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The subterranean catfish *Phreatobius cisternarum* provides insights into visual adaptations to the phreatic environment

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ABSTRACT Vertebrate eyes share the same general organization, though species have evolved morphological and functional adaptations to diverse environments. Cave-adapted animals are characterized by a variety of features including eye reduction, loss of body pigmentation, and enhanced non-visual sensory systems. Species that live in perpetual darkness have also evolved sensory mechanisms that are independent of light stimuli. The subterranean catfish *Phreatobius cisternarum* lives in the Amazonian phreatic zone and displays a diversity of morphological features that are similar to those observed in cavefish and appear to be adaptations to life in the dark. Here we combine histological and transcriptome analyses to characterize sensory adaptations of *P. cisternarum* to the subterranean environment. Histological analysis showed that the vestigial eyes of *P. cisternarum* contain a rudimentary lens. Transcriptome analysis revealed a repertoire of eleven visual and non-visual opsins and the expression of 36 genes involved in lens development and maintenance. In contrast to other cavefish species, such as *Astyanax mexicanus, Phreatichthys andruzzii, Sinocyclocheilus anophthalmus* and *Sinocyclocheilus microphthalmus,* DASPEI neuromast staining patterns did not show an increase in the number of sensory hair cells. Our work reveals unique adaptations in the visual system of *P. cisternarum* to underground habitats and helps to shed light into troglomorphic attributes of subterranean animals.

KEY WORDS: *opsin, blind catfish, neuromast, sensory system*

Introduction

Hypogean species are characterized by the reduction or loss of eyes and pigments, accompanied by enhancement of other sensory structures (Jeffery, 2009; Mohun *et al*., 2010; Partha *et al*., 2017; Protas and Jeffery, 2012). In cave-adapted species, eye degeneration appears to be controlled by at least three different genetic programs. In the blind cavefish *Astyanax mexicanus*, degeneration is triggered by, among other mechanisms, *shh* overexpression and subsequent induction of lens cells apoptosis (Yamamoto *et al*., 2004). Recent studies have shown that the retinal pigment epithelium (RPE) is critical for eye maintenance (Ma *et al*., 2020). In the blind cave-dwelling fish *Phreatichthys andruzzii*, eye degeneration is controlled by the apoptosis of retinal neurons, whereas in the eyeless golden-line fish *Sinocyclocheilus anophthalmus,* transcriptome analysis showed that retinal degeneration is associated with downregulation of transcription factors controlling opsin expression.

Surface fishes can detect moving and vibrating water objects using the mechanosensory organs, the neuromasts. These units are distributed alongside the lateral line and are also observed in the head, where they compose the cephalic lateral line (Suppl. Fig S2) (Soares and Niemiller, 2018). Notably, to offset the loss of vision, other sensory functions have been enhanced in cavefish

Abbreviations used in this paper: bp, base pairs; DASPEI, 2-[4-(di-methylamino)styryl]-N-ethylpyridinium iodide; RPE, retinal pigment epithelium.

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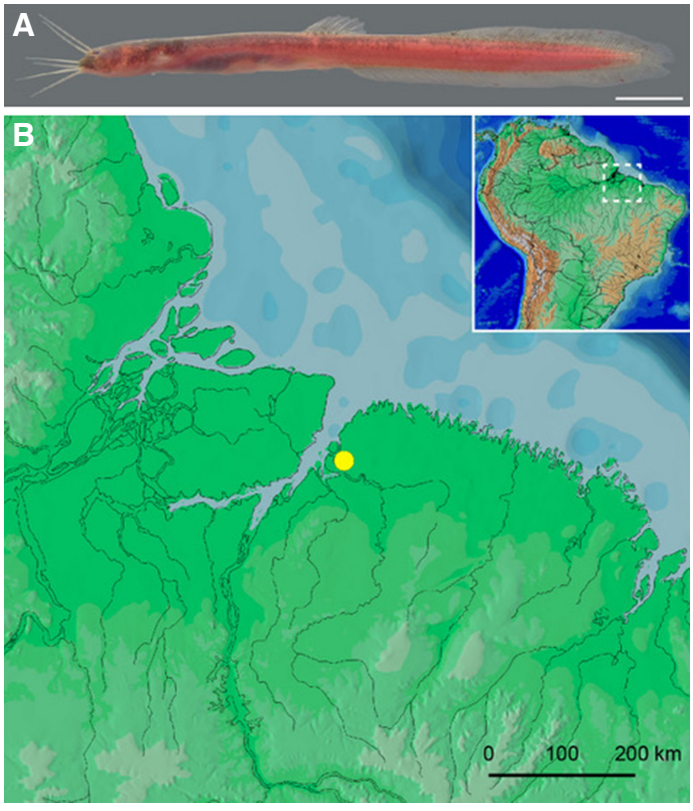

