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RESEARCH

Museomic analyses clarify species diversity in the icefish genus *Channichthys*

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Abstract

The rapid diversification of notothenioid fishes in Antarctic waters is a prime example of the process of adaptive radiation. Within around 10 million years, Antarctic notothenioids have diversified into over 100 species with a broad range of lifestyles and ecological adaptations. However, the exact number of species within this radiation has long been unclear. Particularly challenging is the taxonomy of the genus *Channichthys*, for which between one and nine species have been recognized by different authors. The putative species of this genus are known from a limited number of specimens, of which most were sampled decades ago. Here, we investigated the mitochondrial genomes of museum specimens representing the four species Unicorn Icefish (*C. rhinoceratus*), Red Icefish (*C. rugosus*), Sailfish Pike (*C. velifer*), and Charcoal Icefish (*C. panticapaei*), complemented by morphological analyses. All analyzed specimens were collected in the 1960s and 1970s and fixed in formaldehyde, and their DNA has been heavily degraded. Applying ancient-DNA protocols for DNA extraction and single-stranded library preparation, we were able to obtain sufficient endogenous DNA to reconstruct the mitochondrial genomes of one specimen per species. These mitochondrial genome sequences were nearly identical for the three specimens assigned to Unicorn Icefish, Red Icefish, and Sailfish Pike, while greater divergence was observed for the Charcoal Icefish specimens. We discuss possible explanations of the contrast between these molecular results and the recognizable morphological variation found among the four species, and recommend that at least the Charcoal Icefish be included in the list of valid notothenioid species.

Keywords Icefish · Antarctica · Species richness · Taxonomy · Museomics · Mitochondrial genome

Introduction

Examples of the process of adaptive radiation—the rapid proliferation of species diversity as the result of ecological opportunity (Simpson 1953; Schluter 2000)—are known almost exclusively from terrestrial and freshwater environments (Grant and Grant 2008; Losos 2009; Lerner et al. 2011; Ronco et al. 2021). One remarkable example of this process in the marine system, however, is found in the freezing waters of Antarctica. Here, fishes of the suborder Notothenioidei—the Antarctic members of which are collectively termed "Cryonotothenioidea" (Near et al. 2015)—have diversified into over 100 species (Eastman and Eakin 2021), characterized by an incomparable array of

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phenotypes and lifestyles (Clarke and Johnston 1996; Eastman 2005; Near et al. 2012; Matschiner et al. 2015; Near et al. 2018; Bista et al. 2023).

The diversity of cryonotothenioids is classified into five families, of which icefishes (family Channichthyidae) are arguably the most iconic. Not only are these fishes the only known vertebrates to lack hemoglobin (Ruud 1954; Eastman 1993; Desvignes et al. 2023), they also carry numerous adaptations, such as antifreeze glycoproteins and enlarged hearts and gills (DeVries and Wohlschlag 1969; Tuta et al. 1991; Rankin and Tuurala 1998; Sidell and O'Brien 2006), making them well suited for the highly oxygenated, freezing waters of the Southern Ocean. Along with their range of adaptations, the family of icefishes has evolved very recently, with genome-based age estimates placing their origin within the past 5 million years (Bista et al. 2023). Despite their young age, they today include at least 16 species classified into eleven genera (Eastman and Eakin 2021).

The numbers of species within each of the eleven genera of the family Channichthyidae are in most cases uncontroversial. One genus, however, has been a topic of discussion for years—the genus *Channichthys*, distributed exclusively around the sub-Antarctic Kerguelen and Heard Islands (Duhamel et al. 2005). First described in 1844 by Richardson , the genus has expanded from being monotypic to including as many as nine classified species (Ahyong et al. 2024; Froese and Pauly 2024). These are the Unicorn Icefish (*Channichthys rhinoceratus* Richardson, 1844), the Red Icefish (*C. rugosus* Regan, 1913), the Sailfish Pike (*C. velifer* Meisner, 1974), the Aelita Icefish (*C. aelitae* Shandikov, 1995b), the Big-eyed Icefish (*C. bospori* Shandikov, 1995b), the Pygmy Icefish (*C. irinae* Shandikov, 1995b), the Charcoal Icefish (*C. panticapaei* Shandikov, 1995a), the Green Icefish (*C. mithridatis* Shandikov, 2008), and the Robust Icefish (*C. richardsoni* Shandikov, 2011). However, in recent years, these classifications have become subject to scrutiny due to new morphological analyses (Nikolaeva 2019, 2020). These analyses led to the synonymization of several of the nine species, leaving only the Unicorn Icefish (*C. rhinoceratus*), the Red Icefish (*C. rugosus*), the Sailfish Pike (*C. velifer*), and the Charcoal Icefish (*C. panticapaei*) listed in Eschmeyer's Catalog of Fishes (Fricke et al. 2024). These four species were found to be distinguishable by phenotypic traits such as their gill rakers, coloration, fin height, fin-ray count, and head and snout shape (Nikolaeva and Balushkin 2019; Nikolaeva 2019, 2020, 2021, 2024).

Genetic analyses corroborating this classification have long been lacking, due to the difficulty of obtaining fresh samples from the Kerguelen-Heard Plateau and the technical challenges of extracting suitable DNA from older, museum specimens. A particular obstacle to molecular analyses of museum specimens has been that such specimens were commonly fixed in formaldehyde, a substance that breaks and alters DNA strands (Straube et al. 2021a). Thus, it was only with the development of ancient-DNA protocols facilitating the extraction and sequencing of DNA from museum specimens (Gansauge et al. 2017, 2020; Straube et al. 2021a), that the first molecular insights were gained into the classification of *Channichthys* icefishes. In 2022, Muschick et al. were able to isolate and sequence sufficient DNA from a formaldehyde-fixed specimen of the Red Icefish (*C. rugosus*) that allowed the assembly of nearly its entire mitochondrial genome (NCBI accession PP319402.1). The comparison of this mitochondrial genome with one that was publicly available for the Unicorn Icefish (*C. rhinoceratus*; NCBI accession NC_057120) revealed great similarity between these two mitochondrial genomes, leading the authors to suggest that the two species might in fact be one and the same (Muschick et al. 2022).

Here, we expand on the investigation by Muschick et al. (2022) with additional museum specimens, representing all of the four *Channichthys* species accepted as valid by Nikolaeva (2020). We apply ancient-DNA protocols to tissue samples of these specimens to extract DNA for molecular analyses based on Illumina sequencing, and complement these analyses with a new morphological assessment of species differences. Of twelve specimens sampled around the Kerguelen Islands in the late 1960s and early 1970s, one of each species yielded sufficient suitable DNA to reconstruct its mitochondrial genome sequence. Our molecular analyses showed that not only the Unicorn Icefish (*C. rhinoceratus*) and the Red Icefish (*C. rugosus*) specimens have nearly identical mitochondrial genomes, but that this is also the case for the Sailfish Pike (*C. velifer*) despite its rather clear phenotypic differentiation. In contrast, the mitochondrial genome of the Charcoal Icefish (*C. panticapaei*) specimen was more distinct, with a degree of separation comparable to that found in a second pair of icefish congeners. We speculate that these results may indicate a smaller number of *Channichthys* species than previously assumed, that inter-specific hybridization might have led to exchange of mitochondrial genomes, or that the genus might be in the process of a new and rapid radiation on the Kerguelen-Heard Plateau.

