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MITOGENOME REPORT

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New mitogenomes of Runcinidae and Facelinidae: two understudied heterobranch families (Mollusca: Gastropoda)

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ABSTRACT

Here, we present the mitochondrial sequences of two sea slugs (Heterobranchia): *Runcina aurata* and *Facelina auriculata*, the latter being the type species of the family. The mitochondrial genomes are 14,282 and 14,171bp in length, respectively, with a complete set of 13 PCGs, 2 rRNAs, and 22 tRNAs. None of the mitogenomes show gene reorganization, keeping the standard mitogenomic structure of Heterobranchia. Nucleotide composition differs significantly between them, with *R. aurata* showing the most AT-rich mitogenome (25.7% GC content) reported to date in Heterobranchia, and *F. auriculata* showing a rich GC content (35%) compared with other heterobranch mitochondrial genomes.

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Mitochondrial genome; phylogenomics; neglected taxa; type taxon; systematics; GC content; specific primer design

Introduction

Close to 10 million species are predicted to inhabit the Earth (Mora et al. 2011). Nevertheless, most species remain undescribed, with special underestimation on those of small size or living in unreachable habitats. This issue can also be attributed to the field of genetics and genomics because, while some groups are extensively studied, such as primates (Kuderna et al. 2023), this is not the case for most groups, including mollusks (Sigwart et al. 2021). Among molluscan neglected taxa, the order Runcinida englobes close to 80 small-sized described species (1–4 mm), such as *Runcina aurata* (Figure 1), and it is thought to include many more cryptic ones (Araujo et al. 2022, 2023). Members of this order feed on algae and dead marine phanerogams, contributing to the decomposition of organic matter and, therefore, being an invaluable element in carbon and nitrogen cycling in oceans (Araujo et al. 2019). Due to their small size and difficulties in finding them in their natural environment, very few studies have focused on them so far. On the other hand, the family Facelinidae, which is considered one of the most diverse heterobranch families, with more than 200 species (Karmeinski et al. 2021), is represented here by its type species *Facelina auriculata* (Figure 1). As most aeolid nudibranchs, *F. auriculata* is characterized by its ability to incorporate the nematoblasts from their cnidarian prey, using them as a defensive tool (Goodheart et al. 2018). Here, we present the reference mitogenomes for the two species, *Runcina aurata* and *Facelina auriculata,* to allow for mitogenome comparisons among molluscan orders, and provide new templates for designing specific primers.

Material and methods

The specimen of *Runcina aurata* was collected in Cadiz (S Spain, 36°31′59″N 6°18′31″W), whereas the *Facelina auriculata* individual was collected in Lagos (S Portugal, 37°05′00″N 8°39′57"W). The diagnosis of *R. aurata* and *F. auriculata* were concluded by using morphological traits as described in Araujo et al. (2022) and García-Gómez & Cervera (2011), respectively. For *R. aurata* we confirmed the presence of white regions behind the eyes and anterior to the notum end, and four large rounded gills. On the other hand, we validated *F. auriculata* identification through the coloration of its cerata, which are iridescent blue with red and white tips (Figure 1). Both specimens were collected at intertidal depth

