mammals. For example, *EDAR* (see above section) regulates hair development in mammals and adult structures such as scales and fins in fish (Aman et al., 2018; Brunsdon and Patton, 2018). Similarly, *FGF5* is a regulator of hair growth in mammals (Daverio et al., 2017; He et al., 2016; Higgins et al., 2014; Ozawa et al., 1998). We speculate that a blunted *FGF5* axis contributes to the retention of larval features by *P. chinensis* but appreciate the need for further studies.

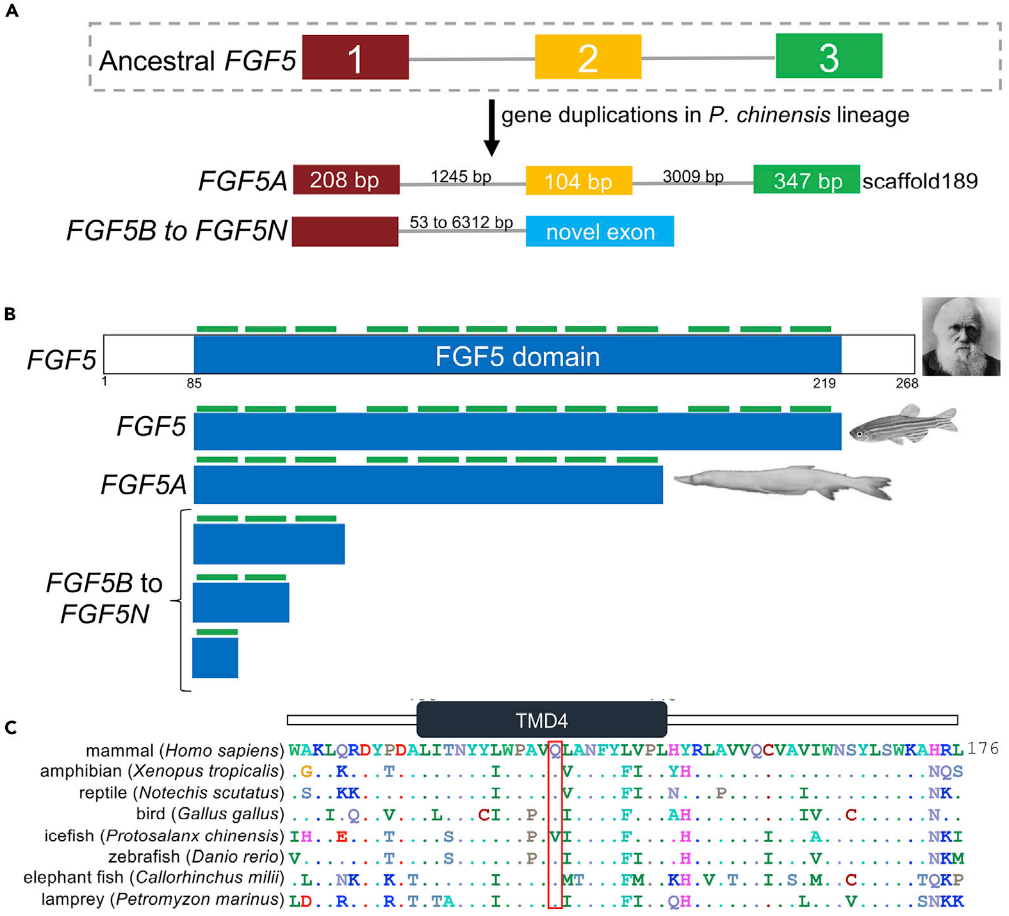
A
Ancestral FGF5
1
2
3
gene duplications in P. chinensis lineage
FGF5A
208 bp
1245 bp
104 bp
3009 bp
347 bp
scaffold189
FGF5B to FGF5N
53 to 6312 bp
novel exon
B
FGF5
FGF5 domain
1
85
219
268
FGF5
FGF5A
FGF5B to FGF5N
C
TMD4
mammal (Homo sapiens) WAKLQRDYPDALITNYYLWPAVQLANFYLVPLHYRLAVVQCVAVIWNSYLSWKAHRL 176
amphibian (Xenopus tropicalis) .G..K...T........I.....V....FI..YH....................NQS
reptile (Notechis scutatus) .S..KK...........I.....V....FI..N...P......I..........NK.
bird (Gallus gallus) ...I.Q..V...L...CI..P..I....F...AH.........IV..C......N..
icefish (Protosalanx chinensis) IH..E...T....S......P.VI....F....H......I...A.........NKI
zebrafish (Danio rerio) V.......T....S......P..I....FI...H......I...V.........NKM
elephant fish (Callorhinchus milii) .L..NK..K.T......I.....MT...FM..KH.V.T..I.S.M..C.....TQKP
lamprey (Petromyzon marinus) LD..R...R.T.TA...I.....I....FI..QH.........L...V.....SNKK

Figure 4. Fibroblast Growth Factor 5 Gene Copy Number Increase and a Unique Amino Acid Change of the Fish Pigmentation Gene Mitochondrial Inner Membrane Protein 17 in *P. chinensis*

(A) One of the 86 gene families gained by *P. chinensis* include fibroblast growth factor 5-like genes. *P. chinensis* has two distinct *FGF5* gene types: a three-exon ortholog to *FGF5* of other fish species was found on scaffold189 (denoted *FGF5A*), whereas duplicated genes are part of a novel gene (*FGF5B* to *FGF5N*).

(B) The domain structure of human, zebrafish, and *P. chinensis* FGF5-derived proteins is shown. The canonical FGF5 domain (shown in blue) has a highly conserved core region with 12 β strands (shown by green bars) within the core region of FGF family polypeptides. If translated, all *P. chinensis FGF5* genes (denoted *FGF5A* to *FGF5N*) would encode a C-terminally truncated FGF5 form.

(C) Partial alignment of mitochondrial inner membrane protein 17 (*MPV17*) sequences in vertebrates. MPV17 transmembrane domain four is shaded in green. An amino acid change (Gln142Val) unique to *P. chinensis* is highlighted in red. Representative species from fishes (zebrafish, *Danio rerio*; icefish, *Protosalanx chinensis*; Australian ghostshark; *Callorhinchus milii*; sea lamprey, *Petromyzon marinus*), amphibians (western clawed frog, *Xenopus tropicalis*), reptiles (mainland tiger snake, *Notechis scutatus*), birds (chicken, *Gallus gallus*), and mammals (human, *Homo sapiens*) are shown. See also Figures S6 and S7 and Tables S18 and S19 and Data S1.

Contraction of Immune System Genes

Fish larvae have a poorly developed immune system (Vadstein et al., 2013). We found no evidence of positive selection of immune-associated genes, or the skeletal system and other gene ontologies and pathways, in *P. chinensis* (Table S20). In order to gain additional insights into the immune system of *P. chinensis*, we performed gene family gain-and-loss analysis using CAFE (De Bie et al., 2006) and observed four expanded and 69 contracted gene families in *P. chinensis* (Figure 1B; Tables S21 and S22). The contracted gene families include the immune signaling pathways NOD-like receptor signaling ($p = 9.65\times10^{-218}$), autoimmune thyroid disease ($p = 5.82\times10^{-60}$), NF-kappa β signaling ($p = 5.68\times10^{-48}$), and B cell (antigen) receptor signaling ($p = 3.76\times10^{-45}$). *P. chinensis* and other teleosts have a similar number of genes in most immune system signaling pathways, except for three pathways

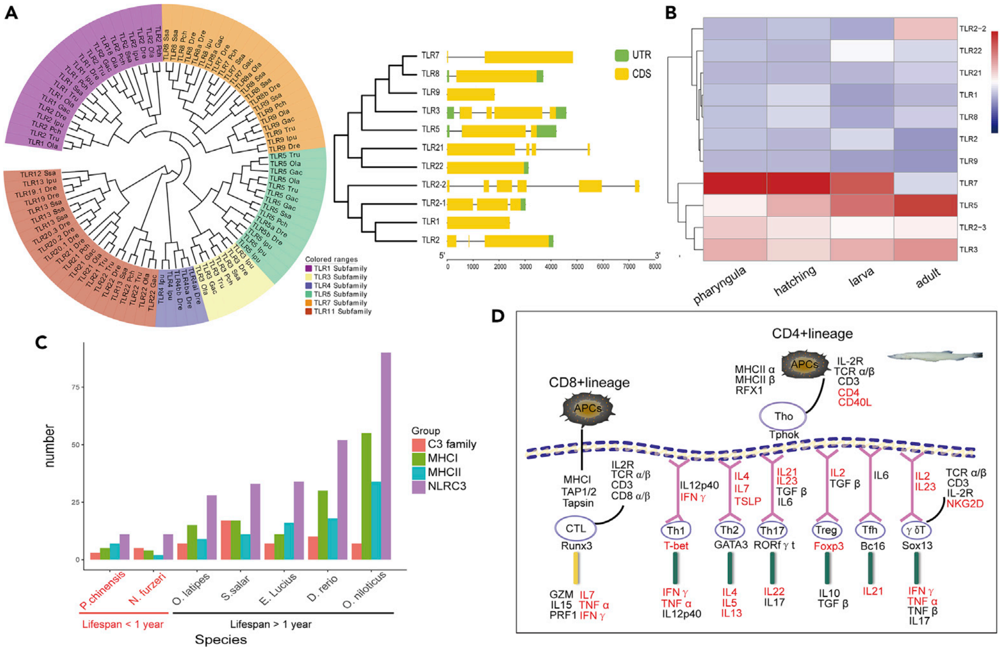

Figure 5. Overview of the *P. chinensis* Immune System Repertoire

(A) Left: phylogenetic relationship of the Toll-like receptor (TLR) family genes of *Protosalanx chinensis* (Pch), *Salmo salar* (Sasa), *Danio rerio* (Dre), *Ictalurus punctatus* (Ipu), *Oryzias latipes* (Ola), and *Takifugu rubripes* (Tru). Right: structure of *P. chinensis* TLR genes. Exons are shown as boxes, with coding sequences (CDSs) shown in yellow and untranslated regions (UTRs) in green.

(B) Expression in *P. chinensis* of TLR genes at four development stages: pharyngula, hatching, larva, and adult. Gene expression was quantified as reads per kilobase of gene per million mapped reads (RPKM).

(C) Overview of the number of genes involved in the complement system C3 family, MHC I protein complex, MHCII protein complex, and NOD-like receptor family (NLRC3) in seven teleost species.

(D) Schematic diagram summarizing genes related to different T cell lineages in *P. chinensis*. Genes absent in the genome assembly are indicated in red.

See also Figure S8 and Tables S20 and S21–S24.

where *P. chinensis* has a lower number of genes: complement and coagulation cascades (KEGG pathway map04610; 61 genes), antigen processing and presentation (map04612; 61 genes), and intestinal immune network for IgA production (map04672; 28 genes) (Table S23).

Toll-like receptors (TLRs) of the innate immune system recognize various pathogen-associated molecular patterns (PAMPs) to activate downstream immune responses (Rebl and Goldammer, 2018). The TLR multigene family comprises a large and variable number (10–15) of genes, and there are substantial sequence differences within and between vertebrate groups, including within teleost fish species (Rebl and Goldammer, 2018; Roach et al., 2005). *P. chinensis* is no exception. For example, *TLR4* is highly divergent in zebrafish and is lost in most teleost species, including, albeit distant, sister taxa to *P. chinensis* (i.e., salmon, trout) (Rebl and Goldammer, 2018; Roach et al., 2005). Based on homology alignment and RefSeq annotations, 11 TLR genes in five sub-families were identified in the *P. chinensis* genome: *TLR1*, *TLR2*, *TLR2-1*, *TLR2-2*, *TLR3*, *TLR5*, *TLR7*, *TLR8*, *TLR9*, *TLR21*, and *TLR22* (Figure 5A). We assessed the expression of *P. chinensis* TLR genes by RNA sequencing from four different development stages (pharyngula, hatching, larva, and adult). *TLR2-3*, *TLR3*, *TLR5*, and *TLR7* were the most highly expressed TLR genes at all stages, suggesting that they play essential roles in the innate immune system of *P. chinensis* (Figure 5B).

The immune response is costly (energy demanding) and comes with life-history trade-offs. Consequently, some small, short-lived animals may have a suppressed or poorly developed immune system and employ terminal investment strategies, i.e., produce as many offspring as possible before an inevitable death (Brace et al., 2017). We counted the genes immune-related families. Short-lived fish species *P. chinensis* (lifespan ~1 year) and the African turquoise killifish (*Nothobranchius furzeri*; lifespan ~4 months) have a smaller number of genes in the major histocompatibility complex (MHC) I and II of the adaptive immune system and the NOD-like receptor family of the innate immune system (Figure 5C; Table S24). T cells are at the center of the adaptive immune system. The MHC I machinery allows activation of CD8$^+$ T cells upon bacterial infection. IFN-γ (*IFNG*), TNF-α (*TNFA*), and interleukin 7 (*IL-7*) are absent in *P. chinensis* (Figure 5D), whereas *IFNG* and *IL-7* are absent in *N. furzeri* (Figure S8). Genes encoded by T helper cells (T_h or CD4$^+$) that recognize MHC class II molecules (*IL-2, IL-4, IL-5, IL-13, IL-21, IL-22, IL-23, TSLP, FOXP3*, and *NKG2D*) are lost in both *P. chinensis* and *N. furzeri* (and *CD40* and *CD40L* are also lost in *P. chinensis*) (Figures 5D and S8). We speculate that the loss of central immune-related genes and lack of associated immunological innovation, as observed in longer-lived teleost (e.g., Malmstrom et al., 2016), reflects the annual life-history strategy of *P. chinensis* and *N. furzeri*. However, given the plasticity of the vertebrate immune system, phylogenetic distances, and the limited number of species examined in this study, larger-scale studies are warranted.

A Unique Amino Acid Change in the Fish Pigmentation Gene *MPV17* of *P. chinensis*

One of the striking features of Asian icefishes is their transparent body, appearing white postmortem (Roberts, 1984). Loss of pigmentation, a complete loss of pigmentation of either skin and eyes (albinism) or skin alone (leucism), is observed in various fish. These include cave-dwelling species (Borowsky, 2018), as well as lines of zebrafish (D'Agati et al., 2017; Krauss et al., 2013; Tsetskhladze et al., 2012) and medaka (Fukamachi et al., 2001). Melanin-based pigmentation genes are highly conserved in vertebrates (Hubbard et al., 2010), offering an opportunity for comparative genomics analyses. We employed the *P. chinensis* genome assembly and whole-body transcriptome data to examine various genes previously associated with pigmentation loss in fish, including *SLC45A2* (also known as *AIM1* or *MATP*) (Fukamachi et al., 2001; Tsetskhladze et al., 2012), *OCA2* (Gross and Wilkens, 2013; Protas et al., 2006; Yang et al., 2016), *LYST* (Link et al., 2004), and MPV17 (D'Agati et al., 2017; Krauss et al., 2013; Yang et al., 2016). MPV17 (mitochondrial inner membrane protein 17) encodes a mitochondrial channel-forming protein (Calvo et al., 2006; Spinazzola et al., 2006). MPV17 transmembrane domain missense mutations are pathogenic in mammals and cause mitochondrial disorders with which affected individuals die at a young age (El-Hattab et al., 2018; Kim et al., 2016; Lollgen and Weiher, 2015). Mutations of the gene appear to be better tolerated in fish, where they also affect melanin-containing cells (Borowsky, 2018; D'Agati et al., 2017; Martorano et al., 2019). In *P. chinensis* MPV17, we found an amino acid substitution (Q142V) in the terminal fourth transmembrane domain. The glutamine residue is conserved in all other vertebrates examined, from sea lamprey to humans, species with a common ancestor approximately 500 mya (Smith et al., 2013) (Figure 4C). MPV17 transcripts are expressed but have unique changes in transparent zebrafish lines (D'Agati et al., 2017; Krauss et al., 2013) and the cavefish *Sinocyclocheilus anshuiensis* (Yang et al., 2016). A 19-bp deletion of MPV17 coding exons 1 and 2 likely results in pigmentation loss in zebrafish (D'Agati et al., 2017; Krauss et al., 2013). Cavefish in genus *Sinocyclocheilus* have two copies of MPV17, one of which has an in-frame exon deletion and codes for a protein lacking transmembrane four in the albino *S. anshuiensis* (Yang et al., 2016). Similarly, *P. chinensis MPV17* is transcribed (data not shown). The *P. chinensis* MPV17 mutation, a change from a polar glutamine to a non-polar valine, is predicted to affect protein stability by I-Mutant 2.0 (Capriotti et al., 2005) and protein function by PANTHER-PSEP (Tang and Thomas, 2016), PolyPhen2 (Adzhubei et al., 2010), and SIFT (Kumar et al., 2009). Missense residue mutations in transmembrane domains may cause membrane protein disassembly (Ng et al., 2012). Taken together, in particular, given the highly conserved nature of MPV17 Gln142 in vertebrates, we speculate that the unique amino acid change in *P. chinensis* contributes to its ostensibly transparent, pigmentless, skin phenotype by encoding a non-functional or dysfunctional protein in the melanin synthesis pathway.