Materials and methods

Sampling

Twelve *Channichthys* icefish specimens were selected for molecular analysis. All of these are part of the collection of the Zoological Institute of the Russian Academy of Sciences in Saint Petersburg, Russia, and were collected during cruises by Soviet and—in one case (ZIN 53007)—French scientists in the late 1960s and early 1970s. All specimens were fixed in formaldehyde after capture and eventually

transferred to 70% ethanol. They were assigned to the four species *C. rhinoceratus*, *C. rugosus*, *C. velifer*, and *C. panticapaei* following the updated identification key of Nikolaeva (2020) (Table 1).

DNA extraction and sequencing

DNA extraction, library preparation, and quality checks (qPCR, concentration measurement, automated electrophoresis) were performed at the molecular laboratory of the University Museum of Bergen. As far as possible, all laboratory procedures followed recommendations detailed in Fulton and Shapiro (2019), such as UV-C irradiation, strict decontamination protocols, and positive pressure HEPA filtration.

For all twelve specimens, DNA was extracted from muscle tissue (between 28 and 54 mg dry weight per sample), applying the same treatment to all samples, as well as two extraction negatives. DNA extraction followed the guanidine treatment described in Straube et al. (Appendix S1; 2021a) which is based on and modified from Sambrook and Russell (2001), Rohland and Hofreiter (2007a), Rohland et al. (2010), Dabney et al. (2013), Basler et al. (2017a, b, 2018). Before amplification and simultaneous dual indexing of the single-stranded libraries, qPCR was used to determine the ideal individual number of amplification cycles. Final concentrations were measured in a Qubit dsDNA High-Sensitivity assay (Thermo Fisher Scientific), and fragment length distributions and maximum peak sizes were assessed with Bioanalyzer High Sensitivity DNA Analysis (Agilent). All libraries were used in shallow shotgun sequencing for initial data generation to check for the presence of target DNA (see "Molecular analyses"). Initial sequencing was performed on an Illumina MiniSeq system at the University Museum of Bergen. Deeper sequencing for specimens identified to contain sufficient target DNA (ZIN 56275, ZIN 56538, and ZIN 56638) was performed on the Illumina NextSeq system at the University of Potsdam (Adaptive Genomics Group). Additional sequencing efforts were guided by initial mapping experiments to a mitochondrial reference genome (see "Molecular analyses"). The numbers of reads mapping to this genome were then used to estimate the additional amount of mitochondrial reads necessary to allow for reconstructing the mitochondrial genome (see Straube et al. 2021b).

All sequencing used Illumina single-end high output kits following Paijmans et al. (2017). For those samples for which additional sequencing was performed, the resulting sets of reads were combined. Similarly, for the specimen that had previously been sequenced in Muschick et al. (2022) (ZIN 56294), new sequencing data generated herein were merged with those generated previously. Illumina's bcl2fastq2 tool (https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html) was used for demultiplexing.

Molecular analyses

Sequence reads were processed with fastp v.0.23.2 (Chen et al. 2018) to remove adapter sequences, the first base of the tail of forward reads, bases with Phred-scaled quality below 25 at both ends of reads, and bases in poly-G regions with a length greater than 10 bp. We then excluded reads if they carried more than 10 undetermined bases, if they were shorter than 20 bp, or if more than 40% of bases had a Phred-scaled quality below 20.

To verify the presence of endogenous DNA and estimate its quantity, we assigned each sequence read to a taxonomic group by mapping to the NCBI non-redundant (NR) sequence database (downloaded on 21 November 2023). This assignment used the BLASTX search

Table 1 *Channichthys* icefish specimens analyzed

Specimen	Species	Sampling date	Sampling location	Depth
ZIN 56635	*C. rhinoceratus*	16 Mar 1969	NA	280 m
ZIN 56636	*C. rhinoceratus*	13 Jan 1970	NA	NA
ZIN 56638	*C. rhinoceratus*	24 Dec 1970	48° 04′ S, 70° 15′ E	145–150 m
ZIN 53007	*C. rugosus*	Jan 1964	NA	NA
ZIN 56294	*C. rugosus*	28 Jun 1974	48° 34′ S, 70° 37′ E	115–120 m
ZIN 56292	*C. rugosus*	1967	NA	NA
ZIN 56273	*C. velifer*	26 Jan 1970	NA	NA
ZIN 56274	*C. velifer*	20 Feb 1970	NA	NA
ZIN 56275	*C. velifer*	20 Feb 1970	48° 58′ S, 67° 27′ E	195–207 m
ZIN 56536	*C. panticapaei*	24 Feb 1969	NA	NA
ZIN 56537	*C. panticapaei*	16 Mar 1969	NA	280 m
ZIN 56538	*C. panticapaei*	26 Dec 1970	48° 00′ S, 70° 32′ E	148 m

Specimen ZIN 56294 was previously used for molecular analyses in Muschick et al. (2022)

NA not available

algorithm as implemented in the program Diamond v.2.1.6 (Altschul et al. 1990; Buchfink et al. 2015). The frequencies of different taxon assignments were then plotted with MEGAN v.6.25.3 (Huson et al. 2016).

To identify endogenous mitochondrial reads, we prepared a chimeric reference sequence from the mitochondrial genome of *Channichthys rhinoceratus* (NCBI accession NC_057120) and the nuclear genome of the closely related species *Pseudochaenichthys georgianus* (EBI accession GCF_902827115.1) (Bista et al. 2023). We mapped reads to this reference using the Paleomix (v.1.3.8) BAM pipeline (Schubert et al. 2014) with BWA MEM (Li and Durbin 2010) selected as aligner software. The pipeline included the application of the program AdapterRemoval (Schubert et al. 2016), excluding reads with a minimum length 25 bp or a mismatch rate greater than 3. Also as part of the Paleomix pipeline, we invoked the program mapDamage (Jónsson et al. 2013) to assess DNA degradation. The resulting files in BAM format were used for variant calling with GATK's Germline Short Variant Discovery (v.4.5.0.0) pipeline (McKenna et al. 2010), a method suitable for the identification of variants from haploid data such as mitochondrial sequences. We focused on the four specimens that yielded most mitochondrial reads per species (ZIN 56638, ZIN 56294, ZIN 56275, and ZIN 56538) in this and the subsequent analyses. Following variant calling, the resulting variant dataset was filtered using bcftools v.1.18 (Li 2011), considering only variants with Phred-scaled quality score above 30 and a read depth greater than 3. We further verified the identified mitochondrial variants through manual inspection with the Integrative Genome Viewer v.2.18.2 (Robinson et al. 2011), confirming that the single nucleotide polymorphisms (SNPs) identified were not artifacts from post mortem DNA damage (e.g., variants near the ends of individual reads). Based on the confirmed variant calls and the mitochondrial reference sequence, we reconstructed the full mitochondrial genome sequences for the four focal specimens, ensuring that sites that had insufficient coverage for variant calling were coded as missing data. We then annotated the mitochondrial genome sequences with the MitoAnnotator pipeline (Zhu et al. 2023).

For phylogenetic analyses, we generated an alignment with MAFFT v.7.520 (Katoh and Standley 2013) from the four reconstructed mitochondrial genome sequences, together with the available reference sequence for *C. rhinoceratus* (NC_057120). Based on this alignment, we calculated the pairwise genetic distances among all five sequences, and performed maximum-likelihood phylogenetic inference with IQ-TREE v.2.2.2.7 (Minh et al. 2020). This inference included IQ-TREE's substitution model selection and the application of 1000 ultrafast bootstrap replicates (Minh et al. 2013).

