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Genome skimming elucidates the evolutionary history of Octopoda

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ABSTRACT

Phylogenies for Octopoda have, until now, been based on morphological characters or a few genes. Here we provide the complete mitogenomes and the nuclear 18S and 28S ribosomal genes of twenty Octopoda specimens, comprising 18 species of Cirrata and Incirrata, representing 13 genera and all five putative families of Cirrata (Cirroctopodidae, Cirroteuthidae, Grimpoteuthidae, Opisthoteuthidae and Stauroteuthidae) and six families of Incirrata (Amphitretidae, Argonautidae, Bathypolypodidae, Eledonidae, Enteroctopodidae, and Megaleledonidae) which were assembled using genome skimming. Phylogenetic trees were built using Maximum Likelihood and Bayesian Inference with several alignment matrices. All mitochondrial genomes had the 'typical' genome composition and gene order previously reported for octopodiforms, except *Bathypolypus ergasticus,* which appears to lack ND5, two tRNA genes that flank ND5 and two other tRNA genes. Argonautoidea was revealed as sister to Octopodidae by the mitochondrial protein-coding gene dataset, however, it was recovered as sister to all other incirrate octopods with strong support in an analysis using nuclear rRNA genes. Within Cirrata, our study supports two existing classifications suggesting neither is likely in conflict with the true evolutionary history of the suborder. Genome skimming is useful in the analysis of phylogenetic relationships within Octopoda; inclusion of both mitochondrial and nuclear data may be key.

1. Introduction

Modern cephalopods, excluding the ancient lineage of shelled nautiluses, comprise two superorders: Decapodiformes, which includes all squids and cuttlefishes, and Octopodiformes, which includes the monospecific vampire "squid" (order Vampyroteuthida) and the speciose order Octopoda. Octopoda comprises two suborders, Cirrata and Incirrata. Previous molecular work has provided strong support for the monophyly of Cirrata and Incirrata and the sister-taxon relationship between these suborders (Strugnell et al., 2005, 2004, 2014; Lindgren et al., 2012), which are well established on morphological grounds, supported by multiple characters in cladistic analyses (Young and Vecchione, 1996; Voight, 1997).

Incirrata is divided into two superfamilies, Argonautoidea and Octopodoidea (WoRMS Editorial Board, 2022). Several phylogenetic analyses place Argonautoidea as sister to all other incirrate octopuses (Strugnell et al., 2004; Lindgren et al., 2012); however, none of these were well supported and additional analyses have yielded alternative topologies (Strugnell et al., 2005, 2014). Argonautoidea is a morphologically highly diverse superfamily united by multiple characteristics, including a holopelagic lifestyle, sexual dimorphism, and a detachable hectocotylus in males, and is generally agreed to be monophyletic (e.g.,

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Bizikov, 2004; Strugnell and Allcock, 2010). However, its relationship to other incirrate octopuses is not well understood. Several authors recognize that argonautoids evolved from benthic ancestors; for example, Naef (1923) recognizes their similarity to benthic octopodids, while Bizikov (2004) traces reduction of stylets in argonaut lineages. Cladistic analyses have placed argonautoids as sister to some other pelagic (ctenoglossan) octopuses (Voight 1997), or in a three-way polytomy with benthic octopods and ctenoglossans (Young and Vecchione, 1996).

Previously, all benthic Octopodoidea were placed in the family Octopodidae, with the pelagic genera in separate families (Sweeney and Roper 1998). Early molecular evidence based on few genes suggested that Octopodidae was not monophyletic (Carlini and Graves, 1999; Carlini et al., 2001) and a more comprehensive molecular study suggested neotenous origins for the pelagic ctenoglossan families Vitreledonellidae and Bolitaenidae, placing these in a clade of benthic octopods rendering Octopodidae paraphyletic (Strugnell et al., 2004). A subsequent molecular analysis with broad taxon coverage and using seven genes (Strugnell et al., 2014) provided a revised taxonomy combining the ctenoglossans into one family, Amphitretidae, and splitting the benthic octopods into five families: Bathypolypodidae, Eledonidae, Enteroctopodidae, Megaleledonidae and Octopodidae. A strict consensus tree of the multiple analyses in this latter study resulted in unresolved relationships among families.

Cirrate octopods inhabit the deep sea and are known from the upper slope to hadal depths (Jamieson and Vecchione, 2021). They are characterized by the presence of paired cirri along a single series of arm suckers, and paired fins supported by a cartilaginous internal shell (Voss, 1988, Collins and Villanueva, 2006). Morphologically, cirrates are considered to be primitive (Young et al., 1998).

Within Cirrata, there is still no consensus on the number of families and their divisions (Piertney et al., 2003; Collins and Villanueva, 2006; Vecchione et al., 2016; Pardo-Gandarillas et al., 2021). Gaps in knowledge concerning their biology, ecology and behaviour make understanding their systematics and phylogeny difficult. Our knowledge is limited because cirrates are caught in small numbers and are extremely fragile, meaning that trawled specimens are often too damaged to yield useful morphological taxonomic information. Cirrates are also hard to sample due to their depth of occurrence, although the extension of trawling into deeper waters has yielded additional specimens, occasionally in high numbers (Vecchione et al., 1998; Golikov et al., 2022) and ROV surveys are yielding high-quality images and occasional captures. However, because cirrates are mostly captured at low densities, sampling tends to be opportunistic. New data for cirrates, such as presented in this article, are therefore vitally important to further our understanding of the group.

The most recent, thorough review of Cirrata (Collins and Villanueva, 2006), which considered both morphology and a phylogenetic analysis with broad taxon coverage based on mitochondrial 16S rRNA (Piertney et al., 2003), proposed a four-family division: Cirroteuthidae, Grimpoteuthidae, Opisthoteuthidae and Cirroctopodidae. Other family arrangements have since been proposed: Vecchione et al. (2016) suggested a different four-family division, of Cirroteuthidae, Opisthoteuthidae, Cirroctopodidae and Stauroteuthidae, which is the classification currently used by the World Register of Marine Species (WoRMS Editorial Board, 2022), while Pardo-Gandarillas et al. (2021) supported a three-family division into Cirroteuthidae, Cirroctopodidae and Opisthoteuthidae. These classifications are not widely different (Table 1). The only two phylogenetic studies that have focused on cirrates both used only the 16S rRNA gene (Piertney et al., 2003; Pardo-Gandarillas et al., 2021). Further molecular work with additional markers could aid in resolving the true evolutionary history of this clade.

Whole mitochondrial genomes are increasingly used in phylogenetic analyses as, in many taxonomic groups, mitochondrial genomes appear to resolve deep relationships (Boore et al., 2005; Cameron et al., 2007; Kayal et al., 2013; Wang and Lavrov, 2007). In addition to the analysis of

Table 1
Summary history of Cirrata systematics. Bold text indicates taxon erected during that study.

