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GENOME SEQUENCING

The complete genome sequence of the protogynous hermaphrodite North American annual killifish *Millerichthys robustus* (Rivulidae, Cyprinodontiformes)

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Biodiversity Genomes

The Mexican Rivulus, *Millerichthys robustus*, is an enigmatic species of seasonal killifish endemic of the Southeast Mexico that has changed paradigms on the evolution of annualism in killifishes. This species survives in ephemeral environments that experience a period of seasonal drought that causes the death of all adult fish. However, populations persist due to their drought-resistant embryos capable of arresting their development in diapause until the next the rainy season. *Millerichthys* evolved unique characteristics within annual killifishes as a functional sequential hermaphroditism, in which females change to males (protogynous) under perceived conditions of mate competition. Also, *M. robustus* express different color phenotypes in both sexes: five-color phenotypes continuously distributed in various perceptual units between yellow and red in males, and different number of ocelli disposed in caudal peduncle in females. The phylogenetic relationships of *M. robustus* revealed that it is a sister clade to two non-annual species found exclusively in Cuba (*Rivulus cylindraceus* and *Rivulus berovidesi*), indicating that the annual life cycle, through the acquisition of embryonic diapause, has evolved independently in this species. Here, we present the complete genome sequences for the North American annual killifish *Millerichthys robustus*. The raw data and assembled genome are available in GeneBank.

Introduction

The Mexican Rivulus, *Millerichthys robustus,* is an enigmatic species of seasonal killifish that has changed paradigms on the evolution of annualism among killifishes (Domínguez-Castanedo, Luna-González, et al. 2024), a group of small freshwater and brackish fishes that integrate the sub-orders Aplocheiloidei and Cyprinodontoidei (Nelson 2006). Annual killifishes (only present in the families Rivulidae, Aplocheilidae and Notobranchidae of Aplocheiloidei; Loureiro and de Sá 2016) are known from the American and African continents, and adapted their life cycle within a single year, following the seasonal changes that occur in the temporary water bodies that they inhabit (Furness 2016). The annual life cycle is made up of a set of extreme life history strategies that allow fish to persist permanently in temporary pools that present alternately a favorable rainy phase for development, and a hostile one of drought along the year (Williams 2006). During the rainy seasons, fish hatch, grow quickly, and mature early to reproduce before perishing due to the seasonal drying of the ponds. However, their populations persist in these environments with highly contrasting changes only by drought-resistant embryos capable of arresting its development by diapause stages, allowing them to survive until the next rainy season (Loureiro and de Sá 2016).

The knowledge about *M. robustus* life cycle has been historically wrapped in controversy regarding its annual nature. Traditionally, an annual life cycle in killifishes was considered to have evolved only in killifish species from Africa and South America (Costa 1995, 1998; Miller 2009). However, the annual life cycle of *Millerichthys*, with the full expression of three diapauses along its embryo development, was recently revealed, making *Millerichthys robustus* the only known species in North America with this unusual life history (Domínguez-Castanedo, Mosqueda-Cabrera, and Valdesalici 2013). These findings challenged historical paradigms of the distribution, and biogeographic history of annual killifishes (Costa 2013; Furness 2016; Loureiro and de Sá 2016). The presence of *Millerichthys robustus* in North America makes also this species the northern mostly distributed annual killifish in the world (Wildekamp, Romand, and Scheel 1986; Valdesalici 2013; Reichard 2016), separated by > 2, 000 kilometers from the closest South American species of annual fishes distributed in Colombia and Venezuela (Loureiro and de Sá 2016).

