First draft genome of loach (Orenectus shuilongensis; Cypriniformes: Nemacheilidae) provide insights into the evolution of cavefish

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Abstract

Background

Loaches of the superfamily Cobitoidea (Cypriniformes, Nemacheilidae) are small elongated bottom-dwelling freshwater fishes with several barbels near the mouth. The genus Oreonectes with 18 currently recognized species contains representatives for all three key stages of the evolutionary process (a surface-dwelling lifestyle, facultative cave persistence, and permanent cave dwelling). Some Oreonectes species show typical cave dwelling-related traits, such as partial or complete leucism and regression of the eyes, rendering them as suitable study objects of micro-evolution. Genome information of Oreonectes species is therefore an indispensable resource for research into the evolution of cavefishes.

Results

Here we assembled the genome sequence of O. shuilongensis, a surface-dwelling species, using an integrated approach that combined PacBio single-molecule real-time sequencing and Illumina X-ten paired-end sequencing. Based on in total 50.9 Gb of sequencing data, our genome assembly from Canu and Pilon spans approximately 515.64 Mb (estimated coverage of 100 ×), containing 803 contigs with N50 values of 5.58 Mb. 25,247 protein-coding genes were predicted, of which 95.65% have been functionally annotated. We also performed genome re-sequencing of three additional cave-dwelling Oreonectes fishes. Twenty-nine pseudogenes annotated using DAVID showed significant enrichment for the GO terms of “eye development” and “retina development in camera-type eye”. It is presumed that these pseudogenes might lead to eye degeneration of semi/complete cave-dwelling Oreonectes species. Furthermore, Mc1r (melanocortin-1 receptor) is a pseudogenization by a deletion in O. daqikongensis, likely blocking biosynthesis of melanin and leading to the albino phenotype.

Conclusions

We here report the first draft genome assembly of Oreonectes fishes, which is also the first genome reference for Cobitidea fishes. Pseudogenization of genes related to body color and eye development may be responsible for loss of pigmentation and vision deterioration in cave-dwelling species. This genome assembly will contribute to the study of the evolution and adaptation of fishes within Oreonectes and beyond (Cobitidea).

Background

Cavefishes are successful vertebrate colonizers in subterranean habitats and usually process some regressive features, such as the rudimentary eyes and loss of pigmentation. Meanwhile, some compensative traits, such as elongated appendages and reinforced non-visual sensory systems, have evolved in cavefishes. Since uncovering the genetic basis of phenotypic adaptations of animals to a specific environment is a key goal in evolutionary study, cavefishes have attracted interests from biologist and certain cavefishes (Astyanax mexicanus and Sinocyclocheilus spp.) have been well studied. However, there is few data available to unravel the genomic mechanism under the evolution and adaptation to subterranean life among other groups of fishes.

Loaches (Cypriniformes: Cobitoidea, Nemacheilidae) are small elongated bottom-dwelling freshwater fishes with several barbels near the mouth, distributed in Eurasia and Africa. Within this group, Oreonectes fishes are distributed only in southwestern China and northern Vietnam, most of which dwell in underground rivers in the karst environment[1]. Oreonectes fishes contain 18 species representing the three key stages of the evolutionary
process including a surface-dwelling lifestyle, facultative cave persistence, and permanent cave dwelling. Almost all Oreonectes species show some cave-related traits, such as part or complete eye degeneration and leucism, which makes this genus a good study system of micro-evolution\textsuperscript{2}. \textit{O. shuilongensis} is a surface-dwelling species which was newly discovered in the Shuilong Township in Guizhou Province of China\textsuperscript{3}. A genome assembly of \textit{O. shuilongensis} would facilitate research into key aspects of the evolutionary history of cave versus surface dwelling in Oreonectes, including the role of environmental changes in the seemingly rapid diversification and speciation in underground caves\textsuperscript{2}.

Here we present a \textit{de novo} genome assembly for \textit{O. shuilongensis}, the first genome constructed for the family Nemacheilidae and the superfamily Cobitidea. The completeness and continuity of the genome provided valuable genomic resources for studies on the evolutionary history of the rapid speciation processes of family Nemacheilidae.

