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Original Research Articles

Mitogenome characterization of the marine annelid *Hyalinoecia robusta* (Annelida: Onuphidae) and its preliminary phylogenetic assessment

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The deep-sea quill worm *Hyalinoecia robusta* (Annelida: Onuphidae) is an ecologically important benthic species, yet its mitochondrial genomic resources remain extremely limited. In this study, we assembled and characterized the complete mitochondrial genome of *H. robusta* using Illumina short-reads, and performed a preliminary phylogenetic analysis based on concatenated mitochondrial protein-coding genes (PCGs). The circular mitogenome is 15,029 bp in length and contains the standard set of 37 metazoan mitochondrial genes, including 13 PCGs, 22 transfer RNA genes, and 2 ribosomal RNA genes. The overall nucleotide composition shows a strong A+T bias (66.45%), with negative AT and GC skews. Maximum-likelihood phylogenetic analyses using both nucleotide and amino-acid sequences of the 13 PCGs consistently recovered *H. robusta* as the sister group to two *Diopatra* species (Onuphidae), while three *Marphysa* species (Eunicidae) formed a separate lineage. The newly characterized mitogenome provides a valuable genetic resource for molecular identification, comparative mitogenomic studies, and biodiversity assessment of marine annelids, and offers preliminary mitogenomic support for the close affinity between *Hyalinoecia* and *Diopatra* within Onuphidae.

INTRODUCTION

Marine annelids are important components of benthic ecosystems and contribute substantially to sediment bioturbation, nutrient cycling, and community dynamics across coastal to deep-sea habitats.[1,2] Within the order Eunicida, the family Onuphidae represents a diverse and ecologically significant group of tube-dwelling polychaetes that inhabit soft sediments and play pivotal roles in benthic structuring and energy flow.[3] Members of the subfamily Hyalinoeciinae, including the genus Hyalinoecia, are distinctive for their free-living epibenthic lifestyle; unlike most Onuphinae, they are mobile epibenthic species that construct quill-like tubes from which they protrude and move in a caterpillar-like fashion known as epibenthic crawling, thereby facilitating mobility on the seafloor.[2,4] *Hyalinoecia robusta* Southward, 1977, a deep-sea quill worm often found at depths of 400–1,600 m in muddy sediments, exhibits unique life-history traits such as simultaneous hermaphroditism with an adolescent male phase and annual iteroparous reproduction.[2,5] Despite its ecological relevance in deep-sea benthic communities, genomic resources for *H. robusta* and many other onuphid taxa remain scarce, limiting insights into their evolutionary history and functional adaptations.

Animal mitochondrial genomes (mitogenomes) typically comprise a compact circular molecule that encodes the standard set of 37 genes, including 13 protein-coding genes (PCGs), 22 transfer RNA genes, and 2 ribosomal RNA genes.[6] These genomes are widely valued for phylogenetics and species identification owing to their maternal inheritance, absence of introns, relatively high evolutionary rate, and highly conserved gene content across metazoans. In annelids, mitogenomes have proven particularly useful for resolving relationships at the family and genus levels, revealing gene-order variations in certain lineages, and fa-

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cilitating comparative analyses of evolutionary patterns.[7,8] However, gene-order conservation is generally high in Annelida, with most rearrangements occurring in specific clades, and mitogenomic data often provide stronger signal for shallower phylogenetic nodes than for deep splits.[9] Recent systematic studies have substantially expanded annelid mitogenome datasets, yet taxon sampling remains highly uneven, with many families, including the ecologically important Onuphidae, still severely underrepresented.