To embed the *Channichthys* sequences within the broader context of notothenioid diversification, we downloaded mitochondrial genome sequences for 34 additional notothenioid species (Online Resource 1; e.g. Papetti et al. 2021). We extracted the sequences of the 13 mitochondrial protein-coding genes from these genomes as well as from the five *Channichthys* genomes. For each of these genes, we then aligned all 39 sequences, with MAFFT. From these alignments, we extracted all first, second, and third codon positions, and concatenated these into three codon-specific partitions. The mitochondrial phylogeny of the included notothenioid species was then reconstructed in a partitioned maximum-likelihood analysis with IQ-TREE that otherwise had the same settings as before.

Finally, we aimed to compare sequences of individual regions from the four reconstructed *Channichthys* mitochondrial genomes to publicly available sequences of orthologous regions, isolated from other specimens of the genus. The only mitochondrial marker with sufficient available sequences, however, was cytochrome c oxidase I (*mt-co1*), for which twelve *C. rhinoceratus* sequences were available (NCBI accessions JN640603–JN640614; Smith et al. 2012). We downloaded these twelve sequences and aligned these to those of the four reconstructed genomes and the reference, again using MAFFT. From this alignment, a haplotype genealogy graph was produced with the program Hapsolutely v.0.2.2 (Vences et al. 2024), selecting maximum parsimony as the method for tree inference and Fitchi (Matschiner 2016) for the graph reconstruction.

Morphological analyses

We complemented the molecular analyses of the four *Channichthys* specimens ZIN 56638 (*C. rhinoceratus*), ZIN 56294 (*C. rugosus*), ZIN 56275 (*C. velifer*), and ZIN 56538 (*C. panticapaei*) with a detailed assessment of morphological variation within the genus. This assessment focused on the four above-named specimens, but also incorporated a broad set of further specimens from the Zoological Institute of the Russian Academy of Sciences, Saint-Petersburg (ZIN), the National Museum of Natural History, National Academy of Sciences of Ukraine, Kyiv (IZANU), and the Natural History Museum, London (BMNH). A total of 280 specimens were studied, including the holotypes of *Channichthys rugosus* and *C. panticapaei*.

- *Channichthys rhinoceratus*: 160 specimens with *SL* from 150 to 395 mm, sampled around Kerguelen Island at known depths from 140 to 420 m. These specimens included ZIN 53006, ZIN 55586, ZIN 55587, and ZIN 56631–56644. For more details, see Nikolaeva (2020).
- *Channichthys rugosus*: 10 specimens with *SL* from 215 to 370 mm, sampled around Kerguelen Island at known

depths from 64 to 120 m. These specimens included ZIN 53007 and ZIN 56291–56994, as well as the holotype BMNH 1876.3.23.4 with *SL* 370 mm. For more details, see Nikolaeva (2021).

- *Channichthys velifer*: 45 specimens with *SL* from 134 to 490 mm, sampled around Kerguelen Island at known depths from 130 to 157 m. These specimens included ZIN 53005, ZIN 54807, ZIN 56271, and ZIN 56273–56290. For more details, see Nikolaeva and Balushkin (2019).
- *Channichthys panticapaei*: 61 specimens with *SL* from 138 to 398 mm, sampled around Kerguelen Island at known depths from 80 to 380 m. These specimens included ZIN 56520–56538 as well as the holotype IZANU 5109 with *SL* 348 mm. For more details, see Nikolaeva (2019).

The morphological analyses used a protocol developed and optimized for the study of *Channichthys* icefishes (Balushkin and Spodareva 2015; Nikolaeva and Balushkin 2019; Nikolaeva 2016, 2017, 2019, 2020, 2021). For each specimen, 50 different morphological characters and indices were recorded (10 morphometric characters, 19 meristic characters, and 21 indices; see section "Morphological analysis"). The features of the gill apparatus, seismosensory system, skin granulation (the degree of its development), and coloration were also examined. Additionally, the axial skeleton was studied using a digital microfocus X-ray diagnostic unit "PRDU-02" ("ELTECH-MED", Saint-Petersburg, Russia) in the Centre of Collective Use "TAXON" at the Zoological Institute of the Russian Academy of Sciences.

All measurements were made visually, using a caliper, or in the case of X-ray images, with the program PRDU, developed by the platform provider. All calculations were performed with Excel (Microsoft) and Statistica (TIBCO Software). Based on our morphological analysis, a revised diagnosis of the genus and the four species accepted as valid by Nikolaeva (2020) was developed.

Results

DNA extraction and sequencing

The application of ancient-DNA protocols to the formaldehyde-fixed museum specimens of the genus *Channichthys* led to mixed results across the twelve specimens. The extracts had DNA concentrations between 1.43 ng/µl (ZIN 56273) and values below the Qubit High Sensitivity kit detection threshold of 0.05 ng/µl (ZIN 56538) (Table 2). Due to the sample-specific amplification step during library preparation, these libraries were more homogeneous in their post-amplification DNA concentrations, ranging from 3.4 to 19.7 ng/µl. Maximum peak sizes in the fragment length distributions were detected between 153 and 175 bp.

Table 2 Summary of DNA extraction and Illumina MiniSeq sequencing results

Specimen	Species	Concentration (ng/µl)		MiniSeq read numbers	
		Extract	Library	Total	Mapping
ZIN 56635	*C. rhinoceratus*	0.20	19.7	1,150,880	0
ZIN 56636	*C. rhinoceratus*	0.42	12.5	1,496,964	0
ZIN 56638	*C. rhinoceratus*	0.80	4.6	1,701,466	131
ZIN 53007	*C. rugosus*	0.34	4.3	1,701,825	3
ZIN 56294	*C. rugosus*	1.10	3.6	1,753,908	109
ZIN 56292	*C. rugosus*	0.94	3.4	1,530,944	51
ZIN 56273	*C. velifer*	1.43	6.9	2,051,860	16
ZIN 56274	*C. velifer*	0.81	9.0	1,627,555	29
ZIN 56275	*C. velifer*	0.84	6.5	1,164,653	1079
ZIN 56536	*C. panticapaei*	0.22	4.7	1,208,445	0
ZIN 56537	*C. panticapaei*	0.36	11.8	915,238	1
ZIN 56538	*C. panticapaei*	NA[1]	5.6	2,212,835	646

Concentrations were measured in a Qubit dsDNA High-Sensitivity assay. The numbers of reads mapping refers to those that map to the mitochondrial genome (using NC_057120 as reference)

[1]Concentration too low for measurement with Qubit High-Sensitivity assay (notably, despite a comparatively large number of reads mapping to the mitochondrial genome)

Sequencing on the Illumina MiniSeq platform produced between 915,238 and 2,215,285 reads per specimen (Table 2). Initial mapping to the reference showed that only few of the libraries contained at least moderate numbers (> 100) of mitochondrial reads. Based on these results, the libraries for ZIN 56275, ZIN 56538, and ZIN 56638 were re-sequenced for additional data on the Illumina NextSeq platform. For the specimen that had previously been sequenced in Muschick et al. (2022) (ZIN 56294), new sequencing data generated herein were combined with those generated previously. Similarly, unpublished NexSeq sequencing data previously generated for specimen ZIN 56292 were also combined with the new MiniSeq data.

Assigning sequencing reads to taxonomic groups with Diamond and MEGAN revealed that most DNA libraries consisted primarily of non-endogenous DNA, dominated by bacterial (such as *Burkholderia*) sequences (Online Resource 2). Human contamination seemed to be minimal, as only three specimens (ZIN 53007, ZIN 56292, and ZIN 56275) had few reads assigned to Catarrhini (Old World monkeys). Out of the twelve specimens, four contained reads assigned to Notothenioidei, and for each of these four specimens, those reads were present in substantial proportions: ZIN 56638 (*C. rhinoceratus*), ZIN 56294 (*C. rugosus*), ZIN 56275 (*C. velifer*), and ZIN 56538 (*C. panticapaei*) (Figs. 1,

2). Thus, all further molecular analyses focused on these four specimens.