**Results**

**Whole Genome and RNA Sequencing**

After removal of < 500 bp PacBio subreads, 5 million subreads (total 50.9 Gb) remained, with an average length of 10.2 kb (Table 1 and Suppl. Tables S2 and S3). Additionally, a total of 11.7 Gb transcriptome data were obtained from RNA-sequencing (Table 1).

| Type   | Libraries      | Insert size (bp) | Read length (bp) | Clean data (Gb) | Coverage (X) |
|--------|----------------|------------------|------------------|-----------------|--------------|
| DNA    | HiSeq X Ten    | 270              | 150              | 120.94          | 234.55       |
| DNA    | PacBio         | 20,000           | 10,187           | 50.94           | 98.79        |
| RNA    | HiSeq X Ten    | 270              | 150              | 11.7            | -            |

The coverage was calculated using an estimated genome size of 515.64 Mb.

**Estimation of the Genome Size and Sequencing Coverage**

The genome size of \textit{O. shuilongensis} was estimated at approximately 515.66 Mb based on k-mer analysis (Suppl. Figure S1), and our \textit{O. shuilongensis} genome assembly spans 521.68 Mb (803 contigs, contig N50 of 5.58 Mb; Table 2 and Suppl. Table S4). The completeness of the \textit{O. shuilongensis} genome assembly was evaluated using CEGMA v2.5\textsuperscript{4} and BUSCO v2\textsuperscript{5}. CEGMA analysis suggested that 99.78% of conserved Core Eukaryotic Genes (CGEs) proteins are present in our assembled genome, and BUSCO analysis showed that 97.58% of vertebrate Benchmarking Universal Single-Copy Orthologs have been assembled, implying a high completeness of our \textit{O. shuilongensis} genome assembly (Suppl. Tables S5 and S6). We found around 99.52% of the reads properly mapped to the genome assembly (Suppl. Table S7). All these results indicate that the assembly of the \textit{O. shuilongensis} genome is characterized by a high level of accuracy.
Table 2

Summary of the loach (O. shuilongensis) genome assembly and annotation.

| Genome quality                          | values  |
|-----------------------------------------|---------|
| Contig N50 size (Mb)                    | 5.58    |
| Estimated genome size (Mb)              | 515.64  |
| Assembled genome size (Mb)              | 521.68  |
| Genome coverage (fold)                  | 234.55 X|
| Content of transposable elements (%)    | 23.42   |
| Total GC content (%)                    | 38.51   |
| Protein-coding gene numbers             | 25,247  |
| Average gene length (kb)                | 234.62  |
| Average CDS length (bp)                 | 2,010   |

Annotation of Repeat Sequences, Protein Coding Genes and Noncoding RNA

We found that repetitive elements comprised 23.42% of the O. shuilongensis genome (Table 3). 25,247 protein-coding genes were identified. The average transcript length, CDS length, and intron length were 9,744 bp, 2,010 bp, and 7,734 bp, respectively (Suppl. Table S8). Among these annotated genes, 64.51% of encoded proteins showed homology to proteins in the KOG database, 95.53% were identified in the NCBI non-redundant database, 46.72% were identified in the KEGG database, 94.90% were identified in the TrEMBL database, and 95.65% could be mapped onto the functional databases (Suppl. Table S9). Finally, 947 miRNAs, 561 rRNAs and 417 tRNAs were discovered from the O. shuilongensis (Suppl. Table S10).
Table 3
Annotation of repeat sequences in the loach (O. shuilongensis) genome assembly.

| Type               | Number | Length(bp)  | Percentage (%) |
|--------------------|--------|-------------|----------------|
| ClassI/DIRS        | 1,566  | 1,563,184   | 0.3            |
| ClassI/LINE        | 6,300  | 11,594,987  | 2.22           |
| ClassI/LTR         | 5,947  | 4,877,346   | 0.93           |
| ClassI/LTR/Copia   | 909    | 528,739     | 0.1            |
| ClassI/LTR/Gypsy   | 9,340  | 6,473,497   | 1.24           |
| ClassI/PLE|LARD   | 7,579  | 15,588,001  | 2.99           |
| ClassI/SINE        | 4,322  | 591,181     | 0.11           |
| ClassI/TRIM        | 3,358  | 3,025,034   | 0.58           |
| ClassI/Unknown     | 342    | 35,272      | 0.01           |
| ClassII/Crypton    | 11,782 | 2,048,568   | 0.39           |
| ClassII/Helitron   | 8,994  | 1,095,311   | 0.21           |
| ClassII/MITE       | 4,176  | 596,316     | 0.11           |
| ClassII/Maverick   | 2,145  | 374,478     | 0.07           |
| ClassII/TIR        | 71,957 | 21,304,786  | 4.08           |
| ClassII/Unknown    | 178,045| 22,476,697  | 4.31           |
| PotentialHostGene  | 32,601 | 4,535,251   | 0.87           |
| SSR                | 797    | 112,798     | 0.02           |
| Unknown            | 178,496| 25,369,612  | 4.86           |
| Total without overlap: | 528,656 | 122,191,689 | 23.42         |