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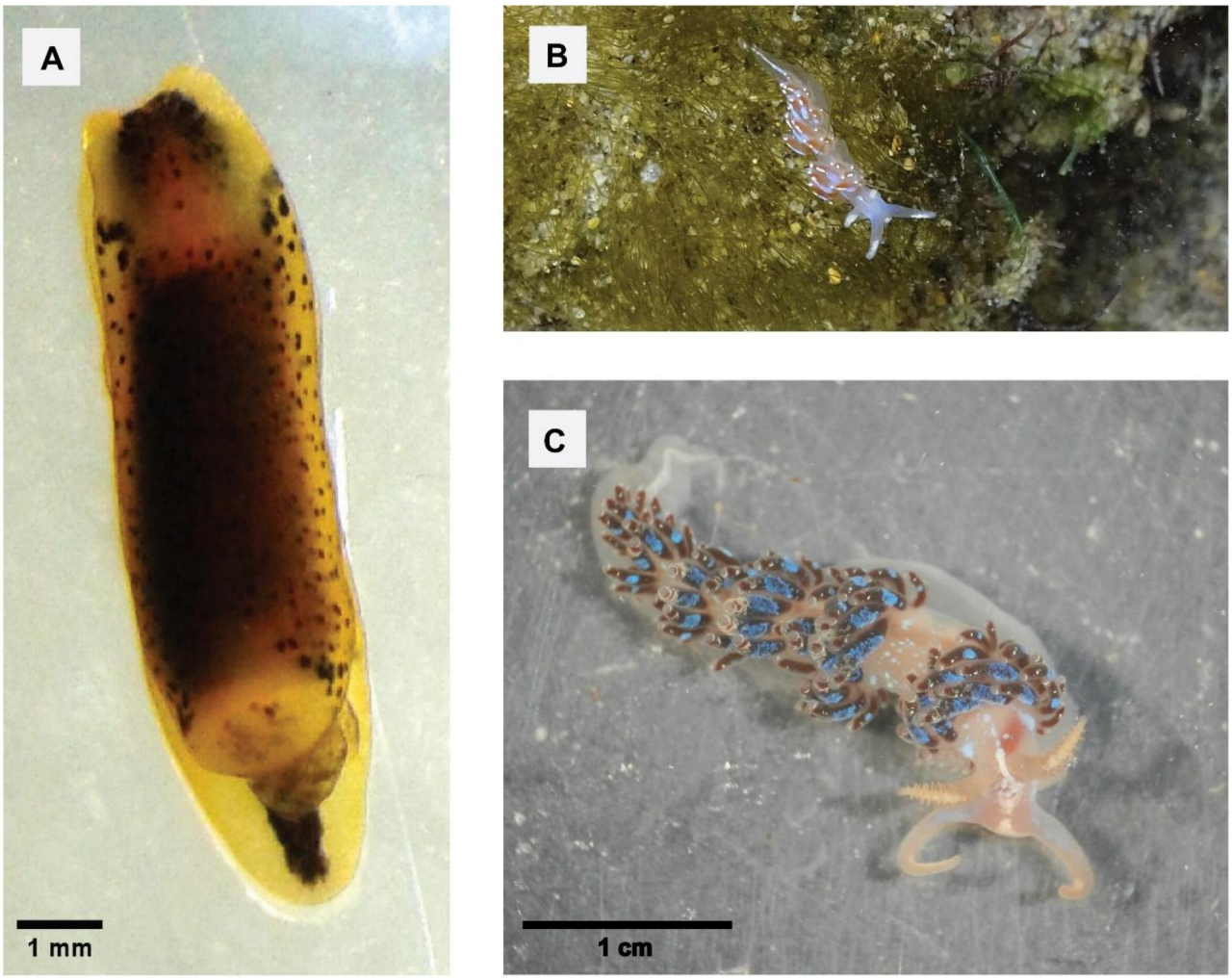

Figure 1. Species used in the present study. (a) *Runcina aurata* under the binocular lens. (b) *Facelina auriculata* in its natural environment. (c) *Facelina auriculata* under the binocular lens. Note that due to the small size of *Runcina aurata*, it was not possible to take a picture in nature. *Runcina aurata* picture was taken by Ana Karla Araujo and *F. auriculata* pictures were taken by Patricia Pérez García.