Fig. 1. *Phreatobius cisternarum* characterization. (A) *A* P. cisternarum *adult specimen with miniaturization body, a slender head, and six long thin barbels.* **(B)** *Map showing collecting site of* P. cisternarum *in Benevides-Para-Brazil (the yellow circle indicates Benevides city). Scale bar: 0.5 cm.*

species, including those involving neuromasts, barbels, and taste buds (Meng *et al*., 2013; Stemmer *et al*., 2015).

The genus *Phreatobius* (Siluriformes) includes three species: *P. sanguijuela, P. dracunculus* and *P. cisternarum* (Fernandez *et al*., 2007; Muriel-Cunha and Pinna, 2005; Shibatta *et al*., 2007; Trajano, 2007). These species occur in the Amazon and Paraguay river basins, however, reports regarding their biology, reproductive cycle, ecology, and physiology remain scarce (Fernandez *et al*., 2007; Muriel-Cunha and Pinna, 2005; Ohara *et al*., 2016). *Phreatobius cisternarum* inhabits the superficial phreatic layer, which corresponds to the upper saturated stratum of soil found in hand-dug wells. This species is characterized by small eyes and reduction in both body size and pigmentation (Fig. 1 A,B, Suppl. Fig. S1 A,B). Besides the six protruding barbels characteristic of catfishes, *P. cisternarum* has a paddle-shaped caudal region formed by a caudal fin extended dorsally and ventrally by numerous large procurrent rays, ventrally confluent with the anal fin, a strongly prognathous lower jaw, hypertrophied jaw muscles, and a bright red coloration (Shibatta *et al*., 2007).

Opsins are a diverse group of G-protein-coupled receptors (GPCRs) broadly categorized as 'visual' or 'non-visual'. The best known opsins are visual opsins found in the rod and cone photoreceptor cells of the retina. These opsins detect light when their ligand, a chromophore called 11-*cis* retinal, undergoes photoisomerization. Fish are notable in having large visual and non-visual opsin repertoires compared to other vertebrates (Beaudry *et al*., 2017; Lin *et al*., 2017; Rennison *et al*., 2012). For example, the four-eyed fish, *Anableps anableps*, and the distantly related zebrafish (*Danio rerio*), have ten visual opsins (Davies *et al*., 2015; Owens *et al*., 2009). Zebrafish also have 32 non-visual opsins (Davies *et al*., 2015). It is clear that such a large repertoire is not essential for vision or circadian rhythm regulation, as other animals have much smaller repertoires (Scholtyssek and Kelber, 2017). By surveying the transcriptome of *P. cisternarum*, we characterized the molecular as well as the morphological response of the visual system to life in perpetual darkness. Furthermore, we used a set of histological analysis, immunofluorescence, and neuromast staining approaches to study adaptations of the sensory system in this subterranean species.