Naef, 1923	Robson, 1932	Voss, 1988	O'Shea, 1999*(regional)	Piertney et al., 2003**	Collins & Villanueva, 2006	Vecchione et al., 2016	Pardo-Gandarillas et al., 2021
Cirroteuthidae	Cirroteuthidae	Cirroteuthidae	Cirroteuthidae	Cirroteuthidae	Cirroteuthidae	Cirroteuthidae	Cirroteuthidae
Cirroteuthis	*Cirroteuthis*	*Cirroteuthis*	*Cirroteuthis*	*Cirroteuthis*	*Cirroteuthis*	*Cirroteuthis*	*Cirroteuthis*
Cirrothauma	*Cirrothauma*	*Cirrothauma*	*Cirrothauma*	*Cirrothauma*	*Cirrothauma*	*Cirrothauma*	*Cirrothauma*
Stauroteuthis	**Stauroteuthidae**	Staurotheuthidae	**Luteuthididae**	*Stauroteuthis*	*Stauroteuthis*	Staurotheuthidae	*Stauroteuthis*
Froekenia	*Stauroteuthis*	*Stauroteuthis*	***Luteuthis***	Opisthoteuthidae	= *Chunioteuthis*[b]	*Stauroteuthis*	Opisthoteuthidae
Chunioteuthis	***Grimpoteuthis***	= *Cirroctopus*[a]	Opisthoteuthidae	*Opisthoteuthis*	*Froekenia*[c]	Opisthoteuthidae	*Opisthoteuthis*
Cirroctopus	= *Cirroctopus*[a]	Opisthoteuthidae	***Grimpoteuthididae***	Grimpoteuthidae	Opisthoteuthidae	*Opisthoteuthis*	*Grimpoteuthis*
Opisthoteuthidae	*Chunioteuthis*	*Grimpoteuthis*	*Grimpoteuthis*	***Enigmatiteuthis***	*Opisthoteuthis*	*Cryptoteuthis*	*Luteuthis*
Opisthoteuthis	*Froekenia*	= *Cirroctopus*[a]	***Enigmatiteuthis***	*Grimpoteuthis*	Grimpoteuthidae	*Grimpoteuthis*	Cirroctopodidae
	Cirrothauma	*Opisthoteuthis*	Opisthoteuthidae	*Luteuthis*	*Grimpoteuthis*	*Luteuthis*	*Cirroctopus*
	Opisthoteuthidae	*Chunioteuthis*	*Opisthoteuthis*	New family	*Luteuthis*	= *Enigmatiteuthis*[d]	
	Opisthoteuthis	*Froekenia*	*Cirroctopus*	*Cirroctopus*	= *Enigmatiteuthis*[d]	Cirroctopodidae	
	Incertae sedis	*Cirroteuthopsis*			*Luteuthis*	*Cirroctopus*	
	Cirroteuthopsis				*Cryptoteuthis*		
					Cirroctopodidae		
					Cirroctopus		

Notes: *Regional study, only includes genera found in New Zealand waters. **Molecular study, only includes genera sequenced. [a]*Cirroctopus mawsoni* treated as *Grimpoteuthis*. [b]*Chunioteuthis ebersbachi* treated as junior synonym of *Stauroteuthis syrtensis*. [c]Considered nomen dubium. [d]*Enigmatiteuthis innominata* treated as *Grimpoteuthis*.

nucleotide data, analyses of mitochondrial gene order may also be phylogenetically informative (Boore and Brown, 1998; Yokobori et al., 2004; Akasaki et al., 2006; Allcock et al., 2011). Genome skimming is a shallow next-generation sequencing approach that allows for comparatively deep sequencing of high-copy genomes such as the mitogenome and complete nuclear ribosomal cluster (Straub et al., 2012; Dodsworth, 2015). It is relatively cheap, does not require fresh tissue, and can provide whole mitogenomes using low-concentration DNA. Despite these advantages, only two studies have used this method to investigate the phylogeny of cephalopod groups (Sanchez et al., 2021; Fernández-Álvarez et al., 2022). In this study we use genome skimming to recover the whole mitochondrial genomes and nuclear 18S rRNA and 28S rRNA genes from diverse octopod taxa, particularly focusing on groups where whole mitochondrial genomes have not previously been recovered, such as cirrates. We use these data to explore evolutionary relationships within Octopoda.

2. Methods

Tissue samples were obtained from colleagues and museums of twenty Octopoda specimens, comprising 18 species (two species were sequenced twice: *Luteuthis dentatus* and *Opisthoteuthis massyae)* of Cirrata and Incirrata, representing 13 genera and all four families of Cirrata (*sensu* Vecchione et al., 2016: Cirroctopodidae, Cirroteuthidae, Opisthoteuthidae and Stauroteuthidae) and six families of Incirrata (Amphitretidae, Argonautidae, Bathypolypodidae, Eledonidae, Enteroctopodidae, and Megaleledonidae). Collection locations are globally distributed to ensure good taxon coverage (Table 2).

2.1. DNA extraction, library preparation and sequencing

DNA was extracted using a standard phenol-chloroform protocol with additional liquid nitrogen and salt steps. Muscle tissue was placed in a prefrozen microtube which was then placed into liquid nitrogen. After 1 min, the muscle tissue was crushed using a sterilized micropestle. To digest muscle tissue, 500 μL of genomic digestion buffer (Invitrogen, California, US) and 55.5 μL Proteinase K (Invitrogen, California, US) were added and the microtube incubated for two hours at 55 °C. Once digested, 555.5 μL of 6 M NaCl was added and the sample was centrifuged at max speed in a minifuge for 10 min. Two 2 ml tubes were prepared with 550 μL of the supernatant and 55.5 μL RNase. After incubation at room temperature for 2 min, 500 μL phenol:chloroform: isoamyl was added to each microtube and the samples briefly inverted and centrifuged in a minifuge for 5 min at 13,000 RPM. The clear, aqueous phase was transferred to a tube containing 50 μL of sodium acetate and 1000 μL of 100% ethanol. The samples were placed in a −80° freezer for 90 min until a precipitate formed and then spun at 13,000 RPM at 4 °C for 30 min in a minifuge. The supernatant was discarded and the ethanol allowed to evaporate for 10 min. The purified DNA was eluted in 50 μL Elution buffer (Invitrogen, California, US) and the sample incubated at 37 °C overnight.

Quality control by Novogene indicated the total amount of DNA extracted per sample was low (from 0.08755 to 2.9964 μg). Genomic libraries were constructed by Novogene. The Novogene protocol sonicates the genomic DNA into random fragments of 350 bp. These fragments are then end polished, A-tailed, and ligated with the full-length adapters of Illumina sequencing, and further amplified with P5 and indexed P7 oligos. AMPure XP system is used to purify PCR products for final library construction. Library size distribution is checked with an

Table 2
Specimens sequenced in this study. Museum catalogue numbers provided where available.