and were preserved in 96% ethanol. A piece of the foot of *F. auriculata* and the whole individual of *R. aurata* were used for DNA extraction, which was carried out with the QIAGEN QIAamp DNA mini Kit (QIAGEN Iberia; Barcelona, Spain) following the manufacturer's recommendations. The remaining tissue of *F. auriculata* was deposited in the Museo Nacional de Ciencias Naturales de Madrid (MNCN) with the code MNCN15.05/94859 (Curator: Francisco Javier de Andrés Cobeta, mail: javiermol@mncn.csic.es), and the *R. aurata* DNA extraction was deposited in the same museum with the code MNCN:ADN118948 (Curator: Isabel Rey Fraile, mail: isabel.rey@csic.es). The quantity and quality of the genomic DNA were checked with a NanoDrop One device (ThermoScientific, Waltham MA, USA) and QUBIT HighSensitivity DNA kit (Invitrogen, Waltham MA, USA). Mitochondrial genomes were enriched for both species by performing long PCR amplification approach (White et al. 2011). In short, different combinations of the universal primers for the genes *cox1*, *cox3*, *cob*, *rrnS*, and *rrnL* (see Boore et al. 2005 for primer details) were used to amplify fragments from ~3Kb to ~10Kb. Long PCRs were carried out in 25 μL reaction mixes of Takara LA PCR Buffer II (Otsu, Japan) supplemented with 0.5 mM $MgCl_2$, 0.4 μM of each primer, 12 mM dNTP Mixture, 0.04 U/μL Takara Z-Taq™ DNA polymerase (Otsu, Japan), and ~4 ng/μL of template DNA. Long PCR cycles consisted of one minute at 98 °C, followed by 30 cycles of 98 °C for 10 s (denaturalization), 45°C for 10 s (annealing) and 68 °C for 15 min (elongation), with a final elongation of 10 min at 72 °C. Amplified fragments were purified with ethanol precipitation prior to library construction and sequencing, carried out at Get-PlaGe core facilities of GenoToul (Toulouse, France). For each species DNA sample, the Illumina TruSeq Nano DNA Library Prep Kit (Illumina, San Diego CA, USA) was used for library construction, and both libraries were sequenced in 2x150pb paired-ends on Illumina HiSeq3000 platform aiming for a 40 Mb output each. Paired-end reads of both genomic libraries were then subjected to quality inspection using the FastQC-0.12.0 software (Andrews, 2010), and the Velvet-1.2.10 *de novo* assembler (Zerbino & Birney, 2008) was used on each dataset independently to obtain genomic contigs (long sequences). For each species, the single contigs including complete mitochondrial genomes were identified with BLAST by using a local database including nudibranch mitochondrial genomes available in GenBank. Also, the raw reads used to get the assembly were mapped against the mitogenomes with BWA-MEM (Li &

Durbin, 2009), and the resulting coverage along the assembly was checked to verify the quality of the mitogenomes (Figure S1). Mitogenomes were annotated using MITOS2 (Al Arab et al. 2017; Donath et al. 2019). The annotation files were manually checked and curated in Geneious Prime 2022.1.1 (Geneious, Boston MA, USA) (Kearse et al. 2012). The mitogenome sequences and their respective annotations were uploaded to the National Center for Biotechnology Information (NCBI) (*F. auriculata*: OP661154, *R. aurata*: OP661155). We downloaded 10 heterobranch mitogenomes from NCBI. For comparisons against *F. auriculata* we downloaded all verified Aeolidioidea mitogenome sequences due to their close phylogenetic position to the study taxon. For comparisons against *R. aurata,* since there was no other mitogenome sequences of the order Runcidina in NCBI, we also used all verified Cephalaspidea and the *Aplysia californica* J. G. Cooper, 1863 mitogenome sequences due to their close phylogenetic relationship (Table 1). We generated two independent matrices. The first one included the nucleotide sequence of 13 protein coding genes (PCG) + 2 ribosomal RNA genes (rRNA) for the six Aeolidioidea species and *R. aurata* as the outgroup. The second matrix included the same set of genes (13 PCG + 2 rRNA) for all the Cephalaspidea species, *A. californica,* and *F. auriculata,* the latter used as the outgroup. Maximum likelihood software IQ-TREE v2 (Minh et al. 2020) was used to reconstruct phylogenetic relationships, partitioning the coding genes according to their codon position. The resulting phylogenies were visualized and edited with the iTOL (Letunic & Bork, 2021) web server.