Results and Discussion

Opsin repertoire in P. cisternarum

The transcriptome assembly process generated 1,350,426 contigs with median length of 329 bp (N50 = 668 bp) (Suppl. Tables S1 and S2). Our BLAST survey of this assembly using *D. rerio* query sequences uncovered 11 *P. cisternarum* opsins: three visual opsins (*rh1.1*, *rh1.2,* and *lws*), and eight non-visual opsins (*exorh*, *va1*, *va2*, *parietopsin*, *tmt3a*, *opn4m3*, *peropsin*, and *rgr1*).

Twenty-three opsins were identified in *Ictalurus punctatus*, and no additional *P. cisternarum* genes were detected when *I. punctatus* opsins were employed as query sequences. The entire *P. cisternarum rh1.1* gene (a single-exon gene) was contained in one contig. The other ten *P. cisternarum* opsins were incomplete; each was represented by one or more contigs that included between 44 bp and 747 bp of coding sequence that could be aligned to all other opsins. Despite this length variation, *P. cisternarum* opsins were placed into well-supported clades with *I. punctatus* orthologs (Fig. 2, Suppl. File S1).

The observations that only *rh1.1* was full-length and that introns were present in all *P. cisternarum* opsins other than *rh1.1* and *rh1.2* suggest that most transcripts are not functional in the specimen studied. However, it is possible that intron retention (IR) is a form of translational regulation providing the potential to generate opsin proteins rapidly in response to light stimulation by splicing pre-existing transcripts. Another possibility is that opsin IR in *P. cisternarum* influences eye development by generating opsin mRNAs with premature termination codons (PTCs). Opsin

TABLE 1

LENS GENES EXPRESSED IN THE SURFACE POPULATION OF *ASTYANAX MEXICANUS* AND RNASEQ FROM THE HEAD OF *PHREATOBIUS CISTERNARUM*

ID	Gene symbol	Gene name	TPM
TRINITY_DN133315_c2_g7_i1	Rom1	Retinal outer segment membrane protein 1	0,11575
TRINITY_DN145112_c3_g7_i3	Gnat1	Guanine nucleotide-binding protein (G protein), alpha transducing activity polypeptide 1	0,30194
TRINITY_DN138747_c6_g1_i2	Pde6	Phosphodiesterase 6G, cGMP-specific, rod	0,24273
TRINITY_DN139252_c5_g3_i1	Crygm1	Crystallin, gamma M1	0,13917
TRINITY_DN304038_c0_g1_i1	Crygm3	Crystallin, gamma M3	0,06959
TRINITY_DN131980_c0_g1_i2	Lhx1b	LIM class homeodomain protein	0,21923
TRINITY_DN146039_c1_g1_i1	Arr3a	Arrestin 3, retinal (X-arrestin), like	0,08267
TRINITY_DN143563_c2_g1_i9	Atp6ap1	ATPase, H+ transporting, lysosomal accessory protein 1	1,63144

loss can lead to retinal degeneration in humans (Silverman *et al.*, 2020), thus nonsense-mediated decay (NMD) of intron-containing *P. cisternarum* opsin transcripts might play a role in eye degeneration.

P. cisternarum retains a rudimentary lens and shows reduced hair cells

Our histological analysis showed that the small eye of *P. cisternarum* is composed of an RPE and a rudimentary lens, we did not observe the cornea or sclera and the retina layers were absent in all specimens analyzed in this study. The lack of photoreceptor cells is interesting considering that we found opsin expression in the transcriptome. In addition to the histological analysis of RPE, we found RPE transcripts encoding for proteins such as retinoid isomer hydrolase (RPE65), GTP cyclohydrolase 1-like, L-dopachrome tautomerase, RPE-retinal G protein-coupled receptor-like, among others, in the transcriptome (Fig. 3A, Suppl. Table S3). Immunostaining revealed that among the lens-specific genes identified in our transcriptome, gamma-crystallin, a typical lens protein, was mainly expressed in border of the lens (Fig. 3B). In addition, our transcriptome analysis showed the expression of 36 genes that are critical for lens development and maintenance. This suggests that *P. cisternarum* retained a rudimentary lens, and that ocular reduction might be due to lower levels of expression of these genes, however, to test this hypothesis, we would need to perform additional gene expression analysis (Suppl. Table S4) (Alunni *et al.*, 2007; Atukorala and Franz-Odendaal, 2018; Cavalheiro *et al.*, 2017; Hooven *et al.*, 2004; Yamamoto *et al.*, 2004).