Within Onuphidae, complete mitogenomes are particularly scarce. To date, only a few have been fully characterized, primarily from Diopatra species, which severely constrains robust phylogenetic assessments of intrafamilial relationships.[5,10] Previous molecular phylogenies of Onuphidae, based on nuclear and mitochondrial markers, have revealed complex relationships, supported the monophyly of the subfamilies Onuphinae and Hyalinoeciinae, and suggested the presence of potential cryptic diversity within several genera.[10,11] Mitogenome-level data could therefore provide additional resolution for closely related taxa, while also revealing evolutionary signals in gene content, nucleotide composition bias, and codon usage.[12] In Onuphidae, several key questions remain unresolved, including the precise intergeneric relationships within subfamilies such as Hyalinoeciinae and the phylogenetic placement of Hyalinoecia relative to other onuphid lineages.

Hyalinoecia robusta is an ideal candidate for mitogenome characterization given its significant ecological role in deep-sea soft-sediment habitats, its distinctive morphology and epibenthic crawling behavior within the subfamily Hyalinoeciinae, and the taxonomic ambiguities surrounding onuphid diversity.[2,10] The scarcity of genomic resources for this species and the subfamily Hyalinoeciinae contributes to broader challenges in understanding onuphid evolution, including potential cryptic speciation and physiological adaptations to deep-sea conditions. By generating and analyzing the complete mitogenome of *H. robusta*, this study addresses a clear and longstanding knowledge gap in annelid mitochondrial genomics. In this study, we assembled and annotated the complete mitochondrial genome of *H. robusta* and evaluated its phylogenetic position within Onuphidae using concatenated sequences of the 13 mitochondrial protein-coding genes. Our objectives were to characterize the genomic features of the H. robusta mitogenome and to provide a preliminary phylogenetic framework for this species based on currently available annelid mitochondrial genomes. The newly generated mitogenome also offers a valuable genetic resource for future molecular identification, comparative mitogenomic analyses, and biodiversity assessment of deep-sea benthic annelids.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Specimens of *Hyalinoecia robusta* were collected from a single station in the South China Sea (17.29°N, 110.82°E) using a grab sampler during a research cruise on 6 April 2025, at a water depth of 1,627 m. The sampling site was characterized by soft, muddy sediment with a bottom water temperature of 2.73°C. A total of nine specimens was obtained from the sediment samples (Figure 1). All specimens were immediately preserved in 95% ethanol on board and transported to the laboratory under chilled conditions. One individual was morphologically identified as *H. robusta* based on key taxonomic characters. The voucher specimen (Holotype: TIO-BTS-ONU-2201) was deposited in the Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, China. Total genomic DNA was extracted from muscle tissue using a standard cetyltrimethylammonium bromide (CTAB) protocol. The quantity and quality of the DNA were checked on a standard agarose gel electrophoresis and a Qubit Fluorometer (Invitrogen, USA), respectively.

Figure 1. Tubes of *H. robusta*.

SEQUENCING, ASSEMBLY, AND ANNOTATION

To obtain the complete mitochondrial genome sequence, approximately 1 μg of high-molecular-weight genomic DNA was used to construct a paired-end library. The DNA was purified using the TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The purified DNA was then fragmented to an average insert size of 350 bp using a Covaris M220 sonicator. Sequencing libraries were prepared using the NEBNext Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. After adapter ligation, libraries were amplified with 8 PCR cycles. Library quality and insert size were validated using agarose gel electrophoresis and a Qubit Fluorometer before sequencing on the Illumina NovaSeq 6000 platform. Raw reads were trimmed to remove low-quality bases and adapter sequences using fastp with the following parameters: -q 10 -u 50 -y -g -Y 10 -e 20 -l 100 -b 150 -B 150.[13] Approximately 6 Gb of Illumina short

reads (clean data) were generated, yielding an average mitogenome coverage of >1,200×, with mitochondrial reads comprising about 0.8–1.2% of total reads.