GenBank Accession Number			Species ID	Paired-end reads	Length of mitogenome (bp)	Collection location	Museum Number
Mitogenome	**18S**	**28S**					
ON367810	ON156550	ON524400	*Grimpoteuthis* sp.	32,652,665	16,416	N Hebrides Terrace	
ON367811	ON156546	ON524405	*Opisthoteuthis* sp.	36,086,921	16,080	Monterey Bay, NE Pacific	USNM 1660928
ON367799	ON156531	ON524403	*Opisthoteuthis californiana* Berry, 1949	49,494,508	16,135	Bering Sea	
ON367807	ON156540	ON524402	*Opisthoteuthis massyae* (Grimpe, 1920)	51,026,516	16,410	N Hebrides Terrace	
ON367808	ON156541	ON524393	*Stauroteuthis syrtensis* Verrill, 1879	48,616,454	15,908	N Rockall Trough	
ON367818	ON156536	ON524390	*Eledone cirrhosa* (Lamarck, 1798)	61,371,420	16,135	Off Sant Carles de la Ràpita, NW Mediterranean	
ON367814	ON156543	ON524394	*Graneledone verrucosa* (Verrill, 1881)	56,866,544	17,000	W St Kilda Slope	
ON367815	ON156544	ON524395	*Muusoctopus johnsonianus* (Allcock et al., 2006)	50,158,236	16,306	W St Kilda Slope	
ON367800	ON156532	ON524386	*Cirroctopus glacialis* (Robson, 1930)	45,643,810	16,474	Heard Island	
ON367804	ON156537	ON524391	*Cirrothauma* sp.	41,427,824	15,968	Gorda Ridge, N. Pacific	FMNH 309245
ON367802	ON156534	ON524388	*Cirroteuthis muelleri* Eschricht, 1838	62,983,914	15,988	N. Atlantic HB, 2009 (Sta 28)	
ON367812	ON156542	ON524401	*Opisthoteuthis grimaldii* (Joubin, 1903)	47,408,440	16,465	N Hebrides Terrace	NHMUK 20200402
ON367809	ON156545	ON524396	*Luteuthis dentatus* (O'Shea, 1999)	48,029,470	16,676	Tasman Sea	NIWA 50746
ON367806	ON156539	ON524392	*Luteuthis dentatus* (O'Shea, 1999)	63,664,450	16,302	Tasman Sea	NIWA 95257
ON367801	ON156533	ON524387	*Cirrothauma murrayi* Chun, 1911	59,813,694	15,757	N. Atlantic; HB, 2009 (Sta 29)	
ON367803	ON156535	ON524389	*Cirrothauma magna* (Hoyle, 1885)	49,989,690	15,943	N. Atlantic HB, 2009 (Sta 27)	USNM 1502926
ON367805	ON156538	ON524404	*Opisthoteuthis massyae* (Grimpe, 1920)	50,455,296	16,561	S Hebrides Terrace	NHMUK 20200399
ON367813	ON156547	ON524399	*Bathypolypus ergasticus* (R. Fischer & H. Fischer, 1892)	66,983,054	14,046	SE Rosemary Slope	
ON367817	ON156548	ON524398	*Argonauta argo* Linnaeus, 1758	67,898,798	15,728	Off Tarragona, NW Mediterranean	ICMC000131
ON367816	ON156549	ON524397	*Bolitaena pygmaea* (Verrill, 1884)	70,669,190	16,083	Off the coast of Western Sahara	ICMC000124

Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Libraries are quantified by real-time PCR (to meet the criterion of 3 nM), pooled according to concentration and expected data volume, and sequenced on an Illumina Novaseq 6000 (150 bp paired-end run). Illumina reads are demultiplexed by Novogene and adapters are trimmed.

Assuming the estimated genome size of 2.7 GB reported for *Octopus bimaculoides* (Albertin et al., 2015), the Novogene Illumina protocol generated around 3.6–7.8X coverage per species (Table 2).

2.2. Assembly

2.2.1. Mitochondrial genes

The mitochondrial genome was assembled de novo for each species using NOVOplasty 4.2 (Dierckxsens et al., 2016). We used a short sequence, which varied according to availability but was usually a partial sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (COX1) or 16S rRNA genes, of the same, or of a closely related, species as a seed. We found that a k-mer value of 30, with the estimated genome size set to 12000–18000, consistently yielded a circularized genome in the absence of a reference sequence and without extending directly from the seed. Exceptions were *Opisthoteuthis grimaldii, Luteuthis dentatus* (NIWA 50746), and *Stauroteuthis syrtensis*. Using the same parameters stated above but extending directly from the seed, yielded a circularized genome for *O. grimaldii*, while using *O. massyae* as a reference genome produced a circularized genome for the *L. dentatus* sample. The *Stauroteuthis* sample was consistently problematic. Several long and overlapping contigs were produced using a k-mer of 20 and varying seeds, and these contigs appear to provide the entire genome (as presented here) but we were not able to generate an automatically circularized genome from Novoplasty for this sample.

In addition, mitochondrial genomes were downloaded from GenBank from an additional 19 species of Octopoda (Table 3). These mostly represent the family Octopodidae, but also included Enteroctopodidae, Tremoctopodidae, Argonautidae, and Vampyroteuthidae, providing an outgroup (*Vampyroteuthis*) and increasing our taxon sampling of incirrates by two families.

Genes and the order in which they occurred were identified for all 39 mitochondrial genomes using the MITOS Web Server (Donath et al., 2019). Gene boundaries for protein-coding genes were identified using MITOS and by checking for the presence of common start codons, such as ATG, and stop codons. The protein-coding genes were individually aligned using the MAFFT alignment server (Katoh et al., 2019) using the E-INS-i iterative refinement methods and the alignment was checked using the amino-acid translation. Where genes of particular species varied in length (such that the extremes of the alignment of each gene added no useful phylogenetic signal), genes were manually trimmed prior to phylogenetic analyses. All 13 protein coding genes were combined into a single FASTA file using FaBox (Villensen, 2007).

All mitochondrial genomes had thirteen protein coding genes (COX1-3, ATP6, ATP8, CYTB, ND1-6 and ND4L), except *Bathypolypus ergasticus* which was missing ND5 (see results).

The gene boundaries of the two ribosomal RNA subunits (12S rRNA and 16S rRNA) were identified using MITOS. Poorly aligned bases were removed using Gblocks (Castresana, 2000). We generated two alignments: a less stringent selection (allowing smaller final blocks, gap positions and less strict flanking positions), and a more stringent selection (not allowing many contiguous non-conserved positions).

2.2.2. Nuclear genes

The nuclear ribosomal genes 28S rRNA and 18S rRNA were also assembled from our 20 samples using NOVOPlasty 4.2, seeding with conserved regions of an alignment constructed from multiple 18S and 28S Octopoda sequences retrieved from GenBank. Multiple seeds recovered overlapping contigs used to assemble complete genes and partial flanking intergenic spacers. The gene was extracted from this assembly using RNammer 1.2 (Lagesen et al., 2007).

With the exception of *Vampyroteuthis*, no species of Octopodiformes had complete sequences of both 18S and 28S available on GenBank. However, we extracted 18S for *Octopus sinensis* by blasting a conserved region of 18S against this genome and extracting 18S from the returned genomic scaffold (NW_021824442.1) using RNammer 1.2. We were unable to retrieve a sequence recognized as 28S rRNA by RNammer using this method.

Nuclear ribosomal genes were aligned in MAFFT (using the E-INS-i iterative refinement methods). Poorly aligned regions were removed using Gblocks (Castresana, 2000). We again generated two alignments: a less stringent selection (allowing smaller final blocks, gap positions and less strict flanking positions), and a more stringent selection (not allowing many contiguous non-conserved positions).

Table 3
Details of complete mitochondrial genome sequences downloaded from GenBank, except last three rows which indicate nuclear ribosomal gene downloads: *=18S rRNA, **=28S rRNA.