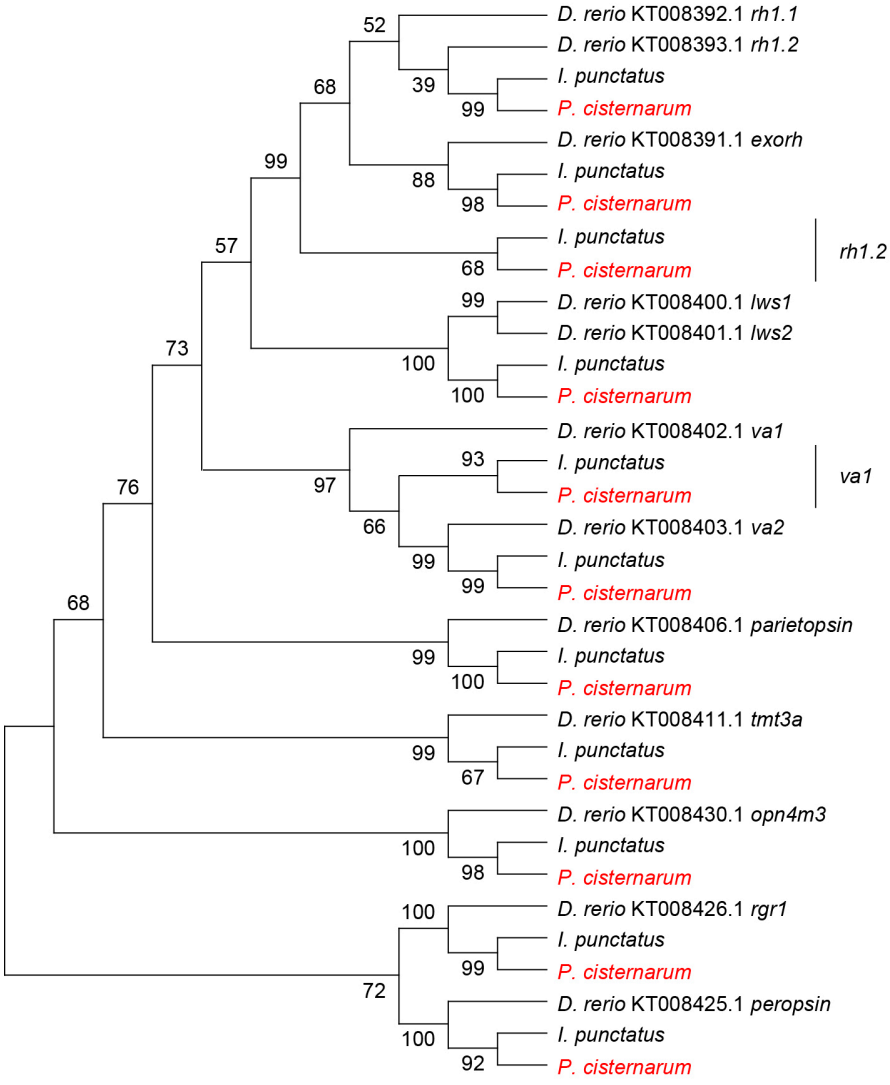

Fig. 2. Phylogenetic tree showing the visual and non-visual opsins *of Phreatobius cisternarum* (in red), *Danio rerio* and *Ictalurus punctatus*. *Maximum parsimony tree showing relationships among* Phreatobius cisternarum, Ictalurus punctatus *and* Danio rerio *opsins. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. Sequences were truncated at the 5′ and 3′ ends as these regions could not be aligned for all opsins with confidence.* rh1.2 *and* va1 *sequences from* P. cisternarum *clustered with orthologs from* I. punctatus, *but for these two genes the truncated alignment (Table S1) contained insufficient data to show their respective relationships to* D. rerio *orthologs. Monophyly of* rh1.2 *and* va1 *clades was wellsupported in analyses of longer subfamily-only alignments (not shown).* Danio rerio *has 42 opsins (Davies* et al.*, 2015) and* I. punctatus *has 23 opsins. All were included in a preliminary analysis but only orthologs of* P. cisternarum *opsins were included in these analysis.*

Gross *et al.*, have shown previously that a set of genes important for lens maintenance and development is exclusively expressed in *A. mexicanus* surface population (lost in cave population). Comparative analysis of *A. mexicanus* natural populations identified 16 genes exclusively expressed in surface, but not cave populations (Gross *et al.*, 2013). Among those, we found 8 genes (*Rom1*, *Gnat1*, *Pde6*, *Crygm1*, *Crygm3*, *Lhx1b*, *Arr3a* and *Atp6ap1*) in the transcriptome of *P. cisternarum* (Table 1, Suppl. Files S2 - S9). These results suggest that the genetic mechanism of ocular reduction in *P. cisternarum* may be different from that described for *A. mexicanus.*

In cavefish, an increase in the number and size of neuromasts is one of the mechanisms used to offset eye degeneration (Soares and Niemiller, 2018; Van Trump *et al.*, 2010). Our results showed the presence of hair cells along the reduced lateral line and on the head, however, not in abundance, as previously reported for *A. mexicanus* (Van Trump *et al.*, 2010) (Fig. 3C,D). The lack of supernumerary neuromasts in *P. cisternarum* could be linked to low water currents in these confined water streams of phreatic environments.

Conclusions

Previous studies in cavefish have uncovered genetic and developmental mechanisms underlying eye degeneration in dark-adapted fish. Here, we present evidence that eye degeneration in *P. cisternarum* might occur via mechanisms distinct from those described for cavefish species. Histological and molecular