All the clean reads were used for *de novo* assemblies using GetOrganelle v1.7.7.0 with the parameter: -R 10 -k 21,45,65,85,105 -F animal_mt.[14] Protein-coding genes were initially annotated using MitoZ v3.6 and then manually checked by comparison with related annelid mitochondrial genomes available in GenBank.[15] Transfer RNA genes and ribosomal RNA genes were identified using the MITOS web server with the invertebrate mitochondrial genetic code.[16] Gene boundaries, start and stop codons were manually verified and refined by comparing the sequences with closely related onuphid and eunicid mitogenomes (e.g., *Diopatra* and *Marphysa* species) using MEGA X.[17] Overlapping regions between adjacent genes were also checked visually to ensure annotation accuracy. Base composition and relative synonymous codon usage (RSCU) were calculated using the PhyloSuite v1.2.3.[18] The AT and GC skews were calculated according to the following formulae: AT-skew = (A−T)/(A+T) and GC-skew = (G−C)/(G+C) (Perna and Kocher. 1995). The mitogenome of *H. robusta* was visualized using OGDRAW v1.3.1 with default parameters.[19]

PHYLOGENETIC ANALYSIS

To elucidate the phylogenetic position of *H. robusta* within the order Eunicida, phylogenetic analyses were conducted using a concatenated mitochondrial dataset comprising 13 shared PCGs from 7 species within Eunicida, with *Cryptonome barbada* used as the outgroup. The nucleotide sequences of the 13 PCGs were extracted, aligned using MAFFT v7.310,[20] and trimmed with TrimAl v1.4 prior to concatenation.[21] The aligned genes were then concatenated into a single nucleotide dataset. In parallel, the corresponding amino-acid sequences translated from the same 13 PCGs were also concatenated into a protein dataset. The optimal substitution model was identified using ModelFinder v2.2.0 based on the Bayesian Information Criterion (BIC).[22] Maximum-likelihood phylogenetic analyses were performed using IQ-TREE v2.2.0 with 1,000 ultrafast bootstrap replicates.[23] We used iTOL v5 (https://itol.embl.de) to visualize the derived ML trees,[24] with the nucleotide-based topology as the main tree and support values from both the nucleotide and amino-acid analyses were displayed together at corresponding nodes.

RESULTS

MITOGENOME ORGANIZATION OF *H. ROBUSTA*

The complete mitogenome of *H. robusta* is a circular molecule of 15,029 bp in length (GenBank accession number PP790749) (Figure 2), which is longer than that of *Diopatra* sp. but shorter than those of other Eunicidae species (Table 1). The total length of intergenic regions is 526 bp, constituting 3.50% of the mitogenome, which is comparable to that observed in other Eunicida species, reflecting a compact mitochondrial genome organization in this order. The mitogenome of *P. amurensis* contains the standard set of 37 genes, including 13 PCGs, 22 transfer RNA genes, and 2 ribosomal RNA genes, together with a putative control region of 541 bp. All genes are encoded on the heavy strand.

The mitogenome exhibited a nucleotide composition of 30.92% A, 35.53% T, 21.42% C, and 12.13% G, yielding an A+T content of 66.45%, which significantly exceeded the C+G content (33.55%; Table 1). Interestingly, the GC content of three Eunicidae species (*H. robusta*, *Diopatra* sp., *Diopatra cuprea*) was significantly lower than that of species from other families. The *H. robusta* mitogenome showed a clear bias in nucleotide composition with a negative AT skew (-0.069) and a negative GC skew (-0.277). The base composition and skewness are consistent with most studies in polychaete annelids. The RSCU analysis of the PCGs in the *H. robusta* mitogenome revealed that 31 codons have RSCU values greater than 1.00, among which 31end with either A or U, suggesting a strong AT bias in the third codon (Figure 3)

All PCGs encoded by the *H. robusta* mitogenome have a methionine (ATG) as the start codon. Four PCGs stop with codon "TAG" (namely, *ND3*, *ND4*, *COX3*, and *CYTB*), while the others stop with codon "TAA" (Table 2). Additionally, a 39 bp overlap is present between *rrn16* and *trnL(uag)*, an 8 bp overlap between *ND6* and *CYTB*, and a 7 bp overlap between *ND4L* and *ND4* in the *H. robusta* mitogenome.