Limitations of the Study

There are currently no genome assemblies of other species in the neotenic Asian salmoniform family Salangidae (salangids; Asian icefishes), somewhat limiting the scope of current comparative genomic analyses. The neotenic salamander the Mexican axolotl (*Ambystoma mexicanum*) has been studied in the laboratory for centuries and has amassed a significant body of research (including population genomic studies of wild-type and mutant strains) that can be supported by comparative genome research (Crowner et al., 2019; Nowoshilow et al., 2018; Smith et al., 2019). In contrast, no such studies of Asian icefishes exist. Fortunately, as the number of high-quality fish genomes is increasing, with more than 10,000 species projected (including several species of order Osmeriformes) to be sequenced by 2030 (Fan et al., 2019), the genetic basis of enigmatic but less studied species, such as Asian icefishes, is sure to be realized. Karyotype data are not available for *P. chinensis* or other Asian icefish species, but future efforts will include such data and provide chromosome-level genome assemblies. Finally, although gene loss was called after examining the genome assembly and transcriptome data (assembled from short reads), additional methods, such *de novo* assembly of long-read RNA sequencing reads (e.g., on the PacBio or Nanopore platforms) (de la Rubia et al., 2020), should be performed to further validate our results. Our improved *P. chinensis* genome assembly provides a valuable resource and steppingstone toward this goal. With a new genome assembly in hand, the use of *P. chinensis* as a laboratory animal can proceed in earnest.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ming Li (lim@ioz.ac.cn).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The NCBI BioProject accession number for the *P. chinensis* genome project reported in this paper is PRJNA604876. The accession numbers for *FGF5* gene PCR amplicon sequences are GenBank: MT416578–MT416594. The accession numbers for *Hox* gene clusters gene PCR amplicon sequences are GenBank: MT394613–MT394616.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101267.

ACKNOWLEDGMENTS

The project was supported by the Strategic Priority Research Program of Chinese Academy of Sciences (XDB31000000, XDA19050200, and XDA19050202), the National Natural Science Foundation of China (31670383), the GDAS Special Project of Science and Technology Development (2019GDASYL-0302007), and the Planning Funds for Science and Technology of Guangdong Province (2016B070701016). I.S. is supported by the National Natural Science Foundation of China (31950410545), the Priority Academic Program Development of Jiangsu Higher Education Institutions (IS), and a Young Foreign Experts in Economic and Technological Sector grant. R.T. is a recipient of a National Natural Science Foundation of China postdoctoral fellowship (31900310). We appreciate valuable suggestions from Professors Xuming Zhou, Hanlin Wu, Yong Ni, and Chunguang Zhang. The authors thank Mr. Xinming Feng, Mr. Jianhua Gu, Mr. Feng Lin, Mr. Zhong Yi, Ms. Kechong Qi, and Mr. Yuehai Zhao, who either helped us with sampling or provided fertilized eggs from Hongze Lake, and Dr. Zhijin Liu, Ms. Xinxin Tan, Mr. Liye Zhang, and Mr. Ziming Wang for assistance with data analysis.

AUTHOR CONTRIBUTIONS

M.L., J.Z., I.S., and J.C. supervised the study and managed the project. J.Z., J.Q., and F.S. collected samples. F.S., I.S., H.P., H.L., R.T., Y.G., Y.Q., M.L., and J.C. performed genome sequencing, assembly, annotation, and genetic data analyses. I.S., F.S., J.Q., J.Z., H.L., H.P., and J.C. wrote the drafted manuscript. I.S., F.S., J.Q., J.Z., H.L., H.P., J.C., and M.L. discussed the data. I.S., J.Q., and F.S. finished the final manuscript with contributions from M.L., J.Z., I.S., and J.C. All authors contributed to data interpretation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 18, 2019
Revised: April 28, 2020
Accepted: June 8, 2020
Published: July 24, 2020

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iScience, Volume *23*

Supplemental Information

Insights into the Evolution of Neoteny from the Genome of the Asian Icefish *Protosalanx chinensis*

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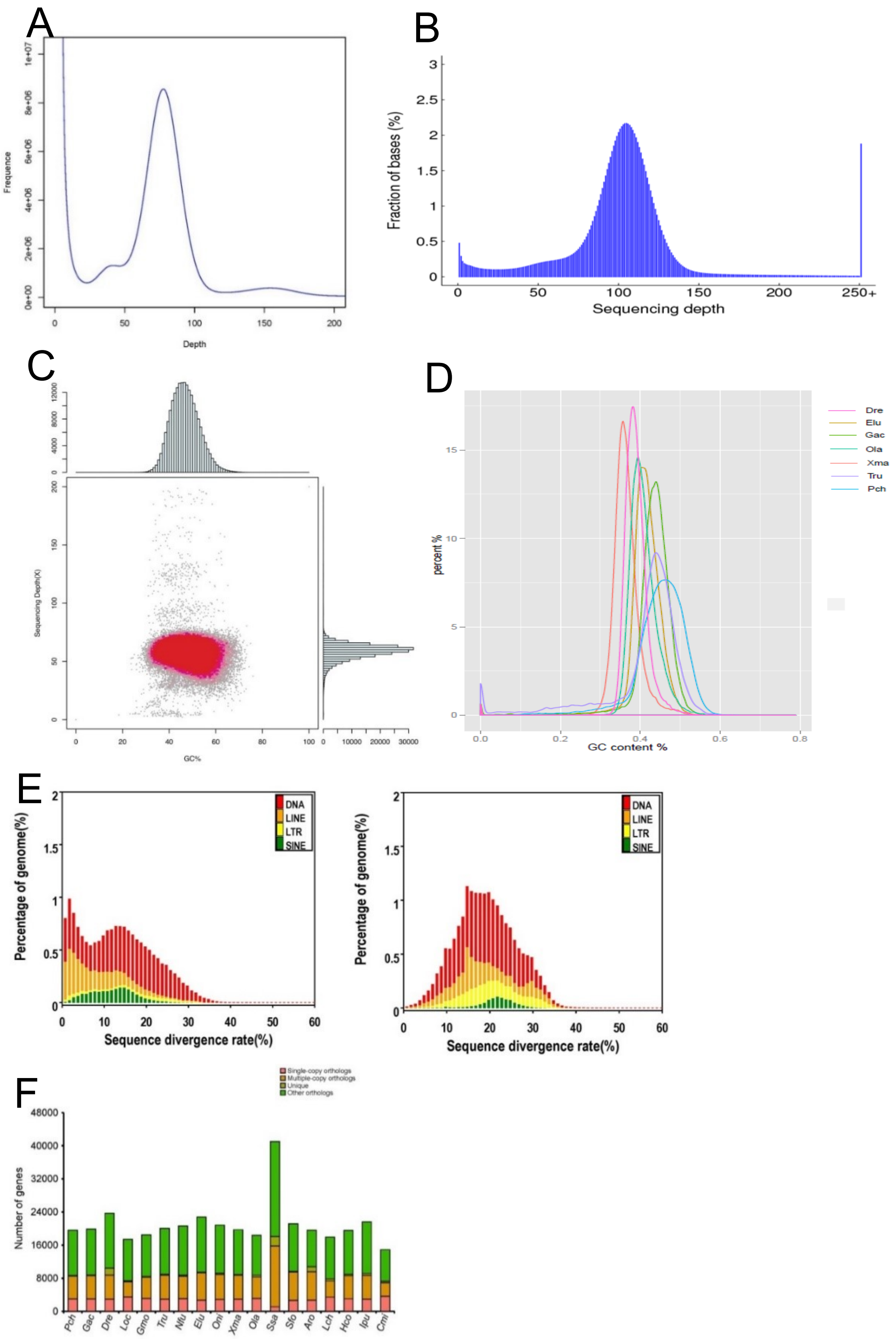
SUPPLEMENTAL DATA ITEMS

Supplemental figures



Supplemental Figure S1. ***P. chinensis*** **genome assembly assessment, Related to Figure 1.**

(A) Distribution of 17-mer frequency of filtered Illumina reads mapped to the *P. chinensis* genome. The *y*-axis shows frequency (in millions); the *x*-axis, *k*-mer depth.

(B) Depth distribution of fraction bases. Short-insert reads were mapped to the *P. chinensis* genome assembly using bwa [82]. The *x*-axis shows sequencing depth, the *y*-axis the fraction of bases.

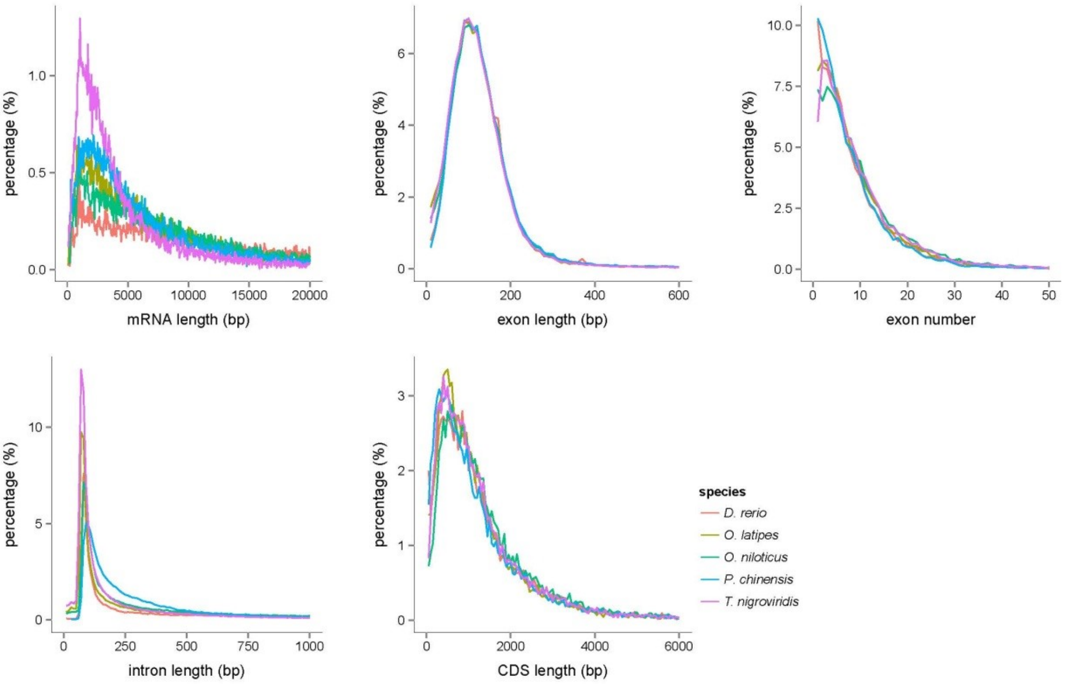
(C) The GC content of the *P. chinensis* genome. The *x*-axis represents GC content; the *y*-axis average sequencing depth. We used a 50 kb non-overlapping sliding window. A lower depth ‘island’ on the scatter plot is due to sex chromosomes with half the sequencing depth of autosomes.

(D). GC content in the genomes of *P. chinensis* and six other fish species

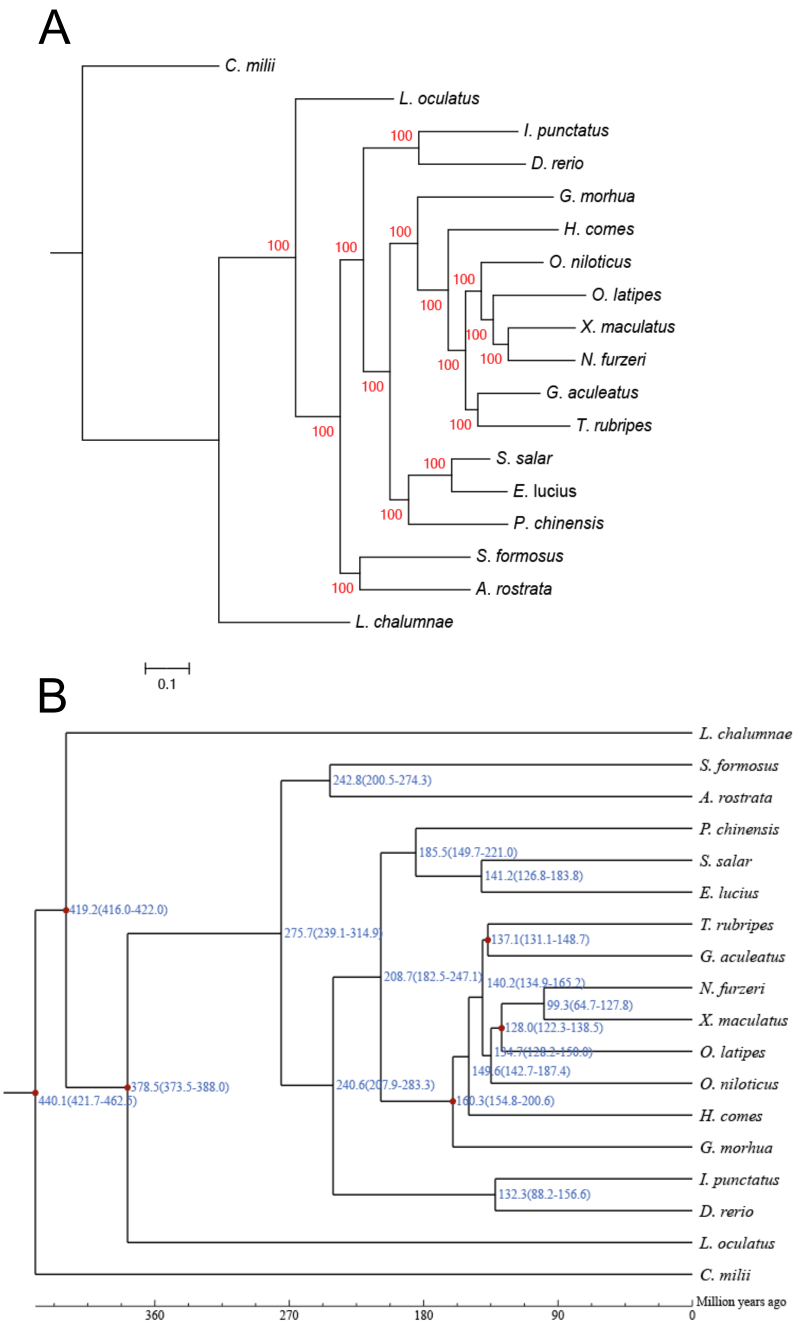
Pch, *P. chinensis*; Dre, *Danio rerio*; Elu, *Esox lucius*; Gac, *Gasterosteus aculeatus*; Ola, *Oryzias latipes*; Xma, *Xiphophorus maculatus*; Tru, *Takifugu rubric*.

(E) Divergence distribution of transposable elements families in the *P. chinensis* genome. The divergence rate was calculated based on an alignment between RepeatMasker-annotated repeat copies and the consensus sequence in the repeat library.

(F). Orthology delineation among the protein-coding gene family repertoires of *P. chinensis* and 18 other fish species. Pch, *Protosalanx chinensis*; Gac, *Gasterosteus aculeatus*; Dre, *Danio rerio*; Loc, *Lepisosteus oculatus*; Gmo, *Gadus morhu*; Tru, *Takifugu rubripes*; Nfu, *Nothobranc hius furzeri*; Elu, *Esox lucius*; Oni, *Oreochromis niloticus*; Xma, *Xiphophorus maculatus*; Ola, *Oryzias latipes*; Ssa, *Salmo salar*; Sfo, *Scleropages formosus*; Aro, *Anguilla rostrata*; Lch, *Latimeria chalumnae*; Hco, *Hippocampus comes*; Ipu, *Ictalurus punctatus*; Cmi, *Callorhinchus milii*.



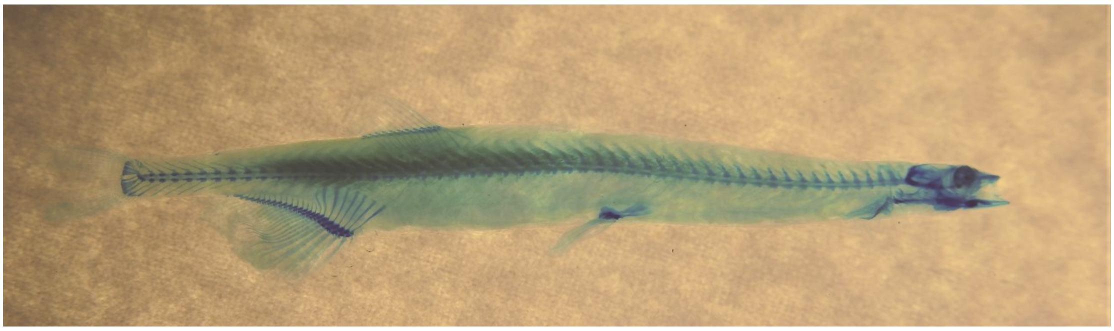
Supplemental Figure S2. A comparison of teleost gene parameters. Characteristic of predicted protein-coding genes in *P. chinensis* genome, Related to Figure 1. Note that mRNA includes untranslated regions (UTRs).