Results

The complete mitochondrial genomes of *Runcina aurata* and *Facelina auriculata* could be identified as a single contig of an extremely high coverage in comparison to the other contigs. Mitogenomes were of approximately 14 Kb, with a mean depth coverage above 1500x in both cases (Figure S1), with successful circularization of the mitochondrial sequence as indicated by the software Velvet. The length of the mitochondrial genome of *Facelina auriculata* is 14,171 bp, comprising 13 PCGs, 2 ribosomal RNAs, and the 22 tRNAs (Figure 2(a)). The overall GC% content of the mitogenome is 35%. The PCGs of *F. auriculata* mitogenome comprise 35.5% GC, the rRNAs have 32% GC and the tRNAs have 35.8% GC. The mitogenome of *Runcina aurata* has a total length of 14,282 bp. This mitogenome also contains the structure 13 PCG + 2 rRNA + 22 tRNA genes (Figure 2(b)). The overall GC% content is 25.7%. When focusing on PCGs, rRNAs, and tRNAs the GC content shifts to 26.3%, 23.6%, and 25.5%, respectively. The order of genes in both mitogenomes is the same, with two clusters of five and eight PCGs separated by both rRNA genes (Figure 2). Nine PCGs and the 16S rRNA are found in the forward chain, and four PCGs and the 12S rRNA are found in the reverse chain (Figure 2). The tRNA are interspersed along the mitochondrial genome (Figure 2), and can be found alone (5 times) or aggregated in clusters of 2 tRNA (4 times), 3 tRNA (1 time) or 4 tRNA (1 time). The phylogenetic analyses show *Facelina auriculata* clustered with *Facelina bostoniensis* with a bootstrap support value of 100, and the genus *Facelina* being the sibling taxon to the other Facelinidae species *Sakuraeolis japonica* (Figure 3(a)). *Runcina aurata,* and therefore, Runcinida, results in the sibling taxon to Aplysiida and Cephalaspidea with a bootstrap support value of 100 (Figure 3(b)).

Discussion and conclusions

The mitogenomes presented in this work help us complete the picture of the heterobranchs. Both mitogenomes have the common set of eukaryotic mitochondrial genes (Garesse & Vallejo 2001), with 13PCGs + 2rRNA + 22tRNA, and these are arranged following the common structure of heterobranchs (Varney et al. 2021). The compaction of heterobranchs mitogenomes, resulting in relatively small-sized mitochondrial genomes, could have contributed to maintaining gene order across members of the Superorder (Ghiselli et al. 2021; Varney et al. 2021).

The GC content of both species is different, with the mitogenome of *R. aurata* being the one with the lowest GC content reported so far within heterobranchs, decreasing the GC content of *Phyllidiopsis krempfi* from 28.1% (Kim et al. 2021) to 25.7%. The mitogenome of *R. aurata* also presents a region of unexpectedly low coverage, which may indicate that primers were suboptimal or a region of high complexity that the assembly software could not resolve due to repetitive elements or a high abundance of tRNA (Kinkar et al. 2020). This result highlights the relevance of generating new mitogenome data of runcinid species, as it would allow to better resolve by comparison the evolutionary processes and nature of this highly-complex mitogenomic region.

Table 1. List of the 12 species used to construct mitogenome trees, their taxonomic position, NCBI accession numbers, authors and year.

Mitogenome assembly	Order	Family	Accession number	Reference
Hermissenda emurai	Nudibranchia	Myrrhinidae	MK279704	Dinh Do et al. (2019)
Berghia stephanieae	Nudibranchia	Aeolidiidae	MW027646	Melo Clavijo et al. (2021)
Protaeolidiella atra	Nudibranchia	Pleurolidiidae	MN911169	Do et al. (2020a)
Sakuraeolis japonica	Nudibranchia	Facelinidae	KX610997	Karagozlu et al. (2016)
Facelina bostoniensis	Nudibranchia	Facelinidae	OQ772261	GenBank
Aplysia californica	Aplysiida	Aplysiidae	AY569552	Knudsen et al. (2006)
Philine kinglipini	Cephalaspidea	Philinidae	OQ579153	GenBank
Bullacta caurina	Cephalaspidea	Haminoeidae	MH924166	GenBank
Haloa japonica	Cephalaspidea	Haminoeidae	MN911170	Do et al. (2020b)
Haminoea flavescens	Cephalaspidea	Haminoeidae	MW590258	GenBank
Facelina auriculata	Nudibranchia	Facelinidae	OP661154	Present study
Runcina aurata	Runcinida	Runcinidae	OP661155	Present study