PRELIMINARY PHYLOGENETIC ASSESSMENT

Phylogenetic reconstruction based on the concatenated 13 mitochondrial protein-coding genes yielded a consistent major topology across the nucleotide and amino-acid datasets (Figure 4). The tree was rooted with *C. barbada*. The ingroup was clearly divided into two highly supported clades corresponding to Eunicidae (three *Marphysa* species) and Onuphidae (*H. robusta* and two *Diopatra* species) (Figure 4). Within the onuphid clade, *Diopatra* sp. and *D. cuprea* formed a sister pair with maximal support (100/100), and *H. robusta* was recovered as their sister with strong support (100/95). Within the eunicid clade, *M. sanguinea* and *M. victori* grouped together (100/99), and this subclade was sister to *M. tamurai* (100/100). These results indicate that *H. robusta* is more closely related to *Diopatra* than to *Marphysa* within the present taxon sampling. The concordant clustering pattern obtained from the nucleotide and amino-acid datasets, together with the high node support values from the topology, supports the preliminary phylogenetic placement of *H. robusta* within the currently sampled onuphid taxa.

DISCUSSION

In this study, we assembled and characterized the complete mitogenome of *H. robusta*, a deep-sea onuphid annelid, and performed a preliminary phylogenetic analysis based on concatenated mitochondrial PCGs. The *H. robusta* mitogenome is a circular molecule of 15,029 bp containing the standard set of 37 metazoan mitochondrial genes (13 PCGs, 22 tRNAs, and 2 rRNAs). Its overall organization, compact

Table 1. General features of mitogenomes in the order Eunicida.

Genome Features	*Hyalinoecia robusta*	*Diopatra* sp.	*Diopatra cuprea*	*Marphysa victori*	*Marphysa tamurai*	*Marphysa sanguinea*	*Cryptonome barbada*
Genome Size (bp)	15,029	14,985	15,050	15,891	15,163	15,159	15,013
GC Content (%)	33.55	30.25	32.22	40.94	39.37	39.04	43.23
rRNA/tRNA/PCGs/Total	2/22/13/37	2/22/13/37	2/22/13/37	2/22/13/37	2/22/13/37	2/22/13/37	2/22/13/37
PCG Total Length (bp)	11,169	11,214	11,126	11,112	11,114	11,114	11,089
PCG Average Length (bp)	859	863	856	855	855	855	853
PCG's GC Content (%)	34.43	30.79	33.11	42.55	40.44	40.19	44.32
% of Genome (PCGs)	74.32	74.83	73.93	69.93	73.30	73.32	73.86
Intergenic Region Length (bp)	526	419	614	1,380	660	572	426
AT-skew/GC-skew	-0.069/-0.277	-0.059/-0.300	-0.120/-0.354	-0.004/-0.350	-0.003/-0.361	0.007/-0.357	0.028/-0.323
GenBank Accession	PP790749	PX116062	NC_058588	NC_060759	NC_037236	NC_023124	NC_037947