Supplemental Figure S3. Phylogenetic relationship of *P. chinensis* with 17 other fish species, Related to Figure 1.

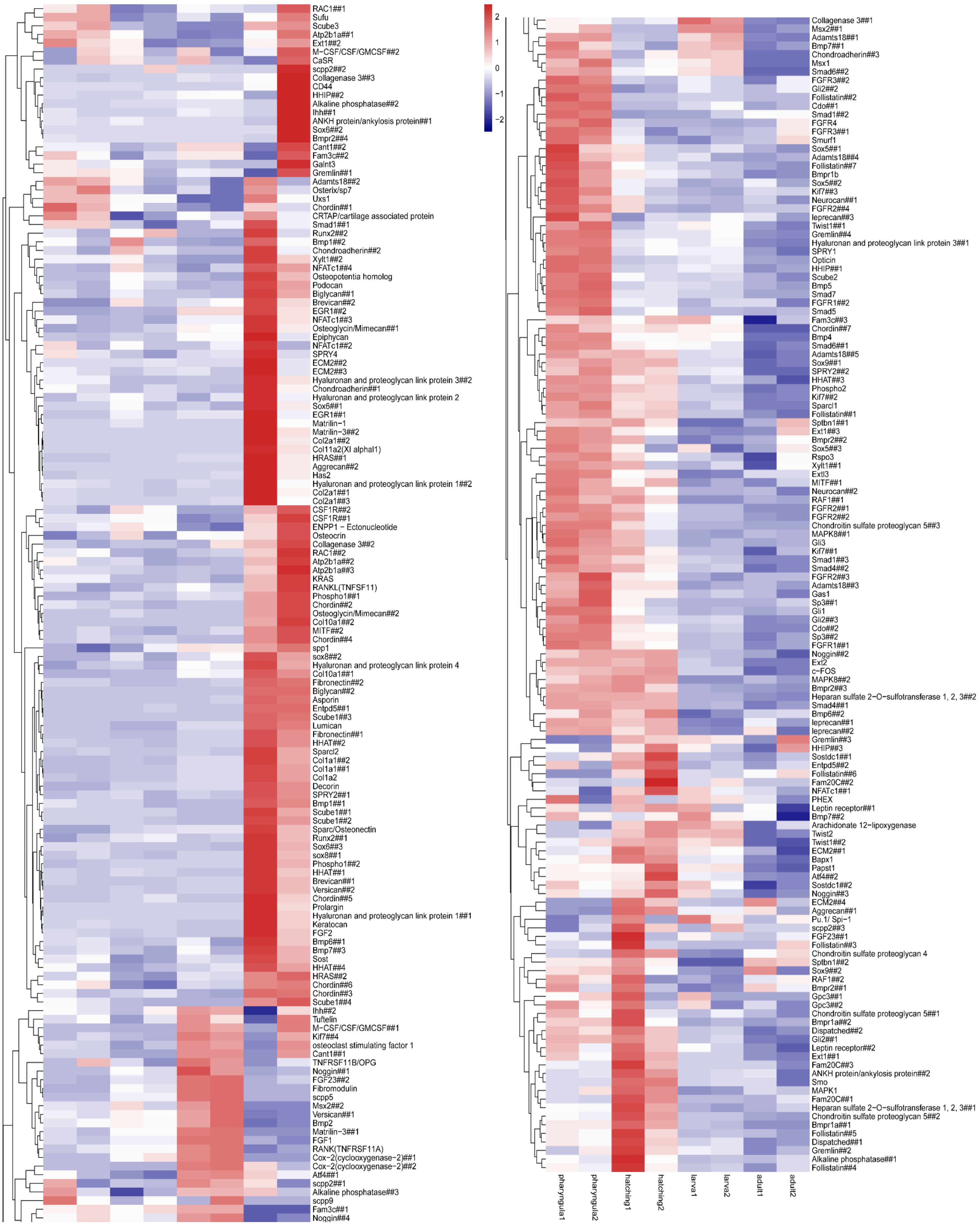
(A) A maximum likelihood tree generated using RaxML (100 bootstrap replicates) from a 627 single-copy orthologs (coding sequence, CDS) concatenated into a 1,414,350 bp alignment.

(B) Estimated divergence times of 18 fish species. The numbers on nodes represent the divergence times from present (million years ago, Mya).



Supplemental Figure S4. ***P. chinensis*** **transparent bone stained specimens, Related to Figure 2.**

After alizarin red and alcian blue staining, endoskeleton of *P. chinensis* is composed of cartilage stained with alcian blue.


RAC1##1
Sufu
Scube3
Atp2b1a##1
Ext1##2
M−CSF/CSF/GMCSF##2
CaSR
scpp2##2
Collagenase 3##3
CD44
HHIP##2
Alkaline phosphatase##2
Ihh##1
ANKH protein/ankylosis protein##1
Sox6##2
Bmpr2##4
Cant1##2
Fam3c##2
Galnt3
Gremlin##1
Adamts18##2
Osterix/sp7
Uxs1
Chordin##1
CRTAP/cartilage associated protein
Smad1##1
Runx2##2
Bmp1##2
Chondroadherin##2
Xylt1##2
NFATc1##4
Osteopotentia homolog
Podocan
Biglycan##1
Brevican##2
EGR1##2
NFATc1##3
Osteoglycin/Mimecan##1
Epiphycan
NFATc1##2
SPRY4
ECM2##2
ECM2##3
Hyaluronan and proteoglycan link protein 3##2
Chondroadherin##1
Hyaluronan and proteoglycan link protein 2
Sox6##1
EGR1##1
Matrilin−1
Matrilin−3##2
Col2a1##2
Col11a2(XI alphal1)
HRAS##1
Aggrecan##2
Has2
Hyaluronan and proteoglycan link protein 1##2
Col2a1##1
Col2a1##3
CSF1R##2
CSF1R##1
ENPP1 − Ectonucleotide
Osteocrin
Collagenase 3##2
RAC1##2
Atp2b1a##2
Atp2b1a##3
KRAS
RANKL(TNFSF11)
Phospho1##1
Chordin##2
Osteoglycin/Mimecan##2
Col10a1##2
MITF##2
Chordin##4
spp1
sox8##2
Hyaluronan and proteoglycan link protein 4
Col10a1##1
Fibronectin##2
Biglycan##2
Asporin
Entpd5##1
Scube1##3
Lumican
Fibronectin##1
HHAT##2
Sparcl2
Col1a1##2
Col1a1##1
Col1a2
Decorin
SPRY2##1
Bmp1##1
Scube1##1
Scube1##2
Sparc/Osteonectin
Runx2##1
Sox6##3
sox8##1
Phospho1##2
HHAT##1
Brevican##1
Versican##2
Chordin##5
Prolargin
Hyaluronan and proteoglycan link protein 1##1
Keratocan
FGF2
Bmp6##1
Bmp7##3
Sost
HHAT##4
HRAS##2
Chordin##6
Chordin##3
Scube1##4
Ihh##2
Tuftelin
M−CSF/CSF/GMCSF##1
Kif7##4
osteoclast stimulating factor 1
Cant1##1
TNFRSF11B/OPG
Noggin##1
FGF23##2
Fibromodulin
scpp5
Msx2##2
Versican##1
Bmp2
Matrilin−3##1
FGF1
RANK(TNFRSF11A)
Cox−2(cyclooxygenase−2)##1
Cox−2(cyclooxygenase−2)##2
Atf4##1
scpp2##1
Alkaline phosphatase##3
scpp9
Fam3c##1
Noggin##4
2
1
0
−1
−2
Collagenase 3##1
Msx2##1
Adamts18##1
Bmp7##1
Chondroadherin##3
Msx1
Smad6##2
FGFR3##2
Gli2##2
Follistatin##2
Cdo##1
Smad1##2
FGFR4
FGFR3##1
Smurf1
Sox5##1
Adamts18##4
Follistatin##7
Bmpr1b
Sox5##2
Kif7##3
Neurocan##1
FGFR2##4
Ieprecan##3
Twist1##1
Gremlin##4
Hyaluronan and proteoglycan link protein 3##1
SPRY1
Opticin
HHIP##1
Scube2
Bmp5
Smad7
FGFR1##2
Smad5
Fam3c##3
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Bmp4
Smad6##1
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SPRY2##2
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Phospho2
Kif7##2
Sparcl1
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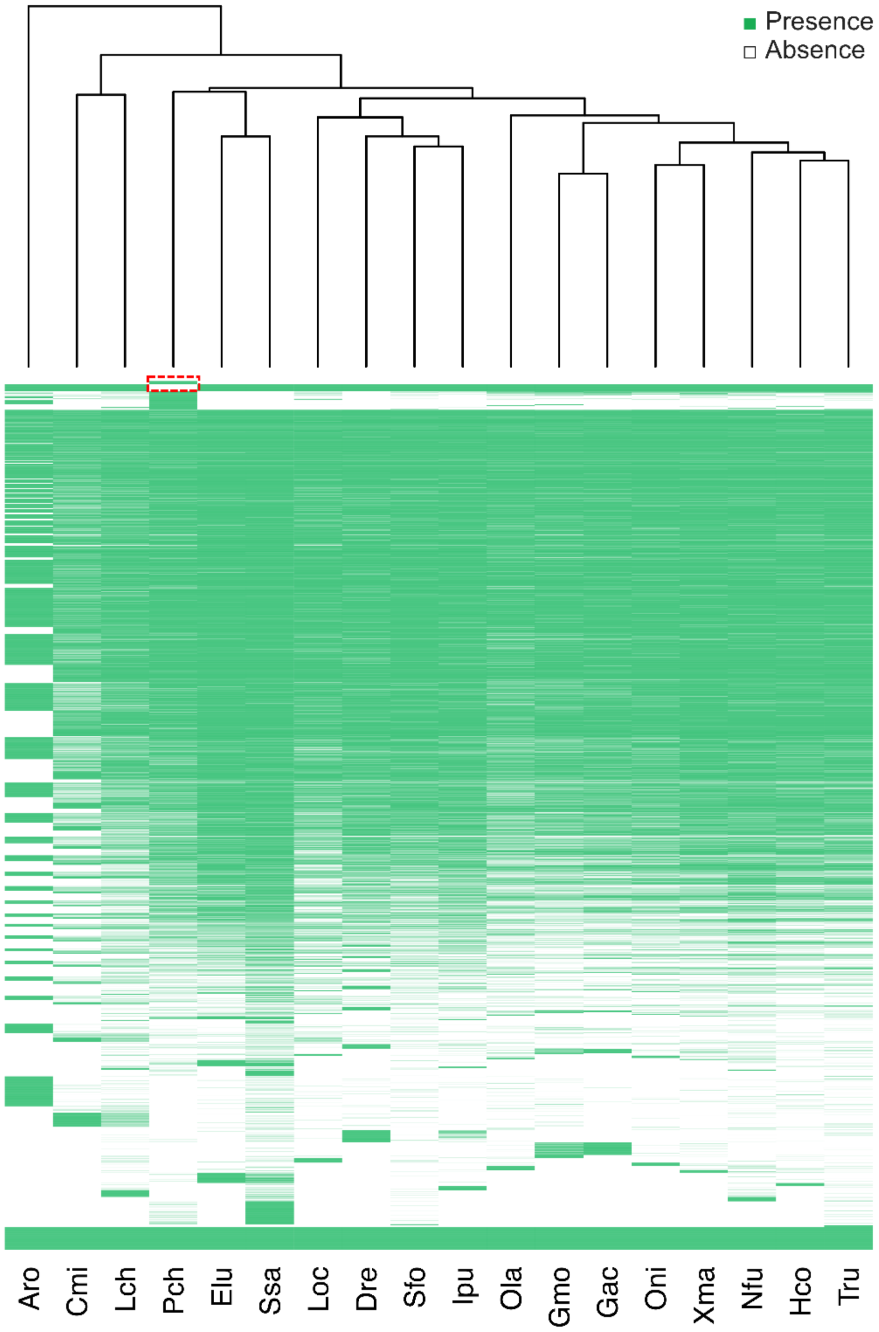
Sptbn1##1
Ext1##3
Bmpr2##2
Sox5##3
Rspo3
Xylt1##1
Extl3
MITF##1
Neurocan##2
RAF1##1
FGFR2##1
FGFR2##2
Chondroitin sulfate proteoglycan 5##3
MAPK8##1
Gli3
Kif7##1
Smad1##3
Smad4##2
FGFR2##3
Adamts18##3
Gas1
Sp3##1
Gli1
Gli2##3
Cdo##2
Sp3##2
FGFR1##1
Noggin##2
Ext2
c−FOS
MAPK8##2
Bmpr2##3
Heparan sulfate 2−O−sulfotransferase 1, 2, 3##2
Smad4##1
Bmp6##2
Ieprecan##1
Ieprecan##2
Gremlin##3
HHIP##3
Sostdc1##1
Entpd5##2
Follistatin##6
Fam20C##2
NFATc1##1
PHEX
Leptin receptor##1
Bmp7##2
Arachidonate 12−lipoxygenase
Twist2
Twist1##2
ECM2##1
Bapx1
Papst1
Atf4##2
Sostdc1##2
Noggin##3
ECM2##4
Aggrecan##1
Pu.1/ Spi−1
scpp2##3
FGF23##1
Follistatin##3
Chondroitin sulfate proteoglycan 4
Sptbn1##2
Sox9##2
RAF1##2
Bmpr2##1
Gpc3##1
Gpc3##2
Chondroitin sulfate proteoglycan 5##1
Bmpr1a##2
Dispatched##2
Gli2##1
Leptin receptor##2
Ext1##1
Fam20C##3
ANKH protein/ankylosis protein##2
Smo
MAPK1
Fam20C##1
Heparan sulfate 2−O−sulfotransferase 1, 2, 3##1
Chondroitin sulfate proteoglycan 5##2
Bmpr1a##1
Follistatin##5
Dispatched##1
Gremlin##2
Alkaline phosphatase##1
Follistatin##4
pharyngula1
pharyngula2
hatching1
hatching2
larva1
larva2
adult1
adult2

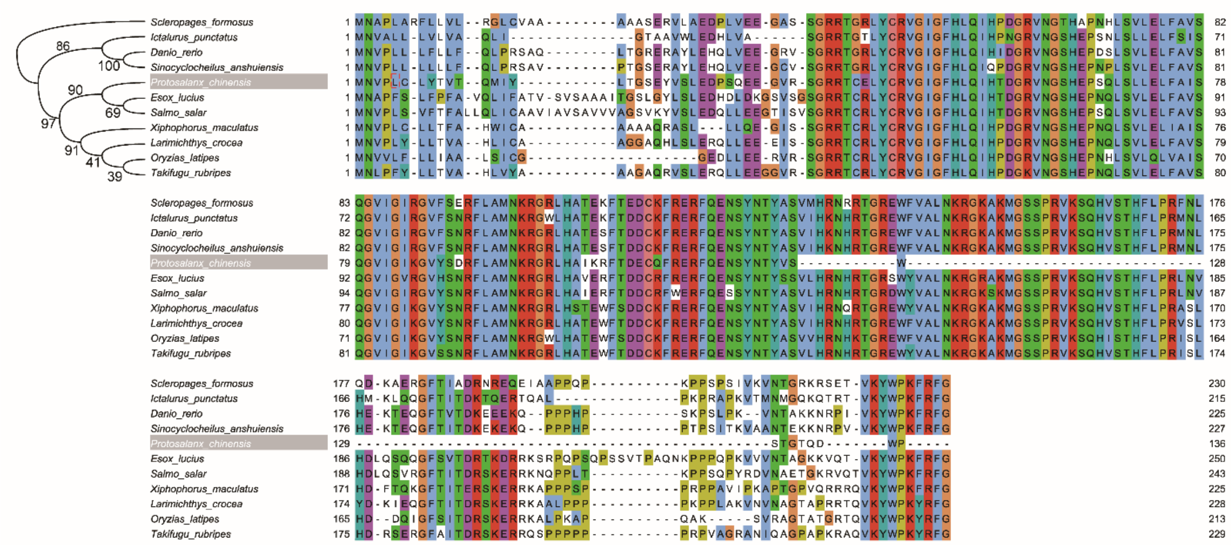
Supplemental Figure S5. Heat map of the expression of 276 involved in the regulation of bone at four *P. chinensis* development stages, Related to Figure 2.