Sample	Species ID	Family	Reference
NC028547	*Octopus bimaculatus* Verrill, 1883	Octopodidae	Domínguez-Contreras et al., 2016
NC044093	*Octopus mimus* Gould, 1852	Octopodidae	Magallon-Gayon et al., unpublished
NC029723	*Octopus bimaculoides* Pickford & McConnaughey, 1949	Octopodidae	Farfan et al., unpublished
KF017606	*Cistopus chinensis* Zheng et al., 2012	Octopodidae	Cheng et al., 2013
AB240156	*Amphioctopus fangsiao* d'Orbigny, 1839–1841	Octopodidae	Akasaki et al., 2006
NC023257	*Cistopus taiwanicus* Liao & Lu, 2009	Octopodidae	Cheng et al., 2013
NC029702	*Amphioctopus aegina* (Gray, 1849)	Octopodidae	Zhang et al., 2017
NC036354	*Argonauta hians* Lightfoot, 1786	Octopodidae	Chiu et al., 2018
NC006353	*Octopus vulgaris* Cuvier, 1797	Octopodidae	Yokobori et al., 2004
NC039847	*Octopus cyanea* Gray, 1849	Octopodidae	Ma et al., unpublished
NC039848	*Callistoctopus luteus* (Sasaki, 1929)	Octopodidae	Ma et al., unpublished
NC048475	*Argonauta nodosa* Lightfoot, 1786	Argonautidae	Zhang unpublished
MH899749	*Amphioctopus neglectus* (Nateewathana & Norman, 1999)	Octopodidae	Tang et al., 2019
NC015896	*Octopus minor* (Sasaki, 1920)	Octopodidae	Cheng et al., 2012
NC029747	*Octopus conispadiceus* (Sasaki, 1917)	Enteroctopodidae	Ma et al., 2016
NC036351	*Amphioctopus marginatus* (Taki, 1964)	Octopodidae	Tang et al., 2018
MK450541	*Octopus fitchi* Berry, 1953	Octopodidae	Magallon-Gayon et al., unpublished
KY649286	*Tremoctopus violaceus* delle Chiaje, 1830	Tremoctopodidae	Shen unpublished
NC038213	*Octopus variabilis* (Sasaki, 1929)	Octopodidae	Du unpublished
NC009689	*Vampyroteuthis infernalis* Chun, 1903	Vampyroteuthidae	Yokobori et al., 2007
KY387929*	*Vampyroteuthis infernalis* Chun, 1903	Vampyroteuthidae	Francis and Haddock unpublished
AH012197**	*Vampyroteuthis infernalis* Chun, 1903	Vampyroteuthidae	Passamaneck et al., 2004
NW021824442*	*Octopus sinensis* d'Orbigny, 1834	Octopodidae	Yellow Sea Fisheries Research Institute unpublished

2.3. Phylogenetic analysis

Five alignment matrices were created: (1) mitochondrial protein-coding genes, (2) mitochondrial ribosomal genes less stringent selection, (3) mitochondrial ribosomal genes more stringent selection, (4) nuclear ribosomal genes less stringent selection only, and (5) nuclear ribosomal genes more stringent selection. Two partition schemes were implemented on the protein-coding genes: by gene and codon (full partition model), and by codon only. Ribosomal genes were always partitioned by gene. *Vampyroteuthis infernalis* was included in each matrix as an outgroup and used to root trees.

For each matrix, we constructed a maximum likelihood (ML) phylogeny in IQ-TREE multicore version 1.6.10 for Mac OS (Nguyen et al., 2015) with automatic model selection and implementation, and with 1000 non-parametric bootstraps. For the protein-coding gene matrix full model partition, IQTree was set to merge partitions to find the best-fit partitioning scheme, to reduce over-parameterization and increase model fit. For presentation, we collapsed nodes with less than 70% support using TreeCollapseCL4 (https://emmahodcroft.com/TreeCollapseCL.html). Families are visualized using colour palettes generated from the R package viridis (Garnier et al., 2021). Family nomenclature follows Strugnell et al. (2014) for Incirrata. For Cirrata, we recognize the smallest family divisions proposed by Collins and Villanueva (2006) and Vecchione et al. (2016). This results in a five-family scheme that is not widely recognized but is best for visualizing how the molecular phylogeny fits with existing classification schemes (c.f. Table 1).

Bayesian trees were built in MrBayes 3.2.7 (Ronquist et al., 2012), using the same partition schemes as our ML analyses, but implementing the most parameter-rich model GTR + I + G, since MrBayes has a limited selection of models and recent analyses show model selection has little influence on outcomes when inferring evolutionary relationships (Abadi et al., 2019). MrBayes was run for 1 million generations, increased to 10 million where necessary (see Supplementary S1), sampling every 100 generations, and with 25% burn in. We checked Bayesian output in Tracer v.1.7.1 (Rambaut et al., 2018) to ensure stationarity and appropriate burn-in. We do not present Bayesian Trees separately but indicate posterior probability (PP) support for nodes in Bayesian analysis alongside the bootstrap support (BS) on the presented ML trees. Posterior probabilities are given to 2 decimal places, but rounded down, to avoid submaximal support appearing as maximal.

To investigate the influence of a closer root, we subdivided the mitochondrial protein-coding gene matrix into Incirrata and Cirrata matrices, with limited individuals of the other suborder as an outgroup. To investigate the influence of potential saturation in these protein coding genes, we deleted the 3rd codon positions of the Cirrata and Incirrata matrices (importing the alignments into R as DNAbin objects and manipulating them therein). In each case, we implemented ML and BI analyses as above, partitioned by codon. Finally we combined protein coding genes and the nuclear ribosomal genes (less stringent Gblocks) into a single alignment with protein coding genes partitioned by codon and each nuclear ribosomal gene assigned its own partition and implemented ML and BI analyses as described previously.

3. Results

3.1. Assembly

Mitochondrial genomes and complete sequences of 18S rRNA and 28S rRNA were assembled from all twenty samples (Genbank Accession Numbers ON367799-ON367818; ON156531-ON156550; and ON524386-ON524405). Eighteen of the assembled mitochondrial genomes contained the typical 37 genes: 13 protein coding genes, 22 tRNA genes, and two ribosomal RNA subunits (Fig. 1). The gene order matched the standard octopod arrangement as first described by Yokobori et al. (2004) for *Octopus vulgaris* and consequently confirmed by Akasaki et al. (2006), Yokobori et al. (2007), Cheng et al. (2012, 2013), Domínguez-Contreras et al. (2016), Ma et al. (2016), Zhang et al. (2017), Chiu et al. (2018) and Tang et al. (2018, 2019) for other octopodiform taxa.

The *Bathypolypus ergasticus* mitochondrial genome is missing one protein-coding gene, *ND5,* and four tRNA genes, *tRNA-Phe*, *tRNA-His*, *tRNA-Glu* and *tRNA-Ala*. These genes were not detected in MITOS and were not present when all mitochondrial genomes were aligned. Furthermore, seeding Novoplasty with a conserved region of *ND5* did not yield any contigs, strongly suggesting *ND5* is not present in the mitochondrial genome of *B. ergasticus*. The complete mitochondrial genome of *B. ergasticus* is the shortest by 1682 bp at 14046 bp.

The *Stauroteuthis syrtensis* mitochondrial genome could not be recovered by NOVOPlasty as a single circular contig and is missing two tRNA genes: *tRNA-His* and *tRNA-Glu.* These were not detected in MITOS and were not present when all mitochondrial genomes were aligned.

3.2. Phylogenetic analysis

For alignment lengths of matrices, number of ingroup taxa, and partition information, see Supplementary Information S1.

3.2.1. Mitochondrial gene trees

Partition scheme, model selection and tree-building method had

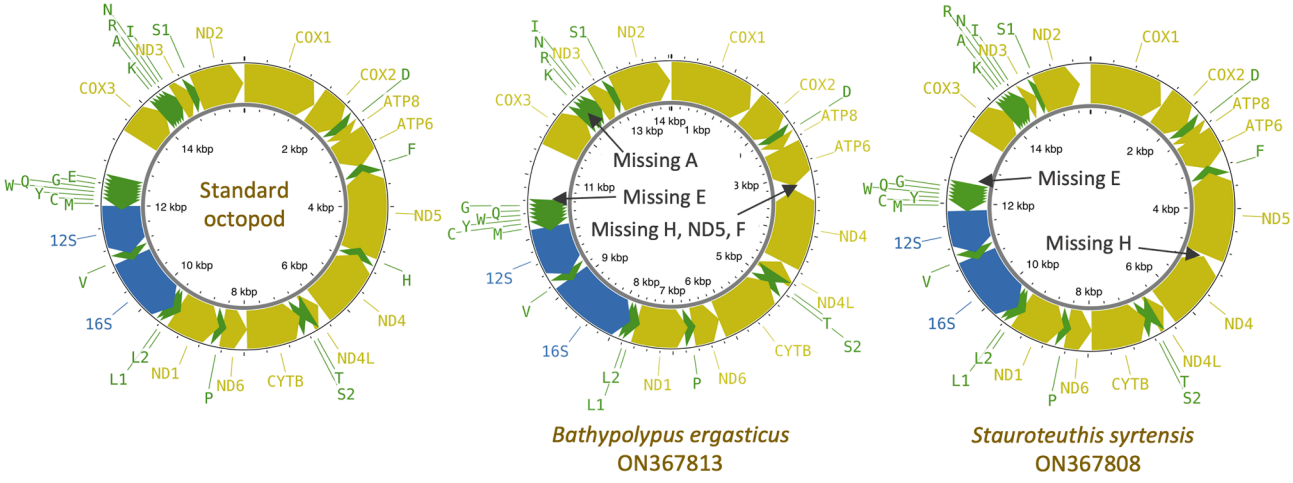

Fig. 1. Gene order of octopod sequence recovered here indicating missing genes in *Bathypolypus ergasticus* and *Stauroteuthis syrtensis.* Light green = protein coding genes; Dark green = transfer RNAs (tRNAs); Blue = ribosomal RNAs (rRNAs). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