Molecular analysis

An assessment of DNA degradation with mapDamage showed elevated rates of C to T substitutions near the ends of reads, indicating deamination of cytosine residues to uracil. However, this deamination appeared to affect only the terminal 1–2 bp of each read (Online Resource 3). For the four focal specimens ZIN 56638 (*C. rhinoceratus*), ZIN 56294 (*C. rugosus*), ZIN 56275 (*C. velifer*), and ZIN 56538 (*C. panticapaei*), Paleomix retained 11,711,034, 39,994,409, 20,801,663, and 35,235,998 reads, respectively. These sets of reads had mean lengths of 47.9, 35.9, 41.8, and 44.9 bp. Of the reads per specimen, 1,856, 10,873, 10,279, and 7,081 reads mapped to the mitochondrial genome of the *C. rhinoceratus* reference (NC_05712), respectively (Table 3). These numbers corresponded to 0.0158%, 0.0272%, 0.0494%, and 0.0201% of the retained reads per specimen.

Analysis with GATK identified 140 variants, of which one was an indel—a deletion of one guanine residue at position 16,596 of the mitochondrial genome (within the D-loop/control region) in ZIN 56538 (*C. panticapaei*). The remaining 139 variants were SNPs. All variants had a Phred-scaled quality score greater than 30 and a coverage of at least 3 reads per specimen, and thus passed the filtering with bcftools. Manual inspection with Integrative Genome Viewer confirmed all 140 variants. Of the 139 SNPs, 119 were transitions and 20 were transversions, resulting in a transition/transversion ratio of 5.95. The number of C to T substitutions (22) was comparable to other transitions (A to G: 43; G to A: 24; T to C: 30). The mitochondrial genome sequence generated in this way for ZIN 56294 (*C. rugosus*) was nearly identical to that published by Muschick et al. (2022) for the same specimen (NCBI accession PP319402.1). The differences between the two versions were limited to the exact beginning and end positions of stretches of missing information (coded as "N") in the D-loop region and for a single site in the same region (position 17,347) that was coded as "C" in the previous version of this genome, but now appeared to be more likely a "T". The other three new mitochondrial genome sequences were uploaded to NCBI with accessions PQ333928 (ZIN 56275; *C. velifer*), PQ333929 (ZIN 56538; *C. panticapaei*), and PQ333930 (ZIN 56638; *C. rhinoceratus*). Annotation with the MitoAnnotator pipeline identified all 13 coding genes, the two rRNA genes, and the 22 tRNA genes in each of the reconstructed mitochondrial genomes.

Genetic distances were low for all pairs of specimens except those involving ZIN 56538 (*C. panticapaei*), with 13–34 changes among the mitochondrial genomes of ZIN 56638 (*C. rhinoceratus*), ZIN 56294 (*C. rugosus*), and ZIN 56275 (*C. velifer*) while 110–114 changes separated the mitochondrial genome of ZIN 56538 (*C. panticapaei*) from all other specimens and the reference (NC_057120; *C. rhinoceratus*) (Table 4). The phylogeny inferred with IQ-TREE from the five full mitochondrial genome sequences of *Channichthys* specimens (including the reference) illustrated this pattern with a long branch separating ZIN 56538 (*C. panticapaei*) from a cluster formed by the other four specimens (Fig. 3a).

The partitioned alignment of mitochondrial coding sequences for the four newly reconstructed mitochondrial genomes, the *C. rhinoceratus* reference, and 34 further notothenioid species had a length of 11,460 bp with a high level of completeness (99.4%). Maximum-likelihood phylogenetic analysis based on this alignment produced a tree that was highly congruent with previous studies of notothenioid relationships (Near et al. 2012, 2018; Colombo et al. 2015; Dornburg et al. 2017). Focusing on Channichthyidae, we recovered the genus *Champsocephalus* (with the two representatives *C. esox* and *C. gunnari*) as the sister clade to all other included icefishes (Fig. 3b), with full bootstrap support (BS: 100). The remaining members of the family formed three strongly supported clades (BS: 100); one combining the genera *Pagetopsis*, *Neopagetopsis*, and *Pseudochaenichthys*, a second grouping *Chaenodraco* and *Chionodraco*,

Table 3 Summary of Illumina NextSeq sequencing results

Specimen	Species	NextSeq read numbers	Combined read numbers	
		Total	Total	Mapping
ZIN 56638	*C. rhinoceratus*	24,342,106	26,043,572	1856
ZIN 56294	*C. rugosus*	63,158,791[a]	64,912,699	10,873
ZIN 56273	*C. velifer*	10,731,926[b]	12,783,786	231
ZIN 56275	*C. velifer*	28,746,984	29,911,637	10,279
ZIN 56538	*C. panticapaei*	42,672,855	44,885,690	7081

The numbers of reads mapping refers to those mapping to the mitochondrial genome (using NC_057120 as reference). The four focal specimens are underlined

[a]Sequenced previously in Muschick et al. (2022)

[b]Sequenced previously as described in Muschick et al. (2022); unpublished

and a third clustering *Chionobathyscus*, *Pagetodes*, and *Channichthys*. All of these three clades have also been supported by nuclear data (Dornburg et al. 2017; Near et al. 2018). In line with phylogenies based on nuclear data, we also recovered the genus *Chaenocephalus* as the sister to the third of the three clades, albeit with weak support from our mitochondrial data (BS: 73). Within *Channichthys*, this phylogeny based on mitochondrial coding sequences (Fig. 3b) slightly differed from the one based on full mitochondrial genomes (Fig. 3a), as the two sequences assigned to *C. rhinoceratus* (ZIN 56538 and NC_057120) formed a clade in the latter, but not the former. However, the relationships among these two sequences were not strongly supported in either of the two phylogenies (BS: 73, 81). Concordant with the calculated pairwise genetic distances, the branches connecting the four *Channichthys* sequences assigned to *C. rhinoceratus*, *C. rugosus*, and *C. velifer* were shorter (below 0.0008 substitutions per site) than any other branches on the tree. The branch leading to ZIN 56538 (*C. panticapaei*) was still short compared to most other terminal branches, but comparable to those connecting the two congeners within the genus *Champsocephalus* (*C. esox* and *C. gunnari*; branch lengths ranging from 0.0038 to 0.0041 substitutions per site). In comparison, the three congeners within the genus *Chionodraco* had branch lengths that were more than twice as long (at least 0.0085 substitutions per site).

Cytochrome c oxidase I (*mt-co1*) haplotypes varied little among the newly reconstructed *Channichthys* genomes, the *C. rhinoceratus* mitochondrial reference genome (NC_057120), and twelve additional *mt-co1* sequences available from NCBI (which had all been assigned to *C. rhinoceratus*) (Fig. 4). A central haplotype was shared by 13 specimens, including those of the three specimens ZIN 56275 (*C. velifer*), ZIN 56294 (*C. rugosus*), and ZIN 56638 (*C. rhinoceratus*). This haplotype differed by one single mutation from that found in the *C. rhinoceratus* reference (NC_057120) and from two further haplotypes found in other sequences assigned to *C. rhinoceratus*. In contrast, the haplotype of specimen ZIN 56538 (*C. panticapaei*) was the most divergent, separated by five substitutions from the central haplotype.