Expression of 276 genes in regulation of bone at four development stages (pharyngula, hatching, larva, and adult). Numbered suffixes (e.g., pharyngula1 and pharyngula2) indicate biological replicates. Mark "#" after gene symbol (e.g., RAC1##1) indicate numbered gene copy number. Gene expression values (TMM-normalized RPKM) were scaled across all samples for each gene. The gene set was obtained from (Venkatesh *et al.*, 2014). The picture is split into two columns.



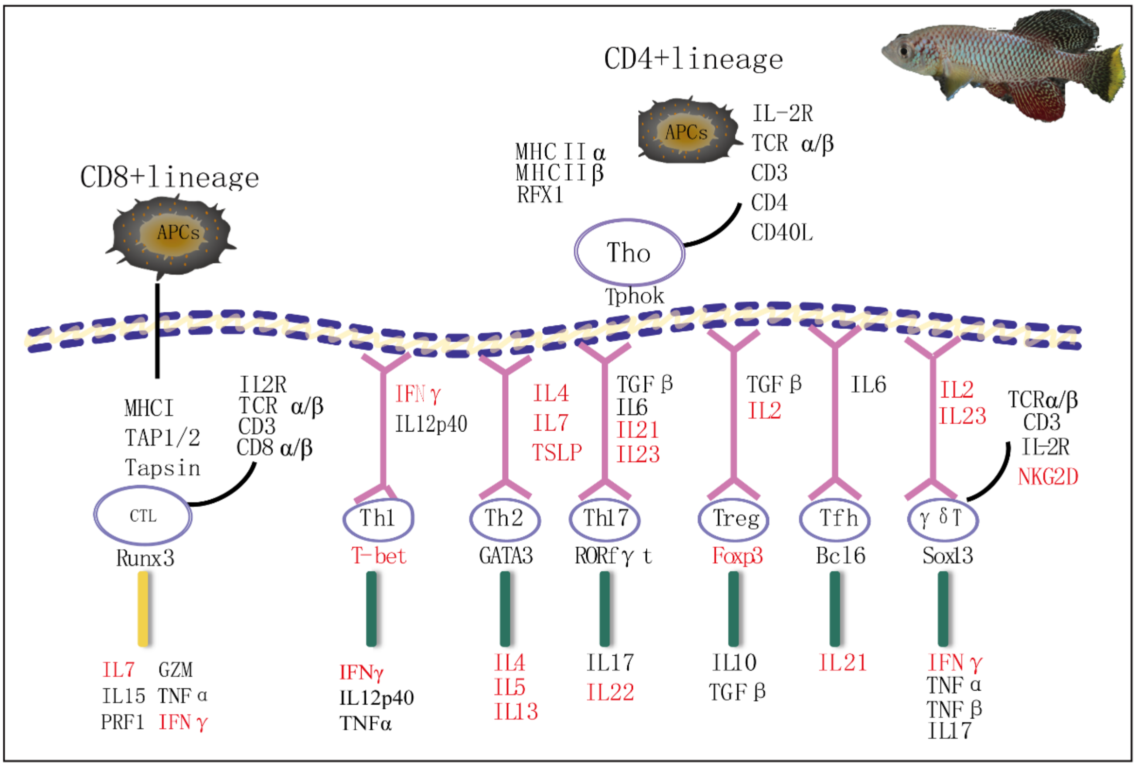
Supplemental Figure S6. Gene family profiles of 18 fish species reveals that 86 single-copy gene families are unique to P. chinensis, Related to Figure 4.

Each column represents a species. Gene family presence is indicated in green; absence in white. Pch, *Protosalanx chinensis*; Gac, *Gasterosteus aculeatus*; Dre, *Danio rerio*; Loc, *Lepisosteus oculatus*; Gmo, *Gadus morhu*; Tru, *Takifugu rubripes*; Nfu, *Nothobranchius furzeri*; Elu, *Esox lucius*; Oni, *Oreochromis niloticus*; Xma, *Xiphophorus maculatus*; Ola, *Oryzias latipes*; Ssa, *Salmo salar*; Sfo, *Scleropages formosus*; Aro, *Anguilla rostrata*; Lch, *Latimeria chalumnae*; Hco, *Hippocampus comes*; Ipu, *Ictalurus punctatus*; Cmi, *Callorhinchus milii*. Clustering was performed using Euclidean distance and average linkage parameters.



Supplemental Figure S7. ***FGF5 phylogenetic tree and multiple sequence alignment of fish FGF5 proteins*, Related to Figure 4.**

The tree was generated from FGF5 protein sequences. The numbers above branches are ML bootstrap proportion. Protein sequences were aligned using MAFFT.



Supplemental Figure S8. Overview of the *N. furzeri* Toll-like receptor family, Related to Figure 5.

Schematic diagram summarizing genes related to different T-cell lineages in *N. furzeri*. Genes absent in the genome assembly are indicated in red.

Supplemental tables

Supplemental Table S1. Summary of genome sequencing strategy, Related to Figure 1.

Paired-end libraries	mate distance	Total data (Gb)	Read length (bp)	Sequence coverage (x)
Illumina	250	26.03	150	53.77
	350	10.67		22.04
	500	14.59		30.14
	2K	32.86		67.88
	5K	12.08		24.95
	10K	17.74		36.64
PacBio	—	10.57	—	21.83
10X Genomics	—	81.92	150	169.22
Total	—	206.46	—	426.48

Supplemental Table S2. Estimated genome size of *P. chinensis* from *k*-mer analysis, Related to Figure 1.

***k*-mer**	**No. *k*-mers**	***k*-mer Depth**	**Genome Size (Mbp)**	**Revised Genome Size (Mbp)**	**Heterozygous Ratio (%)**	**Repeat (%)**
17	38,879,079,724	78	498.45	484.10	0.38	35.43

Supplemental Table S3. ***P. chinensis*** **genome assembly summary statistics, Related to Figure 1.**

	Length (bp)		**Number**	
	Contig (bp)	**Scaffold(bp)**	**Contig**	**Scaffold**
Total	444,877,745	466,695,321	20,856	1,776
Max	2,137,849	44,188,582	-	-
Number>=2000	-	-	16,493	1,087
N50	103,007	5,188,763	876	23
N60	60,712	4,029,931	1,443	33
N70	34,288	2,574,500	2,425	48
N80	17,547	1,626,991	4,260	71
N90	8,371	794,666	7,943	110

Supplemental Table S4. DNA base composition of the *P. chinensis* genome, Related to Figure 1.

	Number (bp)	% of genome
A	117,329,580	25.14
T	117,357,538	25.14
C	105,042,150	22.51
G	105,148,477	22.53
N	21,817,576	4.67
Total (bp)	466,695,321	100
GC	210,190,627	47.25

Supplemental Table S5. Alignment information of reads mapping to the *P. chinensis* genome, Related to Figure 1.

Reads	Mapping rate	97.89%
Genome	Average sequencing depth	105.77×
	Coverage	92.99%
	Coverage at least 4×	92.27%
	Coverage at least 10×	91.71%
	Coverage at least 20×	91.10%
SNP	Heterozygosis	Percent (%)
	656,664	0.16534
	Homology	Percent (%)
	19,671	0.00164

Supplemental Table S6. Proportion of repeats in the *P. chinensis* genome estimated by various methods, Related to Figure 1.

Type	**Repeat size (bp)**	**% of genome**
TRF	60,936,119	13.06
RepeatMasker	123,163,090	26.39
RepeatProteinMask	13,882,973	2.97
Total	149,204,790	31.97

Supplemental Table S7. Statistic of repeat content in the *P. chinensis* genome, Related to Figure 1.

	Repeatmasker (*de novo* + Repbase)		**TE Proteins**		**Combined TEs**	
	Length (bp)	**%in Genome**	**Length (bp)**	**% in Genome**	**Length (bp)**	**% in Genome**
DNA	48,992,395	10.50	3,856,759	0.83	50,280,383	10.77
LINE	28,924,483	6.20	7,805,974	1.67	30,957,635	6.63
SINE	5,878,163	1.26	0	0.00	5,878,163	1.26
LTR	15,363,100	3.29	2,261,950	0.48	15,787,959	3.38
Other	0	0.00	0	0.00	0	0.00
Satellite	13,959,798	2.99	0	0.00	13,959,798	2.99
Simple repeat	29,638,635	6.35	0	0.00	29,638,635	6.35
Unknown	4,635,326	0.99	0	0.00	4,635,326	0.99
Total	123,163,090	26.39	13,882,973	2.97	125,206,960	26.83

Supplemental Table S8. Assessment of *P. chinensis* genome assembly by mapping of *de novo* assembled transcripts, Related to Figure 1.

Dataset	Number	Total length (bp)	Sequences Covered by assembly (%)	with >90% sequence in one scaffold		with >50% sequence in one scaffold	
				Number	Percent (%)	Number	Percent (%)
>0bp	63,983	44,981,816	98.040	59,229	92.679	62,365	97.471
>200bp	63,983	44,981,816	98.040	59,299	92.679	62,365	97.471
>500bp	26,362	33,379,243	99.560	24,270	92.064	26,083	98.942
>1k	12,418	23,668,832	99.903	11,261	90.683	12,333	99.316
>2k	3,939	11,805,674	99.949	3,464	87.941	3,911	99.289

Supplemental Table S9. CEGMA (Core Eukaryotic Genes Mapping Approach) analysis of *P. chinensis* assemblies, Related to Figure 1.

Species	Reference	complete		complete + partial	
		# Proteins	score (%)	# Proteins	score (%)
Protosalanx chinensis	this study	230	92.74	235	94.76
Protosalanx chinensis	Liu *et al*. (2017)	209	84.27	216	87.10

Supplemental Table S10. BUSCO (Benchmarking Universal Single-Copy Orthologs) analysis of *P. chinensis* assemblies, Related to Figure 1.

Species	Reference	Size (Mbp)	Gene number	BUSCO notation assessment results
Protosalanx chinensis	This study	466.70	23,587	C:93.7%[S:89.6%,D:4.1%],F:2.7%,M:3.6%,n:4584
Protosalanx chinensis	Liu et al. (2017)	536.56	19,884	C:85.6%[S:79.8%,D:5.8%],F:3.5%,M:10.9%,n:4584

Supplemental Table S11. Functional annotation of protein coding genes in the *P. chinensis* genome, Related to Figure 1.

		Number	Percent (%)
Total		23,645	-
Swiss-Prot		21,757	92.0
NR		22,891	96.8
KEGG		19,864	84.0
InterPro	all	21,299	90.1
	Pfam	18,987	80.3
	GO	15,558	65.8
Annotated		22,936	97.0

Supplemental Table S12. Summary of the predicted protein-coding genes in *P. chinensis* genome, Related to Figure 1.

Note that the final gene set includes untranslated (UTR) regions.

Gene set		Number	Average transcript length (bp)	Average CDS length (bp)	Average exons per gene	Average exon length (bp)	Average intron length (bp)
De novo	Augustus	27,786	5,123.06	1,124.17	6.3	178.44	752.31
	Geneid	27,556	11,428.85	1,213.68	6.0	202.28	2,026.79
	Genscan	21,391	15,855.64	1,759.49	9.8	179.54	1,601.94
	GlimmerHMM	84,488	4,703.75	680.05	3.8	178.96	1,418.51
	SNAP	66,823	7,053.04	820.44	5.5	149.17	1,397.18
Homology	*Danio rerio*	21,052	7,802.52	1,515.81	8.1	188.09	890.60
	Oryzias latipes	22,166	6,470.85	1,334.08	7.2	186.35	834.00
	Oreochromis niloticus	22,250	7,392.61	1,452.00	7.9	184.56	865.03
	Gasterosteus aculeatus	22,689	6,821.71	1,334.68	7.5	178.78	848.65
	Takifugu rubripes	20,467	7,572.05	1,456.79	8.0	182.77	877.30
	Cynoglossus semilaevis	20,645	8,144.30	1,572.06	8.4	187.66	890.88

	Tetraodon nigroviridis		19,276	7,630.55	1,445.56	8.2	176.21	858.61
	Larimichthys crocea		21,743	7,832.77	1,531.19	8.2	186.01	871.36
	Salmo salar		26,808	6,669.82	1,417.00	7.1	199.53	860.88
RNASeq		Cufflinks	41,385	11,009.96	2,757.41	10.1	273.73	909.52
		PASA	26,639	7,428.80	1,365.34	8.4	162.64	819.93
EVM			30,832	7,011.59	1,261.33	7.2	175.72	930.75
PASA			30,187	7,327.46	1,318.74	7.5	175.63	923.22
Final set			23,645	8,529.84	1,509.75	8.7	173.09	909.08

Supplemental Table S13. Summary of predicted RNA genes and their characteristics, Related to Figure 1.

Type		Copy	Average length (bp)	Total length (bp)	% of genome
miRNA		1,327	127.20	168,800	0.036169
tRNA		1,382	75.64	104,534	0.022399
rRNA	rRNA	95	249.18	23,672	0.005072
	18S	29	331.69	9,619	0.002061
	28S	46	265.02	12,191	0.002612
	5.8S	4	148.25	593	0.000127
	5S	16	79.31	1,269	0.000272
snRNA	snRNA	520	175.74	91,387	0.019582
	CD-box	176	106.92	18,818	0.004032
	HACA-box	231	240.58	55,574	0.011908
	splicing	98	143.31	14,044	0.003009

Supplemental Table S14. Divergence time between species, Related to Figure 1.

Mya denotes million years ago.

Taxon A	**Taxon B**	**Time-range （Mya）**
Gasterosteus aculeatus	*Takifugu rubripes*	97-151
Scleropages formosus	*Lepisosteus oculatus*	374-390
Nothobranchius furzeri	*Oryzias latipes*	128-153
Gadus morhua	*Gasterosteus aculeatus*	139-158
Latimeria chalumnae	*Danio rerio*	416-422
Callorhinchus milii	*Latimeria chalumnae*	422-463

Supplemental Table S16. Expression of genes involved in scale formation in *P. chinensis*, Related to Figure 2.

Transcripts were assessed by interrogating raw RNA-seq reads and a Trinity assembly.

Gene ID	Gene symbol	Name	Transcript detected?
evm.model.scaffold110.338	*EDA*	ectodysplasin A	✓
evm.model.scaffold5.21_evm.model.scaffold5.24	*EDA*	ectodysplasin A	✓
evm.model.scaffold93.206	*EDAR*	ectodysplasin A receptor	✗
evm.model.scaffold22.181	*FGFR1A*	fibroblast growth factor receptor 1a	✓
evm.model.scaffold171.50	*LEF1*	lymphoid enhancer-binding factor 1	✓
evm.model.scaffold34.272	*TCF7*	transcription factor 7 (T-cell specific, HMG-box)	✓
evm.model.scaffold1622.4	*LAMB3*	laminin, beta 3	✓
evm.model.scaffold159.552	*COL7A1*	collagen, type VII, alpha 1	✓

Supplemental Table S19. Summary of BLAST of *FGF5* exon sequences against raw RNA-seq reads from mixed *P. chinensis* tissue, Related to Figure 4.

Sequences were obtained from a multiple sequence alignment (see Supplemental Data 1). Raw RNA-seq reads (150 bp) were queried using a local instance of sequenceserver v1.0.11[131] and various regions of *P. chinensis* FGF5 genes: the 142 bp 3' region of exon 1, the 142 bp 5' region of exon 2, and their exon intron junction (82 bp of the 3' region of exon 1 and 47 bp of exon 2). Note that *FGF5A* exon 2 is distinct from exon 2 of *FGF5B* to *FGF5N*.

gene	exon 1	exon 2	exon 1-exon 2 junction
FGF5A	4	12	2
FGF5B	0	0	0
FGF5C	0	0	0
FGF5D	12	0	4
FGF5E	8	4	4
FGF5F	2	4	4
FGF5G	12	4	4
FGF5H	0	4	0
FGF5I	0	0	0
FGF5J	0	0	0
FGF5K	0	0	0
FGF5L	0	0	0
FGF5M	0	0	0
FGF5M	0	0	0

Supplemental Table S21. KEGG enrichment of gene families contracted in *P. chinensis*, Related to Figure 5.