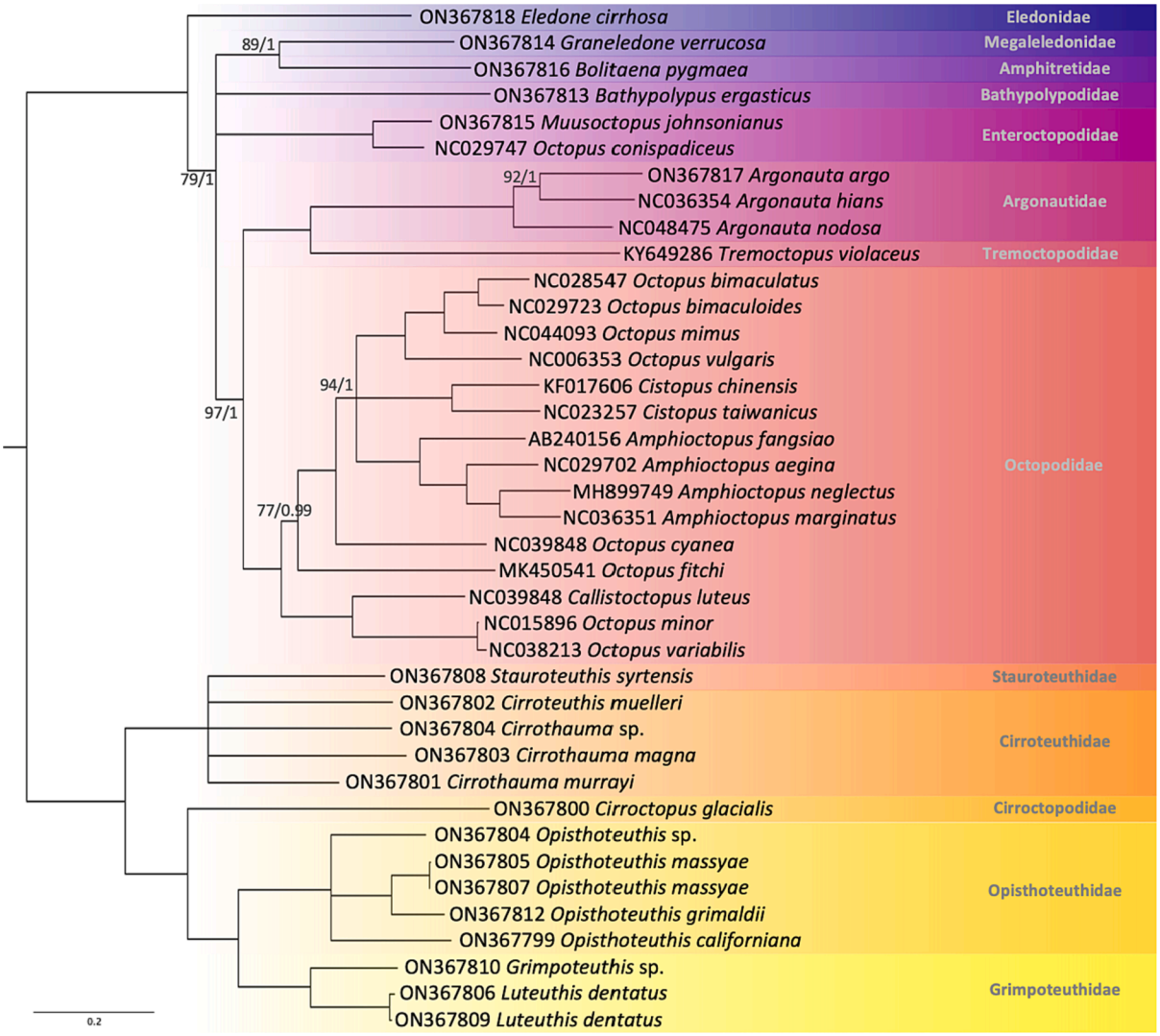

Fig. 2. Maximum likelihood tree of 13 mitochondrial protein-coding genes partitioned by codon. Root (*Vampyroteuthis infernalis*) not shown. Nodes with less than 70% bootstrap support collapsed. All remaining nodes have 100% bootstrap support and Bayesian posterior probabilities of 1 unless otherwise indicated.

little effect on the topology of the protein-coding gene analysis (Fig. 2. vs. Fig. S2). Incirrata and Cirrata are monophyletic (both BS = 100, PP = 1). Within Incirrata, families represented by more than one individual are recovered as monophyletic (all BS = 100, PP = 1). Argonautidae and Tremoctopodidae are sister taxa, supporting the monophyly of Argonautoidea (BS = 100, PP = 1). Octopodoidea (all non-argonautoid incirrates) is not supported, since Argonautoidea and Octopodidae are sister taxa (BS = 97, PP = 1), with a cluster of taxa representing Eledonidae, Megaleledonidae, Amphitretidae, Bathypolypodidae, and Enteroctopodidae outside this clade. Relationships among these taxa are not well resolved except that *Eledone cirrhosa* is sister to all other incirrates, which form a reasonably well-supported clade (BS = 79, PP = 1).

Within Cirrata, Opisthoteuthidae (sensu Collins and Villanueva, 2006) is monophyletic (BS = 100, PP = 1) and sister to a monophyletic Grimpoteuthidae (sensu Collins and Villanueva, 2006) with maximum support (BS = 100, PP = 1). Our single representative of Cirroctopodidae is sister to this clade (BS = 100, PP = 1). Representatives of *Cirroteuthis*, *Cirrothauma* and *Stauroteuthis* form a clade (BS = 100, PP = 1) but relationships within that clade are unresolved.

Analysing incirrates and cirrates separately to allow use of a closer root had little impact on topology, although it resolved relationships among *Opisthoteuthis* species (Fig. S3). Similarly removing 3rd codon positions had little impact, suggesting saturation was not affecting topology (Fig. S3).

Trees built with mitochondrial ribosomal genes also recovered Cirrata and Incirrata as monophyletic but relationships were generally less well resolved within those clades (Figs. S4 & S5). When less stringent G-block settings were applied to the alignment, the topology of Cirrata mirrored that found in protein-coding gene analyses, but with lower node support (Fig. S4). Incirrata, however, was characterized by numerous polytomies and neither Argonautoidea, nor Octopodidae was recovered as monophyletic. When more stringent G-block settings were applied to the alignment, further resolution was lost (Fig. S5) and the resulting topology yields little useful information.

