Genome of the Rio Pearlfish (*Nematolebias whitei*), a bi-annual killifish model for Eco-Evo-Devo in extreme environments

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Abstract

The Rio Pearlfish, *Nematolebias whitei*, is a bi-annual killifish species inhabiting seasonal pools in the Rio de Janeiro region of Brazil that dry twice per year. Embryos enter dormant diapause stages in the soil, waiting for the inundation of the habitat which triggers hatching and commencement of a new life cycle. Rio Pearlfish represents a convergent, independent origin of annualism from other emerging killifish model species. While some transcriptomic datasets are available for Rio Pearlfish, thus far, a sequenced genome has been unavailable. Here, we present a high quality, 1.2 Gb chromosome-level genome assembly, genome annotations, and a comparative genomic investigation of the Rio Pearlfish as representative of a vertebrate clade that evolved environmentally cued hatching. We show conservation of 3D genome structure across teleost fish evolution, developmental stages, tissues, and cell types. Our analysis of mobile DNA shows that Rio Pearlfish, like other annual killifishes, possesses an expanded transposable element profile with implications for rapid aging and adaptation to harsh conditions. We use the Rio Pearlfish genome to identify its hatching enzyme gene repertoire and the location of the hatching gland, a key first step in understanding the developmental genetic control of hatching. The Rio Pearlfish genome expands the comparative genomic toolkit available to study convergent origins of seasonal life histories, diapause, and rapid aging phenotypes. We present the first set of genomic resources for this emerging model organism, critical for future functional genetic, and multiomic explorations of “Eco-Evo-Devo” phenotypes of resilience and adaptation to extreme environments.

Keywords: *Nematolebias whitei*; Rio Pearlfish; diapause; aging; hatching; extreme environments; Eco-Evo-Devo; teleost

Introduction

Apocheiloid killifishes inhabit tropical freshwater habitats around the world. Some African and Neotropical species live in ephemeral waters that are subject to seasonal desiccation (Myers 1952, Simpson 1979). Desiccation kills the adults, but embryos survive inside specialized eggs (Thompson et al. 2017a) buried in the soil via 3 diapause stages (DI, DII, DIII; Wourms 1972a, 1972b, 1972c). DI occurs as a migratory dispersion of blastomeres, DII occurs during somitogenesis when organs are rudimentary, and DIII occurs after organogenesis when the embryo is fully formed and poised to hatch upon habitat inundation. This seasonal life history is a remarkable example of convergent evolution (Furness et al. 2015) with 7 gains across killifish evolution (Thompson et al. 2021a). Additionally, annual killifishes show rapid aging due to relaxed selection on lifespan (Cui et al. 2019) and are an important emerging model system for the study of senescence (Valenzano et al. 2011, 2015; Harel et al. 2015; Reichwald et al. 2015).

The Rio Pearlfish, *Nematolebias whitei*, is a seasonal killifish endemic to the coastal plains of the Rio de Janeiro region in Brazil, inhabiting pools that dry twice annually, from July–August and February–March (Fig. 1a; Myers 1942; Costa 2002). Pearlfish represents a separate origin of seasonality from other killifish model species, i.e. *Nothobranchius furzeri* and *Austrofundulus limnaeus* (Thompson et al. 2021a). In *N. whitei*, DI and DII are facultative, and DIII is a “prolonged,” “deep” stasis compared to hatching delay and DIII in other killifishes, occurring just before environmentally cued hatching upon submersion in water (Wourms 1972c, Thompson and Ortí 2016; Thompson et al. 2017b). Pearlfish was suggested as a top candidate species for killifish models in the seminal work of developmental biologist John P. Wourms in 1967. They are small, prolific, and hardy, and spawn in sand (Wourms 1967), making them easily reared laboratory animals that are furthermore amenable to genetic manipulation like other killifishes (Aluru et al. 2015; Harel et al. 2015). Pearlfish has also been an emergent system to study aging (Ruijter 1987), environmental...
influences on development (Ruijter et al. 1984), the role of prolactin in hatching control (Schoots et al. 1983; Ruijter and Creuwels 1988), resilience to perturbations in development with the ability to develop normally from diblastomeric eggs (Carter and Wourms 1993), and the transcriptional control of diapause and hatching (Thompson and Ortı 2016).

Here, we construct a chromosome-level genome assembly for the Rio Pearlfish, utilizing Hi-C contact maps, genome annotations, and gene expression analyses to characterize genomic evolution and hatching biology in this extremophile vertebrate.

Materials and methods
Genome sequencing and assembly
All animal work was approved by the Michigan State University Institutional Animal Care and Use Committee (PROTO202000108).