Phylogenetic relationship and genomic comparison

As a result, 16,708 gene families were constructed for the O. shuilongensis. Among the families, there were 144 families unique to O. shuilongensis (Fig. 2 and Suppl. Table S11). The constructed phylogenetic tree indicated that O. shuilongensis were clustered closely to Cyprinidae species, which is inconsistent with their putative evolutionary relationships (Fig. 3). The divergence between O. shuilongensis and cyprinid fishes (Cyprinidae) occurred ca. 91.31 million years ago (95% HPD, 82.58-108.26). When comparing this with the other seven fish (S. salar, I. punctatus, A. mexicanus, C. carpio, S. rhinocerous, D. rerio and L. crocea), the expansion and contraction of gene ortholog clusters showed 77 gene families were expanded and 282 gene families contracted significantly in the O. shuilongensis (Fig. 3).

Genomic mechanism underlying the degeneration of eyes and body color
Genome re-sequencing were performed for facultative cave-dwelling *O. jiarongensis* (three individuals) and cave-dwelling *O. daqikongensis* (two individuals) and *O. dongliangensis* (one individual) at a high average depth of 28.06 ± 5.08×, with an overall average genome coverage of 93.77% of the *O. shuilongensis* genome assembly (Table 4). A total of 12,534,348 SNPs was identified in these three species, and the number of SNPs per individual ranged from 6.0 to 6.2 M (Table 4). The reconstructed phylogeny indicates that the ancestor of cave-dwelling *O. daqikongensis*, *O. dongliangensis* and facultative cave-dwelling *O. jiarongensis* first diverged from the surface-dwelling *O. shuilongensis* about 9.31 million years ago (95% HPD, 12.54–7.12) (Fig. 4).

### Table 4

Re-sequencing analysis of cave-dwelling *Orenectus* fishes.

| Species          | *O. jiarongensis* | *O. daqikongensis* | *O. dongliangensis* |
|------------------|-------------------|--------------------|---------------------|
| Sample ID        | R01               | R02                | R03                 |
|                  | R04               | R05                | R06                 |
| **Total_reads**  | 154919048         | 145879318          | 136865354           |
|                  | 147160730         | 126896868          | 123201446           |
| **Clean_Reads**  | 77459524          | 72939659           | 68432677            |
|                  | 73580365          | 63448434           | 61600723            |
| **GC(%)**        | 39.06             | 39.02              | 38.92               |
|                  | 38.92             | 38.92              | 38.95               |
|                  | 39.38             | 39.38              | 38.26               |
| **Mapping_rate**| 90.49%            | 91.15%             | 91.83%              |
|                  | 90.82%            | 91.63%             | 92.26%              |
| **Ave_depth**    | 29                | 28                 | 26                  |
|                  | 30                | 26                 | 24                  |
| **Cov_ratio_1X(%)** | 87.44            | 87.76              | 87.62               |
|                  | 87.26             | 86.98              | 87.46               |
| **Cov_ratio_5X(%)** | 83.46          | 83.74              | 83.5                |
|                  | 83.76             | 82.76              | 83.01               |
| **Cov_ratio_10X(%)** | 80.35           | 80.56              | 80.07               |
|                  | 80.44             | 79.3               | 79.05               |
| **Total_SNP**    | 6201592           | 6207932            | 6201733             |
|                  | 6064030           | 6049874            | 6189968             |
| **Transition**   | 3544204           | 3547586            | 3544063             |
|                  | 34885910          | 3478106            | 3539101             |
| **Transversion** | 2657388           | 2660346            | 2657670             |
|                  | 2578120           | 2571768            | 2650867             |
| **Ti/Tv**        | 1.33              | 1.33               | 1.33                |
|                  | 1.35              | 1.35               | 1.35                |
| **Het_snp**      | 199131            | 193488             | 191382              |
|                  | 183450            | 182330             | 248330              |
| **Hom_snp**      | 6002461           | 6014444            | 6010351             |
|                  | 5880580           | 5867544            | 5941638             |
| **Het-ratio**    | 3.21%             | 3.11%              | 3.08%               |
|                  | 3.02%             | 3.01%              | 4.01%               |
| **Total_Indel**  | 2335694           | 2340858            | 2336570             |
|                  | 2218308           | 2208883            | 2321897             |
| **Het_Indel**    | 113531            | 111329             | 108840              |
|                  | 112158            | 110204             | 135160              |
| **Hom_Indel**    | 2222163           | 2229529            | 2227730             |
|                  | 2106150           | 2098679            | 2186737             |