MapID	MapTitle	P-value
map04621	NOD-like receptor signaling pathway	9.65E-218
map05133	Pertussis	6.17E-168
map04740	Olfactory transduction	2.30E-107
map05164	Influenza A	2.59E-100
map05322	Systemic lupus erythematosus	4.83E-66
map04640	Hematopoietic cell lineage	2.61E-63
map05320	Autoimmune thyroid disease	5.82E-60
map05416	Viral myocarditis	1.23E-54
map05323	Rheumatoid arthritis	2.45E-49
map04064	NF-kappa B signaling pathway	5.68E-48
map04662	B cell receptor signaling pathway	3.76E-45
map05162	Measles	3.86E-37
map04672	Intestinal immune network for IgA production	1.41E-35
map05310	Asthma	1.58E-35
map05140	Leishmaniasis	2.04E-31
map04145	Phagosome	4.53E-31
map05330	Allograft rejection	1.35E-28
map05150	Staphylococcus aureus infection	2.32E-28
map04650	Natural killer cell mediated cytotoxicity	4.42E-27
map05202	Transcriptional misregulation in cancer	1.11E-22
map05143	African trypanosomiasis	1.60E-22
map05340	Primary immunodeficiency	1.60E-22
map04666	Fc gamma R-mediated phagocytosis	9.62E-21
map05146	Amoebiasis	8.77E-20
map05414	Dilated cardiomyopathy	2.00E-18
map04020	Calcium signaling pathway	8.52E-18
map04664	Fc epsilon RI signaling pathway	1.17E-17
map04072	Phospholipase D signaling pathway	2.75E-12
map05169	Epstein-Barr virus infection	8.48E-12
map05152	Tuberculosis	7.58E-11
map04530	Tight junction	8.15E-06
map04514	Cell adhesion molecules (CAMs)	2.65E-05
map05130	Pathogenic Escherichia coli infection	5.67E-05
map05144	Malaria	9.38E-05
map05332	Graft-versus-host disease	0.0001135
map04940	Type I diabetes mellitus	0.0003764
map05321	Inflammatory bowel disease (IBD)	0.0004392

map04612	Antigen processing and presentation	0.0022873
map04151	PI3K-Akt signaling pathway	0.0022942
map04540	Gap junction	0.0028647
map04360	Axon guidance	0.0138875

Supplemental Table S22. Gene Ontology enrichment of gene families contracted in *P. chinensis*,

Related to Figure 5.

GO_ID	GO_Term	GO_Class	P-value	Adjusted P-value
GO:0006915	apoptotic process	BP	0.002995896	0.009705454
GO:0005488	Binding	MF	5.13E-20	1.99E-18
GO:0030246	carbohydrate binding	MF	4.57E-25	2.84E-23
GO:0007155	cell adhesion	BP	1.83E-11	1.68E-10
GO:0007049	cell cycle	BP	0.001330008	0.004809681
GO:0007166	cell surface receptor signaling pathway	BP	7.54E-26	5.87E-24
GO:0034622	cellular macromolecular complex assembly	BP	1.03E-06	5.70E-06
GO:0044430	cytoskeletal part	CC	4.28E-13	5.11E-12
GO:0015074	DNA integration	BP	0.000686831	0.002670054
GO:0048013	ephrin receptor signaling pathway	BP	2.81E-27	2.91E-25
GO:0004930	G-protein coupled receptor activity	MF	1.55E-30	2.40E-28
GO:0007186	G-protein coupled receptor signaling pathway	BP	5.77E-21	2.56E-19
GO:0005525	GTP binding	MF	6.11E-09	4.63E-08
GO:0003924	GTPase activity	MF	0.008251113	0.021746576
GO:0020037	heme binding	MF	0.000103518	0.000487787
GO:0005833	hemoglobin complex	CC	0.00123812	0.00463922
GO:0007156	homophilic cell adhesion	BP	5.24E-18	1.25E-16
GO:0043232	intracellular non-membrane-bounded organelle	CC	0.000520827	0.002131278
GO:0044446	intracellular organelle part	CC	0.001858952	0.006569705
GO:0043167	ion binding	MF	3.56E-13	4.42E-12
GO:0005506	iron ion binding	MF	0.000706633	0.002713122
GO:0046872	metal ion binding	MF	1.26E-16	2.60E-15
GO:0005874	Microtubule	CC	4.22E-07	2.38E-06
GO:0003774	motor activity	MF	8.13E-11	7.23E-10
GO:0016459	myosin complex	CC	5.37E-14	7.95E-13
GO:0003956	NAD(P)+-protein-arginine ADP-ribosyltransferase activity	MF	1.76E-07	1.09E-06
GO:0017111	nucleoside-triphosphatase activity	MF	0.013076943	0.032277217
GO:0000786	Nucleosome	CC	0.007361972	0.019737702
GO:0006334	nucleosome assembly	BP	0.014565938	0.03511633
GO:0004984	olfactory receptor activity	MF	3.14E-135	9.77E-133
GO:0019825	oxygen binding	MF	0.003111349	0.009880125
GO:0015671	oxygen transport	BP	0.00221137	0.007484359
GO:0006471	protein ADP-ribosylation	BP	3.17E-06	1.73E-05
GO:0005515	protein binding	MF	1.61E-11	1.57E-10
GO:0006461	protein complex assembly	BP	0.000371058	0.001580808
GO:0051258	protein polymerization	BP	2.71E-07	1.62E-06

GO:0032550	purine ribonucleoside binding	MF	0.016652344	0.038875768
GO:0035639	purine ribonucleoside triphosphate binding	MF	0.016410404	0.038875768
GO:0032555	purine ribonucleotide binding	MF	0.017907593	0.039780438
GO:0004872	receptor activity	MF	2.77E-15	5.06E-14
GO:0042981	regulation of apoptotic process	BP	0.003208658	0.009880125
GO:0003964	RNA-directed DNA polymerase activity	MF	0.022639537	0.047897251
GO:0007165	signal transduction	BP	0.00609246	0.017069867
GO:0005200	structural constituent of cytoskeleton	MF	1.47E-10	1.20E-09
GO:0001594	trace-amine receptor activity	MF	6.36E-08	4.50E-07
GO:0046914	transition metal ion binding	MF	0.00626301	0.017391036
GO:0004888	transmembrane signaling receptor activity	MF	3.58E-22	1.86E-20

Supplemental Table S23. Number of genes related to KEGG immunity pathways in *P. chinensis* and eight fish species, Related to Figure 5.

Bold denotes lower number of genes in *P. chinensis*

		P.	***D.***	***I.***	***T.***	***T.***	***L.***	***M.***	***O.***	***X.***
	Immune pathway	***chinensis***	***rerio***	***punctatus***	***rubripes***	***nigroviridis***	***crocea***	***zebra***	***latipes***	***maculatus***
Map04640	Hematopoietic cell lineage	71	71	97	83	70	90	127	96	75
Map04610	Complement and coagulation cascades	61	80	114	100	81	90	111	99	88
Map04611	Platelet activation	202	171	176	182	195	204	197	192	189
Map04620	Toll and Imd signaling pathway	66	49	52	46	55	53	56	49	49
Map04624	Toll-like receptor signaling pathway	110	97	106	103	97	122	142	100	102
Map04621	NOD-like receptor signaling pathway	175	157	213	172	165	212	244	177	192
Map04622	RIG-I-like receptor signaling pathway	76	63	71	68	68	86	85	66	65
Map04623	Cytosolic DNA-sensing pathway	53	42	51	40	43	54	66	46	50
Map04650	Natural killer cell mediated cytotoxicity	126	98	132	121	107	131	146	117	132
Map04660	T cell receptor signaling pathway	156	125	131	125	140	149	144	127	125
Map04612	Antigen processing and presentation	61	70	94	70	54	72	110	76	73
Map04658	Th1 and Th2 cell differentiation	106	98	101	108	102	114	133	108	99
Map04659	Th17 cell differentiation	137	112	133	123	124	137	159	125	118
Map04657	IL-17 signaling pathway	104	81	102	94	85	114	119	95	95

Map04662	B cell receptor signaling pathway	105	87	101	97	109	108	108	100	94
Map04664	Fc epsilon RI signaling pathway	92	78	72	85	98	91	82	72	75
Map04666	Fc gamma R-mediated phagocytosis	149	119	127	129	150	134	139	126	136
Map04670	Leukocyte transendothelial migration	202	147	182	196	194	232	212	207	207
Map04672	Intestinal immune network for IgA production	28	41	62	37	38	43	73	39	33
Map4062	Chemokine signaling pathway	247	199	219	206	219	237	268	215	236
	Total	2327	1985	2336	2185	2194	2473	2721	2232	2233

Supplemental Table S24. Overview of the number of genes in four immunity families in *P. chinensis* and seven fish species, Related to Figure 5.

Species	**MHCI**	**MHCII**	**NLRCs**	**C3 family**
P. chinensis	5	7	11	3
E. lucius	11	16	34	7
N. furzeri	4	2	11	5
S. salar	17	11	33	17
D. rerio	30	18	52	10
O. latipes	15	9	28	7
O. niloticus	55	34	90	7

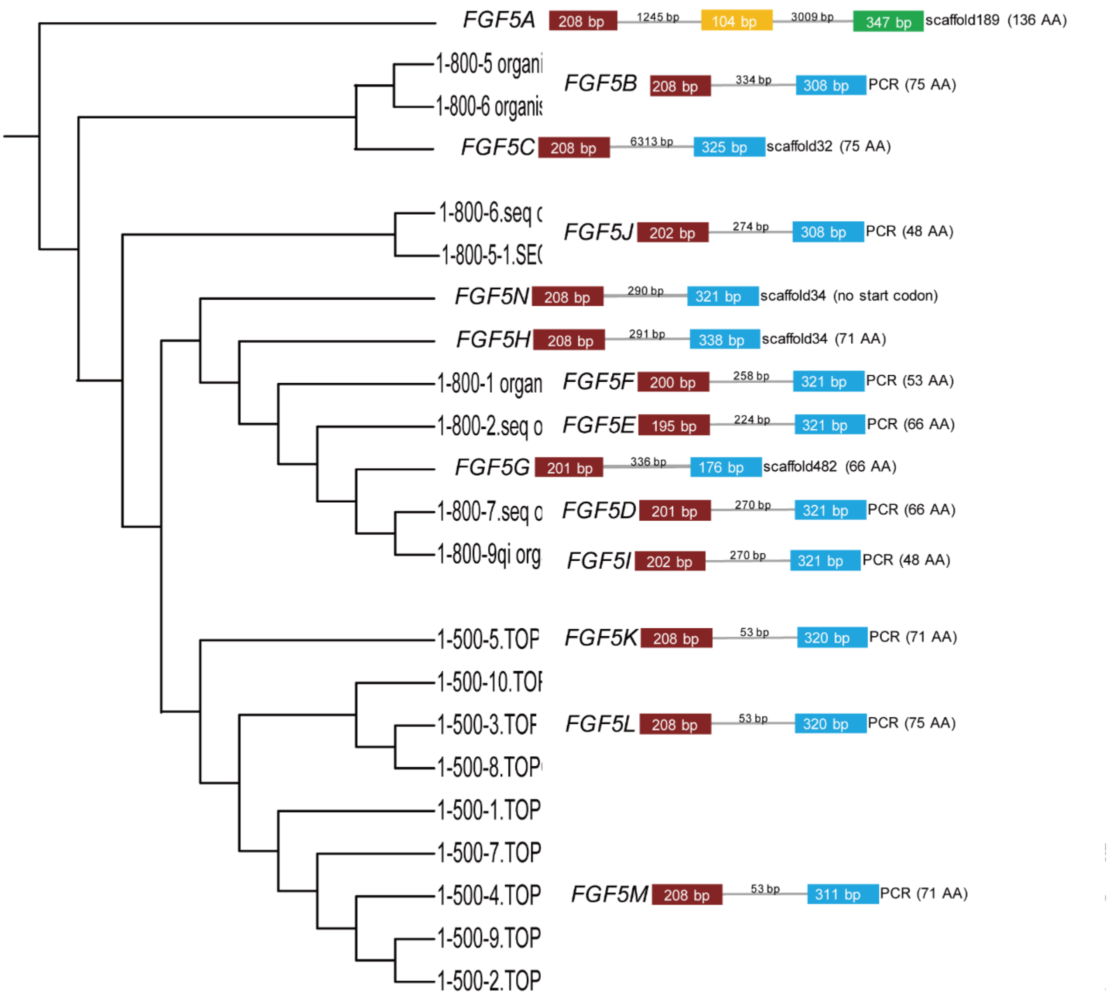
Supplemental Data 1. Duplicated fibroblast growth factor 5 genes in *Protosalanx chinensis*, Related to Figure 4.

(A) Overview of P. *chinensis* FGF5 genes . A neighbor joining tree was generated from conserved sites of a multiple sequence alignment using MAFFT. *FGF5A* denotes the canonical FGF5 gene (exon 1 in brown; exon 2 in green; exon 3 in yellow). Duplicated FGF5 genes (*FGF5B* to *FGFBN*) have a novel exon 2 (shown in blue).

(B) Alignment of proteins encoded by fibroblast growth factor 5 genes in *P. chinensis* and related species in superorder Protacanthopterygii, zebrafish, and human . *Homo sapiens* denotes human; *Danio rerio*, zebrafish, *Salmo salar*, Atlantic salmon; *Esox lucius*, Northern pike. All other sequences are *P. chinensis* genome scaffolds or PCR amplicons. Scaffold189 is the *P. chinensis FGF5A*, the ortholog to teleost *FGF5*, while the sequences below indicate various duplicated *FGF5* genes with a novel exon 2 [see (B)]. The FGF5 domain is indicated by dark blue line underneath the alignment (reside 85 to 219). The location of the 12 -strands are indicted by green boxes. Four residues shared by all FGF genes are indicated in red. Annotations derived from (Mohammadi *et al*., 2005).

(C) Multiple sequence alignment of *P. chinensis FGF5* genes. MAFFT (using the G-INS-i Iterative refinement method) was used to generate multiple sequence alignments of PCR amplicons and genome scaffolds. Exon 1, common to all FGF5 genes, is highlighted in yellow. Alignments from exon 1 onwards exclude *FGF5A*, which employs a different exon 2. The aligned sequence corresponds to the region amplified by PCR of genomic DNA (see transparent Methods). Part of the intron has been omitted.

A



FGF5A 208 bp 1245 bp 104 bp 3009 bp 347 bp scaffold189 (136 AA)
1-800-5 organi
1-800-6 organis
FGF5B 208 bp 334 bp 308 bp PCR (75 AA)
FGF5C 208 bp 6313 bp 325 bp scaffold32 (75 AA)
1-800-6.seq (
1-800-5-1.SE(
FGF5J 202 bp 274 bp 308 bp PCR (48 AA)
FGF5N 208 bp 290 bp 321 bp scaffold34 (no start codon)
FGF5H 208 bp 291 bp 338 bp scaffold34 (71 AA)
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FGF5F 200 bp 258 bp 321 bp PCR (53 AA)
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FGF5E 195 bp 224 bp 321 bp PCR (66 AA)
FGF5G 201 bp 336 bp 176 bp scaffold482 (66 AA)
1-800-7.seq o
FGF5D 201 bp 270 bp 321 bp PCR (66 AA)
1-800-9qi org
FGF5I 202 bp 270 bp 321 bp PCR (48 AA)
1-500-5.TOF
FGF5K 208 bp 53 bp 320 bp PCR (71 AA)
1-500-10.TOI
1-500-3.TOF
FGF5L 208 bp 53 bp 320 bp PCR (75 AA)
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1-500-1.TOP
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1-500-4.TOP
FGF5M 208 bp 53 bp 311 bp PCR (71 AA)
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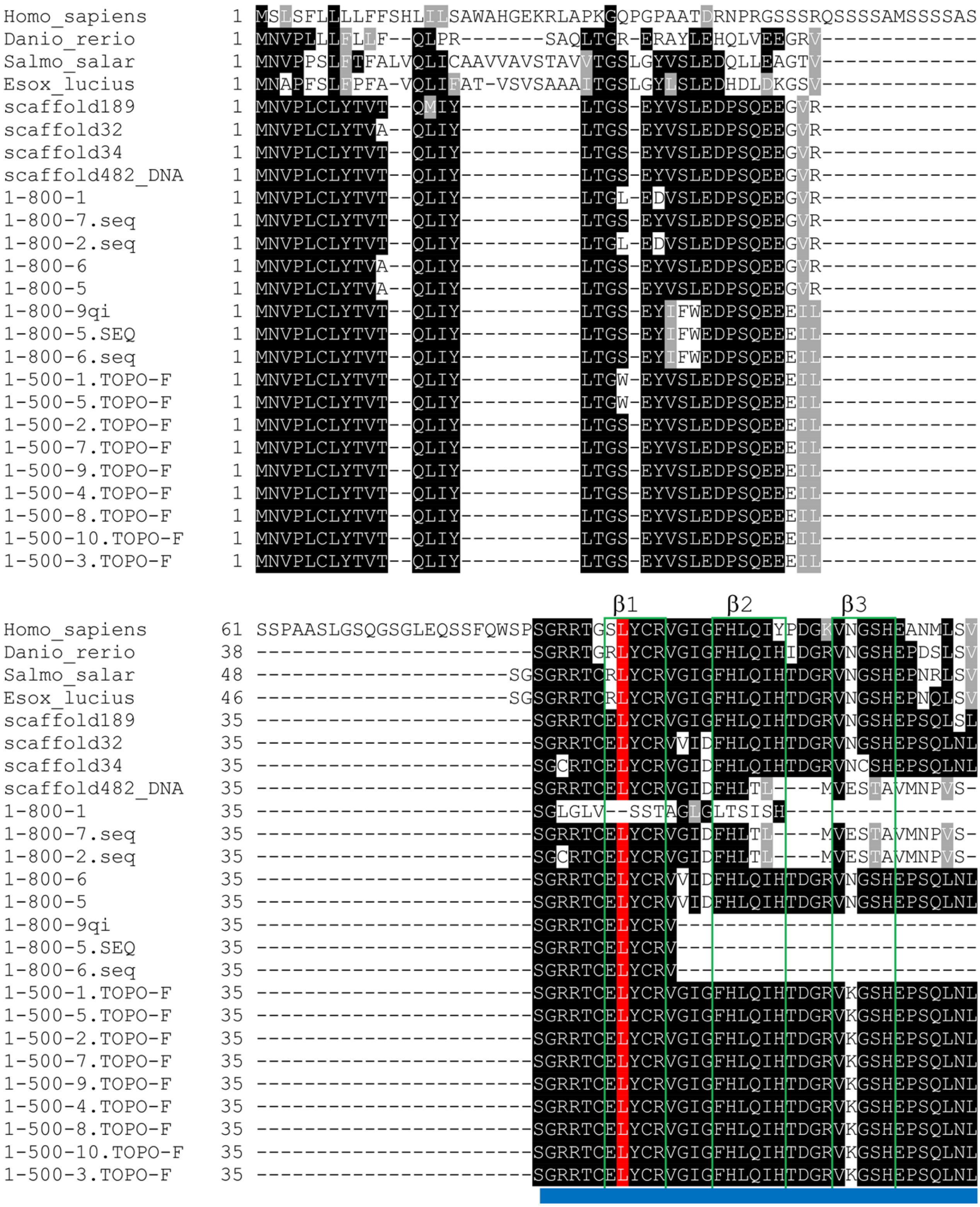
1-500-2.TOP