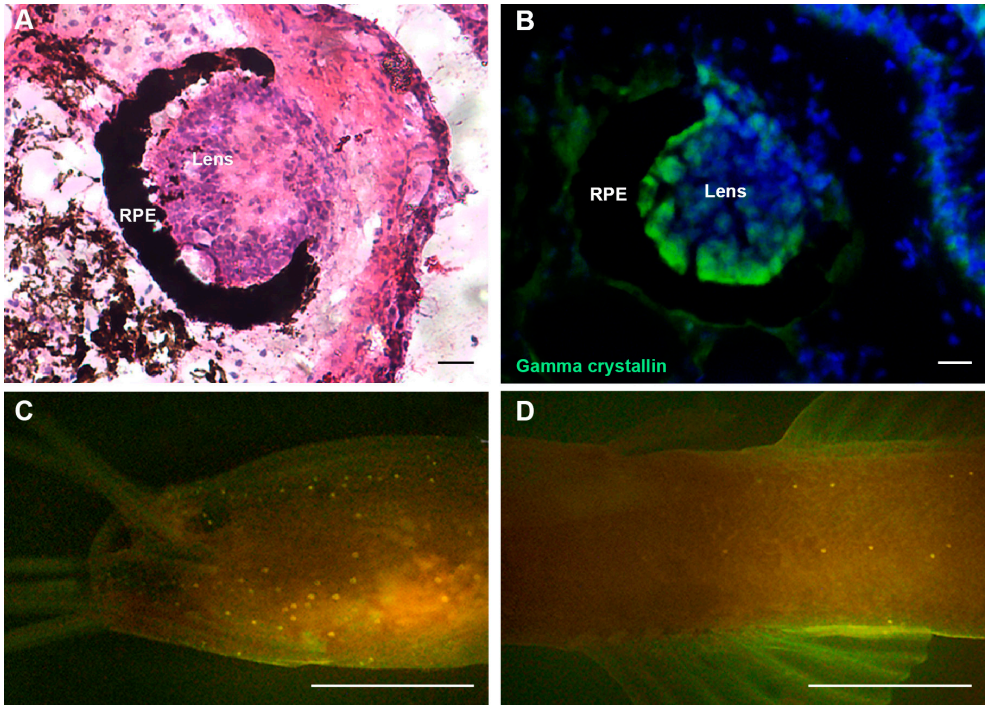

Fig. 3. *Phreatobius cisternarum* remaining eye structures. *Hematoxylin and eosin staining of head sections of* P. cisternarum *showing a retinal pigment epithelium (RPE) and lens* **(A)**. *Immunofluorescence assay showing gamma crystallin expression on the lens (green) and DNA staining with DAPI (blue)* **(B)**. *The distribution of superficial neuromasts (yellow dots) on the anterior lateral line (head)* **(C)** *and posterior lateral line (trunk)* **(D)** *of* P. cisternarum *specimens with DASPEI staining. Cryosections are 20 μm thick. Scale bars: 200 μm (A,B) and 2 mm (C,D).*

analyses in adult fish showed that *P. cisternarum* maintains eye structures such as RPE and a small lens. Our RNA-seq analyses revealed the visual and non-visual opsin repertoire in *P. cisternarum*, we identified a set of transcripts implicated in the development and maintenance of the RPE, retina as well as lens. Finally, adaptation to a subterranean environment in *P. cisternarum* appears to have occurred without concomitant enhancement of sensory neuromasts. In sum, our results provide the groundwork for future studies aimed at identifying the genetic and developmental underpinnings of eye degeneration in *P. cisternarum* and dark-adapted species in general.

Material and Methods

Specimen collection

Fifteen *P. cisternarum* adults, ranging from 30 mm to 58 mm, were collected from artificial wells in Benevides (Pará, Brazil). In addition to the light protected tanks, the light in the room was controlled to keep the fish in the perpetual darkness. During daily feeding, tank protection was removed and lights were kept off. Three specimens of *Poecilia reticulata* were obtained in the pet trade and were maintained in individual tanks in a