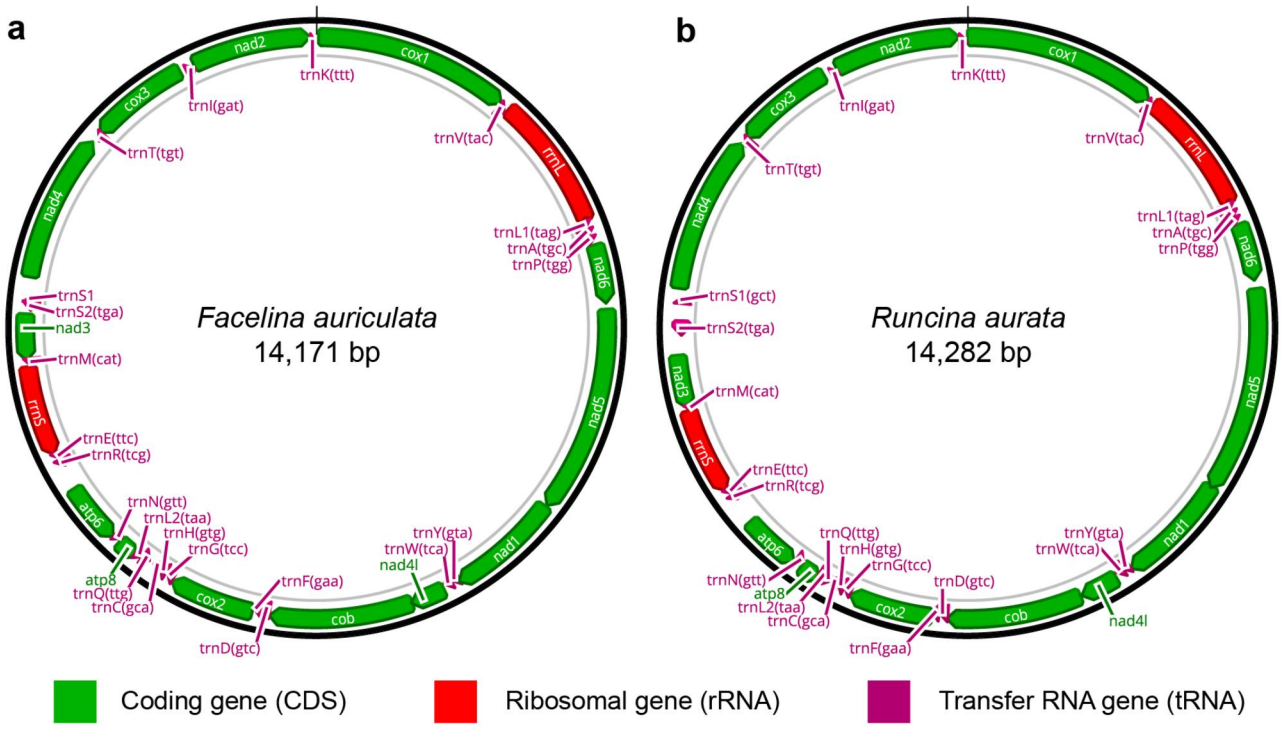

Figure 2. Gene map of the two newly circularized mitogenomes. (a) Gene map of *Facelina auriculata*. (b) Gene map of *Runcina aurata*.