B



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Homo_sapiens        1 MSLSFLLLLFFSHLILSAWAHGEKRLAPKGQPGPAATDRNPRGSSSRQSSSSAMSSSSAS
Danio_rerio         1 MNVPLLLFLLF--QLPR-------SAQLTGR-ERAYLEHQLVEEGRV-------------
Salmo_salar         1 MNVPPSLFTFALVQLICAAVVAVSTAVVTGSLGYVSLEDQLLEAGTV-------------
Esox_lucius         1 MNAPFSLFPFA-VQLIFAT-VSVSAAAITGSLGYLSLEDHDLDKGSV-------------
scaffold189         1 MNVPLCLYTVT--QMIY----------LTGS-EYVSLEDPSQEEGVR-------------
scaffold32          1 MNVPLCLYTVA--QLIY----------LTGS-EYVSLEDPSQEEGVR-------------
scaffold34          1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEGVR-------------
scaffold482_DNA     1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEGVR-------------
1-800-1             1 MNVPLCLYTVT--QLIY----------LTGL-EDVSLEDPSQEEGVR-------------
1-800-7.seq         1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEGVR-------------
1-800-2.seq         1 MNVPLCLYTVT--QLIY----------LTGL-EDVSLEDPSQEEGVR-------------
1-800-6             1 MNVPLCLYTVA--QLIY----------LTGS-EYVSLEDPSQEEGVR-------------
1-800-5             1 MNVPLCLYTVA--QLIY----------LTGS-EYVSLEDPSQEEGVR-------------
1-800-9qi           1 MNVPLCLYTVT--QLIY----------LTGS-EYIFWEDPSQEEEIL-------------
1-800-5.SEQ         1 MNVPLCLYTVT--QLIY----------LTGS-EYIFWEDPSQEEEIL-------------
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1-500-5.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGW-EYVSLEDPSQEEEIL-------------
1-500-2.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------
1-500-7.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------
1-500-9.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------
1-500-4.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------
1-500-8.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------
1-500-10.TOPO-F     1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------
1-500-3.TOPO-F      1 MNVPLCLYTVT--QLIY----------LTGS-EYVSLEDPSQEEEIL-------------

                                                    β1        β2        β3
Homo_sapiens       61 SSPAASLGSQGSGLEQSSFQWSPSGRRTGSLYCRVGIGFHLQIYPDGKVNGSHEANMLSV
Danio_rerio        38 -----------------------SGRRTGRLYCRVGIGFHLQIHIDGRVNGSHEPDSLSV
Salmo_salar        48 ---------------------SGSGRRTCRLYCRVGIGFHLQIHTDGRVNGSHEPNRLSV
Esox_lucius        46 ---------------------SGSGRRTCRLYCRVGIGFHLQIHTDGRVNGSHEPNQLSV
scaffold189        35 -----------------------SGRRTCELYCRVGIGFHLQIHTDGRVNGSHEPSQLSL
scaffold32         35 -----------------------SGRRTCELYCRVVIDFHLQIHTDGRVNGSHEPSQLNL
scaffold34         35 -----------------------SGCRTCELYCRVGIDFHLQIHTDGRVNCSHEPSQLNL
scaffold482_DNA    35 -----------------------SGRRTCELYCRVGIDFHLTL----MVESTAVMNPVS-
1-800-1            35 -----------------------SGLGLV--SSTAGLGLTSISH----------------
1-800-7.seq        35 -----------------------SGRRTCELYCRVGIDFHLTL----MVESTAVMNPVS-
1-800-2.seq        35 -----------------------SGCRTCELYCRVGIDFHLTL----MVESTAVMNPVS-
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1-800-5            35 -----------------------SGRRTCELYCRVVIDFHLQIHTDGRVNGSHEPSQLNL
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1-500-8.TOPO-F     35 -----------------------SGRRTCELYCRVGIGFHLQIHTDGRVKGSHEPSQLNL
1-500-10.TOPO-F    35 -----------------------SGRRTCELYCRVGIGFHLQIHTDGRVKGSHEPSQLNL
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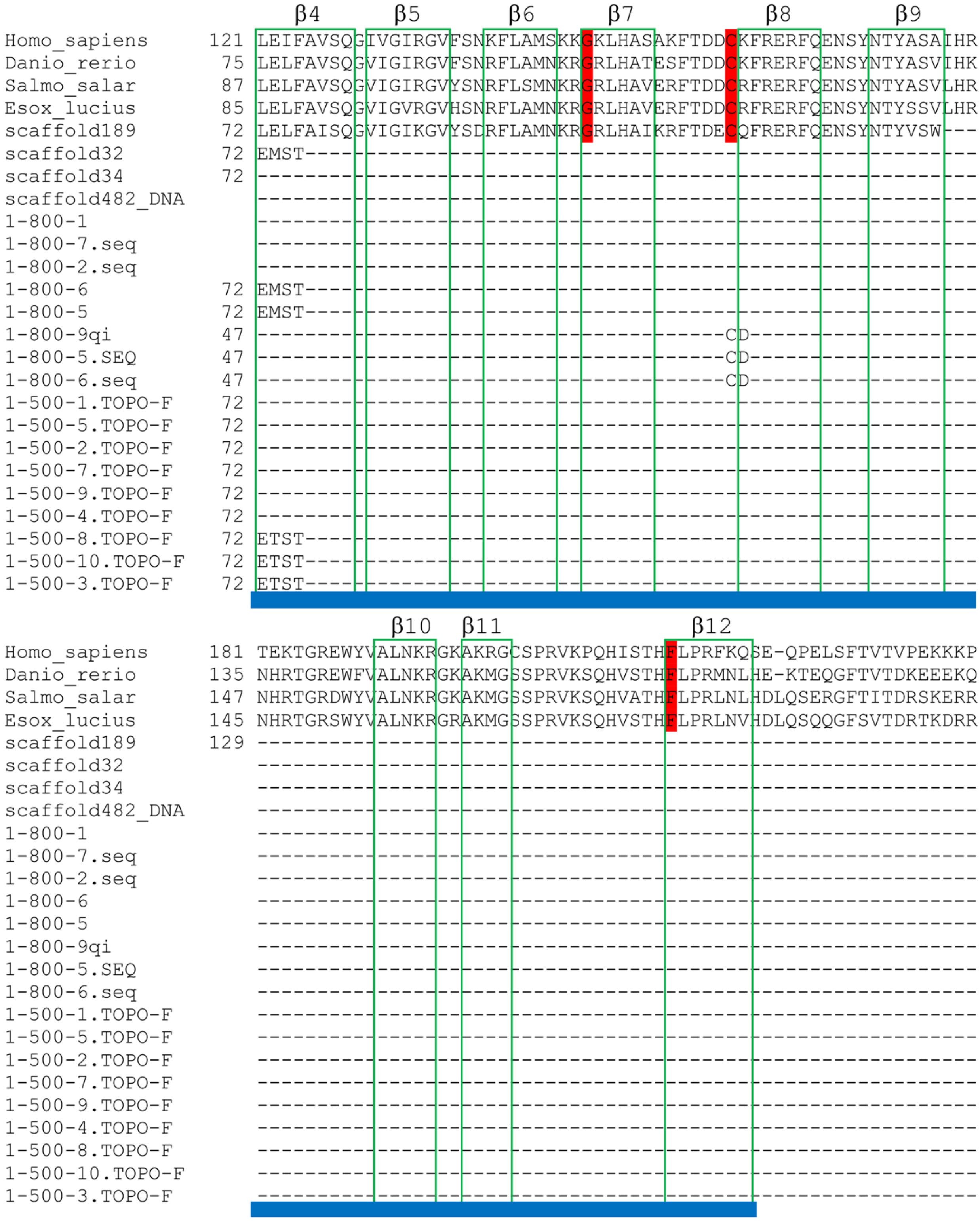

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                            β4      β5        β6      β7           β8         β9
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Danio_rerio           75 LELFAVSQGVIGIRGVFSNRFLAMNKRGRLHATESFTDDCKFRERFQENSYNTYASVIHK
Salmo_salar           87 LELFAVSQGVIGIRGVYSNRFLSMNKRGRLHAVERFTDDCRFRERFQENSYNTYASVLHR
Esox_lucius           85 LELFAVSQGVIGVRGVHSNRFLAMNKRGRLHAVERFTDDCRFRERFQENSYNTYSSVLHR
scaffold189           72 LELFAISQGVIGIKGVYSDRFLAMNKRGRLHAIKRFTDECQFRERFQENSYNTYVSW---
scaffold32            72 EMST--------------------------------------------------------
scaffold34            72 ------------------------------------------------------------
scaffold482_DNA          ------------------------------------------------------------
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1-800-2.seq              ------------------------------------------------------------
1-800-6               72 EMST--------------------------------------------------------
1-800-5               72 EMST--------------------------------------------------------
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1-500-8.TOPO-F        72 ETST--------------------------------------------------------
1-500-10.TOPO-F       72 ETST--------------------------------------------------------
1-500-3.TOPO-F        72 ETST--------------------------------------------------------

                                    β10   β11                β12
Homo_sapiens         181 TEKTGREWYVALNKRGKAKRGCSPRVKPQHISTHFLPRFKQSE-QPELSFTVTVPEKKKP
Danio_rerio          135 NHRTGREWFVALNKRGKAKMGSSPRVKSQHVSTHFLPRMNLHE-KTEQGFTVTDKEEEKQ
Salmo_salar          147 NHRTGRDWYVALNKRGKAKMGSSPRVKSQHVATHFLPRLNLHDLQSERGFTITDRSKERR
Esox_lucius          145 NHRTGRSWYVALNKRGRAKMGSSPRVKSQHVSTHFLPRLNVHDLQSQQGFSVTDRTKDRR
scaffold189          129 ------------------------------------------------------------
scaffold32               ------------------------------------------------------------
scaffold34               ------------------------------------------------------------
scaffold482_DNA          ------------------------------------------------------------
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1-800-2.seq              ------------------------------------------------------------
1-800-6                  ------------------------------------------------------------
1-800-5                  ------------------------------------------------------------
1-800-9qi                ------------------------------------------------------------
1-800-5.SEQ              ------------------------------------------------------------
1-800-6.seq              ------------------------------------------------------------
1-500-1.TOPO-F           ------------------------------------------------------------
1-500-5.TOPO-F           ------------------------------------------------------------
1-500-2.TOPO-F           ------------------------------------------------------------
1-500-7.TOPO-F           ------------------------------------------------------------
1-500-9.TOPO-F           ------------------------------------------------------------
1-500-4.TOPO-F           ------------------------------------------------------------
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1-500-10.TOPO-F          ------------------------------------------------------------
1-500-3.TOPO-F           ------------------------------------------------------------
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C

```
[EXON 1]
M gttcctctttgtctttataccgtcacccagttgatttacctgactggatgggagtatgtt
M gttcctctttgtctttataccgtcacccagttgatttacctgactggatcggagtatgtt
M gttcctctttgtctttataccgtcacccagttgatttacctgactggatcggagtatgtt
M gttcctctttgtctttataccgtcacccagttgatttacctgactggatcggagtatgtt
M gttcctctttgtctttataccgtcacccagttgatttacctgactggatcggagtatgtt
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L gttcctctttgtctttataccgtcactcagttgatttacctgactggatcggagtatgtt
K gttcctctttgtctttataccgtcactcagttgatttacctgactggatgggagtatgtt
I gttcctctttgtctttataccgtcactcagttgatttacctgactggatcggagtatatt
D gttcctctttgtctttataccgtcactcagttgatttacctgactggatcggagtatgtt
E gttcctctttgtctttataccgtcactcagttgatttacctgactggattggaggatgtt
F gttcctctttgtctttataccgtcactcagttgatttacctgactggattggaggatgtt
H gttcctctttgtctttataccgtcactcagttgatttacctgactggatcggagtatgtt
B gttcctctttgtctttataccgtcgctcagttgatttacctgactggatcggagtatgtt
B gttcctctttgtctttataccgtcgctcagttgatttacctgactggatcggagtatgtt
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J gttcctctttgtctttataccgtcactcagttgatttacctgactggatcggagtatatt
G gttcctctttgtctttataccgtcacccagttgatttacctgactggatcggagtatgtt
N ctcctgtgtgggcccgttaccgaagacgccgtgccccaaggagcctgttgccttgacttc
C gttcctctttgtctttataccgtcgctcagttgatttacctgactggatcggagtatgtt
A gttcctctttgtctttataccgtcactcagatgatttacctgactggatccgagtacgtt
  *.*. . * * *..  ***** . .   ** ...*   ..*. * *     *. *.

M tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
M tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
M tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
M tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
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L tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
L tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
L tctttggaagacccttctcaggaagaggagatcctctcaggacgcaggacttgtgagctc
K tctttggaagacccttctcaggaagaggaaatcctctcaggacgcaggacttgtgagctc
I ttttgggaagacccttctcaggaggaggagatcctctcaggacgcaggacttgtgagctc
D tctttggaagacccttctcaggaggaggggtccgctcaggacgcaggacttgtgagctc
E tctttggaagacccttctcaggaggaggggtccgctcaggatgcaggacttgtgagctc
F tctttggaagacccttctcaggaggaggggtccgctcagggt-taggacttgtgagctc
H tctttggaagacccttctcaggaggaggggtccgctcaggatgcaggacttgtgagctc
B tctttggaagacccttctcaggaggaggggtgcgctcaggacgcaggacttgtgagctc
B tctttggaagacccttctcaggaggaggggtgcgctcaggacgcaggacttgtgagctc
```

```
J ttttgggaagacccttctcaggaggaggagatcctctcaggacgcaggacttgtgagctc
J ttttgggaagacccttctcaggaggaggagatcctctcaggacgcaggacttgtgagctc
G tctttggaagacccttctcaggaggagggggtccgctcaggacgcaggacttgtgagctc
N tcacctgatgaaggggttagggataaccctagccctaaccctaaccctaaccctagccct
C tctttggaagacccttctcaggaggagggggtgcgctcaggacgcaggacttgtgagctc
A tctttggaagacccttctcaggaggagggggtccgctcaggacgcaggacttgtgagctc
  *. .  ** **     .* .*** .*    .  * .       .   * .. *.. *..

M tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
M tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
M tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
M tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
M tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
L tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
L tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
L tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
K tactgcaggg-ttgggattggcttccatcttcagattcacaccgatggtagagtcaaagg
I tactgcagggtttgtgattgacttccatc-------tcacactgatggtagagtcaacgg
D tactgcaggg-ttgggattgacttccatc-------tcacactgatggtagagtcaacgg
E tactgcaggg-ttgggattgacttccatc-------tcacactgatggtagagtcaacgg
F tactgcaggg-ttgggattgacttccatc-------tcacactgatggtagagtcaacgg
H tactgcaggg-ttgggattgacttccatcttcagattcacaccgatggtagagtcaactg
B tactgcaggg-ttgtgattgacttccatcttcagattcacaccgatggtagagtcaacgg
B tactgcaggg-ttgtgattgacttccatcttcagattcacaccgatggtagagtcaacgg
J tactgcagggtttgtgattgacttccatc-------tcacaccgatggtagagtcaacgg
J tactgcagggtttgtgattgacttccatc-------tcacaccgatggtagagtcaacgg
G tactgcaggg-ttgggattgacttccatc-------tcacactgatggtagagtcaacgg
N aaccctaacc-atggagaggatcaccatgatcagattcacaccgatggtagagtcaacgg
C tactgcaggg-ttgtgattgacttccatcttcagattcacaccgatggtagagtcaacgg
A tactgcagag-ttgggattggcttccatcttcagattcacaccgatggtagagtcaacgg
   **. .*.    ** ..  *... ****        ******.**************  *

M cagtcatgaacccagtcagttaagtg----------------------------------
M cagtcatgaacccagtcagttaagtg----------------------------------
M cagtcatgaacccagtcagttaagtg----------------------------------
M cagtcatgaacccagtcagttaagtg----------------------------------
M cagtcatgaacccagtcagttaagtg----------------------------------
L cagtcatgaacccagtcagttaagtg----------------------------------
L cagtcatgaacccagtcagttaagtg----------------------------------
L cagtcatgaacccagtcagttaagtg----------------------------------
K cagtcatgaacccagtcagttaagtg----------------------------------
I cagtcatgaatccagtcagttaagtaagtttcattcatttattacatgcatccaacaggt
D cagtcatgaatccagtcagttaagtaagtttcattcatttattacatgcatccaacaggt
E cagtcatgaatccagtcagttaagtaaatttcattcatttattacatgcatccaacaggt
```

```
F cagtcatgaatccagtcagttaagtaagtttcattcatttattacatgcatccaacaggt
H cagtcatgaacccagtcagttaagtaagtttcattcatttattacatgcatccaacaggt
B cagtcatgaacccagtcagttaagtacgtttcattcattcattacatgcatccaacaggt
B cagtcatgaacccagtcagttaagtacgtttcattcattcattacatgcatccaacaggt
J cagtcatgaacccagtcagttaagtaagtttcattcattcattacatgcatccaacaggt
J cagtcatgaacccagtcagttaagtaagtttcattcattcattacatgcatccaacaggt
G cagtcatgaatccagtcagttaagtaagtttcattcatttattacatgcatccaacaggt
N cagtcatgaacccagtcagttaagtaagtttcattcattcattacatgcatccaacaggt
C cagtcatgaacccagtcagttaagtacgtttcattcattcattacatgcatccaacaggt
A cagtcatgaacccagtcagttaagtaagtttcatttattcattacatgcatccaacaggt
  **********.**************.

M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
K ------------------------------------------------------------
I tattgaaccaagccactcctctccactcctcccctccactcct-----------------
D tattgaaccaagccactcctctccactcctcccctccactcct-----------------
E tattgaaccaagccactcctctgcactcctcccctccactcct-----------------
F tattgaaccaagccactcctctccactcctcccctcctctcct----------tctctcc
H tattgaactccaccccactcctcccctcctctcctccactcctccccttcactcctcccc
B tattgaactccaccccactcctcccctcctctcctccactcctccccttcactcctcccc
B tattgaactccaccccactcctcccctcctctcctccactcctccccttcactcctcccc
J tattgaaccaagccactcctctc--ctccattcctgccctcctctcctccactcctcccc
J tattgaaccaagccactcctctc--ctccattcctgccctcctctcctccactcctcccc
G tattgaaccaagccactcctctccactcctcccctccactcctctccttctctcctcccc
N tattgaactccaccccactcctcccttcctctccttcactcctcccctcctctcctccac
C tattgaactccaccccactcctcccctcctctcctccactcctccccttcactcctcccc

M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
K ------------------------------------------------------------
I -------------------------------------------------ctccttc---t
```

```
D -------------------------------------------------ctccttc---t
E -------------------------------------------------ctccttc---t
F tc---------------------------------------------ccctcctcc---a
H tc---------------------------------------------ctctcctcc---a
B tc---------------------------------------------ctctcctcc---c
B tc---------------------------------------------ctctcctcc---c
J tc---------------------------------------------ctctcctccactc
J tc---------------------------------------------ctctcctccactc
G tcctccactcctcccctcctctcctcctctcctccccttctctcctcccctcctcc---a
N cc---------------------------------------------cactcctcc---c
C tc---------------------------------------------ctctcctcccctc

M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
K ------------------------------------------------------------
I ctcctcc-----------------------------------------------------
D ctcctcc-----------------------------------------------------
E ctcctcc-----------------------------------------------------
F ctcctcc-----------------------------------------------------
H ctcctct-----------------------------------------------------
B ctcctctcctc-------------------------------------------------
B ctcctctcctc-------------------------------------------------
J ctcccctcctc-------------------------------------------------
J ctcccctcctc-------------------------------------------------
G ctcctcc-----------------------------------------------------
N ctcctctcctc-------------------------------------------------
C ctctcctcctcaaagtcaatactttgagttttcctaattagagggaaagattcacagtgt
```