*Ave_depth is average sequencing depth; Cov_ratio_1X, Cov_ratio_5X and Cov_ratio_10X are XXX respectively; Ti/Tv is Transition/Transversion; Het_snp is heterozygosis SNP number; Hom_snp is homozygosis SNP number; Het-ratio is the percentage of heterozygosis SNPs in total SNPs; Het_Indel is heterozygosis indel numbers; Hom_Indel is homozygosis indel numbers.

Annotation of all sequence variants in SnpEff[6] (Fig. 5) suggested that 1,541 SNPs and 438 indels were located in 401 genes, likely resulting in pseudogenization in semi cave-dwelling and cave-dwelling species. Twenty-nine
pseudogenes annotated using DAVID\textsuperscript{[7]} showed significant enrichment for the GO terms of “eye development (0001654)” and “retina development in camera-type eye (0060041)” (Table 5, Fig. 6 and Suppl. Table S12). For example, the expression of \textit{six7}, \textit{six6a} and \textit{six6b} is required for optic primordium development and the specification and proliferation of the eye field in vertebrate embryos\textsuperscript{[8]}. The function-lost mutation of \textit{aldh1a3} and \textit{tfap2a} caused eye and retinal defects in zebrafish\textsuperscript{[9]}. It is presumed that these pseudogenes might lead to eye degeneration of semi/complete cave-dwelling \textit{Oreonectes} species. Furthermore, \textit{Mc1r} (melanocortin-1 receptor), a key gene regulating the biosynthesis of melanin in most vertebrates, is a pseudogenization caused by a deletion in \textit{O. daqikongensis} (Fig. 7), likely blocking biosynthesis of melanin and leading to the albino phenotype (Fig. 1). Remaining pseudogenes are enriched for the GO terms of “potassium channel activity”, “regulation of axon extension involved in axon guidance”, “G-protein coupled receptor activity” and KEGG pathway of “neuroactive ligand-receptor interaction” (Table 5).
Table 5
Functional classification and enrichment analysis of pseudogenes in cave-dwelling *Oreonecetes* fishes. Gray boxed GOs are associated with retina and eye development.

| GO_ID       | GO_Term                                                                 | GO Class | Gene num | Adjusted P-value |
|-------------|--------------------------------------------------------------------------|----------|----------|------------------|
| GO:0060041  | retina development in camera-type eye                                    | BP       | 9        | 2.8E-9           |
| GO:0001654  | eye development                                                          | BP       | 8        | 8.9E-9           |
| GO:0051260  | protein homooligomerization                                              | BP       | 4        | 0.0031           |
| GO:0007186  | G-protein coupled receptor signaling pathway                             | BP       | 11       | 0.0033           |
| GO:0007165  | signal transduction                                                      | BP       | 13       | 0.0043           |
| GO:0034765  | regulation of ion transmembrane transport                               | BP       | 4        | 0.0085           |
| GO:0006813  | potassium ion transport                                                  | BP       | 4        | 0.013            |
| GO:0071805  | potassium ion transmembrane transport                                    | BP       | 5        | 0.013            |
| GO:0021785  | branchiomotor neuron axon guidance                                       | BP       | 2        | 0.025            |
| GO:0048841  | regulation of axon extension involved in axon guidance                  | BP       | 2        | 0.033            |
| GO:1902287  | semaphorin-plexin signaling pathway involved in axon guidance           | BP       | 2        | 0.033            |
| GO:0005267  | potassium channel activity                                               | MF       | 5        | 0.00012          |
| GO:0004930  | G-protein coupled receptor activity                                      | MF       | 10       | 0.0083           |
| GO:0005251  | delayed rectifier potassium channel activity                            | MF       | 3        | 0.011            |
| GO:0005244  | voltage-gated ion channel activity                                       | MF       | 4        | 0.0038           |
| GO:0005249  | voltage-gated potassium channel activity                                | MF       | 4        | 0.0038           |
| GO:0046332  | SMAD binding                                                            | MF       | 2        | 0.044            |
| GO:0008076  | voltage-gated potassium channel complex                                  | CC       | 4        | 0.0038           |
| GO:0002116  | semaphorin receptor complex                                              | CC       | 2        | 0.075            |
| GO:0000276  | mitochondrial proton-transporting ATP synthase complex, coupling factor  | CC       | 2        | 0.049            |
| dre04080*   | Neuroactive ligand-receptor interaction                                  | KEGG     | 5        | 0.0088           |