Morphological analysis

Based on our morphological analysis, species of the genus *Channichthys* can be divided into two groups according to the structure of the gill apparatus (Fig. 5): (1) that with "double-rowed gill rakers", having two rows of gill rakers on the gill arches (*sp.br.*) on the outer side of the ceratobranchial (*sp.br.a*) and on the inner side of the hypobranchial (*sp.br.b*); and (2) those with "single-rowed gill rakers", having only one row of gill rakers on the gill arches on the outer side of the ceratobranchial (*sp.br.a*), but no gill rakers the inner side of the hypobranchial (*sp.br.b*). Only a single species belongs to the "double-rowed gill rakers" group (*C. panticapaei*), while the "single-rowed gill rakers" group includes three species (*C. rhinoceratus*, *C. rugosus* and *C. velifer*). Thus, these three species are characterized by the first branchial arch bearing only one row of gill rakers on the outer side of the ceratobranchial, while rakers are usually absent on the inner side of the arch (rarely, single rakers can be found in the angle of the arch).

Of all other morphological characteristics (Tables 5 and 6), it is primarily the meristic counts (Table 6) that are of importance for diagnosis, such as: the number of rays in the first (*D1*) and second (*D2*) dorsal fins, in the pectoral fins (*P*), and in the anal fin (*A*); the number of gill rakers on the first gill arches (*sp.br.*), the number of scales in the dorsal (*Dll*) and medial (*Mll*) lateral lines. Additionally, head measurements are important: head length (*c*), head height through the middle of the eye (*ho*), snout length (*ao*), longitudinal diameter orbit of the eye (*o*), interorbital space (*io*), length of the upper (*lmx*) and lower (*lmd*) jaws (Table 5). Finally, certain indices (Table 6) and the external coloration of the body (Fig. 1) can be informative.

Channichthys rhinoceratus is characterized by a first dorsal fin (*D1*) with 5–9 (mean 7) rays, a fin membrane that does not reach the tips of the largest rays, a granulation that is poorly or moderately developed, and marbled color.

Channichthys rugosus has a first dorsal fin with 7–9 (mean 8) rays, a fin membrane reaching the tips of the largest rays, a granulation that is well developed, and is plain reddish in color.

Channichthys velifer has a first dorsal fin with 9–12 (mean 11) rays and a characteristic sail-like shape, a fin membrane that does not reach the tips of the largest rays, poorly developed granulation, and is of lighter spotted color.

As described above, *Channichthys panticapaei* is characterized, in contrast to all other species, by the first branchial arch bearing two rows of gill rakers located on the outer and inner sides of the cerato- and hypobranchial. The outside rakers count 12–20 and the inside rakers 4–14; the total number of rakers is 18–32. The first dorsal fin has 6–8 (mean 7) rays, the fin membrane does not reach the tips of the largest rays, the granulation is highly developed, and the body is of darker brownish-black color.

Thus, according to morphological characteristics (Tables 5 and 6), four species can be distinguished in the genus *Channichthys*: *C. rhinoceratus*, *C. rugosus*, *C. velifer*, and *C. panticapaei*. A detailed diagnosis of these four species is presented in the Appendix (Online Resource 4).

Discussion

Endogenous DNA contents showed large differences among the extracts from the twelve specimens we tested. These differences are surprising given the similar ages of

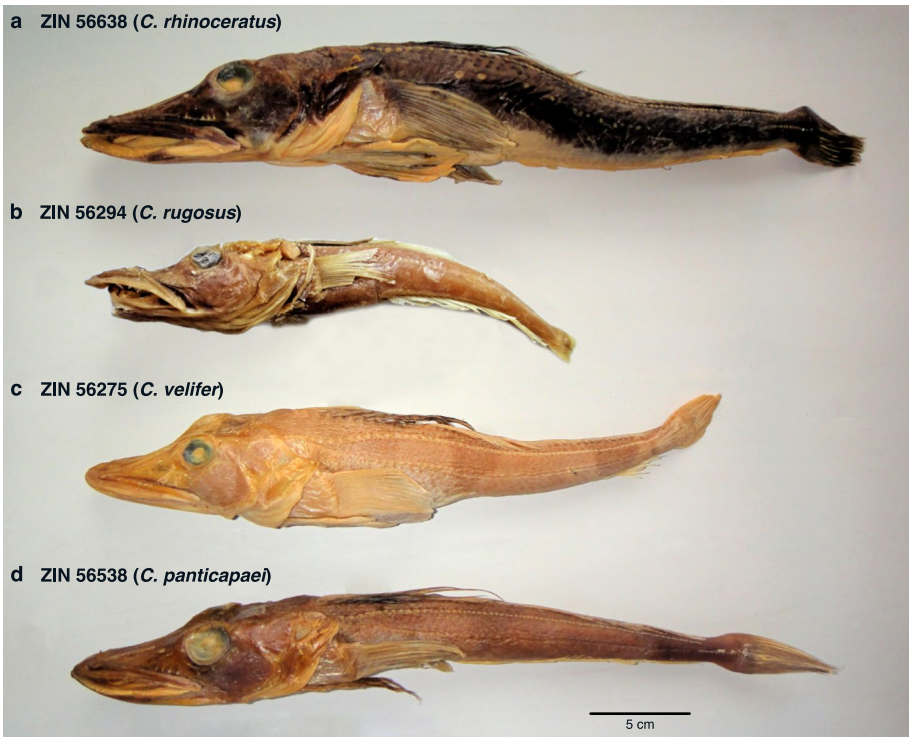

Fig. 1 Specimens analyzed of the four *Channichthys* species. **a** ZIN 56638 (*C. rhinoceratus*), **b** ZIN 56294 (*C. rugosus*), **c** ZIN 56275 (*C. velifer*), **d** ZIN 56538 (*C. panticapaei*)

the specimens (50–55 years) and the presumably comparable conditions under which they were fixed and preserved at the Zoological Institute of the Russian Academy of Sciences. These results therefore suggest that DNA degradation may still vary strongly between specimens, likely due to unknown parameters influencing individual degradation levels. Among these could be concentration differences of formaldehyde used for fixation, the fixation duration, the temperature to which specimens were exposed, or variation of ethanol concentration over time in different storage units. Our results, however, show another case, where a DNA concentration below the Qubit High Sensitivity kit detection threshold of 0.05 ng/µl (specimen ZIN 56538) was not impeding successful single-stranded library construction and ultimately obtaining sufficient reads for reconstruction of the mitochondrial genome sequence (Table 2). This has previously been reported (Straube et al. 2021b) and suggests that samples with undetectable DNA concentration can still be successfully sequenced. In other cases, when shotgun sequencing data indicated low amounts of endogenous DNA (Table 2), target gene capture can be a suitable alternative for future endeavors (e.g., Straube et al. 2021; Agne et al.2022a, b).

Our molecular analyses revealed high similarity, with no more than 34 substitutions in pairwise comparisons, among the mitochondrial genomes of specimens from three of the four species within the genus *Channichthys*: Unicorn Icefish (*C. rhinoceratus*), Red Icefish (*C. rugosus*), and Sailfish Pike (*C. velifer*). Only the specimen representing the Charcoal Icefish (*C. panticapaei*) had a more divergent mitochondrial genome, differing from its congeners at 110–114 sites. These results extend those obtained by Muschick et al. (2022), which had already indicated great similarity between the mitochondrial genomes of Unicorn Icefish (*C. rhinoceratus*) and Red Icefish (*C. rugosus*) specimens.

The lack of substantial mitochondrial variation among three of the four species seems to contrast with their notable morphological differences, in fin ray counts, gill arch structure, size proportions, and coloration, among other traits. The most obvious phenotypic difference among the three species may be the tall sail-shaped first dorsal fin of Sailfish Pike (*C. velifer*) that is reflected in its Latin and English names. Similarly, the Red Icefish (*C. rugosus*) differs markedly from other members of the genus by its reddish body coloration, and is easily recognized by this trait. Further morphological differences among all four species are described in the Appendix (Online Resource 4). We see three possible explanations for the contrast between these clear morphological differences and the similarity of the three mitochondrial genomes.