recirculating freshwater system at 24 to 28 °C with aeration at the Genomics and Systems Biology Center. Experiments and animal care were performed following animal care guidelines approved by the Animal Care Committee at the Universidade Federal do Pará (protocol no. 037-2015).

Library preparation and Illumina sequencing

One specimen of *P. cisternarum* was anesthetized in 0.1% tricaine solution (Sigma-Aldrich). Total RNA was extracted from its head using TRIzol® (Life Technologies), following manufacturer's protocol. Paired-end 150bp libraries were generated with NEXTSeq Mid Output, according to standard protocol (Illumina) and sequenced on Illumina NEXTSeq 500 platform (NCBI Sequence Read Archive project: PRJNA491408 and run: SRR7878036).

De novo transcriptome assembly and gene annotation

Over 190 million raw sequence reads were obtained. Trimmomatic was used with default parameters to remove adapters (Bolger *et al.*, 2014). *De novo* transcriptome assembly was performed using standard parameters in Trinity (Grabherr *et al.*, 2011). The completeness of the assembled transcripts was assessed through the Benchmarking Universal Single-Copy Orthologs tool (BUSCO) (Seppey *et al.*, 2019). Mapping was performed by means of Bowtie (Langmead and Salzberg, 2012) using the constructed transcriptome as a reference. StringTie (Pertea *et al.*, 2015) was used to estimate the abundance of transcripts in Transcripts Per Million (TPM). The identification of lens and RPE genes was performed via tBLASTn search tool, and each contig of interest was searched with manual queries of the consensus sequences from Uniprot (Priyam *et al.*, 2019), and Expasy Translate predicted Open Reading Frames (ORFs) (Gasteiger *et al.*, 2005). BLASTp (NCBI) was performed to confirm protein homology.