A total of 1.25 ng of template genomic DNA extracted from the liver of a single adult female N. whitei was loaded on a Chromium Genome Chip. Whole-genome sequencing libraries were prepared using 10× Genomics Chromium Genome Library & Gel Bead Kit v.2, Chromium Genome Chip Kit v.2, Chromium i7 Multiplex Kit, and Chromium controller according to the manufacturer’s instructions with one modification. Briefly, gDNA was combined with Master Mix, a library of Genome Gel Beads, and partitioning oil to create Gel Bead-in-Emulsions (GEMs) on a Chromium Genome Chip. The GEMs were isothermally amplified. Prior to Illumina library construction, the GEM amplification product was sheared on a Covaris E220 Focused Ultrasonicator to ~350 bp and then converted to a sequencing library following the standard operating procedure. A total of 679.43 million read pairs were sequenced on an Illumina HiSeqX sequencer, and a de novo assembly was constructed with Supernova 2.1.1 (Weisenfeld et al. 2018).

A Chicago library was prepared as described previously (Putnam et al. 2016). Briefly, ~500 ng of HMW gDNA was reconstituted into chromatin in vitro and fixed with formaldehyde. Fixed chromatin was digested with DpnI, the 5’ overhangs were filled in with biotinylated nucleotides, and then free blunt ends were ligated. After ligation, crosslinks were reversed, and the DNA was purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was then...
were aligned, and scaffolds were generated following the same perspective. Joins were identified and broken using a tool designed for this purpose. The DNA was then sheared to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The libraries were sequenced on an Illumina HiSeqX to produce 242 million 2×150 bp paired end reads.

A Dovetail Hi-C library was prepared in a similar manner as described previously (Lieberman-Aiden et al. 2009). For each library, chromatin was fixed in place with formaldehyde in the nucleus and then extracted. Fixed chromatin was digested with DpnII, the 5' overhangs filled in with biotinylated nucleotides, and then free blunt ends were ligated. After ligation, crosslinks were reversed, and the DNA purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was then sheared to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The libraries were sequenced on an Illumina HiSeqX to produce 179 million 2×150 bp paired end reads.

The Supernova de novo assembly built from 10× Chromium data, Chicago library reads, and Dovetail Hi-C library reads were used as input data for assembly scaffolding with HiRise v1 (Putnam et al. 2016). An iterative analysis was conducted. First, Chicago library sequences were aligned to the draft input assembly using a modified SNAP read mapper (http://snap.cs.berkeley.edu). The separations of Chicago read pairs mapped within draft scaffolds were analyzed by HiRise v1 to produce a likelihood model for genomic distance between read pairs, and the model was used to identify and break putative misjoins, to score prospective joins, and make joins above a threshold. After aligning and scaffolding Chicago data, Dovetail Hi-C library sequences were aligned, and scaffolds were generated following the same approach.

**Genome annotation**

The Rio Pearlfish genome was annotated with the NCBI Euakryotic annotation pipeline v9.0 (Thibaud-Nissen et al. 2016) and with MAKER 2.31 (Cantarel et al. 2008, Campbell et al. 2014, Bowman et al. 2017) using protein evidence from 15 fish species (Supplementary Table 1) and transcriptome evidence from Rio Pearlfish DIII embryos and hatched larvae (Thompson and Ortí 2016). Genome assembly and annotation completeness (Supplementary Table 2) were analyzed with BUSCO v5 (Simão et al. 2015) and CEGMA 2.4 (Parra et al. 2007) via the gVolante server (Nishimura et al. 2017, https://gvolante.riken.jp).

**Phylogenetics and orthology**

To confirm species identification, we extracted and concatenated the barcoding marker genes cox1 and cytb from our genome assembly, aligned them with orthologous sequences from all 3 described Nematolebias species (Costa et al. 2014) and inferred a phylogeny partitioned by codon and gene with RAXML ( Stamatakis 2006, 2014) with the following parameters: -T 4 -N autoMRE -m GTRCAT -c 25 -p 12345 -f a -x 12345 --asc-corr-lower. We used Orthofinder v2.4.1 (Emms and Kelly 2015) to identify orthologous protein sequences between N. whitei and 35 other vertebrates genomes (Supplementary Table 3) as well as protein sequences obtained from Cui et al. (2019), Hara et al. (2018), and the longest isoforms of other species available on NCBI RefSeq (last accessed 2021 September 22) downloaded with ortholog’s retrieve longest isoforms function (Drost et al. 2015). The output of Orthofinder (Supplementary Table 4) was examined to identify Pearlfish-specific orthogroups. Genes in these orthogroups were used as queries in BLAST searches (e value cutoff of e–3) against Japanese medaka (HdR strain, assembly ASM223467v1) protein sequences downloaded from Ensembl (last accessed 2022 January 17, Supplementary Table 5). We performed a statistical overrepresentation test with a Fisher’s exact test and a false discovery rate correction on the Gene Ontologies (GOs) of these medaka genes using Panther v.16.0 (Mi et al. 2021) with the GO biological processes complete database (Supplementary Table 6).