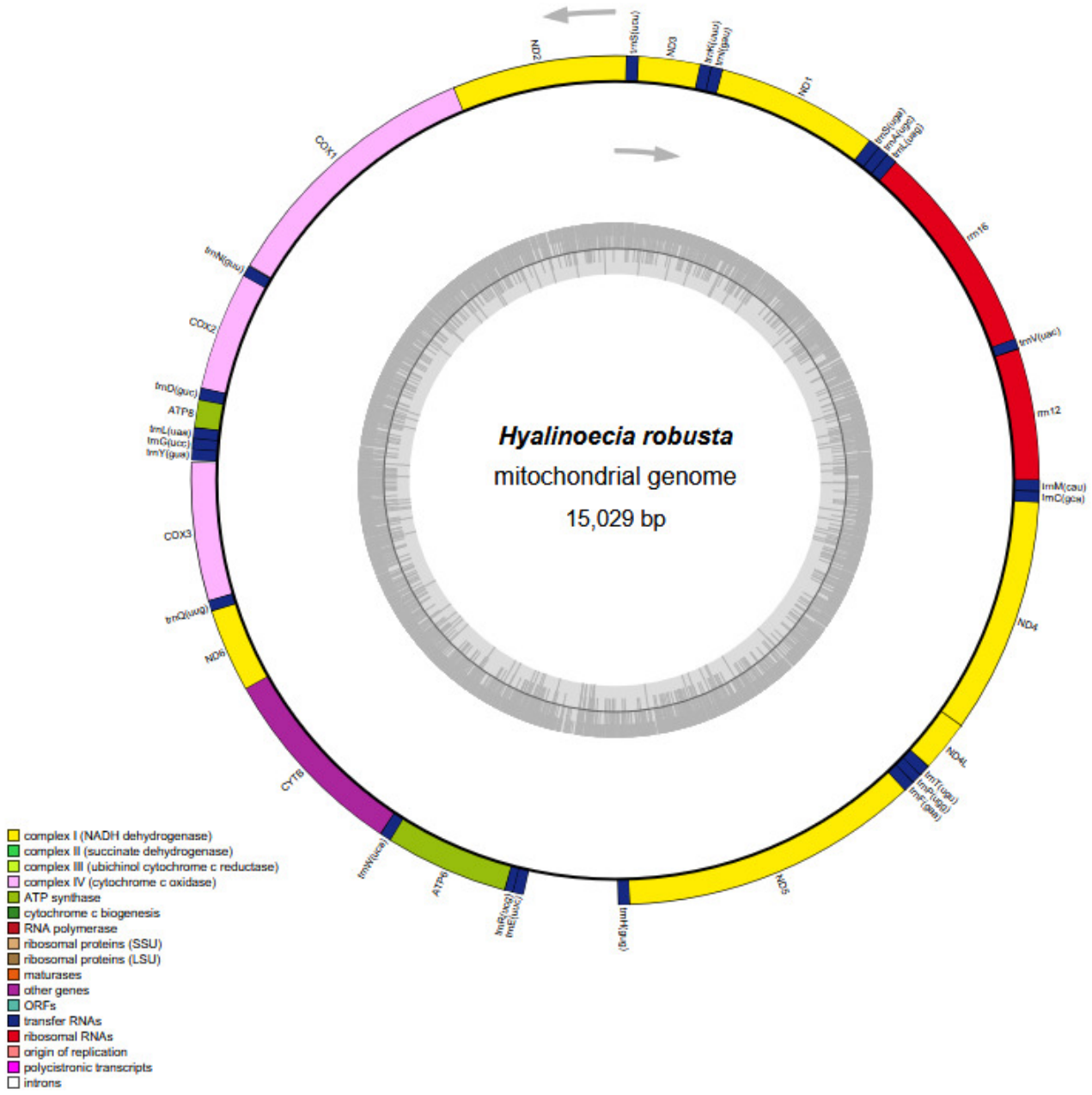

Figure 2. The circular diagram of the *H. robusta* mitogenome.

The outer ring represents the H-strand, and the inner ring represents the L-strand. Within the inner grey circle, the distribution of GC and AT contents is illustrated, with dark areas indicating GC content and light areas indicating AT content.

size, and gene content are highly consistent with those reported for other annelids, particularly members of Eunicida.[5,12,25] The total length falls within the range observed in Onuphidae (14,985-15,050 bp; Table 1) and is slightly shorter than that of most Eunicidae species, which typically exceed 15,150 bp. The intergenic region length (526 bp, 3.50% of the genome) is comparable to that of *Diopatra* sp. and *D. cuprea*,[5,12] reflecting a generally compact mitogenome architecture in this family.