Characterization of visual and non-visual opsins

Forty-two zebrafish *(Danio rerio)* opsins (Davies *et al.*, 2015) were used as query sequences in a tBLASTx survey of the *P. cisternarum* transcriptome (E-value of 10^{-6}). *D. rerio* opsins were also used to survey the transcriptome of another species in the order Siluriformes, the channel catfish (*Ictalurus punctatus* (accession GCF_001660625.1_IpCoco_1.2). Opsins from *P. cisternarum*, *I. punctatus* and the 42 *D. rerio* opsins were aligned using ClustalW in BioEdit (Hall, 1999; Thompson *et al.*, 1994). Introns in the *P. cisternarum* opsin transcripts that were obvious in this alignment were removed; precise intron-exon boundaries could be identified by comparing the *P. cisternarum* sequences to *D. rerio* orthologs on the NCBI Graphical Sequence Viewer. The alignment was then truncated at the 5' and 3' ends because the degree of sequence divergence in these regions made alignments (even when translated into amino acids sequences) unreliable. Maximum Parsimony (MP) phylogenetic trees were reconstructed using MegaX version 10.1.8 (Stecher *et al.*, 2020) and confidence in the topology was evaluated by Bootstrap re-analyses.

DASPEI staining

The fluorescent dye 2-[4-(di-methylamino)styryl]-N-ethylpyridinium iodide (DASPEI; Invitrogen) was used as a vital dye to stain hair cells within neuromasts. Three *P. cisternarum* and three *P. reticulata* specimens were incubated in an embryo medium containing 0.005% DASPEI for 15 min, anesthetized in 0.1% tricaine solution (Sigma-Aldrich) for 5 minutes, and rinsed once in fresh embryo medium according to a previously established protocol (Yoshizawa *et al.*, 2010). The specimens were analyzed and

photographed with a NIKON-Eclipse 80i fluorescence microscope using the NIS-Elements imaging software.

Histological analysis

Twelve specimens of *P. cisternarum* were anesthetized in 0.1% tricaine solution (Sigma-Aldrich). The heads were collected and flash-frozen in TissueTek embedding medium (Sakura). Cryosections (20 μm) were obtained on a Leica CM1850 UV cryostat (Leica Biosystems), captured on Color Frost Plus microscope slides (Thermo Fisher Scientific), fixed in 3% paraformaldehyde, and stored at -80°C for further use. Slides were stained with hematoxylin and eosin following standard protocol (Kiernan, 2008).

Immunostaining

Slides were incubated with gamma-crystallin antibody (a gift from Dr. Martins) overnight at 4°C following previously established protocol (Cavalheiro *et al*., 2014). Immunofluorescence reaction was performed with an anti-rabbit secondary antibody (1:200, Sigma-Aldrich, cat# F6005), incubated for 2 hours at room temperature. Fluorescence nuclear counterstaining was performed with DAPI (Sigma-Aldrich, cat# F6057). Images were captured with a NIKONEclipse 80i fluorescence microscope and NIS-Elements imaging software.

Ethics

This study was approved by IBAMA/SISBIO under license number 66015-1 and by the Ethics Committee for Animal Research at the Universidade Federal do Pará (protocol number 037-2015).

Data accessibility

Sequence data have been deposited in GenBank with the following BioProject accession number: PRJNA491408 and SRA file: SRR7878036.

Authors' contributions

PNS conceived the ideas and experimental design. LNP, BRM and JL collected the data; LNP, JST, RAPM and PNS analyzed the data; MPS and JT performed the transcriptome assembly, JT and AL performed opsin analysis; PNS, BRM and LNP led the writing of the manuscript. All authors contributed critically to the drafts, gave final approval for publication and agree to be held accountable for the work performed therein.

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Conflict of Interest statement. None declared.

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