**Synteny and genome 3D structure**

We examined conservation of synteny using genome assemblies and NCBI annotations for Rio Pearlfish, medaka (oryLat2, UCSC), and zebrafish (GCF_000002035.5_GRCz10, NCBI) as input for SynMap in the online CoGe database and toolkit (Lyons and Freeling 2008; last accessed October 14, 2021). Bwa v 0.7.17 (Li and Durbin 2009) was used to independently map Rio Pearlfish Hi-C read pairs to the genome assembly with the following parameters: bwa mem -A1 -B4 -E50 -L0, and HiCExplorer 3.6 (Ramírez et al. 2018) was used to construct a Hi-C matrix with the resulting bam files as follows: hicBuildMatrix –binSize 10000 –restrictionSequence GATC –danglingSequence GATC. The matrix was corrected via hicCorrectMatrix correct –filterThreshold -1.5. 5. The matrix was binned depending on preferred resolution for viewing. Contact maps were visualized with hicPlotMatrix –log1p, and compared with contact maps of syntenic regions in medaka and zebrafish (Nakamura et al. 2021).

**Repeat content and repeat landscape**

We constructed a species-specific repeat database with Repeat Modeler 2.0.1 (Smit and Hubley 2008). This library as well as vertebrate Repbase annotations (Jurka 2000) (downloaded 15 November 2017), and repeat libraries from playfish (Schartl et al. 2013), coelacanth (Arnesiata et al. 2013), bowfin (Thompson et al. 2021b), and spotted gar (Braasch et al. 2016) were combined to annotate repeat elements with RepeatMasker v4.0.5 (Smit et al. 2013). CalcDivergenceFromAlign.pl and createRepeatLandscape.pl in the RepeatMasker package were used to generate a repeat landscape. We graphically compared the repeat landscape of Rio Pearlfish to those described for other sequenced killifish species (Reichwald et al. 2015; Valenzano et al. 2015; Rhee et al. 2017; Cui et al. 2019) to identify similarities and difference in the magnitude and location of peaks at different Kimura distances in the histograms.

**Hatching enzyme gene expression**

Aquatic vertebrates hatch by secreting choriolytic enzymes from hatching gland cells (HGCs) that break down the egg chorion (Yamagami 1988, Hong and Saint-Jeannet 2014). Teleost fishes underwent hatching enzyme gene duplications followed by divergence and functional divergence into the high choriolytic enzyme (hce) and low choriolytic enzyme (lce) genes (Yasumasa et al. 1992, Kawaguchi et al. 2006, 2010; Sano et al. 2014). We used BLAST to search the well-studied medaka hatching enzyme paralogs (hce and lce) against the annotated Pearlfish genome. We used the Pearlfish gene sequences from these BLAST hits as well as other metalloprotease gene sequences from medaka, Astrodendrulus, Kryptolebias, and Nothobranchius (accession numbers in Supplementary Table 7) to infer gene trees. The identified Pearlfish hce and lce genes are orthologous to those of other teleosts (data not shown). Pearlfish hatching enzyme orthologs were examined for transcript evidence from DIII embryos (Thompson 2015).
Results and discussion

Genome sequencing and assembly

We report a high-quality, 1.2 Gb chromosome-level genome assembly of *N. whitei*. The Rio Pearlfish genome assembly consists of 24 chromosomal pseudomolecules represented by 24 superscaffolds that match the described karyotype (Post, 1965). The assembly has an N50 of over 49.98 Mb and an L50 of 11 scaffolds (Table 1). BUSCO and CEGMA scores for different core gene databases indicate a high-quality assembly with an average of 94% complete BUSCOs and CEGs across all relevant databases (Table 1, Supplementary Table 2).