Discussion And Conclusions

Organisms that have colonized underground caves encounter vastly different selective pressures than their relatives in above-ground habitats. In the present study, we report the first whole genome sequencing, assembly, and annotation of the *O. shuilongensis*, encompassing a total of predicted 25,247 protein-coding genes and 7,041 noncoding RNAs. We anticipate that this genome assembly will serve as a basis for in-depth biological studies of evolution and adaptation of cavefishes. With the availability of these genomic data, genomic/transcriptomics
differences between surface-dwelling and cave-dwelling loaches can be studied at the genomic scale. More broadly, our assembly will facilitate evolutionary and genomics research of Cobitoidea fishes and beyond.

**Materials And Methods**

**Animals captured and DNA extracted**

*O. shuilongensis*, *O. jiarongensis* (three individuals), *O. dongliangensis* (two individuals) and *O. daqikongensis* (one individuals) (Fig. 1) were captured from Guizhou Province, China. Each individual was over-exposed and executed by anesthesia, and quickly frozen in liquid nitrogen for one hour before storing at −80 °C. Genomic DNA was extracted from a muscle sample using DNeasy Blood & Tissue Kit (Qiagen). The present study was approved by the Animal Ethics Committee of Guizhou Normal University. The procedure of sample collection was in strict accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China.

**Whole Genome Sequencing of O. slonglongensis**

Three small-insert libraries (270 bp) were constructed by using Illumina's paired-end kits according to the manufacturer's instructions. The libraries were sequenced on Illumina Hiseq X Ten platform. For the raw reads, sequencing adaptors were removed. Contaminated reads (such as chloroplast, mitochondrial, bacterial and viral sequences, etc.) were screened by alignment to the NCBI-NR database using BWA\(^{[10]}\) with default parameters. Duplicated read pairs were removed by FastUniq v1.12\(^{[11]}\), and low-quality reads were filtered under the following conditions: (1) reads with ≥ 10% unidentified nucleotides (N), (2) reads with >10 nucleotides aligned to the adapter, allowing ≤10% mismatches, (3) reads with >50% bases having Phred quality < 5.

**RNA Sequencing**

Tissues of skin, muscle, intestine, liver and kidney of the same loach individual were collected and RNAs were extracted with TRIZOL Reagent (Invitrogen, USA). RNAs were then balanced mixed for the sequencing. The absorbance of 1.90 at 260 nm/280 nm and the RIN of 9.1 were obtained for the purified RNA sample by Nanodrop ND-1000 spectrophotometer (LabTech, USA) and 2100 Bioanalyzer (Agilent Technologies, USA), respectively. One microgram of RNA was reverse transcribed using Clontech SMARTer cDNA synthesis kit, and was further fragmented using divalent cations for the sequencing. The paired-end library was prepared following the manual of the Paired-End Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). Then the library with an insert length of 270 bp was sequenced by Illumina HiSeq X Ten in 150 bp paired-end mode (Illumina Inc., San Diego, CA, USA).

**Estimation of the Genome size and Sequencing Coverage**

The 19-mer frequency distribution analysis was performed on the remaining clean reads to estimate the genome size of the *O. shuilongensis* using the formula: Genome size = kmer_Number/Peak_Depth. Corrected Illumina reads were selected to perform genome size estimation.