3.2.2. Nuclear gene trees

The maximum likelihood tree resulting from the alignment of nuclear ribosomal RNA subunits (18S and 28S) using a less stringent Gblocks selection (alignment length 7213 base pairs) (Fig. 3), contains fewer terminals, as fewer nuclear ribosomal RNA subunit sequences were available. However, all families represented in previous trees, except Tremoctopodidae, are present.

Incirrata and Cirrata are each monophyletic (BS = 100, PP = 1). The topology of Incirrata differs from that seen in the mitochondrial trees. Representatives of Megaleledonidae, Amphitretidae, Enteroctopodidae,

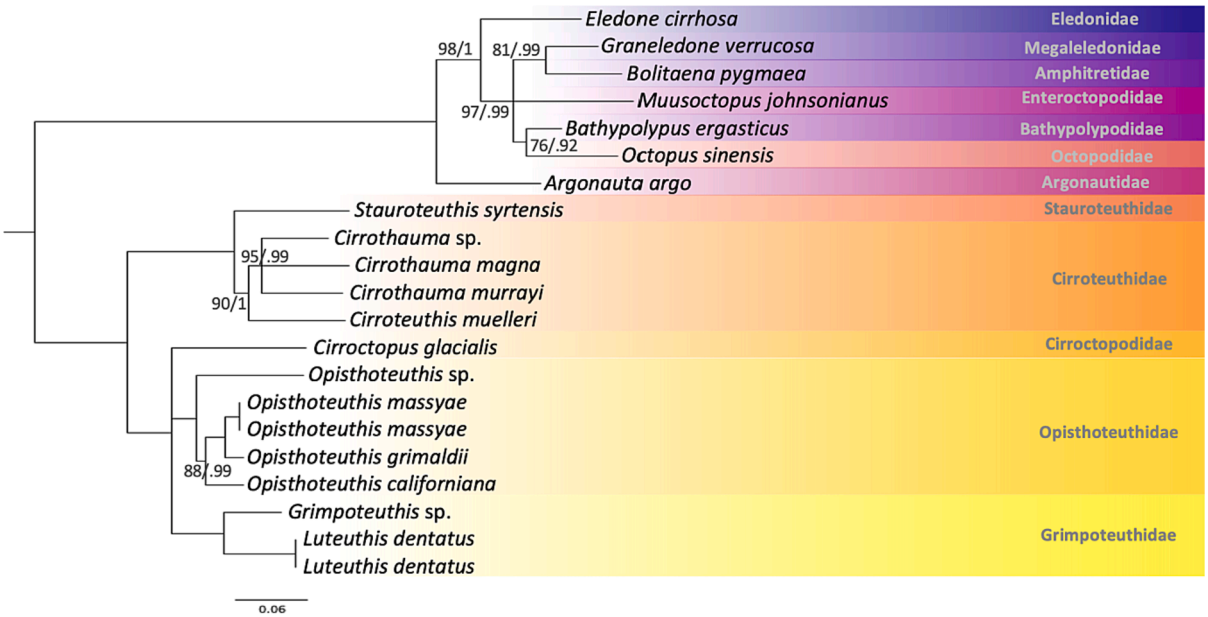

Fig. 3. Maximum likelihood tree of nuclear ribosomal genes (18S rRNA and 28S rRNA) with poorly aligned positions removed under a less stringent Gblocks selection procedure, partitioned by gene. Root (*Vampyroteuthis infernalis*) not shown. Nodes with less than 70% bootstrap support collapsed. All remaining nodes have 100% bootstrap support and Bayesian posterior probabilities of 1 unless otherwise indicated.

Bathypolypodidae, and Octopodidae formed a well-supported clade (BS = 97, PP = 0.99), with *Eledone cirrhosa* sister to this clade (BS = 98, PP = 1). *Argonauta* is the first branching incirrate lineage in this topology and is sister to all other incirrates.

Within Cirrata, there are two large, fully-supported clades. One is composed of the families Opisthoteuthidae and Grimpoteuthidae (both sensu Collins and Villanueva, 2006) plus our single representative of Cirroctopodidae (BS = 100, PP = 1). In contrast to the mitochondrial trees, the relationship between Opisthoteuthidae and Grimpoteuthidae is not resolved. The second large, fully-supported clade (BS = 100, PP = 1), which is sister to the first, contains our single representative of Stauroteuthidae, sister to Cirroteuthidae, which in contrast to the mitochondrial trees is here recovered as monophyletic (BS = 90, PP = 1). Within the monophyletic Cirroteuthidae clade, *Cirroteuthis muelleri* is sister to a well-supported clade containing all *Cirrothauma* species (BS = 95, PP = 0.99).

The maximum likelihood tree resulting from the alignment of nuclear ribosomal RNA subunits (18S and 28S) using a more stringent Gblocks selection (alignment length 5135 base pairs) (Fig. S6) recovers Cirrata and Incirrata as monophyletic (BS = 100, PP = 1). It provides no resolution on relationships within Incirrata. The topology of Cirrata is very similar to that recovered with the less stringent G-blocks selection, except that it fails to recover Opisthoteuthidae as monophyletic.

3.2.3. Combined analysis

Combining mitochondrial protein coding genes with nuclear ribosomal genes did not further resolve the Incirrata part of the tree, but did increase support values for a monophyletic Cirroteuthidae (Fig. S7).

4. Discussion

The first Octopodiformes mitochondrial genomes to be published were those of *O. vulgaris* (Yokobori et al., 2004) and *V. infernalis* (Yokobori et al., 2007). The 18 additional octopod mitochondrial genomes sequenced since (see Table 3), have all been incirrate species. No studies have sequenced the whole mitochondrial genome of any cirrate species, and our understanding of cirrate phylogeny has barely increased in this period; it continues to be based on the 16S rRNA study of Piertney et al. (2003). This, the first study to sequence whole cirrate mitochondrial genomes, includes representatives of all currently recognized cirrate families: Cirroteuthidae, Stauroteuthidae, Cirroctopodidae, Opisthoteuthidae (all sensu Vecchione et al., 2016), allowing a more thorough understanding of relationships within Cirrata.

Almost all existing mitochondrial genomes of Incirrata are from members of the most speciose family Octopodidae. The exceptions are *O. conispadiceus* (Ma et al., 2016), which based on the structure of the male hectocotylus, is an enteroctopodid (Allcock, pers. obs.), and three mitochondrial genomes of argonauts (Table 3). We herein add representatives of the families Bathypolypodidae, Eledonidae, Megaleledonidae, Amphitretidae, and an additional representative of Enteroctopodidae, providing the first comprehensive review of Octopodoidea systematics since Strugnell et al. (2014).

4.1. Genome composition

Despite low total genomic DNA concentration, Novoplasty assembled a circular genome for 19 out of 20 samples suggesting that this technique can yield useful data even with low DNA concentrations. For the most part, the genome composition and gene order recovered in our assemblies mirror those first reported by Yokobori et al. (2004, 2007) for *O. vulgaris* and *V. infernalis* and reported for all subsequent octopus mitochondrial genomes. Herein, we report *Bathypolypus ergasticus* as the first exception. For *Stauroteuthis syrtensis*, we suspect that missing tRNA genes are a consequence of poor assembly, since we were unable to recover a circularized genome.