The nucleotide composition of the *H. robusta* mitogenome exhibits a marked A+T bias (66.45%), with negative AT skew (-0.069) and GC skew (-0.277). Such strong AT enrichment is a common feature in annelid mitochondrial genomes, especially in polychaetes.[7] More interestingly, when comparing across Eunicida, the three Onuphidae species (*H. robusta*, *Diopatra* sp., *D. cuprea*) show significantly lower GC contents (30.25-33.55%) than the representatives of Eunicidae (39.04-40.94%) and the outgroup *Cryptonome barbada* (43.23%). This consistent difference suggests that Onuphidae may have experienced distinct evolutionary pressures that have shaped their mitochondrial base composition. Lower GC content is often associated with reduced energetic costs for replication and transcription. It could reflect adaptation to specific environmental conditions, such as hypoxic or deep-sea habitats where energetic efficiency is at a premium.[26] However, the

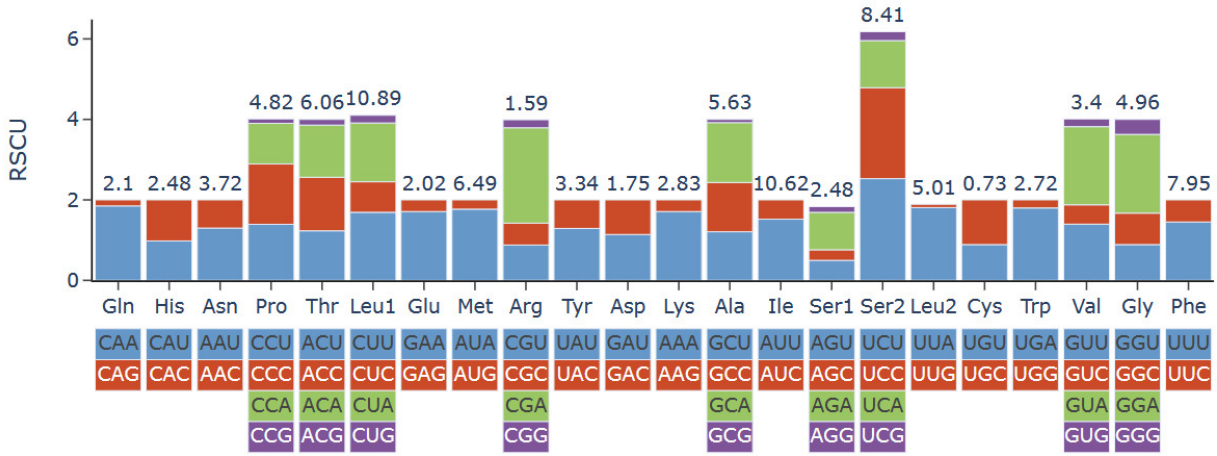

Figure 3. RSCU of the *H. robusta* mitogenome.

Numbers to the left refer to the total number of the RSCU values. Codon families are plotted on the x-axis.

precise ecological or molecular mechanisms driving this GC disparity among eunicidan families remain to be explored. The RSCU analysis further supports the strong A+T bias, as the majority of codons with RSCU > 1 end with A or U, a pattern typical of AT-rich annelid mitogenomes.

Phylogenetic analyses based on concatenated nucleotide and amino acid sequences of the 13 mitochondrial PCGs consistently recovered *H. robusta* as the sister group to the two Diopatra species, with strong bootstrap support (100/95 for the nucleotide and amino acid datasets, respectively). The three Marphysa species (family Eunicidae) formed a separate, well-supported clade. This topology aligns with the current taxonomic recognition of Onuphidae and Eunicidae as distinct families within Eunicida,[10] and provides preliminary mitogenomic evidence for a close affinity between *Hyalinoecia* and *Diopatra*. Previous phylogenetic studies employing nuclear and mitochondrial markers (16S and 18S rDNA) also supported the monophyly of Onuphidae and placed Hyalinoecia within a clade containing *Diopatra* and other onuphid genera.[10] Thus, our results offer independent mitogenomic support for these findings and extend the currently limited mitogenomic framework for Onuphidae, which previously included only *Diopatra* species.[5,12]