**De novo Genome Assembly and Quality Assessment of O. shuilongensis Genome**

The single-molecule sequencing (SMS) data are assembled through Canu v1.5\(^{[12]}\) a comprehensive and scalable pipeline for SMS data assembly (https://github.com/marbl/canu), and the draft assembly polished through Pilon\(^{[13]}\). In the correction step, we used Canu to first select longer seed reads with the settings ‘genomeSize =
520M’ and ‘corOutCoverage = 90’, then to detect raw reads overlapping through a highly sensitive over lapper MHAP (mhap-2.1.2, option ‘corMhapSensitivity = normal’), and finally to perform error correction through the falcon sense method (option ‘corrected Error Rate = 0.045’). Next, using default parameter settings, error-corrected reads were trimmed of unsupported bases and hairpin adapters to obtain their longest supported range. In the last step, Canu generated the draft assembly using trimmed reads. The draft assembly was polished to obtain the final assembly, adopting Pilon\textsuperscript{13} (https://github.com/broadinstitute/pilon) using Illumina data with the parameters ‘--mindepth 10 --changes --threads 4 --fix bases’. To further evaluate the accuracy of the \textit{O. shuilongensis} genome, we aligned the high-quality short reads from short insert size pair-ended libraries against the genome assembly using \textit{BWA}\textsuperscript{14}.

**Annotation of Repeat Sequences**

The repeat composition of the assemblies was estimated by building a repeat library employing the \textit{de novo} prediction programs LTR FINDER v1.05\textsuperscript{15}, MITE-Hunter\textsuperscript{16}, RepeatScout v1.0.5\textsuperscript{17} and PILER-DF v2.4\textsuperscript{18}. The database was classified using PASTEClassifier\textsuperscript{19} and was combined with the Repbase\textsuperscript{20} database to create the final repeat library. Repeat sequences in the \textit{O. shuilongensis} genome were identified and classified using the RepeatMasker v4.0.6\textsuperscript{21} program.

**Annotation of Protein Coding Genes**

Protein-coding genes were predicted based on \textit{de novo}, protein homology-based and RNA-Seq method. We first used five \textit{de novo} gene prediction tools including Genscan\textsuperscript{22}, Augustus v2.4\textsuperscript{23}, GlimmerHMM v3.0.4\textsuperscript{24}, GeneID v1.4\textsuperscript{25} and SNAP\textsuperscript{12} to predict protein-coding genes in the \textit{O. shuilongensis} genome. For homology-based gene prediction, protein sequences from four closely related teleost species including \textit{Cyprinus carpio}, \textit{Danio rerio}, \textit{Sinocyclocheilus rhinocerous} and \textit{Astyanax mexicanus} were downloaded from Ensembl database and aligned against the \textit{O. shuilongensis} genome using GeMoMa v1.3.1\textsuperscript{26} software; the RNA-Seq reads were assembled into contigs \textit{de novo} into unigenes using Trinity and the unigenes were aligned to the repeat-masked assemblies using BLAT\textsuperscript{27}, and subsequently the gene structures of BLAT alignment results were modeled using PASA v2.02\textsuperscript{28}. Additionally, the RNA-Seq reads were also assembled into transcripts through mapping to the assembled genome using Hisat2 v2.0.4\textsuperscript{29} and StringTie v1.3.0\textsuperscript{30}, and the protein-coding regions were identified with TransDecoder v2.0\textsuperscript{31} and GeneMarkS-T v5.1\textsuperscript{32}, respectively. All these consensus gene models were generated by integrating the \textit{de novo} predictions, protein alignments and transcripts data using EVidenceModeler\textsuperscript{33}.

**Gene Functional Annotation**

Annotation of the predicted genes was performed by local \textit{BLAST}\textsuperscript{27} programs blasting their sequences against a number of nucleotide and protein sequence databases, including NCBI-NR\textsuperscript{20}, KOG\textsuperscript{34}, KEGG\textsuperscript{35}, and TrEMBL\textsuperscript{36} with an E-value cutoff of 1e-5. We then searched the Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases using the software Blast2GO\textsuperscript{37}.

**Noncoding RNA Annotation**

Non-coding RNAs play important roles in a great variety of processes, such as the rRNAs and tRNAs involved in mRNA translation. The rRNA fragments were identified by aligning the rRNA template sequences using BLAST with E-value at 1e-10 and identity cutoff at 95% or more. The tRNAscan-SE v1.3.1\textsuperscript{38} algorithms with default parameters were applied to the prediction of tRNA genes. The tRNA and miRNA genes were predicted by Infenal.
against the Rfam database and miRbase with cutoff score at 30 or more. The minimum a cutoff score was based on the settings which yield a false positive rate of 30 bits.