In *B. ergasticus*, our circularized assembly lacked ND5, two tRNA genes that flank ND5, and two other tRNAs. Novoplasty yielded a single 14046 bp contig that circularized, suggesting that the assembly was accurate. tRNA loss is not uncommon (Lavrov et al., 2016), protein-coding gene loss more so. Nonetheless, evidence suggests mitochondrial protein-coding gene loss in various taxa, as summarized by Lavrov et al. (2016) in nonbilaterian groups. ATP6 has been transferred to the nuclear genome in two ctenophores (Pett et al., 2011; Kohn et al., 2012), and ATP8 has been lost from the mitochondrial genome of those ctenophores (Pett et al., 2011; Kohn et al., 2012), placozoans (Signorovitch et al., 2007; Burger et al., 2009), calcareous sponges (Lavrov et al., 2013, 2016), and some glass sponges (Haen et al., 2007, 2014; Rosengarten et al., 2008). ATP8 has similarly been reported to be lost

from diverse groups, including Bivalvia and Platyhelminthes (e.g., Gissi et al., 2008). However, manual genome curation has often subsequently revealed the presence of ATP8. ATP8 is actually present in bivalves (Lubośny et al., 2018), and in flatworms it is now thought only to be lost in the parasitic Neodermata (Egger et al., 2017). Manual curation did not reveal ND5 in *Bathypolypus ergasticus*. Neodermatans lacking ATP8 have smaller mitochondrial genomes; our *Bathypolypus ergasticus* genome that putatively lacks ND5 is, perhaps notably, 1682 bp shorter than the next smallest that we assembled. Sanger sequencing using a primer pair that spans the standard location of ND5 in the octopod genome (e.g., primers placed in ND4 and ATP6) could provide further support for its absence. Given the functional importance of ND5 in oxidative phosphorylation, its complete absence is unlikely; in *Bathypolypus ergasticus* ND5 may have been transferred to the nuclear genome, as seen for ATP6 in ctenophores. Our sequencing coverage likely would not be high enough to detect it in this scenario, even using a conserved region of ND5 as a seed. Interestingly, the mitogenome of the congeneric species *Bathypolypus sponsalis* has the complete set of genes (Sánchez-Márquez et al., 2022), and we must acknowledge that given genome skimming is a method that aims to pull high copy number genes from low coverage sequencing, it is also possible that our sequencing simply did not cover the mitochondrial genome of *Bathypolypus ergasticus* adequately and that subsequent work could show the missing elements to be present.

4.2. Incirrate phylogenetic relationships

Incirrate octopod systematics has long been in a state of flux. The current accepted classification (WoRMS, 2021) is based on the phylogenetic analyses of Strugnell et al. (2014), who sequenced three nuclear (rhodopsin, octopine dehydrogenase and pax-6) and four mitochondrial genes (12S rRNA, 16S rRNA, COXI, and COXIII) and built six trees. Three trees were based on nucleotide data and comprised: (i) mitochondrial genes, (ii) nuclear genes, and (iii) combined mitochondrial and nuclear genes. The other three trees were based on the same gene composition, but with data coded as amino acids. Three of the six trees recovered Argonautoidea as sister taxon to Octopodoidea; three trees did not resolve the position of Argonautoidea. More recently, a study of whole mitochondrial genomes did recover Argonautoidea and Octopodoidea as monophyletic sister taxa (Hirota et al., 2021), but in that study Octopodoidea was represented only by members of the family Octopodidae. Another study that has included a significant number of both genes and incirrate taxa (Lindgren et al., 2012) also recovered Argonautoidea as sister to Octopodoidea but without support for Octopodoidea monophyly. That study included six nuclear genes and four mitochondrial genes, but tolerated more missing data, with the number of genes per incirrate taxon varying between one and six. Our protein-coding gene tree and the nuclear RNA tree with less stringent Gblocks selection were quite well resolved, but conflicted (Fig. 4). The protein-coding gene tree placed Argonautoidea in a derived position as sister to Octopodidae; the nuclear RNA tree with less stringent Gblocks selection placed Argonautoidea as the first branching taxon, with Eledonidae as sister to a clade containing all other Octopodoidea families. Such conflicting results make it difficult to determine the true phylogenetic position of both Argonautoidea and Eledonidae, and a combined analysis did not provide further resolution (Fig. S7). It is not possible to conclude a 'right' answer. Maternal inheritance and lack of recombination in the mitochondrion may cause the evolution of the mitochondrial genome to diverge from the nuclear genome (Edwards and Bensch, 2009), and, given known problems with nuclear ribosomal genes in cephalopods (e.g., Bonnaud et al., 2002), the nuclear ribosomal tree might not reflect one built with a wider selection of nuclear genes. The adjacency of genes in both the mitochondrial genome and the nuclear ribosomal cluster means that each data set is effectively a single locus. Thus high support values may be obtained even when the tree topology reflects the evolutionary history of that set of genes rather than the organism. Where

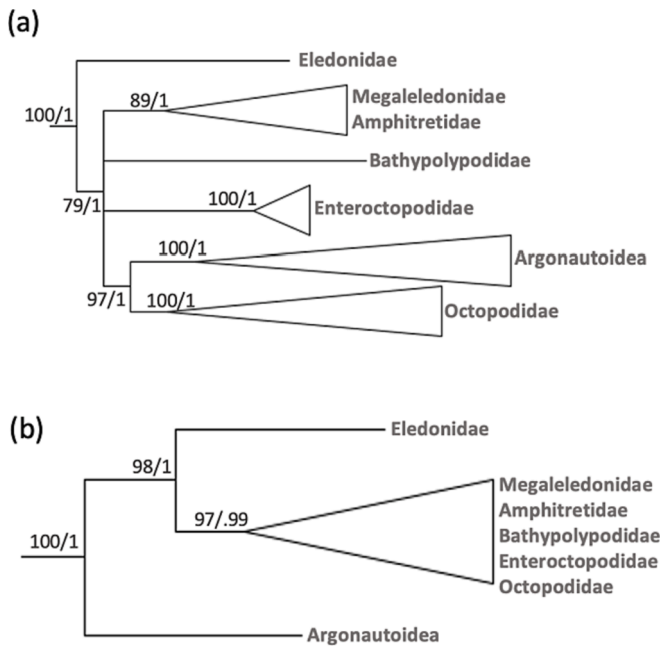

Fig. 4. The conflicting positions of Argonautoidea and Eledonidae in (a) the protein-coding gene tree (b) the nuclear RNA gene tree with less stringent Gblocks selection.

trees concur, we can be confident of the topology, but, where they do not, drawing conclusions from molecular data is difficult.

Both Argonautoidea and Eledonidae are highly derived morphologically: the suckers on the arm tips (excepting the hectocotylized arm) are modified in eledonids to form small pads (Naef, 1923), while argonautoids exhibit a range of apomorphies (e.g., holopelagic lifestyle, sexual dimorphisms, detachable hectocotylus), some of which are also exaptations (Bello, 2012). Naef (1923) suggested that eledonids "probably developed from a typical form of *Octopus*" and that the ancestral form of argonautoids also had to be "assumed to resemble *Octopus*". There are no known fossil eledonids. The earliest fossil argonaut egg cases date from the Oligocene, and peak diversity is known from the Miocene (Tomida et al., 2006). Hence, neither lineage appears early in the fossil record to support a hypothesis of its early separation from other incirrate octopods.

The other potential anomaly in this tree is the placement of *Octopus conispadiceus* sister to *Muusoctopus johnsonianus* in Enteroctopodidae. However, it has long been noted (e.g., Robson, 1929) that the long ligula of *O. conispadiceus* suggests an affinity to *Enteroctopus* and previous phylogenetic works (e.g., Takumiya et al., 2005) have confirmed a close relationship. Current placement in the taxon *Octopus* (WoRMS Editorial Board, 2022) simply reflects a historical position that has not yet been formally corrected.

4.3. Cirrate phylogenetic relationships

Our analysis included representatives of all families of Cirrata recognized in recent classifications, including multiple genera and species from each family where diversity and sample availability allowed. The families are well distinguished on morphological grounds. Vecchione et al. (2016) recognized a sister-taxon relationship between the demersal families Opisthoteuthidae (including *Opisthoteuthis*, *Grimpoteuthis* and *Luteuthis*) and Cirroctopodidae, and between the more pelagic families Cirroteuthidae and Stauroteuthidae, reflecting the phylogenetic analysis of Piertney et al. (2003). We consistently found wide divergence between these pairs of families, and the two clades containing these family pairs had high support in all analyses even though the monophyly of individual families was not evident in all cases.