<The 5940bp sequences of intron is omitted here>

```
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
M ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
L ------------------------------------------------------------
K ------------------------------------------------------------
```

```
I ----------------------------------------tcctccactcctgc------
D ----------------------------------------tcctccactcctgc------
E ----------------------------------------tcctccactcctgc------
F ----------------------------------------tcctccactcctgc------
H ----------------------------------------tcctccactcctgc------
B ----------------------------------------tcctccactcctccactcct
B ----------------------------------------tcctccactcctccactcct
J ----------------------------------------tcctccactcctccgct---
J ----------------------------------------tcctccactcctccgct---
G ----------------------------------------tcctccactcctgc------
N ------------------------------------------tcctcctcctccactcct
C acttctctgagggagcaaggggccagccagctcaagtccagcctagccttgcctaggggc

M ----------------------ctctcctcccctcccctttactcctctcctccttcact
M ----------------------ctctcctcccctcccctttactcctctcctccttcact
M ----------------------ctctcctcccctcccctttactcctctcctccttcact
M ----------------------ctctcctcccctcccctttactcctctcctccttcact
M ----------------------ctctcctcccctcccctttactcctctcctccttcact
L ----------------------ctctcctcccctcccctttactcctctcctccttcact
L ----------------------ctctcctcccctcccctttactcctctcctccttcact
L ----------------------ctctcctcccctcccctttactcctctcctccttcact
K ----------------------ctctcctcccctcccctttactcctctcctccttcact
I -----cctccacttctcccctcctctcctcccctcccctttactcctctcctccttcact
D -----cctccacttctcccctcctctcctcccctcccctttactcctctcctccttcact
E -----cctccacttctcccctcctctcctcccctcccctttattcctctcctccttcact
F -----cctccacttctccc-----------------------ctcctctcctccttcact
H -----cctccacttctcccctcctctcctcccctcccctttactcctctcctccttcact
B cccctcctcccctcctctcctccactcctcccctcccctttactcctctcctccttcact
B cccctcctcccctcctctcctccactcctcccctcccctttactcctctcctccttcact
J ---------------------------cctcccccccctttattcctctcctccttcact
J ---------------------------cctcccccccctttattcctctcctccttcact
G -----cctccacttctcccctcctctcctcccctcccctttactcctctcctccttcact
N ccactcatcccctcctctcctccactcctcccctcccctttactcctctcctccttcact
C cagcccctcccctcctctcctccactcctcccctcccctttactcctctcctccttcact

[EXON 2]
M ctc---------tgctttcagacttataaacgagtacatgaaaaaaggagaccccatcct
M ctc---------tgctttcagacttataaacgagtacatgaaaaaaggagaccccatcct
M ctc---------tgctttcagacttataaacgagtacatgaaaaaaggagaccccatcct
M ctc---------tgctttcagacttataaacgagtacatgaaaaaaggagaccccatcct
M ctc---------tgctttcagacttataaacgagtacatgaaaaaaggagaccccatcct
L ctc---------tgctttcagacttagaaacgagtacatgaaaaaaggagaccccatcct
L ctc---------tgctttcagacttagaaacgagtacatgaaaaaaggagaccccatcct
L ctc---------tgctttcagacttagaaacgagtacatgaaaaaaggagaccccatcct
```

```
K ctc---------tgctttcagacttataaacgagtacatgaaaaaaggagaccccatcct
I ctc---------tgctttcagatttataaacgagtacatg-aagaaggagaccccatcct
D ctc---------tgctttcagatttataaacgagtacatg-aagaaggagaccccatcct
E ctctactttcagtgctttcagatttataaacgagtacatg-aagaaggagaccccatcct
F ctc---------tgctttcagatttataaacgagtacatg-aagaaggagaccccatcct
H ctc---------tgctttcagatttataaacgagtacatg-aagaaggagaccccatcct
B ctctactttcagtgctttcagatttagaaatgagtacatgaaaaaaggagaccccatcct
B ctctactttcagtgctttcagatttagaaatgagtacatgaaaaaaggagaccccatcct
J ctctactttcagtgctttcagatttcgaaacgagtacatgaaaaaaggagaccccagcct
J ctctactttcagtgctttcagatttcgaaacgagtacatgaaaaaaggagaccccagcct
G ctc---------tgctttcagatttataaacgagtacatg-aagaaggagaccccatcct
N ctctactttcagcgctttcagacttagaaatgagtacatg-aagaaggagaccccatcct
C ctctactttcagtgctttcagatttagaaatgagtacatgaaaaaaggagaccccatcct
  ***         .*********.**  ***.********* **.************ ***

M tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
M tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
M tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
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I tccctcctcaaggacagctggaggatcagggggattaaggtccatcaaactatcatttca
D tccctcctcaaggacagctggaggatcagggggattaaggtccatcaaactatcatttca
E tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
F tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
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G tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
N tccctcctcaaggacagctggaggatcagggggactaaggtccatcaaactatcatttca
C tccctcctcaaggacagctgggggatcagggggactaaggtccatcaaactatcatttca
  **.******************.************.*************************

M ctcctcccagagggtcgagatgccccaccagtcaggtgcccagtgctggag---------
M ctcctcccagagggtcgagatgccccaccagtcaggtgcccagtgctggag---------
M ctcctcccagagggtcgagatgccccaccagtcaggtgcccagtgctggag---------
M ctcctcccagagggtcgagatgccccaccagtcaggtgcccagtgctggag---------
M ctcctcccagagggtcgagatgccccaccagtcaggtgcccagtgctggag---------
L ctcctcccggagggtcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
```

```
L ctcctcccggagggtcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
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I ctcctcccagaggatcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
D ctcctcccagaggatcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
E ctcctcccagaggatcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
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H ctcctcccagaggatcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
B ctcctcccagaggatcgagatgccccaccagtcagttgcccagtgctggagaggaccccc
B ctcctcccagaggatcgagatgccccaccagtcagttgcccagtgctggagaggaccccc
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J ctcctcccagaggatcgagataccccaccagtcaggtgcccagtgctggagaggaccccc
G ctcctcccagaggatcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
N ctcctcccagaggatcgagatgccccaccagtcaggtgcccagtgctggagaggaccccc
C ctcctcccagaggatcgagatgccccaccagtcagttgcccagtgctggagaggaccccc
  ********.****.*******.************* ***************

M aggacccccaggtgtatccgatggcagacagctgcgtctgtcgaacgggagttcagatta
M aggacccccaggtgtatccgatggcagacagctgcgtctgtcgaacgggagttcagatta
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L aggacccccaggtgtatccgatggcagacagctgcgtctgtcgaacgggatttcagatta
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K aggacccccaggtgtatccgatggcagacagctgcgtctgtcgaacgggagttcagatta
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B aggacccccaggtgtatccgatggcagacagcggcgtctgtcgaaggggagttcagattt
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G aggacccccaggtgtatc------------------------------------------
N aggacccccaggtgtatccgatggcagacagctgcgtctgtcgaaggggagttcagatta
C aggacccccaggtgtatccgatggcagacagctgcgtctgtcgaaggggagttcagattt
  ******************

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M gattagattaacccaaacccagcaccctcacaac--tgtattgtgcttgtatcatttagg
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M gattagattaacccaaacccagcaccctcacaac--tgtattgtgcttgtatcatttagg
```

```
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L gattagattaacccaaacccagcaccctcacaac--tgtattgtgcttgtatcatttagg
K gattagattaacccaaacccagcaccctcacaac--tgtattgtgcttgtatcatttagg
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D gattagattaacctaaacccagcaccctcacaactatgtattgtgccagtatcattttgg
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F gattagattaacctaaacccagcaccctcacaactatgtattgtgccagtatcattttgg
H gattagattaacctaaacccagcaccctcacaactatgtattgtgccagtatcattttgg
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B gatttgattaacccaaacccagcagcctcacaactatgtattgtgcctgtatcattttgg
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J gatttgattaacccaaacccagcagcctcacaactatgtattgtgcctgtatcattttgg
G ------------------------------------------------------------
N gattagattaacctaaacccagcaccctcacaactatgtattgtgccagtatcattttgg
C gatttgattaacccaaacccagcagcctcacaactatgtattgtgcctgtatcattttgg

M attttcaaagcagcaactgatttgagttgatcaagttgttcac
M attttcaaagcagcaactgatttgagttgatcaagttgttcac
M attttcaaagcagcaactgatttgagttgatcaagttgttcac
M attttcaaagcagcaactgatttgagttgatcaagttgttcac
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E attttcaaagcaacaactgatttgagttgatcaagttgttcac
F attttcaaagcaacaactgatttgagttgatcaagttgttcac
H attttcaaagcaacaactgatttgagttgatcaagttgttcac
B --------------aactgatttgagttgatcaagttgttcac
B --------------aactgatttgagttgatcaagttgttcac
J --------------aactgatttgagttgatcaagttgttcac
J --------------aactgatttgagttgatcaagttgttcac
G -------------------------------------------
N attttcaaagcaacaactgatttgagttgatcaagttgttcac
C --------------aactgatttgagttgatcaagttgttcac
```

TRANSPARENT METHODS

Sample collection and identification.

The methods were carried out in accordance with the approved guidelines of the Good Experimental Practices adopted by the Institute of Zoology, Chinese Academy of Sciences (CAS). All procedures described in this study were approved by the Committee for Animal Experiments at the Institute of Zoology, Chinese Academy of Sciences. An adult male *P. chinensis* was collected from the Guanting Reservoir in Hebei province for de novo sequencing. The specimen was confirmed to be *P. chinensis* by DNA barcoding analysis [as outlined by (Ward et al., 2005)]. Specimens for transcriptome (RNA-seq) sequencing were collected from Hongze Lake, Jiangsu province. We followed the method of Kimmel (Kimmel et al., 1995) to define four development stages: the pharyngula stage, the hatching stage, larval fish stage, and the adult stage.

Genome sequencing and assembly

Genomic DNA was extracted from a whole-animal using a DNeasy Blood & Tissue Kit (QIAGEN). To sequence the *P. chinensis* genome, we employed PacBio Sequel long-read sequencing and 10X Genomics Chromium linked reads sequencing, coupled with short-read sequencing of 250bp, 350bp, 2kb, 5kb, 10kb paired-end libraries on the Illumina platform (Table S1). Pacific Biosciences SMRTbell libraries were prepared using 10 kb and 20 kb preparation protocols. The main steps for library preparation were: (1) gDNA shearing; (2) DNA damage repair; (3) blunt end-ligation with hairpin adapters from the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences); (4) size selection; and (5) binding to polymerase. Sequencing was performed on a PacBio Sequel instrument with Sequel Sequencing Kit 1.2.1. A total of 10.57 Gb (21.83×) PacBio reads were generated. 10X Genomics Chromium sequencing (also known as the Chromium Genome Solution) allows long-range sequence information to be generated on a short-read Illumina sequencer by barcoding long DNA molecules before preparation of short-read fragment DNA libraries (Zheng et al., 2016). The barcodes (also known as linked-reads) can be used to obtain 'synthetic long reads'. A total of 81.92 Gb (169.22×) 10X Genomics linked-reads were generated. The initial assembly was generated using Allpaths-LG v44080 (Butler et al., 2008) and 250 bp, 350 bp, 2 kb, 5 kb, and 10 kb Illumina data, followed by PacBio data and gap filling using PBJelly v14.1 (English et al., 2012) and two-rounds of polishing using Pilon v1.18 (Walker et al., 2014) and Illumina reads. Finally, 10X Genomics linked-reads were used to link scaffolds using fragScaff v140324 (Adey et al., 2014). Genome completeness was assessed by mapping de novo assembled transcripts to the genome (see below), and by CEGMA v2.5 (Parra et al., 2007) and BUSCO v1.1 (Waterhouse et al., 2017) evolutionary conserved gene set analysis.