The phylogenetic interpretation of the present dataset should remain cautious. Complete mitogenomes are still scarce for Onuphidae, and taxon sampling within the family remains sparse. Previous phylogenetic studies based on nuclear and mitochondrial markers have shown that onuphid relationships are more complex than can be fully resolved with a few representative taxa alone.[27] In the present study, both nucleotide- and amino-acid-based analyses recovered the same major clustering pattern, which supports the robustness of the principal relationships observed here. However, slight differences in node support between the two datasets suggest that deeper relationships among the sampled lineages are not yet fully stabilized. Accordingly, the phylogeny presented here is best regarded as a preliminary assessment of the placement of *H. robusta*, rather than a comprehensive reconstruction of intrafamilial relationships within Onuphidae. Beyond its phylogenetic implications, the mitogenome reported here also represents a useful genetic resource for marine annelid research. Onuphid worms are ecologically important members of benthic communities and, in some cases, are relevant to aquaculture and bait-worm use. Additional mitochondrial genome data from this group will be valuable for molecular identification, comparative mitogenomics, and biodiversity assessment of marine benthic annelids. Future studies incorporating broader taxon sampling, additional onuphid mitogenomes, and nuclear genomic markers will be necessary to test the stability of the relationships recovered here and to establish a more complete phylogenetic framework for Onuphidae and related annelid lineages.

CONCLUSION

This study reports the complete mitochondrial genome of *H. robusta*, a marine annelid of the family Onuphidae. The mitogenome is 15,029 bp in length and contains the typical set of 37 mitochondrial genes, with a pronounced A+T bias of 66.45%. Phylogenetic analyses based on concatenated nucleotide and amino-acid sequences of the 13 mitochondrial PCGs consistently support a close relationship between *H. robusta* and Diopatra species. The newly characterized mitogenome provides a useful genetic resource for molecular identification and future comparative studies of marine annelids.

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Table 2. The organization of the mitogenome of *H. robusta*.

Gene	Location		Codon		Length (bp)	Strand	Intergenic nucleotides[a]
	From	To	Start	Stop			
rrn12	1	754			754	+	0
trnV(uac)	755	809			55	+	1
rrn16	811	2068			1258	+	-39
trnL(uag)	2030	2089			60	+	0
trnA(ugc)	2090	2150			61	+	0
trnS(uga)	2151	2216			66	+	0
ND1	2217	3146	ATG	TAA	930	+	1
trnI(gau)	3148	3208			61	+	0
trnK(uuu)	3209	3271			63	+	1
ND3	3273	3626	ATG	TAG	354	+	1
trnS(ucu)	3628	3692			65	+	0
ND2	3693	4691	ATG	TAA	999	+	1
COX1	4693	6243	ATG	TAA	1551	+	9
trnN(guu)	6253	6311				+	0
COX2	6312	6992	ATG	TAA	681	+	0
trnD(guc)	6993	7055			63	+	0
ATP8	7056	7217	ATG	TAA	162	+	1
trnL(uaa)	7219	7275			57	+	2
trnG(ucc)	7278	7337			60	+	0
trnY(gua)	7338	7399			62	+	16
COX3	7416	8204	ATG	TAG	678	+	1
trnQ(uug)	8206	8263			58	+	0
ND6	8264	8740	ATG	TAA	477	+	-8
CYTB	8733	9869	ATG	TAG	1137	+	1
trnW(uca)	9871	9930			60	+	0
ATP6	9931	10642	ATG	TAA	712	+	1
trnR(ucg)	10644	10686			43	+	0
trnE(uuc)	10687	10748			62	+	542
trnH(gug)	11291	11351			61	+	0
ND5	11352	13072	ATG	TAA	1721	+	1
trnF(gaa)	13074	13133			60	+	0
trnP(ugg)	13134	13195			60	+	0
trnT(ugu)	13196	13256			61	+	0
ND4L	13257	13553	ATG	TAA	297	+	-7
ND4	13547	14905	ATG	TAG	1359	+	1
trnC(gca)	14907	14965			59	+	0
trnM(cau)	14966	15029			64	+	0

a: the positive number indicates interval base pairs between genes, while the negative number indicates the overlapping base pairs between genes.