Genome annotation

The NCBI *N. whitei* Annotation Release 100.20210725 contains 23,038 genes, with 21,341 protein-coding genes, similar to other chromosome-level killifish genome assemblies from *N. furzeri* and *Kryptolebias marmoratus* (Reichwald et al. 2015; Valenzano et al. 2015; Kelley et al. 2016; Rhee et al. 2017) (Supplementary Table 8). Minor differences in gene numbers among killifish species are likely due to annotation methods and minor species-specific gene losses or expansions. The number and content of annotated genes can be influenced by evidence used for annotation, differences in gene model prediction likelihoods, and post-annotation filtering (Holt and Yandell 2011; Campbell et al. 2014). MAKER annotated 26,016 protein-coding genes, on par with the NCBI annotation. See Table 1 and Supplementary Tables 2 and 8 for Rio Pearlfish genome annotation statistics. Although our BUSCO analyses show fewer genes missed by the NCBI annotation (Supplementary Table 2), our additional MAKER annotation provides additional, valid gene models missed by the NCBI pipeline. For example, MAKER annotates 28 vertebrate and 27 actinopterygian BUSCOs missed by the NCBI annotation pipeline (Supplementary Table 9).

Phylogenetics and orthology

Our orthofinder analysis illustrates the phylogenetic position of Rio Pearlfish among vertebrates (Fig. 1b) and identified 31,317 orthogroups across 36 vertebrate species with 99.2% of Rio Pearlfish genes within orthogroups (Table 1; Supplementary Table 4). We identified 7,287 orthogroups present across all species from sharks to human to Rio Pearlfish, highlighting the utility of the Rio Pearlfish genome to connect species with extreme developmental phenotypes to other vertebrates, including traditional vertebrate model species such as mouse, *Xenopus*, zebras, and *Drosophila*. We confirmed the identity of our genome specimen with barcoding and a molecular phylogeny of *cox1* and *cytb* with its position located within the *N. whitei* clade of Nemateleotris killifishes (Fig. 1c). We found a total of 17 Pearlfish-specific orthogroups comprising a total of 42 protein sequences. For 39 of these, we established homology to a medaka gene class by BLAST (Supplementary Table 5) and found an overrepresentation for GO terms related to glycolysis (Supplementary Table 6). This may indicate an adaptive expansion of metabolic genes in this species as annual killifishes tolerate anoxia (Podrabsky et al. 2007, 2012; Wagner et al. 2018), severely depress metabolic rate during diapause (Podrabsky and Hand 1999), and drastically increase metabolic rate during fast maturation (Vrtilek et al. 2018) necessary for an annual life cycle. In a separate study, we have also found that genes involved in cell respiration, specifically oxidative phosphorylation, show higher ratios of nonsynonymous/synonymous codon changes in annual killifishes compared to their nonannual counterparts (Thompson et al. 2021). Together, these observations point to potential positive selection on genes involved in cell respiration in annual killifishes.

Synteny and genome 3D structure

Three-dimensional chromatin structure impacts gene regulation and can manifest as topologically associated domains (TADs) that could represent higher order gene regulatory regions conserved across evolution (Krefting et al. 2018). However, 3D genome structure has thus far remained uncharacterized in annual killifishes. To confirm the quality of the genome assembly and assess the utility of the chromatin conformation data to interrogate 3D genome structure and gene regulation, we constructed a Hi-C contact map showing higher contact frequency within the 24 pearlfish chromosomes (Fig. 1d) than between chromosomes.
Using the genome sequence and gene annotations for Rio Pearlfish in synteny comparisons to another atherinomorph teleost, the medaka (separated by ~85 million years), and the ostariophysian teleost zebrafish (separated by ~224 million years), we reveal largely conserved synteny with these species (Fig. 2, e and f) across millions of years of teleost evolution (Hughes et al. 2018; Thompson et al. 2021a). We examined a TAD previously shown to be conserved from zebrafish to medaka (Nakamura et al. 2021) and found high frequency of contacts in the syntenic region between rasa1a and mctp1a in Rio Pearlfish liver tissue that resembles contact maps both in a medaka fibroblast cell line and zebrafish whole embryos (Fig. 1g). Hi-C analyses thus confirm the high-quality of our genome assembly as well as the strikingly conserved nature of 3D genome interactions across teleost evolution, developmental stages, and among cell and tissue types.