First, the genus *Channichthys* might not include as many species as assumed by recent studies (Nikolaeva 2020, 2024), even after the synonymization of five formerly recognized taxa (Shandikov 1995b; Nikolaeva 2020). This possibility was suggested by Muschick et al. (2022), following their analysis of the mitochondrial genome of *C. rugosus*, and it would mirror conclusions made for another formerly species-rich notothenioid genus, *Pogonophryne* (Parker

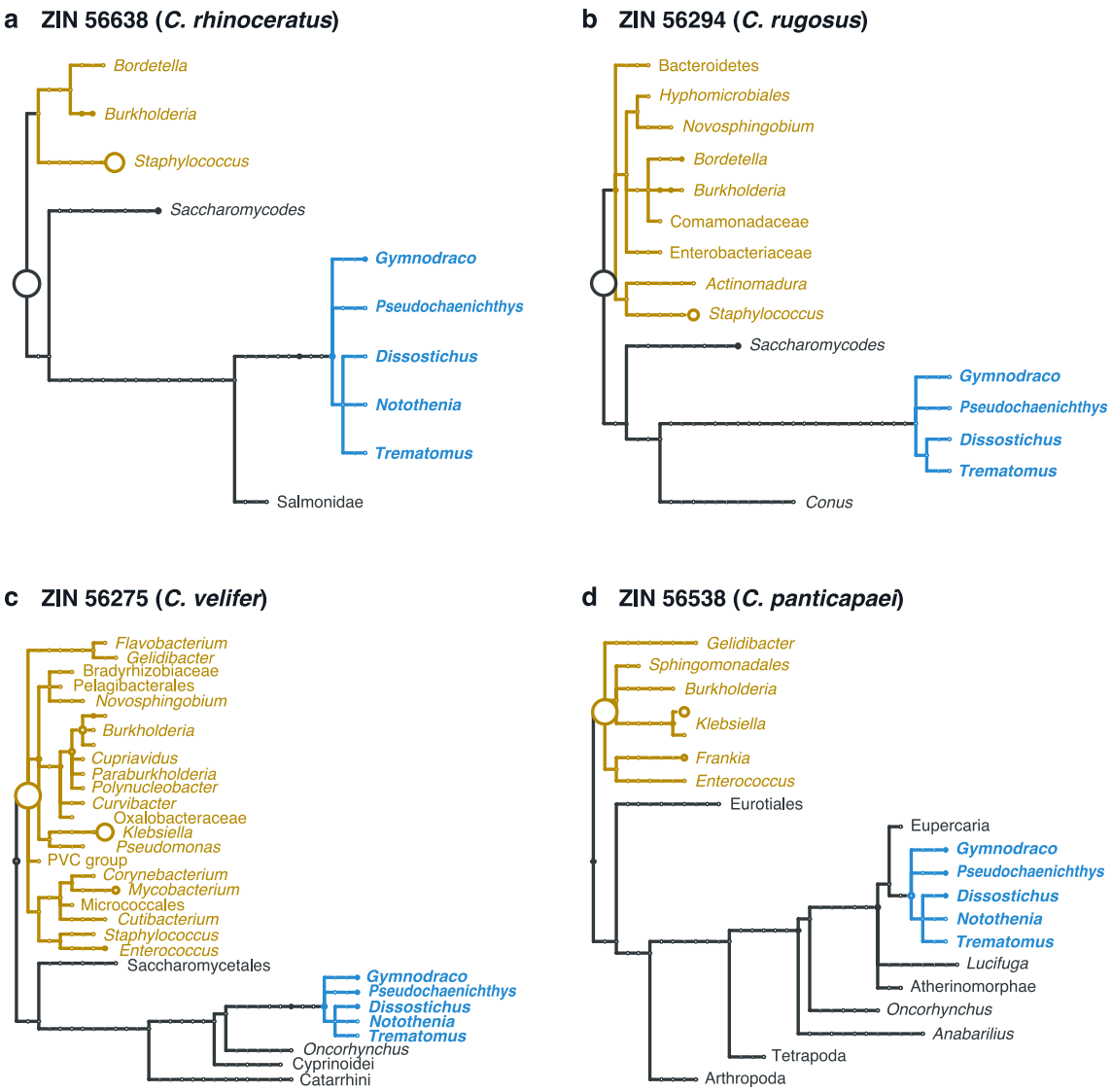

Fig. 2 Taxonomic assignments of reads extracted from the four focal *Channichthys* specimens. Circles on nodes indicate frequencies of assignments. Bacterial taxa are colored in brown; notothenioid taxa are shown in blue. Assignments were made with the programs Diamond and MEGAN

Table 4 Pairwise genetic distances

Specimen	Species	ZIN 56638	ZIN 56294	ZIN 56275	ZIN 56538
ZIN 56638	*C. rhinoceratus*	0	31	34	110
ZIN 56294	*C. rugosus*	31	0	13	113
ZIN 56275	*C. velifer*	34	13	0	114
ZIN 56538	*C. panticapaei*	110	113	114	0
NC_057120	*C. rhinoceratus*	25	26	29	109

Pairwise genetic distances were calculated as the numbers of sites differing between two sequences

et al. 2022). In Muschick et al. (2022), comparisons of mitochondrial genome sequences revealed that specimens within notothenioid species generally had fewer than 100 genetic differences, whereas between-species comparisons typically exceeded 400 differences. However, the comparisons of Muschick et al. (2022) did not include the closely related, but undoubtedly distinct species pair *Champsocephalus gunnari* and *C. esox* (Rivera-Colón et al. 2023), as

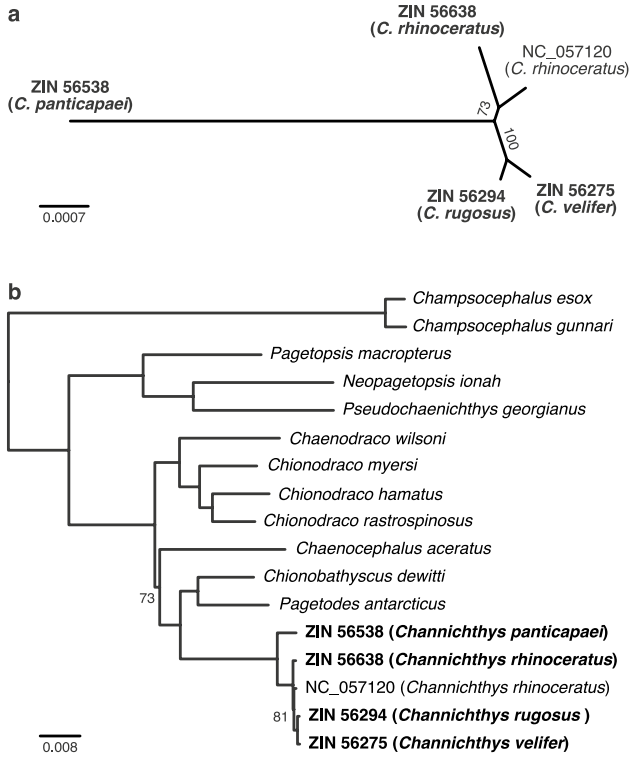

Fig. 3 Maximum-likelihood phylogenies inferred for the genus *Channichthys* and the family *Channichthyidae*. **a** Unrooted phylogeny based on the four newly reconstructed mitochondrial *Channichthys* genomes (ZIN 56638, ZIN 56294, ZIN 56275, and ZIN 56538) and the *C. rhinoceratus* reference (NC_057120). **b** Rooted phylogeny for all species of Channichthyidae with available mitochondrial genomes, based on an alignment of coding sequences. In **a** and **b**, branch lengths mark substitutions per site, and labels on branches indicate bootstrap support. In **b**, bootstrap support is only shown for nodes that did not receive full support (BS < 100). Newly reconstructed mitochondrial genomes are marked in bold font