Millerichthys robustus inhabit diverse natural environments, such as coastal floodplains, induced grasslands, intermittent wetland habitat, low evergreen forests, and even urbanized grasslands on the Papaloapan and Coatzacoalcos River basins of Veracruz and Oaxaca, in México. This species was originally described as *Rivulus robustus*, within the family Rivulidae (Miller and Hubbs 1974). This description included several unique morphological traits, such as a frontal pattern of squamation, distinctive color phenotypes in both sexes, and a deeper body than any other species in the genus *Rivulus*, motivating its inclusion in a newly erected genus "*Millerichthys*", and placed it within Cynolebiatinae, a group integrated only by South American annual killifishes (Costa 1995). However, Murphy and Collier (1996) challenged that classification, showing that the meristic of *M. robustus* were similar to those of the Rivulus endemic to the Antilles, suggesting a closer phylogenetic affinity of *M. robustus* with these non-annual *Rivulus* species.

The controversy about the phylogenetic position of *M. robustus* extended over long period of time, particularly due to the difficulties to access samples for genetic analyses to test between these two contrasting phylogenetic hypotheses. Recently, the phylogenetic relationships of *M. robustus* were clarified using molecular analyses, revealing that *Millerichthys robustus* is a sister clade to two non-annual species found exclusively in Cuba (*Rivulus cylindraceus* and *Rivulus berovidesi*). Ancestral state reconstruction analysis also indicated that the annual life cycle, through the acquisition of embryonic diapause, has evolved independently in *Millerichthys robustus* (Domínguez-Castanedo, Thompson, et al. 2024). These results also suggest, as Murphy and Collier (1996) originally suggested, that *M. robustus* ancestor may have been isolated at the southern tip of North America following the migration of the proto-Antilles during the late Cretaceous, Therefore, it is a remarkable case of the

convergent evolution of the annual life history in North America, representing an independet instance of annualism evolution in the suborder Aplocheiloidei, with traits shared with all other annual killifishes of the world.

All annual killifishes share traits such as (*i*) three diapauses during its embryonic development (an early diapause during epiboly, a diapause during somitogenesis, and a pre-hatching diapause; Wourms 1972a, 1972b, 1972c); (*ii*) rapid growth and early sexual maturity (Furness 2016); and (*iii*) high metabolic rates accompanied by voracious eating behaviors that allow them to sustain the accelerated life cycle (Careau et al. 2008; Laufer et al. 2009; Careau and Garland 2012; Furness 2016; Genade et al. 2005; Ijumba and Kilama 1991; Polacik, Donner, and Reichard 2011; Domínguez-Castanedo, Uribe, and Rosales-Torres 2017; Domínguez-Castanedo, Luna-González, et al. 2024). In addition, *M. robustus* also evolved unique characteristics within annual killifishes. First, *M. robustus* have functional sequential hermaphroditism, in which females change to males (protogynous) under perceived conditions of mate competition. Protogyny also was observed in female-only environments, where high levels of physical aggression among females seem to be a trigger for the sex change (Domínguez-Castanedo, Valdez-Carbajal, Muñoz-Campos, et al. 2022). Additionally, mature females under a perceived environment of mate competition, increase their reproductive effort and become more selective, choosing males of larger size than those chosen without this perceived risk (Domínguez-Castanedo 2021). Collectively, all these studies demonstrated that the life history of *M. robustus* females is plastic and sensitive to social context, responding adaptively to limitations of male access (Domínguez-Castanedo 2021; Domínguez-Castanedo, Valdez-Carbajal, Muñoz-Campos, et al. 2022). Second, *Millerichthys robustus* express different color phenotypes in both sexes: different coloration in the anal fin, and to less extent in the dorsal and ventral fins of the males, and ocelli in caudal peduncle in females (Miller and Hubbs 1974; Miller 2009). Males occur in five-color phenotypes continuously distributed in various perceptual units between yellow and red, based on the RGB system: yellow, moderate orange, dark orange, strong orange, and red. However, there are only three male color phenotypes perceived by the human eye: orange, red, and yellow (**Figure 1**). The color-polymorphism of females varies among 1 to 15 ocelli that are arranged in the upper region of the caudal peduncle, and to a lesser extent in the dorsal region (**Figure 2**; Domínguez-Castanedo, Muñoz-Campos, Valdesalici, et al. 2021).