**Global Gene Family Classification**

In order to identify gene families among fish species in this work, proteins of the longest transcripts of each individual gene from *O. shuilongensis* and other sequenced species, including *Salmo salar, Ictalurus punctatus, A. mexicanus, C. carpio, S. rhinocerous, D. rerio and Larimichthys crocea* were analyzed. All data was downloaded from NCBI. Gene family analysis based on the homolog of gene sequences in related species was initially implemented by the alignment of an “all against all” BLASTP with a cutoff of 1e-5 and subsequently followed by alignments with high-scoring segment pairs conjoined for each gene pair by Solar. To identify homologous gene pairs, we required more than 30% coverage of the aligned regions in both homologous genes. Finally, homologous genes were clustered into gene families by OrthoMCL with the inflation parameter set at 1.5.

**Phylogenetic Relationship and Genomic Comparison**

Evolutionary analysis was performed using the single-copy protein-coding genes among all species. Amino acid and nucleotide sequences of the ortholog genes were aligned using the multiple alignment software MUSCLE with default parameters. A total number of 724 single-copy ortholog alignments were concatenated into a super alignment matrix of 1,692,085 nucleotides. A maximum likelihood method deduced tree was inferred based on the matrix of nucleotide sequences using PhyML package with the JTT + G + F model. Clade support was assessed using bootstrapping algorithm in the PhyML package with 100 alignment replicates. The constructed phylogenetic tree indicated that *O. shuilongensis* were clustered closely to Cyprinidae species, which is inconsistent with their putative evolutionary relationships (Fig. 3).

We determined the expansion and contraction of the orthologous gene families by comparing the cluster size differences between the ancestor and each of the *O. shuilongensis* and seven other fish species using the CAFÉ program. A random birth and death model were used to study changes of gene families along each lineage of the 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 identify families that were significantly expanded in *O. shuilongensis* genome.

**Dating the Divergence among Oreonectes Fishes and Assessing the Genomic Mechanism Underlying the Degeneration of Eyes and Body Color**

*O. shuilongensis* is a surface-dwelling species with intact eyes and a unique color pattern consisting of fine black marks on the body except on the abdomen. To explore the genomic changes resulting in the degeneration of eyes and body color of semi cave-dwelling and cave-dwelling *Oreonectes* fishes, the whole genome re-sequencing was performed for facultative cave-dwelling *O. jiarongensis* (three individuals) and cave-dwelling *O. daqikongensis* (two individuals) and *O. dongliangensis* (one individual). For each individual, ~3 µg of DNA was sheared into fragments of 350 bp with the Covaris v1.8 system. DNA fragments were then processed and sequenced using the Illumina HiSeq 4000 platform. Filtered sequence reads were mapped to the *O. shuilongensis* reference genome using BWA-MEM with default parameters (0.7.10-r789). Alignment bam files were imported to SAMtools (v0.1.19) for sorting and Picard (http://broadinstitute.github.io/picard/) was used to remove duplicated reads. Following mapping, we performed variant calling using the GATK package with default
parameters. To explore the phylogenetic relationships among *Oreonectes* fishes, phylogenetic tree was inferred using the Neighbor-Joining (NJ) algorithm as implemented in RAxML software with 1000 bootstraps[48] and the divergence times of the taxa analyzed were estimated with mcmctree[15]. The outgroup sequences were chosen from the zebrafish genome assembly GRCz11, with the genome alignment to the obtained *Oreonectes* genome assembly using LASTZ[49]. We employed calibration points from the dated Cyprinidae-Nemacheilidae divergence to place a lognormally distributed prior on the age of the root of a tree containing all samples of *Oreonectes* species and outgroup. To explore the genomic mechanism underlying the degeneration of eyes and body color, the SNPs and Indels obtained through GATK pipeline were annotated using the software SnpEff[6].

**Abbreviations**

BLAST: Basic Local Alignment Search Tool; KEGG: Kyoto Encyclopedia of Genes and Genomes; NCBI: National Center for Biotechnology Information.

**Declarations**

**Declaration of Interest Statement**

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Written informed consent for publication was obtained from all participants.

**Availability of supporting data**

Supporting data is available in the NCBI database. Raw data has been deposited in NCBI with the project accession PRJNA505902. BioSample accessions: SAMN10438768, SAMN10438757-60, SAMN10438763 and SAMN10438766.

**Competing interests**

The authors declare that they have no competing interests.

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Author contributions

ZL, XQ, HW, and LH, performed the experiments, XQ, and ZW, analyzed the data, ZL and HW, wrote the paper, and participated in the design of the study. XQ, HW, and Z W, participated in data statistics and the draft manuscript. JZ, and ZL, conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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