4.3.1. Cirroteuthidae and Stauroteuthidae

Previous phylogenetic analyses recovered Cirroteuthidae (sensu Vecchione et al., 2016) as paraphyletic, with Stauroteuthidae falling within that clade (Piertney et al., 2003). Our protein-coding gene analysis also failed to recover Cirroteuthidae as monophyletic, similarly placing *Stauroteuthis* within Cirroteuthidae. However, our analysis of nuclear ribosomal genes recovered these families as monophyletic regardless of the stringency of Gblocks selection used.

The placement of *Stauroteuthis* has varied through time (Table 1). Naef (1923) used a two-family system and placed *Stauroteuthis* in Cirroteuthidae with *Cirroteuthis*, *Cirrothauma* and three other genera. Robson (1932) erected a new family, Stauroteuthidae. He left only the genus *Cirroteuthis* in Cirroteuthidae, and placed *Stauroteuthis*, *Cirrothauma*, *Grimpoteuthis* and several genera now considered to be junior synonyms of these in Stauroteuthidae. Voss (1988) placed *Cirroteuthis* and *Cirrothauma* in Cirroteuthidae and *Stauroteuthis* in Stauroteuthidae, as currently proposed by Vecchione et al. (2016). All three genera share long arms and cirri, and a complex web, with secondary web linking the arms to the primary web. *Cirroteuthis* and *Cirrothauma* have large saddle-shaped and butterfly-shaped shells respectively while *Stauroteuthis* has a simple U-shaped shell. *Cirroteuthis* and *Cirrothauma* have a traditional sepioid-form gill while the gills of *Stauroteuthis* have highly branching lamellae that do not form a symmetrical series along the gill, and do not have a large afferent vessel dominating the upper surface (Collins et al., 2008). Vecchione et al. (2016) believed that these morphological differences were large enough to maintain the separate families Stauroteuthidae and Cirroteuthidae. Given that combining mitochondrial and nuclear genes increased support for families Stauroteuthidae and Cirroteuthidae, it appears molecular data support this conclusion. Our taxon sampling includes five of the six known described species in these families: inclusion of *Stauroteuthis gilchristi*, providing a second representative of Stauroteuthidae, might increase support further.

4.3.2. Cirroctopodidae and Opisthoteuthidae

Piertney et al. (2003) found that *Cirroctopus* formed a distinct monophyletic grouping outside of Opisthoteuthidae and believed it should be considered as a separate family based on this, and differences in the morphological configuration of the shell and number of optic nerves. Collins and Villanueva (2006) consequently erected a new family, Cirroctopodidae, to accommodate *Cirroctopus*. Our analyses support this. Morphologically, Cirroctopodidae is further distinguished by the V-shaped shell that tapers into fine points (Collins and Villanueva, 2006) in combination with very large fins and a thick, single web. Like Opisthoteuthidae, its gills are of the 'half-orange' form.

Opisthoteuthidae, as recognized by Vecchione et al. (2016), includes the genera *Opisthoteuthis*, *Grimpoteuthis*, *Luteuthis* and *Cryptoteuthis* (the latter not included in our molecular analysis). *Luteuthis,* described as a new genus by O'Shea (1999), was initially placed in its own family Luteuthididae. O'Shea (1999) also erected a new family Grimpoteuthididae. Collins and Villanueva (2006), supported by molecular data (Piertney et al., 2003), considered that *Grimpoteuthis* and *Luteuthis* were closely related and placed both within Grimpoteuthidae, treating the genus *Opisthoteuthis* in the separate family Opisthoteuthidae. The characters that unite *Opisthoteuthis, Grimpoteuthis* and *Luteuthis* are their U-shaped shell, although the ends of the lateral walls differ, tapering to fine points in *Opisthoteuthis* and ending bluntly or in two lobes in *Grimpoteuthis* and *Luteuthis* (Collins and Villanueva, 2006), 'half-orange' form gills, and a deep, single web. Arms are short in *Opisthoteuthis* and short to moderate in *Grimpoteuthis* and *Luteuthis*.

All our analyses recovered *Luteuthis* as very closely related to *Grimpoteuthis* in a highly supported sister-taxon relationship. This Grimpoteuthidae (sensu Collins and Villanueva, 2006) clade was sister to *Opisthoteuthis* species in most analyses, forming a monophyletic Opisthoteuthidae *sensu* Vecchione et al. (2016); the nuclear analyses with the less stringent Gblocks selection were the exception, which could reflect poor alignment in this matrix.

4.4. Gblocks selection

Current practice in phylogenetics is to remove poorly aligned nucleotides to prevent introducing misinformation into phylogenetic analyses and this has commonly been applied in cephalopod phylogenetics (Lindgren et al., 2004; Yokobori et al., 2004; Uribe and Zardoya, 2017; Sanchez et al., 2018). The degree of filtering will impact the topology of the resulting phylogenetic tree. Very stringent filtering removes phylogenetic signal and the resulting tree may be based on insufficient data and consequently be poorer, as demonstrated by simulation studies (Tan et al., 2015). Although light filtering might leave unreliable alignments, tree inference may be reasonably robust to a small number of alignment errors (Tan et al., 2015).

Filtering through Gblocks resulted in alignments of greatly differing lengths (Table S1). Both matrices composed of mitochondrial ribosomal RNA genes provided limited resolution. In the nuclear ribosomal RNA gene alignments, the less stringent selection resulted in an alignment of total length 7213 base pairs compared with an alignment length of 5135 base pairs in the more stringent selection. The topology of the trees resulting from a more stringent Gblocks selection has less resolution (Fig. S6), particularly within Incirrata, probably reflecting less retained signal.

5. Conclusion

We successfully assembled mitochondrial genomes from 20 octopod specimens comprising 18 species using Illumina sequence on low concentrations of total genomic DNA, confirming the usefulness of genome skimming. Our assembly of one genome appeared to be incomplete. One species appeared to lack a protein-coding gene, but this could simply reflect low coverage in the sequencing. Our study is the first to yield mitochondrial genomes for cirrate octopods.

Although Argonautoidea is traditionally regarded as sister to all other incirrate octopods, this relationship has rarely been well supported (e.g. Strugnell et al., 2014). Our nuclear RNA tree with the less stringent Gblocks selection provides strong support for this accepted relationship, but our mitochondrial protein-coding tree recovered Argonautoidea as sister to Octopodidae with Eledonidae as the first branching lineage within Incirrata. Our study shows the importance of using data from both mitochondrial and nuclear genomes, which are under different evolutionary pressures, and illustrates how useful genome skimming can be in achieving this.

Our data support the classifications of both Collins and Villanueva (2006) and Vecchione et al. (2016); each is a four-family classification. Vecchione et al. (2016) combines Grimpoteuthidae and Opisthoteuthidae of Collins and Villanueva (2006) into a single family Opisthoteuthidae, whereas Collins and Villanueva combine Stauroteuthidae and Cirroteuthidae of Vecchione et al. (2016) into a single family Cirroteuthidae. Our mitochondrial tree supports all proposed families except Cirroteuthidae sensu Vecchione et al. (2016). Our nuclear trees find all proposed families to be monophyletic. Thus, neither classification likely conflicts with the true evolutionary history, and only the level of dissimilarity required to distinguish families needs to be determined. Additional taxon sampling, and further evaluation of morphological characters, may help resolve this.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107729.

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