Here we provide whole genome sequencing data for the enigmatic Mexican Rivulus *Millerichthys robustus*, which in addition to having unique biological characteristics, it has been classified as “Endangered” by the IUCN due to its reduced distribution range (- 8,000 km2), and the progressive decline of its extension and habitat quality by municipal and industrial development (Lyons 2019). Also, the Government of Mexico maintains it is classified in the category of “in danger of extinction” (DOF 2010); therefore, it is a species that need focused conservation efforts.

Figure 1. Male color phenotypic variations perceibed by the human eye: Red, Orange and Yellow (from top to down). Bar = 0.50 cm. Modified from Domínguez-Castanedo, Muñoz-Campos, Valdesalici, et al. (2021).

Methods

We used the tissue of a single wild-collected *Millerichthys robustus* individual in "Playa Las Salinas", near Alvarado, Veracruz, México (18°54'39.58"N, 95°56'41.05"W), under the authorization GPA/DGVS/02404/14 of the Subsecretaría de Gestión para la Protección Ambiental, Dirección General de Vida Silvestre of SEMARNAT. DNA extraction was performed with the Qiagen DNAeasy Genomic Extraction Kit using a standard process. A paired-end sequencing library was constructed using the Illumina TruSeq kit, according to the manufacturer's instructions. The library was sequenced on an Illumina Hi-Seq platform in a paired-end, 2 × 150bp format. The resulting fastq files were trimmed of adapter/primer sequences and low-quality regions using Trimmomatic v0.33 (Bolger, Lohse, and Usadel 2014). The trimmed sequence was assembled using SPAdes v2.5 (Bankevich et al. 2012) followed by a final step using Zanfona (Kieras M., O'Neill K., and Pirro S. 2021).

Figure 2. Female color phenotypic variations in ocelli number. Bar = 0.50 cm. Modified from Domínguez-Castanedo, Muñoz-Campos, Valdesalici, et al. (2021).

Genome assembly statistics

Genome assembly and annotation completeness were analyzed with BUSCO v5 (Simão et al. 2015) and CEGMA 2.4 (Parra, Bradnam, and Korf 2007) using the gVolante server (Nishimura, Hara, and Kuraku 2017, https://gvolante.riken.jp).

Results and Data Availability

All raw read data and the assembled genome for *Millerichthys robustus* is available in the GenBank database (Raw data: SRR25700366; Assembled Genome: JAVKLF000000000). Both BUSCO and CEGMA scores for different core gene databases indicated a high-quality complete assembly, with an average of 92.65% complete BUSCOs and CEGs across eukaryotic, vertebrate, and ray-finned fish databases (**Table 1**).

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Table 1. Mexican rivulus (*Millerichthys robustus*) genome assembly (ASM3478349v1) statistics. Complete BUSCOs or CEGs (C); Complete and single-copy BUSCOs (S); Complete and duplicated BUSCOs (D); Fragmented BUSCOs or partial CEGs (F); Missing BUSCOs or CEGs (M); Total BUSCOs or CEGs queried (N). CEGMA scores are shown for vertebrate ortholog database (CVG) and eukaryotic ortholog database (CEG).

No. of scaffolds	255,480
No. of base pairs	616,408,618
N50	19,168,897
L50	14
GC content (%)	40%
Average genome coverage	60x
BUSCO scores	Vertebrata: C:93.2% (S:92.9%, D:0.3%); F:4.6%; M:2.2%; N:3354 Actinopterygiii: C:93.8% (S:93.2%, D:0.6%); F:3.0%; M:3.2%; N:3640
CEGMA scores	CVG: C: 88.84%; C+F: 98.71%; M: 1.29%; N:233 CEG C: 94.76%; C+F: 99.60%; M: 0.40%; N: 248

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