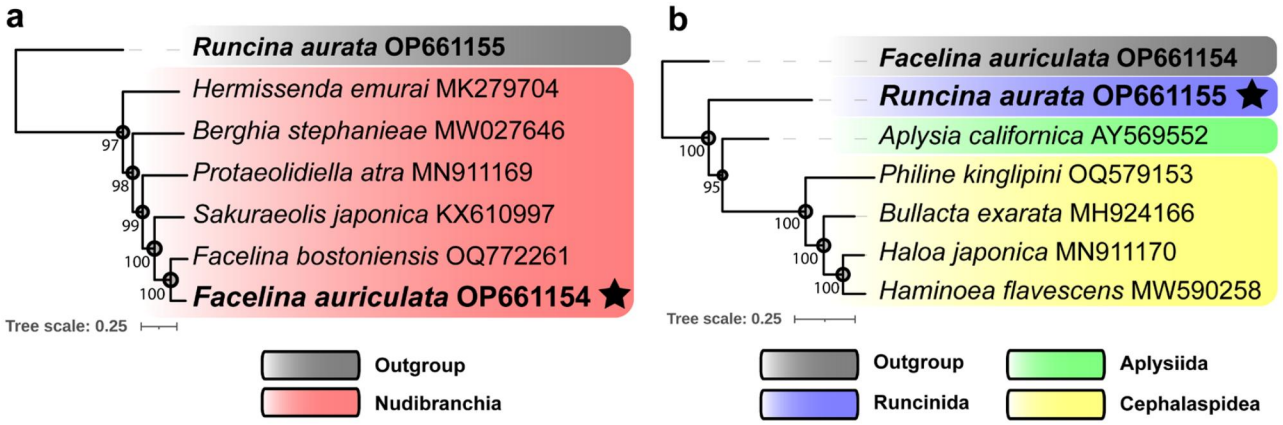

Figure 3. Best maximum likelihood phylogeny recovered for both mitochondrial genomes. Bootstrap node supports above 95 are signaled with a circle. Bold font indicate the species newly included in this study. (a) Aeolidioidea (Mollusca: Heterobranchia: Nudibranchia) phylogeny including all available mitogenomes of the family. (b) Tectipleura (Mollusca: Heterobranchia) phylogeny including available mitogenomes from Aplysiida and Cephalaspidea. *Bullacta exarata* is a synonym for *Bullacta caurina*, and *Haminoea flavescens* is a synonym for *Haloa japonica*. Note that we have not changed the species' names to keep NCBI's nomenclature. References for mitogenomes used in this study can be found at Table 1.

The mitogenomes of *R. aurata* and *F. auriculata* will be an invaluable new reference for exploring the phylogenetic relationships within heterobranchs, given that Runcinida is a largely unexplored taxon and *F. auriculata* is the type taxon of the family Facelinidae. Although our results corroborate previous knowledge generated using mitogenomes (Varney et al. 2021), our study also makes evident the lack of Runcinid and other related taxa mitogenome sequences in NCBI. Deeper efforts in the sequencing of neglected taxa are essential to improve public databases that may allow to fully resolve the phylogenetic position of these taxa. Moreover, two of the mitogenomes included in the analysis have recently been synonymized (Oskars et al. 2019; Oskars & Malaquias 2022), being *Bullacta exaracta* now *Bullacta caurina*, and being *Haminoea flavescens* now *Haloa japonica*. Cephalaspideans, as well as Runcinida, are a highly neglected taxonomic group, and therefore their members are subjected to continuous taxonomic changes as molecular revisions are carried out. The mitogenome of *Haminoea flavescens*, one of the few assemblies currently available for Cephalaspidea in NCBI, turned out to be redundant (Oskars & Malaquias 2022), limiting inter-specific studies although it will allow comparison at the species level.

Finally, the sequences provided here offer a unique opportunity to design specific primers for mitochondrial-targeted markers, as in some cases it has been shown that the standard set of primers for cytochrome oxidase I (Folmer et al. 1994), and the standard set of primers for 16S rRNA (Palumbi et al. 1991) lack specificity and hamper PCR amplification when used for the obtention of sequences of specimens

belonging to Facelinidae and Runcinida (Carmona et al. 2013; Araujo et al. 2023).

Ethics statements

None of the species used in the present study is included in any protected or threatened species on the IUCN Red List or the Spanish and Portuguese governments. Therefore, no specific permissions or licenses were needed for the sampling. We followed ethical procedures to ensure no substantial harm to the collecting individual.

Authors' contributions

FP, MP, and JLC conceived the study. FP carried out the molecular analyses prior to sequencing. CG, FP, LC, AKA, and MRM analyzed the data. CG drafted the manuscript. All authors critically reviewed the article. All authors approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data that support the findings of this study are openly available in GenBank of NCBI at https://www.ncbi.nlm.nih.gov/genbank/, under the accession numbers OP661154 for *Facelina auriculata* and OP661155 for *Runcina aurata*. The associated BioProject, SRA, and Bio-Samples numbers are PRJNA947946, SRR23947280 (*Facelina auriculata*) and SRR23947281 (*Runcina aurata*), and SAMN33870547 (*Facelina auriculata*) and SAMN33870546 (*Runcina aurata*) respectively. GaliaCamps_MainBody_preprint.docx available at: https://www.authorea.com/users/492660/articles/617135-runcinidae-and-facelinidae-two-complete-mitogenomes-of-understudied-and-misleading-heterobranch-families-gastropoda-mollusca

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