Table 5 Absolute and relative measurements for the four focal *Channichthys* specimens

Character	ZIN 56638 (*C. rhinoceratus*)	ZIN 56294 (*C. rugosus*)	ZIN 56275 (*C. velifer*)	ZIN 56538 (*C. panticapaei*)
TL (mm)	420	265	332	363
SL (mm)	380	252	300	320
ho (mm)	43	36	39	35
hD1 (mm)	78	72	73	91
lP_l (mm)	69	43	54	59
lV_l (mm)	65	43	55	63
c (mm)	142	93	108	116
o (mm)	20	15	18	20
io (mm)	28	14	19	20
ao (mm)	70	48	52	52
lmx (mm)	80	51	60	60
lmd (mm)	97	65	73	77
ho/SL	0.113	0.143	0.130	0.109
hD1/SL	0.205	0.286	0.243	0.284
lP_l/SL	0.182	0.171	0.180	0.184
lV_l/SL	0.171	0.171	0.183	0.197
c/SL	0.374	0.369	0.360	0.363
o/SL	0.053	0.060	0.060	0.063
io/SL	0.074	0.056	0.063	0.063
ao/SL	0.184	0.190	0.173	0.163
lmx/SL	0.211	0.202	0.200	0.188
lmd/SL	0.255	0.258	0.243	0.241

TL total body length, *SL* standard body length, *c* head length, *hD1* maximum height of the 1st dorsal fin, lP_l and lP_d length of the left and right pectoral fins, lV_l and lV_d length of the left and right pelvic fins, *ho* head height through the middle of the eye, *lmx* length of the upper jaw, *lmd* length of the lower jaw, *ao* snout length, *o* longitudinal diameter of the eye orbit, *io* interorbital space

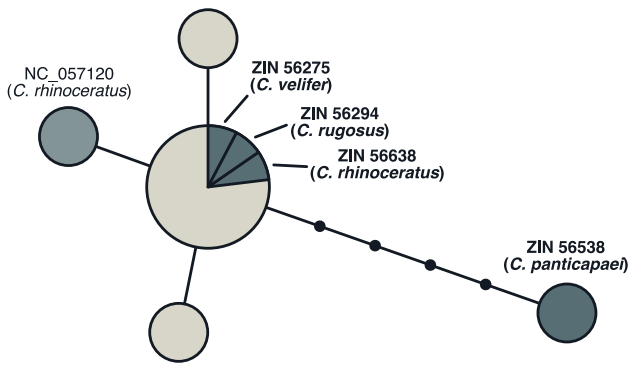

Fig. 4 Haplotype genealogy graph for *mt-co1* sequences of the genus *Channichthys*. The three specimens ZIN 56275 (*C. velifer*), ZIN 56294 (*C. rugosus*), and ZIN 56638 (*C. rhinoceratus*) shared the central haplotype with ten other sequences; the four other haplotypes were each represented by a single sequence

no mitochondrial genome was available for the latter at the time of analysis. The now available mitochondrial genome sequences of the two species (NCBI accessions NC_018340 and NC_063099) are separated by 167 substitutions, a number that is far below the over 400 substitutions found in other cross-species comparisons. On the other hand, this number is still not comparable to the 13–34 substitutions separating the mitochondrial genomes of the here analyzed Unicorn Icefish (*C. rhinoceratus*), Red Icefish (*C. rugosus*), and Sailfish Pike (*C. velifer*) specimens. If these three taxa should in fact be part of one and the same species, this species—which, based on priority, would be *C. rhinoceratus* (Richardson 1844)—would exhibit substantial morphological variation. This variation could perhaps in part be due to phenotypic plasticity, e.g., resulting from life at different depths in the water column influencing food composition or parasite exposure. Such factors are known to affect body shapes (Hetzel and Forsythe 2023), but are unlikely to influence

Table 6 Indices and meristic counts for the four focal *Channichthys* specimens

Character	ZIN 56638 (*C. rhinoceratus*)	ZIN 56294 (*C. rugosus*)	ZIN 56275 (*C. velifer*)	ZIN 56538 (*C. panticapaei*)
ho/c	0.303	0.387	0.361	0.302
ao/c	0.493	0.516	0.481	0.448
o/c	0.141	0.161	0.167	0.172
io/c	0.197	0.151	0.176	0.172
lmx/c	0.563	0.548	0.556	0.517
lmd/c	0.683	0.699	0.676	0.664
o/io	0.714	1.071	0.947	1.000
io/o	1.400	0.933	1.056	1.000
ao/io	2.500	3.429	2.737	2.600
io/ao	0.400	0.292	0.365	0.385
o/ao	0.286	0.313	0.346	0.385
ao/o	3.500	3.200	2.889	2.600
c/o	7.100	6.200	6.000	5.800
c/ao	2.029	1.938	2,077	2.231
ho/o	2.150	2.400	2,167	1.750
o/ho	0.465	0.417	0.462	0.571
ho/io	1.536	2.571	2.053	1.750
io/ho	0.651	0.389	0.487	0.571
c/ho	3.302	2.583	2.769	3.314
D1	7	9	10	7
D2	33	33	32	34
P_l	21	19	20	20
A	31	30	31	31
sp.br	12	15	12	26
sp.br.a	12	15	12	17
sp.br.b	0	0	0	9
Dll_l	76	68	68	66
Dll_d	77	67	67	68
Mll_l	34	42	13	28
Mll_d	29	43	13	32

D1, *D2*, P_l, *A* number of rays in the first dorsal, second dorsal, left pectoral, and anal fins, respectively, *sp.br.* total number of gill rakers on the first gill arch, *sp.br.a* number of gill rakers on the outer side of the first gill arch, *sp.br.b* number of gill rakers on the inner side of the first gill arch, Dll_l and Dll_d number of pores and scales in the left and right dorsal lateral lines of the seismosensory system, Mll_l and Mll_d number of pores and scales in the left and right medial lateral lines of the seismosensory system, *vert.* the total number of vertebrae in the spine. See Table 5 for other abbreviations

meristic characters. Better knowledge of the biology of the three taxa, including their (overlapping or not) depth distributions, would be required to assess the extent of phenotypic plasticity in *Channichthys*. Alternatively, large-effect genetic features such as inversion-linked supergenes can produce distinct phenotypes within species (Jeong et al. 2022; Lamichhaney et al. 2016). Such features could in principle be identified with population-level nuclear genomic data, but these data are so far unavailable for the genus *Channichthys*. The most relevant source of information to evaluate *Channichthys* species would be experimental data on potential reproductive barriers; however, the remoteness of their habitat, the difficulty of raising them in captivity, and their late age at sexual maturity render such experiments hard to perform conclusively (Desvignes et al. 2019, 2024). In addition, a few studies have revealed the potential for reproductive isolation between species to be incomplete (Marino et al. 2013; Desvignes et al. 2019; Schiavon et al. 2021) and hybridization barriers to be permissive and potentially driven more by differing reproduction seasons and nesting behavior instead of intrinsic gametic incompatibility (Desvignes et al. 2019).