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AUTHORS' CONTRIBUTION

Formal Analysis: Yaqin Huang (Equal), Site Luo (Equal), Heshan Lin (Equal). Funding acquisition: Yaqin Huang (Equal), Xuebao He (Equal). Methodology: Yaqin Huang (Lead). Project administration: Yaqin Huang (Equal), Xuebao He (Equal). Writing – original draft: Yaqin Huang (Lead). Investigation: Xuebao He (Equal), Junhui Lin (Equal), Kun Liu (Equal), Jianfeng Mou (Equal). Resources: Qianyong Liang (Lead). Supervision: Zhongbao Li (Lead). Writing – review & editing: Zhongbao Li (Equal).

COMPETING OF INTEREST – COPE

No competing interests were disclosed.

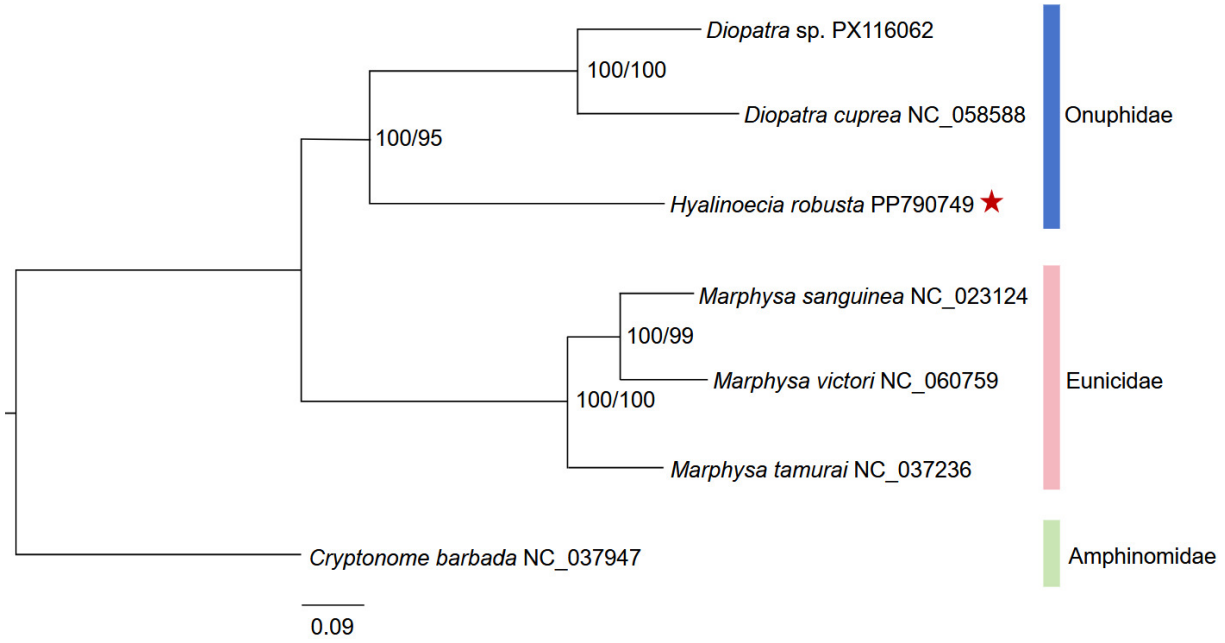

Figure 4. Maximum-likelihood phylogeny inferred from the concatenated 13 mitochondrial PCGs.

The displayed topology is based on the nucleotide dataset, and support values at the nodes are shown as nucleotide bootstrap support/amino-acid bootstrap support. *C. barbada* was designated as the outgroup.

ETHICAL CONDUCT APPROVAL – IACUC

The sample collection and animal experiments adhered to regulations and guidelines for the care and use of laboratory animals, and received approval from the Animal Care and Use Committee of Third Institute of Oceanography, Ministry of Natural Resources (protocol code TIO-IACUC-01-2023-03-20).

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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