Repeat content and transposable element landscape

Transposable elements (TEs) are hypothesized to generate novel genetic substrate for adaptations (Casacuberta and González 2013; Feiner 2016; Esnault et al. 2019). Some annual killifish species have expanded TE content compared to nonannual relatives (Cui et al. 2019) and the link between TEs, aging, and human diseases (Bravo et al. 2020) coupled with the rapid senescence of annual killifishes highlights the importance of examining the Pearlfish “mobilome.” We found that the Rio Pearlfish genome is highly repetitive with a repeat content of ~57% (Fig. 2a and Table 1; Supplementary Table 10) which is substantially elevated compared to a nonannual member of the same South American family, K. marmoratus, with a repeat content of around ~27% (Rhee et al. 2017; Choi et al. 2020). Similarly, African annual Nothobranchius killifishes have higher TE repeat content than their nonannual relatives (Cui et al. 2019). This pattern might be the result of adaptation to extreme environments as animals, fungi, and plants have co-opted TEs for environmental adaptations to harsh conditions (Casacuberta and González 2013; Esnault et al. 2019) and TEs may play roles in vertebrate adaptive radiations (Feiner 2016). Our findings further highlight the expanded repeat content in annual killifish genomes and the Pearlfish genome provides novel resources to study the role of mobile DNA in extremophiles.

Hatching enzyme gene expression and hatching gland location

While hatching from the egg is a critical time point during animal development, little is known about its genetic regulation and the integration of environmental cues. Additionally, development of HGCs is dynamic among fishes (Inohaya et al. 1995; 1997) as they migrate and localize in different anatomical locations in different species (Korwin-Kossakowski 2012; Shimizu et al. 2014; Nagasawa et al. 2016). Pinpointing HGC location in seasonal killifishes is necessary for understanding the regulation of hatching in extreme environments.
Rio Pearlfish is a tractable model for studying hatching regulation since hatching is easily induced in this species by exposing DIII embryos to water (Thompson and Ortí 2016). Thus, we examined the hatching enzyme gene repertoire and HGC locations in Pearlfish. We identified five expressed hatching enzyme genes (Fig. 2b, 3 hce and 2 lce genes) upon mapping DIII mRNA reads from Thompson and Ortí (2016) to our reference genome assembly. We annotated hce1 and hce2 on chromosome 2 (corresponding to NCBI genes LOC119423801, LOC119423789), and hce3 on chromosome 20 (LOC119426643) and the adjacent lce.1 and lce.2 genes (LOC119418488, LOC119418489) on chromosome 12 that are species-specific tandem duplicates (Fig. 2b) supported by transcript evidence (Thompson and Ortí 2016). Using whole mount RNA in situ hybridization for lce.2 in DIII embryos, we identified HGC locations in the buccal and pharyngeal cavity in Rio Pearlfish (Fig. 2, c and d) similar to HGC localization in the related mummichog or Atlantic killifish (Fundulus heteroclitus) (Kawaguchi et al. 2005) and in medaka (Inohaya et al. 1995).

A pattern of expanded hce genes is also found in other Atherinomorph fishes like medaka. High choriolytic enzyme genes in this clade of teleosts have lost introns (Kawaguchi et al. 2010) and subfunctionalized post duplication with some hce genes performing better at higher or lower salinities in the euryhaline medaka Oryzias javanicus (Takehana et al. 2020) and the Atlantic killifish Fundulus heteroclitus (Kawaguchi et al. 2013b). Furthermore, the duplication of the lce gene in Rio Pearlfish is an example of convergent evolution within teleosts with another lce duplication in stickleback fishes (Kawaguchi et al. 2013a). These findings underscore the commonality of hatching enzyme gene duplications in teleost fishes that provides a model system for studying convergent gene duplications and functional divergence by sub- and neofunctionalizations.

Conclusions

Our chromosome-level, dually annotated genome assembly of the Rio Pearlfish provides a valuable comparative genomics resource strengthening the utility of killifishes for studying aging, suspended animation, and response to environmental stress. The Rio Pearlfish is an emerging “Extremo” Eco-Evo-Devo research organism, and this reference genome will be a substrate for future organism, and this reference genome will be a substrate for future adaptation to extreme environmental conditions in a changing world.

Data availability

The genome sequence, annotation, and sequence read data are available on NCBI under accession GCA_014905685.2 and Bioproject PRJNAS60526. The genome assembly and annotation has also been integrated to the University of California Santa Cruz Genome Browser (https://hgdownload.soe.ucsc.edu/hubs/fish/index.html). The MAKER genome annotation is available on github (https://github.com/AndrewWT/NematolebiasGenomics).

Supplemental material is available at G3 online.

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AWT and IB conceived the project, wrote the manuscript, and acquired funding; genome sequencing and assembly was performed with Dovetail Genomics; MD, HW, AWT, and IB analyzed hatching enzyme genes; HW and AWT performed RNA in situ hybridization; AWT performed comparative genomics analyses, and genome structure analyses; AWT and IB analyzed the repeat content.

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Conflicts of interest

None declared.

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