Second, the three taxa Unicorn Icefish (*C. rhinoceratus*), Red Icefish (*C. rugosus*), and Sailfish Pike (*C. velifer*) might all be distinct and valid species, even though their separation is not reflected in their mitochondrial genomes. Mitochondrial genomes are known to cross species boundaries through hybridization, as long as reproductive barriers remain incomplete (Sloan et al. 2017). This introgression can lead to mitochondrial capture, the complete replacement of the mitochondrial lineage of one species by that of a closely related one (Willis et al. 2013). Thus, two species that are reproductively largely isolated could have little or no mitochondrial divergence even when nuclear genomes are clearly separated. Again, nuclear genomic data or experimental data on reproductive barriers would be required to evaluate this possibility.

Third, the three *Channichthys* taxa might be valid species that have radiated extremely recently on the Kerguelen-Heard Plateau, in which case both mitochondrial and nuclear divergence would be minimal. Indeed, all four *Channichthys* species inhabit the same geographic area and seemingly the same bathymetric range on the Kerguelen-Heard Plateau, which suggests that the four *Channichthys* species would have diversified in sympatry. This would contrast most other examples of recent divergence in Antarctic notothenioids, that are usually considered to occur in allopatry or parapatry (Dornburg et al. 2016; La Mesa et al. 2017; Desvignes et al. 2020; Corso et al. 2024). We know from other cases of adaptive radiation that large numbers of recognizable species (even when not completely reproductively isolated) can be generated over extremely short time scales, such as the Lake Victoria cichlid fish radiation that produced hundreds of species in no more than 15,000 years (Ngoepe et al. 2023; Meier et al. 2023). However, even this, perhaps most extreme, example of rapid species diversification, resulted in greater mitochondrial variation than present among the three *Channichthys* specimens. While few complete mitochondrial genomes are available for the Lake Victoria cichlid radiation, focusing on the NADH dehydrogenase subunit 2 gene (*mt-nd2*), a marker that has been sequenced for many

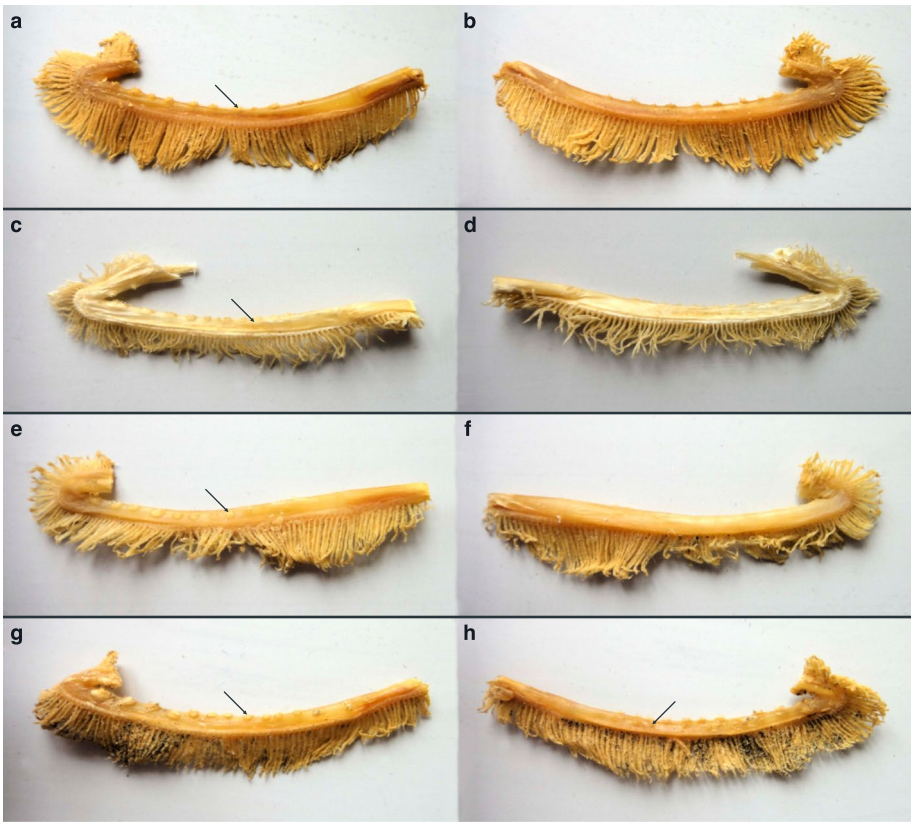

Fig. 5 The first gill arches of specimens representing the four species of the genus *Channichthys* accepted as valid by Nikolaeva (2020). **a**, **b** *C. rhinoceratus*, external (**a**) and internal (**b**) sides, **c**, **d** *C. rugosus*, external (**c**) and internal (**d**) sides; **e**, **f** *C. velifer*, external (**e**) and internal (**f**) sides; **g**, **h** *C. panticapaei*, external (**g**) and internal (**h**) sides. Arrows indicate gill rakers in single-rowed gill arches in *C. rhinoceratus* (**a**), *C. rugosus* (**c**), and *C. velifer* (**e**) and the double-rowed gill arch in *C. panticapaei* (**g**, **h**)

cichlid species (Wagner et al. 2012), allows a comparison. At this marker, the three *Channichthys* specimens for the Unicorn Icefish (*C. rhinoceratus*), Red Icefish (*C. rugosus*), and Sailfish Pike (*C. velifer*) differ by no more than 1–2 substitutions. In contrast, representatives of the Lake Victoria cichlid radiation, like *Haplochromis sauvagei*, *Paralabidochromis* sp. 'rock kribensis', and *Pundamilia pundamilia*, differ by 4–8 substitutions in their *mt-nd2* sequences (NCBI accessions KJ955418, JQ950392, and KY366728). Thus, unless the radiation of *Channichthys* species would be even more recent than that of Lake Victoria cichlids, their low mitochondrial divergence is not likely to be explained by this scenario.

Regardless of the explanation for the low mitochondrial variation among the Unicorn Icefish (*C. rhinoceratus*), Red Icefish (*C. rugosus*), and Sailfish Pike (*C. velifer*) specimens, much more pronounced divergence was observed for the mitochondrial genome of the analyzed Charcoal Icefish (*C. panticapaei*) specimen. This genome differed at 110–114 sites from those of its congeners, a range comparable to the separation of the *Champsocephalus gunnari* and *C. esox* species pair with 167 substitutions and beyond the diversity usually found within a single notothenioid species (Muschick et al. 2022). In light of this molecular separation, together with the distinct morphological variation exhibited by the Charcoal Icefish (*C. panticapaei*), we consider the support for its species status sufficient to recommend its inclusion in the list of validated icefish and more broadly notothenioid species (Eastman and Eakin 2021). These insights gained from the analyzed museum specimens highlight the importance of natural history collections in biodiversity studies, especially in rarely accessed areas, such as Antarctica (Hilton et al. 2021; O'Brien et al. 2022).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00300-025-03349-x.

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Author contributions BGA contributed to lab work, performed molecular analyses, and wrote a partial draft of the manuscript. EN contributed tissue samples, performed all morphological analyses, and contributed to the draft manuscript. TD contributed conceptually. NS led the lab work and contributed conceptually. MM contributed to lab work, performed molecular analyses, completed the draft manuscript, and contributed conceptually. EN, TD, NS, and MM contributed to the final version of the manuscript.

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Data availability Mitochondrial genome sequences generated for this study have been deposited at NCBI GenBank with accession numbers PQ333928-PQ333930.

Declarations

Conflict of interest The authors declare that no conflict of interest exists, as well as that there is no financial support or relationships that may pose any kind of conflict.

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