Experimental model and subject details

A broodstock of *P. chinensis* was collected from Hongze Lake, Jiangsu province. After artificial insemination, eggs were transported (at 4-10°C) to the Chinese Academy of Sciences Institute of Zoology in Beijing for incubation experiments on 7 Jan 2017. The eggs were hatched in an experimental glass tank (200-300 eggs per 3L tank), with a 12:12-hour light:dark regime. The water temperature was 10-15°C. Water changes were performed daily (70% exchanged).

Transcriptome sequencing

Total RNAs was isolated from four different development stages: the pharyngula stage, the hatching stage, larval fish stage, and the adult stage. RNA sequencing libraries were constructed using the Illumina mRNA-Seq Prep Kit. Briefly, oligo(dT) magnetic beads were used to mRNA molecules. Paired-end libraries were sequenced on the Illumina HiSeq platform, and 150 bp paired-end reads were generated. Raw sequencing reads were filtered for base quality >15 and read length >30 bp using the Novogene-developed application ng_QC v2.0 with default parameters (i.e., L:5 -p:0.5 -N:0.1). We used TopHat v1.3.1 (Trapnell et al., 2009) to align RNA-seq reads to the genome. Gene expression was quantified as reads per kilobase of gene per million mapped reads (RPKM). RPKM values were scaled using the TMM (trimmed mean of M values; M values mean the log expression ratios) method (Robinson and Oshlack, 2010). A *P. chinensis* transcriptome was also generated (*de novo* assembled) from pooled RNA-seq samples using Trinity v2.1.1 (Haas et al., 2013) with the parameters '-ss 0.5 -jc 0 -minkmercov 2 -minglue 2'.

Estimation of genome size using *k*-mer method

Genome size can be estimated by *k*-mer frequency analysis (Liu et al., 2013). Error-corrected [NGS QC Toolkit v2.3.3 (Patel et al., 2012)] 180 bp to 270 bp Illumina genome sequencing reads (~229.86 Gb data) were used to estimate the genome size of *P. chinensis*. The distribution of 17 *k*-mers showed a major peak at 78-fold depth (Table S2; Figure S1A). Based on the total number of reads (38,879,079,724) and corresponding to a *k*-mer depth of 78, the *P. chinensis* genome size was estimated to be ~484.10Mbp using the formula ‘Genome size= kmer_Number/Peak_Depth’.

Genome assembly assessment

The *P. chinensis* assembly was evaluated by mapping Illumina short-insert library genome sequencing reads (see the section above) to the assembly using BWA v0.7.8 (Li and Durbin, 2010) (Table S5; Figure

S1B). De novo transcriptome reads were mapped to the assembly using BLAT v0.35 (Kent, 2002)(Table S8). We also employed two methods which employ core gene sets to assess genome completeness (Table S9 and S10): CEGMA v2.5 (Core Eukaryotic Genes Mapping Approach) (Parra et al., 2007) compares a set of 248 core eukaryotic genes to an assembled genome, while BUSCO v1.1 (Benchmarking Universal Single-Copy Orthologs) (Seppey et al., 2019; Simao et al., 2015) compares near-universal single-copy orthologs. To assess GC bias, we plotted the distribution of GC content against sequencing depth (Figure S1C and S1D).

Genome annotation

Repeats, including repetitive sequences and transposable elements, were identified using RepeatMasker v4.0.5 (Tarailo-Graovac and Chen, 2009) and either the RepBase vertebrate library (Bao et al., 2015) or a de novo repeat library [built using RepeatModeler] (Figure S1E). Tandem repeats were identified by searching for two or more contiguous, approximate copies of a pattern of nucleotides using Tandem Repeats Finder v407 (Benson, 1999).

Homology-based predictions, de novo predictions, and transcriptome-based prediction methods were used to annotate the protein-coding genes of *P. chinensis*. For homology-based gene prediction, protein sequences from nine other sequenced teleost genomes [*Danio rerio* (zebrafish), *Tetraodon nigroviridis* (pufferfish), *Gasterosteus aculeatus* (stickleback), *Oryzias latipes* (medaka), *Salmo salar* (salmon), *Cynoglossus semilaevis* (flatfish), *Takifugu rubripes* (fugu), *Oreochromis niloticus* (tilapia), and *Larimichthys crocea* (yellow croaker)] were used to query the *P. chinensis* genome using tBLASTn v2.2.26 (E-value $\leq 10^{-5}$) (Camacho et al., 2009). Next, the homologous genome sequences were aligned against the matching proteins using GeneWise V2.4.1(Birney et al., 2004) to take into account splice site variation. Three de novo gene prediction tools Augustus v3.1 (Stanke et al., 2006), GlimmerHMM v3.0.4 (Majoros et al., 2004), and SNAP (Korf, 2004) were employed to predict genes in the repeat-masked *P. chinensis* genome. RNA-seq reads from *P. chinensis* [whole-fish from four development stages Given the small size of P. chinensis, several individuals were pooled for each sample type] were aligned to the genome using TopHat v2.0.11 (Trapnell et al., 2009) and Cufflinks v2.1.1 (Trapnell et al., 2014) was used to produce assembled transcripts and predict transcript structures. Data from the three prediction methods were merged into CDS models using EVM v1.1.1 (Haas et al., 2008), and untranslated (UTR) and isoforms were constructed using PASA v2.0.2 (Haas et al., 2003).

We next performed functional annotation of protein-coding genes in the *P. chinensis* genome. The predicted protein sequences of *P. chinensis* were assessed using publicly available databases – Swiss-Prot (Artimo et al., 2012), NR (non-redundant nucleotides) (O'Leary et al., 2016), KEGG (Kanehisa et al., 2017), and InterPro (Zdobnov and Apweiler, 2001) using BLASTp (Camacho et al., 2009) (*E*-value $\leq 10^{-5}$) – and the best hit for each query retained. For each gene, its Gene Ontology (GO) term(s) and Pfam accession were used to query various additional databases (ProDom, HAMAP, PANTHER, TIGRFAMs, PRINTS, PIRSF, Gene3D, COILS, PROSITE, Pfam, and SMART) (Attwood et al., 2003; Corpet et al., 2000; Falquet et al., 2002; Haft et al., 2003; Lees et al., 2014; Lupas et al., 1991; Punta et al., 2012; Schultz et al., 1998; Tania et al., 2009; Thomas et al., 2003; Wu et al., 2004). Non-coding RNA genes were also identified. The tRNAscan-SE (Lowe and Eddy, 1997) software (v1.3.1) was used to predict tRNA sequences. We aligned the *P. chinensis* genome to the rRNA sequences of *Homo sapiens* using BLASTn (*E*-value $\leq 10^{-5}$) (Camacho et al., 2009). The miRNA and snRNA genes of *P. chinensis* were extracted using v1.1rc4 Infernal (Nawrocki and Eddy, 2013) and against the Rfam database (Griffiths-Jones et al., 2005).

Orthology and phylogenomics

A total of 18 fish species, including *P. chinensis*, were selected for orthology analysis. Orthology was determined using the OrthoMCL (Li et al., 2003) pipeline. Briefly, we first filtered out redundant splice variants – retaining the longest isoform of each protein set – followed by all-against-all protein comparisons using BLASTp (Camacho et al., 2009) (*E*-value ≤ 10^{-5}). High-scoring Segment Pair (HSPs) were processed by MCL v10-201 (Enright et al., 2002) to define orthologs, inparalogs, and co-orthologs. Alignments with high-scoring segment pairs (HSPs) were conjoined for each gene pair using SOLAR (Sorting Out Local Alignment Results). More than 30% coverage of the aligned region in both homologous genes was required to assign homologous gene-pairs.

To generate a phylogenetic tree, 627 single-copy ortholog nucleotide alignments (coding sequence; CDS) from 18 species (*P. chinensis*, *Gasterosteus aculeatus*, *Danio rerio*, *Lepisosteus oculatus*, *Gadus morhu*, *Takifugu rubripes*, *Nothobranchius furzeri*, *Esox lucius*, *Oreochromis niloticus*, *Xiphophorus maculatus*, *Oryzias latipes*, *Salmo salar*, *Scleropages formosus*, *Anguilla rostrata*, *Latimeria chalumnae*, *Hippocampus comes*, *Ictalurus punctatus*, and *Callorhinchus milii*) were concatenated into a super-alignment. Multiple alignments of coding sequences (CDS) for each ortholog group were performed using MUSCLE v3.7 (Edgar, 2004). jModelTest v2.1.2 was used to select the best substitution model by Akaike information criterion (AIC). The species tree was obtained using RAxML v704 (Stamatakis, 2014) and the GTR+GAMMA model, with 100 replicates of bootstrap analysis. Species divergence times were inferred using MCMCTree (Donoghue et al., 2009), included in PAML v4.7a (Yang, 2007), with the parameters ‘RootAge = <500 model = REV (GTR) alpha = 0.666853 clock = 3’, and the calibration points as prior [obtained from (Benson, 1999; Bian et al., 2016; Schartl et al., 2013; Yang et al., 2016)] are provided in (Table S14).

Expansion and contraction of gene families

We determined the expansion and contraction of gene families by comparing the cluster size differences between the of the *P. chinensis* and 17 other fish species using CAFE (Version 1.6) (De Bie et al., 2006). A random birth and death model was used to study changes of gene families along each lineage of phylogenetic tree. A probabilistic graphical model (PGM) was introduced to calculate the probability of transitions in gene family size from parent to child nodes in the phylogeny. Using conditional likelihoods as the test statistics, we calculated the corresponding *P*-values in each lineage. A *P*-value of 0.05 was used to denote families significantly expanded in the *P. chinensis* genome.

Identification of single-copy gene families gained by *P. chinensis*

We clustered paralogs and orthologs using the OrthoMCL method (Li et al., 2003) (BLASTp *E*-value ≤ 10^{-5}) and 18 sequenced fish species (*P. chinensis*, *Gasterosteus aculeatus*, *Danio rerio*, *Lepisosteus oculatus*, *Gadus morhu*, *Takifugu rubripes*, *Nothobranchius furzeri*, *Esox lucius*, *Oreochromis niloticus*, *Xiphophorus maculatus*, *Oryzias latipes*, *Salmo salar*, *Scleropages formosus*, *Anguilla rostrata*, *Latimeria chalumnae*, *Hippocampus comes*, *Ictalurus punctatus*, and *Callorhinchus milii*).

Identification of positively selected genes

Positive selection on an ORF-wide level was estimated using in-frame codon alignments and the Branch-site Unrestricted Statistical Test for Episodic Diversification (BUSTED) method implemented in HyPhy v2.5.9 (Pond et al., 2005). BUSTED requires a prior partitioning of branches into foreground and background branches and considered to more accurately identify episodic (acting only on particular lineages) positive selection (Murrell et al., 2015; Spielman et al., 2019). In the species tree, the *P. chinensis* lineage was marked as ‘foreground’ and the rest of the fish species (*Gadus morhua*, *Gasterosteus aculeatus*, *Danio rerio*, *Oreochromis niloticus*, *Esox lucius*, *Oryzias latipes*, *Xiphophorus maculatus*, and *Scleropages formosus*) as ‘background’.

Prediction of bone and scale genes

Genes involved in vertebrate bone formation were obtained from (Venkatesh et al., 2014). If a gene could not be found by searching *P. chinensis* gene names and symbols, we obtained the gene (CDS and protein sequence) from a dataset of *O. latipes*, *G. aculeatus*, *T. rubripes*, *E. Lucius*, *I. punctatus*, and *D. rerio* and queried the *P. chinensis* genome using BLAST (Camacho et al., 2009) or GeneWise (Birney et al., 2004) [using protein sequences as query]. Predictions were also made using *ab initio* methods, such as FGENESH (Solovyev et al., 2006), when no *P. chinensis* sequence could be obtained. All predictions were manually curated.

Staining of the *P. chinensis* skeleton

Adult bones and cartilage were stained with Alizarin red and Alcian blue, respectively. Briefly, a fish specimen was fixed in formalin (10% formaldehyde), briefly dehydrated in 70% ethanol, decolorized with 3% hydrogen peroxide for 6 hours, and placed in Alcian blue for 12 hours. The specimen was dehydrated using 50% ethanol for 48 hours, and bones were next stained with 2 g/l Alizarin red and detained with 1% KOH until background stain was lost.

Hox gene analysis

Hox genes from zebrafish, as well as Atlantic salmon (*Salmo salar*) and Northern pike (*Esox Lucius*) were used to query the *P. chinensis* genome using GeneWise v2.4.1 (Birney et al., 2004). *Hox* gene clusters were next manually curated. We performed PCR and Sanger sequencing of the following *P. chinensis* pseudogenes to rule out genome sequencing or assembly errors: *HOXB3b* (5'-AGAGATTGACAGGGGCATGG-3' and 5'-TGATAGATGTAGGTCCACTGTTG-3'; T_a=56 °C), *HOXB8b* (5'-CCTAAGTGTATCTAAAACGT-3 and 5'-ATTCTACATTCTACATTTCC-3; T_a=55 °C), *HOXC3a* (5'-CCACACAGACATTTAGAGGC-3 and 5'-TAAGGGCATAATCCAGTCGA-3; T_a=55 °C), and *HOXC5a* (5'-CCTGGATTATTTGGGGCAGG-3 and 5'-TGAAATTCACAACCGTTCAACA-3; T_a=58 °C).

Sequencing and analysis of _P. chinensis_ fibroblast growth factor 5 genes

P. chinensis FGF5-derived genes were amplified from whole-fish genomic DNA using PrimeSTAR HS DNA Polymerase (TaKaRa) – with a forward primer in exon 1 and a reverse primer in an exon unique to a novel *P. chinensis FGF5* exon (5'-GTTCCTCTTTGTCTTTATACCGTC-3' and 5'-GTGAACAACTTGATCAACTCAAATC-3', T_a=52 °C) – on a ABI-9700 (ABI) thermal cycler and Sanger sequenced. The MAFFT online server v7.452 (http://mafft.cbrc.jp) (Katoh et al., 2019; Kuraku et al., 2013) was used to generate multiple sequence alignments of amplicons and *FGF5* genome scaffolds (using the G-INS-i Iterative refinement method). The settings for local instances of MAFFT are 'mafft --threadtb 5 --threadit 0 --reorder --leavegappyregion --maxiterate 1000 --retree 1 --globalpair') and a phylogenetic tree [neighbor joining tree generated from conserved sites, maximum likelihood (ML) analysis was generated using RaxML v8 (Stamatakis, 2014) with the search strategy set to rapid bootstrapping and 1,000 bootstrap replicates]. The deduced amino acid sequences were predicted using the ExPASy translate tool (https://web.expasy.org/translate) (Artimo et al., 2012). In addition, the *FGF5* phylogenetic tree was generated from *FGF5* protein sequences of *P. chinensis* and other 10 species (*Scleropages Formosus*, *Letalurus punctatus*, *Danio rerio*, *Sinocyclocheilus anshuiensis*, *Esox lucius*, *Salmo salar*, *Xiphophorus maculatus*, *Larimichthys crocea*, *Oryzias latipes*, *Takifugu rubripes*). The maximum likelihood (ML) analysis was generated using RaxML v8 (Stamatakis, 2014) with the ProtCAT model search strategy set to rapid bootstrapping and 1,000 bootstrap replicates. Protein sequences were aligned using MAFFT with default parameter.

Immune system analysis

We retrieved immunity-related genes in the *P. chinensis* from our annotation pipeline as well as by manual curation. Sequence alignments were obtained using ClustalX v2.1 (Larkin et al., 2007). A neighbor-joining phylogenetic tree of the TLR gene family was conducted from multiple sequence alignments of proteins using MAGE6 (Tamura et al., 2013).

Assessment of pigmentation genes

Pigmentation genes of interest in *P. chinensis* were interrogated by BLAST (Camacho et al., 2009) analysis of the genome assembly and whole-fish RNA-seq data (raw reads and Trinity assembly) on a local instance of sequenceserver v1.1.0 (Priyam et al., 2015), using zebrafish gene sequences as the query. Regions with unique changes in *P. chinensis* were next investigated by BLAST searches against the NCBI databases RefSeq (curated genomes, transcripts, and proteins) and NR (O'Leary et al., 2016), and the ~15,000-proteome database UniProt (The UniProt, 2017) [the number of output alignments was set to 1,000]. Obtained sequences were aligned with the *P. chinensis* query using the MAFFT web server (Katoh et al., 2019). The impact of amino acid residue changes on protein function, structure, and stability was assessed using the online tools PANTHER-PSEP (Tang and Thomas, 2016), PolyPhen2 (Adzhubei et al., 2010), SIFT (Kumar et al., 2009), and I-Mutant 2.0 (Capriotti et al., 2005).

Quantification and statistical analyses

Statistics details are provided in the Methods Details section.

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